This is to certify that the

dissertation entitled

BIOCHEMICAL AND GENETIC INVESTIGATION OF THE CHLOROPLASTIC PROTEIN IMPORT APPARATUS

presented by

Diane Therese Jackson Constan

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Genetics

Kenneth Keegstra
Major professor

Date Dec 19, 2001

DATE DUE	DATE DUE	DATE DUE

BIOCHEMICAL AND GENETIC INVESTIGATION OF THE CHLOROPLASTIC PROTEIN IMPORT APPARATUS

By

Diane Therese Jackson Constan

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Genetics Program

2001

ABSTRACT

BIOCHEMICAL AND GENETIC INVESTIGATION OF THE CHLOROPLASTIC PROTEIN IMPORT APPARATUS

By

Diane Therese Jackson Constan

Although plastids contain their own genome, almost all of the proteins needed within the organelle are encoded in the nuclear genome, synthesized on cytoplasmic ribosomes, and imported into the plastid posttranslationally. Protein import into chloroplasts is mediated by a proteinaceous complex localized within the two membranes of the plastid envelope. Several components of this translocation machinery have been identified from pea (*Pisum sativum*) chloroplasts, but little evidence exists concerning the individual functions of these proteins during the import process. Thus, the goal of this dissertation research has been to study the function of three import complex subunits (Tic110, Toc34, and Hsp93) in more detail. By means of experiments designed to elucidate the topology of pea Tic110 within the chloroplast inner envelope membrane, we have determined that the large C-terminal domain of Tic110, which contains the functional residues of this protein, is localized within the chloroplast stroma. Thus, Tic110 is likely involved in recruiting stromal factors, such as molecular chaperones, to the site of precursor protein translocation. The availability of the genome sequence for *Arabidopsis thaliana* allowed us to establish that all of the known components of the chloroplastic import apparatus are present in this species. Most of the import components have multiple homologs in *Arabidopsis*, suggesting that import complex composition may vary within *Arabidopsis* plastids. Having identified *Arabidopsis* homologs for the

subunits of the translocation apparatus, we isolated knockout mutant lines for two putative *Arabidopsis* import components: AtToc34 and AtHsp93-V. Plants lacking expression of the gene encoding AtToc34 appear similar to wild-type plants, both visually and at the level of chloroplast structure and composition. In addition, *in vitro* import of precursor proteins is not impaired for chloroplasts isolated from the AtToc34 mutant line. Overall, we could detect no significant differences between wild-type and mutant plants. However, double mutants that lack both AtToc34 and AtToc33, a homolog of AtToc34, were not viable, indicating that AtToc33/AtToc34 function may be essential within *Arabidopsis* chloroplasts. A knockout mutant line for AtHsp93-V, on the other hand, is viable but much smaller and paler than wild-type plants. Chloroplasts isolated from these mutant plants contain less thylakoid membrane than do wild-type chloroplasts. These results suggest that AtHsp93-V function is necessary for normal chloroplast development. Experiments addressing whether chloroplast protein import is altered in the AtHsp93-V mutant line have given equivocal results. The rate of import of at least one precursor into isolated chloroplasts *in vitro* is significantly decreased in the mutant line. However, the levels of endogenous chloroplastic proteins appear to be unaffected in AtHsp93-V knockout mutant plants, suggesting that import may not be significantly impaired *in vivo*. This dissertation research has provided insight into the possible functions of three subunits of the chloroplastic import complex, although much still remains to be learned. It is anticipated that the tools developed during this research, especially the knockout mutant lines for AtToc34 and AtHsp93-V, will be useful for future investigations of the plastid protein import process.

ACKNOWLEDGMENTS

First and foremost, I would like to thank my advisor, Kenneth Keegstra. I honestly cannot imagine having had a better person to serve as my major professor. He has challenged me in my work yet has always shown a willingness to help at any time and with any problem, no matter how trivial. Most importantly, he has constantly served as an example of what a successful scientist is and should be. Working in his lab over the past five years has been a rewarding experience.

My time in the Keegstra lab has been greatly enriched by the fact that I shared it with a group of outstanding individuals: Mitsuru Akita, Jenny Davila-Aponte, Amy DeRocher, Ahmed Faik, Lynda Fitzpatrick, John Froehlich, Kentaro Inoue, Erik Nielsen, Robyn Perrin, Sigrun Reumann, and Weiqing Zeng. They have always assisted me in my work, providing me with solutions to whatever scientific problem I was facing. In addition, they have been a source of entertainment and companionship, one that I truly appreciate. I would especially like to thank John Froehlich, who patiently guided me through my early years in the lab. Also, I would like to thank Ana Kelly, an undergraduate who provided me with skilled assistance in screening for T-DNA insertional mutants.

I would also like to thank the entire Plant Research Lab at Michigan State University. I could not even begin to name all the people who have helped me over the years. The support staff of both the Plant Research Lab and the Genetics Program deserve a special mention for all of their assistance. They have taken care of many a crisis for me, and I am very grateful for their efforts.

Finally, I would like to thank my family for their support during my graduate studies, especially my parents, Jim and Sharon Jackson, who have never questioned my decision to become a scientific researcher. No matter what choices I have made in my life, they have supported me and allowed me to do what I want without interference. In addition, I would like to acknowledge my husband, Zachary Constan, who has been a great source of comfort to me. Thanks for doing all the "dirty work" whenever I asked you to!

TABLE OF CONTENTS

LIST OF TABLES.....viii

LIST OF FIGURES.....ix

CHAPTER 1
INTRODUCTION.....1
Transport of precursor proteins across the two membranes of the chloroplast envelope.....6
Proteinaceous components of the chloroplast protein import machinery.....11
Comparisons to posttranslational protein import into other organelles.....16
Experimental investigation of protein transport systems.....24
The use of reverse genetics to study chloroplast protein import.....28
Statement of problem and attribution.....37
References.....40

CHAPTER 2
THE HYDROPHILIC DOMAIN OF TIC110, AN INNER ENVELOPE MEMBRANE COMPONENT OF THE CHLOROPLASTIC PROTEIN TRANSLOCATION APPARATUS, FACES THE STROMAL COMPARTMENT.....49
Abstract.....50
Introduction.....51
Materials and Methods.....54
Results.....58
Tic110 is resistant to digestion by adequately quenched trypsin.....58
Tic110 is degraded by insufficiently quenched trypsin only after chloroplast lysis.....59
Trypsin degrades proteins exposed to the intermembrane space but not Tic110.....62
Tic110 is exposed on the same face of inner envelope membrane vesicles as ClpC, a stroma-facing protein.....70
Discussion.....73
References.....78

CHAPTER 3
ARABIDOPSIS GENES ENCODING COMPONENTS OF THE CHLOROPLASTIC PROTEIN IMPORT APPARATUS.....81
Abstract.....82
Introduction.....83
Materials and Methods.....86
Results and Discussion.....88
Outer envelope membrane proteins.....88
Inner envelope membrane proteins.....95

Soluble factors....98
Conclusions....103
References....107

CHAPTER 4
AN OUTER ENVELOPE MEMBRANE COMPONENT OF THE CHLOROPLASTIC PROTEIN IMPORT APPARATUS IS ESSENTIAL IN *ARABIDOPSIS*....112
Abstract....113
Introduction....114
Materials and Methods....117
Results....124
Isolation of a knockout mutant line for AtToc34....124
Chloroplasts of *ppi3* plants are similar to wild-type chloroplasts....127
ppi1/ppi3 double homozygous mutants are not viable....145
Discussion....152
References....157

CHAPTER 5
AN HSP100 CHAPERONE IS REQUIRED FOR NORMAL CHLOROPLAST DEVELOPMENT AND FUNCTION IN *ARABIDOPSIS*....160
Abstract....161
Introduction....162
Materials and Methods....166
Results....172
AtHsp93-V knockout mutant plants are much smaller and paler than wild-type plants....172
Chloroplast structure and composition in the AtHsp93-V knockout mutant line....176
Import into AtHsp93-V knockout mutant chloroplasts is impaired....186
Discussion....195
References....201

CHAPTER 6
CONCLUSIONS AND FUTURE DIRECTIONS....205
References....216

APPENDIX....219
Introduction....220
Materials and Methods....221
Results....223
Discussion....229
References....231

LIST OF TABLES

Table 3-I. Putative *Arabidopsis* homologs for the components of the pea chloroplast protein import machinery.....89

Table 3-II. Comparison of the acidic properties of various members of the outer envelope membrane import complex.....90

LIST OF FIGURES

Figure 1.1. Schematic representation of pathways involved in protein targeting to chloroplasts.....4

Figure 1.2. Current model describing the process of protein import into pea chloroplasts.....7

Figure 1.3. Summary of the strategy used for PCR screening of T-DNA mutagenized *Arabidopsis* populations.....31

Figure 2.1. Tic110 is not degraded by trypsin as long as the protease is adequately quenched.....60

Figure 2.2. Tic110 is degraded by insufficiently quenched trypsin only after chloroplast lysis.....63

Figure 2.3. The trypsin sensitivity of newly imported Tic110 constructs does not mimic that of an intermembrane space marker protein.....66

Figure 2.4. Trypsin digestion of inner envelope membrane vesicles.....71

Figure 3.1. Multiple sequence alignment for the putative chloroplast-localized *Arabidopsis* Hsp70 isoforms.....100

Figure 4.1. Characteristics of the *ppi3* mutation.....125

Figure 4.2. Visible phenotype of the *ppi3* mutant line.....128

Figure 4.3. Chlorophyll levels present in *ppi3* plants do not differ significantly from those present in wild-type plants.....130

Figure 4.4. Chloroplast ultrastructure in the *ppi3* mutant line.....133

Figure 4.5. Endogenous levels of various chloroplastic proteins are not decreased in the *ppi3* mutant.....135

Figure 4.6. Total protein profiles of wild-type and *ppi3* mutant plants.....138

Figure 4.7. Chloroplasts isolated from *ppi3* mutant plants are able to import precursor proteins representing a variety of chloroplastic subcompartments.....141

Figure 4.8. The rate of import of prSS into chloroplasts isolated from the *ppi3* mutant line is not significantly impaired.....143

Figure 4.9. Plants that are homozygous for a T-DNA insert in the gene encoding AtToc33 and heterozygous for an insert in the gene encoding AtToc34 are much smaller and paler than either *ppi3* or *ppi1* mutant plants.....147

Figure 4.10. Siliques of *ppi1/ppi1/PPI3/ppi3* plants contain significantly more failed ovules than do wild-type siliques.....149

Figure 5.1. AtHsp93-V knockout mutant plants express a truncated mRNA from the *AtHSP93-V* gene and may produce a truncated AtHsp93-V protein.....174

Figure 5.2. AtHsp93-V knockout mutant plants are much smaller and paler than wild-type plants.....177

Figure 5.3. Chlorophyll levels are reduced in the AtHsp93-V knockout mutant line...179

Figure 5.4. Chloroplasts isolated from AtHsp93-V mutant plants are slightly smaller and contain less thylakoid membrane in comparison to wild-type chloroplasts.....182

Figure 5.5. Endogenous levels of various chloroplastic proteins are unaffected in the AtHsp93-V knockout line.....184

Figure 5.6. Total protein profiles of wild-type and AtHsp93-V mutant plants.....187

Figure 5.7. Chloroplasts isolated from AtHsp93-V mutant plants are able to import a variety of precursor proteins, although not as efficiently as do wild-type chloroplasts..190

Figure 5.8. The rate of import of prSS into AtHsp93-V mutant chloroplasts is decreased in comparison to import into chloroplasts isolated from wild-type plants.....192

Figure A.1. Tic110 is not labeled by Sulfo-NHS-Biotin, a membrane-impermeable biotinylation reagent, as long as chloroplasts remain intact.....225

Figure A.2. Sulfo-NHS-Biotin can cross the chloroplast inner envelope membrane and label stromal proteins.....227

CHAPTER 1

Introduction

One of the characteristic features of higher plants is the presence, within virtually every cell, of plastids. These plastids exist in a variety of forms, including proplastids, leucoplasts, chloroplasts, and chromoplasts. In most instances, the function of a particular plant tissue is determined by the type of plastid that it contains (i.e. chloroplasts in photosynthetic tissues, amyloplasts in starch-storing organs; Kirk and Tilney-Bassett, 1978). Plastids carry out several essential metabolic processes within plant cells, including photosynthesis, starch formation and breakdown, amino acid and fatty acid biosynthesis, and nutrient assimilation. However, although plastids contain a genome that encodes approximately 100 polypeptides, relatively few of the enzymes needed to catalyze these processes are encoded or synthesized within the plastid itself (Sugiura, 1989; Abdallah et al., 2000). Instead, the vast majority of plastid proteins are encoded in the nuclear genome, synthesized on cytoplasmic ribosomes, and imported into the organelle posttranslationally (reviewed in Chen and Schnell, 1999; Keegstra and Cline, 1999; Keegstra and Froehlich, 1999; May and Soll, 1999; Schleiff and Soll, 2000; Vothknecht and Soll, 2000).

Most nuclear-encoded plastid proteins are initially synthesized as higher molecular weight precursors, containing an N-terminal transit peptide that is both necessary and sufficient for their import into the organelle (reviewed in Keegstra and Cline, 1999; Bruce, 2000; Schleiff and Soll, 2000; Vothknecht and Soll, 2000). These presequences range in size from relatively small (~30 amino acids) to quite large (>100 residues; Keegstra and Cline, 1999; Schleiff and Soll, 2000). Transit peptides from a variety of precursor proteins appear to share a few general features, such as a predominance of serine and threonine residues and a lack of acidic amino acids, which

result in the transit peptide having an overall basic pI and net positive charge (Keegstra et al., 1989). However, no consensus sequence for plastid protein transit peptides has yet been recognized (von Heijne et al., 1989). A similar situation exists for the targeting sequences of mitochondrial precursor proteins. Instead of a conserved primary structure, mitochondrial targeting sequences appear to display a common secondary structure: an amphipathic helix (von Heijne, 1986; Roise et al., 1988). While the transit peptides of most plastid proteins are predicted to form random coils instead of any regular secondary structure (von Heijne and Nishikawa, 1991), it is possible that there might be some shared structural feature by which proteins destined for the plastid are specifically selected to complete the import process.

Despite their apparent lack of conservation, transit peptides from various chloroplastic proteins can be functionally substituted for one another in *in vitro* import reactions (Keegstra et al., 1995). In addition, all chloroplastic precursor proteins that have been tested appear to compete for the same proteinaceous import apparatus (Keegstra et al., 1995). These observations have led to the proposal that there is a general translocation machinery within the chloroplast envelope that all preproteins utilize to enter the organelle (Figure 1.1; Keegstra and Cline, 1999). However, it should be noted that only a relatively small number of chloroplastic precursors have been tested in this manner so it is possible that additional pathways into the plastid interior may exist.

Once a precursor protein has been imported into the plastid stroma, the transit peptide is cleaved off by the stromal processing peptidase, and the protein is folded and assembled into its mature form (Oblong and Lamppa, 1992; VanderVere et al., 1995; Richter and Lamppa, 1998). Some imported proteins are further sorted to compartments

Figure 1.1. Schematic representation of pathways involved in protein targeting to chloroplasts. Most chloroplastic proteins are synthesized within the cytoplasm as higher molecular weight precursors containing an N-terminal transit peptide that targets them to the general import machinery of the chloroplast envelope. Translocation of precursor proteins into the plastid stroma is assisted by molecular chaperones (MC) and requires hydrolysis of stromal ATP. Once the protein has been imported, the transit peptide is removed. Proteins without additional targeting information remain in the chloroplast stroma (path 1). If a protein has transmembrane domains, it can be inserted into either the thylakoid membrane (path 2) or the inner envelope membrane (not shown). Precursors destined for the thylakoid lumen contain a second targeting sequence, which is activated upon cleavage of the stromal-targeting transit peptide. Following import of the protein into the thylakoid lumen (path 3), this second targeting domain is also removed. Most outer envelope membrane proteins do not have transit peptides. Instead, these proteins are inserted directly into the outer membrane from the cytoplasm, without the assistance of additional factors (path 4). [Figure adapted from Keegstra and Cline (1999).]

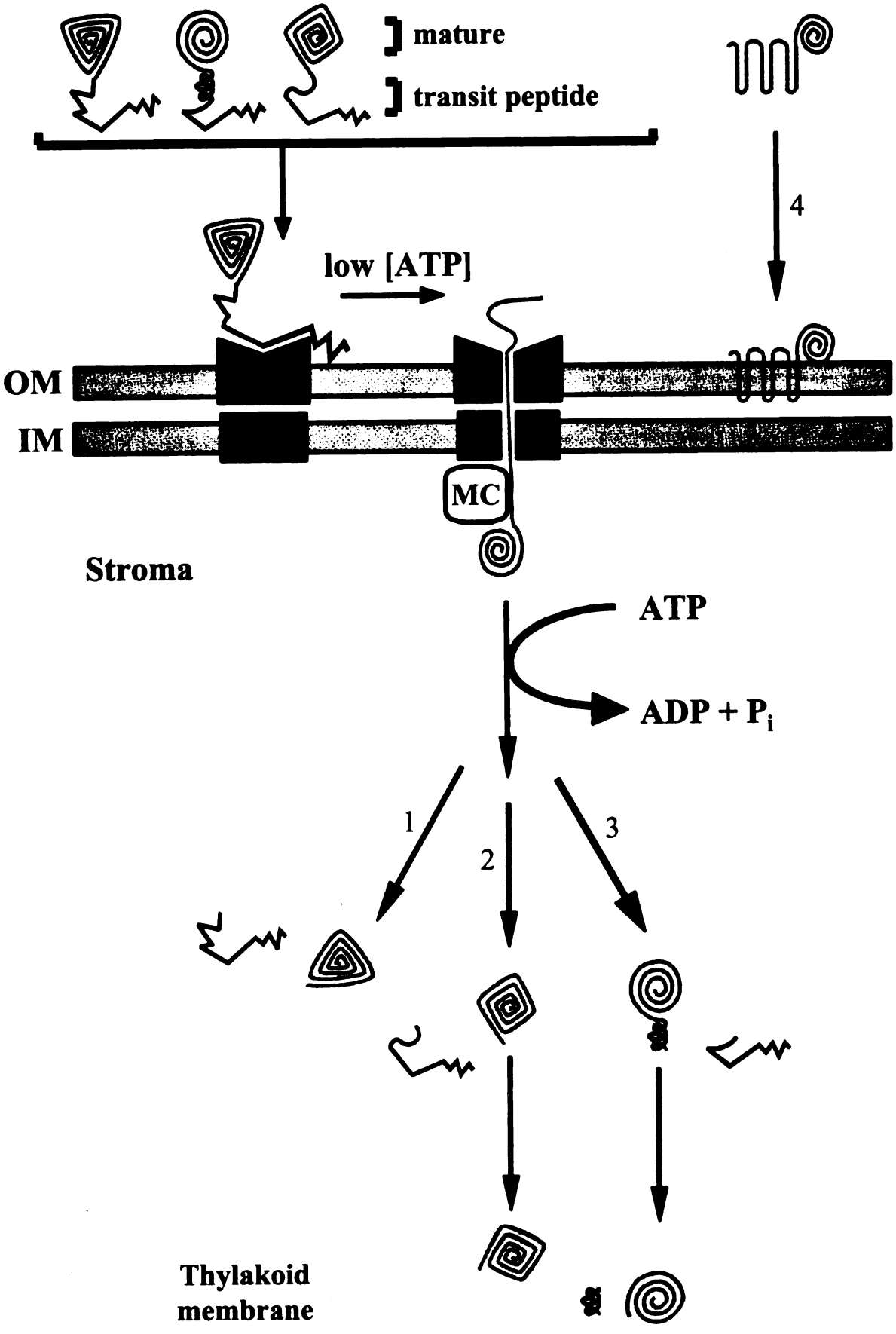

mature
transit peptide
4
low [ATP]
OM
IM
MC
Stroma
ATP
ADP + P_i
1
2
3
Thylakoid membrane

Figure 1.1

other than the stroma: the inner envelope membrane, or in chloroplasts, the thylakoid lumen or thylakoid membrane (Figure 1.1). At least one outer envelope membrane protein also utilizes the general import apparatus (although the vast majority of outer membrane proteins are inserted into this membrane directly from the cytoplasm [Figure 1.1]), but it is unlikely that the protein completely enters the stroma before being targeted to the outer membrane (Tranel and Keegstra, 1996; Keegstra and Cline, 1999; Vothknecht and Soll, 2000). A sixth compartment, the intermembrane space between the two envelope membranes, also exists; however, it is not clear what the targeting mechanism to this compartment might be.

Transport of precursor proteins across the two membranes of the chloroplast envelope

Studies on the mechanism of protein import into plastids have primarily utilized isolated pea (*Pisum sativum*) chloroplasts as a model system. With this system, it is possible to import precursor proteins into the organelle *in vitro*, presumably with similar kinetics and molecular requirements as *in vivo* import. Results from these investigations suggest that precursor proteins initially interact with a proteinaceous complex located within the chloroplast outer envelope membrane (Figure 1.2, step b; Ma et al., 1996; Akita et al., 1997; Nielsen et al., 1997). These early events involve the hydrolysis of GTP, most likely mediated by two GTP-binding proteins present in the outer membrane import complex (Olsen and Keegstra, 1992; Kessler et al., 1994; Seedorf et al., 1995).

Subsequent transport of the preprotein into the chloroplast can be further subdivided into two distinct stages, based on their differing nucleoside triphosphate requirements (Olsen et al., 1989). The first stage, referred to as the "binding" or

Figure 1.2. Current model describing the process of protein import into pea chloroplasts. Nuclear-encoded chloroplastic proteins are initially synthesized in the cytoplasm with a transit peptide (teal) that targets them to the plastid surface (a). In a process stimulated by GTP, the precursor protein associates with the components (blue) of the outer envelope membrane translocon (b). Hydrolysis of ATP in the cytoplasm and/or intermembrane space causes the precursor to interact with the components (green) of the inner membrane translocon as well (c). It is postulated that this step may be assisted by chaperones residing in the intermembrane space (purple). Hydrolysis of stromal ATP results in the complete translocation of the precursor protein into the chloroplast interior, where the transit peptide is removed (d). This final step is mediated at least in part by stromal factors (red). The numbers within the components of the outer and inner membrane translocons refer to the calculated molecular mass of each subunit. OM = outer membrane; IM = inner membrane. Images in this dissertation are presented in color.

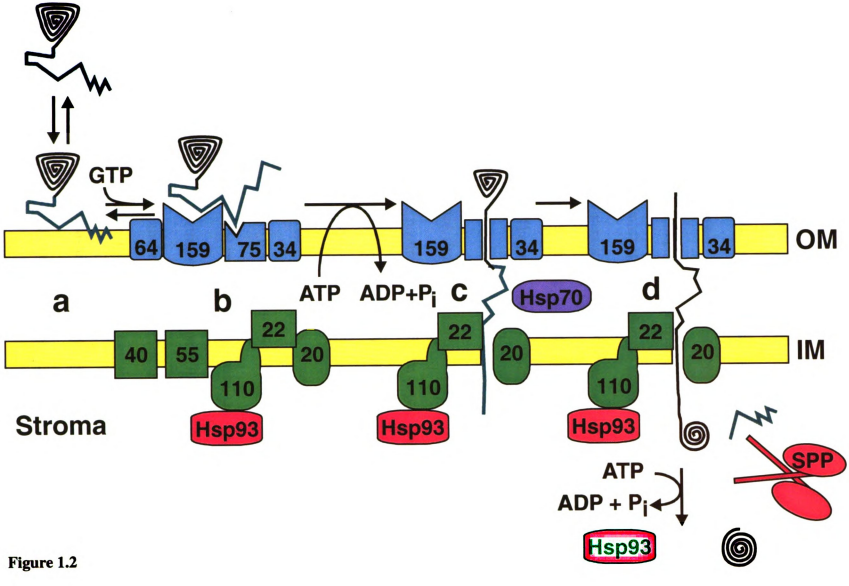

GTP
64
159
75
34
159
34
159
34
OM
a
b
ATP
ADP+P$_i$
c
Hsp70
d
40
55
22
20
110
22
20
110
20
22
20
110
IM
Stroma
Hsp93
Hsp93
Hsp93
SPP
ATP
ADP + P$_i$
Hsp93

Figure 1.2

"docking" stage of import, requires low concentrations (0.1 mM) of ATP to be present within the intermembrane space of the chloroplast envelope (Figure 1.2, step c; Olsen et al., 1989; Olsen and Keegstra, 1992). Hydrolysis of ATP at this stage results in the irreversible association of a precursor protein with the translocation machinery of both the outer and inner envelope membranes (Olsen et al., 1989; Schnell and Blobel, 1993; Nielsen et al., 1997). The second import stage is distinguished from the first by a requirement for high levels (>1 mM) of ATP within the chloroplast stroma (Theg et al., 1989). Stromal ATP hydrolysis permits the complete translocation of the preprotein into the interior of the organelle (Figure 1.2, step d), leading this stage to be termed the "translocation" phase of import (Theg et al., 1989; Chen and Schnell, 1999; Keegstra and Cline, 1999; Vothknecht and Soll, 2000). ATP hydrolysis at this stage is presumably mediated by molecular chaperones, similar to the situation known for the mitochondrial and endoplasmic reticulum (ER) posttranslational protein import systems (reviewed in Jensen and Johnson, 1999; Pilon and Schekman, 1999; Rapoport et al., 1999; Herrmann and Neupert, 2000; Strub et al., 2000).

Analysis of protein import into mitochondria, in which precursors must cross a double membrane system similar to that found in plastids, has revealed that ATP hydrolysis and a membrane potential are required for translocation into the interior of the organelle (Pfanner et al., 1997; Herrmann and Neupert, 2000). A corresponding requirement for a membrane potential at the chloroplast envelope is not present (Theg et al., 1989). However, as mentioned above, there is evidence to indicate that ATP may not be the sole energy source for the plastid protein import process. Studies into the effects of nucleoside triphosphates on precursor import have shown that GTP, by itself or in

cooperation with ATP, can stimulate binding of precursor proteins to the chloroplast envelope (Olsen and Keegstra, 1992). GTP-γS can inhibit both binding and translocation, even in the presence of high concentrations of ATP (Olsen and Keegstra, 1992; Young et al., 1999). Furthermore, it has been demonstrated that the stimulation of precursor binding by GTP is not the result of conversion of the nucleotide to ATP (Olsen and Keegstra, 1992). Thus, it appears that GTP hydrolysis may be important for the binding of preproteins to chloroplasts (Olsen and Keegstra, 1992; Young et al., 1999).

Recently, work in our lab has further explored the role of GTP during the import process. In addition to confirming that GTP stimulates and GTP-γS inhibits precursor binding, it has been demonstrated that GDP and GDP-βS do not significantly affect the binding of preproteins to the chloroplast surface in the presence of ATP (Young et al., 1999). These observations indicate that it is GTP hydrolysis, rather than merely binding of the nucleotide, that is important during the binding stage of import (Young et al., 1999).

While it is apparent from the above discussion that, at least in the *in vitro* system, GTP is playing a role in chloroplast protein import, the molecular nature of its function during the import process is still unclear. Current speculation revolves around the possibility that GTP hydrolysis has a regulatory role while ATP hydrolysis is responsible for providing the driving force for protein import to occur (Chen and Schnell, 1999; Keegstra and Froehlich, 1999; Vothknecht and Soll, 2000). It is known that GTP can regulate protein import in other systems, specifically in the cotranslational insertion of proteins into the ER (Rapoport et al., 1996). During import of proteins into the ER lumen, binding of GTP is linked with a transfer of the precursor protein-ribosome

complex to the translocation channel (Connolly and Gilmore, 1989). It is possible that GTP may be playing a similar regulatory role during chloroplast protein import, perhaps signaling the import channel to open and translocation to commence or controlling the formation of contact sites between the translocation machineries of the outer and inner envelope membranes (Chen and Schnell, 1999; Keegstra and Froehlich, 1999). Further experiments will be necessary to test these hypotheses in detail.

Proteinaceous components of the chloroplast protein import machinery

A significant amount of work in the field of plastid protein import has focused on the identification and characterization of the membrane-bound components that mediate the transport process (Figure 1.2). The first members of the import apparatus to be extensively studied, Toc159 (translocon at the outer envelope membrane of chloroplasts, 159 kD), Toc75, and Toc34, form a complex in the chloroplast outer envelope membrane (Waegemann and Soll, 1991; Hirsch et al., 1994; Kessler et al., 1994; Perry and Keegstra, 1994; Schnell et al., 1994; Seedorf et al., 1995; Tranel et al., 1995; Ma et al., 1996). All three of these components are integral proteins of the outer membrane (Hirsch et al., 1994; Kessler et al., 1994; Schnell et al., 1994; Seedorf et al., 1995; Tranel et al., 1995). Chemical crosslinking experiments have demonstrated that Toc159 is in close association with precursor proteins even in the absence of added nucleotides (i.e. GTP, ATP; Perry and Keegstra, 1994; Ma et al., 1996). In addition, antibodies against Toc159 are able to inhibit the binding of preproteins to the chloroplast surface (Hirsch et al., 1994). These data indicate that Toc159 is the first of the known translocation components encountered

by a preprotein, likely acting as the receptor for proteins that are to be imported (Hirsch et al., 1994; Perry and Keegstra, 1994).

In the presence of low levels of ATP, preproteins are crosslinked predominantly to Toc75 (Perry and Keegstra, 1994; Ma et al., 1996). Antibodies against Toc75 have been found to block protein translocation (Tranel et al., 1995). Also, Toc75 is predicted to form a porin-like structure within the outer envelope membrane (Schnell et al., 1994), and *Escherichia coli*-overexpressed Toc75 has been observed to form a channel in an artificial lipid bilayer (Hinnah et al., 1997). These results have led to the hypothesis that Toc75 may form the channel through which a precursor protein passes as it crosses the outer membrane (Schnell et al., 1994; Tranel et al., 1995; Hinnah et al., 1997). The presence of a channel in the outer membrane that is involved in protein import has been established (van den Wijngaard and Vredenberg, 1997). What still remains to be definitively shown, however, is that this observed import channel is composed of Toc75 subunits.

Recently, it has been shown that Toc34 can be crosslinked to a precursor protein in a manner that is regulated by the binding, but not hydrolysis, of GTP (Kouranov and Schnell, 1997). Both Toc34 and Toc159 contain GTP-binding domains that are exposed to the cytoplasm and have been shown to bind and hydrolyze GTP *in vitro* (Kessler et al., 1994; Seedorf et al., 1995). The significance of these results *in vivo*, though, is unknown. It is likely, however, that these two GTP-binding proteins are responsible for the observed GTP-mediated stimulation of preprotein binding to the chloroplast surface (see above; Olsen and Keegstra, 1992; Young et al., 1999). Further experiments need to be

done in order to confirm this hypothesis and to determine the exact function that GTP binding and/or hydrolysis by Toc159 and Toc34 may have during the import process.

A recent report by Sohrt and Soll (2000) has implicated a fourth component, Toc64, as being a member of the outer membrane import machinery. The amino acid sequence for this subunit contains an amidase domain, but the protein itself has no measurable amidase activity (Sohrt and Soll, 2000). In addition, Toc64 contains three tetratricopeptide repeats (TPR), which are predicted to be involved in protein-protein interactions with cytosolic factors complexed with a precursor protein and/or with the precursor itself, perhaps serving as a docking site for the incoming protein (Sohrt and Soll, 2000).

Five proteins have been identified as components of the inner membrane import apparatus based on their ability to interact with both a translocating precursor and the Toc complex (Schnell et al., 1994; Wu et al., 1994; Kessler and Blobel, 1996; Lübeck et al., 1996; Ma et al., 1996; Caliebe et al., 1997; Kouranov and Schnell, 1997). Tic110 (translocon at the inner envelope membrane of chloroplasts, 110 kD), the first of these proteins to be cloned and characterized, is an integral protein of the inner envelope membrane, containing a large hydrophilic domain that is oriented toward the chloroplast stroma (Kessler and Blobel, 1996; Lübeck et al., 1996). Based on this topology, it has been proposed that Tic110 may be involved in recruiting stromal factors, in particular molecular chaperones, to the site of protein import (Kessler and Blobel, 1996). Preliminary evidence suggests that Tic110 may indeed physically interact with at least one stromal molecular chaperone (M. Akita and K. Keegstra, unpublished observations).

Less evidence exists concerning the function of the other four inner membrane import complex components (Tic20, Tic22, Tic40, and Tic55). Tic20, an integral protein of the inner envelope membrane, is believed to form at least a portion of the channel through which chloroplastic precursors traverse the inner membrane (Kouranov and Schnell, 1997; Kouranov et al., 1998). Tic22 is localized within the intermembrane space of the chloroplast envelope and appears to be only peripherally associated with the inner envelope membrane (Kouranov et al., 1998). Due to its localization, it has been proposed that Tic22 may be involved in the formation of contact sites between the import complexes of the outer and inner envelope membranes (Kouranov and Schnell, 1997; Kouranov et al., 1998). Tic55 is an iron-sulfur protein that may play a regulatory role during chloroplast protein import (Caliebe et al., 1997). Tic40 is hypothesized to recruit chaperones to the site of precursor protein translocation, based on the presence of an Hsp70-interacting domain within its amino acid sequence (Stahl et al., 1999).

It is thought that molecular chaperones within the chloroplast provide the driving force, through the hydrolysis of ATP, for the translocation of precursor proteins into the organelle (Chen and Schnell, 1999; Keegstra and Cline, 1999; Keegstra and Froehlich, 1999), analogous to the mitochondrial and ER posttranslational protein import systems (Jensen and Johnson, 1999; Pilon and Schekman, 1999; Rapoport et al., 1999; Herrmann and Neupert, 2000; Strub et al., 2000). To date, only one stromal molecular chaperone has been conclusively demonstrated, via its association with both translocating precursor molecules and the Toc/Tic complex throughout all stages of preprotein transport, to be involved in chloroplast protein import (Akita et al., 1997; Nielsen et al., 1997; Kouranov et al., 1998). Heat shock protein 93 (Hsp93), a stromal Hsp100 homolog, is primarily a

soluble protein. However, a small, but significant, percentage of Hsp93 proteins can be found in tight association with the import complex at the inner envelope membrane, presumably through an interaction with an integral membrane protein, such as Tic110 (Moore and Keegstra, 1993; Nielsen, 1997; M. Akita and K. Keegstra, submitted). It has been proposed that Hsp93, which contains two ATP-binding domains within its amino acid sequence (Schirmer et al., 1996), may mediate the stromal requirement for ATP hydrolysis during preprotein translocation (Akita et al., 1997; M. Akita and K. Keegstra, submitted). This chaperone may "pull" the precursor protein into the organelle, thus providing the driving force for import.

It has been reported that two additional molecular chaperones localized at the outer envelope membrane of pea chloroplasts are necessary to assist the import process (Schnell et al., 1994; Wu et al., 1994; Kourtz and Ko, 1997). One of them, chloroplast outer membrane protein 70 (Com70), is exposed on the cytoplasmic side of the membrane (Ko et al., 1992; Kourtz and Ko, 1997). The other, Hsp70-import associated protein (Hsp70-IAP), faces the intermembrane space between the outer and inner envelope membranes (Marshall et al., 1990; Schnell et al., 1994). Based on its localization, it is possible that Hsp70-IAP mediates the observed requirement for ATP hydrolysis within the intermembrane space during the early stages of precursor protein transport (Olsen et al., 1989; Olsen and Keegstra, 1992). To date, however, neither of these chaperones has been definitively shown to be part of the translocation complex. In addition, cytoplasmic chaperones may be required to guide precursor proteins to the chloroplast surface and maintain them in an unfolded state prior to their transport across

the plastid envelope (Schatz and Dobberstein, 1996), although this hypothesis is still under debate.

Comparisons to posttranslational protein import into other organelles

As is apparent from the above discussion, identification of components of the chloroplastic translocation apparatus has not, in most cases, been accompanied by an experimental determination of the molecular functions of these proteins. Proposals for the roles of various components during the import process have relied mostly on knowledge of the topology of these factors in relation to the envelope membranes and of the sequence motifs contained within the proteins themselves. In contrast to the relative uncertainty concerning the function of the chloroplastic import components, several pieces of evidence exist concerning the functions of the factors involved in the posttranslational import of preproteins into mitochondria and the ER. (Although cotranslational import of proteins into the ER is well known (Rapoport et al., 1996) and may also occur, in some cases, for transport into mitochondria (Lithgow, 2000), cotranslational import across the plastid envelope membranes has not been observed.)

Chloroplast protein import shares several general features with both the mitochondrial and ER posttranslational transport systems. First of all, all three systems utilize cleavable, N-terminally localized amino acid sequences to target precursor proteins to the relevant organelle: transit peptides for chloroplastic precursors, targeting sequences for mitochondrial preproteins, and signal sequences for proteins destined for the ER (Schatz and Dobberstein, 1996). ER signal sequences, which are quite similar to the protein export signal sequences of Gram-negative bacteria, consist of a recognizable

sequence motif containing a hydrophobic core region (Emanuelsson et al., 2000). No consensus sequence, on the other hand, has been detected for the targeting signals of either chloroplastic or mitochondrial precursor proteins (von Heijne et al., 1989), making targeting to these organelles much more difficult to predict. At the primary sequence level, chloroplastic transit peptides and mitochondrial targeting sequences are similar. Both lack acidic amino acids, resulting in a net positive charge (Keegstra et al., 1989; Pfanner et al., 1997). Thus, an interesting question in the field of protein targeting concerns how plant cells, which contain both chloroplasts and mitochondria, are able to differentiate between precursors destined to one or the other of these two organelles and target them to their proper destination.

Second, like import into mitochondria and (in some cases) the ER, protein import into chloroplasts occurs after precursors have been completely translated in the cytoplasm. Thus, as is true for the mitochondrial and ER posttranslational transport systems, it has been predicted that newly-synthesized, chloroplast-targeted proteins need to be maintained, by molecular chaperones, in an import-competent, partially unfolded state after emerging from the ribosome (Schatz and Dobberstein, 1996). Both ER and mitochondrial precursor proteins are guided to their respective organelles by cytoplasmic Hsp70 proteins (Schatz and Dobberstein, 1996). Mitochondrial preproteins also utilize a cytoplasmic14-3-3 protein, mitochondrial import stimulating factor, to assist in precursor targeting (Hachiya et al., 1993; Komiya et al., 1994). It is possible that chloroplastic precursor proteins are targeted to the plastid envelope via similar factors, and accordingly, both Hsp70 proteins and 14-3-3 proteins have been reported to bind at least one chloroplastic precursor *in vitro* (May and Soll, 2000).

Even though it has been predicted that chloroplastic preproteins must be maintained within the cytoplasm in an unfolded state, there is evidence that suggests that precursors in a native, enzymatically-active conformation can still be imported into plastids. For example, della-Cioppa and co-workers (1986) demonstrated that the precursor form of 5-*enol*pyruvylshikimate-3-phosphate synthase had enzymatic activity, but was still capable of being imported into chloroplasts. Similarly, it has been shown that a chimeric precursor containing dihydrofolate reductase was properly folded and capable of binding methotrexate, yet was also still able to be imported into chloroplasts (Guéra et al., 1993; America et al., 1994). One possible explanation of these results is that the import apparatus of chloroplasts generates sufficient pulling force that it is capable of causing the unfolding of precursors that have already been folded in the cytoplasm (Guéra et al., 1993; America et al., 1994).

Finally, the posttranslational protein import systems of chloroplasts, mitochondria, and the ER all involve proteinaceous, membrane-bound complexes, consisting of, at a minimum, a receptor for the incoming preproteins, a channel through which precursors enter the organelle, and an ATPase motor that provides the driving force for protein translocation to occur (Schatz and Dobberstein, 1996). The receptor for signal sequences during the posttranslational import of precursors into the ER has not been identified, although the leading candidates for this role are the members of the Sec62/63p tetrameric complex (Sec62p, Sec63p, Sec71p, and Sec72p) or the Sec61p channel complex (Ogg et al., 1992; Jungnickel and Rapoport, 1995; Schatz and Dobberstein, 1996). The mitochondrial outer membrane import complex contains four receptor subunits, arranged as a Tom37-Tom70 heterodimer and a Tom20-Tom22

heterodimer (Tom = translocase of the outer membrane; Pfanner et al., 1997; Herrmann and Neupert, 2000). However, only one of these proteins, Tom22, is essential for cell viability in the yeast *Saccharomyces cerevisiae*, suggesting that this subunit is the major receptor to which all or most precursor proteins must bind prior to being imported into the mitochondrial matrix (Pfanner et al., 1997). In chloroplasts, the best candidate to serve as the receptor for incoming transit peptides is Toc159 (discussed above). This hypothesis is based on the fact that precursor proteins can be crosslinked to Toc159 even in the absence of ATP, suggesting that Toc159 interacts with precursors before they have become committed to the import pathway, as would be expected for a receptor component (Perry and Keegstra, 1994; Ma et al., 1996).

Early in the study of membrane transport, it was not known whether precursor proteins spontaneously inserted across the lipid bilayer or whether channels existed within the membranes through which these proteins could pass. Due to the hydrophilic nature of most precursors, however, it was believed that the latter situation would turn out to be true, and subsequent investigation confirmed this hypothesis (Schatz and Dobberstein, 1996). The protein-conducting channel across the single ER membrane is composed of three subunits: Sec61p, Sss1p, and Sbh1p (Rapoport et al., 1999). The same channel complex is likely utilized for the transport of proteins via either the posttranslational or the cotranslational import pathway (Rapoport et al., 1996; Rapoport et al., 1999). The two membranes of the mitochondrial envelope each contain a multimeric protein-conducting channel. The outer envelope membrane import channel is composed of four subunits: Tom40, Tom22, Tom7, and Tom6 (Herrmann and Neupert, 2000). Tom40 crosses the outer membrane of the mitochondrial envelope via a series of

β-strands, similar to the structure known for bacterial porins (Mannella et al., 1996). Since Tom40 is also an essential component of the yeast mitochondrial import machinery, it is likely that it is the main constituent of the protein-conducting channel within the outer membrane (Pfanner et al., 1997; Hill et al., 1998; Herrmann and Neupert, 2000). The translocation pore of the mitochondrial inner envelope membrane contains at least two subunits: Tim23 and Tim17 (Pfanner et al., 1997; Herrmann and Neupert, 2000). Interestingly, it has been observed that the translocation channels of the outer and inner mitochondrial membranes can act independently of one another (Segui-Real et al., 1993; Pfanner et al., 1997). It is not known whether this is the case for the import channels of the chloroplast envelope as well. The major subunit of the plastid outer envelope membrane protein-conducting channel is Toc75 (discussed above). Like Tom40, Toc75 crosses the membrane via a series of β-strands (Schnell et al., 1994). In addition, Toc75 has been shown to form a voltage-gated, peptide-sensitive channel in artificial lipid bilayers (Hinnah et al., 1997). Within the inner envelope membrane of chloroplasts, Tic20 is believed to form part of the translocation pore (Kouranov and Schnell, 1997; Kouranov et al., 1998), although there is no direct experimental evidence to support this hypothesis. It is also still unknown whether additional proteins interact with Tic20 to create the inner membrane import channel.

ATP hydrolysis is the major energy source for the movement of precursor proteins into the ER, mitochondria, and chloroplasts (Pfanner et al., 1997; Chen and Schnell, 1999; Keegstra and Cline, 1999; May and Soll, 1999; Rapoport et al., 1999; Herrmann and Neupert, 2000; Vothknecht and Soll, 2000). Mitochondria also utilize a second major energy source, an electrical potential [$\Delta\Psi$], to power translocation across

the inner envelope membrane (Pfanner et al., 1997; Herrmann and Neupert, 2000). Chloroplasts and the ER, however, appear to require only ATP hydrolysis to drive precursor import (Theg et al., 1989; Rapoport et al., 1999). In all three of these systems, ATP hydrolysis is mediated by molecular chaperones within the organelle interior. Kar2p, also known as BiP, a member of the Hsp70 family of chaperones, is located within the ER lumen (Rapoport et al., 1999). It is localized to the site of precursor translocation via its interaction with Sec63p, a component of the import complex of the ER membrane (Sanders et al., 1992; Brodsky and Schekman, 1993). In mitochondria, preprotein translocation is driven by the action of mitochondrial Hsp70 (mt-Hsp70), a matrix protein (Herrmann and Neupert, 2000). Like Kar2p, mt-Hsp70 interacts with a member of the membrane-bound import complex, in this case Tim44 (Kronidou et al., 1994; Rassow et al., 1994; Schneider et al., 1994). As discussed above, all evidence currently points to Hsp93, a stromal member of the Hsp100 chaperone family, as the mediator of ATP hydrolysis for protein import into chloroplasts (Akita et al., 1997; Nielsen et al., 1997; Kouranov et al., 1998). It is most likely localized to the site of precursor translocation through its interaction with Tic110 (Kouranov et al., 1998; M. Akita and K. Keegstra, submitted).

