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Review

Molecular chaperones involved in chloroplast protein import

Diane Jackson-Constan [a,b,1], Mitsuru Akita [e,1], Kenneth Keegstra [a,c,d,*]

[a] *Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, MI 48824-1312, USA*
[b] *Genetics Program, Michigan State University, East Lansing, MI 48824, USA*
[c] *Department of Plant Biology, Michigan State University, East Lansing, MI 48824, USA*
[d] *Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824, USA*
[e] *Plant Biophysics/Biochemistry Research Laboratory, College of Agriculture, Ehime University, 3-5-7 Tarumi, Matsuyama, Ehime, 790-8566, Japan*

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Abstract

Transport of cytoplasmically synthesized precursor proteins into chloroplasts, like the protein transport systems of mitochondria and the endoplasmic reticulum, appears to require the action of molecular chaperones. These molecules are likely to be the sites of the ATP hydrolysis required for precursor proteins to bind to and be translocated across the two membranes of the chloroplast envelope. Over the past decade, several different chaperones have been identified, based mainly on their association with precursor proteins and/or components of the chloroplast import complex, as putative factors mediating chloroplast protein import. These factors include cytoplasmic, chloroplast envelope-associated and stromal members of the Hsp70 family of chaperones, as well as stromal Hsp100 and Hsp60 chaperones and a cytoplasmic 14-3-3 protein. While many of the findings regarding the action of chaperones during chloroplast protein import parallel those seen for mitochondrial and endoplasmic reticulum protein transport, the chloroplast import system also has unique aspects, including its hypothesized use of an Hsp100 chaperone to drive translocation into the organelle interior. Many questions concerning the specific functions of chaperones during protein import into chloroplasts still remain that future studies, both biochemical and genetic, will need to address.

Keywords: Chloroplast; Protein targeting; Precursor protein; Molecular chaperone; Heat shock protein

1. Introduction

Plastids import the vast majority of their resident proteins post-translationally from the cytoplasm [1–5]. Most of the knowledge concerning the transport of proteins into plastids has been obtained through experiments with isolated pea chloroplasts, although it is assumed that all types of plastids utilize the same general import apparatus. The import process requires a variety of membrane-bound and soluble factors. Membrane proteins of the chloroplast envelope that mediate import are discussed in an accompanying review by Jarvis and Soll. This review will focus on a major class of soluble factors important in chloroplast protein import, the molecular chaperones.

Chloroplast protein import can be divided into two stages, based on their differing nucleotide triphosphate requirements. The 'binding' or 'docking' stage of import involves the hydrolysis of low levels

* Corresponding author, at address a. Fax: +1-517-353-9168.
E-mail address: keegstra@msu.edu (K. Keegstra).
[1] These authors contributed equally to this work.

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(0.1 mM) of ATP in the cytoplasm and/or intermembrane space of the chloroplast envelope [6,7]. The second stage of import, termed 'translocation', requires hydrolysis of high levels (>1 mM) of ATP within the plastid stroma in order to fully move a precursor into the organelle interior [8]. It has been suggested that chloroplast protein import should actually be subdivided into three stages: binding, translocation across the outer membrane, and translocation across the inner membrane [9]. Each of these three stages requires ATP hydrolysis, in increasing concentrations as the precursor moves from initial binding to inner membrane translocation [9]. It is probable that molecular chaperones are involved during each of the stages of chloroplast protein import, at the steps where energy in the form of ATP hydrolysis is needed. However, that hypothesis has not yet been proven.

More evidence exists for the role of chaperones during the transport of proteins across the endoplasmic reticulum (ER) membrane and into mitochondria (for recent reviews, see [10–14]). During mitochondrial protein import, chaperones have been implicated in maintaining precursors in an import-competent state prior to their transport, in guiding precursors to the membrane-bound import apparatus, in preventing retrograde movement of translocating precursor proteins, in providing the driving force for precursor translocation via ATP hydrolysis, and in refolding proteins once they have been fully imported into the organelle [10,11,13,14]. These functions have also been proposed to be accomplished by molecular chaperones during post-translational import into the ER [10–12]. It is likely that similar functions are mediated by chaperones during chloroplast protein import as well.

One similarity between the chloroplast and mitochondrial protein import processes is that precursors must cross two membranes in order to reach the organelle interior. However, while mitochondria utilize two major energy sources (ATP hydrolysis and an electrical potential ($\Delta\Psi$)) to accomplish this step [13,14], chloroplasts appear to require only ATP hydrolysis as the major energy source driving precursor translocation [8]. This suggests that plastids may have a greater requirement for ATPases, presumably molecular chaperones, than do some other protein import systems.