Two models have been proposed to describe how molecular chaperones act to import proteins into either the ER or mitochondria (reviewed in Jensen and Johnson, 1999; Pilon and Schekman, 1999; Strub et al., 2000). The first, known as the molecular ratchet or "trapping" model, proposes that precursor proteins move through protein-conducting channels within the membrane via random Brownian motion, capable of movement either into or out of the organelle (Neupert et al., 1990; Simon et al., 1992).

However, as a portion of the preprotein enters the organelle interior, a molecular chaperone binds to the emerging section, preventing the backwards motion of the precursor. Thus, the random motion of the precursor protein is transformed into a unidirectional movement into the organelle. This model assumes that proteins presented to the organelle for import are either kept in a completely unfolded state within the cytoplasm or are capable of spontaneously unfolding once they arrive at the organelle surface. However, since it has been observed that mitochondria can import folded proteins more rapidly than these proteins could spontaneously unfold (Gambill et al., 1993; Glick et al., 1993; Stuart et al., 1994), the molecular ratchet model likely does not provide a complete explanation of the translocation process. The second model describing chaperone function during preprotein import is known as the translocation motor or "pulling" model (Glick, 1995; Pfanner and Meijer, 1995). This model states that once a chaperone binds an incoming precursor protein, the ATP hydrolyzing ability of the chaperone is stimulated. ATP hydrolysis results in a conformational change within the chaperone, which causes it to pull a portion of the precursor further into the organelle interior. In this scenario, the pulling force produced by the chaperone would actively unfold highly folded proteins. It is still not known which of these two models best explains how chaperones work during protein translocation *in vivo*. It is possible that both are true, depending on the protein being imported. Loosely folded or unfolded proteins may enter the organelle through the mechanism proposed by the molecular ratchet model while more tightly folded proteins would require the mechanism described by the translocation motor model (Schatz and Dobberstein, 1996; Jensen and Johnson, 1999; Pilon and Schekman, 1999; Strub et al., 2000). Although this matter has been

extensively investigated for the ER and mitochondrial protein import systems, no experiments have been reported that address this question for protein transport into chloroplasts.

One aspect of chaperone function in which chloroplasts appear to be unique is their predicted use of an Hsp100 protein, rather than an Hsp70 protein, to drive translocation into the organelle interior. As mentioned above, in both the ER and mitochondria, Hsp70 chaperones are thought to assist in "pulling in" incoming precursors (Rapoport et al., 1999; Herrmann and Neupert, 2000). However, most of the evidence for the chloroplastic import system indicates that Hsp93, rather than one of the stromal Hsp70s, is accomplishing this task (Akita et al., 1997; Nielsen et al., 1997; Kouranov et al., 1998). In the bacterial protein export system, the energy for protein translocation is provided by SecA (Manting and Driessen, 2000), a protein that shares some features with Hsp93 (M. Akita and K. Keegstra, submitted). Thus, Hsp93, and perhaps the entire chloroplastic import machinery, may be more similar in function, but working in the opposite direction, to the bacterial export system than to either the ER or mitochondrial import systems.

Besides this difference, more chaperones seem to be involved during chloroplast protein import than in either of the other two systems, perhaps because ATP hydrolysis is the only energy source driving import across the two membranes of the chloroplast envelope (Theg et al., 1989). In the ER and mitochondrial import systems, chaperones are needed both in the cytoplasm to guide precursors to the organelle and in the organelle interior to drive translocation (Rapoport et al., 1999; Herrmann and Neupert, 2000). Within chloroplastic import complexes, however, two additional chaperones have been

found in association with the outer envelope membrane (Marshall et al., 1990; Ko et al., 1992; Schnell et al., 1994; Wu et al., 1994). Apparently, there is a need for chaperone function during a step at this membrane that is either not required or is mediated by a non-chaperone factor during transport across the mitochondrial outer membrane or the ER membrane.

Experimental investigation of protein transport systems

As mentioned, the study of precursor protein translocation into the ER and mitochondria has led to a better understanding of these systems than is the case for the plastid protein import system. Application of the results obtained from the investigation of ER and mitochondrial protein transport to the analysis of chloroplast protein import is hampered by the fact that virtually all the known components of the chloroplastic translocation apparatus show no significant homology to the comparable ER/mitochondrial proteins (Reumann and Keegstra, 1999). In fact, with the exception of the molecular chaperone, the subunits of the chloroplastic import complex are not homologous to any proteins of known function outside of common motif regions, such as nucleotide-binding domains (Reumann and Keegstra, 1999).

For the most part, the results described above have taken advantage of the fact that both ER and mitochondrial protein import can be studied in yeast. This organism provides a relatively simple system in which knockout mutations of various import components can be generated and analyzed. For the ER protein transport system, several of the essential components of the import complex, including Sec61p, Sec62p, and Sec63p, were first identified using a temperature-sensitive mutant screen that searched

for individuals that accumulated ER precursor proteins within the cytoplasm when the cells were shifted to the non-permissive temperature (Rothblatt et al., 1989). Similar strategies have been employed to identify the essential components of the mitochondrial import complex (Yaffe and Schatz, 1984). Proteins isolated in these screens compose the non-redundant "core" of their respective import pathways (Baker and Schatz, 1991). Interestingly, in the mitochondrial protein import system, which is composed of two separate but interconnected import complexes (similar to the chloroplastic protein transport system), the inner membrane import complex has more essential components than does the outer membrane complex (Pfanner et al., 1997). Within the outer membrane import complex, only two of the eight subunits are indispensable: Tom22, which is assumed to be the receptor that all incoming precursor proteins must bind, and Tom40, the core constituent of the translocation channel (Pfanner et al., 1997). For the inner membrane complex, however, four of the five components are necessary, including the two channel subunits, Tim23 and Tim17, and the protein that serves as the interaction site for mt-Hsp70, Tim44 (Pfanner et al., 1997).

At the time this dissertation was begun, a comparable genetic system for *in vivo* analysis of the chloroplastic transport machinery, which is primarily studied in multicellular plants, was not yet developed. Consequently, the primary method to study plastid protein import was biochemical experimentation. Such experiments have also been widely used to investigate the ER and mitochondrial transport systems, in particular for non-essential proteins that would not be detected by the genetic screens described above (Baker and Schatz, 1991). One of the major techniques used to isolate components of the import machinery is chemical crosslinking. A precursor protein is halted, via a

variety of methods, while it is undergoing import into the organelle and covalently linked to any proteins in close physical contact with it. The crosslinked complex can then be separated from other organellar proteins and analyzed to determine the subunits it contains. Using this method, several components of the chloroplastic import apparatus were identified, including Toc159, Toc75, Tic110, and Hsp93 (Perry and Keegstra, 1994; Wu et al., 1994; Lübeck et al., 1996; Akita et al., 1997; Kouranov and Schnell, 1997; Sohrt and Soll, 2000). A similar method involves the immunoprecipitation of import complexes that contain a halted preprotein (Schnell et al., 1994; Wu et al., 1994; Seedorf et al., 1995; Lübeck et al., 1996; Nielsen et al., 1997).

Chemical crosslinking and immunoprecipitation techniques have been successful not only in the identification of components of the chloroplastic import complex, but also in providing clues to the functions of these proteins during precursor transport. For example, insight can be gained into when during import various subunits are needed by halting precursor proteins at different stages of the transport process (i.e. binding stage, translocation stage) and determining which proteins are complexed with the preprotein at each of these stages. From such experiments, it has been predicted that Toc159 is the receptor for incoming chloroplastic proteins because it is the first import component to be crosslinked to precursors (Perry and Keegstra, 1994; Ma et al., 1996). Antibody inhibition experiments have been used to support these hypotheses. Experiments showing that antibodies against Toc159 inhibit the initial binding of preproteins to the chloroplast surface again suggest that Toc159 may be the receptor for the outer membrane import complex (Hirsch et al., 1994). Other biochemical methods that have been used to investigate possible functions for the subunits of the chloroplastic transport

machinery include topology determination and *in vitro* reconstitution of overexpressed proteins into artificial lipid bilayers.

While all of these biochemical investigations have been crucial for the development of the current model of chloroplast protein import (Figure 1.2), they have only indirectly addressed the question of component function. During the course of this dissertation, however, another method for addressing import complex component function became available: genome analysis. Prior to the publication of the genome sequence for *Synechocystis* sp. PCC6803, a cyanobacterium, no proteins with significant homology to the chloroplastic import components could be detected (Reumann and Keegstra, 1999). Following sequencing of this cyanobacterial genome (Kaneko et al., 1996), however, a homolog for Toc75, the predicted chloroplast outer envelope membrane import channel, was found (Bölter et al., 1998; Reumann and Keegstra, 1999; Reumann et al., 1999). Identification of this homolog, designated SynToc75, permitted the study of Toc75 function in a simple genetic system. These investigations established that SynToc75 is an essential protein of *Synechocystis* (Reumann et al., 1999). It is localized to the cyanobacterial outer membrane (Bölter et al., 1998; Reumann et al., 1999), which is believed to have evolved into the outer membrane of the chloroplast envelope (Keegstra et al., 1984; Cavalier-Smith, 1987; Joyard et al., 1991). Interestingly, SynToc75 has been found to form a cation-selective channel within artificial lipid bilayers, giving support to the hypothesis that pea Toc75 is a channel-forming protein that permits the translocation of positively-charged transit peptides (Bölter et al., 1998). Homology searches using SynToc75 as the query have shown that this cyanobacterial protein is related to proteins that secrete proteinaceous virulence factors in other Gram-

negative bacteria, again lending support to the prediction that chloroplastic Toc75 is a protein-conducting channel (Reumann and Keegstra, 1999; Reumann et al., 1999).

The *Synechocystis* genome also contains clear homologs for Tic55, Tic22, and Tic20, three components of the chloroplast inner envelope membrane import machinery (Reumann and Keegstra, 1999). Homology searches using the cyanobacterial homolog of Tic20, Sll1737, reveal that this protein has sequence similarity to branched amino acid transporters of other bacterial species (Reumann and Keegstra, 1999). This finding bolsters the prediction that Tic20 may be the protein-conducting channel of the inner membrane (Kouranov and Schnell, 1997; Kouranov et al., 1998). Significantly, Tim23 and Tim17, the two subunits of the mitochondrial inner membrane import channel, are also similar to bacterial branched amino acid transporters (Rassow et al., 1999), suggesting that Tic20 may be playing the same role as Tim23/Tim17 in the chloroplastic transport system (Reumann and Keegstra, 1999). The cyanobacterial homologs of Tic55 and Tic22 have not yet been investigated in detail, but the continued publication of genomes from a variety of species should assist in the prediction of functions for these two components, in addition to the other members of the chloroplastic import complex, as well.

The use of reverse genetics to study chloroplast protein import

One of the most recent genome sequences to be published is that of *Arabidopsis thaliana* (The Arabidopsis Genome Initiative, 2000), a model system for genetic studies in plants. This has allowed the identification of *Arabidopsis* homologs for a variety of proteins that have been studied in other systems. In conjunction with the publication of

its genome, a technique has been developed by which an *Arabidopsis* "knockout" mutant line for any gene of interest can be isolated and studied in detail (McKinney et al., 1995; Krysan et al., 1996; Krysan et al., 1999). This reverse genetic technique is based upon a T-DNA insertional mutagenesis strategy. T-DNA is derived from the Ti (tumor-inducing) plasmid of the bacterial species *Agrobacterium tumefaciens* (Zambryski, 1988). In nature, this bacterium infects several plant species, transferring the T-DNA into a plant's cells. Within the plant cell, the T-DNA inserts randomly into the genome, and the genes contained within it are expressed, providing the invading bacteria with nutrients (Zambryski, 1988).

The T-DNA is flanked by border regions of known nucleotide sequence (Zambryski, 1988). Any genes that are placed in between these border sequences are transferred into a plant cell and integrated into its genome. Consequently, this system has been utilized to introduce foreign genes, including selectable markers, into plant tissues (Koncz et al., 1992). In addition, the T-DNA can be used as an effective, random insertional mutagen (Feldmann et al., 1994; Krysan et al., 1999). Since the T-DNA is very large, approximately 5-25 kilobases (kb), any genes into which it inserts are generally either no longer able to be expressed or are expressed but non-functional (Krysan et al., 1999). These gene disruptions are often recessive; thus, heterozygous knockouts of essential genes are viable and can be propogated (Krysan et al., 1996).

Recently, populations of several thousand T-DNA mutagenized *Arabidopsis* lines have been generated (Forsthoefel et al., 1992; Krysan et al., 1999). The mutant screening strategy for these populations is based on the fact that the sequences are known for both the T-DNA border regions and for the gene in which an insertional mutation is desired.

PCR primers based on these sequences are used to amplify fragments from the *DNA of* plants that contain a T-DNA insert within the gene of interest (Figure 1.3; McKinney *et* al., 1995; Krysan et al., 1996; Krysan et al., 1999). Because performing several thousand, individual PCR reactions would be prohibitive, PCR is done using genomic DNA from pools of plants rather than from individual plants. For example, initial screening of a mutagenized population containing 100,000 lines, with a primer to one of the T-DNA border sequences and a gene-specific primer, would be done on pools of genomic DNA from 1500 plants, a total of about sixty-five PCR reactions. Usually, a fragment will be amplified from only a single pool of DNA; the identity of this fragment can be confirmed by Southern blot analysis using the wild-type gene as a probe (McKinney et al., 1995; Krysan et al., 1996; Krysan et al., 1999). The pool of 1500 plants that contained the fragment of interest is then separated into fifteen pools of 100 plants each, which can then be screened in a similar fashion. This process is repeated until the individual line containing a T-DNA insert within the desired gene is obtained. This screening procedure is very sensitive, able to detect a single, T-DNA tagged *line in a* background of several hundred lines containing other T-DNA inserts, and reproducible (McKinney et al., 1995; Krysan et al., 1996; Krysan et al., 1999). Thus, it is possible to detect virtually any T-DNA insertional mutation of interest, if it is present in the mutagenized population (Krysan et al., 1996; Krysan et al., 1999).

This method has several advantages over other common procedures to obtain knockout mutations in *Arabidopsis*. A similar method is based on transposon insertion rather than T-DNA insertion (Martienssen, 1998; Wisman et al., 1998). Because these transposons are often still active, however, stable mutants are not necessarily obtainable

Figure 1.3. Summary of the strategy used for PCR screening of T-DNA mutagenized *Arabidopsis* populations. The filled boxes represent the gene into which the T-DNA has inserted; the T-DNA is depicted as a thin line. Screening for a T-DNA insert within any particular gene is accomplished by performing a PCR reaction on genomic DNA from a mutagenized *Arabidopsis* line. The primers used for such a reaction include one specific for one of the T-DNA border sequences (L or R) and one specific for the gene of interest (5' or 3'). In the scenario depicted, PCR products could possibly be generated when the 5' and R primers are used in combination and/or when the 3' and L primers are used in combination. [Figure adapted from Krysan *et al.* (1996).]

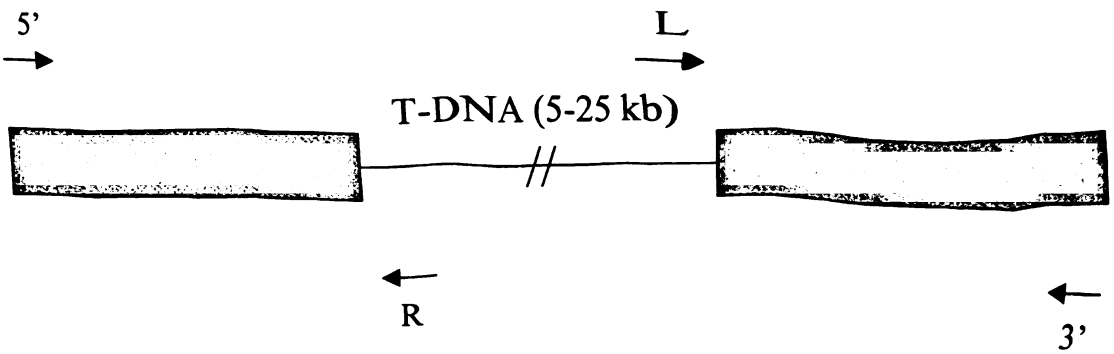


Figure 1.3

(Krysan et al., 1999). In addition, not as many transposon-tagged lines are currently available as exist for T-DNA tagged lines. Thus, the probability of obtaining an insertional mutation in the gene of interest is higher for the T-DNA tagged lines. Another common strategy for obtaining knockout mutants is based on antisense technology. Inhibition of wild-type gene expression by antisense RNA has been demonstrated in several cases (Flavell, 1994). However, this method is not completely effective, possibly resulting in a downregulation rather than a complete suppression of gene expression. Consequently, the likelihood of obtaining a knockout is lower than for the T-DNA insertion procedure.

During the time this dissertation research was being done, reverse genetic techniques were used successfully to isolate knockout mutant lines for the two GTP-binding components of the chloroplastic protein import machinery, Toc34 and Toc159 (Jarvis et al., 1998; Bauer et al., 2000). The knockout mutant line for one of the two *Arabidopsis* Toc34 homologs, AtToc33, was originally isolated from a mutant screen looking for genes affecting the expression of photosynthetic proteins (Jarvis et al., *1998*). This recessive mutant is known as *ppi1* (plastid protein import 1). The T-DNA insert within the gene encoding AtToc33 completely disrupts expression of the gene, as detected by RT-PCR (Jarvis et al., 1998). The knockout mutant plants are very pale early in development, containing between ten and twenty percent of the chlorophyll levels observed for wild-type plants (Jarvis et al., 1998). Later in development, leaves of the *ppi1* mutant appear similar in color to the wild type (Jarvis et al., 1998). This phenotype is paralleled at the level of plastid ultrastructure. Chloroplasts isolated from young *ppi1* leaves are smaller and contain fewer thylakoids than do chloroplasts isolated from wild-

type leaves (Jarvis et al., 1998). Chloroplasts from older *ppi1* leaves, however, appear more like wild-type chloroplasts (Jarvis et al., 1998). These results suggest that *AtToc33* is involved early in chloroplast development; its function may be less important in more mature plastids (Jarvis et al., 1998). In addition, chloroplasts isolated from young *ppi1* plants are impaired in their ability to import a variety of precursor proteins (Jarvis et al., 1998), lending support to the assignment of AtToc33 (and thus, pea Toc34) as a component of the chloroplastic protein import apparatus.

There are two homologs of pea Toc34 in *Arabidopsis* chloroplasts, AtToc33 and AtToc34, both of which are expressed (Jarvis et al., 1998; Gutensohn et al., 2000). These two proteins share >60% sequence identity with one another (Jarvis et al., 1998). Accordingly, the phenotype of *ppi1* plants can be complemented by the gene for either protein (Jarvis et al., 1998). This result indicates that the two homologs perform similar, if not identical, functions (Jarvis et al., 1998). However, endogenous AtToc34 cannot completely substitute for AtToc33 function in the *ppi1* mutant. One explanation for this observation may be related to the relative expression levels of AtToc33 and AtToc34. Both are expressed at their highest levels early in *Arabidopsis* development, and their expression levels decrease rapidly approximately one week after germination (Jarvis et al., 1998). However, AtToc34 is expressed at relatively low levels at all ages, while AtToc33 is expressed at much higher levels (Jarvis et al., 1998). Thus, in the *ppi1* mutant line, the low endogenous levels of *AtTOC34* gene expression may not be enough to compensate for the lack of AtToc33.

The second import component to be studied via reverse genetic techniques was Toc159, the predicted transit peptide receptor protein. In the recessive mutant, *ppi2*, the

gene for one of the *Arabidopsis* homologs of pea Toc159, AtToc159, has been disrupted by a T-DNA insert (Bauer et al., 2000). This disruption results in the absence of both the mRNA and the protein for AtToc159 within mutant plants (Bauer et al., 2000). The *ppi2* mutant has an albino, seedling-lethal phenotype (Bauer et al., 2000). Plastids of mutant plants do not differentiate past the proplastid stage, the earliest stage of plastid development (Bauer et al., 2000). The levels of photosynthetic, plastid-imported proteins, and their corresponding mRNAs, are greatly reduced in *ppi2* plants (Bauer et al., 2000). Interestingly, however, these proteins are still present in *ppi2* protein extracts, and they are correctly localized to the plastid (Bauer et al., 2000). Thus, plastid protein import is still occurring in the mutant cells, but the overall levels of import have been greatly reduced. Surprisingly, the mRNA and protein levels of nonphotosynthetic, plastid-imported proteins are unaffected in *ppi2* plants (Bauer et al., 2000). (The exception being AtToc34, which has increased mRNA levels in the mutant [Bauer et al., 2000].) Thus, the defect in the *ppi2* mutant line appears to be specific for photosynthetic proteins.

Arabidopsis has three homologs of pea Toc159, designated AtToc159, AtToc132, and AtToc120 based on their predicted molecular masses (Bauer et al., 2000). These homologs are ~40% identical to each other at the amino acid level, most of which is concentrated in the C-terminal halves of the proteins (~65% sequence identity in this region; Bauer et al., 2000). All three of these proteins are expressed and localized to the outer envelope membrane import complex of *Arabidopsis* chloroplasts (Bauer et al., 2000). However, AtToc159 is expressed five to ten times more abundantly than either AtToc132 or AtToc120 (Bauer et al., 2000). All three are expressed twice as highly in

light-grown plants, in which proplastids would be differentiating into chloroplasts, than in dark-grown seedlings, which would be forming etioplasts (Bauer et al., 2000).

Based on the phenotype of the *ppi2* mutant, it is obvious that AtToc159 plays an essential role early in *Arabidopsis* plastid development, and that AtToc132 and AtToc120 are unable to compensate for its loss in mutant plants. As for the *ppi1* mutant discussed above, it is possible that, in *ppi2* plants, the low endogenous levels of the two homologs, AtToc132 and AtToc120, are not high enough to compensate for the absence of AtToc159. Another interesting possibility is that AtToc159 may be the import receptor specific for photosynthetic proteins (Bauer et al., 2000). In this model, AtToc132 and AtToc120 would be receptors specific for non-photosynthetic proteins that are normally localized to plastids (Bauer et al., 2000). Thus, in the *ppi2* mutant line, AtToc132 and AtToc120 are unable to substitute for AtToc159 because of their different specificities for incoming precursor proteins. Because these three proteins are highly divergent from one another in the N-terminal halves of their amino acid sequences, it is likely that this is region is responsible for the predicted differences in substrate specificity (Bauer et al., 2000).

The application of reverse genetics to the process of chloroplast protein import has already led to several conclusions regarding the possible roles of AtToc33 and AtToc159 during precursor transport. Continuing investigations on these mutant lines will likely lead to even more insights regarding component function. In addition, work is ongoing in several laboratories to isolate and characterize knockout mutants for virtually all of the other known subunits of the chloroplastic protein transport machinery (P. Jarvis, personal communication; D. Schnell, personal communication). Thus, the hypotheses

concerning import component function should receive more evidence, either for or against, in the near future.

Statement of problem and attribution

Almost all chloroplast-localized proteins are encoded in the nuclear genome, synthesized on cytoplasmic ribosomes, and imported into the organelle posttranslationally. The process of transporting proteins into the chloroplast interior is mediated, with the assistance of several molecular chaperones, by a proteinaceous complex located within the two membranes of the plastid envelope. When this dissertation research was begun, five subunits of the pea chloroplastic import complex had been identified: Toc159, Toc75, Toc34, Tic110, and Hsp93. Several hypotheses concerning the role of these proteins during precursor transport had been proposed, based mainly on chemical crosslinking, antibody inhibition, and topology determination experiments. Knowledge of the motifs, such as nucleotide-binding domains, contained within the amino acid sequences of these components had led to additional hypotheses regarding subunit function. However, most of these techniques were unable to directly test the functional predictions. Thus, the goal of this dissertation research has been to utilize the experimental tools available to further expand our knowledge of the molecular functions of a few of these components, specifically Tic110, Toc34, and Hsp93. Initial experimentation was limited to the same types of biochemical investigation that had been done by previous researchers, but during the course of this dissertation, additional tools, including genome analysis and reverse genetic techniques, became available and were employed. Identification of the functions of the individual subunits of the chloroplastic

import complex will undoubtedly provide a deeper understanding of the overall process of plastid protein import.

Chapter 2 describes work done on Tic110, the first component of the chloroplast inner envelope membrane import complex to be identified (Kessler and Blobel, 1996; Lübeck et al., 1996). The experiments presented in this chapter were performed in order to determine the topology of Tic110 within the inner envelope membrane. All of the work described has been published (Jackson DT, Froehlich JE, Keegstra K [1998] J Biol Chem 273: 16583-16588). The experiment presented in Figure 2.3 was performed in collaboration with John Froehlich. I performed all the other experiments and wrote the manuscript.

Chapter 3 presents an analysis of the genome of *Arabidopsis thaliana*, which was completed in December 2000 (The Arabidopsis Genome Initiative, 2000), for homologs of the pea chloroplastic protein import apparatus. This work has also been published (Jackson-Constan D, Keegstra K [2001] Plant Physiol 125: 1567-1576). I wrote the manuscript and performed all the work reported in this chapter.

Chapter 4 describes the isolation and characterization of a knockout mutant line for AtToc34, one of the two *Arabidopsis* homologs of pea Toc34, a GTP-binding component of the chloroplast outer envelope membrane import complex. This work is being submitted to the journal Plant Cell for publication. The experiments describing the generation and characterization of a double mutant in which the genes encoding both AtToc33 and AtToc34 have been disrupted were performed by Paul Jarvis and Ramesh Patel of the University of Leicester, England. I performed all the other experiments presented and wrote the manuscript.

Chapter 5 describes the isolation and characterization of a knockout mutant line for AtHsp93-V, one of the two *Arabidopsis* homologs of pea Hsp93, the predicted translocation motor for chloroplast protein import. The isolation of the AtHsp93-V knockout mutant was done in collaboration with Ana Kelly, an undergraduate who worked under my supervision. I performed all the other experiments presented. This work has not yet been submitted for publication.

Conclusions based on this dissertation research and possible directions for further investigation are discussed in Chapter 6.

REFERENCES

Abdallah F, Salamini F, Leister D (2000) A prediction of the size and evolutionary origin of the proteome of chloroplasts of *Arabidopsis*. Trends Plant Sci 5: 141-142

Akita M, Nielsen E, Keegstra K (1997) Identification of protein transport complexes in the chloroplastic envelope membranes via chemical cross-linking. J Cell Biol 136: 983-994

America T, Hageman J, Guéra A, Rook F, Archer K, Keegstra K, Weisbeek P (1994) Methotrexate does not block import of a DHFR fusion protein into chloroplasts. Plant Mol Biol 24: 283-294

Arabidopsis Genome Initiative, The (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408: 796–815

Baker KP, Schatz G (1991) Mitochondrial proteins essential for viability mediate protein import into yeast mitochondria. Nature 349: 205-208

Bauer J, Chen K, Hiltbunner A, Wehrli E, Eugster M, Schnell D, Kessler F (2000) The major protein import receptor of plastids is essential for chloroplast biogenesis. Nature 403: 203–207

Bölter B, Soll J, Schulz A, Hinnah S, Wagner R (1998) Origin of a chloroplast protein importer. Proc Natl Acad Sci USA 95: 15831-15836

Brodsky JL, Schekman R (1993) A Sec63p-BiP complex from yeast is required for protein translocation in a reconstituted proteoliposome. J Cell Biol 123: 1355–1363

Bruce BD (2000) Chloroplast transit peptides: structure, function and evolution. Trends Cell Biol 10: 440-447

Caliebe A, Grimm R, Kaiser G, Lübeck J, Soll J, Heins L (1997) The chloroplastic protein import machinery contains a Rieske-type iron-sulfur cluster and a mononuclear iron-binding protein. EMBO J 16: 7342-7350

Cavalier-Smith T (1987) The simultaneous symbiotic origin of mitochondria, chloroplasts, and microbodies. Ann NY Acad Sci 503: 55-71

Chen X, Schnell DJ (1999) Protein import into chloroplasts. Trends Cell Biol 9: 222-227

Connolly T, Gilmore R (1989) The signal recognition particle receptor mediates the GTP-dependent displacement of SRP from the signal sequence of the nascent polypeptide. Cell 57: 599-610

della-Cioppa G, Bauer SC, Klein BK, Shah DM, Fraley RT, Kishore GM (1986) Translocation of the precursor of 5-enolpyruvylshikimate-3-phosphate synthase into chloroplasts of higher plants *in vitro*. Proc Natl Acad Sci USA 83: 6873-6877

Emanuelsson O, Nielsen H, Brunak S, von Heijne G (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J Mol Biol 300: 1005-1016

Feldmann KA, Malmberg RL, Dean C (1994) Mutagenesis in *Arabidopsis*. *In* Meyerowitz EM, Sommerville CR, eds, *Arabidopsis*. Cold Spring Harbor Press, Plainview, NY, pp 137-172

Flavell RB (1994) Inactivation of gene expression in plants as a consequence of specific sequence duplication. Proc Natl Acad Sci USA 91: 3490-3496

Forsthoefel NR, Wu Y, Schulz B, Bennett MJ, Feldmann KA (1992) T-DNA insertion mutagenesis in *Arabidopsis*: prospects and perspectives. Aust J Plant Physiol 19: 353-366

Gambill BD, Voos W, Kang PJ, Miao B, Langer T, Craig EA, Pfanner N (1993) A dual role for mitochondrial heat shock protein 70 in membrane translocation of preproteins. J Cell Biol 123: 109-117

Glick BS (1995) Can Hsp70 proteins act as force-generating motors? Cell 80: 11-14

Glick BS, Wachter C, Reid GA, Schatz G (1993) Import of cytochrome b_2 to the mitochondrial intermembrane space: the tightly folded heme-binding domain makes import dependent upon matrix ATP. Protein Sci 2: 1901-1917

Guéra A, America T, van Waas M, Weisbeek PJ (1993) A strong protein unfolding activity is associated with the binding of precursor chloroplast proteins to chloroplast envelopes. Plant Mol Biol 23: 309-324

Gutensohn M, Schulz B, Nicolay P, Flügge U-I (2000) Functional analysis of the two *Arabidopsis* homologues of Toc34, a component of the chloroplast protein import apparatus. Plant J 23: 771-783

Hachiya N, Alam R, Sakasegawa Y, Sakaguchi M, Mihara K, Omura T (1993) A mitochondrial import factor purified from rat liver cytosol is an ATP-dependent conformational modulator for precursor proteins. EMBO J 12: 1579-1586

Herrmann JM, Neupert W (2000) Protein transport into mitochondria. Curr Opin Microbiol 3: 210-214

Hill K, Model K, Ryan MT, Dietmeier K, Martin F, Wagner R, Pfanner N (1998) Tom40 forms the hydrophilic channel of the mitochondrial import pore for preproteins. Nature 395: 516-521

Hinnah SC, Hill K, Wagner R, Schlicher T, Soll J (1997) Reconstitution of a chloroplast protein import channel. EMBO J 16: 7351-7360

Hirsch S, Muckel E, Heemeyer F, von Heijne G, Soll J (1994) A receptor component of the chloroplast protein translocation machinery. Science 266: 1989-1992

Jarvis P, Chen L-J, Li H-M, Peto CA, Fankhauser C, Chory J (1998) An *Arabidopsis* mutant defective in the plastid general protein import apparatus. Science 282: 100-103

Jensen RE, Johnson AE (1999) Protein translocation: is Hsp70 pulling my chain? Curr Biol 9: R779-R782

Joyard J, Block MA, Douce R (1991) Molecular aspects of plastid envelope biochemistry. Eur J Biochem 199: 489-509

Jungnickel B, Rapoport TA (1995) A posttargeting signal sequence recognition event in the endoplasmic reticulum membrane. Cell 82: 261-270

Kaneko T, Sato S, Kotani H, Tanaka A, Asamizu E, Nakamura Y, Miyajima N, Hirosawa M, Sugiura M, Sasamoto S, *et al.* (1996) Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Res 3: 109-136

Keegstra K, Werner-Washburne M, Cline K, Andrews J (1984) The chloroplast envelope: is it homologous with the double membranes of mitochondria and Gram-negative bacteria? J Cell Biochem 24: 55-68

Keegstra K, Bruce B, Hurley M, Li H-M, Perry S (1995) Targeting of proteins into chloroplasts. Physiol Plant 93: 157-162

Keegstra K, Cline K (1999) Protein import and routing systems of chloroplasts. Plant Cell 11: 557-570

Keegstra K, Froehlich JE (1999) Protein import into chloroplasts. Curr Opin Plant Biol 2: 471-476

Keegstra K, Olsen LJ, Theg SM (1989) Chloroplastic precursors and their transport across the envelope membranes. Annu Rev Plant Physiol Plant Mol Biol 40: 471-501

Kessler F, Blobel G (1996) Interaction of the protein import and folding machineries in the chloroplast. Proc Natl Acad Sci USA 93: 7684-7689

Kessler F, Blobel G, Patel HA, Schnell DJ (1994) Identification of two GTP-binding proteins in the chloroplast protein import machinery. *Science 266: 1035-1039*

Kirk JTO, Tilney-Bassett RAE (1978) The plastids: *their chemistry, structure, growth and* inheritance. Elsevier Press, Amsterdam

Ko K, Bornemisza O, Kourtz L, Ko ZW, Plaxton WC, Cashmore AR (1992) Isolation and characterization of a cDNA clone encoding a cognate 70-kDa heat shock protein of the chloroplast envelope. J Biol Chem 267: 2986-2993

Komiya T, Hachiya N, Sakaguchi M, Omura T, Mihara K (1994) Recognition of mitochondria-targeting signals by a cytosolic import stimulation factor, MSF. J Biol Chem 269: 30893-30897

Koncz C, Németh K, Rédei GP, Schell J (1992) T-DNA insertional mutagenesis in *Arabidopsis*. Plant Mol Biol 20: 963-976

Kouranov A, Chen X, Fuks B, Schnell DJ (1998) Tic20 and Tic22 are new components of the protein import apparatus at the chloroplast inner envelope membrane. J Cell Biol 143: 991-1002

Kouranov A, Schnell DJ (1997) Analysis of the interactions of preproteins with the import machinery over the course of protein import into chloroplasts. J Cell Biol 139: 1677-1685

Kourtz L, Ko K (1997) The early stage of chloroplast protein import involves Com70. J Biol Chem 272: 2808-2813

Kronidou NG, Oppliger W, Bolliger L, Hannavy K, Glick BS, Schatz G, Horst M *(1994)* Dynamic interaction between Isp45 and mitochondrial hsp70 in the protein import system of the yeast mitochondrial inner membrane. Proc Natl Acad Sci USA *91:12818-12822*

Krysan PJ, Young JC, Sussman MR (1999) T-DNA as an insertional mutagen in Arabidopsis. Plant Cell 11: 2283-2290

Krysan PJ, Young JC, Tax F, Sussman MR (1996) Identification of transferred DNA insertions within *Arabidopsis* genes involved in signal transduction and ion transport. Proc Natl Acad Sci USA 93: 8145-8150

Lithgow T (2000) Targeting of proteins to mitochondria. FEBS Lett 476: 22-26

Lübeck J, Soll J, Akita M, Nielsen E, Keegstra K (1996) Topology of IEP110, a component of the chloroplastic protein import machinery present in the inner envelope membrane. EMBO J 15: 4230-4238

Ma Y, Kouranov A, LaSala SE, Schnell DJ (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*

Mannella CA, Neuwald AF, Lawrence CE (1996) Detection of likely transmembrane beta strand regions in sequences of mitochondrial pore proteins using the Gibbs sampler. J Bioenerg Biomembr 28: 163-169

Manting EH, Driessen AJM (2000) *Escherichia coli* translocase: the unravelling of a molecular machine. Mol Microbiol 37: 226-238

Marshall JS, DeRocher AE, Keegstra K, Vierling E (1990) Identification of heat shock protein hsp70 homologues in chloroplasts. Proc Natl Acad Sci USA 87: 374-378

Martienssen RA (1998) Functional genomics: probing plant gene function and expression with transposons. Proc Natl Acad Sci USA 95: 2021-2026

May T, Soll J (1999) Chloroplast precursor protein translocon. FEBS Lett 452: 52-56

May T, Soll J (2000) 14-3-3 proteins form a guidance complex with chloroplast precursor proteins in plants. Plant Cell 12: 53-63

McKinney EC, Ali N, Traut A, Feldmann KA, Belostotsky DA, McDowell JM, Meagher RB (1995) Sequence-based identification of T-DNA insertion mutations in *Arabidopsis*: actin mutants *act2-1* and *act4-1*. Plant J 8: 613-622

Moore T, Keegstra K (1993) Characterization of a cDNA clone encoding a chloroplast-targeted Clp homologue. Plant Mol Biol 21: 525-537

Neupert W, Hartl F-U, Craig EA, Pfanner N (1990) How do polypeptides cross the mitochondrial membranes? Cell 63: 447-450

Nielsen EE (1997) The role of stromal molecular chaperones in chloroplastic protein translocation. Ph.D. Dissertation, Michigan State University, East Lansing, MI

Nielsen E, Akita M, Davila-Aponte J, Keegstra K (1997) Stable association of chloroplastic precursors with protein translocation complexes that contain proteins from both envelope membranes and a stromal Hsp100 molecular chaperone. EMBO J 16: 935-946

Oblong JE, Lamppa GK (1992) Identification of two structurally related proteins involved in proteolytic processing of precursors targeted to the chloroplast. EMBO J 11: 4401-4409

Olsen LJ, Keegstra K (1992) The binding of precursor proteins to chloroplasts requires nucleoside triphosphates in the intermembrane space. *J Biol Chem 267: 433-439*

Olsen LJ, Theg SM, Selman BR, Keegstra K (1989) *ATP is required for the binding of* precursor proteins to chloroplasts. J Biol Chem *264: 6724-6729*

Ogg SC, Poritz MA, Walter P (1992) Signal recognition particle receptor is important for cell growth and protein secretion in *Saccharomyces cerevisiae*. Mol Biol Cell 3: 895-911

Perry SE, Keegstra K (1994) Envelope membrane proteins that interact with chloroplastic precursor proteins. Plant Cell 6: 93-105

Pfanner N, Craig EA, Hönlinger A (1997) Mitochondrial preprotein translocase. Annu Rev Cell Dev Biol 13: 25-51

Pfanner N, Meijer M (1995) Protein sorting: pulling in the proteins. Curr Biol 5: 132-135

Pilon M, Schekman R (1999) Protein translocation: how Hsp70 pulls it off. Cell 97: 679-682

Rapoport TA, Matlack KES, Plath K, Misselwitz B, Staeck O (1999) Posttranslational protein translocation across the membrane of the endoplasmic reticulum. Biol Chem 380: 1143-1150

Rapoport TA, Rolls MM, Jungnickel B (1996) Approaching the mechanism of protein transport across the ER membrane. Curr Opin Cell Biol 8: 499-504

Rassow J, Maarse AC, Krainer E, Kübrich M, Müller H, Meijer M, Craig EA, Pfanner N (1994) Mitochondrial protein import: biochemical and genetic evidence for interaction of matrix hsp70 and the inner membrane protein MIM44. J Cell Biol *127: 1547-1556*

Rassow J, Dekker PJT, van Wilpe S, Meijer M, Soll J (1999) The preprotein translocase of the mitochondrial inner membrane: function and evolution. J Mol Biol *286: 105-120*

Reumann S, Davila-Aponte J, Keegstra K (1999) The evolutionary origin of the protein-translocating channel of chloroplastic envelope membranes: identification of a cyanobacterial homolog. Proc Natl Acad Sci USA 96: 784-789

Reumann S, Keegstra K (1999) The endosymbiotic origin of the protein import machinery of chloroplastic envelope membranes. Trends Plant Sci 4: 302-307

Richter S, Lamppa GK (1998) A chloroplast processing enzyme functions as the general stromal processing peptidase. Proc Natl Acad Sci USA 95: 7463-7468

Roise D, Theiler F, Horvath SJ, Tomich JM, Richards JH, Allison DS, Schatz G (1988) Amphiphilicity is essential for mitochondrial presequence function. EMBO J 7:649-653

Rothblatt JA, Deshaies RJ, Sanders SL, Daum G, Schekman R (1989) Multiple genes are required for the proper insertion of secretory proteins into the endoplasmic reticulum in yeast. J Cell Biol 109: 2641-2652

Sanders SL, Whitfield KM, Vogel JP, Rose MD, Schekman RW (1992) Sec61p and BiP directly facilitate polypeptide translocation into the ER. Cell 69: 353-365

Schatz G, Dobberstein B (1996) Common principles of protein translocation across membranes. Science 271: 1519-1526

Schirmer EC, Glover JR, Singer MA, Lindquist S (1996) HSP100/Clp proteins: a common mechanism explains diverse functions. Trends Biochem Sci 21: 289-296

Schleiff E, Soll J (2000) Travelling of proteins through membranes: translocation into chloroplasts. Planta 211: 449-456

Schneider H-C, Berthold J, Bauer MF, Dietmeier K, Guiard B, Brunner M, Neupert W (1994) Mitochondrial Hsp70/MIM44 complex facilitates protein import. Nature 371: 768-774

Schnell DJ, Blobel G (1993) Identification of intermediates in the pathway of protein import into chloroplasts and their localization to envelope contact sites. J Cell Biol 120: 103-115

Schnell DJ, Kessler F, Blobel G (1994) Isolation of components of the chloroplast protein import machinery. Science 266: 1007-1012

Seedorf M, Waegemann K, Soll J (1995) A constituent of the chloroplast import complex represents a new type of GTP-binding protein. Plant J 7: 401-411

Segui-Real B, Kispal G, Lill R, Neupert W (1993) Functional independence of the protein translocation machineries in mitochondrial outer and inner membranes: passage of preproteins through the intermembrane space. EMBO J 12: 2211-2218

Simon SM, Peskin CS, Oster GF (1992) What drives the translocation of proteins? Proc Natl Acad Sci USA 89: 3770-3774

Sohrt K, Soll J (2000) Toc64, a new component of the protein translocon of chloroplasts. J Cell Biol 148: 1213-1221

Stahl T, Glockmann C, Soll J, Heins L (1999) Tic40, a new "old" subunit of the chloroplast protein import translocon. J Biol Chem 274: 37467-37472

Strub A, Lim JH, Pfanner N, Voos W (2000) The mitochondrial protein import motor. Biol Chem 381: 943-949

St
rec
m

Stuart RA, Gruhler A, van der Klei I, Guiard B, Koll H, Neupert W (1994) The requirement of matrix ATP for the import of precursor proteins into the mitochondrial matrix and intermembrane space. Eur J Biochem 220: 9-18

Sugiura M (1989) The chloroplast chromosomes in land plants. Annu Rev Cell Biol 5: 51-70

Theg SM, Bauerle C, Olsen LJ, Selman BR, Keegstra K (1989) Internal ATP is the only energy requirement for the translocation of precursor proteins across chloroplastic membranes. J Biol Chem 264: 6730-6736

Tranel PJ, Froehlich J, Goyal A, Keegstra K (1995) A component of the chloroplastic protein import apparatus is targeted to the outer envelope membrane via a novel pathway. EMBO J 14: 2436-2446

Tranel PJ, Keegstra K (1996) A novel, bipartite transit peptide targets OEP75 to the outer membrane of the chloroplastic envelope. Plant Cell 8: 2093-2104

van den Wijngaard PWJ, Vredenberg WJ (1997) A 50-picosiemens anion channel of the chloroplast envelope is involved in chloroplast protein import. J Biol Chem 272: 29430-29433

VanderVere PS, Bennett TM, Oblong JE, Lamppa GK (1995) A chloroplast processing enzyme involved in precursor maturation shares a zinc-binding motif with a recently recognized family of metalloendopeptidases. Proc Natl Acad Sci USA 92: 7177-7181

von Heijne G (1986) Mitochondrial targeting sequences may form amphiphilic helices. EMBO J 5:1335-1342

von Heijne G, Nishikawa K (1991) Chloroplast transit peptides: the perfect random coil? FEBS Lett 278: 1-3

von Heijne G, Steppuhn J, Herrmann RG (1989) Domain structure of mitochondrial and chloroplast targeting peptides. Eur J Biochem 180: 535-545

Vothknecht UC, Soll J (2000) Protein import: the hitchhikers guide into chloroplasts. Biol Chem 381: 887-897

Waegemann K, Soll J (1991) Characterization of the protein import apparatus in isolated outer envelopes of chloroplasts. Plant J 1: 149-158

Wisman E, Hartmann U, Sagasser M, Baumann E, Palme K, Hahlbrock K, Saedler H, Weisshaar B (1998) Knock-out mutants from an *En-1* mutagenized *Arabidopsis thaliana* population generate phenylpropanoid biosynthesis phenotypes. Proc Natl Acad Sci USA 95: 12432-12437

Wu C, Seibert FS, Ko K (1994) Identification of chloroplast envelope proteins in close physical proximity to a partially translocated chimeric precursor protein. J Biol Chem 269: 32264-32271

Yaffe MP, Schatz G (1984) Two nuclear mutations that block mitochondrial protein import in yeast. Proc Natl Acad Sci USA 81: 4819-4823

Young ME, Keegstra K, Froehlich JE (1999) GTP promotes the formation of early-import intermediates but is not required during the translocation step of protein import into chloroplasts. Plant Physiol 121: 237-243

Zambryski P (1988) Basic processes underlying *Agrobacterium*-mediated DNA transfer to plant cells. Annu Rev Genet 22: 1-30

CHAPTER 2

The hydrophilic domain of Tic110, an inner envelope membrane component of the chloroplastic protein translocation apparatus, faces the stromal compartment

The work presented in this chapter has been published:

Jackson DT, Froehlich JE, Keegstra K (1998) J Biol Chem 273: 16583-16588

The experiment presented in Figure 2.3 was done in collaboration with John Froehlich.