This review will discuss recent efforts to identify the chaperones that work during each stage of protein import into chloroplasts. These include soluble factors from the cytoplasm and the plastid stroma as well as chaperones that appear to be peripherally associated with both membranes of the chloroplast envelope (Fig. 1). While the number of chaperones believed to be involved in chloroplast protein import continues to increase, studies addressing their specific functions during precursor transport are still lacking. Comparisons with the protein targeting systems of other organelles, especially mitochondria, can be useful in developing hypotheses about the roles of chaperones during chloroplast protein import that can

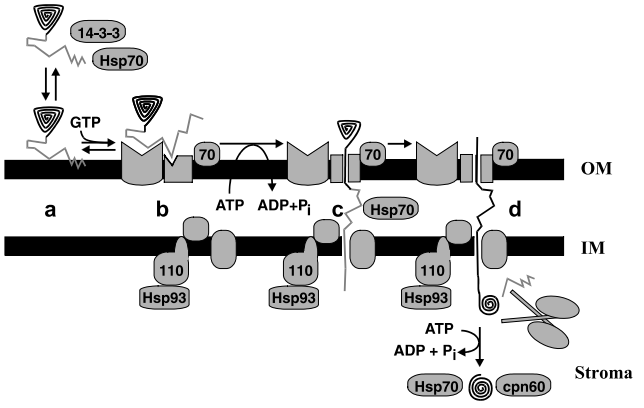

Fig. 1. Current model depicting molecular chaperones predicted to act at each stage of protein import into chloroplasts. Nuclear-encoded chloroplast proteins are initially synthesized in the cytoplasm with a transit peptide that, possibly in conjunction with cytoplasmic Hsp70 and 14-3-3 proteins, targets them to the plastid surface (a). In a process stimulated by GTP, the precursor protein associates with the components of the outer envelope translocon (b), which may include Com70 (70). Hydrolysis of ATP in the intermembrane space causes the precursor to interact with the components of the inner membrane translocon (c). It is postulated that this step may be assisted by an Hsp70 protein residing in the intermembrane space. The complete translocation of the precursor protein into the chloroplast interior, where the transit peptide is removed, is accomplished via stromal ATP hydrolysis (d), presumably mediated by Hsp93. Finally, chaperones within the chloroplast stroma, including possibly Hsp70 and cpn60, assist the newly imported protein in folding into its native conformation. A more complete model and information concerning the remaining components (Tic110 (110) and unlabeled) shown in this figure can be obtained from a review by Jarvis and Soll in this issue or from Jackson-Constan and Keegstra [30]. OM, outer membrane; IM, inner membrane.

then be tested experimentally in order to correct this lack of knowledge.

2. Interaction of molecular chaperones with transit peptides

Models depicting the process of chloroplast protein import usually show the N-terminal transit peptides of precursors, which target chloroplast proteins to the organelle (reviewed by Bruce in this issue), in direct contact with one or more chaperones [1–5]. Due to the proposed random coil nature of chloroplast transit peptides, it has long been thought that they could act as substrates for the binding of molecular chaperones [15]. Recently, two lines of evidence have been offered in support of this model. First, an analysis of precursor protein sequences by two different statistical algorithms [16,17] has revealed that they contain putative high affinity binding sites for DnaK, an *Escherichia coli* heat shock protein (Hsp) 70, within their transit peptides [18,19]. There is some disparity over whether the highest affinity sites may exist within the N-terminal or central domains of most transit peptides, but there is general agreement that over 75% of all plastid precursor proteins contain at least one site predicted to bind DnaK, and most likely homologous plant Hsp70s, within their transit peptides [18,19]. In addition, it appears that the majority of transit peptides contain at least two predicted mid- to high-affinity Hsp70-binding sites [18]. Mitochondrial precursor proteins also have been found to contain possible binding sites for the Hsp70 class of molecular chaperones within their presequences [20], so this phenomenon may be a general aspect of organellar import.

A second line of evidence supporting an interaction between chaperones and transit peptides comes from experiments describing a direct physical association of Hsp70s and precursor proteins. Two separate groups have published reports demonstrating that DnaK can bind to the transit peptides of two different chloroplast precursor proteins [18,19,21]. Direct binding was observed both in vitro and in *E. coli* [21]. The addition of ATP to the in vitro binding reaction caused DnaK to release the bound peptide, and mutations that disrupted the predicted chaperone-binding site within the synthetic transit peptide decreased the efficiency of the DnaK interaction [19]. Similar experiments done with plant Hsp70 proteins normally found in the cytoplasm [19] or plastid stroma [18] also showed binding between these plant Hsp70s and transit peptide sequences.