ABSTRACT

It has previously been found that Tic110, an integral protein of the chloroplast inner envelope membrane, is a component of the chloroplastic protein import apparatus. However, conflicting reports exist concerning the topology of this protein within the inner envelope membrane. In this report, we provide evidence that indicates that the large (>90 kD) hydrophilic domain of Tic110 is localized within the chloroplast stroma. Trypsin, a protease that cannot penetrate the permeability barrier of the inner envelope membrane, degrades neither Tic110 nor other proteins exposed to the stromal compartment but is able to digest proteins exposed to the intermembrane space between the two envelope membranes. Previous reports indicating that trypsin is able to degrade Tic110 were influenced by incomplete quenching of protease activity. When trypsin is not sufficiently quenched, it is able to digest Tic110, but only after chloroplasts have been ruptured. It is therefore necessary to employ adequate quenching protocols, such as the one reported here, whenever trypsin is utilized as an analytical tool. Based on a stromal localization for the majority of Tic110, we propose that this protein may be involved in the recruitment of stromal factors, possibly molecular chaperones, to the translocation apparatus during protein import.

INTRODUCTION

The majority of chloroplastic proteins are encoded within the nuclei of plant cells and are synthesized on cytoplasmic ribosomes. As a result, these proteins must be imported into the chloroplast posttranslationally, usually via an unfolded, higher molecular weight precursor form containing a N-terminal transit peptide (Chua and Schmidt, 1978; Highfield and Ellis, 1978; Schmidt et al., 1979; Kouranov and Schnell, 1996). Once a precursor protein has entered the chloroplast stroma, the transit peptide is cleaved off by the stromal processing peptidase, and the protein is folded into its mature form (Kouranov and Schnell, 1996). Protein import into chloroplasts is mediated by a proteinaceous translocation apparatus that spans both the outer and inner envelope membranes of this organelle. Several components of the translocation apparatus have been identified, including the outer membrane components Toc86 (translocon at the outer envelope membrane of chloroplasts, 86 kD), Toc75, and Toc34; an inner membrane component, Tic110 (translocon at the inner envelope membrane of chloroplasts, 110 kD); and a primarily stromal component, ClpC, a heat shock protein 100 (Hsp100) homolog (Waegemann and Soll, 1991; Hirsch et al., 1994; Kessler et al., 1994; Perry and Keegstra, 1994; Schnell et al., 1994; Wu et al., 1994; Seedorf et al., 1995; Tranel et al., 1995; Kessler and Blobel, 1996; Lübeck et al., 1996; Akita et al., 1997; Nielsen et al., 1997).

The first component of the inner envelope membrane translocation apparatus to be cloned was Tic110. Using chemical crosslinking and coimmunoprecipitation techniques, two separate laboratories have found Tic110 in a complex with both a translocating precursor and components of the outer membrane translocation apparatus (Kessler and Blobel, 1996; Lübeck et al., 1996). Tic110 is an integral protein of the inner

envelope membrane of chloroplasts, with either one or two putative, hydrophobic, transmembrane domains located near its N-terminus (Kessler and Blobel, 1996; Lübeck et al., 1996). The overall topology of Tic110 within the inner envelope membrane, however, remains a point of debate. Lübeck *et al.* (1996) reported that Tic110 spans the membrane once and that its large (>90 kD) hydrophilic domain is oriented toward the intermembrane space between the outer and inner envelope membranes. On the other hand, Kessler and Blobel (1996) proposed that Tic110 spans the membrane twice and that its hydrophilic domain is contained within the chloroplast stroma. To date, no evidence has been presented that satisfactorily resolves this controversy.

Knowing the topology of Tic110 will be important in assigning a putative function to this protein. For instance, if Tic110 is oriented towards the chloroplast intermembrane space, then it may function by interacting with the outer membrane translocation apparatus, promoting the formation of contact sites between the two envelope membranes (Lübeck et al., 1996). However, if Tic110 is instead exposed to the stromal compartment, then it is more likely that the protein acts by recruiting stromal proteins, for example molecular chaperones, to the protein import apparatus (Kessler and Blobel, 1996).

In previous studies, the topology of Tic110 was investigated by analyzing the protease sensitivity of the protein within intact chloroplasts, a technique that has been used for other chloroplastic membrane proteins and for membrane proteins of other organelles (Etemadi, 1980; Kessler et al., 1994; Seedorf et al., 1995; Kessler and Blobel, 1996; Lübeck et al., 1996; Tranel and Keegstra, 1996). Two of the most widely used proteases in such studies are thermolysin and trypsin. Thermolysin has been used to

selectively degrade outer envelope membrane proteins exposed on the surface of chloroplasts, since this protease, at moderate concentrations, does not penetrate the outer membrane (Cline et al., 1984). Trypsin, however, does penetrate the chloroplast outer envelope membrane, but it does not, at moderate concentrations, destroy the permeability barrier of the inner membrane (Cline et al., 1981; Cline et al., 1984; Marshall et al., 1990). Thus, trypsin is useful in defining the topology of inner envelope membrane proteins and in localizing soluble proteins to the intermembrane space of the chloroplast.

In this paper, we report on the topology of Tic110, attempting to resolve the controversy that currently exists concerning the orientation of this protein within the chloroplast inner envelope membrane. When steps are taken to adequately quench proteases, Tic110 is degraded by neither trypsin nor thermolysin, indicating that the large hydrophilic domain of Tic110 is contained within the chloroplast stromal compartment. In addition, when trypsin is insufficiently quenched, Tic110 is degraded, but only after chloroplasts are broken open. Comparison of the protease sensitivity of Tic110 with those of proteins of established topology lends further support to the conclusion that Tic110 is indeed oriented toward the chloroplast stroma.

MATERIALS AND METHODS

Materials

Pea seeds (*Pisum sativum* var. *little marvel*) were supplied by the Olds Seed Company (Madison, Wisconsin, USA). Percoll silica gel, trypsin (from bovine pancreas), N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), soybean trypsin inhibitor, and Mg-ATP were obtained from Sigma (St. Louis, Missouri, USA). Phenylmethanesulfonyl fluoride (PMSF) and aprotinin were purchased from Boehringer Mannheim (Indianapolis, Indiana, USA). ^{35}S-methionine was obtained from NEN Life Science Products (Boston, Massachusetts, USA).

Isolation of chloroplasts

Chloroplasts were isolated from 8 to 12-day-old pea seedlings over Percoll gradients as described previously (Bruce et al., 1994). Final resuspension was in import buffer (50 mM HEPES-KOH [pH 7.7], 0.33 M sorbitol) at a concentration of 1 mg chlorophyll/mL.

Trypsin digestion of intact chloroplasts

Purified intact chloroplasts (50 μg chlorophyll) were incubated with trypsin (6300 BAEE U/mg; 10 to 1000 μg trypsin/mg chlorophyll) in import buffer containing calcium chloride at a final concentration of 0.1 mM. The final reaction volume for these digestions was 300 μL. After incubation with the protease for either 10 minutes or 60 minutes at room temperature, trypsin activity was quenched by adding either PMSF at a final concentration of 1 mM or by adding a mixture of protease inhibitors to a final

concentration of 1 mM PMSF, 0.05 mg/mL TLCK, 0.1 mg/mL soybean trypsin inhibitor, and 2 μg/mL aprotinin. Chloroplasts were incubated with the quenching reagents for 10 minutes on ice.

After quenching, intact chloroplasts were reisolated over a 40% (v/v) Percoll cushion. The recovered chloroplasts were lysed hypotonically and fractionated into crude membrane and soluble fractions as described previously (Bruce et al., 1994), except that the lysis buffer contained either PMSF at a final concentration of 1 mM or a protease inhibitor mixture at final concentrations of 5 mM PMSF, 0.05 mg/mL TLCK, and 0.1 mg/mL soybean trypsin inhibitor. Fractions were analyzed by SDS-PAGE and immunoblotting with either Tic110 or Toc75 antibodies essentially as described by Tranel *et al.* (1995). Variations to this protocol are given in the figure legends.

Protease digestion of newly imported precursor proteins

Precursor proteins were synthesized in the presence of ^{35}S-methionine using the TNT®-coupled translation system from Promega (Madison, Wisconsin, USA). Rabbit reticulocyte lysate-translated ^{35}S-prToc75 (Tranel et al., 1995), ^{35}S-tp110-110N (Lübeck et al., 1997), ^{35}S-tpSS-110N (Lübeck et al., 1997), ^{35}S-tpToc75-mSS (Tranel and Keegstra, 1996), or ^{35}S-prSS (Olsen and Keegstra, 1992) was imported into purified chloroplasts (100 μg chlorophyll) essentially as previously reported (Bruce et al., 1994). After import, intact chloroplasts were reisolated over a 40% (v/v) Percoll cushion and resuspended in 600 μL import buffer. Each import reaction was then split into four equal samples for further analysis.

One sample from each import reaction was lysed hypotonically, as described previously (Bruce et al., 1994), without any further treatment. A second sample was incubated on ice for 30 minutes with 0.2 mg/mL thermolysin. Protease activity was quenched by adding EDTA to a final concentration of 5 mM. Intact chloroplasts were then reisolated over a 40% (v/v) Percoll cushion containing 5 mM EDTA and lysed hypotonically. The remaining two samples from each import reaction were incubated in the presence of trypsin (6000 BAEE U/mg) at a concentration of 500 μg trypsin/mg chlorophyll for 60 minutes at room temperature. Trypsin activity was quenched for 10 minutes on ice either with PMSF at a final concentration of 1 mM or with a mixture of protease inhibitors at final concentrations of 1 mM PMSF, 0.05 mg/mL TLCK, 0.1 mg/mL soybean trypsin inhibitor, and 2 μg/mL aprotinin. Intact chloroplasts from the two trypsin treatments were then reisolated and lysed as described above. Lysed chloroplasts from all four treatments were fractionated into crude membrane and soluble fractions as described by Bruce *et al.* (1994). The protein concentration of each fraction was determined by the Lowry protein assay (Lowry et al., 1951). Equal amounts of protein from each fraction were analyzed by SDS-PAGE and fluorography or immunoblotting (Tranel et al., 1995).

Trypsin digestion of purified inner envelope membrane vesicles

Inner envelope membranes were purified from intact chloroplasts essentially as described by Keegstra and Yousif (1986), except that the purified inner membranes were resuspended in lysis buffer (25 mM HEPES-KOH [pH 8.0], 4 mM $MgCl_2$) at a concentration of 0.5 mg/mL. Protein concentration was determined by the Bradford

protein assay (Bio-Rad, Hercules, California, USA). Purified inner envelope membranes (20 μg protein) were incubated with trypsin (6000 BAEE U/mg; 1 to 10000 ng trypsin/mg protein) in the presence of 0.1 mM calcium chloride for 10 minutes at room temperature. The final reaction volume for these digestions was 200 μL. Trypsin activity was quenched by the addition of a mixture of protease inhibitors at final concentrations of 1 mM PMSF, 0.05 mg/mL TLCK, 0.1 mg/mL trypsin inhibitor, and 2 μg/mL aprotinin. The quenched reactions were incubated for 10 minutes on ice. Inner envelope membranes were then recovered by centrifuging the samples at 250000 *g* for 10 minutes. Samples were analyzed by SDS-PAGE and immunoblotting with antibodies against either Tic110 or ClpC as described previously (Tranel et al., 1995).

Antibodies

All antibodies used in this investigation were polyclonal and raised in rabbits. Antiserum to Tic110 was generated as described by Akita *et al.* (1997). Antiserum against Toc75 was raised as discussed by Tranel *et al.* (1995). Antiserum to Toc34 (Schnell et al., 1994) was a gift from D. Schnell. Affinity-purified anti-ClpC antibodies (Shanklin et al., 1995) were a gift from J. Shanklin.

RESULTS

Tic110 is resistant to digestion by adequately quenched trypsin

It has been reported that certain proteases, most notably trypsin, are able to destroy the permeability barrier of the outer envelope membrane of chloroplasts and thereby degrade outer membrane proteins, as well as inner envelope membrane proteins exposed to the intermembrane space, while leaving stromally exposed proteins undigested (Cline et al., 1981; Cline et al., 1984; Marshall et al., 1990). Consequently, this method can be used to selectively degrade inner envelope membrane proteins that are oriented towards the intermembrane space while leaving stromally exposed inner membrane proteins intact. Such selective proteolysis techniques have previously been utilized to analyze the location and topology of various chloroplast envelope membrane proteins, including Tic110 (Kessler and Blobel, 1996; Lübeck et al., 1996; Tranel and Keegstra, 1996).

During efforts to repeat and extend these previous studies, we observed that Tic110 was resistant to degradation when intact chloroplasts were incubated with a range of trypsin concentrations (data not shown), indicating that this protein was not exposed to the chloroplast intermembrane space. These results were in direct contrast with the trypsin sensitivity of Tic110 reported by Lübeck *et al.* (1996). However, several differences in protocol existed between the two experiments, including the length of time used for trypsin digestion and the reagents used to quench trypsin activity. Consequently, we sought to determine whether these protocol differences could explain the contrasting results.

Intact chloroplasts were incubated with trypsin for either 10 minutes (Figure 2.1, lanes 1-3 and lanes 7-9) or for 60 minutes (Figure 2.1, lanes 4-5 and lanes 10-11), as described by Lübeck *et al.* (1996). After digestion for the specified period of time, trypsin activity was quenched either with a mixture of protease inhibitors (Figure 2.1, lanes 1-5) or with 1 mM PMSF (Figure 2.1, lanes 7-11), as reported by Lübeck *et al.* (1996). Degradation of Tic110 was not significantly affected by the duration of incubation with the protease (Figure 2.1, compare lanes 2-3 with lanes 4-5). On the other hand, the quench protocol had a dramatic effect on Tic110 digestion (Figure 2.1, compare lanes 2-5 and lanes 8-11). Tic110 remained undigested when trypsin was quenched with the mixture of protease inhibitors but was completely degraded when 1 mM PMSF was used to quench trypsin activity. These observations indicated that 1 mM PMSF was insufficient to quench protease activity. This result was supported by the finding that when chloroplasts were incubated with 1 mM PMSF prior to trypsin addition, Tic110 was still completely degraded (Figure 2.1, lane 12). Other differences (i.e. number of washes) between our protease digestion protocol and that of Lübeck *et al.* (1996) were also tested for their effects on Tic110 degradation. However, none affected the pattern of Tic110 digestion (data not shown). We concluded, therefore, that the difference in results could be completely explained by differences in the methods used to quench trypsin activity.

Tic110 is degraded by insufficiently quenched trypsin only after chloroplast lysis

We next sought to determine at what stage of the protease digestion protocol trypsin degraded Tic110 when 1 mM PMSF was used as the quenching reagent. Specifically, we wanted to determine whether Tic110 was degraded before chloroplast

Figure 2.1. Tic110 is not degraded by trypsin as long as the protease is adequately quenched. Intact chloroplasts were incubated for the times indicated without (lanes 1 and 7) or with (lanes 2-6 and 8-12) trypsin. Protease concentrations used are indicated. The concentration of trypsin used in the lanes marked "Q" (lanes 6 and 12) was 500 μg protease/mg chlorophyll. Trypsin activity was quenched as indicated either after (lanes 2-5 and 8-11) or before (lanes 6 and 12) trypsin addition. Intact chloroplasts were reisolated, lysed, and separated into membrane and soluble protein fractions. Equivalent volumes of each membrane protein fraction were analyzed by SDS-PAGE and immunoblotting with antibodies against either Tic110 or Toc75.

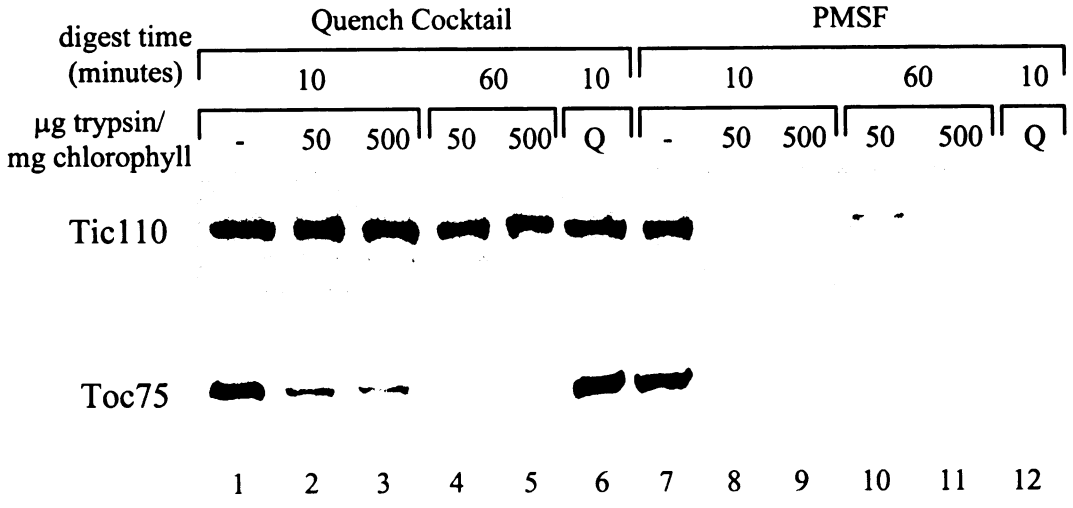

digest time
(minutes)
Quench Cocktail
PMSF
10
60
10
10
60
10
µg trypsin/
mg chlorophyll
-
50
500
50
500
Q
-
50
500
50
500
Q
Tic110
Toc75
1
2
3
4
5
6
7
8
9
10
11
12

Figure 2.1

lysis, when the permeability barrier of the inner membrane was still intact, or after lysis, when the inner membrane had been ruptured. There were three stages during our protocol in which degradation of Tic110 by trypsin could occur: before chloroplasts were broken open (incubation of chloroplasts with trypsin, quenching of protease activity, and reisolation of intact chloroplasts), during chloroplast lysis, or during post-lysis steps (membrane sedimentation and incubation of the membranes in SDS-PAGE sample buffer). To distinguish among these possibilities, we quenched trypsin-treated chloroplasts with the mixture of protease inhibitors before lysis, during lysis, and/or after lysis. During those steps when the protease inhibitor mixture was not added, 1 mM PMSF was added in its place.

Figure 2.2 shows the results from this experimental approach. When the quench mixture was added at all three stages or just during and after lysis, Tic110 was not significantly degraded (Figure 2.2A, lanes 1-2). Tic110 was completely digested only when the quenching mixture was added just during the post-lysis stage (Figure 2.2A, lane 3), indicating that it was most likely degraded by active trypsin during chloroplast lysis. In addition, as long as the protease inhibitor mixture was added before and/or during chloroplast lysis, Tic110 was not digested by trypsin (Figure 2.2B). Thus, it appeared that unless trypsin was adequately quenched before or at the time of lysis, Tic110 was digested by the protease once chloroplasts were broken open.

Trypsin degrades proteins exposed to the intermembrane space but not Tic110

Recently, several investigators have utilized protease digestion techniques to analyze the location and topology of newly imported chloroplastic proteins (Tranel and

Figure 2.2. Tic110 is degraded by insufficiently quenched trypsin only after chloroplast lysis. (A,B) Intact chloroplasts (50 μg chlorophyll) were incubated for 10 minutes with trypsin (500 μg protease/mg chlorophyll). Protease activity was quenched either with a mixture of protease inhibitors (+) or with 1 mM PMSF (-) at the stages indicated above and as outlined in "Results." Intact chloroplasts were reisolated, lysed, and separated into membrane and soluble protein fractions. Half of each membrane fraction was analyzed by SDS-PAGE and immunoblotting with antiserum against Tic110.

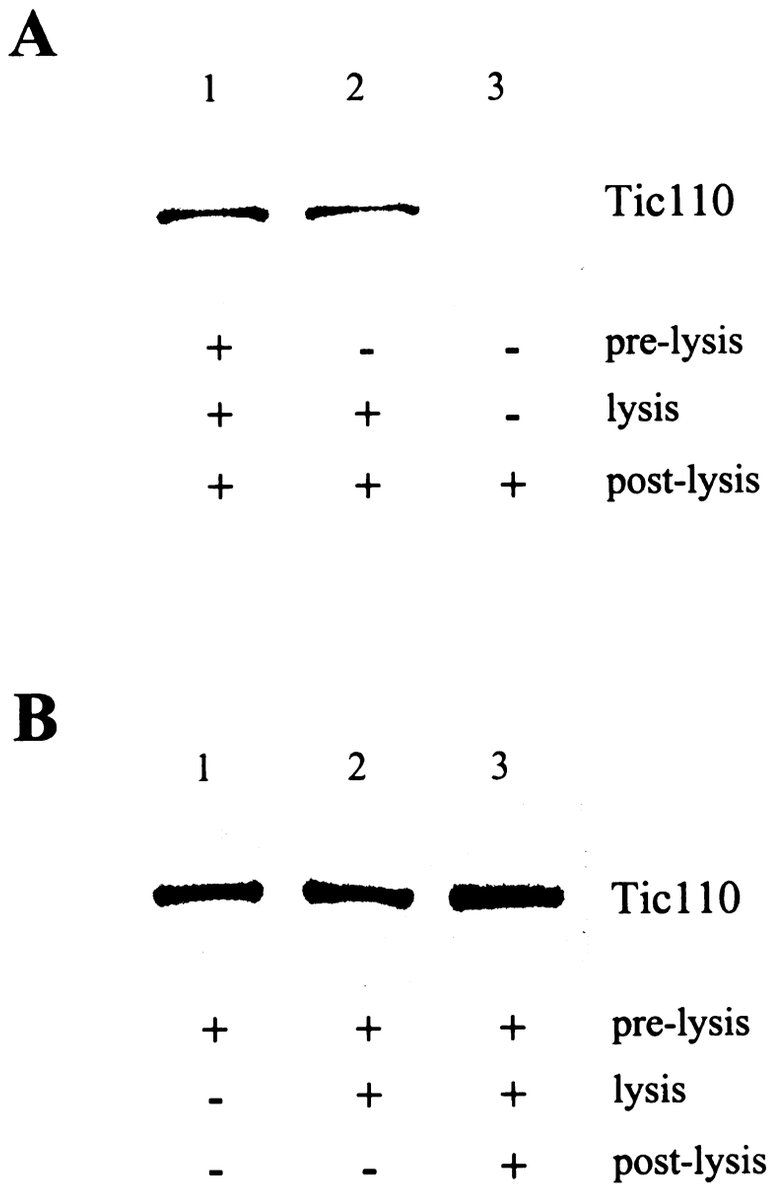

A
1
2
3
Tic110
+
-
-
pre-lysis
+
+
-
lysis
+
+
+
post-lysis
B
1
2
3
Tic110
+
+
+
pre-lysis
-
+
+
lysis
-
-
+
post-lysis

Figure 2.2

Keegstra, 1996; Lübeck et al., 1997). We obtained these precursor constructs in order to determine whether the quenching protocol used had an effect on the results and to compare the protease sensitivity of constructs with known topology to that of Tic110 constructs. Intact chloroplasts were subjected to an import assay with one of five different precursor proteins: prToc75 (Tranel et al., 1995); tp110-110N, a truncated version of prTic110 containing the putative transmembrane domain(s) and approximately one-fifth (<20 kD) of the hydrophilic domain (Lübeck et al., 1997); tpSS-110N, a chimeric precursor containing the transit peptide of the small subunit of ribulose 1,5-bisphosphate carboxylase (SS) attached to the truncated version of mature Tic110 (Lübeck et al., 1997); tpToc75-mSS, a chimeric precursor consisting of the transit peptide of Toc75 attached to the mature form of SS (Tranel and Keegstra, 1996); and prSS (Olsen and Keegstra, 1992). After import, intact chloroplasts were reisolated and digested with either thermolysin or trypsin. Trypsin-treated chloroplasts were quenched with either the protease inhibitor mixture (trypsin I protocol) or 1 mM PMSF (trypsin II protocol). Following protease digestion, intact chloroplasts were reisolated, lysed, and separated into membrane and soluble protein fractions. The proteins from these fractions were then analyzed by SDS-PAGE and fluorography to detect the newly imported, radiolabeled proteins (Figure 2.3A) or immunoblotting to detect endogenous proteins (Figure 2.3B).

The processed forms of prToc75 (mToc75 and iToc75), which were used as markers for the outer envelope membrane, were degraded by trypsin but not by thermolysin (Figure 2.3A, row 1). Because Toc75 is deeply embedded in the chloroplast outer envelope membrane, thermolysin could not access the protein. However, because trypsin is able to penetrate the outer membrane, it was able to completely digest Toc75

Figure 2.3. The trypsin sensitivity of newly imported Tic110 constructs does not mimic that of an intermembrane space marker protein. ^{35}S-labeled prToc75 (row 1), tp110-110N (row 2), tpSS-110N (row 3), tpToc75-mSS (row 4), and prSS (row 5) were imported into isolated chloroplasts (100 μg chlorophyll). Intact chloroplasts were reisolated and divided into four equal samples. The four samples from each import reaction were protease-treated as indicated above and as outlined in "Materials and Methods." Trypsin-treated samples were quenched either with a mixture of protease inhibitors (Trypsin I) or with 1 mM PMSF (Trypsin II). Intact chloroplasts were reisolated from each sample, lysed, and separated into membrane (P) and soluble (S) protein fractions. Equivalent protein from each fraction was analyzed by SDS-PAGE and either fluorography (A) or immunoblotting (B) with antibodies against Tic110, Toc75, and Toc34.

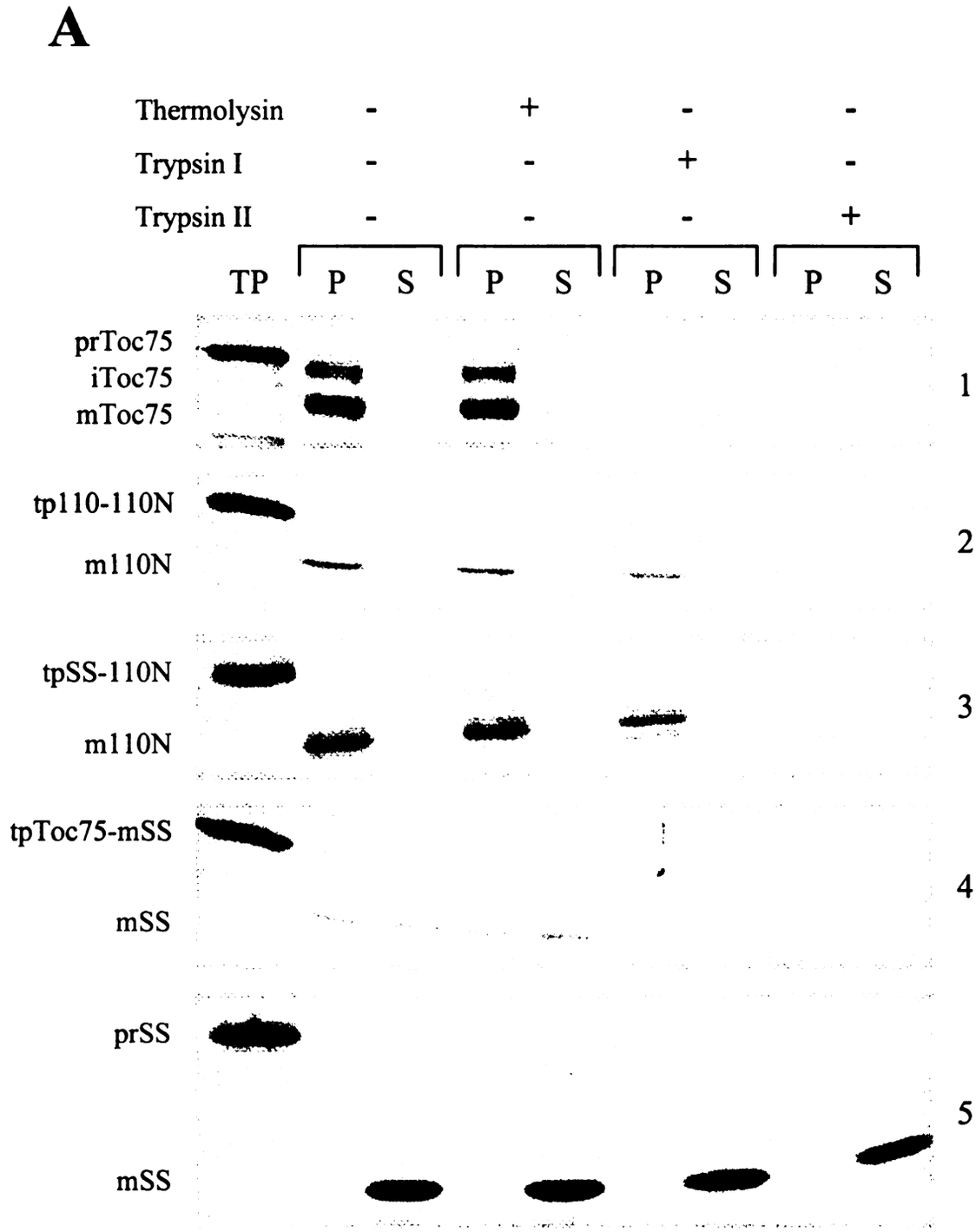

A
Thermolysin - + - -
Trypsin I - - + -
Trypsin II - - - +
TP P S P S P S P S
prToc75
iToc75
mToc75
tp110-110N
m110N
tpSS-110N
m110N
tpToc75-mSS
mSS
prSS
mSS
1
2
3
4
5

Figure 2.3

B

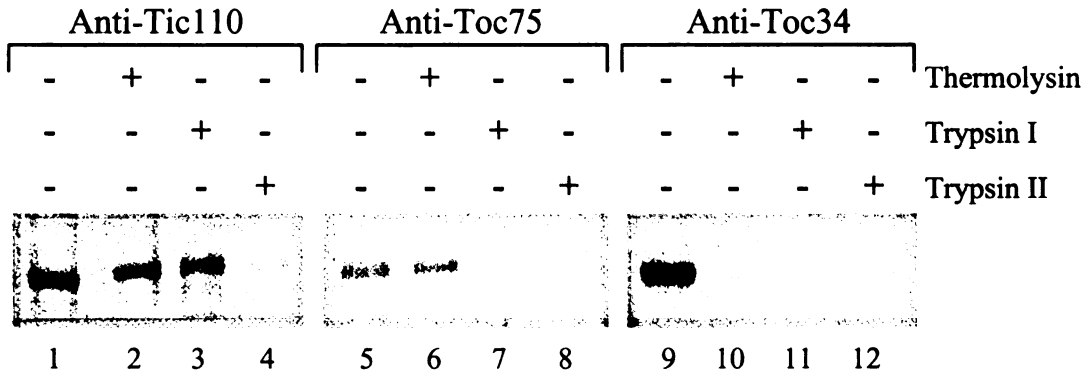


Figure 2.3 (continued)

(as in Figure 2.1). In contrast, because neither thermolysin nor trypsin can penetrate the inner envelope membrane, prSS, a stromal marker, was not digested by either protease (Figure 2.3A, row 5).

It has previously been reported that when tpToc75-mSS is imported into chloroplasts, the processed product is exposed to the chloroplast intermembrane space in both soluble and membrane-bound forms (Tranel and Keegstra, 1996). Thus, we utilized this construct as a marker for the intermembrane space. Accordingly, we found that both the soluble and membrane-bound products generated from tpToc75-mSS were degraded by trypsin but not by thermolysin (Figure 2.3A, row 4). If Tic110 was also exposed to the intermembrane space, we would have expected it to have a protease sensitivity similar to tpToc75-mSS. However, neither of the Tic110 constructs, tp110-110N and tpSS-110N, which were expected to have the same topology as Tic110 itself (Lübeck et al., 1997), were degraded by trypsin as long as protease activity was sufficiently quenched by the protease inhibitor mixture (Figure 2.3A, rows 2 and 3, compare trypsin I and trypsin II protocols). We interpreted these results to indicate that these Tic110 constructs were not exposed to the intermembrane space. Since it has been previously demonstrated that these two constructs are inserted in the inner envelope membrane (Lübeck et al., 1997), we concluded that they must be oriented towards the chloroplast stroma.

We also examined the protease sensitivity of three endogenous proteins in these chloroplasts (Figure 2.3B). Tic110 was not significantly degraded by either thermolysin or trypsin as long as trypsin was adequately quenched (Figure 2.3B, lanes 1-4). This is similar to the results obtained in our previous experiments and those seen for the imported Tic110 constructs. Toc75 was degraded by trypsin but not by thermolysin

(Figure 2.3B, lanes 5-8), consistent with the results obtained for imported Toc75. On the other hand, Toc34 was degraded by both proteases (Figure 2.3B, lanes 9-12). These results are consistent with the fact that the cytosolic domain of Toc34 is exposed on the outer surface of chloroplasts (Kessler et al., 1994; Seedorf et al., 1995).

Tic110 is exposed on the same face of inner envelope membrane vesicles as ClpC, a stroma-facing protein

The results presented above suggest that within intact chloroplasts, Tic110 is oriented toward the stromal compartment. In order to extend and confirm this conclusion, we analyzed the topology of Tic110 in a second system, isolated inner envelope membrane vesicles. Specifically, we compared the trypsin sensitivity of Tic110 to that of ClpC, a stromal Hsp100 homolog. ClpC is primarily a soluble protein; however, it is known that a significant portion of the ClpC molecules in the chloroplast are associated with the stromal side of the inner envelope membrane (Moore and Keegstra, 1993; Nielsen, 1997). Therefore, if Tic110 is indeed exposed on the stromal face of the inner envelope membrane, it should display the same trypsin sensitivity as ClpC. This indeed was what we observed upon analysis of inner membrane vesicles (Figure 2.4). Both Tic110 and ClpC were resistant to degradation at low protease concentrations and susceptible at higher levels of trypsin. In addition, both proteins began to be significantly degraded at the same trypsin concentration (Figure 2.4A, lane 5 and 2.4B, lane 5), indicating that Tic110 and ClpC were exposed on the same side of the vesicles. Consequently, we concluded that Tic110, like ClpC, was oriented toward the chloroplast stroma.

Figure 2.4. Trypsin digestion of inner envelope membrane vesicles. (A,B) Inner envelope vesicles (20 μg protein) were incubated for 10 minutes without (lane 1) or with (lanes 2-7) trypsin. Trypsin concentrations used are indicated. The concentration of trypsin used in the lane marked "Q" (lane 7) was 1000 ng protease/mg inner envelope membrane protein. Trypsin activity was quenched with a mixture of protease inhibitors as described in "Materials and Methods" either after (lanes 2-6) or before (lane 7) incubation with the protease. Half of each sample was analyzed by SDS-PAGE and immunoblotting with antibodies against Tic110 (A) or ClpC (B). The position of an apparent trypsin degradation product of Tic110 is indicated by an asterisk (*).

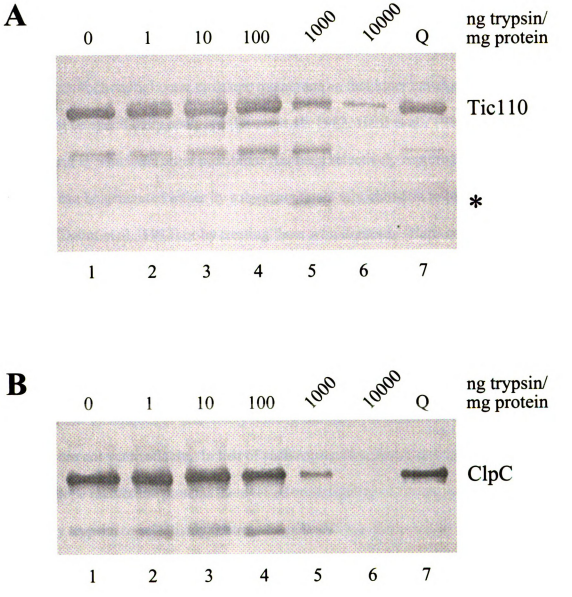

A
0
1
10
100
1000
10000
Q
ng trypsin/
mg protein
Tic110
*
1
2
3
4
5
6
7
B
0
1
10
100
1000
10000
Q
ng trypsin/
mg protein
ClpC
1
2
3
4
5
6
7

Figure 2.4

DISCUSSION

To investigate the process of protein import into chloroplasts in detail, it will be necessary to study the translocation machineries of the outer and inner envelope membranes separately, as has been done for the mitochondrial protein import system (Pfanner et al., 1994). Mitochondria, like chloroplasts, are surrounded by an envelope composed of two separate membranes. Techniques have been developed to physically remove the mitochondrial outer envelope membrane so that inner envelope membrane proteins can be specifically analyzed (Daum et al., 1982; Hartl et al., 1986). Mitoplasts, mitochondria in which the outer membrane has been selectively ruptured and/or dissolved, can be generated either by subjecting intact mitochondria to osmotic shock treatment (Daum et al., 1982) or by treating them with digitonin (Hartl et al., 1986). These two methods have been used successfully to study the location and topology of mitochondrial inner envelope membrane proteins and the mechanism of mitochondrial protein import (for example, see Ohba and Schatz, 1987; Hwang et al., 1991; Glick et al., 1992; Beasley et al., 1993; Rospert et al., 1994; Horst et al., 1995).

Similar techniques to selectively remove the outer membrane of chloroplast envelopes are not yet available. In lieu of such approaches, investigations on chloroplast inner envelope membrane proteins have relied on the ability of certain proteases, specifically trypsin, to selectively destroy the permeability barrier of the outer membrane and degrade inner membrane proteins that are exposed to the intermembrane space while leaving stromally exposed proteins intact (Cline et al., 1981; Cline et al., 1984; Marshall et al., 1990). This technique can thus be used to differentiate between an intermembrane space and a stromal localization for both soluble and membrane proteins (Kessler and

Blobel, 1996; Lübeck et al., 1996; Tranel and Keegstra, 1996; Lübeck et al., 1997), as we have done in this study.

Two independent investigations have provided evidence indicating that Tic110 is a component of the chloroplast protein translocation apparatus localized in the inner envelope membrane (Kessler and Blobel, 1996; Lübeck et al., 1996). However, no function for Tic110 during protein translocation has been clearly established. Elucidating the topology of Tic110, about which the original reports disagreed (Kessler and Blobel, 1996; Lübeck et al., 1996), will be an important first step toward understanding the role of this protein in the import process. In this investigation, we have provided evidence indicating that the large (>90 kD) hydrophilic domain of Tic110 was oriented toward the stromal compartment. Because the one or two predicted transmembrane domains of Tic110 are near the N-terminus (within the first 10% of the mature protein), it is likely that the regions of Tic110 that are important for its function reside within the large hydrophilic domain, which we have localized.

Previous investigations have proposed that Tic110 may be involved in mediating the interaction between outer and inner envelope membrane translocation components during protein import (Lübeck et al., 1996). However, our evidence does not support this view. The stromal orientation of the major portion of Tic110 would probably not allow this protein to interact with outer envelope membrane proteins. Instead, it is more likely that Tic110 interacts with stromal components of the translocation apparatus. For instance, Tic110 may be involved in the recruitment of molecular chaperones, including ClpC, to the site of protein import.

This study has demonstrated that Tic110 is degraded by trypsin only when trypsin-treated chloroplasts are insufficiently quenched. Incomplete quenching of trypsin activity with PMSF is the most likely explanation for previous reports concluding, based on trypsin analysis, that Tic110 is degraded by the protease and thus is oriented toward the intermembrane space (Lübeck et al., 1996). An investigation reported by Kessler and Blobel (1996) on the topology of Tic110 also utilized trypsin digestion data to analyze this problem. These investigators, who found that trypsin does not degrade Tic110, quenched protease activity with a combination of inhibitors. Their results support our claim that when trypsin is adequately quenched, Tic110 remains largely undigested after protease treatment of intact chloroplasts and provide further evidence for the conclusion that the large hydrophilic domain of Tic110 is exposed to the chloroplast stroma rather than the intermembrane space.

In the case where trypsin was insufficiently quenched, it is possible that active trypsin either bound to the chloroplast envelope membranes or was trapped in the intermembrane space and, consequently, was retained during reisolation of intact chloroplasts. Then, during or after chloroplast lysis, trypsin was able to gain access to proteins exposed on the stromal face of the inner envelope membrane and digest them. In this investigation, we analyzed the protease sensitivity of newly imported, radiolabeled SS, a stromally localized protein. This protein did not seem to be significantly degraded by trypsin, regardless of the method used to quench protease activity. To explain these observations, we propose that incompletely quenched trypsin is "trapped" inside envelope vesicles that form upon chloroplast lysis and is thus unable to significantly degrade soluble proteins, including SS. During separation of membrane and soluble proteins, the

protease would be sedimented with the membrane vesicles away from soluble molecules, including any quenching reagents added during or after the lysis stage. When membrane proteins are subsequently solubilized in SDS-PAGE sample buffer, active trypsin can be released from the vesicles and degrade Tic110 and perhaps other membrane proteins as well. It is thus necessary to adequately quench trypsin either before or at the time of chloroplast lysis in order to prevent active protease from being released after chloroplasts have been ruptured (Figure 2.2).

It should be noted that in this investigation we did not completely repeat the results of Lübeck *et al.* (1997). During the trypsin digestion of the newly imported, radiolabeled precursor constructs, we utilized a lower trypsin concentration (500 μg trypsin/mg chlorophyll vs. 1000 μg trypsin/mg chlorophyll) and a different quenching protocol (protease inhibitor mixture or PMSF vs. PMSF and trypsin inhibitor). The protocol we utilized during these import experiments was the same used in all of the other experiments presented in this report. Although we did not completely repeat the protocol of Lübeck *et al.* (1997), we do not believe this significantly affected our results or the conclusions we have drawn from them, since all other observations indicate that Tic110 does indeed face the chloroplast stroma.