These results have several interesting implications. For instance, because the transit peptide is likely the first part of a precursor protein presented to the import machinery of both the outer and inner envelope membranes, it has been suggested that binding of the transit peptide to a chaperone represents the stage at which precursors become committed to the import pathway [18]. It is also possible that the binding of a cytoplasmic Hsp70 protein to the transit peptide assists in the guidance of a precursor to the chloroplast surface in a 'transit peptide-first' manner. Another intriguing proposition stems from the observation that in transit peptides containing two predicted chaperone-binding sites, these sites are separated by approx. 26 amino acids, long enough to have a chaperone-binding site exposed on either side of a lipid bilayer [18]. Thus, a transit peptide may be able to bind multiple chaperones in different chloroplast subcompartments at one time [18], assisting in preventing retrograde movements and in driving the translocation process.

One puzzling question that remains is whether mature regions of the precursor protein can also bind Hsp70s or other chaperones. In the one study that addressed this question, no binding of Hsp70s to a mature chloroplast protein was seen unless an Hsp70-binding site was artificially introduced [19]. Current models of chloroplast protein import assume that chaperones driving precursor translocation would do so through repeated cycles of binding and release throughout the length of the protein [2]. Thus, it is uncertain at this time how to resolve that model with these observations, although it is possible that, in vivo, chaperones may bind translocating precursors without the assistance of high-affinity binding sites or that chaperones other than Hsp70s are involved in binding the translocating proteins. It is also unclear whether Hsp70s or other chaperones bind to the 25% of transit peptides without detectable Hsp70-binding sites [19]. In addition, it is not known at exactly which stage of the import

process interactions between transit peptides and Hsp70s are important or whether binding sites, in either the transit peptide or the mature regions of precursor proteins, exist for other classes of chaperones, such as Hsp100s or Hsp60s. Further experiments will be needed to learn more about these matters.

3. From the cytoplasm to chloroplasts

Protein import into chloroplasts occurs after precursors have been completely translated in the cytoplasm. Based on what is known from the mitochondrial and ER transport systems, it is likely 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 [22]. However, there is evidence that supports the conclusion that precursors in a native, enzymatically active form can still be imported into the chloroplast. For example, della-Cioppa and co-workers [23] demonstrated that the precursor form of 5-enolpyruvylshikimate-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 [24,25]. 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 [24,25].

3.1. Cytoplasmic Hsp70s

Hsp70 proteins, as well as proteins from other chaperone families, are known to interact with nascent chains in order to prevent their aggregation and misfolding [22,26]. In yeast, it has been observed that precursors targeted to the mitochondria and ER will accumulate in the cytoplasm when a subset of Hsp70 proteins is depleted from the cells [27]. At least 12 Hsp70-related polypeptides have been reported to be expressed when *Arabidopsis* plants are treated with heat [28]. Within the *Arabidopsis* genome sequence database, several of these different Hsp70 homologues can be identified. Based upon their similarity to known Hsp70 proteins, most can be categorized into the following four groups: (1) eukaryotic cytoplasmic heat shock cognate (Hsc) 70 homologues, (2) mitochondrial Hsp70 homologues, (3) chloroplastic Hsp70 homologues, and (4) homologues to the ER-localized Hsp70, BiP [29,30].

As is the case in other organisms, it is expected that newly synthesized proteins associate with Hsc70-homologous Hsp70 proteins in the plant cytoplasm. Miernyk and co-workers [31] have shown that an Hsp70 protein present in a wheat germ cell-free translation system can associate with newly synthesized proteins in an ATP-dependent manner. May and Soll [32] have reported that the precursor to the small subunit of Rubisco (prSS) can associate with Hsp70s in either the rabbit reticulocyte lysate or wheat germ translation systems. In addition, it has been reported that the efficiency of import into chloroplasts of the precursor to the light harvesting chlorophyll *a*/*b*-binding protein (prLHCP) was significantly increased when leaf extract was added to the import assay [33]. A purified Hsp70 protein also stimulated precursor import, although not to the same extent as when leaf extract was used [33]. Based on these results, Waegemann and colleagues [33] hypothesized that Hsp70 is at least one of the cytosolic factors involved in maintaining the import competence of prLHCP. On the other hand, import of prSS and the precursor to ferredoxin (prFd) was not stimulated by the addition of plant extract to the import assay [34,35]. Because the mature forms of SS and Fd are both soluble proteins, it is possible that factors within the translation systems themselves are enough to maintain these precursors in an import-competent state, without the need for additional factors to stimulate import. For the import of an integral membrane protein like LHCP, however, these additional factors may be more important.