During the course of this investigation, we also attempted to specifically label proteins exposed to the intermembrane space with biotinylation reagents that supposedly could not permeate the inner envelope membrane. However, we observed that these reagents were able to enter the chloroplast stroma in small but significant quantities, and we were unable to develop reaction conditions to prevent labeling of stromal proteins. Thus, it appears that at the current time, trypsin digestion analysis is the most reliable

method to analyze the topology of inner envelope membrane proteins. Our experiments have revealed that the protocol used to quench trypsin activity during such studies can have a major effect on the results of experiments, and thus, the conclusions drawn from these results (Figure 2.1). Consequently, it is important that measures be taken to ensure that trypsin is adequately quenched whenever this protease is used as an analytical tool. When our quench mixture is added to intact chloroplasts prior to trypsin addition, Tic110 and Toc75 (an outer envelope membrane protein normally susceptible to trypsin action) are left largely intact (Figure 2.1). Thus, we concluded that the protease inhibitor mixture used in this investigation was sufficient to quench trypsin activity. Such a mixture of inhibitors should be useful in studying the topology of membrane proteins or in any other investigations where analysis by trypsin digestion plays a pivotal role.

REFERENCES

Akita M, Nielsen E, Keegstra K (1997) Identification of protein transport complexes in the chloroplastic envelope membranes via chemical crosslinking. J Cell Biol 136: 983-994

Beasley EM, Müller S, Schatz G (1993) The signal that sorts yeast cytochrome b_2 to the mitochondrial intermembrane space contains three distinct functional regions. EMBO J 12: 2303-2311

Bruce BD, Perry S, Froehlich J, Keegstra K (1994) *In vitro* import of proteins into chloroplasts. Plant Mol Biol Manual J1: 1-15

Chua N-H, Schmidt GW (1978) Post-translational transport into intact chloroplasts of a precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase. Proc Natl Acad Sci USA 75: 6110-6114

Cline K, Andrews J, Mersey B, Newcomb EH, Keegstra K (1981) Separation and characterization of inner and outer envelope membranes of pea chloroplasts. Proc Natl Acad Sci USA 78: 3595-3599

Cline K, Werner-Washburne M, Andrews J, Keegstra K (1984) Thermolysin is a suitable protease for probing the surface of intact pea chloroplasts. Plant Physiol 75: 675-678

Daum G, Böhni PC, Schatz G (1982) Import of proteins into mitochondria: cytochrome b_2 and cytochrome *c* peroxidase are located in the intermembrane space of yeast mitochondria. J Biol Chem 257: 13028-13033

Etemadi AH (1980) Membrane asymmetry. A survey and critical appraisal of the methodology. I. Methods for assessing the asymmetric orientation and distribution of proteins. Biochim Biophys Acta 604: 347-422

Glick BS, Brandt A, Cunningham K, Müller S, Hallberg RL, Schatz G (1992) Cytochromes c_1 and b_2 are sorted to the intermembrane space of yeast mitochondria by a stop-transfer mechanism. Cell 69: 809-822

Hartl F-U, Schmidt B, Wachter E, Weiss H, Neupert W (1986) Transport into mitochondria and intramitochondrial sorting of the Fe/S protein of ubiquinol-cytochrome c reductase. Cell 47: 939-951

Highfield PE, Ellis RJ (1978) Synthesis and transport of the small subunit of chloroplast ribulose bisphosphate carboxylase. Nature 271: 420-424

Hirsch S, Muckel E, Heemeyer F, von Heijne G, Soll J (1994) A receptor component of the chloroplast protein translocation machinery. Science 266: 1989-1992

Horst M, Hilfiker-Rothenfluh S, Oppliger W, Schatz G (1995) Dynamic interaction of the protein translocation systems in the inner and outer membranes of yeast mitochondria. EMBO J 14: 2293-2297

Hwang ST, Wachter C, Schatz G (1991) Protein import into the yeast mitochondrial matrix: a new translocation intermediate between the two mitochondrial membranes. J Biol Chem 266: 21083-21089

Keegstra K, Yousif AE (1986) Isolation and characterization of chloroplast envelope membranes. Methods Enzymol 118: 316-325

Kessler F, Blobel G (1996) Interaction of the protein import and folding machineries in the chloroplast. Proc Natl Acad Sci USA 93: 7684-7689

Kessler F, Blobel G, Patel HA, Schnell DJ (1994) Identification of two GTP-binding proteins in the chloroplast protein import machinery. Science 266: 1035-1039

Kouranov A, Schnell DJ (1996) Protein translocation at the envelope and thylakoid membranes of chloroplasts. J Biol Chem 271: 31009-31012

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193: 265-275

Lübeck J, Heins L, 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

Lübeck J, Soll J, Akita M, Nielsen E, Keegstra K (1996) Topology of IEP110, a component of the chloroplastic protein import machinery present in the inner envelope membrane. EMBO J 15: 4230-4238

Marshall JS, DeRocher AE, Keegstra K, Vierling E (1990) Identification of heat shock protein hsp70 homologues in chloroplasts. Proc Natl Acad Sci USA 87: 374-378

Moore T, Keegstra K (1993) Characterization of a cDNA clone encoding a chloroplast-targeted Clp homologue. Plant Mol Biol 21: 525-537

Nielsen EE (1997) The role of stromal molecular chaperones in chloroplastic protein translocation. Ph.D. Dissertation, Michigan State University, East Lansing, MI

Nielsen E, Akita M, Davila-Aponte J, Keegstra K (1997) Stable association of chloroplastic precursors with protein translocation complexes that contain proteins from both envelope membranes and a stromal Hsp100 molecular chaperone. EMBO J 16: 935-946

Ohba M, Schatz G (1987) Disruption of the outer membrane restores protein import to trypsin-treated yeast mitochondria. EMBO J 6: 2117-2122

Olsen LJ, Keegstra K (1992) The binding of precursor proteins to chloroplasts requires nucleoside triphosphates in the intermembrane space. J Biol Chem 267: 433-439

Perry SE, Keegstra K (1994) Envelope membrane proteins that interact with chloroplastic precursor proteins. Plant Cell 6: 93-105

Pfanner N, Craig EA, Meijer M (1994) The protein import machinery of the mitochondrial inner membrane. Trends Biochem Sci 19: 368-372

Rospert S, Müller S, Schatz G, Glick BS (1994) Fusion proteins containing the cytochrome b_2 presequence are sorted to the mitochondrial intermembrane space independently of hsp60. J Biol Chem 269: 17279-17288

Schmidt GW, Devillers-Thiery A, Desruisseaux H, Blobel G, Chua N-H (1979) NH_2-terminal amino acid sequences of precursor and mature forms of the ribulose-1,5-bisphosphate carboxylase small subunit from *Chlamydomonas reinhardtii.* J Cell Biol 83: 615-622

Schnell DJ, Kessler F, Blobel G (1994) Isolation of components of the chloroplast protein import machinery. Science 266: 1007-1012

Seedorf M, Waegemann K, Soll J (1995) A constituent of the chloroplast import complex represents a new type of GTP-binding protein. Plant J 7: 401-411

Shanklin J, DeWitt ND, Flanagan JM (1995) The stroma of higher plant plastids contain ClpP and ClpC, functional homologs of *Escherichia coli* ClpP and ClpA: an archetypal two-component ATP-dependent protease. Plant Cell 7: 1713-1722

Tranel PJ, Froehlich J, Goyal A, Keegstra K (1995) A component of the chloroplastic protein import apparatus is targeted to the outer envelope membrane via a novel pathway. EMBO J 14: 2436-2446

Tranel PJ, Keegstra K (1996) A novel, bipartite transit peptide targets OEP75 to the outer membrane of the chloroplastic envelope. Plant Cell 8: 2093-2104

Waegemann K, Soll J (1991) Characterization of the protein import apparatus in isolated outer envelopes of chloroplasts. Plant J 1: 149-158

Wu C, Seibert FS, Ko K (1994) Identification of chloroplast envelope proteins in close physical proximity to a partially translocated chimeric precursor protein. J Biol Chem 269: 32264-32271

CHAPTER 3

Arabidopsis Genes Encoding Components of the Chloroplastic Protein Import Apparatus

The work presented in this chapter has been published:

Jackson-Constan D, Keegstra K (2001) Plant Physiol 125: 1567-1576

ABSTRACT

The process of protein import into plastids has been studied extensively using isolated pea (*Pisum sativum*) chloroplasts. As a consequence, virtually all of the known components of the proteinaceous apparatus that mediates import were originally cloned from pea. With the recent completion of the *Arabidopsis thaliana* genome sequencing project, it is now possible to identify putative homologs of the import components in this species. Our analysis has revealed that *Arabidopsis* homologs with high sequence similarity exist for all of the pea import complex subunits, making *Arabidopsis* a valid model for further study of this system. Multiple homologs can be identified for over one-half of the components. In all but one case, it is known that more than one of the putative isoforms for a particular subunit are expressed. Thus, it is possible that multiple types of import complexes are present within the same cell, each having a unique affinity for different chloroplastic precursor proteins, depending upon the exact mix of isoforms it contains. Sequence analysis of the putative *Arabidopsis* homologs for the chloroplast protein import apparatus has revealed many questions concerning subunit function and evolution. It should now be possible to use the genetic tools available in *Arabidopsis*, including the generation of knockout mutants and antisense technology, to address these questions and learn more about the molecular functions of each of the components during the import process.

INTRODUCTION

The availability of the sequence for the entire genome of *Arabidopsis thaliana* allows a detailed analysis of all the genes involved in a particular biological process, regardless of the plant species in which the system was first identified. One such process is the import of cytoplasmically synthesized precursor proteins into chloroplasts. Most of the current information regarding this process, including the identification of components of the import apparatus that mediates it, has come from biochemical studies in pea (*Pisum sativum*; see Figure 1.2; Chen and Schnell, 1999; Keegstra and Cline, 1999; Keegstra and Froehlich, 1999; May and Soll, 1999; Schleiff and Soll, 2000). From these studies, it has been determined that nuclear-encoded, chloroplast-localized enzymes are synthesized in the cytoplasm as precursors containing an N-terminal transit peptide not seen in the mature protein within the chloroplast (for review, see Bruce, 2000). A precursor protein initially interacts with a complex located within the outer membrane of the chloroplast envelope that consists of at least three subunits: Toc159 (translocon at the outer envelope membrane of chloroplasts, 159 kD), Toc75, and Toc34 (Waegemann and Soll, 1991; Hirsch et al., 1994; Kessler et al., 1994; Perry and Keegstra, 1994; Schnell et al., 1994; Seedorf et al., 1995; Tranel et al., 1995). These early events involve the hydrolysis of GTP, presumably by Toc159 and Toc34, which are known to be GTP-binding proteins (Kessler et al., 1994; Seedorf et al., 1995). A recent report by Sohrt and Soll (2000) has also implicated a fourth component, Toc64, as being a member of the outer membrane import machinery. Hydrolysis of low concentrations of ATP in the cytoplasm or intermembrane space results in the irreversible association of precursor proteins with the translocation machinery of both the outer and inner envelope

membranes (Olsen et al., 1989; Olsen and Keegstra, 1992). The import complex of the chloroplastic inner envelope membrane also consists of at least three subunits: Tic110 (translocon at the inner envelope membrane of chloroplasts, 110 kD), Tic20, and Tic22 (Kessler and Blobel, 1996; Lübeck et al., 1996; Kouranov and Schnell, 1997; Kouranov et al., 1998). Two additional components, Tic55 and Tic40, have also been reported to be a part of this translocon, but their inclusion is more controversial (Wu et al., 1994; Ko et al., 1995; Caliebe et al., 1997; Stahl et al., 1999). Complete translocation of precursor proteins into the chloroplast interior is accomplished via the hydrolysis of ATP within the stroma (Theg et al., 1989). This ATP hydrolysis is presumably mediated by stromal molecular chaperones, at least one of which, heat shock protein 93 (Hsp93; a member of the Hsp100 family of molecular chaperones), has been found to interact with the import complex (Akita et al., 1997; Nielsen et al., 1997). As the precursor enters the chloroplast, the transit peptide is cleaved off by the stromal processing peptidase (SPP), and the mature protein begins the process of folding and assembly (Oblong and Lamppa, 1992; VanderVere et al., 1995; Richter and Lamppa, 1998).

Although virtually all of the conclusions described above were derived from work done with pea chloroplasts, expressed sequence tags (ESTs) for homologs of the various import components can be identified in the databases for a variety of monocots and dicots, including maize, tomato, and *Arabidopsis*. More importantly, the recent completion of the *Arabidopsis* genome sequencing project (The Arabidopsis Genome Initiative, 2000) has made it possible to find, in this species, homologs of those components for which no ESTs exist. In addition to establishing the general significance of the components of the import apparatus, identification of *Arabidopsis* homologs for

the subunits of the pea import complex will allow the use of this species to perform molecular work that is not practical and/or possible with pea, including isolation of "knockout" mutants and generation of transgenic plants expressing sense or antisense copies of the genes encoding one or more of these components.

In this paper, we analyze the *Arabidopsis* genomic, cDNA, and EST information currently available in GenBank concerning each of the known and putative subunits of the chloroplast protein import machinery. All of these components have homologs of high sequence identity within the *Arabidopsis* genome that are expressed and likely act as functional counterparts to the pea proteins. For several of these translocation components, multiple putative homologs are present in the *Arabidopsis* genome. However, in most cases, it is unclear whether all copies are expressed, or if they are, whether they are all acting as functional homologs within *Arabidopsis* chloroplasts. The information revealed by this analysis will allow important new questions to be raised, and further experimental work can then be designed to answer them in the near future.

MATERIALS AND METHODS

All sequence comparisons were done using the BLASTN, BLASTP, and TBLASTN programs (versions 2.0) available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST; Altschul et al., 1990; Altschul et al., 1997). The weight matrix used was the blosum62 matrix, and no settings were changed from the default. The database searched was the Arabidopsis thaliana Database Project, found at The Arabidopsis Information Resource (http://www.arabidopsis.org/blast), which contains genomic and EST sequences. This database was checked for the final time between October 30, 2000 and November 5, 2000, just before manuscript submission. During manuscript revision, a recheck of the database between January 11, 2001 and January 18, 2001 found no additional homologs.

A sequence was considered a homolog only if the following conditions were met, unless otherwise noted: (1) using the pea (*Pisum sativum*) sequence as the query, one of the BLAST programs used detected this sequence with an expect value of less than or equal to 0.0001; (2) using the putative *Arabidopsis* homolog as the query, one of the BLAST programs used detected the pea sequence and other *Arabidopsis* isoforms with an expect value of less than or equal to 0.0001; (3) the region of similarity between the pea protein and the putative *Arabidopsis* homolog extended for approximately 50% or more of the sequence lengths; (4) the region of similarity to the pea protein extended beyond common motifs (i.e. nucleotide-binding domains) and (5) the putative *Arabidopsis* homolog was not already annotated as being more similar to another protein of known function. Levels of identity between different amino acid sequences were calculated with the MegAlign program (Lipman-Pearson algorithm; ktuple = 2, gap penalty = 4, gap

length penalty = 12) of the Lasergene software package (DNASTAR, Inc., Madison, Wisconsin, USA). Predictions concerning chloroplast targeting were made using the TargetP program (version 1.01), available at http://www.cbs.dtu.dk/services/TargetP (Emanuelsson et al., 2000).

RESULTS AND DISCUSSION

Outer envelope membrane proteins

Toc159, a GTP-binding protein, is postulated to be the first subunit of the import complex with which an incoming precursor protein interacts, serving as the receptor for transit peptides (Waegemann and Soll, 1991; Hirsch et al., 1994; Kessler et al., 1994; Perry and Keegstra, 1994; Ma et al., 1996). There are three homologs of this protein in *Arabidopsis* (Table 3-I), designated AtToc159, AtToc132, and AtToc120 based on their predicted molecular masses (Bauer et al., 2000). All three are expressed, as demonstrated by the presence of at least one *Arabidopsis* EST for each and by RT-PCR experiments (Bauer et al., 2000).

The pea Toc159 protein is composed of three domains: an N-terminal acidic region, a central domain encompassing the GTP-binding motifs, and a C-terminal domain containing the membrane-spanning regions (Chen et al., 2000a). AtToc159 shares ~48% identity with the pea protein, most of which is concentrated in the central and C-terminal domains (~69% identity in these regions). Both pea Toc159 and AtToc159 are highly acidic, especially in their N-terminal regions (Bölter et al., 1998a; Bauer et al., 2000; Chen et al., 2000a). Approximately 30% and 27%, respectively, of the amino acids in this domain are either aspartate or glutamate (Table 3-II). This is in contrast to the other members of the outer membrane import complex (Toc75, Toc34, Toc64) in which the percentage of acidic residues ranges from 9% to 11% for the *Arabidopsis* isoforms. One of the defining features of transit peptides is that they lack acidic amino acids, resulting in an overall basic pI and net positive charge (Keegstra et al., 1989). Thus, it is interesting to speculate that the N-terminal acidic domain of Toc159, which is localized on the

Table 3-I. Putative *Arabidopsis* homologs for the components of the pea chloroplast protein import machinery

Pea Import Component	*Arabidopsis* Homologs[a]	Chromosome[b]	GenBank Designation[c]	EST in Database	Number of Introns[d]
Toc159	AtToc159	IV	T14P8.24	Yes	1
	AtToc132	II	At2g16640	Yes	0
	AtToc120	III	MGL6.8	Yes	0
Toc75	AtToc75-III	III	T6H20.230	Yes	6
	AtToc75-I	I	F10O5.4	No	ND[e]
	AtToc75-IV	IV	At4g09080	No	5
Toc34	AtToc34	V	MUG13.14	Yes	6
	AtToc33	I	T7I23.11	Yes	6
Toc64	AtToc64-III	III	MEB5.17	Yes	12
	AtToc64-V	V	T5E8_220	Yes	12
	AtToc64-I	I	F7G19.15	Yes	ND[e]
Tic110	AtTic110	I	F10K1.33 and F4H5.1[f]	Yes	14
Tic20	AtTic20-I	I	F13M7.7	Yes	ND[e]
	AtTic20-IV	IV	F4C21.25	Yes	2
Tic22	AtTic22-IV	IV	F17M5.110	Yes	7
	AtTic22-III	III	MYM9.5	Yes	7
Tic55	AtTic55	II	At2g24820	Yes	2
Tic40	AtTic40	V	MTG13.6	Yes	13
Hsp93[g, h]	AtHsp93-V	V	K3K7.7	Yes	8
	AtHsp93-III	III	T21J18_140	Yes	8
SPP (CPE)	AtCPE	V	MDH9.8	Yes	23
Hsp70[g]	AtHsp70-V	V	K9P8.5	Yes	7
	AtHsp70-IVa	IV	At4g24280	Yes	7
	AtHsp70-IVb	IV	At4g37910	Yes	5

[a]See "Materials and Methods" for an explanation of the criteria used in designating a sequence as a homolog of the pea import apparatus. [b]The chromosomal location for the genes encoding each of the putative *Arabidopsis* chloroplast protein import components is indicated. [c]The entries in this column refer to the bacterial artificial chromosome (BAC) or P1 clone designation in GenBank for each of the putative homologs. [d]Gene structure predictions are based on the annotations given in the GenBank entry indicated in this table, unless otherwise noted. [e]ND, not determined. The current annotations given in the database for these homologs are predicted to be incorrect because the regions of sequence identity to the pea proteins are very different from the predicted boundaries of the open reading frame. [f]This coding region is split between two BACs, which overlap by 200 bp. [g]*Arabidopsis* homologs given for these components are only those predicted to have a chloroplastic targeting sequence (see "Materials and Methods" for an explanation of how this was determined). [h]*Arabidopsis* homologs given for this protein are only those predicted to belong to the ClpC class of the Hsp100 family of chaperones.

Table 3-II. Comparison of the acidic properties of various members of the outer envelope membrane import complex

Import Component	% acidic residues in whole protein[a]	pI of whole protein	% acidic residues in N-terminal domain[a]	pI of N-terminal domain
Pea Toc159	20	4.2	30	3.6
AtToc159	19	4.3	27	3.8
AtToc132	17	4.7	28	3.8
AtToc120	16	4.9	26	3.9
AtToc75-III	10	8.8	N/A	N/A
AtToc34	10	9.4	N/A	N/A
AtToc64-III	10	8.2	N/A	N/A

N/A, not applicable.

[a]Acidic residues are aspartate (D) and glutamate (E).

cytoplasmic face of the chloroplast, is involved in an electrostatic interaction with positively charged transit peptides, increasing the overall efficiency of precursor protein binding (Bölter et al., 1998a). This is similar to the situation described by the acid chain hypothesis for the early interaction of basic mitochondrial targeting sequences with their acidic receptors (Komiya et al., 1998).

AtToc132 and AtToc120 show less overall identity with pea Toc159 (~37% and ~39%, respectively), the majority of which is again concentrated in the central and C-terminal domains (~50% for each). In addition, their levels of identity to AtToc159 are also relatively low (~37% and ~38%, respectively). On the other hand, the two proteins share ~70% amino acid identity with each other. This suggests that AtToc132 and AtToc120 share a common ancestor that diverged from AtToc159 before these two proteins diverged from one another.

AtToc132 and AtToc120 are also highly acidic in their N-terminal regions (approximately 28% and 26% acidic residues, respectively). In fact, this is the main feature shared between the *Arabidopsis* homologs at their N-termini. There is very little conservation of primary structure between the three proteins before the GTP-binding domain (Bauer et al., 2000). However, despite a maintenance of the overall percentage of acidic residues within the N-terminal domains, the pI of the N-termini and the whole proteins differs between the three isoforms (Table 3-II). Thus, the question arises of whether these subtle changes in size and overall charge between the *Arabidopsis* Toc159 homologs reflect differences in the types of precursors with which these proteins interact (Bauer et al., 2000). It is interesting to note that mutant *Arabidopsis* plants that lack AtToc159 are still able to import some, but not all, chloroplastic proteins, suggesting that

some other factor, perhaps AtToc132 and/or AtToc120, is substituting for AtToc159 in the import of some precursors (Bauer et al., 2000).

Toc75 has been shown to form a voltage-gated, peptide-sensitive channel in artificial lipid bilayers (Hinnah et al., 1997). Thus, it is hypothesized that this protein forms the channel through which precursor proteins cross the outer envelope membrane (Perry and Keegstra, 1994; Schnell et al., 1994; Tranel et al., 1995; Hinnah et al., 1997). Analysis of the *Arabidopsis* genome sequence reveals at least three coding regions that have strong similarity to pea *TOC75*: *AtTOC75-III*, *AtTOC75-I*, and *AtTOC75-IV*, named according to their chromosomal location. Only one of these genes, *AtTOC75-III*, is represented by an EST. More than 10 ESTs for this gene can be found, but none currently exist for the other two homologs. In addition, of the three, AtToc75-III shows the highest levels of identity with the pea protein (~74%). Consequently, it is likely that AtToc75-III is the major Toc75 isoform in *Arabidopsis* cells.

AtToc75-III and AtToc75-I are quite similar to one another in size and amino acid sequence, sharing >60% identity throughout their lengths. On the other hand, AtToc75-IV displays some striking differences from its two homologs. First of all, the protein encoded by *AtTOC75-IV* is much smaller at 407 amino acids in length versus 818 amino acids for the protein encoded by *AtTOC75-III*. Furthermore, the region of similarity between AtToc75-IV and the other two *Arabidopsis* homologs is confined to the C-termini of the larger proteins. It appears that *AtTOC75-IV* may represent just the last six exons of *AtTOC75-III*. In fact, this gene seems to be an extreme case of a more common phenomenon. For a few components, including Toc75 and Toc159, BLAST searches reveal several small regions with high levels of sequence similarity to these subunits

throughout the genome. Although these putative open reading frames do show similarity to the import components outside of commonly found motifs (i.e. nucleotide-binding domains), the regions of similarity are not extensive. In general, they constitute less than one-quarter of the total length of the queried import component, not enough to really be considered a possible functional homolog. One possible explanation for the occurrence of these presumably unexpressed regions of similarity is that these short open reading frames are an example of the evolutionary process of exon shuffling in progress.

In the case of AtToc75-IV, the region of similarity extends for ~50% of the length of the larger Toc75 homologs. It is possible that this may be enough for the protein made by *AtTOC75-IV* to be functional. Future research should address this problem, but some observations suggest that it may indeed be needed in *Arabidopsis* cells. First of all, it is interesting to note that the levels of identity between this coding region and its "parent" are quite high, both at the amino acid level (~65% with AtToc75-III) and the nucleotide level. Moreover, the predicted splicing pattern of *AtTOC75-IV* is identical to that seen in the 3' region of *AtTOC75-III*, implying that selection pressure on *AtTOC75-IV* may still be relatively high.

Toc34, another GTP-binding protein of the translocation apparatus, is hypothesized to have a regulatory function during precursor import (Kessler et al., 1994; Seedorf et al., 1995; Kouranov and Schnell, 1997). This subunit has two homologs in *Arabidopsis*, named AtToc34 and AtToc33 based on their predicted molecular masses (Jarvis et al., 1998). ESTs are present for both of these homologs within the *Arabidopsis* database, and their expression has been verified via Northern and Western blot analysis (Jarvis et al., 1998; Gutensohn et al., 2000). It appears that the two proteins, which are

>60% identical to each other and to the pea protein, can at least partially substitute for one another within plant cells. *Arabidopsis* mutants that lack AtToc33 display a delayed greening phenotype and reduced levels of chloroplast protein import early in their development, but are otherwise normal (Jarvis et al., 1998; Gutensohn et al., 2000).

The genes for AtToc34 and AtToc33 provide an example of the evolutionary process of gene duplication. Each coding region consists of six introns and seven exons; five of the seven exons are exactly the same size between the two genes. In addition, in every case, the exon-intron junctions occur at homologous positions within the sequences. Thus, it appears that these two coding regions have diverged from one another only relatively recently after the duplication of a common ancestral gene.

A fourth putative subunit of the outer envelope membrane import apparatus, Toc64, was recently isolated (Sohrt and Soll, 2000). The amino acid sequence for this component contains an amidase domain, but the protein itself has no measurable amidase activity (Sohrt and Soll, 2000). In addition, Toc64 contains three tetratricopeptide repeats (TPR), which are hypothesized to be involved in protein-protein interactions with cytosolic factors complexed with a precursor protein and/or with the precursor itself, perhaps serving as a docking site for the incoming protein (Sohrt and Soll, 2000). Within the *Arabidopsis* genome, there are three coding regions that display extensive similarity with the pea protein outside of the amidase domain and/or the TPR motifs. These homologs have been designated AtToc64-III, AtToc64-V, and AtToc64-I. For all three isoforms, cognate ESTs have been isolated. However, only AtToc64-III and AtToc64-V contain regions similar to both the amidase domain and the TPR motifs of pea Toc64 (~67% and ~50% identical, respectively). Thus, although it is likely that the proteins

encoded by *AtTOC64-III* and *AtTOC64-V* could serve as functional homologs of pea Toc64 within *Arabidopsis* cells, further experiments will need to be done to determine whether AtToc64-I, which lacks the TPR motifs, is playing a similar role.

Inner envelope membrane proteins

The first component of the inner membrane import complex to be cloned and characterized was Tic110 (Kessler and Blobel, 1996; Lübeck et al., 1996). This subunit consists of a large globular domain localized in the chloroplast stroma and anchored to the envelope by a membrane-spanning α-helix at the N-terminus (Kessler and Blobel, 1996; Jackson et al., 1998). Based on this topology, it has been proposed that Tic110 acts as an anchor for stromal molecular chaperones involved in precursor protein import (Kessler and Blobel, 1996; Jackson et al., 1998). Preliminary evidence suggests that Tic110 may indeed physically interact with at least one molecular chaperone (M. Akita and K. Keegstra, unpublished observations). BLAST searches on the *Arabidopsis* genome sequence reveal only one coding region, *AtTIC110*, similar to the pea gene (Table 3-I). The protein encoded by *AtTIC110* is expressed and displays high levels of identity (~68%) to pea Tic110. In addition, it appears to have the same overall structure as the pea protein, with a predicted transmembrane helix at the N-terminus followed by a large hydrophilic domain. Thus, it is reasonable to conclude that AtTic110 acts as a functional homolog of pea Tic110 within *Arabidopsis* cells.

The gene structure for *AtTIC110* is quite complicated; the coding region consists of 15 exons and 14 introns. Overall, the coding region is 5261 bp in length, with 42% of this length comprising the introns. This complexity is in contrast to the genes encoding

the *Arabidopsis* Toc159 isoforms. The coding regions for these proteins are also quite long, ranging from 3270 bp (*AtTOC120*) to 4595 bp (*AtTOC159*) in length. However, they contain only one small intron (83 bp; *AtTOC159*) or none at all (*AtTOC132* and *AtTOC120*). This diversity in gene structure is seen for the other components of the import complex as well. The genes encoding the *Arabidopsis* homologs of Tic20 and Tic55 are relatively simple (two or fewer introns), whereas the genes for the remaining subunits are more complicated, containing between six and 23 introns (Table 3-I).

Tic20, an integral protein of the inner envelope membrane, is believed to form at least a portion of the channel through which chloroplast precursors traverse the inner membrane (Kouranov and Schnell, 1997; Kouranov et al., 1998). The *Arabidopsis* genome contains two genes encoding proteins, AtTic20-I and AtTic20-IV (designated according to the chromosomal locations of the genes), that are similar to pea Tic20. Both of these genes have corresponding ESTs within the *Arabidopsis* database. AtTic20-I is highly similar to the pea protein, sharing >60% identity with pea Tic20. As a consequence, it is likely to act as the functional counterpart to the pea protein in *Arabidopsis* chloroplasts. On the other hand, AtTic20-IV is only ~33% identical to pea Tic20 and ~40% identical to AtTic20-I. Although these levels of identity are relatively high, it is quite low for this system; most of the putative *Arabidopsis* homologs for the other import components show much higher levels of identity to their pea counterparts and related *Arabidopsis* isoforms. Thus, it appears that these two Tic20 isoforms may have diverged from one another earlier in evolution than is the case for isoforms of some of the other subunits of the import complex.

BLAST searches for *Arabidopsis* homologs of pea Tic20 reveal a third putative isoform on chromosome II. However, this protein is much smaller (by ~70 amino acids) than the other two *Arabidopsis* homologs. More importantly, BLAST searches using this putative isoform as the query sequence fail to detect either AtTic20-I or AtTic20-IV. Thus, it was concluded that this coding region, despite sharing ~26% identity with pea Tic20 at the amino acid level, should not be considered an *Arabidopsis* homolog of the pea protein.

Tic22 is localized in the intermembrane space of the chloroplast envelope and appears to be peripherally associated with the inner envelope membrane (Kouranov and Schnell, 1997; Kouranov et al., 1998). Due to its localization, it has been proposed that Tic22 may be involved in the formation of contact sites between the import complexes of the outer and inner membranes (Kouranov and Schnell, 1997; Kouranov et al., 1998). Within the *Arabidopsis* genome, there are at least two coding regions, *AtTIC22-IV* and *AtTIC22-III*, of high similarity to pea *TIC22*. These genes are expressed, as determined by the presence of several ESTs for each in the database. The encoded proteins share ~62% and ~41% identity, respectively, with pea Tic22.

Tic55, an iron-sulfur protein believed to play a regulatory role during chloroplast protein import (Caliebe et al., 1997), and Tic40, which is proposed to recruit chaperones to the site of precursor protein import (Wu et al., 1994; Ko et al., 1995; Stahl et al., 1999), each have one clear homolog of high similarity in *Arabidopsis*. ESTs exist for both AtTic55 and AtTic40. The proteins display ~78% identity and ~52% identity, respectively, with their pea counterparts. Thus, it is likely that they serve as functional homologs to the corresponding pea proteins.

Soluble factors

It is thought that molecular chaperones within the chloroplast stroma provide the driving force, through the hydrolysis of ATP, for the translocation of precursor proteins into the chloroplast interior (Chen and Schnell, 1999; Keegstra and Cline, 1999; Keegstra and Froehlich, 1999). At the present time, the best candidate for this role is Hsp93, a member of the Hsp100 family of chaperones that is consistently found in import complexes isolated from pea chloroplasts (Akita et al., 1997; Nielsen et al., 1997; Kouranov et al., 1998). This chaperone has at least two homologs (Table 3-I) predicted to be present in *Arabidopsis* chloroplasts, AtHsp93-V (~88% identity to pea Hsp93) and AtHsp93-III (~83% identity to the pea protein; Nakabayashi et al., 1999). These two proteins, along with pea Hsp93, belong to the ClpC class of Hsp100 chaperones. Hsp100 proteins of other classes, specifically the ClpB and ClpD classes, that are predicted to be chloroplast-localized can also be detected in the *Arabidopsis* genome, as can potentially chloroplastic members of the Hsp70 and Hsp60 chaperone families. This diversity of stromally localized chaperones raises the question of whether Hsp93 is the only chaperone that interacts with the protein import complex or whether other types of chaperones could substitute for it in different species. Further work will be needed to confirm that the AtHsp93 homologs directly interact with the import complex in *Arabidopsis* chloroplasts as Hsp93 does in pea chloroplasts.

Although no stromal Hsp70 proteins have been found to interact with import complexes (Akita et al., 1997; Nielsen et al., 1997), there is evidence to suggest that Hsp70 molecules do bind to precursor proteins before and/or during envelope translocation (Schnell et al., 1994; Wu et al., 1994; Kourtz and Ko, 1997; Ivey et al.,

2000; May and Soll, 2000). Furthermore, an outer membrane-associated Hsp70 protein, which faces the intermembrane space of the chloroplast envelope, is believed to interact with precursor proteins as they move between the outer and inner membrane translocons (Marshall et al., 1990; Schnell et al., 1994). Within the *Arabidopsis* genome, there are several coding regions that encode proteins similar to known Hsp70 molecules from other species. These *Arabidopsis* Hsp70 proteins can be classified into one of four groups: (1) proteins of approximately 650 residues that likely represent cytosolic Hsp70 molecules, (2) proteins that are 668 or 669 residues long and contain an obvious signal peptide at their N-termini, (3) molecules with clear chloroplastic (two proteins) or mitochondrial (one protein) targeting motifs, and (4) proteins that do not fit into any of the previous three groups. Of the proteins within the last group, only one shows some characteristics of a chloroplast transit peptide at its N-terminus. Sequence alignment between this protein (AtHsp70-IVb) and the two obvious chloroplast-targeted Hsp70 molecules (AtHsp70-V and AtHsp70-IVa) is shown in Figure 3.1.

The only known intermembrane space protein that has been cloned is Tic22 (Kouranov et al., 1998). An analysis of the transit peptide for pea Tic22 reveals that it has a relatively high incidence of acidic amino acids: three within the 50 residues of its length (Kouranov et al., 1998). AtTic22 has five acidic residues within the same region. The paradigm for chloroplast transit peptides is that they are deficient in acidic amino acids, having no more than two over their length (Keegstra et al., 1989). Thus, the transit peptides for both pea and *Arabidopsis* Tic22 are somewhat unusual, and this fact may account for why these proteins are targeted to the intermembrane space of the chloroplast envelope rather than the stroma, although this has not been experimentally verified. We

Figure 3.1. Multiple sequence alignment for the putative chloroplast-localized *Arabidopsis* Hsp70 isoforms. Shaded residues designate sequence identities between two or more of the proteins. The predicted transit peptide is indicated (>). The predicted cleavage site is based on sequence identity to a pea chloroplastic Hsp70 (accession number L03299) and has not been experimentally verified. The alignment was created using the PileUp program from the Wisconsin package of sequence analysis tools (Genetics Computer Group, Madison, Wisconsin, USA).

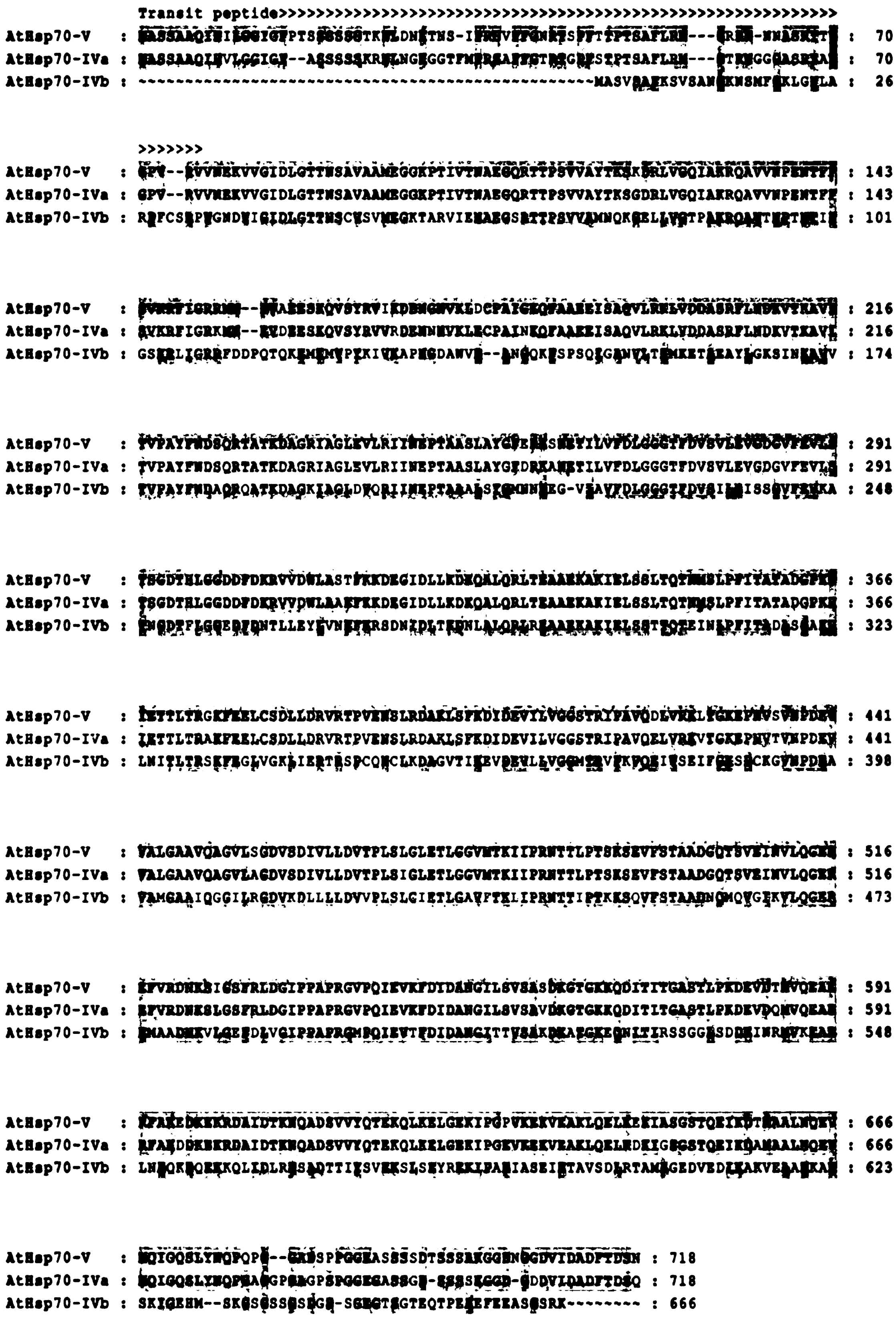

Transit peptide
AtHsp70-V : 70
AtHsp70-IVa : 70
AtHsp70-IVb : 26
AtHsp70-V : 143
AtHsp70-IVa : 143
AtHsp70-IVb : 101
AtHsp70-V : 216
AtHsp70-IVa : 216
AtHsp70-IVb : 174
AtHsp70-V : 291
AtHsp70-IVa : 291
AtHsp70-IVb : 248
AtHsp70-V : 366
AtHsp70-IVa : 366
AtHsp70-IVb : 323
AtHsp70-V : 441
AtHsp70-IVa : 441
AtHsp70-IVb : 398
AtHsp70-V : 516
AtHsp70-IVa : 516
AtHsp70-IVb : 473
AtHsp70-V : 591
AtHsp70-IVa : 591
AtHsp70-IVb : 548
AtHsp70-V : 666
AtHsp70-IVa : 666
AtHsp70-IVb : 623
AtHsp70-V : 718
AtHsp70-IVa : 718
AtHsp70-IVb : 666

Figure 3.1

analyzed the transit peptides of the possible chloroplastic Hsp70 proteins to see if we could detect, based on what is observed from the transit peptide of Tic22, which one (or ones) might be targeted to the intermembrane space. However, all three of these proteins display a low incidence of acidic amino acids within their presumed transit peptides (Figure 3.1). Thus, either the presence of acidic amino acids within the transit peptide is not the determining factor for intermembrane space targeting or *Arabidopsis* may not contain an intermembrane space-localized Hsp70 protein as has been suggested for pea (Marshall et al., 1990; Schnell et al., 1994). Further experimental work will be needed to differentiate between these possibilities.

The SPP (also known as the chloroplast processing enzyme [CPE]) is a metalloendopeptidase that cleaves transit peptides off precursor proteins as they enter the chloroplast stroma (Oblong and Lamppa, 1992; VanderVere et al., 1995; Richter and Lamppa, 1998). This component has one homolog in *Arabidopsis*, named AtCPE, which shares ~75% identity with the pea protein (Richter and Lamppa, 1998). Currently, the SPP is the only constituent of the import machinery whose molecular function has been studied in enough detail to be unequivocally assigned (Richter and Lamppa, 1998; Richter and Lamppa, 1999).

CONCLUSIONS

Analysis of the *Arabidopsis* sequence database has revealed that homologs of high sequence similarity can be found for each of the chloroplast protein import components that were originally identified in pea. This suggests that the protein import system is conserved between pea and *Arabidopsis*, making *Arabidopsis* a valid model for its study. It is likely that the import complex is conserved in other plant species as well. EST sequences similar to the known import components can be found in many species, including maize, soybean, and rice. In addition, antibody cross-reactivity studies on species as diverse as mosses and tomato have suggested that at least some of the subunits of the import machinery can be found in all chloroplast-containing eukaryotes (J. Davila-Aponte and K. Keegstra, unpublished observations). Various lines of evidence have also indicated that cyanobacteria contain homologs of at least some of the import components (Bölter et al., 1998b; Reumann and Keegstra, 1999; Reumann et al., 1999). Thus, the chloroplast protein import system is likely to be conserved, at least in part, in all plant (and related) species.

For at least seven (Toc159, Toc75, Toc34, Toc64, Tic20, Tic22, and Hsp93) of the 11 known import components, multiple homologs can be found within the *Arabidopsis* genome. In all but one of these cases, it is known that more than one of these homologs is expressed within *Arabidopsis* cells (Jarvis et al., 1998; Bauer et al., 2000; Gutensohn et al., 2000). This observation immediately suggests that multiple isoforms of the same subunit may be present in the same cells at the same time (Jarvis et al., 1998; Bauer et al., 2000; Chen et al., 2000b; Gutensohn et al., 2000). If this is the case, then one may imagine the existence of multiple types of import complexes within

the chloroplast envelope, each with their own particular precursor specificity. For example, if all three *Arabidopsis* Toc159 homologs are expressed within the same cell, then the chloroplasts within that cell may have a mixture of import complexes: some containing AtToc159, others containing AtToc132, and still others containing AtToc120. However, because the stoichiometry of the subunits within the outer membrane translocon is not known, it is also possible that all three may exist within the same import complex. Obviously, such questions cannot be answered by sequence analysis alone, and further experiments will be needed to address these issues.

The possibility of multiple isoforms for some of the protein import components within *Arabidopsis* chloroplasts also raises the question of whether the same situation is present in pea plants. Is *Arabidopsis* "unusual" in having multiple genes for at least some of the subunits of the import complex or is this the case in pea as well? So far, only one isoform has been identified for each component of the pea import apparatus. However, this fact does not mean that additional homologs do not exist within the pea genome. Since the pea import components were all initially isolated via biochemical means, it is possible that isoforms not present at high concentrations or at the particular stage of development studied would be missed. At this time, there is not enough pea sequence information in GenBank to determine if multiple genes for the import components may indeed also be found in this species.