3.2. 14-3-3 proteins

Recent studies have implicated 14-3-3 proteins as molecular chaperones interacting with precursor proteins destined for either chloroplasts or mitochondria [32,36]. 14-3-3 proteins are found throughout eukaryotic phylogenies [37]. Their major known function is

in cellular regulation, via an interaction with a variety of proteins at a phosphoserine or phosphothreonine residue [37,38]. 14-3-3 proteins can be found in diverse subcellular locations, including the cytoplasm [37], the plasma membrane [39], the nucleus [40], the inner membrane of mitochondria [41], and the chloroplast stroma [42].

Mitochondrial import stimulating factor (MSF) is a 14-3-3 protein that interacts with a subset of precursor proteins destined for the mitochondria, possibly in a targeting sequence-dependent manner [36,43]. MSF directs these precursor proteins to the Tom70–Tom37 receptor complex on the surface of the mitochondrial outer membrane to form a tetrameric MSF–precursor–Tom70–Tom37 complex [44]. MSF is released from this precursor–receptor complex upon the addition of ATP [44,45]. Once MSF is released, precursors are transferred to a second outer membrane-localized receptor complex, Tom20–Tom22 [44]. Another subset of precursor proteins is targeted to the outer mitochondrial membrane via a different pathway, mediated through an association with an Hsp70 protein [45]. These precursors apparently bypass the Tom70–Tom37 receptor complex and are directly targeted to the Tom20–Tom22 complex [44,45].

Soll and his colleagues [5,32,46] have put forward a somewhat different model regarding the possible role of 14-3-3 proteins during chloroplast protein import. Their model proposes that: (1) after synthesis, chloroplast precursor proteins are phosphorylated by a kinase; (2) a phosphorylated precursor forms a complex with a 14-3-3 protein, an Hsp70, and possibly additional, unidentified factors; (3) this complex is targeted to the appropriate receptor of the chloroplast outer envelope membrane; and (4) precursor translocation through the envelope membranes is initiated via dephosphorylation of the precursor by a phosphatase. In support of this model, it has been shown that a 14-3-3 protein in the wheat germ translation system can co-immunoprecipitate two different chloroplast precursor proteins in a transit peptide-dependent manner [32]. Interactions between precursor proteins and the 14-3-3 protein were observed only when the transit peptides of the precursors were phosphorylated on a serine residue contained within their predicted 14-3-3-binding motif [32].

Because MSF, the mitochondrial 14-3-3 protein, can recognize unphosphorylated precursors [47] while the presumed chloroplastic 14-3-3 chaperone does not [32], this model may provide one hypothesis to explain the regulation process by which precursors are directed to the correct organelle in plant cells. In order to test this hypothesis, it would be useful to study precursor proteins that normally can be imported into both chloroplasts and mitochondria, such as glutathione reductase [48] or ferrochelatase I [49]. Both of these proteins have a putative 14-3-3 protein-binding motif in their targeting sequences. When these proteins are destined for chloroplasts, a putative targeting sequence-dependent kinase may phosphorylate the precursors, allowing them to associate with the chloroplast targeting-specific 14-3-3 protein. In the absence of phosphorylation, however, the model predicts that these precursors would be targeted to the mitochondria with the assistance of MSF, which can recognize unphosphorylated precursor proteins [47].

Not all chloroplast-targeted precursor proteins have a predicted 14-3-3 protein-binding motif. In fact, the same precursor from different species may differ in the presence or absence of the motif. One example of this is seen for prSS. In the experiments on which the model described above is based, only prSS from tobacco, which has this motif, was used. However, prSS from soybean, pea, and wheat does not have a serine (or a threonine) at the presumed phosphorylation site. Mutant precursors without a serine or threonine at this position cannot be phosphorylated, nor are they able to form a complex with the 14-3-3 protein [32,46]. However, they still can be imported into isolated chloroplasts [46], as can prSS from soybean, pea, and wheat. Thus, it is unclear whether phosphorylation of precursor proteins and the interaction between phosphorylated precursors and 14-3-3 proteins are significant in the chloroplast import process, although it is also possible that there are different interaction sites present in precursors that do not have the predicted 14-3-3-binding motif or that the in vivo situation differs from the in vitro one.