It is interesting to note that none of the coding regions for the *Arabidopsis* import components are found close to one another within the genome. Even for the components that have multiple putative isoforms, the genes encoding these proteins are located on separate chromosomes (see Table 3-I). This is in contrast to the situation known for

several other gene families (Lin et al., 1999; Mayer et al., 1999; The Arabidopsis Genome Initiative, 2000). Often, homologs of a particular coding region can be found nearby in the genome, if not in tandem (Lin et al., 1999; Mayer et al., 1999; The Arabidopsis Genome Initiative, 2000). In the case of the chloroplast protein import complex, however, the genes encoding the various subunits are found scattered throughout the genome. The explanation for this observation is not clear. Perhaps recombination in the areas immediately surrounding the genes for the import components is suppressed due to the essential nature of either the import complex genes themselves or other genes in their local environment. Additional work will be needed to test this hypothesis.

It has been known for many years that the components of the pea chloroplast protein import complex show little sequence similarity to proteins of known function from other organisms (with the exception of the molecular chaperones and the SPP), including the subunits of the protein import systems of other organelles (Reumann and Keegstra, 1999; Reumann et al., 1999). Thus, it has not been possible to use information gained from the genetic study of other protein import systems to learn more about the functions of the individual subunits in the chloroplast import complex. Identification of the *Arabidopsis* homologs for the pea import components has now made it practical to analyze the functions of these proteins genetically, especially through the use of knockout mutants and antisense technology. Such experiments are already being carried out in several laboratories, and three reports have recently emerged from these investigations (Jarvis et al., 1998; Bauer et al., 2000; Gutensohn et al., 2000). The study of knockout mutants and antisense plants for each of the import components should lead to a better

understanding of their molecular functions. Cross-complementation studies in knockout mutants will also be useful in determining whether the putative *Arabidopsis* import complex isoforms are the functional homologs of the corresponding pea proteins, as is predicted. However, it should be noted that since several of these proteins appear to have multiple isoforms within *Arabidopsis* cells, double and triple mutants may need to be constructed in some cases before component function can be analyzed in detail. Despite this limitation, the genetic study of chloroplast protein import in *Arabidopsis* should provide a great deal of information concerning this system in the coming years.

REFERENCES

Akita M, Nielsen E, Keegstra K (1997) Identification of protein transport complexes in the chloroplastic envelope membranes via chemical cross-linking. J Cell Biol 136: 983-994

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403-410

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389-3402

Arabidopsis Genome Initiative, The (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408: 796-815

Bauer J, Chen K, Hiltbunner A, Wehrli E, Eugster M, Schnell D, Kessler F (2000) The major protein import receptor of plastids is essential for chloroplast biogenesis. Nature 403: 203-207

Bölter B, May T, Soll J (1998a) A protein import receptor in pea chloroplasts, Toc86, is only a proteolytic fragment of a larger polypeptide. FEBS Lett 441: 59-62

Bölter B, Soll J, Schulz A, Hinnah S, Wagner R (1998b) Origin of a chloroplast protein importer. Proc Natl Acad Sci USA 95: 15831-15836

Bruce BD (2000) Chloroplast transit peptides: structure, function and evolution. Trends Cell Biol 10: 440-447

Caliebe A, Grimm R, Kaiser G, Lübeck J, Soll J, Heins L (1997) The chloroplastic protein import machinery contains a Rieske-type iron-sulfur cluster and a mononuclear iron-binding protein. EMBO J 16: 7342-7350

Chen K, Chen X, Schnell DJ (2000a) Initial binding of preproteins involving the Toc159 receptor can be bypassed during protein import into chloroplasts. Plant Physiol 122: 813-822

Chen K, Chen X, Schnell DJ (2000b) Mechanism of protein import across the chloroplast envelope. Biochem Soc Trans 28: 485-491

Chen X, Schnell DJ (1999) Protein import into chloroplasts. Trends Cell Biol 9: 222-227

Emanuelsson O, Nielsen H, Brunak S, von Heijne G (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J Mol Biol 300: 1005-1016

Gutensohn M, Schulz B, Nicolay P, Flügge U-I (2000) Functional analysis of the two *Arabidopsis* homologues of Toc34, a component of the chloroplast protein import apparatus. Plant J 23: 771-783

Hinnah SC, Hill K, Wagner R, Schlicher T, Soll J (1997) Reconstitution of a chloroplast protein import channel. EMBO J 16: 7351-7360

Hirsch S, Muckel E, Heemeyer F, von Heijne G, Soll J (1994) A receptor component of the chloroplast protein translocation machinery. Science 266: 1989-1992

Ivey RA, Subramanian C, Bruce BD (2000) Identification of a Hsp70 recognition domain within the Rubisco small subunit transit peptide. Plant Physiol 122: 1289-1299

Jackson DT, Froehlich JE, Keegstra K (1998) The hydrophilic domain of Tic110, an inner envelope membrane component of the chloroplastic protein translocation apparatus, faces the stromal compartment. J Biol Chem 273: 16583-16588

Jarvis P, Chen L-J, Li H-M, Peto CA, Fankhauser C, Chory J (1998) An *Arabidopsis* mutant defective in the plastid general protein import apparatus. Science 282: 100-103

Keegstra K, Cline K (1999) Protein import and routing systems of chloroplasts. Plant Cell 11: 557-570

Keegstra K, Froehlich JE (1999) Protein import into chloroplasts. Curr Opin Plant Biol 2: 471-476

Keegstra K, Olsen LJ, Theg SM (1989) Chloroplastic precursors and their transport across the envelope membranes. Annu Rev Plant Physiol Plant Mol Biol 40: 471-501

Kessler F, Blobel G (1996) Interaction of the protein import and folding machineries in the chloroplast. Proc Natl Acad Sci USA 93: 7684-7689

Kessler F, Blobel G, Patel HA, Schnell DJ (1994) Identification of two GTP-binding proteins in the chloroplast protein import machinery. Science 266: 1035-1039

Ko K, Budd D, Wu C, Seibert F, Kourtz L, Ko ZW (1995) Isolation and characterization of a cDNA clone encoding a member of the Com44/Cim44 envelope components of the chloroplast protein import apparatus. J Biol Chem 270: 28601-28608

Komiya T, Rospert S, Koehler C, Looser R, Schatz G, Mihara K (1998) Interaction of mitochondrial targeting signals with acidic receptor domains along the protein import pathway: evidence for the 'acid chain' hypothesis. EMBO J 17: 3886-3898

Kouranov A, Chen X, Fuks B, Schnell DJ (1998) Tic20 and Tic22 are new components of the protein import apparatus at the chloroplast inner envelope membrane. J Cell Biol 143: 991-1002

Kouranov A, Schnell DJ (1997) Analysis of the interactions of preproteins with the import machinery over the course of protein import into chloroplasts. J Cell Biol 139: 1677-1685

Kourtz L, Ko K (1997) The early stage of chloroplast protein import involves Com70. J Biol Chem 272: 2808-2813

Lin X, Kaul S, Rounsley S, Shea TP, Benito M-I, Town CD, Fujii CY, Mason T, Bowman CL, Barnstead M, *et al.* (1999) Sequence and analysis of chromosome 2 of the plant *Arabidopsis thaliana*. Nature 402: 761-768

Lübeck J, Soll J, Akita M, Nielsen E, Keegstra K (1996) Topology of IEP110, a component of the chloroplastic protein import machinery present in the inner envelope membrane. EMBO J 15: 4230-4238

Ma Y, Kouranov A, LaSala SE, Schnell DJ (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

Marshall JS, DeRocher AE, Keegstra K, Vierling E (1990) Identification of heat shock protein hsp70 homologues in chloroplasts. Proc Natl Acad Sci USA 87: 374-378

May T, Soll J (1999) Chloroplast precursor protein translocon. FEBS Lett 452: 52-56

May T, Soll J (2000) 14-3-3 proteins form a guidance complex with chloroplast precursor proteins in plants. Plant Cell 12: 53-63

Mayer K, Schüller C, Wambutt R, Murphy G, Volckaert G, Pohl T, Düsterhöft A, Stiekema W, Entian K-D, Terryn N, *et al.* (1999) Sequence and analysis of chromosome 4 of the plant *Arabidopsis thaliana*. Nature 402: 769-777

Nakabayashi K, Ito M, Kiyosue T, Shinozaki K, Watanabe A (1999) Identification of *clp* genes expressed in senescing *Arabidopsis* leaves. Plant Cell Physiol 40: 504-514

Nielsen E, Akita M, Davila-Aponte J, Keegstra K (1997) Stable association of chloroplastic precursors with protein translocation complexes that contain proteins from both envelope membranes and a stromal Hsp100 molecular chaperone. EMBO J 16: 935-946

Oblong JE, Lamppa GK (1992) Identification of two structurally related proteins involved in proteolytic processing of precursors targeted to the chloroplast. EMBO J 11: 4401-4409

Olsen LJ, Keegstra K (1992) The binding of precursor proteins to chloroplasts requires nucleoside triphosphates in the intermembrane space. J Biol Chem 267: 433-439

Olsen LJ, Theg SM, Selman BR, Keegstra K (1989) ATP is required for the binding of precursor proteins to chloroplasts. J Biol Chem 264: 6724-6729

Perry SE, Keegstra K (1994) Envelope membrane proteins that interact with chloroplastic precursor proteins. Plant Cell 6: 93-105

Reumann S, Davila-Aponte J, Keegstra K (1999) The evolutionary origin of the protein-translocating channel of chloroplastic envelope membranes: identification of a cyanobacterial homolog. Proc Natl Acad Sci USA 96: 784-789

Reumann S, Keegstra K (1999) The endosymbiotic origin of the protein import machinery of chloroplastic envelope membranes. Trends Plant Sci 4: 302-307

Richter S, Lamppa GK (1998) A chloroplast processing enzyme functions as the general stromal processing peptidase. Proc Natl Acad Sci USA 95: 7463-7468

Richter S, Lamppa GK (1999) Stromal processing peptidase binds transit peptides and initiates their ATP-dependent turnover in chloroplasts. J Cell Biol 147: 33-43

Schleiff E, Soll J (2000) Travelling of proteins through membranes: translocation into chloroplasts. Planta 211: 449-456

Schnell DJ, Kessler F, Blobel G (1994) Isolation of components of the chloroplast protein import machinery. Science 266: 1007-1012

Seedorf M, Waegemann K, Soll J (1995) A constituent of the chloroplast import complex represents a new type of GTP-binding protein. Plant J 7: 401-411

Sohrt K, Soll J (2000) Toc64, a new component of the protein translocon of chloroplasts. J Cell Biol 148: 1213-1221

Stahl T, Glockmann C, Soll J, Heins L (1999) Tic40, a new "old" subunit of the chloroplast protein import translocon. J Biol Chem 274: 37467-37472

Theg SM, Bauerle C, Olsen LJ, Selman BR, Keegstra K (1989) Internal ATP is the only energy requirement for the translocation of precursor proteins across chloroplastic membranes. J Biol Chem 264: 6730-6736

Tranel PJ, Froehlich J, Goyal A, Keegstra K (1995) A component of the chloroplastic protein import apparatus is targeted to the outer envelope membrane via a novel pathway. EMBO J 14: 2436-2446

VanderVere PS, Bennett TM, Oblong JE, Lamppa GK (1995) A chloroplast processing enzyme involved in precursor maturation shares a zinc-binding motif with a recently recognized family of metalloendopeptidases. Proc Natl Acad Sci USA 92: 7177-7181

Waegemann K, Soll J (1991) Characterization of the protein import apparatus in isolated outer envelopes of chloroplasts. Plant J 1: 149-158

Wu C, Seibert FS, Ko K (1994) Identification of chloroplast envelope proteins in close physical proximity to a partially translocated chimeric precursor protein. J Biol Chem 269: 32264-32271

CHAPTER 4

An outer envelope membrane component of the chloroplastic protein import apparatus is essential in *Arabidopsis*

ABSTRACT

Toc34 is a GTP-binding component of the plastid protein import apparatus located within the chloroplast outer envelope membrane. *Arabidopsis* chloroplasts contain two homologs of Toc34, designated AtToc34 and AtToc33. In this report, we describe the isolation and characterization of a knockout mutant line, *ppi3*, that no longer expresses the gene encoding AtToc34. *ppi3* plants appear similar to wild-type plants throughout their development. In addition, no significant differences from the wild type can be detected when chloroplast ultrastructure, endogenous levels of various plastid proteins, or *in vitro* import kinetics are examined in the mutant line. Overall, *ppi3* plants do not appear to be significantly affected by the loss of *AtTOC34* expression, presumably because AtToc33 can substitute for AtToc34 in the mutant line. Attempts to generate a double homozygous mutant lacking both AtToc34 and AtToc33 by crossing *ppi3* and *ppi1*, a knockout mutant line that does not express the gene encoding AtToc33, were unsuccessful, indicating that the function provided by AtToc34 and AtToc33 is essential in *Arabidopsis*.

INTRODUCTION

The recent development of reverse genetic techniques for *Arabidopsis thaliana*, in particular the wide availability of T-DNA mutagenized populations, has permitted the genetic study of a variety of proteins that are specific to plant systems, such as the factors involved in protein import into chloroplasts. Plastids must import the vast majority of their resident proteins posttranslationally from the cytoplasm (Chen and Schnell, 1999; Keegstra and Cline, 1999; Keegstra and Froehlich, 1999; May and Soll, 1999; Schleiff and Soll, 2000; Vothknecht and Soll, 2000). The process of plastid protein transport is mediated by a proteinaceous machinery located within the two membranes of the organellar envelope. Several components of this import complex have been identified in recent years from pea chloroplasts, including four proteins localized to the outer envelope membrane, five inner envelope membrane proteins, and at least one stromal molecular chaperone (Chen and Schnell, 1999; Keegstra and Cline, 1999; Keegstra and Froehlich, 1999; May and Soll, 1999; Schleiff and Soll, 2000; Vothknecht and Soll, 2000).

GTP hydrolysis has been found to stimulate the initial binding of precursor proteins to the chloroplast surface (Olsen and Keegstra, 1992; Kessler et al., 1994; Young et al., 1999). This observation is particularly interesting because two components of the outer membrane import complex, Toc159 (translocon at the outer envelope membrane of chloroplasts, 159 kD) and Toc34, contain GTP-binding motifs within their amino acid sequences and have been shown to both bind and hydrolyze this nucleotide (Kessler et al., 1994; Seedorf et al., 1995). Toc159 is predicted to be the receptor for chloroplastic precursor proteins (Hirsch et al., 1994; Perry and Keegstra, 1994). The possible role of Toc34 during the import process is less clear, however. Current speculation suggests that

this component may regulate the efficiency of precursor protein transport via GTP binding and/or hydrolysis (Kessler et al., 1994; Seedorf et al., 1995).

Toc34 was first identified as a member of the pea chloroplastic protein import complex through its ability to immunoprecipitate, and be immunoprecipitated by, a translocating precursor protein (Schnell et al., 1994; Seedorf et al., 1995). It is an integral protein of the chloroplast outer envelope membrane, anchored in the lipid bilayer by a transmembrane domain near the C-terminus (Kessler et al., 1994; Seedorf et al., 1995). The soluble N-terminus of Toc34, which contains the GTP-binding domain, is localized within the cytoplasm (Kessler et al., 1994; Seedorf et al., 1995). Toc34 forms a stable complex in the outer membrane with at least two other components of the import machinery: Toc159 and Toc75 (Ma et al., 1996). This trimeric complex forms both in the absence and in the presence of precursors (Ma et al., 1996; Akita et al., 1997; Nielsen et al., 1997). Crosslinking of a translocating preprotein to Toc34 can only be observed in the presence of apyrase, suggesting that the Toc34-precursor interaction is very sensitive to nucleotides, in particular the binding of GTP (Kouranov and Schnell, 1997).

There are two homologs of pea Toc34 in *Arabidopsis*, designated AtToc33 and AtToc34 based on their predicted molecular masses (Jarvis et al., 1998). The genes for both AtToc33 and AtToc34 are expressed throughout development and in all tissues, although they are expressed at higher levels in seedlings and young tissues than in older parts of the plant (Jarvis et al., 1998; Gutensohn et al., 2000). Recently, a mutant, in which both copies of the gene encoding AtToc33 are disrupted by a T-DNA insert, was isolated (Jarvis et al., 1998). This mutant displays several defects in young leaves: a pale phenotype, smaller chloroplasts with fewer thylakoids, and reduced levels of import into

isolated chloroplasts (Jarvis et al., 1998). Older leaves, however, are more similar to those of wild-type plants (Jarvis et al., 1998). Antisense plants for AtToc33 also have a pale phenotype that is restricted to the early stages of development (Gutensohn et al., 2000).

Based on the results obtained with the knockout and antisense lines for AtToc33, it is apparent that AtToc34 cannot completely compensate for the loss of its homolog. However, when overexpressed in the AtToc33 knockout line, AtToc34 can complement the mutant phenotype, indicating that the two proteins are functionally similar (Jarvis et al., 1998). It is possible that AtToc33 and AtToc34 have overlapping, but nonidentical, roles within *Arabidopsis* chloroplasts. This hypothesis is supported by the fact that the genes for these two proteins appear to be differentially expressed when analyzed via *in situ* hybridization and promoter-reporter fusions (Gutensohn et al., 2000). In addition, it has been observed that AtToc33 and AtToc34 may have different affinities for a precursor protein substrate (Gutensohn et al., 2000).

In order to learn more about the function of AtToc34 within *Arabidopsis* chloroplasts, we have isolated a mutant line in which both copies of the gene for this protein have been disrupted by a T-DNA insert. Mutant plants appear similar to wild-type plants, both visually and at the level of chloroplast structure and composition. In addition, chloroplasts isolated from the knockout mutant are able to import a variety of precursor proteins with efficiencies similar to those measured for wild-type chloroplasts. Double homozygous mutants lacking both AtToc33 and AtToc34, however, are not viable, indicating that the function provided by these two homologs is essential in *Arabidopsis* chloroplasts.

MATERIALS AND METHODS

Plant material

Arabidopsis thaliana plants, both wild type and mutant, used in this study were of the Wassilewskija (Ws) ecotype, except as noted otherwise. Seeds were surface-sterilized in 30% (v/v) bleach, 0.02% (v/v) Triton-X 100 for 30 minutes, washed three times with water, and imbibed overnight at 4^0C. After imbibition, seeds were either sown on soil or plated on 1X MS salt and vitamin mixture (Gibco BRL, Grand Island, New York, USA), 1% (w/v) sucrose, 0.8% (w/v) phytagar (Gibco BRL, Grand Island, New York, USA). Soil-grown plants were then grown in 12-hour days (12 hours light:12 hours dark) at 20^0C. Plate-grown plants were incubated in long days (16 hours light:8 hours dark) at 22^0C.

Screening of T-DNA mutagenized Arabidopsis populations

Two T-DNA mutagenized *Arabidopsis* populations, available at the Arabidopsis Functional Genomics Consortium Arabidopsis Knockout Facility at the University of Wisconsin-Madison (Krysan et al., 1999), were screened. A PCR-based screening strategy was employed to analyze a total of 133,440 mutagenized lines, as described previously (Krysan et al., 1996; Krysan et al., 1999). The following PCR primers were used: *AtTOC34* (*PPI3*) 5', aaagaaactaatggagacaacggcaaatg; *AtTOC34* (*PPI3*) 3', gcttcgc-aaatatcctcaccactgtcttc; T-DNA left border, cattttataataacgctgcggacatctac; T-DNA right border, tgggaaaacctggcgttacccaacttaat. The *AtTOC34* 5' and *AtTOC34* 3' primers were used in combination with the T-DNA left border primer to screen all 133,440 lines available in both mutagenized populations. Only one population, containing 60,480

mutagenized lines, was screened with the *AtTOC34* 5' and *AtTOC34* 3' primers in combination with the T-DNA right border primer. PCR products generated by positive hits in the screening reactions were sequenced to determine the location of the T-DNA insert within or near the coding region for AtToc34. After a line containing a T-DNA within the coding region for AtToc34 was isolated, plants homozygous for the insertion were generated and used for further study. Homozygosity of the T-DNA insert was confirmed by the inability of the *AtTOC34* 5' and *AtTOC34* 3' primers to amplify the wild-type gene from genomic DNA isolated from the mutant line.

mRNA isolation and RT-PCR

Ground tissue (~1 g) from four-week-old wild-type or *ppi3* mutant plants grown on soil was added to warm (65^{0}C) extraction buffer (5 mL; 2% [w/v] hexadecyltrimethyl-ammonium bromide, 2% [w/v] polyvinylpyrrolidone K 30, 100 mM Tris-HCl [pH 8.0], 25 mM EDTA, 2 M NaCl, 0.5 g/L spermidine, 2% [v/v] β-mercaptoethanol). This solution was extracted twice with an equal volume of chloroform, and the upper phase was precipitated overnight at 4^{0}C with one-quarter volume 10 M LiCl. After centrifugation at ~6000 *g* for 20 minutes, the pellet was resuspended in 1.5 mL 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5% SDS. This solution was then extracted once in an equal volume of phenol:chloroform:isoamyl-alcohol (25:24:1). The upper phase was again precipitated overnight at 4^{0}C with one-quarter volume 10 M LiCl, and the pellet was recovered by centrifugation at ~6000 *g* for 20 minutes. The pellet was resuspended in 400 µL distilled water and then reprecipitated by the addition of one-tenth volume sodium acetate and 2.5 volumes 100% ethanol. Following recovery by centrifugation at

~6000 *g* for 15 minutes, the final pellet was resuspended in 100 µL distilled water and quantitated. The PolyATract mRNA Isolation System (Promega, Madison, Wisconsin, USA) was then used to isolate mRNA from ~150 µg of total RNA.

RT-PCR was performed with the Titan One Tube RT-PCR System (Roche Molecular Biochemicals, Mannheim, Germany). 10 µL of mRNA isolated from wild-type or *ppi3* plants was used as the template. The following primers specific for the gene encoding AtToc34 were used: 5', gttgtcggtgctataactgatg; 3', acttgctaaaccggagtctcg. Primers specific for the gene encoding AtToc33 were also used: 5', acaatgggagggttcacta-tc; 3', tcttctccttgtaatttgctcac. Gene-specificity of the primers was confirmed by using plasmids containing cDNA versions of the *AtTOC34* and *AtTOC33* genes (Arabidopsis Biological Resource Center, Columbus, Ohio, USA) as templates in control PCR experiments.

Chlorophyll isolation and quantitation

Wild-type or *ppi3* seedlings at four, seven, eleven, fourteen, seventeen, or twenty days after planting were weighed and then ground, with sand, in 80% acetone. Ground tissue was centrifuged at ~2000 *g* for 5 minutes to pellet any insoluble material. The absorbance of the extracted chlorophyll, at 645 nm and 663 nm, was then determined. Each sample was measured twice at these two wavelengths. The chlorophyll levels present (µg/mL) in each sample were calculated using the equation given in Arnon (1949).

Transmission electron microscopy

Leaf tissue from soil-grown wild-type or *ppi3* mutant plants was fixed in 2% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M sodium phosphate (pH 7.4) for 90 minutes at room temperature under vacuum, followed by 24 hours at 4^0C. A second fixation in 1% osmium tetroxide, 0.1 M sodium phosphate (pH 7.4) for two hours was then performed. The samples were dehydrated in acetone and embedded in Spurr resin. Thin sections (~70 nm) of each sample were then stained with uranium and lead and examined in a JEOL 100CX electron microscope (JEOL USA, Peabody, Massachusetts, USA). All of these procedures were carried out by the Center for Advanced Microscopy, Michigan State University.

Protein extraction and immunoblotting

Ground tissue (~1 g) from two-week-old or four-week-old soil-grown wild-type or *ppi3* plants was extracted by boiling for 5 minutes in 0.15 M Tris-HCl (pH 6.8), 7.5% β-mercaptoethanol, 3% SDS, 0.2 mM phenylmethylsulfonyl fluoride (PMSF). Insoluble material was pelleted by centrifugation at ~20,000 *g* for 20 minutes, and the soluble extract was used for further study.

SDS-PAGE was performed as described previously (Laemmli, 1970). Total protein extracts were loaded on the basis of equal amounts of starting tissue fresh weight; chloroplast protein samples were loaded according to equal amounts of total chlorophyll. Following SDS-PAGE, the separated proteins were either stained with Coomassie Brilliant Blue R250 or transferred overnight to Immobilon-P PVDF membranes (Millipore, Bedford, Massachusetts, USA). Membranes were incubated in blocking

buffer (0.1% TBS, 1% Tween 20, 5% non-fat dry milk) prior to incubation in 0.1% TBS, 1% Tween 20, 1% non-fat dry milk supplemented with antiserum. Washings were performed with 0.1% TBS, 1% Tween 20. Primary antibody, against all proteins tested except biotin carboxyl carrier protein (BCCP) and plastocyanin (PC), was detected with horseradish peroxidase-conjugated goat anti-rabbit antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA). Secondary antibody was detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, Illinois, USA). Anti-biotin antibodies directly conjugated to alkaline phosphatase (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA) were used to visualize BCCP; alkaline phosphatase-conjugated goat anti-chicken antibodies were used to detect the primary antibody against PC. These antibodies were then detected with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Antibodies against Toc75 were raised as discussed by Tranel *et al.* (1995). Antiserum to Tic110 was generated as described by Akita *et al.* (1997). Antibodies to Hsp93 were made as described by Akita and Keegstra (submitted). Antiserum against S78 was generated as discussed in Nielsen *et al.* (1997). Antibodies to Tic22 and IEP35 were a gift from D. Schnell (Schnell et al., 1994; Kouranov et al., 1998). Antiserum against allene oxide synthase (AOS) was a gift from G. Howe (Howe et al., 2000). Antiserum to FtsZ1 was a gift from K. Osteryoung (Stokes et al., 2000).

Isolation of chloroplasts

Chloroplasts were isolated from four-week-old plate-grown *Arabidopsis* plants, as described previously (Fitzpatrick and Keegstra, 2001). Final resuspension of chloroplasts

was in import buffer (330 mM sorbitol, 50 mM HEPES-KOH, pH 8.0) at a concentration of 1 mg chlorophyll/mL.

In vitro import assays

The precursor proteins used in this study were the precursor to the small subunit of Rubisco (prSS) from pea, the precursor to the light harvesting chlorophyll a/b binding protein (prLHCP) from pea, the precursor to plastocyanin (prPC) from *Silene pratensis*, and a truncated version of the precursor to Tic110 (tp110-110N) from pea (Bauerle and Keegstra, 1991; Lübeck et al., 1997). All precursors were generated with a TNT®-coupled transcription and translation system (Promega, Madison, Wisconsin, USA) containing ^{35}S-methionine and either SP6 RNA polymerase (prSS, prLHCP, and prPC) or T7 RNA polymerase (tp110-110N).

Import reactions were carried out essentially as described previously (Bruce et al., 1994). In brief, chloroplasts (25 μg chlorophyll) were incubated with rabbit reticulocyte lysate-translated precursor in 150 μL import buffer (330 mM sorbitol, 50 mM HEPES-KOH, pH 8.0) supplemented with 4 mM ATP. At the times indicated in the figures, import was stopped by sedimenting intact chloroplasts through a 40% (v/v) Percoll cushion. Pellets were then resuspended in SDS-PAGE sample buffer and analyzed by electrophoresis and fluorography. Results were quantified using a phosphoimager (Molecular Imager FX, Bio-Rad, Hercules, California, USA). Import rates were calculated by determining the slope of the line generated by plotting time after reaction initiation versus % maximum level of import achieved (see Figure 4.8B).

Generation of ppi1/ppi3 double mutants

All of the following work was done by Paul Jarvis and Ramesh Patel at the University of Leicester, England. *ppi3* mutant plants were crossed to the *ppi1* knockout line, which is in the Columbia background. F2 individuals were grown on 1X MS media, supplemented with 0.5% sucrose, under long day conditions (16 hours light:8 hours dark). After seven days, plants were visually examined and classified into one of three phenotypic classes: green, pale, or bleached. After several more days of growth, genomic DNA was extracted (Edwards et al., 1991) from 15 pale individuals and 15 bleached individuals and genotyped. PCR primers used for genotyping were as follows: *AtTOC33* (*PPI1*) 5', ggtctctcgttcgtgaatgg; *AtTOC33* (*PPI1*) 3', ctgagcgcctatgataagag; *AtTOC34* (*PPI3*) 5', taatttgatacgaggtcagcgaatccggc; *AtTOC34* (*PPI3*) 3', tccctgagatcgatcaagggtagc-ttcac; T-DNA left border, ataacgctgcggacatctac; T-DNA right border, tgggaaaacctggcgtt-acccaacttaat. Each DNA sample was tested using four primer combinations: (1) *AtTOC33* 5' and *AtTOC33* 3' primers, (2) *AtTOC33* 5' and T-DNA left border primers, (3) *AtTOC34* 5' and *AtTOC34* 3' primers, and (4) *AtTOC34* 5' and T-DNA right border primers.

ppi1/ppi1/PPI3/ppi3 individuals were backcrossed, as either the male or the female parent, to Columbia wild-type plants. DNA was isolated (Edwards et al., 1991) from 45 F1 individuals (male = *ppi1/ppi1/PPI3/ppi3*) or 40 F1 individuals (female = *ppi1/ppi1/PPI3/ppi3*) and genotyped as described above.

RESULTS

Isolation of a knockout mutant line for AtToc34

In order to investigate the possible role of AtToc34 during protein import into chloroplasts, we isolated a mutant line in which both copies of the gene encoding AtToc34 have been disrupted by a T-DNA insert. This mutant was designated *ppi3* (for plastid protein import 3), according to the nomenclature previously used for mutants of the chloroplast protein import complex (Jarvis et al., 1998; Bauer et al., 2000). *ppi3* was found by screening the T-DNA mutagenized *Arabidopsis* populations housed at the University of Wisconsin-Madison as part of the Arabidopsis Functional Genomics Consortium (AFGC; Krysan et al., 1999). Using the PCR-based screening strategy developed by the AFGC (Krysan et al., 1996; Krysan et al., 1999), a total of 133,440 mutagenized lines were screened using a primer to the left border of the T-DNA along with primers specific for the gene encoding AtToc34. 60,480 of these lines were also screened using a primer to the T-DNA right border in combination with the *AtToc34*-specific primers. A total of three insertions within or near the gene encoding AtToc34 were found: two were slightly downstream of the coding region and one was within the coding region itself. The last of these mutant lines was chosen for further study.

The T-DNA insert in *ppi3* plants was located within the next to last exon of the gene for AtToc34 (Figure 4.1A). This insertion abolished the expression of the full-length mRNA in mutant plants, as determined by RT-PCR (Figure 4.1B). The gene encoding AtToc33, a homolog of AtToc34, was still expressed, however, in *ppi3* plants (Figure 4.1C). The visible phenotype of the knockout mutant did not differ significantly from that of wild-type plants of the same ecotype. At various stages throughout

Figure 4.1. Characteristics of the *ppi3* mutation. (A) Schematic depicting the structure of the *AtTOC34* gene. Exons are represented by filled boxes; introns are symbolized by thin lines. The approximate location of the T-DNA insert within the *AtTOC34* gene in the *ppi3* mutant line is indicated. (B,C) RT-PCR analysis for the gene encoding AtToc34 (B) and the gene encoding AtToc33 (C), using mRNA isolated from either wild-type or *ppi3* mutant plants as a template. Expression of the *AtTOC34* gene is undetectable in the *ppi3* mutant line.

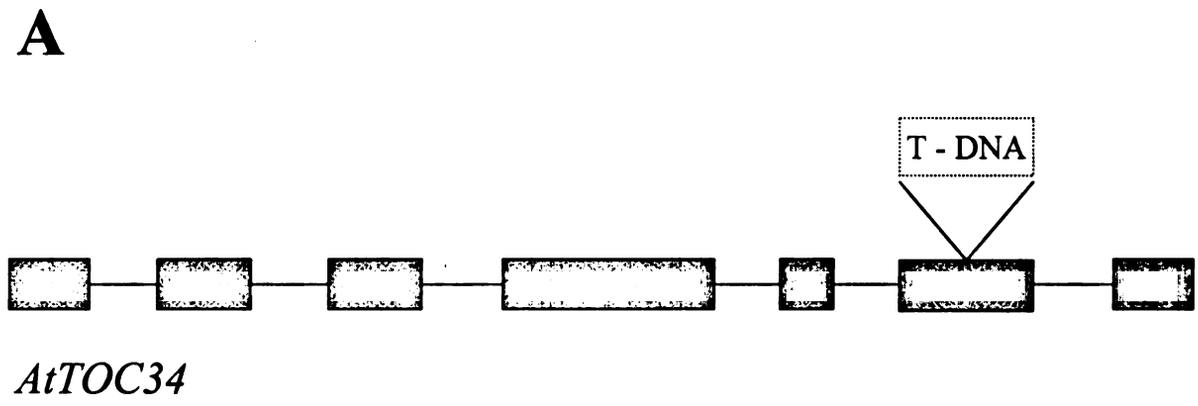


Figure 4.1

development, soil-grown *ppi3* plants were approximately the same size and color as wild-type plants (Figure 4.2). In addition, mutant plants appeared to reach various developmental milestones, such as the emergence of true leaves and bolting, at approximately the same age as wild-type plants grown under the same conditions (compare Figures 4.2E and 4.2F).

It has been reported that plants expressing antisense copies of the gene for AtToc34 are slightly paler than the wild type at four days of age (Gutensohn et al., 2000). However, we did not notice a pale phenotype for four-day-old *ppi3* plants. We quantified chlorophyll levels in soil-grown plants of various ages in order to determine whether there were differences in chlorophyll content between wild-type and *ppi3* mutant lines (Figure 4.3A). On average, *ppi3* plants contained less chlorophyll per gram fresh weight than the wild type during the first week after planting, having ~70% of the chlorophyll levels of wild-type plants grown under the same conditions (Figure 4.3B). However, measurements of the chlorophyll content of four-day-old wild-type plants included a large amount of variation so differences seen at this age are unlikely to be significant. After one week, *ppi3* plants contained at least as much chlorophyll per gram fresh weight as the wild type (Figure 4.3B).

Chloroplasts of ppi3 plants are similar to wild-type chloroplasts

AtToc34 is an integral protein of the plastid outer envelope membrane (Gutensohn et al., 2000). Consequently, *ppi3* mutant plants, which no longer express the gene encoding AtToc34, could possibly have altered chloroplast structure and/or function. In order to determine whether chloroplasts of *ppi3* plants differ from wild-type

Figure 4.2. Visible phenotype of the *ppi3* mutant line. Wild-type (panels A, C, and E) and *ppi3* (panels B, D, and F) plants were grown on soil in 12-hour days (12 hours light:12 hours dark). Individual plants were photographed at two weeks (panels A and B), three weeks (panels C and D), and four weeks (panels E and F) after planting. Note the emergence of flower buds at three weeks and bolting at four weeks. Images in this dissertation are presented in color.

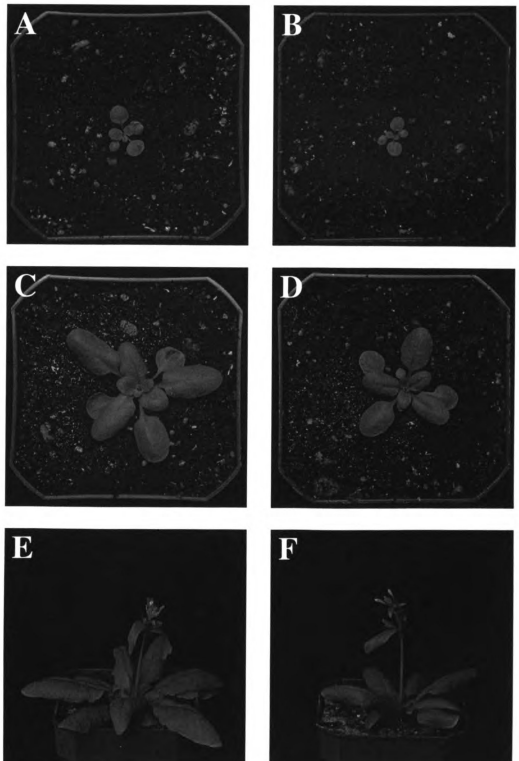
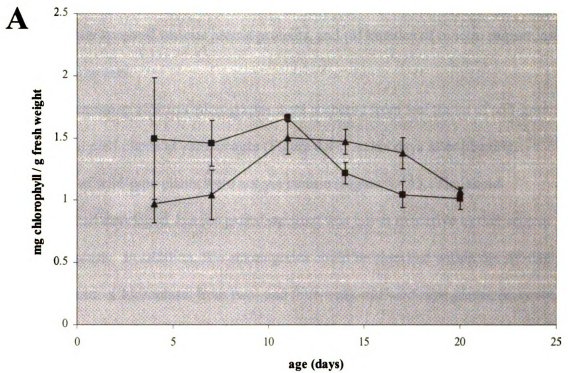


Figure 4.2

Figure 4.3. Chlorophyll levels present in *ppi3* plants do not differ significantly from those present in wild-type plants. (A) Chlorophyll was extracted in 80% acetone from wild-type (square) and *ppi3* mutant (triangle) lines and quantified as described in "Materials and Methods." Values shown are the average of a minimum of three measurements. (B) Average chlorophyll levels present in the *ppi3* mutant, expressed as a percentage of the chlorophyll levels measured for wild-type plants of the same age.


A
mg chlorophyll / g fresh weight
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
age (days)

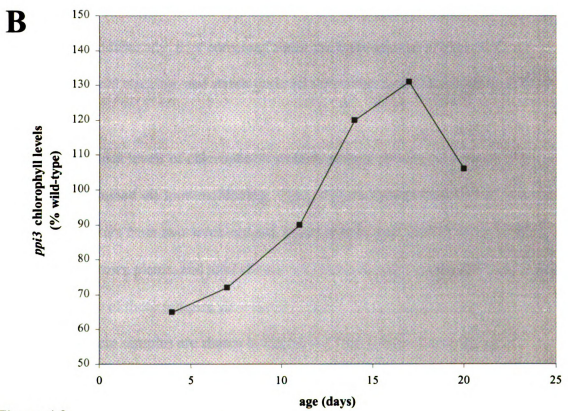

B
ppi3 chlorophyll levels
(% wild-type)
150
140
130
120
110
100
90
80
70
60
50
0
5
10
15
20
25
age (days)

Figure 4.3

chloroplasts, we looked at three aspects of plastid biology: (1) chloroplast ultrastructure, (2) endogenous levels of various plastid proteins, and (3) kinetics of *in vitro* import into isolated chloroplasts.

Transmission electron micrographs were prepared from leaf tissue of soil-grown wild-type and *ppi3* plants at various ages (Figure 4.4). At six days after planting, chloroplasts of wild-type plants were not yet mature (Figure 4.4A). Thylakoid membranes had developed, but the granal stacking was not as extensive as that seen in older chloroplasts. In addition, few starch grains could be observed within the six-day-old chloroplasts. Chloroplasts from two- and four-week-old wild-type plants, however, were fully mature, containing several starch grains and extensive granal stacking (Figures 4.4C and 4.4E). At all ages examined, chloroplasts from *ppi3* mutant plants appeared similar in structure to wild-type chloroplasts of the same ecotype (Figures 4.4B, 4.4D, and 4.4F). *ppi3* chloroplasts were approximately the same size and shape as wild-type chloroplasts. Additionally, they contained about the same amount of thylakoid membranes, granal stacking, and starch grain accumulation as did chloroplasts from wild-type plants.

Endogenous levels of chloroplastic proteins present in wild-type and *ppi3* mutant plants were examined via immunoblotting. Three different tissue samples were analyzed: total protein extract from two-week-old soil-grown plants, total protein extract from four-week-old soil-grown plants, and total chloroplast protein from four-week-old plate-grown plants. All three of these samples gave similar results. Representative data from the chloroplast protein samples are shown in Figure 4.5. For all proteins tested, no significant differences in protein levels were observed between samples isolated from

Figure 4.4. Chloroplast ultrastructure in the *ppi3* mutant line. Leaf tissue was isolated from soil-grown wild-type (panels A, C, and E) and *ppi3* (panels B, D, and F) plants and prepared for transmission electron microscopy by the Center for Advanced Microscopy, Michigan State University. Representative chloroplasts from six- day-old (panels A and B), two-week-old (panels C and D), and four-week-old (panels E and F) plants are shown. Scale bar, 1 μm.

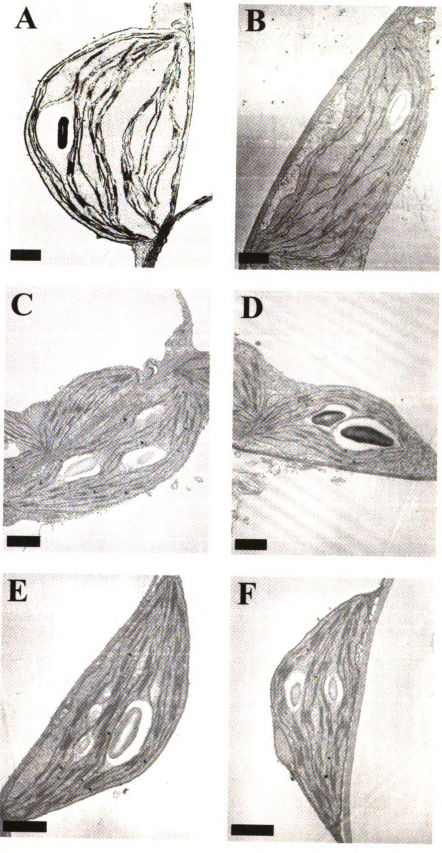


Figure 4.4

Figure 4.5. Endogenous levels of various chloroplastic proteins are not decreased in the *ppi3* mutant. Chloroplasts were isolated from four-week-old plate-grown wild-type and *ppi3* mutant seedlings. Total chloroplast protein equivalent to 10 μg chlorophyll was separated by SDS-PAGE and analyzed by immunoblotting, using antibodies against the proteins listed. Toc = translocon at the outer envelope membrane of chloroplasts; Tic = translocon at the inner envelope membrane of chloroplasts. The number following the Toc or Tic designation refers to the molecular mass of the specified component. Hsp93 = a stromal Hsp100 molecular chaperone; S78 = a stromal Hsp70 molecular chaperone; AOS = allene oxide synthase, an enzyme in the jasmonic acid biosynthetic pathway; FtsZ1 = a plastid division protein; IEP35 = an integral protein of the chloroplast inner envelope membrane; BCCP = biotin carboxyl carrier protein, a protein involved in lipid biosynthesis; PC = plastocyanin, a thylakoid lumen protein.