Because not all precursors have a predicted 14-3-3-binding motif, then, as is the case with mitochondrial protein import, there may be additional factors that guide precursors to the organelle surface. It is known

that there are at least three putative outer membrane import receptors in *Arabidopsis* chloroplasts: Toc159, Toc132, and Toc120 [50]. It will be interesting to learn whether 14-3-3 protein-dependent and 14-3-3 protein-independent pathways have preferred receptors in chloroplasts as they do in mitochondria.

3.3. Other cytoplasmic factors

During mitochondrial protein import, cytoplasmic Hsp40 proteins are thought to assist Hsp70s in targeting precursors to the organelle [51,52]. In addition, Hsp70 proteins are known to interact with both Hsp104 and Hsp40 proteins in yeast cytoplasm [53]. Cytoplasmically localized homologues of these molecular chaperones are also present in plants [29], so it is possible that they may associate with chloroplast precursor proteins in conjunction with plant Hsp70s. Other cytoplasmic factors, including 'targeting factor' [54] and presequence-binding factor [55], have also been found to interact with precursor proteins during transport to mitochondria. Plant homologues of these factors may be involved in protein targeting to chloroplasts as well.

4. Chaperones at the chloroplast outer envelope membrane

4.1. The role of lipids

One important, but often overlooked, subject is the role of lipids during precursor import. A more extensive discussion of the function of lipids during plastid protein transport is available in a review by Bruce in this issue. Lipids can sometimes assist with the insertion of integral membrane proteins. For example, lactose permease (LacY) was found to be misfolded in the membrane of an *E. coli* mutant that lacked the lipid phosphatidylethanolamine (PE) [56]. The binding of lipids to particular regions of unfolded proteins appears to prevent their misfolding and aggregation [57].

One attractive hypothesis is that transit peptides may interact with lipids during targeting to the chloroplast surface. The transit peptides of Fd and SS have been shown to interact with the chloroplast lipids sulfoquinovosyldiacylglycerol (SQDQ), phosphatidylglycerol (PG), and monogalactosyldiacylglycerol (MGDG) [58–61]. In addition, SQDQ, PG, and the non-chloroplast lipid PE can interact with the N-terminal portion of the targeting signal of Toc75, a component of the outer membrane import complex [62]. Interactions between transit peptides and these lipids may affect the structure of the mature portions of precursor proteins.

4.2. Two Hsp70 proteins associated with the outer envelope membrane

Two Hsp70 proteins localized at the outer envelope membrane of pea chloroplasts have been reported to be involved in protein import. One of them, chloroplast outer membrane protein 70 (Com70), is exposed on the cytoplasmic side of the membrane [63]. The other, Hsp70-import associated protein (described below), faces the intermembrane space between the outer and inner envelope membranes [64,65].

Com70 is a eukaryotic cytoplasmic Hsc70 homologue, first isolated in plants from chloroplast envelope membranes [63]. Using chemical cross-linking methods, Com70 was found to associate with a translocating precursor protein [66]. Because Com70 was found to associate with precursors at the earliest stage of import [67], it was suggested that Com70 might function to maintain precursor proteins in an unfolded state during translocation, to prevent the release of translocating precursor proteins from the envelope, and/or to insert them further into the outer envelope membrane [67]. It should be noted that some controversy stills exists concerning whether this protein is a chloroplast outer membrane (Com) factor, calling into question the putative assignment of Com70 as a member of the chloroplast outer membrane import complex.

A second Hsp70 protein, distinct from Com70, has also been found in association with the outer envelope membrane of pea chloroplasts [64]. This Hsp70 was not degraded when isolated chloroplasts were treated with thermolysin [64,65]. Because thermolysin cannot penetrate the outer envelope membrane and thus degrades only surface-exposed proteins [68], it was concluded that this outer membrane-bound Hsp70 is exposed to the intermembrane space (IMS) of the chloroplast envelope [64,65]. Subse-

quent studies identifying proteins associated with a translocating precursor protein showed that this IMS-localized chaperone was a component of the pea import complex [65]. This Hsp70 protein was co-immunoprecipitated with prSS at the earliest stages of the import process [65]. Partial amino acid sequencing of this protein indicated that it was similar, but not identical, to previously characterized Hsp70s [65].

Several possible roles for this IMS-localized Hsp70, termed Hsp70-import associated protein (IAP), during import have been hypothesized, although there are few data available to support any of them. Along with Com70, Hsp70-IAP may be involved in unfolding translocating precursors [1,2,4,69]. As discussed earlier, chloroplasts can import highly folded proteins [23–25]. These two chaperones, one on either side of the outer envelope membrane, may account for this ability, which appears to be weaker in mitochondria [70,71]. Another possible role for Hsp70-IAP may be to bind the transit peptide as it emerges from the outer membrane translocon, acting as an additional recognition site and preventing the backwards movement of the precursor [22,64,65]. In this way, the chloroplastic Hsp70-IAP would be playing a similar role to that accomplished by the intermembrane space acidic receptors (i.e. Tom22) during mitochondrial protein import, ensuring 'one way' transport of the incoming protein across the outer envelope membrane [22,72]. Hsp70-IAP may also assist in guiding precursors from the outer membrane translocon to the protein channel of the inner envelope membrane. Finally, Hsp70-IAP might act as the ATPase for translocation across the outer envelope membrane [2,3,69]. Experiments indicating that outer membrane translocation can be separated from either initial precursor binding or translocation across the inner envelope membrane suggest that the outer membrane may have its own translocation motor [9], a role for which Hsp70-IAP is the best candidate. Hsp70s as translocation motors have precedence in both the ER and mitochondrial import systems [10,11,14].

Regardless of the actual function of Hsp70-IAP, it is likely that this chaperone mediates the requirement for ATP hydrolysis within the IMS observed during the early stages of precursor protein transport [6,7]. Attempts to learn more about the role of Hsp70-IAP during the import process are currently hindered by the fact that the gene encoding this protein has not been identified, despite the fact that the protein was first observed over a decade ago [64]. The recent completion of the *Arabidopsis* genome sequencing project has made the entire complement of Hsp70 sequences in that species now available. However, efforts to determine, through an analysis of transit peptide sequence, which of the many putative Hsp70 isoforms might be localized in the chloroplast IMS, and thus be candidates for Hsp70-IAP, have not been successful [30]. Further research on Hsp70-IAP, therefore, awaits identification of its gene.

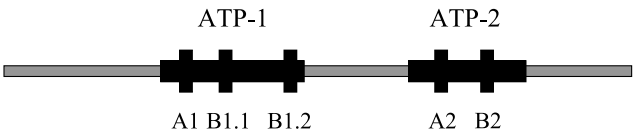

Fig. 2. Structural model of Hsp93, showing the two ATP-binding domains contained within this protein. Hsp93 is a class 1 protein of the Hsp100 family of chaperones. Proteins within this class have two nucleotide-binding domains [76]. These ATP-binding domains are depicted as black boxes. Taller boxes indicate the Walker A (A1, A2) and Walker B (B1.1, B1.2, B2) nucleotide-binding motifs contained within these regions (for more information, see [76]). This figure is adapted from one previously published by Schirmer and colleagues [76].

5. Stromal molecular chaperones

5.1. Hsp93

The major factors driving translocation in the ER and mitochondrial protein transport systems are Hsp70s, which through ATP hydrolysis move translocating precursor proteins into the organelle interior [10,11,14]. Thus, it was believed that an Hsp70 molecule would also be found to drive protein translocation into chloroplasts. 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 [73–75]. This protein, which has two ATP-binding domains (Fig. 2) [76], is known as Hsp93, reflecting its calculated molecular mass of 93 kDa for the mature form of the protein, and formerly was known as ClpC. Hsp93 was found to be a component of import complexes regardless of

whether precursor proteins were present ([74,75]; M. Akita and K. Keegstra, manuscript in preparation). While the majority of Hsp93 molecules are found in soluble form in the chloroplast stroma, a significant proportion of this protein is found associated with the inner envelope membrane, presumably through its interaction with the import complex ([77,78]; M. Akita and K. Keegstra, manuscript in preparation). A similar situation is known to exist for the mitochondrial Hsp70 protein, which is mostly soluble in the matrix, but can be found in a membrane-associated form via an interaction with the inner membrane import complex component, Tim44 [14].

Several lines of evidence indicate that the association of Hsp93 with isolated import complexes is relevant to the process of precursor transport. First, Hsp93 co-immunoprecipitated prSS only under conditions that supported either binding or translocation of the precursor [74]. Secondly, Hsp93 was able to co-immunoprecipitate several precursor proteins that utilize the general import apparatus of the chloroplast envelope but not plastid proteins that do not use this import machinery [74]. The association of Hsp93 with prSS was disrupted by the addition of ATP, but not GTP, to the import reactions [74]. Because Hsp100 chaperones interact with their substrates in an ATP-sensitive fashion [79,80], this ATP dependence indicates that the association between Hsp93 and prSS is physiologically relevant [74]. Finally, the interaction between Hsp93 and prSS decreased with time during an import reaction [74]. This indicates that prSS proteins associated with Hsp93 were functional import intermediates [74].