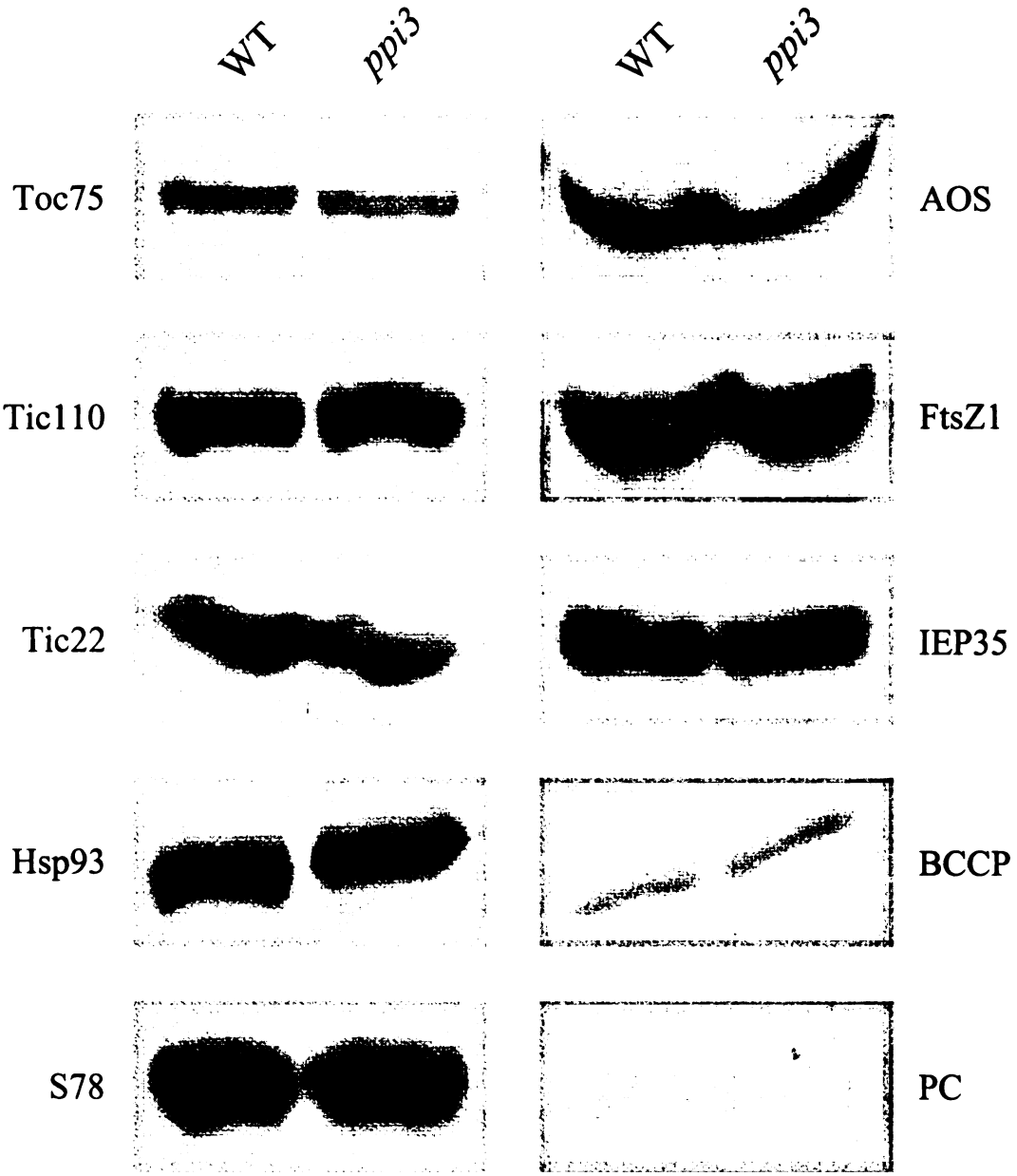

WT
ppi3
WT
ppi3
Toc75
AOS
Tic110
FtsZ1
Tic22
IEP35
Hsp93
BCCP
S78
PC

Figure 4.5

wild-type and *ppi3* plants. Interestingly, the protein levels of several components of the chloroplast protein import complex, including Toc75, Tic110, and Hsp93, appeared to be unaffected by the loss of AtToc34 in *ppi3* plants (Figure 4.5). Several other plastid-localized proteins, representing a variety of metabolic pathways, including allene oxide synthase (AOS; an enzyme in the jasmonic acid biosynthetic pathway), FtsZ1 (a plastid division protein), biotin carboxyl carrier protein (BCCP; a protein involved in fatty acid biosynthesis), and plastocyanin (PC; a component of the chloroplastic electron transport chain), also appeared to be present at normal levels within mutant chloroplasts (Figure 4.5).

A comparison of total protein profiles from wild-type and *ppi3* mutant plants was also conducted. The same three samples that were examined by immunoblotting were subjected to Coomassie staining after separation by SDS-PAGE in order to determine whether any major changes in total leaf or chloroplast protein composition could be observed. As shown in Figure 4.6, no significant differences between the protein profiles of wild-type and *ppi3* plants were detected for total protein extract from two-week-old soil-grown plants or for total chloroplast protein from four-week-old plate-grown plants. Total protein extract from four-week-old soil-grown plants gave similar results (data not shown).

Toc34 has been established to be a component of the outer envelope membrane import complex of pea chloroplasts (Kessler et al., 1994; Schnell et al., 1994; Seedorf et al., 1995). It is assumed that AtToc34, a homolog of pea Toc34, is also a member of this complex within *Arabidopsis* chloroplasts (Jarvis et al., 1998). Therefore, we attempted to determine whether the loss of AtToc34 had any effect on the ability of *ppi3* chloroplasts

Figure 4.6. Total protein profiles of wild-type and *ppi3* mutant plants. Total leaf protein from two-week-old soil-grown plants (wild-type and *ppi3* mutant lines) was extracted by boiling tissue samples in SDS and β-mercaptoethanol. Protein extract equivalent to equal amounts of starting fresh mass was separated by SDS-PAGE and stained with Coomassie Brilliant Blue R250 (lanes 1 and 2). Intact chloroplasts were isolated from four-week-old wild-type and *ppi3* plants that had been grown on plates. Total chloroplasts equivalent to 15 μg chlorophyll were separated by electrophoresis, followed by staining with Coomassie Brilliant Blue R250 (lanes 3 and 4).

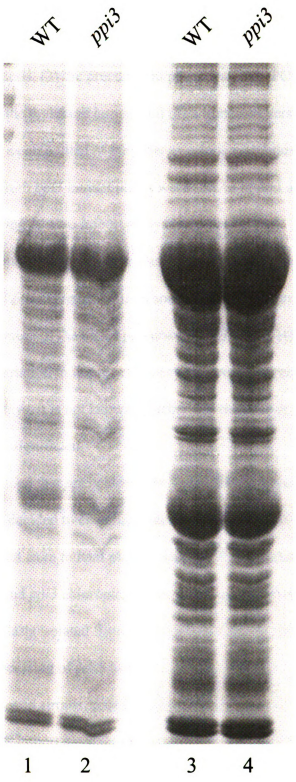


Figure 4.6

to import precursor proteins. Four different precursors were tested: (1) the precursor to the small subunit of Rubisco (prSS), a stromal protein, (2) the precursor to the light harvesting chlorophyll a/b binding protein (prLHCP), a thylakoid membrane protein involved in photosynthesis, (3) the precursor to plastocyanin (prPC), a photosynthetic protein located within the thylakoid lumen, and (4) a truncated version of the precursor to Tic110 (tp110-110N), a component of the inner envelope membrane import complex. Chloroplasts isolated from *ppi3* mutant plants were able to import all of these precursors within ten minutes during *in vitro* import assays (data not shown). Indeed, *ppi3* chloroplasts could import these proteins at least as well as wild-type chloroplasts under the same reaction conditions (Figure 4.7). In some cases, it appeared that *ppi3* chloroplasts imported more of the tested precursor than did wild-type chloroplasts. However, due to the variation in import efficiency observed between chloroplast samples prepared on different days, it is not known whether any increase in import efficiency seen for *ppi3* chloroplasts is significant.

Having determined that *ppi3* chloroplasts were able to import a variety of precursor proteins, we decided to examine in detail the import of one precursor, prSS, into chloroplasts isolated from mutant plants. Specifically, we compared the rate of prSS import into wild-type and *ppi3* chloroplasts over the course of a twenty-minute *in vitro* import assay. Chloroplasts isolated from wild-type plants were able to rapidly import prSS (Figure 4.8). Processing of prSS to SS, an indication that the precursor has at least partially entered the plastid stroma, could be observed at even very early time points. Maximal levels of prSS import were seen within ten minutes. *ppi3* chloroplasts were also able to rapidly import prSS into the organelle (Figure 4.8). Overall, the rate of prSS

Figure 4.7. Chloroplasts isolated from *ppi3* mutant plants are able to import precursor proteins representing a variety of chloroplastic subcompartments. ^{35}S-labeled prSS (precursor to the small subunit of Rubisco, a stromal protein), ^{35}S-labeled prLHCP (precursor to the light harvesting chlorophyll a/b binding protein, a thylakoid membrane protein), ^{35}S-labeled prPC (precursor to plastocyanin, a thylakoid lumen-localized protein), and ^{35}S-labeled tp110-110N (a truncated version of the precursor to Tic110, an inner membrane-localized import component) were imported into chloroplasts (25 μg chlorophyll) isolated from wild-type or *ppi3* knockout mutant plants. Plants had been plate-grown for approximately four weeks prior to chloroplast isolation. After ten or twenty minutes at room temperature in the light, import was stopped, and intact chloroplasts were recovered, by centrifuging the reactions through a 40% Percoll cushion. Equivalent amounts of chlorophyll from each sample were analyzed by SDS-PAGE and fluorography. The amount of radiolabeled precursor proteins imported into the chloroplasts was quantified using a phosphoimager. Values presented depict the amount of precursor imported into *ppi3* mutant chloroplasts as a percentage of the amount imported into wild-type chloroplasts and are the average of at least two independent experiments.

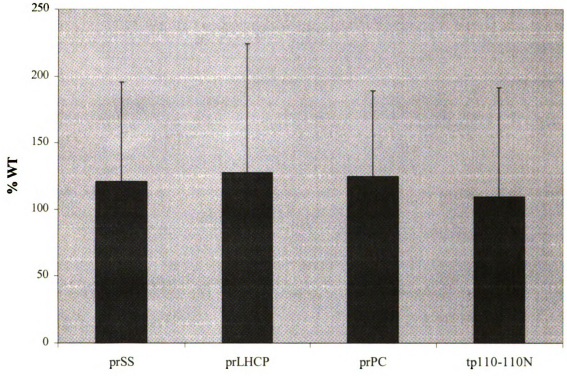


Figure 4.7

Figure 4.8. The rate of import of prSS into chloroplasts isolated from the *ppi3* mutant line is not significantly impaired. (A) ^{35}S-labeled prSS (precursor to the small subunit of Rubisco) was imported into chloroplasts isolated from wild-type or *ppi3* mutant plants. Plants had been plate-grown for approximately four weeks prior to chloroplast isolation. At the times indicated, aliquots equivalent to 25 μg chlorophyll were removed from the import reactions. Import was stopped, and intact chloroplasts were recovered, by centrifuging the aliquots through a 40% Percoll cushion. Equivalent amounts of chlorophyll from each sample were analyzed by SDS-PAGE and fluorography. TP = 1/10 volume of radioactive translation product added to each sample. SS = mature form of prSS. (B) Results from panel A (wild type = square; *ppi3* = triangle) were quantified using a phosphoimager. Values depicted are the average of four independent import reactions.

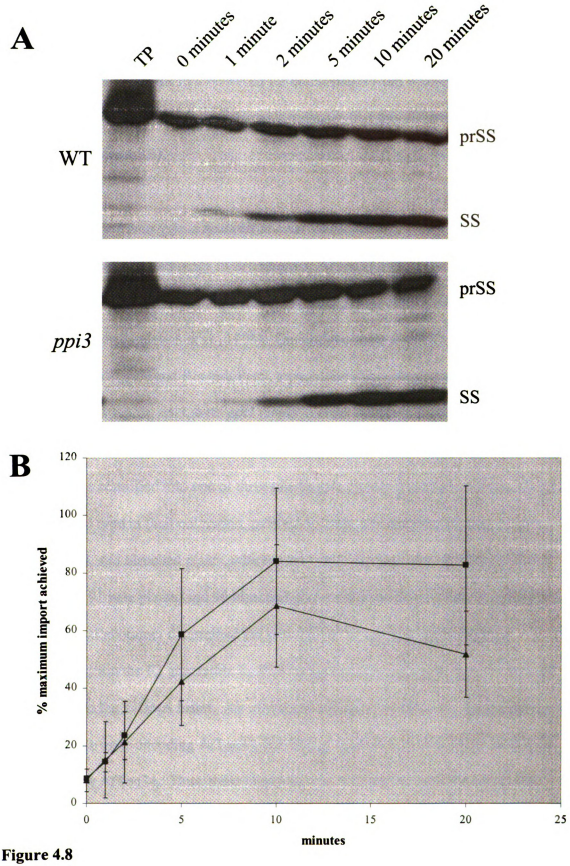

A
TP
0 minutes
1 minute
2 minutes
5 minutes
10 minutes
20 minutes
WT
prSS
SS
ppi3
prSS
SS
B
% maximum import achieved
minutes
0
20
40
60
80
100
120
0
5
10
15
20
25

Figure 4.8

import into chloroplasts isolated from *ppi3* plants did not appear to lag significantly behind the rate of import into wild-type chloroplasts. On average, the rate into *ppi3* chloroplasts was ~80% the rate observed for chloroplasts from wild-type plants, although the variation observed between experiments indicates that this difference is not significant (Figure 4.8B).

ppi1/ppi3 double homozygous mutants are not viable

Toc34 has two homologs in *Arabidopsis*: AtToc34 and AtToc33 (Jarvis et al., 1998). A T-DNA insertional mutant line for AtToc33, known as *ppi1*, has been described previously (Jarvis et al., 1998). Our collaborators at the University of Leicester in England, Paul Jarvis and Ramesh Patel, crossed the knockout mutant line for AtToc34 described in this report, *ppi3*, with *ppi1* plants in order to determine the effect of removing both isoforms of Toc34 from *Arabidopsis* chloroplasts. F2 progeny from this cross could be classified into one of three phenotypic classes: green individuals, which appeared wild type in their coloration, pale individuals, which looked similar to *ppi1* mutant plants, and bleached plants, which were extremely pale overall. A total of 985 green plants, 91 pale plants, and 96 bleached plants were observed in the F2 generation.

Our collaborators determined the genotype of several pale and bleached individuals within the F2 generation by PCR analysis using both gene-specific primers and primers to the T-DNA insert. All of the pale plants examined were homozygous for an insert in the gene encoding AtToc33, but did not contain a T-DNA insert within the gene encoding AtToc34. Thus, these individuals were identical, genotypically and phenotypically, to the *ppi1* mutant line, which lacks AtToc33. Bleached individuals, on

the other hand, were homozygous for a T-DNA insert in the gene encoding AtToc33 (*PPI1*) and hemizygous for an insert within the gene encoding AtToc34 (*PPI3*), having a genotype of *ppi1/ppi1/PPI3/ppi3*. These plants, consequently, were expressing only one copy of the *PPI3* gene, likely producing lower than the normal amount of AtToc34 and no AtToc33. Accordingly, *ppi1/ppi1/PPI3/ppi3* plants were much smaller in size and paler in color than either the *ppi1* or *ppi3* knockout mutant lines (Figure 4.9). Assuming that double homozygous mutants would not be found among the green individuals, these results indicate that no *ppi1/ppi3* double homozygous mutants were present among the 1172 F2 progeny screened ($X^2 = 390.67$, $p = <0.001$). Thus, it appears that removing both AtToc33 and AtToc34 from *Arabidopsis* chloroplasts may be lethal.

Overall, the phenotypic results (985 green plants:91 pale plants:96 bleached plants) approximate a 10:1:1 ratio ($X^2 = 0.62$, $p = >0.5$). Since pale and bleached individuals are both homozygous for an insert in *PPI1* but differ in their insertion status for *PPI3*, this would be the expected ratio if gametes containing a T-DNA insert within both the *PPI1* gene and the *PPI3* gene could be transmitted through either the male or the female parent, but not both. In order to explore this possibility in more detail, our collaborators analyzed siliques generated from the self-fertilization of *ppi1/ppi1/PPI3/ppi3* plants. In contrast to siliques from self-fertilized wild-type individuals, which contained many seeds, siliques from self-fertilized *ppi1/ppi1/PPI3/ppi3* plants contained very few seeds (Figure 4.10A). The scarcity of seed within these mutant siliques apparently resulted, at least partially, from a failure of ovules to develop into seeds (Figure 4.10B). While failed ovules were relatively rare in wild-type siliques (two out of 201 ovules examined in two siliques), approximately half

Figure 4.9. Plants that are homozygous for a T-DNA insert in the gene encoding AtToc33 and heterozygous for an insert in the gene encoding AtToc34 are much smaller and paler than either *ppi3* or *ppi1* mutant plants. Plants were grown on 1X MS media, supplemented with 0.5% sucrose, in long days (16 hours light:8 hours dark). Individual plants were photographed on the days indicated. The *ppi3* knockout (KO) mutant has a genotype of *PPI1/PPI1/ppi3/ppi3*; the *ppi1* KO mutant has a genotype of *ppi1/ppi1/PPI3/PPI3*. Images in this dissertation are presented in color.

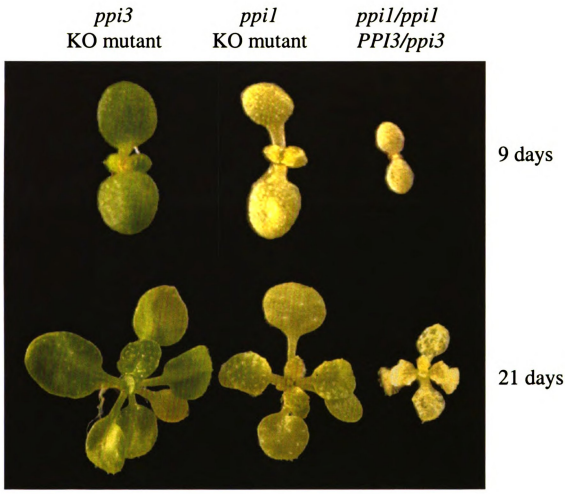

ppi3
KO mutant
ppi1
KO mutant
ppi1/ppi1
PPI3/ppi3
9 days
21 days

Figure 4.9

Figure 4.10. Siliques of *ppi1/ppi1/PPI3/ppi3* plants contain significantly more failed ovules than do wild-type siliques. (A) Mature siliques from wild-type (left) and *ppi1/ppi1/PPI3/ppi3* (right) plants that have been self-fertilized. The silique from a *ppi1/ppi1/PPI3/ppi3* individual is shown at a higher magnification. (B) Close-up of a silique from a *ppi1/ppi1/PPI3/ppi3* plant, showing a failed ovule (arrow). (C) Siliques from wild-type and *ppi1/ppi1/PPI3/ppi3* plants were analyzed to determine the number of ovules that had developed into seeds. Two wild-type siliques and ten siliques from *ppi1/ppi1/PPI3/ppi3* individuals were examined. N = normal seed; FO = failed ovule; AS = aborted seed. Images in this dissertation are presented in color.

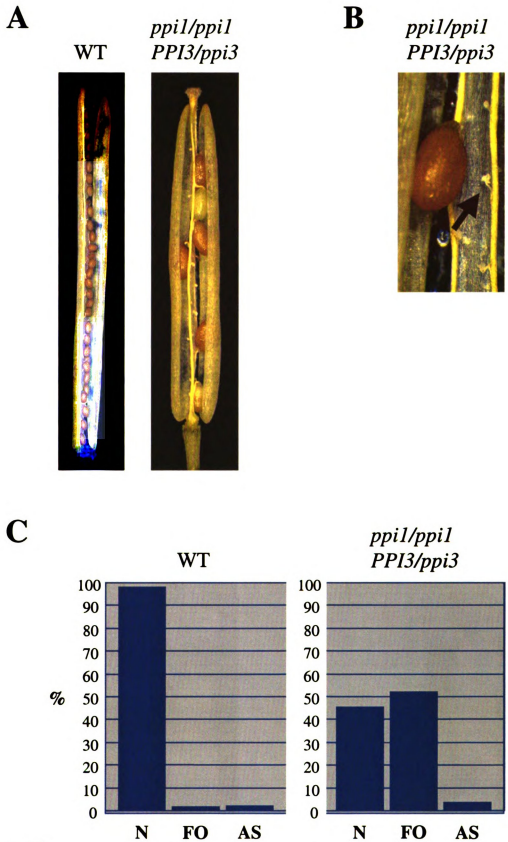

A
WT
ppi1/ppi1
PPI3/ppi3
B
ppi1/ppi1
PPI3/ppi3
C
WT
ppi1/ppi1
PPI3/ppi3
%
100
90
80
70
60
50
40
30
20
10
0
N
FO
AS

Figure 4.10

of the ovules in siliques from *ppi1/ppi1/PPI3/ppi3* individuals did not develop properly: 173 failed ovules were observed in ten siliques containing a total of 335 ovules (Figure 4.10C).

Reciprocal backcrosses to wild-type plants of the Columbia ecotype, using *ppi1/ppi1/PPI3/ppi3* plants as either the male or the female parent, were performed to determine which parent was unable to transmit gametes having a T-DNA insert within the genes encoding both AtToc33 and AtToc34. 18 of 40 F1 progeny from a backcross in which a *ppi1/ppi1/PPI3/ppi3* individual was the female parent received a T-DNA insert in both the *PPI1* and the *PPI3* genes. However, no progeny, out of 45 screened, received both inserts when a *ppi1/ppi1/PPI3/ppi3* plant was used as the male parent. These results would suggest that AtToc33 and AtToc34 may be essential for the proper development and/or function of the male gametophyte.

DISCUSSION

We have isolated a knockout mutant line, *ppi3*, for the gene encoding AtToc34, one of the components of the outer envelope membrane protein import complex of *Arabidopsis* chloroplasts (Kessler et al., 1994; Schnell et al., 1994; Seedorf et al., 1995; Jarvis et al., 1998; Gutensohn et al., 2000). Overall, this mutant did not appear to be significantly altered when compared to wild-type plants of the same ecotype. Analysis of visible phenotype, chloroplast ultrastructure, endogenous levels of plastid proteins, and *in vitro* import of various precursors revealed no major differences between wild-type and *ppi3* plants (Figures 4.2-4.8). Thus, loss of AtToc34 function in the *ppi3* mutant line did not appear to have a significant effect on the development of chloroplasts or the plant as a whole.

The most likely explanation for these results is that AtToc33, a homolog of AtToc34, is able to substitute for the loss of *AtTOC34* expression in *ppi3* mutant plants. It has been determined that these two homologs perform similar, if not identical, functions within *Arabidopsis* plants; both are able to complement the AtToc33 knockout mutant line, *ppi1* (Jarvis et al., 1998). Thus, although the two homologs likely display subtle differences in their individual functions, it is likely that AtToc33 could provide functions normally carried out by AtToc34. In addition, because *AtTOC33* is expressed at higher levels than *AtTOC34* throughout *Arabidopsis* development (Jarvis et al., 1998), it is likely to be present at high enough levels to compensate for the absence of AtToc34 in *ppi3* plants. However, it has also been reported that *AtTOC34* and *AtTOC33* are differentially expressed within various organs of *Arabidopsis* (Gutensohn et al., 2000). Thus, if the plastids of individual organs were analyzed in more detail, then more

significant differences between the phenotypes of wild-type and *ppi3* plants may be observed, especially in those organs, such as roots, where expression of *AtTOC34* is normally higher than *AtTOC33* expression (Gutensohn et al., 2000).

Antisense plants for AtToc34 have recently been generated (Gutensohn et al., 2000). These plants appear similar to wild-type plants, except at very early stages of development when they are slightly paler than the wild type (Gutensohn et al., 2000). These results are similar to those we observed for the AtToc34 knockout mutant line, with the exception of plants at four days of age. The visible phenotype of *ppi3* plants was indistinguishable from that of wild-type plants of the same ecotype at all ages examined (Figure 4.2 and data not shown). Quantitation of chlorophyll concentration in wild-type and mutant plants indicated that the *ppi3* mutant contained chlorophyll levels similar to those observed for wild-type plants throughout development (Figure 4.3). The reason for the differences in phenotype between AtToc34 antisense and knockout mutant lines at four days of age is unknown.

In this investigation, we attempted to construct double mutants between *ppi3*, the AtToc34 knockout mutant line described in this report, and *ppi1*, an AtToc33 knockout mutant line (Jarvis et al., 1998). However, we were unable to recover any individuals that were homozygous for T-DNA disruptions in the genes for both of these proteins. On the other hand, we were able to obtain plants that were homozygous for a disruption in the gene encoding AtToc33 and heterozygous for a disruption in the gene encoding AtToc34. These plants have only one functional copy of a gene encoding a Toc34 homolog, specifically one for AtToc34, the lesser expressed of the two homologs.

Accordingly, this mutant has a more severe phenotype than either *ppi3* or *ppi1* plants alone (Figure 4.9).

Interestingly, there appears to be a progression in severity of phenotype as the number of functional genes encoding Toc34 homologs is decreased. *ppi1* plants, which have both copies of the gene encoding AtToc33 disrupted by a T-DNA insert, are small and pale, especially early in their development (Jarvis et al., 1998). When one copy of the gene encoding AtToc34 was subsequently disrupted, the resulting plants were even smaller and paler (Figure 4.9). This phenotype was also no longer restricted to younger tissues. Attempts to additionally disrupt the second copy of the gene for AtToc34, producing *ppi1/ppi3* double homozygous mutants, have so far yielded no viable plants. Thus, there is an absolute requirement for the function of the Toc34 homologs in *Arabidopsis* plants. At this time, the minimum amount of Toc34 homologs needed for normal development appears to be represented by the expression levels achieved by having at least one functional copy of the gene encoding AtToc33, the more highly expressed of the two *Arabidopsis* Toc34 homologs. *ppi3* plants, which lack AtToc34 expression but have normal expression of AtToc33, were visually indistinguishable from wild-type plants.

The inability to obtain *ppi1/ppi3* double homozygous mutant plants indicates that the function provided by AtToc33 and AtToc34 is essential in *Arabidopsis*, possibly for male gametophyte development and/or function in particular, as no transmission of gametes containing a disruption in the genes for both AtToc33 and AtToc34 from the male parent could be observed. Several components of the mitochondrial protein import machinery have been found to be essential for yeast growth (Pfanner et al., 1997). These

subunits are considered to be part of a core protein import complex, the minimum set of factors required for precursor transport (Baker and Schatz, 1991; Pfanner et al., 1997). Thus, AtToc33/AtToc34 may be a subunit of the core import complex required for the movement of proteins into chloroplasts, or possibly plastids in general.

Current hypotheses concerning AtToc33/AtToc34 function suggest that these proteins play a regulatory role during precursor import (Kessler et al., 1994; Seedorf et al., 1995). Their function is probably controlled by their GTP-binding and hydrolysis abilities, as indicated by the finding that crosslinking between pea Toc34 and an incoming precursor protein can only be observed in the absence of added nucleotides (Kouranov and Schnell, 1997). The essential nature of AtToc33/AtToc34 in *Arabidopsis* would argue that if AtToc33/AtToc34 are indeed regulating an aspect of precursor import, they are more likely affecting an essential process, such as channel formation, rather than simply modulating the efficiency of transport. Alternatively, AtToc33/AtToc34 may be controlling the transfer of precursor proteins from a receptor component to the protein-conducting channel (Kessler et al., 1994; Seedorf et al., 1995). A recent report suggesting that the receptor for precursor proteins, Toc159, actually binds preproteins in the cytoplasm and subsequently inserts with the bound precursor into the chloroplast outer envelope membrane presents an interesting possibility (Hiltbrunner et al., 2001). It is possible that AtToc33/AtToc34 function is required for the recognition of the Toc159-precursor complex at the chloroplast surface and/or for its insertion into the membrane, either of which would likely be essential processes (Hiltbrunner et al., 2001).

The development of the knockout mutant line for AtToc34 described in this report, along with the AtToc33 knockout line (Jarvis et al., 1998) and the antisense lines

for both AtToc34 and AtToc33 (Gutensohn et al., 2000) that are already available, should provide useful tools in the future for exploring hypotheses regarding the role of AtToc33/AtToc34 during precursor transport into chloroplasts. In particular, the function of the GTP-binding domains of AtToc33 and AtToc34 in the import process should be investigated in more detail.

REFERENCES

Akita M, Nielsen E, Keegstra K (1997) Identification of protein transport complexes in the chloroplastic envelope membranes via chemical cross-linking. J Cell Biol 136: 983-994

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24: 1-15

Baker KP, Schatz G (1991) Mitochondrial proteins essential for viability mediate protein import into yeast mitochondria. Nature 349: 205-208

Bauer J, Chen K, Hiltbunner A, Wehrli E, Eugster M, Schnell D, Kessler F (2000) The major protein import receptor of plastids is essential for chloroplast biogenesis. Nature 403: 203-207

Bauerle C, Keegstra K (1991) Full-length plastocyanin precursor is translocated across isolated thylakoid membranes. J Biol Chem 266: 5876-5883

Bruce BD, Perry S, Froehlich J, Keegstra K (1994) *In vitro* import of proteins into chloroplasts. Plant Mol Biol Manual J1: 1-15

Chen X, Schnell DJ (1999) Protein import into chloroplasts. Trends Cell Biol 9: 222-227

Edwards K, Johnstone C, Thompson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res 19: 1349

Fitzpatrick LM, Keegstra K (2001) A method for isolating a high yield of *Arabidopsis* chloroplasts capable of efficient import of precursor proteins. Plant J 27: 59-65

Gutensohn M, Schulz B, Nicolay P, Flügge U-I (2000) Functional analysis of the two *Arabidopsis* homologues of Toc34, a component of the chloroplast protein import apparatus. Plant J 23: 771-783

Hiltbrunner A, Bauer J, Vidi P-A, Infanger S, Weibel P, Hohwy M, Kessler F (2001) Targeting of an abundant cytosolic form of the protein import receptor atToc159 to the outer chloroplast membrane. J Cell Biol 154: 309-316

Hirsch S, Muckel E, Heemeyer F, von Heijne G, Soll J (1994) A receptor component of the chloroplast protein translocation machinery. Science 266: 1989-1992

Howe GA, Lee GI, Itoh A, Li L, DeRocher AE (2000) Cytochrome P450-dependent metabolism of oxylipins in tomato. Cloning and expression of allene oxide synthase and fatty acid hydroperoxide lyase. Plant Physiol 123: 711-724

Jarvis P, Chen L-J, Li H-M, Peto CA, Fankhauser C, Chory J (1998) An *Arabidopsis* mutant defective in the plastid general protein import apparatus. Science 282: 100-103

Keegstra K, Cline K (1999) Protein import and routing systems of chloroplasts. Plant Cell 11: 557-570

Keegstra K, Froehlich JE (1999) Protein import into chloroplasts. Curr Opin Plant Biol 2: 471-476

Kessler F, Blobel G, Patel HA, Schnell DJ (1994) Identification of two GTP-binding proteins in the chloroplast protein import machinery. Science 266: 1035-1039

Kouranov A, Chen X, Fuks B, Schnell DJ (1998) Tic20 and Tic22 are new components of the protein import apparatus at the chloroplast inner envelope membrane. J Cell Biol 143: 991-1002

Kouranov A, Schnell DJ (1997) Analysis of the interactions of preproteins with the import machinery over the course of protein import into chloroplasts. J Cell Biol 139: 1677-1685

Krysan PJ, Young JC, Sussman MR (1999) T-DNA as an insertional mutagen in Arabidopsis. Plant Cell 11: 2283-2290

Krysan PJ, Young JC, Tax F, Sussman MR (1996) Identification of transferred DNA insertions within *Arabidopsis* genes involved in signal transduction and ion transport. Proc Natl Acad Sci USA 93: 8145-8150

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685

Lübeck J, Heins L, 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

Ma Y, Kouranov A, LaSala SE, Schnell DJ (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

May T, Soll J (1999) Chloroplast precursor protein translocon. FEBS Lett 452: 52-56

Nielsen E, Akita M, Davila-Aponte J, Keegstra K (1997) Stable association of chloroplastic precursors with protein translocation complexes that contain proteins from both envelope membranes and a stromal Hsp100 molecular chaperone. EMBO J 16: 935-946

Olsen LJ, Keegstra K (1992) The binding of precursor proteins to chloroplasts requires nucleoside triphosphates in the intermembrane space. J Biol Chem 267: 433-439

Perry SE, Keegstra K (1994) Envelope membrane proteins that interact with chloroplastic precursor proteins. Plant Cell 6: 93-105

Pfanner N, Craig EA, Hönlinger A (1997) Mitochondrial preprotein translocase. Annu Rev Cell Dev Biol 13: 25-51

Schleiff E, Soll J (2000) Travelling of proteins through membranes: translocation into chloroplasts. Planta 211: 449-456

Schnell DJ, Kessler F, Blobel G (1994) Isolation of components of the chloroplast protein import machinery. Science 266: 1007-1012

Seedorf M, Waegemann K, Soll J (1995) A constituent of the chloroplast import complex represents a new type of GTP-binding protein. Plant J 7: 401-411

Stokes KD, McAndrew RS, Figueroa R, Vitha S, Osteryoung KW (2000) Chloroplast division and morphology are differentially affected by overexpression of *FtsZ1* and *FtsZ2* genes in Arabidopsis. Plant Physiol 124: 1668-1677

Tranel PJ, Froehlich J, Goyal A, Keegstra K (1995) A component of the chloroplastic protein import apparatus is targeted to the outer envelope membrane via a novel pathway. EMBO J 14: 2436-2446

Vothknecht UC, Soll J (2000) Protein import: the hitchhikers guide into chloroplasts. Biol Chem 381: 887-897

Young ME, Keegstra K, Froehlich JE (1999) GTP promotes the formation of early-import intermediates but is not required during the translocation step of protein import into chloroplasts. Plant Physiol 121: 237-243

CHAPTER 5

An Hsp100 chaperone is required for normal chloroplast development and function in *Arabidopsis*

ABSTRACT

Molecular chaperones are required for the posttranslational import of proteins into mitochondria and the ER, providing the driving force for the translocation of precursor proteins into the organelle. In the chloroplast protein import system, an Hsp100 protein, known as Hsp93, is hypothesized to be the factor providing the energy for precursor movement, although there is little direct evidence for this hypothesis. In order to learn more about the possible function of Hsp93 during protein import into chloroplasts, we isolated a knockout mutant line that has both copies of *AtHSP93-V*, which encodes one of the two *Arabidopsis* homologs of Hsp93, disrupted by a T-DNA insert. Mutant plants are much smaller and paler than wild-type plants. In addition, mutant chloroplasts contain less thylakoid membrane when compared to the wild type. Plastid protein composition, however, seems to be largely unaffected in AtHsp93-V knockout plants. Chloroplasts isolated from the AtHsp93-V knockout mutant line are still able to import a variety of precursor proteins, but the rate of import of some of these precursors appears to be significantly impaired. These results indicate that AtHsp93-V has an important, but not essential, role in *Arabidopsis* chloroplasts.

INTRODUCTION

The vast majority of plastid proteins are encoded within the nucleus, rather than in the plastid genome. As a consequence, these proteins must be imported into the organelle following their synthesis on cytoplasmic ribosomes (Chen and Schnell, 1999; Keegstra and Cline, 1999; Keegstra and Froehlich, 1999; May and Soll, 1999; Schleiff and Soll, 2000; Vothknecht and Soll, 2000). The process of plastid protein import has been studied extensively using isolated pea chloroplasts as a model system. From these studies, it has been determined that protein import is mediated by a proteinaceous transport machinery located within the two membranes of the chloroplast envelope (Chen and Schnell, 1999; Keegstra and Cline, 1999; Keegstra and Froehlich, 1999; May and Soll, 1999; Schleiff and Soll, 2000; Vothknecht and Soll, 2000).

The energy for precursor protein translocation is provided by ATP hydrolysis within the chloroplast stroma (Theg et al., 1989). ATP hydrolysis has also been implicated in providing the driving force for the posttranslational import of proteins into the endoplasmic reticulum (ER) and mitochondria (Rapoport et al., 1999; Herrmann and Neupert, 2000). The major factors mediating ATP hydrolysis in the ER and mitochondrial protein transport systems are heat shock protein 70s (Hsp70s; Jensen and Johnson, 1999; Pilon and Schekman, 1999; Strub et al., 2000). Thus, it was believed that a stromal Hsp70 would be found to drive protein translocation into chloroplasts as well. However, when isolated import complexes from pea chloroplasts were probed for the presence of stromal molecular chaperones, a member of the Hsp100 family of chaperones was found instead (Akita et al., 1997; Nielsen et al., 1997; Kouranov et al., 1998).

Hsp100 proteins (also known as Clp [caseinolytic protease] proteins) are a diverse class of molecular chaperones involved in a wide variety of essential metabolic processes throughout prokaryotic and eukaryotic phylogenies (Schirmer et al., 1996). They were first identified as the regulatory component of the Clp proteolytic complex in *Escherichia coli* (Hwang et al., 1987; Katayama et al., 1988). Subsequently, they have been found to be involved in regulating the DNA-binding activity of several proteins (Mhammedi-Alaoui et al., 1994; Wickner et al., 1994; Lazazzera and Grossman, 1997) and in providing tolerance to a variety of environmental stresses, including heat (Sanchez and Lindquist, 1990; Squires et al., 1991) and salt (Krüger et al., 1994), among other functions. In general, Hsp100 proteins operate by disassembling protein aggregates or oligomers via the energy provided by ATP hydrolysis (Schirmer et al., 1996).

Two classes of Hsp100 proteins exist. Hsp100s of the ClpA, ClpB, ClpC, ClpD, and ClpE subfamilies are Class 1 proteins (Schirmer et al., 1996; Derré et al., 1999). These proteins have two ATP-binding domains within their amino acid sequences (Schirmer et al., 1996). Higher plants contain homologs of the ClpB, ClpC, and ClpD subfamilies (Schirmer et al., 1996). The plant ClpB proteins are primarily cytoplasmic (Boston et al., 1996) while the plant ClpC and ClpD homologs are found within chloroplasts (Moore and Keegstra, 1993; Shanklin et al., 1995; Boston et al., 1996; Nakashima et al., 1997; Nakabayashi et al., 1999). The molecular chaperone that was found in association with the pea chloroplastic protein import apparatus is a member of the ClpC subfamily and is known as Hsp93, reflecting its calculated molecular mass of 93 kD for the mature form of the protein (Akita et al., 1997; Nielsen et al., 1997; Kouranov et al., 1998; M. Akita and K. Keegstra, submitted). While the majority of

Hsp93 molecules are present in soluble form in the chloroplast stroma, a significant proportion of Hsp93 proteins are found in association with the inner envelope membrane, presumably through their interaction with the import complex (Moore and Keegstra, 1993; Nielsen, 1997; M. Akita and K. Keegstra, submitted). A similar situation exists for the mitochondrial import-associated Hsp70 chaperone, which is mostly soluble in the matrix, but can be found in a membrane-bound form via an interaction with the inner membrane import complex component, Tim44 (Pfanner et al., 1997; Herrmann and Neupert, 2000).

Hsp93 was found to be a component of chloroplastic import complexes regardless of whether precursor proteins were also present (Nielsen et al., 1997; Kouranov et al., 1998). Several lines of evidence indicate that this association of Hsp93 with isolated import complexes is relevant to the process of precursor transport. First, Hsp93 coimmunoprecipitates the precursor to the small subunit of Rubisco (prSS) only under conditions that support either binding or translocation of the preprotein (Nielsen et al., 1997). Secondly, Hsp93 is able to coimmunoprecipitate several precursor proteins that utilize the general import apparatus of the chloroplast envelope but not plastid proteins that do not use this import machinery (Nielsen et al., 1997). The association of Hsp93 with prSS is disrupted by the addition of ATP, but not GTP, to an import reaction (Nielsen et al., 1997). Because Hsp100 chaperones interact with their substrates in an ATP-sensitive manner (Wickner et al., 1994; Wawrzynow et al., 1995), this ATP-dependence suggests that the association between Hsp93 and prSS is physiologically relevant (Nielsen et al., 1997). Finally, the interaction between Hsp93 and prSS decreases with time during an import reaction (Nielsen et al., 1997). This observation

indicates that prSS proteins associated with Hsp93 are functional import intermediates (Nielsen et al., 1997).

Arabidopsis thaliana has two Hsp93 (ClpC) homologs, which we have designated AtHsp93-V and AtHsp93-III according to the chromosomal locations of the genes encoding the proteins, that are ~88% identical to one another at the amino acid level (see Chapter 3). Both homologs contain predicted chloroplastic transit peptides at their N-termini, and antibodies made against AtHsp93-III recognize a protein localized to the chloroplast stroma (Nakabayashi et al., 1999). RNA and protein levels for AtHsp93-III, and ClpC homologs in other species, have been shown to be relatively stable under a variety of environmental conditions (Shanklin et al., 1995; Clarke and Eriksson, 1996; Ostersetzer and Adam, 1996; Nakabayashi et al., 1999). This has led to the hypothesis that the ClpC protein in plants may play a housekeeping role within chloroplasts (Shanklin et al., 1995; Nakabayashi et al., 1999).

In order to learn more about the possible role of Hsp93 during protein import into chloroplasts, we utilized the reverse genetic resources now available for *Arabidopsis* to study this protein genetically. At the time this study was undertaken, the genomic sequence of *AtHSP93-V* was available while that of *AtHSP93-III* was still unknown. Thus, we were able to obtain a knockout mutant line that had both copies of the gene for AtHsp93-V disrupted by a T-DNA insert. Characterization of the visible and chloroplastic phenotype of this AtHsp93-V knockout mutant line is described in this report.

MATERIALS AND METHODS

Plant material

Wild-type *Arabidopsis thaliana* plants used in this study were of the Wassilewskija (Ws) ecotype. AtHsp93-V knockout mutant plants were in the Ws background. For soil-grown plants, seeds were surface-sterilized in 30% (v/v) bleach for 30 minutes, washed three times with water, and imbibed overnight at 4^0C before being sown on soil. Plants were then grown in 12-hour days (12 hours light:12 hours dark) at 20^0C. For plate-grown seedlings, seeds were surface-sterilized in 30% bleach (v/v), 0.02% (v/v) Triton-X 100 for 30 minutes, washed three times with water, and imbibed overnight at 4^0C. After imbibition, seeds were plated on 1X MS salt and vitamin mixture (Gibco BRL, Grand Island, New York, USA), 1% (w/v) sucrose, 0.8% (w/v) phytagar (Gibco BRL, Grand Island, New York, USA) and incubated in long days (16 hours light:8 hours dark) at 22^0C.

Screening of a T-DNA mutagenized Arabidopsis population

A T-DNA mutagenized *Arabidopsis* population, containing a total of 60,480 mutagenized lines, was screened. This population is available at the Arabidopsis Functional Genomics Consortium Arabidopsis Knockout Facility at the University of Wisconsin-Madison (Krysan et al., 1999). A PCR-based screening strategy was employed, as described previously (Krysan et al., 1996; Krysan et al., 1999). The following PCR primers were utilized: *AtHSP93-V* 5', attcggattcttcgttggtttctgttgtt; *AtHSP93-V* 3', tctgccatactatcctctaaaagcctcat; T-DNA left border, cattttataataacgctgcggac-atctac. The location of the T-DNA insert within or near the coding region for AtHsp93-V

was determined by sequencing PCR products produced from positive hits detected in the screening reactions. After a single line containing a T-DNA insert within the coding region for AtHsp93-V was identified, plants homozygous for the insertion were made and used for further study. The homozygous state of the T-DNA insert was confirmed by the inability of the *AtHSP93-V* 5' and *AtHSP93-V* 3' primers to produce a product when used in combination on genomic DNA isolated from the mutant line.

mRNA isolation and RT-PCR

Extraction buffer (5 mL; 2% [w/v] hexadecyltrimethylammonium bromide, 2% [w/v] polyvinylpyrrolidone K 30, 100 mM Tris-HCl [pH 8.0], 25 mM EDTA, 2 M NaCl, 0.5 g/L spermidine, 2% [v/v] β-mercaptoethanol) was warmed to 65^0C in a centrifuge tube. Ground tissue (~1 g) from four-week-old soil-grown wild-type or AtHsp93-V mutant plants was added, and the tube was vortexed. The solution was extracted twice with an equal volume of chloroform. The upper phase was then precipitated overnight at 4^0C with one-quarter volume 10 M LiCl. The pellet was recovered by centrifugation at ~6000 *g* for 20 minutes and resuspended in 1.5 mL 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5% SDS. This solution was then extracted once in an equal volume of phenol:chloroform:isoamyl-alcohol (25:24:1). The upper phase was precipitated overnight at 4^0C with one-quarter volume 10 M LiCl, and the pellet was recovered by centrifugation at ~6000 *g* for 20 minutes. The pellet was then resuspended in 400 μL distilled water and reprecipitated by the addition of one-tenth volume sodium acetate and 2.5 volumes 100% ethanol. The final pellet was recovered by centrifugation at ~6000 *g* for 15 minutes and resuspended in 100 μL distilled water. Total RNA was then

quantitated. ~150 μg of each total RNA sample was used to isolate mRNA using the PolyATract mRNA Isolation System (Promega, Madison, Wisconsin, USA).

RT-PCR was done using the Titan One Tube RT-PCR System (Roche Molecular Biochemicals, Mannheim, Germany). 10 μL of mRNA from wild-type or AtHsp93-V knockout mutant plants was used as the template. The following primers specific for *AtHSP93-V* were used: 5' forward, tcggtgaaaatgatgtgtagtc; 5' reverse, gggttgttcttggttctcc-tg; 3' forward, attgaaaaagcccatcca; 3' reverse, tgccacttccaccatttag. Primers specific for *AtHSP93-III* were also employed: forward, gggcccccagtgcattagattatt; reverse, tccaagacac-gagctgccacac.

Chlorophyll isolation and quantitation

Whole wild-type or AtHsp93-V knockout mutant plants at four, seven, eleven, fourteen, seventeen, or twenty days after planting were weighed and then ground with sand in 80% acetone. Ground tissue was spun at ~2000 *g* for 5 minutes to remove the sand and other debris from the extracted chlorophyll. The supernatant was then spectrophotometrically measured at 645 nm and 663 nm. Each sample was measured twice at these two wavelengths. The amount of chlorophyll (μg/mL) in each sample was determined using the equation given in Arnon (1949).

Transmission electron microscopy

Leaf tissue isolated from soil-grown wild-type and AtHsp93-V knockout mutant plants at six days, two weeks, or four weeks after planting was fixed, under vacuum, for 90 minutes at room temperature in 2% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M

sodium phosphate (pH 7.4). Fixation was then continued for an additional 24 hours at 4^0C, followed by a second fixation in 1% osmium tetroxide, 0.1 M sodium phosphate (pH 7.4) for two hours. After the second fixation, samples were dehydrated in acetone, embedded in Spurr resin, and sectioned. The thin sections (~70 nm) were stained with uranium and lead prior to examination in a JEOL 100CX electron microscope (JEOL USA, Peabody, Massachusetts, USA). This work was performed by the Center for Advanced Microscopy, Michigan State University.

Protein extraction and immunoblotting

Whole two-week-old or four-week-old wild-type or AtHsp93-V knockout plants grown on soil were ground and then extracted in 0.15 M Tris-HCl (pH 6.8), 7.5% β-mercaptoethanol, 3% SDS, 0.2 mM phenylmethylsulfonyl fluoride (PMSF) for five minutes at 100^0C. Following centrifugation at ~20,000 *g* for 20 minutes to pellet insoluble material, the soluble extract was used for further study.

SDS-PAGE was performed as described previously (Laemmli, 1970). Chloroplast protein samples were loaded according to equal amounts of total chlorophyll; total protein extracts were loaded on the basis of equal amounts of starting tissue fresh weight. After separation by SDS-PAGE, the proteins were either stained with Coomassie Brilliant Blue R250 or transferred overnight to Immobilon-P PVDF membranes (Millipore, Bedford, Massachusetts, USA). Membranes were incubated in blocking buffer (0.1% TBS, 1% Tween 20, 5% non-fat dry milk) for 30 minutes, followed by incubation in 0.1% TBS, 1% Tween 20, 1% non-fat dry milk supplemented with antiserum. Washings were done in 0.1% TBS, 1% Tween 20. Primary antibody, against

all proteins examined except biotin carboxyl carrier protein (BCCP) and plastocyanin (PC), was detected with horseradish peroxidase-conjugated goat anti-rabbit antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA). Secondary antibody was visualized with the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, Illinois, USA). Primary antibody against PC was detected with alkaline phosphatase-conjugated goat anti-chicken antibodies; anti-biotin antibodies directly conjugated to alkaline phosphatase (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA) were used to detect BCCP. These antibodies were then visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Antibodies to Toc75 were raised as described by Tranel *et al.* (1995). Antibodies against Tic110 were generated as discussed by Akita *et al.* (1997). Antibodies to Hsp93 were made as described by Akita and Keegstra (submitted). Antiserum to S78 was made as discussed in Nielsen *et al.* (1997). Antiserum against Tic22 and IEP35 was a gift from D. Schnell (Schnell et al., 1994; Kouranov et al., 1998). Antiserum to allene oxide synthase (AOS) was a gift from G. Howe (Howe et al., 2000). Antibodies against FtsZ1 were a gift from K. Osteryoung (Stokes et al., 2000). The generation of antiserum against Tic40 is discussed elsewhere (L. Fitzpatrick et al., manuscript in preparation).

Isolation of chloroplasts

Chloroplasts were isolated from four-week-old *Arabidopsis* plants that had been grown on plates, as described previously (Fitzpatrick and Keegstra, 2001). Final resuspension of chloroplasts, at a concentration of 1 mg chlorophyll/mL, was in import buffer (330 mM sorbitol, 50 mM HEPES-KOH, pH 8.0).

In vitro import assays

Precursor proteins used in this investigation were prSS from pea, the precursor to the light harvesting chlorophyll a/b binding protein (prLHCP) from pea, the precursor to plastocyanin (prPC) from *Silene pratensis*, and a truncated version of the precursor to Tic110 (tp110-110N) from pea (Bauerle and Keegstra, 1991; Lübeck et al., 1997). All precursors were made using the TNT®-coupled transcription and translation system (Promega, Madison, Wisconsin, USA) containing ^{35}S-methionine and either SP6 RNA polymerase (prSS, prLHCP, and prPC) or T7 RNA polymerase (tp110-110N).

Import reactions were performed essentially as described previously (Bruce et al., 1994). In brief, chloroplasts (25 μg chlorophyll) were incubated with radiolabeled precursor in 150 μL import buffer (330 mM sorbitol, 50 mM HEPES-KOH, pH 8.0) supplemented with 4 mM ATP. Import was halted, at the times indicated in the figures, by sedimenting intact chloroplasts through a 40% (v/v) Percoll cushion. Pellets were then resuspended in SDS-PAGE sample buffer and analyzed by electrophoresis and fluorography. Quantification of the amount of radiolabeled precursor imported was done using a phosphoimager (Molecular Imager FX, Bio-Rad, Hercules, California, USA). Import rates were determined by measuring the slope of the line generated when time after reaction initiation versus % maximum level of import achieved was plotted (see Figure 5.8).

RESULTS

AtHsp93-V knockout mutant plants are much smaller and paler than wild-type plants

Molecular chaperones are essential for posttranslational protein import into mitochondria and the ER, providing, among other functions, the driving force for the movement of precursor proteins into the organelle interior (Jensen and Johnson, 1999; Pilon and Schekman, 1999; Rapoport et al., 1999; Herrmann and Neupert, 2000; Strub et al., 2000). In chloroplast protein import, most of the evidence currently points to Hsp93, a stromal Hsp100 protein, as the molecular chaperone assisting in "pulling in" incoming precursors (Akita et al., 1997; Nielsen et al., 1997; Kouranov et al., 1998). However, there is no direct experimental evidence to support this hypothesis. In order to learn more about the function of Hsp93 during protein import into plastids, we isolated a knockout mutant line that has a T-DNA inserted into both copies of the gene encoding AtHsp93-V, one of the two Hsp93 homologs present in *Arabidopsis* chloroplasts. This mutant was obtained from a T-DNA mutagenized *Arabidopsis* population, containing 60,480 mutagenized lines, administered by the Arabidopsis Functional Genomics Consortium at the University of Wisconsin-Madison (Krysan et al., 1999). Using an established PCR screening strategy (Krysan et al., 1996; Krysan et al., 1999), this population was screened using *AtHSP93-V* gene-specific primers in combination with a primer to the T-DNA left border. Three T-DNA inserts within or near the coding region for AtHsp93-V were detected. Two were more than three kilobases downstream of the stop codon. Consequently, it was concluded that these two inserts would have no effect on the expression of the *AtHSP93-V* gene. The third insert that was found was located within the coding region for AtHsp93-V, making it likely that this T-DNA insert would result in

a disruption of *AtHSP93-V* expression. As a consequence, this mutant line was chosen for further study.

The T-DNA insert in the knockout mutant line was located within the last exon of the *AtHSP93-V* gene (Figure 5.1A). The presence of this insert resulted in a truncated mRNA being produced from the *AtHSP93-V* gene (Figure 5.1B). Primers specific to the 5' end of *AtHSP93-V* were able to amplify a product from mRNA isolated from mutant plants, but primers specific to the 3' end of the gene were not. Thus, accumulation of the full-length mRNA for *AtHSP93-V* is abolished in the mutant line. The gene encoding AtHsp93-III, a homolog of AtHsp93-V, was still expressed in mutant plants (Figure 5.1C). Because AtHsp93-V and AtHsp93-III are almost 90% identical to one another at the amino acid level, it is unlikely that antibodies generated against the whole protein, such as those used in this study, would be specific. Thus, when either total protein extract or chloroplast protein from four-week-old AtHsp93-V knockout plants was probed for the presence of Hsp93 proteins, an immunoreactive band was detected (Figure 5.1D). The overall amount of Hsp93 proteins did appear to be slightly reduced in the mutant, however. Interestingly, a smaller immunoreactive band, approximately 32 kD in size, was detectable in protein isolated from the mutant line but not in protein from the wild type. Thus, as is suggested by the RT-PCR results, it is likely that a truncated version of AtHsp93-V is in fact being produced in the knockout plants. This truncated version would likely not be functional, however, because the protein would lack several important regions of AtHsp93-V, including the second nucleotide-binding domain. Similar results were obtained when total protein extract from two-week-old plants was probed with antibodies against Hsp93 proteins (data not shown).

Figure 5.1. AtHsp93-V knockout mutant plants express a truncated mRNA from the *AtHSP93-V* gene and may produce a truncated AtHsp93-V protein.

(A) Schematic depicting the structure of the *AtHSP93-V* gene. Exons are represented by filled boxes; introns are symbolized by thin lines. The approximate location of the T-DNA insert within the *AtHSP93-V* gene in the knockout mutant line is indicated. (B) RT-PCR analysis for the gene encoding AtHsp93-V. Primers specific to the 5' end of the *AtHSP93-V* gene, upstream of the T-DNA insert (lanes 1 and 2), or the 3' end of the gene, downstream of the T-DNA insert (lanes 3 and 4), were used on mRNA isolated from wild-type and AtHsp93-V knockout (KO) mutant plants. (C) RT-PCR analysis for the gene encoding AtHsp93-III, using mRNA isolated from either wild-type or AtHsp93-V mutant plants as a template. (D) Immunoblot analysis for Hsp93 proteins. Total leaf protein from four-week-old soil-grown plants (wild type [lane 1] and AtHsp93-V knockout [KO] mutant [lane 2] lines) was extracted by boiling tissue samples in SDS and β-mercaptoethanol. Protein extract equivalent to equal amounts of starting fresh mass was separated by SDS-PAGE and analyzed by immunoblotting with antibodies to Hsp93 proteins. Intact chloroplasts were isolated from four-week-old wild-type (lane 3) and AtHsp93-V mutant (lane 4) plants that had been grown on plates. Total chloroplasts equivalent to 10 μg chlorophyll were separated by electrophoresis and immunoblotted with antiserum against Hsp93 proteins. A possible truncated protein produced by the *AtHSP93-V* gene in mutant plants is indicated (*).

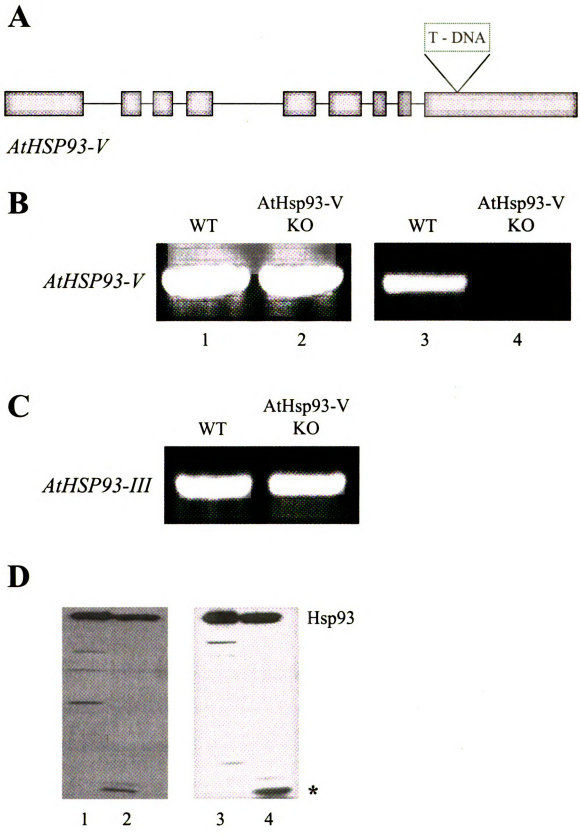

A
T - DNA
AtHSP93-V
B
AtHsp93-V
KO
WT
AtHsp93-V
KO
WT
AtHSP93-V
1
2
3
4
C
AtHsp93-V
KO
WT
AtHSP93-III
D
Hsp93
*
1
2
3
4

Figure 5.1

AtHsp93-V mutant plants were much smaller and paler than wild-type plants of the same ecotype (Figure 5.2). These differences could be observed throughout development, although the size difference in very young seedlings was minor (data not shown). As the plants grew, however, the disparity in size between wild-type and mutant seedlings became more pronounced and was quite apparent by two weeks after planting (compare Figures 5.2A and 5.2B). Despite the alterations in overall size and color, AtHsp93-V mutant plants appeared to reach major developmental milestones, such as the emergence of flower buds and bolting, at approximately the same age as did wild-type plants (compare Figures 5.2C, 5.2D, 5.2E, and 5.2F).

We quantified the chlorophyll levels present in wild-type and AtHsp93-V mutant plants at various ages (Figure 5.3A). Average chlorophyll levels for the wild type ranged from ~1.5 mg chlorophyll/g fresh weight during the first ten days after planting to ~1.0 mg chlorophyll/g fresh weight at later ages. Chlorophyll levels in the AtHsp93-V knockout mutant line were significantly lower. Average values were between ~0.6 and ~0.8 mg chlorophyll/g fresh weight at all ages tested. Overall, the AtHsp93-V mutant plants had ~50-60% of the chlorophyll levels, on a per gram fresh weight basis, observed for wild-type plants throughout development (Figure 5.3B).

Chloroplast structure and composition in the AtHsp93-V knockout mutant line

Disruption of both copies of the *AtHSP93-V* gene by a T-DNA insert resulted in a significant reduction in overall chlorophyll levels in mutant plants. These plants, therefore, could possibly have other aspects of chloroplast physiology altered as well. In order to examine this possibility in more detail, transmission electron microscopy was

Figure 5.2. AtHsp93-V knockout mutant plants are much smaller and paler than wild-type plants. Wild-type (panels A, C, and E) and AtHsp93-V knockout mutant (panels B, D, and F) plants were grown on soil in 12-hour days (12 hours light:12 hours dark). Individual plants were photographed at two weeks (panels A and B), three weeks (panels C and D), and four weeks (panels E and F) after planting. Note the emergence of flower buds at three weeks and bolting at four weeks. Images in this dissertation are presented in color.

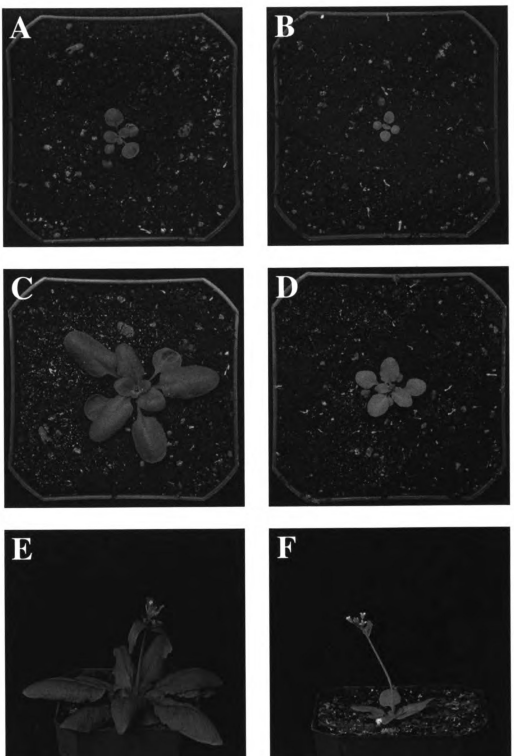


Figure 5.2

Figure 5.3. Chlorophyll levels are reduced in the AtHsp93-V knockout mutant line. (A) Chlorophyll was extracted in 80% acetone from wild-type (square) and AtHsp93-V mutant (triangle) plants and quantified as described in "Materials and Methods." Values shown are the average of four measurements. (B) Average chlorophyll levels present in the AtHsp93-V knockout mutant line, expressed as a percentage of the chlorophyll levels measured for wild-type plants of the same age.

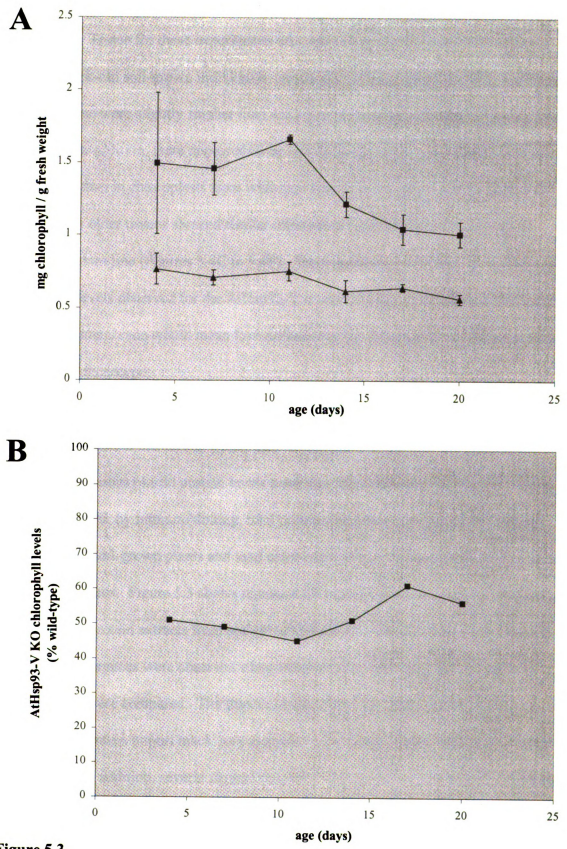

A
mg chlorophyll / g fresh weight
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
age (days)
B
AtHsp93-V KO chlorophyll levels
(% wild-type)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
age (days)

Figure 5.3

performed on leaf tissue isolated from wild-type and AtHsp93-V knockout mutant plants (Figure 5.4). Tissue for these experiments was taken from six-day-old, two-week-old, and four-week-old soil-grown individuals. At six days after planting, chloroplasts from the mutant line were slightly smaller than wild-type chloroplasts (compare Figures 5.4A and 5.4B). In addition, there appeared to be less thylakoid membrane present in mutant chloroplasts than in chloroplasts from wild-type tissue. A comparison of chloroplasts isolated from older tissues showed similar differences between the wild type and the knockout mutant line (Figures 5.4C to 5.4F). These results may explain the decrease in chlorophyll levels observed for the AtHsp93-V mutant plants. A reduction in the amount of thylakoid membrane would mean less surface area for chlorophyll incorporation and thus, a paler phenotype.

Having determined that AtHsp93-V knockout mutant plants have decreased amounts of thylakoid membrane within their chloroplasts, we wanted to determine whether endogenous plastid protein levels were also affected in the mutant line. To do this, we analyzed, by immunoblotting, total protein extracted from two-week-old and four-week-old soil-grown plants and total chloroplast protein isolated from four-week-old plate-grown plants. Figure 5.5 shows representative results from the chloroplast protein samples; total protein extracts from soil-grown plants gave similar results. Overall, no significant differences were observed when samples from wild-type and AtHsp93-V mutant plants were compared. The protein levels of various components of the chloroplastic protein import machinery appeared to be largely unaffected by the loss of AtHsp93-V. In addition, several stromal enzymes, from a variety of metabolic pathways, appeared to be present in mutant chloroplasts at levels comparable to those seen for wild-

Figure 5.4. Chloroplasts isolated from AtHsp93-V mutant plants are slightly smaller and contain less thylakoid membrane in comparison to wild-type chloroplasts. Leaf tissue was isolated from soil-grown wild-type (panels A, C, and E) and AtHsp93-V mutant (panels B, D, and F) plants at six days (panels A and B), two weeks (panels C and D), and four weeks (panels E and F) after planting and prepared for transmission electron microscopy by the Center for Advanced Microscopy, Michigan State University. Scale bar, 1 μm.

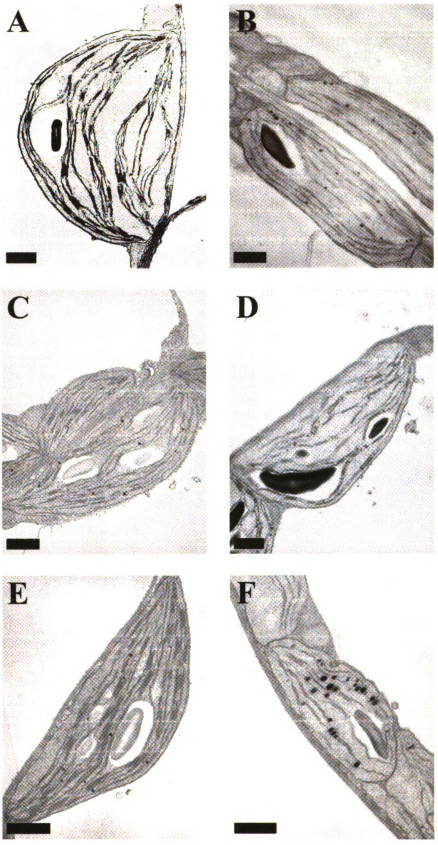


Figure 5.4

Figure 5.5. Endogenous levels of various chloroplastic proteins are unaffected in the AtHsp93-V knockout line. Chloroplasts were isolated from four-week-old plate-grown wild-type and AtHsp93-V knockout (KO) mutant seedlings. Total chloroplast protein equivalent to 10 μg chlorophyll was separated by SDS-PAGE and analyzed by immunoblotting, using antibodies against the proteins listed. Toc = translocon at the outer envelope membrane of chloroplasts; Tic = translocon at the inner envelope membrane of chloroplasts. The number following the Toc or Tic designation refers to the molecular mass of the specified component. S78 = a stromal Hsp70 molecular chaperone; AOS = allene oxide synthase, an enzyme in the jasmonic acid biosynthetic pathway; FtsZ1 = a plastid division protein; IEP35 = an integral protein of the chloroplast inner envelope membrane; BCCP = biotin carboxyl carrier protein, a protein involved in lipid biosynthesis; PC = plastocyanin, a thylakoid lumen protein.

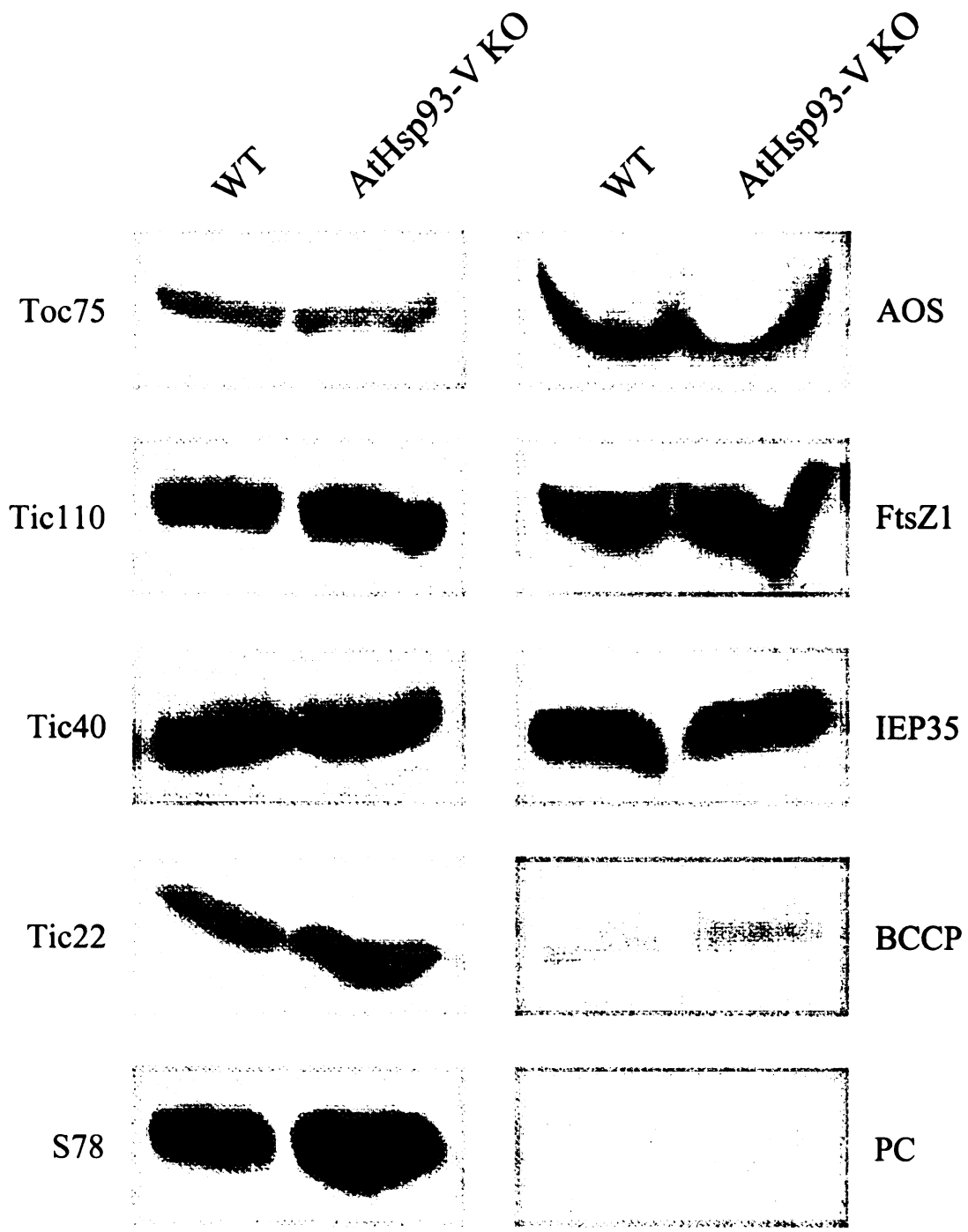

WT
AtHsp93-V KO
WT
AtHsp93-V KO
Toc75
AOS
Tic110
FtsZ1
Tic40
IEP35
Tic22
BCCP
S78
PC

Figure 5.5

type chloroplasts. It is interesting to note that no reduction in protein levels for plastocyanin (PC), a thylakoid lumen protein, and light harvesting chlorophyll a/b binding protein (LHCP), a protein localized to the thylakoid membrane, was observed in AtHsp93-V knockout plants despite the fact that mutant chloroplasts had less thylakoid membrane (Figure 5.5 and data not shown). The one exception to these results was S78, a stromal Hsp70, whose protein levels were slightly increased in samples from the mutant line. Thus, it is possible that the absence of AtHsp93-V from mutant chloroplasts resulted in an upregulation of the protein levels for this molecular chaperone.

The same samples that were analyzed via immunoblotting were also examined by Coomassie staining in order to compare total protein profiles between wild-type and AtHsp93-V knockout mutant lines. No significant differences could be observed when total protein extracts from two-week-old soil-grown wild-type or mutant plants were compared (Figure 5.6). Similar results were obtained when extract from four-week-old soil-grown plants was used for comparison (data not shown). Analysis of chloroplast protein from four-week-old plate-grown plants also revealed no major disparities between the wild type and the AtHsp93-V mutant (Figure 5.6). Again, no reduction in various thylakoid proteins was observed despite the overall decrease in thylakoid membrane within mutant chloroplasts.

Import into AtHsp93-V knockout mutant chloroplasts is impaired

In the pea chloroplast protein import system, an Hsp100 protein, Hsp93, is predicted to be the factor responsible for driving precursor translocation (Akita et al., 1997; Nielsen et al., 1997; Kouranov et al., 1998). Therefore, we analyzed AtHsp93-V

Figure 5.6. Total protein profiles of wild-type and AtHsp93-V mutant plants. Total leaf protein from two-week-old soil-grown plants (wild-type and AtHsp93-V knockout [KO] mutant lines) was extracted by boiling tissue samples in SDS and β-mercaptoethanol. Protein extract equivalent to equal amounts of starting fresh mass was separated by SDS-PAGE and stained with Coomassie Brilliant Blue R250 (lanes 1 and 2). Intact chloroplasts were isolated from four-week-old wild-type and AtHsp93-V mutant plants that had been grown on plates. Total chloroplasts equivalent to 15 μg chlorophyll were separated by electrophoresis, followed by staining with Coomassie Brilliant Blue R250 (lanes 3 and 4).

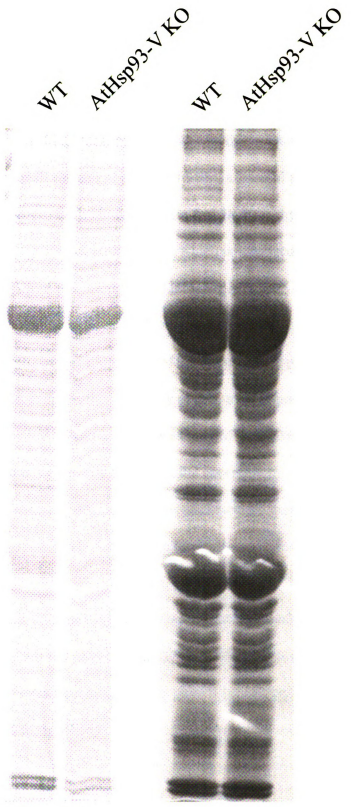


Figure 5.6

knockout plants, which lack one of the two *Arabidopsis* chloroplastic Hsp93 isoforms, to determine whether loss of the chaperone had any effect on import into mutant chloroplasts. First, we determined whether chloroplasts isolated from AtHsp93-V mutant plants were able to import a variety of precursor proteins during an *in vitro* assay. The precursors that were used represent four distinct subcompartments within the chloroplast: the precursor to the small subunit of Rubisco (prSS), a stromal protein; the precursor to light harvesting chlorophyll a/b binding protein (prLHCP), a thylakoid membrane protein; the precursor to plastocyanin (prPC), which is localized to the thylakoid lumen; and a truncated version of the precursor to Tic110 (tp110-110N), an integral protein of the inner envelope membrane. Mutant chloroplasts are able to import all of these precursors, as indicated by the appearance of the mature-sized protein, within ten minutes (data not shown). On average, the efficiency of precursor import into chloroplasts isolated from AtHsp93-V knockout plants was only 65% to 75% of that observed for chloroplasts isolated from the wild type, with the exception of prPC, which was imported with approximately the same efficiency as for wild-type chloroplasts (Figure 5.7). Thus, mutant chloroplasts appear to be impaired in the transport of some, but not all, precursor proteins.

Next, to investigate the import of precursor proteins into chloroplasts isolated from AtHsp93-V mutant plants in more detail, we compared the rate of prSS transport into either wild-type or mutant chloroplasts. prSS was very rapidly imported into chloroplasts isolated from wild-type plants. Conversion of prSS to SS, indicating that the precursor had been at least partially translocated across the chloroplast envelope, was observed even at the very earliest time points tested (Figure 5.8A). The import of prSS

Figure 5.7. Chloroplasts isolated from AtHsp93-V mutant plants are able to import a variety of precursor proteins, although not as efficiently as do wild-type chloroplasts. ^{35}S-labeled prSS (precursor to the small subunit of Rubisco, a stromal protein), ^{35}S-labeled prLHCP (precursor to the light harvesting chlorophyll a/b binding protein, a thylakoid membrane protein), ^{35}S-labeled prPC (precursor to plastocyanin, a thylakoid lumen-localized protein), and ^{35}S-labeled tp110-110N (a truncated version of the precursor to Tic110, an inner membrane-localized import component) were imported into chloroplasts (25 μg chlorophyll) isolated from wild-type or AtHsp93-V mutant plants. Plants had been plate-grown for approximately four weeks prior to chloroplast isolation. After ten or twenty minutes at room temperature in the light, import was stopped, and intact chloroplasts were recovered, by centrifuging the reactions through a 40% Percoll cushion. Equivalent amounts of chlorophyll from each sample were analyzed by SDS-PAGE and fluorography. The amount of radiolabeled precursor proteins imported into the chloroplasts was quantified using a phosphoimager. Values presented depict the amount of precursor imported into AtHsp93-V mutant chloroplasts as a percentage of the amount imported into wild-type chloroplasts and are the average of at least two independent experiments.

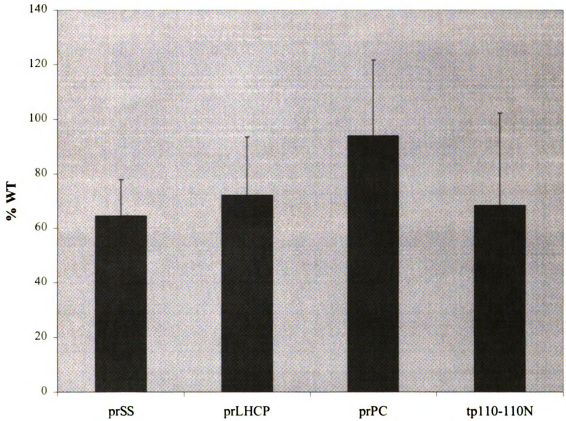


Figure 5.7

Figure 5.8. The rate of import of prSS into AtHsp93-V mutant chloroplasts is decreased in comparison to import into chloroplasts isolated from wild-type plants. (A) ^{35}S-labeled prSS (precursor to the small subunit of Rubisco) was imported into chloroplasts isolated from wild-type or AtHsp93-V knockout (KO) mutant plants. Plants had been plate-grown for approximately four weeks prior to chloroplast isolation. At the times indicated, aliquots equivalent to 25 μg chlorophyll were removed from the import reactions. Import was stopped, and intact chloroplasts were recovered, by centrifuging the aliquots through a 40% Percoll cushion. Equivalent amounts of chlorophyll from each sample were analyzed by SDS-PAGE and fluorography. TP = 1/10 volume of radioactive translation product added to each sample. SS = mature form of prSS. (B) Results from panel A (wild type = square; AtHsp93-V knockout = triangle) were quantified using a phosphoimager. Values depicted are the average of three independent import reactions.

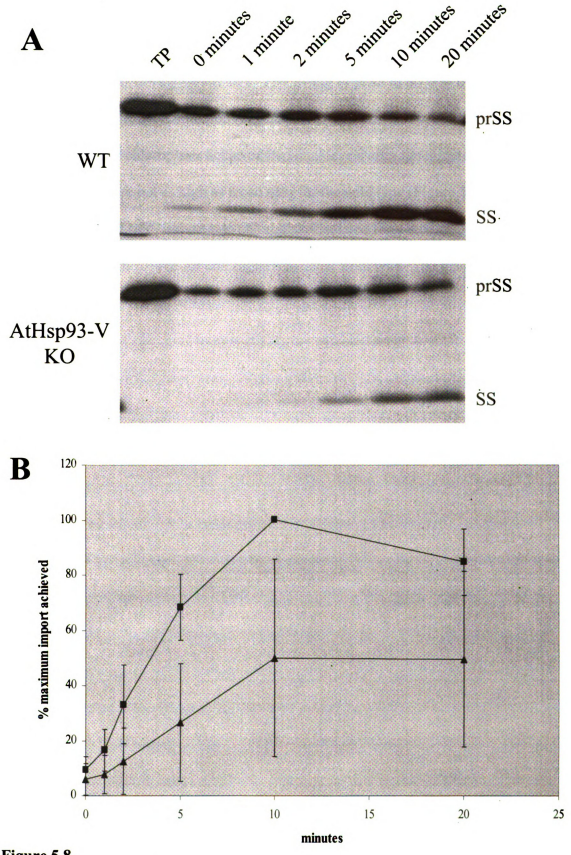

A
TP
0 minutes
1 minute
2 minutes
5 minutes
10 minutes
20 minutes
WT
prSS
SS
AtHsp93-V
KO
prSS
SS
B
% maximum import achieved
minutes
0
20
40
60
80
100
120
0
5
10
15
20
25

Figure 5.8

into wild-type chloroplasts was saturated within ten minutes (Figure 5.8B). Import of prSS into chloroplasts isolated from AtHsp93-V knockout mutant plants, however, proceeded at a significantly slower rate. Processing of prSS to SS was not apparent until two minutes after reaction initiation (Figure 5.8A). As for wild-type chloroplasts, prSS import into mutant chloroplasts achieved maximal levels after ten minutes, although the levels obtained were not as high as those seen for the wild type (Figure 5.8B). On average, the rate of prSS import into chloroplasts isolated from AtHsp93-V mutant plants was ~50% of that measured for chloroplasts isolated from wild-type plants.

DISCUSSION

At least two possibilities exist for the role of Hsp93, an Hsp100 protein of the ClpC subfamily, within chloroplasts. Hsp100s were first identified in *E. coli* as a component of an ATP-dependent protease complex (Hwang et al., 1987; Katayama et al., 1988). The Hsp100 identified, ClpA, in this two-subunit complex acts as a regulatory factor, controlling the action of the proteolytic component, ClpP (Hwang et al., 1987; Katayama et al., 1988). Chloroplasts of higher plants encode a homolog of the ClpP protein within the organellar genome (Shanklin et al., 1995; Boston et al., 1996). It is hypothesized that plastid-localized ClpC proteins, which are homologous to bacterial ClpA, may substitute for the function of ClpA in the plastid Clp proteolytic complex (Shanklin et al., 1995; Boston et al., 1996). Immunological experiments have demonstrated a possible interaction of ClpP and ClpC proteins within barley chloroplasts (Desimone et al., 1997). In addition, Hsp93 (a ClpC protein) isolated from pea chloroplasts has been found to stimulate the *in vitro* activity of bacterial ClpP, although it had no effect on the *in vitro* activity of recombinant pea ClpP (Shanklin et al., 1995).

Hsp93 has also been identified as a component of the protein import apparatus of pea chloroplasts (Akita et al., 1997; Nielsen et al., 1997; Kouranov et al., 1998). It is known that molecular chaperones are essential components of the posttranslational protein import systems of the mitochondria and the ER, providing the driving force to pull the incoming precursor proteins into the organelle (Jensen and Johnson, 1999; Pilon and Schekman, 1999; Rapoport et al., 1999; Herrmann and Neupert, 2000; Strub et al., 2000). Thus, it is predicted that a similar role for a chaperone exists within the chloroplastic protein transport machinery. As Hsp93 is the only chaperone consistently

found to be present within import complexes isolated from chloroplasts, it is currently the best candidate to provide the energy needed for precursor translocation (Akita et al., 1997; Nielsen et al., 1997; Kouranov et al., 1998).

In order to investigate the possible role of Hsp93 within chloroplasts in more detail, we isolated an *Arabidopsis* knockout line containing a disruption in both copies of *AtHSP93-V*, which encodes one of the two *Arabidopsis* chloroplast-localized Hsp93 homologs. The AtHsp93-V mutant line was distinctly paler than wild-type plants of the same ecotype (Figures 5.2 and 5.3). Thus, some aspect of chloroplast development is affected in the mutant plants. This impairment may be related to thylakoid development as AtHsp93-V mutant chloroplasts appeared to contain less thylakoid membrane than did wild-type chloroplasts (Figure 5.4). Interestingly, RNAi plants for AtHsp93-V display a similar phenotype (P. Jarvis, personal communication), confirming that the phenotype observed for the knockout mutant line is indeed due to the disruption in *AtHSP93-V* gene expression.

A reduction in the amount of thylakoid membrane could result from a defect of chloroplast protein import. Many proteins necessary for the development of thylakoids are encoded in the nucleus and must be imported into the organelle (Keegstra and Cline, 1999). Thus, if import into chloroplasts were impaired, thylakoid development would likely be influenced. AtHsp93-V mutant plants may indeed be altered in their capacity to import precursors into chloroplasts. When the rate of import of prSS, a stromal protein, was examined, a decrease of ~50% in the overall translocation rate into mutant chloroplasts was observed (Figure 5.8), suggesting that AtHsp93-V may indeed be important for the movement of precursors into the organelle. The translocation of two

additional chloroplastic proteins, prLHCP and tp110-110N, which also utilize the general import machinery, seemed to be impaired in the AtHsp93-V knockout line as well (Figure 5.7). However, the import of a fourth protein, prPC, into mutant chloroplasts was largely unaffected, indicating that AtHsp93-V function may not be needed for the transport of all precursor proteins. Additional experiments addressing the rate of import of a variety of precursors will be necessary to determine the exact effect the loss of AtHsp93-V function has on the chloroplast protein import process.

The endogenous levels of most plastid proteins were similar within wild-type and AtHsp93-V mutant plants, as measured by immunoblotting and Coomassie staining (Figures 5.5 and 5.6). Therefore, the import of proteins into chloroplasts *in vivo* may not be significantly affected in the mutant line, suggesting that an import defect may not be the cause of the pale phenotype observed for mutant plants. The reduction in import rate by one-half that was measured in this investigation would also probably be insufficient to explain the severity of the visible phenotype. These results would instead suggest that AtHsp93-V has a function unrelated to import *in vivo*, perhaps as part of the Clp proteolytic complex or in assisting imported proteins to achieve their native conformation and suborganellar location. A role for AtHsp93-V in protein import is not ruled out by these results, however. It is possible that this molecular chaperone normally performs multiple functions within *Arabidopsis* chloroplasts.

Molecular chaperones, including the Hsp70 proteins involved in the mitochondrial and ER protein import systems, have been found to be essential in many species (Baker and Schatz, 1991; Boston et al., 1996; Pfanner et al., 1997). Disrupting the gene for AtHsp93-V, a chloroplastic Hsp100 in *Arabidopsis*, however, is not lethal.

One explanation of these results is that the putative truncated version of AtHsp93-V that can be observed on Western blots is at least partially functional in the mutant line. Alternatively, another chaperone, most likely AtHsp93-III, a homolog of AtHsp93-V, is partly substituting for the loss of AtHsp93-V function in mutant plants. Interestingly, although AtHsp93-V and AtHsp93-III are almost 90% identical to one another at the amino acid level, AtHsp93-III cannot completely compensate for the disruption in *AtHSP93-V* gene expression. It is possible that the gene encoding AtHsp93-III is not expressed at the same developmental stages as *AtHSP93-V*, preventing the protein from being present at sufficient levels at the times when AtHsp93-V function is needed within *Arabidopsis* chloroplasts. Northern blots investigating the expression patterns of *AtHSP93-V* and *AtHSP93-III* throughout development are needed to test this hypothesis. On the other hand, AtHsp93-V and AtHsp93-III, despite their overall similarity, could actually perform specialized functions within chloroplasts. Thus, AtHsp93-III may be only able to partially compensate for the loss of functional AtHsp93-V because it can only inefficiently accomplish the task normally done by AtHsp93-V. We have isolated a knockout mutant line for the gene encoding AtHsp93-III in order to learn more about the function of this Hsp93 isoform within chloroplasts. Future work with the AtHsp93-III mutant line will involve characterizing its phenotype in a manner similar to what has been done for the AtHsp93-V mutant and crossing these two mutant lines to determine the effect of disrupting the expression of all Hsp93/ClpC isoforms normally present in *Arabidopsis* chloroplasts.

It is also possible that chaperones of other families, such as Hsp70s, could substitute for AtHsp93-V in mutant plants. This may indeed be the case if the role of

AtHsp93-V is to drive precursor protein translocation. Hsp70 proteins are known to perform this function in other posttranslational import systems (Jensen and Johnson, 1999; Pilon and Schekman, 1999; Rapoport et al., 1999; Herrmann and Neupert, 2000; Strub et al., 2000). In addition, a stromal Hsp70, S78, has been found in import complexes isolated from pea chloroplasts, although not under all conditions as Hsp93 is (Nielsen et al., 1997). Thus, the physiological relevance of the association of S78 with the import machinery is still a matter of debate. In AtHsp93-V knockout mutant plants, the protein levels of S78 appeared to be elevated (Figure 5.5), suggesting there may be a greater need for S78 in the absence of a functional AtHsp93-V protein. More experiments will be necessary to investigate this hypothesis.