These results suggest that Hsp93 is a bona fide component of the import complex in pea chloroplasts [73–75]. In addition, it is the major stromal chaperone found in import complexes during all stages of precursor transport [74]. Thus, it is currently the leading candidate to bind precursor proteins as they enter the chloroplast stoma, preventing their backwards movement, and to act as the translocation motor for precursor protein translocation, presumably through the hydrolysis of stromal ATP [8]. This distinguishes the chloroplast import system from the ER and mitochondrial protein import machineries, which both utilize Hsp70s for these functions [12–14]. In the bacterial protein export system, the energy for protein translocation is provided by SecA [81], a protein that has some features in common with Hsp93 (M. Akita and K. Keegstra, manuscript in preparation). Thus, Hsp93, and perhaps the entire chloroplast 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. Current work on Hsp93 is attempting to confirm these hypotheses via both biochemical and genetic strategies (M. Akita and K. Keegstra, manuscript in preparation).

5.2. Stromal Hsp70s

While Hsp93 appears to be the major stromal molecular chaperone found in isolated import complexes, pea chloroplasts also have at least two stromal Hsp70s [64]. An analysis of the *Arabidopsis* genome sequence suggests that this species also has at least two Hsp70 proteins within the stroma of its plastids [30]. The role of one of the two pea isoforms, S78 or CSS1, during chloroplast protein import has been studied in some detail. This protein bound the transit peptide of prSS in a manner similar to that seen for DnaK, as described above [18]. In addition, S78 was co-immunoprecipitated with prSS under conditions that stimulated binding and translocation of the precursor [74]. This association, like the interaction between Hsp93 and prSS, decreased with time during an import reaction, suggesting that the precursor was part of a functional import intermediate [74].

These results suggest that S78 is part of the import complex in pea chloroplasts. However, several other lines of evidence argue against this conclusion. First, when import complexes were solubilized in mild detergent prior to immunoprecipitation, S78 was no longer found in association with translocating prSS [74]. In addition, when import complexes were isolated via chemical cross-linking methods, S78 was not found in association with either prSS or other transport complex components [73]. Finally, S78 did not co-sediment in linear sucrose gradients with either prSS or various import complex components following detergent solubilization of pea chloroplasts [74]. In all of these situations, however, Hsp93 was still found in association with the import complex [73,74].

Thus, it is not clear whether S78, or the other stromal Hsp70 in pea chloroplasts, either associates

with the import complex or acts as the translocation motor, as had been predicted by analogy to the mitochondrial and ER transport systems. Because Tic40, an import component of the inner envelope membrane, is homologous to Hsp70 interacting protein (Hip), it is possible that Hsp70 proteins are recruited to the site of precursor import [82]. If these Hsp70s are not acting as the translocation motor, as their equivocal association with the import complex would suggest, what other possible roles could they be playing in the import process? Hsp70 proteins may be involved at a slightly later stage in import than is Hsp93, perhaps working to refold proteins as they emerge from the translocation channel [5]. Tsugeki and Nishimura [83] have reported a transient, ATP-dependent association of the stromal Hsp70 of pumpkin, and chaperonin 60 (described below), with newly imported ferredoxin $NADP^+$ reductase, as would be expected if these chaperones were assisting the protein with its folding. In mitochondrial protein import, the matrix hsp70 is believed to act both as the translocation motor and as the mediator of precursor refolding [13,14]. It is possible that these two functions are divided between two different chaperones, Hsp93 and S78, in the chloroplast system [2].

Another possibility for S78 function is in guiding precursor proteins with a thylakoid-targeting signal to this chloroplast subcompartment [64,84]. It has been suggested that some proteins destined for the thylakoid may need to be maintained in an import-competent, unfolded state prior to their translocation across or into the thylakoid membrane [64]. S78 or other soluble, stromal molecular chaperones may perform this function in the chloroplast. Further experiments will need to be done to determine whether any of these hypotheses for the function of stromal Hsp70s are relevant to the in vivo situation.