Previous studies on the *Arabidopsis* chloroplast-localized Hsp100 proteins have indicated that these factors likely play a housekeeping role within the plastid, although the exact nature of this role has yet to be determined (Shanklin et al., 1995; Nakabayashi et al., 1999). We obtained AtHsp93-V knockout mutant plants in order to learn more about the possible functions of this molecular chaperone. Based on our current results, it is apparent that AtHsp93-V is important, but not essential, for normal chloroplast development and function. Additional work on the AtHsp93-V mutant line will attempt to further address what the role of this chaperone within chloroplasts may be, especially whether it directly or indirectly impacts the process of chloroplast protein import. In addition to the experiments mentioned in the above discussion, we plan to complement AtHsp93-V mutant plants with both wild-type and mutated versions of AtHsp93-V and AtHsp93-III. This work should give insight into whether various regions of these proteins, such as the nucleotide-binding domains, are necessary for their function and

whether these two isoforms are indeed functionally equivalent. In combination with the planned studies on the AtHsp93-III mutant line, it is hoped that these experiments will provide evidence regarding the hypothesis that an Hsp100 protein, rather than an Hsp70, provides the energy for protein import into chloroplasts.

REFERENCES

Akita M, Nielsen E, Keegstra K (1997) Identification of protein transport complexes in the chloroplastic envelope membranes via chemical cross-linking. J Cell Biol 136: 983-994

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24: 1-15

Baker KP, Schatz G (1991) Mitochondrial proteins essential for viability mediate protein import into yeast mitochondria. Nature 349: 205-208

Bauerle C, Keegstra K (1991) Full-length plastocyanin precursor is translocated across isolated thylakoid membranes. J Biol Chem 266: 5876-5883

Boston RS, Viitanen PV, Vierling E (1996) Molecular chaperones and protein folding in plants. Plant Mol Biol 32: 191-222

Bruce BD, Perry S, Froehlich J, Keegstra K (1994) *In vitro* import of proteins into chloroplasts. Plant Mol Biol Manual J1: 1-15

Chen X, Schnell DJ (1999) Protein import into chloroplasts. Trends Cell Biol 9: 222-227

Clarke AK, Eriksson M-J (1996) The cyanobacterium *Synechococcus* sp. PCC 7942 possesses a close homologue to the chloroplast ClpC protein of higher plants. Plant Mol Biol 31: 721-730

Derré I, Rapoport G, Devine K, Rose M, Msadek T (1999) ClpE, a novel type of HSP100 ATPase, is part of the CtsR heat shock regulon of *Bacillus subtilis*. Mol Microbiol 32: 581-593

Desimone M, Beiβ-Wichert C, Wagner E, Altenfeld U, Johanningmeier U (1997) Immunological studies on the Clp-protease in chloroplasts: evidence for the formation of a ClpC/P complex. Bot Acta 110: 234-239

Fitzpatrick LM, Keegstra K (2001) A method for isolating a high yield of *Arabidopsis* chloroplasts capable of efficient import of precursor proteins. Plant J 27: 59-65

Herrmann JM, Neupert W (2000) Protein transport into mitochondria. Curr Opin Microbiol 3: 210-214

Howe GA, Lee GI, Itoh A, Li L, DeRocher AE (2000) Cytochrome P450-dependent metabolism of oxylipins in tomato. Cloning and expression of allene oxide synthase and fatty acid hydroperoxide lyase. Plant Physiol 123: 711-724

Hwang BJ, Park WJ, Chung CH, Goldberg AL (1987) *Escherichia coli* contains a soluble ATP-dependent protease (Ti) distinct from protease La. Proc Natl Acad Sci USA 84: 5550-5554

Jensen RE, Johnson AE (1999) Protein translocation: is Hsp70 pulling my chain? Curr Biol 9: R779-R782

Katayama Y, Gottesman S, Pumphrey J, Rudikoff S, Clark WP, Maurizi MR (1988) The two-component, ATP-dependent Clp protease of *Escherichia coli*: purification, cloning, and mutational analysis of the ATP-binding component. J Biol Chem 263: 15226-15236

Keegstra K, Cline K (1999) Protein import and routing systems of chloroplasts. Plant Cell 11: 557-570

Keegstra K, Froehlich JE (1999) Protein import into chloroplasts. Curr Opin Plant Biol 2: 471-476

Kouranov A, Chen X, Fuks B, Schnell DJ (1998) Tic20 and Tic22 are new components of the protein import apparatus at the chloroplast inner envelope membrane. J Cell Biol 143: 991-1002

Krüger E, Völker U, Hecker M (1994) Stress induction of *clpC* in *Bacillus subtilis* and its involvement in stress tolerance. J Bacteriol 176: 3360-3367

Krysan PJ, Young JC, Sussman MR (1999) T-DNA as an insertional mutagen in Arabidopsis. Plant Cell 11: 2283-2290

Krysan PJ, Young JC, Tax F, Sussman MR (1996) Identification of transferred DNA insertions within *Arabidopsis* genes involved in signal transduction and ion transport. Proc Natl Acad Sci USA 93: 8145-8150

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685

Lazazzera BA, Grossman AD (1997) A regulatory switch involving a Clp ATPase. BioEssays 19: 455-458

Lübeck J, Heins L, 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

May T, Soll J (1999) Chloroplast precursor protein translocon. FEBS Lett 452: 52-56

Mhammedi-Alaoui A, Pato M, Gama M-J, Toussaint A (1994) A new component of bacteriophage Mu replicative transposition machinery: the *Escherichia coli* ClpX protein. Mol Microbiol 11: 1109-1116

Moore T, Keegstra K (1993) Characterization of a cDNA clone encoding a chloroplast-targeted Clp homologue. Plant Mol Biol 21: 525-537

Nakabayashi K, Ito M, Kiyosue T, Shinozaki K, Watanabe A (1999) Identification of *clp* genes expressed in senescing *Arabidopsis* leaves. Plant Cell Physiol 40: 504-514

Nakashima K, Kiyosue T, Yamaguchi-Shinozaki K, Shinozaki K (1997) A nuclear gene, *erd1*, encoding a chloroplast-targeted Clp protease regulatory subunit homolog is not only induced by water stress but also developmentally up-regulated during senescence in *Arabidopsis thaliana*. Plant J 12: 851-861

Nielsen EE (1997) The role of stromal molecular chaperones in chloroplastic protein translocation. Ph.D. Dissertation, Michigan State University, East Lansing, MI

Nielsen E, Akita M, Davila-Aponte J, Keegstra K (1997) Stable association of chloroplastic precursors with protein translocation complexes that contain proteins from both envelope membranes and a stromal Hsp100 molecular chaperone. EMBO J 16: 935-946

Ostersetzer O, Adam Z (1996) Effects of light and temperature on expression of ClpC, the regulatory subunit of chloroplastic Clp protease, in pea seedlings. Plant Mol Biol 31: 673-676

Pfanner N, Craig EA, Hönlinger A (1997) Mitochondrial preprotein translocase. Annu Rev Cell Dev Biol 13: 25-51

Pilon M, Schekman R (1999) Protein translocation: how Hsp70 pulls it off. Cell 97: 679-682

Rapoport TA, Matlack KES, Plath K, Misselwitz B, Staeck O (1999) Posttranslational protein translocation across the membrane of the endoplasmic reticulum. Biol Chem 380: 1143-1150

Sanchez Y, Lindquist S (1990) HSP104 required for induced thermotolerance. Science 248: 1112-1115

Schirmer EC, Glover JR, Singer MA, Lindquist S (1996) HSP100/Clp proteins: a common mechanism explains diverse functions. Trends Biochem Sci 21: 289-296

Schleiff E, Soll J (2000) Travelling of proteins through membranes: translocation into chloroplasts. Planta 211: 449-456

Schnell DJ, Kessler F, Blobel G (1994) Isolation of components of the chloroplast protein import machinery. Science 266: 1007-1012

Shanklin J, DeWitt ND, Flanagan JM (1995) The stroma of higher plant plastids contain ClpP and ClpC, functional homologs of *Escherichia coli* ClpP and ClpA: an archetypal two-component ATP-dependent protease. Plant Cell 7: 1713-1722

Squires CL, Pedersen S, Ross BM, Squires C (1991) ClpB is the *Escherichia coli* heat shock protein F84.1. J Bacteriol 173: 4254-4262

Stokes KD, McAndrew RS, Figueroa R, Vitha S, Osteryoung KW (2000) Chloroplast division and morphology are differentially affected by overexpression of *FtsZ1* and *FtsZ2* genes in Arabidopsis. Plant Physiol 124: 1668-1677

Strub A, Lim JH, Pfanner N, Voos W (2000) The mitochondrial protein import motor. Biol Chem 381: 943-949

Theg SM, Bauerle C, Olsen LJ, Selman BR, Keegstra K (1989) Internal ATP is the only energy requirement for the translocation of precursor proteins across chloroplastic membranes. J Biol Chem 264: 6730-6736

Tranel PJ, Froehlich J, Goyal A, Keegstra K (1995) A component of the chloroplastic protein import apparatus is targeted to the outer envelope membrane via a novel pathway. EMBO J 14: 2436-2446

Vothknecht UC, Soll J (2000) Protein import: the hitchhikers guide into chloroplasts. Biol Chem 381: 887-897

Wawrzynow A, Wojtkowiak D, Marszalek J, Banecki B, Jonsen M, Graves B, Georgopoulos C, Zylicz M (1995) The ClpX heat-shock protein of *Escherichia coli*, the ATP-dependent substrate specificity component of the ClpP-ClpX protease, is a novel molecular chaperone. EMBO J 14: 1867-1877

Wickner S, Gottesman S, Skowyra D, Hoskins J, McKenney K, Maurizi MR (1994) A molecular chaperone, ClpA, functions like DnaK and DnaJ. Proc Natl Acad Sci USA 91: 12218-12222

CHAPTER 6

Conclusions and future directions

When this dissertation research was begun, only five components of the chloroplastic protein import machinery had been identified: Toc159, Toc75, Toc34, Tic110, and Hsp93 (Waegemann and Soll, 1991; Hirsch et al., 1994; Kessler et al., 1994; Perry and Keegstra, 1994; Schnell et al., 1994; Seedorf et al., 1995; Tranel et al., 1995; Kessler and Blobel, 1996; Lübeck et al., 1996; Akita et al., 1997; Nielsen et al., 1997). Two of these proteins, Toc159 and Toc75, had been studied in some detail, and several lines of experimental evidence were emerging to support hypotheses concerning their molecular function (Hirsch et al., 1994; Perry and Keegstra, 1994; Tranel et al., 1995; Ma et al., 1996; Hinnah et al., 1997). Less was known about the remaining three proteins, on the other hand, and all of those have been a focus of this dissertation research. More recently, five additional proteins have been found within isolated import complexes, and it is predicted that other, as yet unidentified, subunits are present as well (Caliebe et al., 1997; Kouranov and Schnell, 1997; Kouranov et al., 1998; Stahl et al., 1999; Sohrt and Soll, 2000). Overall, the focus of research in the field of chloroplast protein import, however, has been moving away from identification of components of the import apparatus to studies addressing the possible functions of these proteins during precursor transport. Thus, the goal of this dissertation research has been to use the experimental tools currently available, both biochemical and genetic, to investigate subunit function in more detail.

Initially, we took a biochemical approach to study pea Tic110, which had only recently been identified (Kessler and Blobel, 1996; Lübeck et al., 1996). Our investigation into the topology of this protein within the chloroplast inner envelope membrane confirmed that the large, hydrophilic C-terminal domain of Tic110 faces the

interior of the chloroplast (Chapter 2). Because this C-terminal domain is preceded only by a transmembrane domain and a very short N-terminal hydrophilic region (Kessler and Blobel, 1996; Lübeck et al., 1996), it is likely that the functional residues of Tic110 are localized within the plastid stroma. Current speculation regarding the function of Tic110 during chloroplast protein import assumes that this large C-terminal domain is involved in protein-protein interactions with other members of the import complex (Chen and Schnell, 1999; Keegstra and Cline, 1999; Keegstra and Froehlich, 1999; May and Soll, 1999; Schleiff and Soll, 2000; Vothknecht and Soll, 2000). Specifically, it is believed that Tic110 may be involved in recruiting molecular chaperones to the site of precursor import, similar to the role played by Tim44 in the mitochondrial protein import system (Kessler and Blobel, 1996; Nielsen et al., 1997; Kouranov et al., 1998). Tim44 serves as an anchor for the import associated-mitochondrial Hsp70 protein, which is involved both in providing the energy for precursor translocation and in assisting with the proper folding of newly-imported proteins (Pfanner et al., 1997; Herrmann and Neupert, 2000). Tic110 may interact with chaperones performing either or both of these functions. In a study by Kessler and Blobel (1996), it was found that chaperonin (cpn) 60, a member of the Hsp60 family of chaperones, was the major protein coimmunoprecipitated by Tic110. The Tic110-cpn60 complex is localized in the vicinity of contact sites formed between the outer and inner membranes of the chloroplast envelope, which are presumed to be the sites of precursor protein import (Kouranov et al., 1998). The mature form of the small subunit of Rubisco (mSS) associates with this Tic110-cpn60 complex in a transient, ATP-sensitive manner, suggesting that cpn60 is interacting with mSS in order to assist it in achieving its native conformation as it emerges from the translocation channel (Kessler

and Blobel, 1996). Additional studies have found that Hsp93, which is predicted to be the motor for protein import into chloroplasts, can also associate with Tic110 (Nielsen et al., 1997; Kouranov et al., 1998). This Tic110-Hsp93 association is not dependent on contact site formation (Nielsen et al., 1997; Kouranov et al., 1998).

Future work on the role of Tic110 during chloroplast protein import should focus first on determining whether this protein directly or indirectly interacts with either cpn60 or Hsp93. If the interaction between Tic110 and one or both of these chaperones is indeed direct, this would provide strong evidence for the hypothesis that the function of Tic110 is to recruit chaperones to the protein import complex. Both coimmunoprecipitation and direct binding assays are being conducted in our lab in order to address the nature of the association between Tic110 and Hsp93 (M. Akita and K. Keegstra, unpublished observations). If neither Hsp93 nor cpn60 are found to directly bind to Tic110, then additional studies will be necessary to determine with what proteins the hydrophilic domain of Tic110 can interact. Once the putative partner or partners of Tic110 have been determined, investigations addressing the regions of Tic110 that are important for this protein-protein interaction can be conducted.

Shortly after this dissertation research was initiated, reverse genetic resources in *Arabidopsis thaliana* started to become widely available. As a consequence, a decision was made to shift our research efforts from pea to *Arabidopsis*. In order to do this, we first needed to establish that homologs of the chloroplastic protein import complex components were indeed present in *Arabidopsis* (Chapter 3). Our analysis of the *Arabidopsis* genome sequence revealed that at least one expressed homolog for each of the known members of the pea import machinery was present in this species. Thus, it

was concluded that *Arabidopsis* could indeed serve as a model for the genetic study of chloroplast protein import.

Most of the import components have multiple homologs in *Arabidopsis*. In all but one case, all of these homologs are known to be expressed. Therefore, it is possible that multiple types of import complexes exist within *Arabidopsis* plastids, each containing a specific combination of the various isoforms for each subunit (Jarvis et al., 1998; Bauer et al., 2000; Gutensohn et al., 2000; Yu and Li, 2001). It would be interesting to investigate this phenomenon in more detail, studying whether there is tissue or developmental-specific expression of the homologs for a particular import component. Such experiments have already been carried out for two subunits of the outer envelope membrane import complex, Toc159 and Toc34 (Jarvis et al., 1998; Bauer et al., 2000; Gutensohn et al., 2000; Yu and Li, 2001). Toc159 has three homologs in *Arabidopsis*: AtToc159, AtToc132, and AtToc120. The gene for AtToc159 is most highly expressed in aboveground, light-grown tissue (Bauer et al., 2000; Yu and Li, 2001), supporting the hypothesis that AtToc159 may be the primary receptor for photosynthesis-related proteins, which would be the major group of proteins being imported into chloroplasts present in the aboveground tissues. AtToc132 and AtToc120, on the other hand, could be the primary receptors for nonphotosynthetic proteins imported into all plastid types (Bauer et al., 2000). In contrast to Toc159, Toc34 only has two homologs in *Arabidopsis* chloroplasts, known as AtToc34 and AtToc33. The genes for these two proteins also appear to be differentially expressed, with the gene for AtToc33 being elevated in some tissues while the gene for AtToc34 being upregulated in others (Gutensohn et al., 2000; Yu and Li, 2001). These results, like those for the Toc159 homologs, suggest that there

are in fact multiple types of import complexes present in *Arabidopsis* plastids, some containing primarily one isoform of an import component and others containing primarily a different isoform. Additional work should now be done to see if other components of the import apparatus give similar results and to determine the significance of these observations to the process of chloroplast protein import. One possibility is that each combination of specific isoforms is specialized for the transport of a subset of precursor proteins (Bauer et al., 2000; Gutensohn et al., 2000; Yu and Li, 2001). Different plastid types, such as amyloplasts, chloroplasts, or chromoplasts, would contain one or more isoforms for each subunit depending on the types of precursor proteins that are required within the plastid.

As more genomic and EST sequence information becomes available for species other than *Arabidopsis*, it would be useful to determine whether multiple isoforms for the components of the import machinery exist in these species as well. An analysis of the EST sequence database for homologs of Toc75 and Tic110 has indicated that multiple isoforms of these proteins are present in some higher plant species (J. Davila-Aponte et al., submitted). This work needs to be extended to examine all of the known members of the chloroplastic protein import apparatus. These results can then be used in combination with the expression analysis done in *Arabidopsis* to investigate whether particular isoforms do indeed provide specificity to the process of precursor translocation.

Having established that *Arabidopsis* can indeed serve as a model system for the study of protein import into chloroplasts, we isolated knockout mutant lines for two putative import components: AtToc34 and AtHsp93-V (Chapters 4 and 5). Initial studies with these mutant lines have already allowed us to gain some insight into the possible

functions of these proteins. For example, attempts to generate a double homozygous mutant line that lacks both AtToc34 and its homolog, AtToc33, indicate that AtToc33/AtToc34 function is essential in *Arabidopsis*. This was somewhat surprising because we believed AtToc33/AtToc34 to be regulatory components that modulate the efficiency of the import process. These results would suggest, however, that AtToc33/AtToc34 play a much more important role in chloroplasts. If they are indeed regulatory factors, then the process that they are controlling must be essential for plastid protein import to occur.

One possibility for the function of AtToc33/AtToc34 in *Arabidopsis* chloroplasts is that they could act as docking sites within the outer envelope membrane for cytoplasmic Toc159 (Hiltbrunner et al., 2001). Toc159 is predicted to be the receptor for precursor proteins (Hirsch et al., 1994; Perry and Keegstra, 1994). Originally, it was believed that the recognition of transit peptides by Toc159 occurred at the chloroplast surface. Recent evidence, however, suggests that Toc159 may actually interact with precursor proteins within the cytoplasm and then insert, with the bound preprotein, into the outer membrane of the chloroplast envelope (Hiltbrunner et al., 2001). This putative mechanism is similar to the method used by signal recognition particle (SRP) to bring precursors to the cotranslational import complex of the ER (Rapoport et al., 1996; Hiltbrunner et al., 2001). It is possible that the regulatory function of AtToc33/AtToc34 involves recognizing the Toc159-precursor complex and/or assisting in its insertion into the outer membrane (Hiltbrunner et al., 2001). One way to test this prediction would be to analyze Toc159 localization in the AtToc33 knockout mutant, *ppi1*, which is impaired in the import of proteins into chloroplasts (Jarvis et al., 1998). If, in the mutant, more

Toc159 is found in the cytoplasm and less in the outer envelope membrane than is the case in wild-type plants, this would support the hypothesis that AtToc33, and AtToc34, is important for Toc159 insertion into the chloroplast envelope.

AtToc33 and AtToc34 are both GTP-binding proteins (Kessler et al., 1994; Seedorf et al., 1995; Jarvis et al., 1998). Consequently, it is believed that GTP binding and hydrolysis are important for the function of AtToc33/AtToc34 during chloroplast protein import (Kessler et al., 1994; Seedorf et al., 1995). It would be interesting to explore this hypothesis in more detail with mutated versions of AtToc33 and AtToc34 that have altered GTP-binding domains. These mutated forms of AtToc33 and AtToc34 could be used to attempt to complement the pale phenotype of *ppi1* plants. *ppi3* mutant plants, which lack AtToc34, would not be useful in these studies because they have neither an obvious visible phenotype nor a measurable import defect. Mutations that disrupt normal AtToc33/AtToc34 function would prevent the introduced proteins from complementing the *ppi1* phenotype. Proteins containing mutations that had no effect on AtToc33/AtToc34 function, however, would restore the wild-type phenotype to the mutant plants. In this manner, it could be determined what residues are important for AtToc33/AtToc34 to function properly in *Arabidopsis*. In particular, residues known to be essential for GTP binding and hydrolysis in other GTP-binding proteins could be tested for their effect on AtToc33/AtToc34 function.

Results obtained from studies of the AtHsp93-V knockout mutant line suggest that this protein is important for normal chloroplast development. Mutant plants are paler than wild-type plants, and their growth is severely inhibited. In addition, chloroplasts isolated from the mutant seem to contain less thylakoid membrane than do wild-type

chloroplasts. This decrease in thylakoid membrane content could explain both the pale phenotype and the growth defect of mutant plants. Surprisingly, the function of AtHsp93-V seems to be somewhat unique in *Arabidopsis* because other stromal molecular chaperones, including a homolog, AtHsp93-III, that is almost 90% identical to AtHsp93-V, cannot completely compensate for the disruption in *AtHSP93-V* gene expression. However, because it is assumed that a complete loss of the chaperone function provided by AtHsp93-V would be lethal in *Arabidopsis*, we hypothesize that other chaperones are able to at least partially substitute for AtHsp93-V in the mutant plants. It is possible that an Hsp70 protein, S78, may be performing a task normally done by AtHsp93-V, as the protein levels of S78 are elevated in the mutant. Investigations are ongoing in our lab to address this question as well as to determine possible reasons for the inability of AtHsp93-III to substitute for its homolog.

Unfortunately, the experiments done on the AtHsp93-V knockout mutant line have not allowed us to differentiate between possible functions for this molecular chaperone within chloroplasts. We believe that AtHsp93-V is the translocation motor for chloroplast protein import (Akita et al., 1997; Nielsen et al., 1997). However, at this time, our results with the mutant neither support nor refute this hypothesis. Chloroplasts isolated from AtHsp93-V knockout plants do show an import defect *in vitro*, at least for some precursors. However, analysis of endogenous chloroplastic protein levels in mutant plants suggests that import is unaffected *in vivo*. Thus, it is possible that the *in vivo* phenotype is caused not by a defect in chloroplast protein import. Instead, the phenotype could be related to other putative roles of AtHsp93-V, including as a component of the Clp (caseinolytic protease) proteolytic complex or as a factor assisting protein folding.

Future work on the AtHsp93-V mutant line will attempt to differentiate between these possible functions.

As previously mentioned, there are two chloroplastic Hsp100 homologs in *Arabidopsis*: AtHsp93-V and AtHsp93-III. Thus, it is possible that AtHsp93-V is not the stromal Hsp100 protein required for chloroplast protein import. AtHsp93-III may be involved in precursor protein import, while AtHsp93-V is needed for other tasks within the plastid, such as protein degradation. In order to address this hypothesis, we plan to characterize mutant plants disrupted in the gene encoding AtHsp93-III. Our initial studies of this knockout mutant line will be similar to what has been done for the AtHsp93-V mutant line. In particular, it will be interesting to see if the AtHsp93-III mutant plants have either an *in vivo* or an *in vitro* import defect and if they do, whether that defect is more or less severe than that observed for the AtHsp93-V knockout mutant.

Finally, the AtHsp93-V mutant line, due to its obvious pale phenotype visible even in very young plants, will be a useful tool to study site-directed mutants of Hsp93 proteins. As described above for the AtToc33 and AtToc34 proteins, AtHsp93-V or AtHsp93-III proteins disrupted in their nucleotide-binding domains can be used to attempt to complement the AtHsp93-V phenotype. ATPase assays using mutant pea Hsp93 proteins indicate that the first nucleotide-binding domain of this molecular chaperone is required for ATP hydrolysis (M. Akita and K. Keegstra, submitted). The second nucleotide-binding domain may be important for the interaction of Hsp93 proteins with the chloroplast inner envelope membrane (M. Akita and K. Keegstra, submitted). By transforming AtHsp93-V knockout mutant plants with mutant versions of

AtHsp93-V/AtHsp93-III disrupted in one of these nucleotide-binding regions, we may be able to determine whether ATPase activity and/or membrane association are necessary for the normal function of AtHsp93-V/AtHsp93-III.

Much remains to be learned about the individual roles of the import complex components during the transport of precursor proteins into chloroplasts. This dissertation research has addressed this question in detail for three members of the import machinery: Tic110, Toc34 and Hsp93. In addition, we have analyzed the translocation apparatus on a more global scale by looking at the complete complement of known import components in *Arabidopsis*. Through this research, several important findings regarding component function have been made, including that the putative functional domain of Tic110 is localized within the chloroplast stroma and that AtToc33/AtToc34 function is essential in *Arabidopsis*. In addition, many questions and hypotheses have been generated that will need to be investigated. It is hoped that the tools developed during this research, especially the isolation of T-DNA insertional mutants for AtToc34 and AtHsp93-V, will be useful in future studies addressing the mechanism of protein import into plastids.

REFERENCES

Akita M, Nielsen E, Keegstra K (1997) Identification of protein transport complexes in the chloroplastic envelope membranes via chemical cross-linking. J Cell Biol 136: 983-994

Bauer J, Chen K, Hiltbunner A, Wehrli E, Eugster M, Schnell D, Kessler F (2000) The major protein import receptor of plastids is essential for chloroplast biogenesis. Nature 403: 203-207

Caliebe A, Grimm R, Kaiser G, Lübeck J, Soll J, Heins L (1997) The chloroplastic protein import machinery contains a Rieske-type iron-sulfur cluster and a mononuclear iron-binding protein. EMBO J 16: 7342-7350

Chen X, Schnell DJ (1999) Protein import into chloroplasts. Trends Cell Biol 9: 222-227

Gutensohn M, Schulz B, Nicolay P, Flügge U-I (2000) Functional analysis of the two *Arabidopsis* homologues of Toc34, a component of the chloroplast protein import apparatus. Plant J 23: 771-783

Herrmann JM, Neupert W (2000) Protein transport into mitochondria. Curr Opin Microbiol 3: 210-214

Hiltbrunner A, Bauer J, Vidi P-A, Infanger S, Weibel P, Hohwy M, Kessler F (2001) Targeting of an abundant cytosolic form of the protein import receptor atToc159 to the outer chloroplast membrane. J Cell Biol 154: 309-316

Hinnah SC, Hill K, Wagner R, Schlicher T, Soll J (1997) Reconstitution of a chloroplast protein import channel. EMBO J 16: 7351-7360

Hirsch S, Muckel E, Heemeyer F, von Heijne G, Soll J (1994) A receptor component of the chloroplast protein translocation machinery. Science 266: 1989-1992

Jarvis P, Chen L-J, Li H-M, Peto CA, Fankhauser C, Chory J (1998) An *Arabidopsis* mutant defective in the plastid general protein import apparatus. Science 282: 100-103

Keegstra K, Cline K (1999) Protein import and routing systems of chloroplasts. Plant Cell 11: 557-570

Keegstra K, Froehlich JE (1999) Protein import into chloroplasts. Curr Opin Plant Biol 2: 471-476

Kessler F, Blobel G (1996) Interaction of the protein import and folding machineries in the chloroplast. Proc Natl Acad Sci USA 93: 7684-7689

Kessler F, Blobel G, Patel HA, Schnell DJ (1994) Identification of two GTP-binding proteins in the chloroplast protein import machinery. Science 266: 1035-1039

Kouranov A, Chen X, Fuks B, Schnell DJ (1998) Tic20 and Tic22 are new components of the protein import apparatus at the chloroplast inner envelope membrane. J Cell Biol 143: 991-1002

Kouranov A, Schnell DJ (1997) Analysis of the interactions of preproteins with the import machinery over the course of protein import into chloroplasts. J Cell Biol 139: 1677-1685

Lübeck J, Soll J, Akita M, Nielsen E, Keegstra K (1996) Topology of IEP110, a component of the chloroplastic protein import machinery present in the inner envelope membrane. EMBO J 15: 4230-4238

Ma Y, Kouranov A, LaSala SE, Schnell DJ (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

May T, Soll J (1999) Chloroplast precursor protein translocon. FEBS Lett 452: 52-56

Nielsen E, Akita M, Davila-Aponte J, Keegstra K (1997) Stable association of chloroplastic precursors with protein translocation complexes that contain proteins from both envelope membranes and a stromal Hsp100 molecular chaperone. EMBO J 16: 935-946

Perry SE, Keegstra K (1994) Envelope membrane proteins that interact with chloroplastic precursor proteins. Plant Cell 6: 93-105

Pfanner N, Craig EA, Hönlinger A (1997) Mitochondrial preprotein translocase. Annu Rev Cell Dev Biol 13: 25-51

Rapoport TA, Rolls MM, Jungnickel B (1996) Approaching the mechanism of protein transport across the ER membrane. Curr Opin Cell Biol 8: 499-504

Schleiff E, Soll J (2000) Travelling of proteins through membranes: translocation into chloroplasts. Planta 211: 449-456

Schnell DJ, Kessler F, Blobel G (1994) Isolation of components of the chloroplast protein import machinery. Science 266: 1007-1012

Seedorf M, Waegemann K, Soll J (1995) A constituent of the chloroplast import complex represents a new type of GTP-binding protein. Plant J 7: 401-411

Sohrt K, Soll J (2000) Toc64, a new component of the protein translocon of chloroplasts. J Cell Biol 148: 1213-1221

Stahl T, Glockmann C, Soll J, Heins L (1999) Tic40, a new "old" subunit of the chloroplast protein import translocon. J Biol Chem 274: 37467-37472

Tranel PJ, Froehlich J, Goyal A, Keegstra K (1995) A component of the chloroplastic protein import apparatus is targeted to the outer envelope membrane via a novel pathway. EMBO J 14: 2436-2446

Vothknecht UC, Soll J (2000) Protein import: the hitchhikers guide into chloroplasts. Biol Chem 381: 887-897

Waegemann K, Soll J (1991) Characterization of the protein import apparatus in isolated outer envelopes of chloroplasts. Plant J 1: 149-158

Yu T-S, Li H-M (2001) Chloroplast protein translocon components atToc159 and atToc33 are not essential for chloroplast biogenesis in guard cells and root cells. Plant Physiol 127: 90-96

APPENDIX

INTRODUCTION

Chapter 2 describes our efforts to define the topology of Tic110, a component of the chloroplastic protein import apparatus, within the inner envelope membrane of pea chloroplasts. The experiments reported in Chapter 2, all of which have been published previously (Jackson DT, Froehlich JE, Keegstra K [1998] J Biol Chem 273: 16583-16588), utilize protease digestion techniques to ascertain Tic110 topology. However, during the course of this investigation, we also used a variety of other reagents in an attempt to establish the orientation of Tic110 within the inner membrane. That work, which has never been submitted for publication, is presented here.

MATERIALS AND METHODS

Chloroplasts were isolated from 8 to 12-day-old pea seedlings as described previously (Bruce et al., 1994). Intact chloroplasts were incubated, at room temperature, with Sulfo-NHS-Biotin (Pierce, Rockford, Illinois, USA) at a final concentration of 40 μM. At the times indicated in the figures, the biotinylation reactions were quenched with Tris-HCl (pH 7.5) at a final concentration of 100 mM. Quenched samples were incubated for 10 minutes at room temperature. Intact chloroplasts from each sample were then reisolated by centrifugation through a 40% Percoll cushion and lysed hypotonically, as described previously (Bruce et al., 1994). After a 10 minute incubation on ice, lysed chloroplasts were separated into crude membrane and soluble fractions as described by Bruce *et al.* (1994).

For immunoprecipitation, membrane proteins were solubilized in 25 mM HEPES-KOH (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1% decylmaltoside prior to the addition of antibodies against either Tic110 (Akita et al., 1997) or Toc75 (Tranel et al., 1995). Sepharose beads conjugated to protein A were also included in the immunoprecipitation reactions. After 2 hours at 4^0C, the protein A-sepharose beads were recovered from each sample and washed three times in 25 mM HEPES-KOH (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1% decylmaltoside and once in the same buffer without decylmaltoside. Proteins bound to the beads were eluted by boiling the reactions in SDS-PAGE sample buffer for 5 minutes.

SDS-PAGE was performed essentially as described previously (Laemmli, 1970); samples were loaded on the basis of equal amounts of starting chlorophyll. After SDS-PAGE, the separated proteins were transferred overnight to Immobilon-P PVDF

membranes (Millipore, Bedford, Massachusetts, USA). Biotinylated proteins were detected with avidin conjugated to alkaline phosphatase (Pierce, Rockford, Illinois, USA). The presence of avidin was visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

RESULTS

Our initial efforts to investigate the topology of Tic110 within the chloroplast inner envelope membrane focused not on the sensitivity of the protein to various proteases, as reported in Chapter 2, but rather on our ability to label Tic110 with membrane-impermeable reagents. For this work, we utilized Sulfo-NHS-Biotin, a biotinylation reagent that reportedly cannot cross lipid bilayers (Cole et al., 1987). Sulfo-NHS-Biotin reacts covalently with primary amine groups, thus labeling macromolecules, including proteins, with the biotin moiety. The presence of biotin on a protein can then be detected with various avidin reagents, which bind specifically to the biotin molecules.

Based on the fact that Sulfo-NHS-Biotin is sold as a membrane-impermeable reagent, we believed that it would be unable to cross the inner envelope membrane of chloroplasts. Because the chloroplast outer envelope membrane contains pores that allow the passage of small molecules (Keegstra et al., 1984), however, Sulfo-NHS-Biotin, which has a mass of 443 Daltons, should be able to pass through the outer membrane and gain access to the intermembrane space of the chloroplast envelope. Thus, this biotinylation reagent should be able to react with inner envelope membrane proteins that are exposed to the intermembrane space, but not with those that are largely facing the chloroplast stroma. Our earlier results had indicated that the large hydrophilic domain of Tic110 was oriented toward the intermembrane space (Lübeck et al., 1996). As a consequence, we predicted that Tic110 would be labeled by Sulfo-NHS-Biotin.

In order to determine whether Tic110 was indeed specifically labeled by Sulfo-NHS-Biotin, we incubated intact chloroplasts with this biotinylation reagent. After the biotinylation reaction, we reisolated intact chloroplasts, solubilized membrane proteins in

detergent, and immunoprecipitated Tic110. As can be seen in Figure A.1, Tic110 was not labeled by Sulfo-NHS-Biotin during these reactions. These results would suggest that the large hydrophilic domain of Tic110 is oriented toward the interior of the chloroplast, contrary to our initial prediction. Toc75, an integral protein of the chloroplast outer envelope membrane, was labeled by Sulfo-NHS-Biotin, as expected (Figure A.1).

In order to establish whether Sulfo-NHS-Biotin was in fact unable to cross the chloroplast inner envelope membrane, we analyzed the biotinylation of total chloroplast protein fractions. Following incubation with Sulfo-NHS-Biotin, intact chloroplasts were recovered and separated into membrane and soluble fractions. The biotinylation status of proteins within these fractions was detected with avidin conjugated to alkaline phosphatase. At even the earliest time points, biotin labeling of soluble chloroplastic proteins could be observed (Figure A.2). Because the vast majority of soluble proteins within the chloroplast are localized in the stroma, this result indicated that Sulfo-NHS-Biotin was able to cross the inner envelope membrane, even though it has been reported to be a membrane-impermeable reagent (Cole et al., 1987).

In addition to the experiments described above, we attempted to specifically label inner membrane proteins exposed to the intermembrane space of the chloroplast envelope with other reagents, such as biotin-BMCC, iodoacetyl-LC-biotin, and ^{3}H-iodoacetic acid. However, in all cases, we observed rapid labeling of stromal proteins as well (data not shown). Therefore, we were unable to find a labeling reagent that could not cross the inner membrane of the chloroplast envelope.

Figure A.1. Tic110 is not labeled by Sulfo-NHS-Biotin, a membrane-impermeable biotinylation reagent, as long as chloroplasts remain intact. Intact chloroplasts (50 μg chlorophyll) were incubated with Sulfo-NHS-Biotin at a final concentration of 40 μM (lanes 2-4). As a control for the ability of proteins to be labeled by the biotinylation reagent, one aliquot of chloroplasts was lysed hypotonically prior to incubation with Sulfo-NHS-Biotin (lane 1). At the times indicated, the biotinylation reactions were stopped by the addition of Tris-HCl (pH 7.5) at a final concentration of 100 mM. Intact chloroplasts were then recovered by centrifugation through a 40% Percoll cushion and lysed hypotonically. Following lysis, each sample was separated into crude membrane and soluble fractions. Membrane proteins equivalent to 25 μg starting chlorophyll were immunoprecipitated with antiserum against either Tic110 or Toc75. After immunoprecipitation, the samples were analyzed by SDS-PAGE. The presence of a biotin moiety on the proteins was detected with avidin conjugated to alkaline phosphatase.

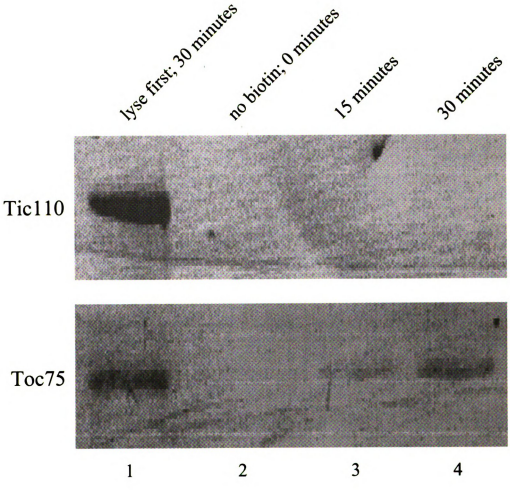

lyse first; 30 minutes
no biotin; 0 minutes
15 minutes
30 minutes
Tic110
Toc75
1
2
3
4

Figure A.1

Figure A.2. Sulfo-NHS-Biotin can cross the chloroplast inner envelope membrane and label stromal proteins. Intact chloroplasts (50 μg chlorophyll) were incubated with Sulfo-NHS-Biotin at a final concentration of 40 μM (lanes 3-12). As a control for the ability of chloroplastic proteins to be labeled by the biotinylation reagent, one aliquot of chloroplasts was lysed hypotonically prior to incubation with Sulfo-NHS-Biotin (lanes 1-2). At the times indicated, the biotinylation reactions were stopped by the addition of Tris-HCl (pH 7.5) at a final concentration of 100 mM. Intact chloroplasts were then recovered by centrifugation through a 40% Percoll cushion and lysed hypotonically. Following lysis, each sample was separated into crude membrane (P) and soluble (S) fractions and analyzed by SDS-PAGE. Biotinylated proteins were detected with avidin conjugated to alkaline phosphatase.

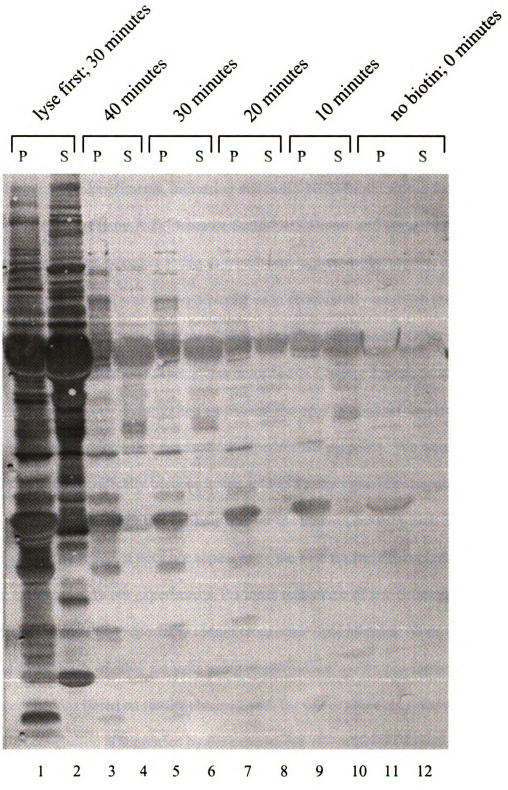

lyse first; 30 minutes
40 minutes
30 minutes
20 minutes
10 minutes
no biotin; 0 minutes
P S P S P S P S P S P S
*
1 2 3 4 5 6 7 8 9 10 11 12

Figure A.2

DISCUSSION

Attempts to label Tic110 with a biotinylation reagent, Sulfo-NHS-Biotin, that supposedly could not cross the chloroplast inner envelope membrane indicated that Tic110 was not accessible to this chemical (Figure A.1). These results suggest that the soluble domain of Tic110 that we were aiming to localize was exposed within the chloroplast stroma. However, soluble proteins of the chloroplast stroma were also labeled during these experiments, indicating that Sulfo-NHS-Biotin was in fact crossing the inner membrane (Figure A.2). Because Sulfo-NHS-Biotin, and several other labeling reagents that we tested, were not acting as membrane-impermeable reagents in our system, we decided that it was not possible for us to conclusively establish the topology of Tic110 within the inner envelope membrane using these chemicals. Other methods, such as the protease digestions experiments described in Chapter 2, were needed instead.

The results of this investigation emphasize the need to confirm the relevant properties of any chemicals that are used for experimental purposes. We were using Sulfo-NHS-Biotin specifically because it was sold as a membrane-impermeable reagent (Cole et al., 1987). Although this is likely true for the membranes on which Sulfo-NHS-Biotin was initially tested, it does not appear to be the case for the chloroplast inner envelope membrane. In our experiments, the inner membrane of pea chloroplasts was permeable to reagents that reportedly cannot cross other lipid bilayers. Thus, it is important to establish whether reagents being employed are working as expected prior to making conclusions based on results obtained with the use of those chemicals.

This conclusion is supported by the experiments described in Chapter 2. We decided to investigate the topology of Tic110 within the chloroplast inner envelope

membrane primarily because there was disagreement in the literature regarding the orientation of this protein (Kessler and Blobel, 1996; Lübeck et al., 1996). We determined the reason for the disparity was that one research group did not adequately quench the protease they were using in their experiments. As a consequence, they falsely concluded that the large hydrophilic domain of Tic110 was contained within the intermembrane space of the chloroplast envelope (Lübeck et al., 1996). Because their reagent was not working the way they assumed it was, they were misled by their results. Once again, this emphasizes the need to do adequate controls to insure all aspects of an experiment are working properly.

REFERENCES

Akita M, Nielsen E, Keegstra K (1997) Identification of protein transport complexes in the chloroplastic envelope membranes via chemical crosslinking. J Cell Biol 136: 983-994

Bruce BD, Perry S, Froehlich J, Keegstra K (1994) *In vitro* import of proteins into chloroplasts. Plant Mol Biol Manual J1: 1-15

Cole SR, Ashman LK, Ey PL (1987) Biotinylation: an alternative to radioiodination for the identification of cell surface antigens in immunoprecipitates. Mol Immunol 24: 699-705

Keegstra K, Werner-Washburne M, Cline K, Andrews J (1984) The chloroplast envelope: is it homologous with the double membranes of mitochondria and Gram-negative bacteria? J Cell Biochem 24: 55-68

Kessler F, Blobel G (1996) Interaction of the protein import and folding machineries in the chloroplast. Proc Natl Acad Sci USA 93: 7684-7689

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685

Lübeck J, Soll J, Akita M, Nielsen E, Keegstra K (1996) Topology of IEP110, a component of the chloroplastic protein import machinery present in the inner envelope membrane. EMBO J 15: 4230-4238

Tranel PJ, Froehlich J, Goyal A, Keegstra K (1995) A component of the chloroplastic protein import apparatus is targeted to the outer envelope membrane via a novel pathway. EMBO J 14: 2436-2446