5.3. cpn60

One additional stromal molecular chaperone has been found in isolated import complexes under certain conditions. This protein is chaperonin (cpn) 60, a member of the Hsp60 family of chaperones and a homologue of the bacterial chaperone GroEL [29]. Cpn60 has long been known to be required for the folding and assembly of several chloroplast proteins [85]. The first indication that it might be involved in precursor import as well came from the observation that cpn60 could form a complex with several newly imported proteins [83,86]. Further support for this hypothesis was reported in a study by Kessler and Blobel [87], which found cpn60 as the major protein immunoprecipitated by Tic110, a component of the inner membrane import apparatus. The interaction of Tic110 with cpn60 was unrelated to the folding state of Tic110 and could be disrupted by the addition of ATP [87]. The mature form of SS (mSS) associated with the Tic110–cpn60 complex in a transient, ATP-sensitive manner, as would be expected if cpn60 was interacting with mSS to assist in its proper folding [87]. The Tic110–cpn60 complex was concentrated 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 active precursor protein import [75]. This is in contrast to the association seen between Tic110 and Hsp93, which is not dependent on contact site formation [74,75]. Thus, it is believed that cpn60 has only an indirect role in chloroplast protein import, assisting in folding proteins as they emerge from the translocation channel [75]. In this model, Tic110 helps to recruit the chaperone to the site of active protein import [87].

5.4. Other stromal factors

Chloroplasts contain a variety of other proteins, including Hsp40s, Hsp10s, and GrpEs, which can act as co-chaperones for Hsp70s and Hsp60s [29,88]. It is possible that these other factors are also involved in precursor protein import, either by assisting in the folding of newly imported proteins or by acting as co-chaperones with the factors described above. In addition, there is a 14-3-3 protein present in the chloroplast stroma [42]. This 14-3-3 protein has been found in association with the thylakoid-localized N-subunit of photosystem I in a targeting signal-dependent manner [42]. The function of this interaction, however, has yet to be established.

6. Conclusions

It has been suggested that at least three ATPases are required for the import of precursor proteins into

the chloroplast interior: one for initial precursor binding, one for translocation across the outer envelope membrane, and one to drive translocation across the inner envelope membrane [9]. At the binding stage, ATP may be required for the transfer of the incoming precursor from cytoplasmic partners to the import complex of the chloroplast outer membrane [89]. This energy requirement could be mediated by either cytoplasmic factors, such as Hsp70 and/or 14-3-3 protein, or by the envelope-bound Com70 chaperone [32,63,89]. There is also a requirement for GTP hydrolysis at this stage, presumably mediated by the two GTP-binding components of the outer membrane import complex [7,90,91]. It is possible that the ATP- and GTP-mediated steps work together to regulate the process of precursor recognition and binding. It has been hypothesized that at least a portion of the precursor protein, most likely the transit peptide, has crossed the outer envelope membrane by the end of the binding stage of import [74]. Thus, it is also possible that the ATPase needed for precursor binding is Hsp70-IAP, localized in the envelope IMS. Hsp70-IAP is also the most likely candidate to be the ATPase mediating outer membrane translocation. Inner membrane translocation could then be accomplished via the ATP-hydrolyzing ability of Hsp93 [73,74], although other stromal molecular chaperones, including an Hsp70 homologue, could possibly be functioning at this stage. After the three ATPases have mediated the import process, other stromal chaperones would then be involved in folding and assembling the protein into its native form and, in some cases, possibly guiding it to the thylakoid membrane for additional intraorganellar targeting. Other scenarios are also possible, including ones in which the same chaperone is involved in more than one step, such as Hsp70-IAP being involved in simultaneously unfolding an incoming precursor and driving its translocation across the outer membrane, or in which chaperones are involved in the regulation of components of the membrane-bound translocon. Experiments addressing the individual functions of each of these chaperones in more detail will be needed to refine this model further.

Regardless of which chaperones are necessary at each individual stage, the overall picture that is emerging suggests that the process of protein import into chloroplasts can be thought of as a 'pathway of chaperones'. Precursor proteins are 'passed' from cytoplasmic factors, including chaperones, that guide the precursor to the chloroplast surface to chaperones associated with the outer envelope membrane to ones bound to the inner envelope membrane to stromal factors that assist the protein in attaining its native conformation. The random coil nature of transit peptides may allow them to interact with multiple chaperones in succession [15], supporting this hypothesis. The various stages of import would be accomplished via conformational changes in the chaperones themselves that are brought about by ATP binding and hydrolysis [92,93]. Translocation would be driven by repeated cycles of binding and release of chaperones with the incoming precursor protein, triggered by the ATP/ADP status of the molecular chaperones [2].

Many aspects of the above model are similar to the well-studied import systems of mitochondria and the ER. In both of these organelles, precursor translocation is also accomplished, at least to some extent, by an ATP-hydrolyzing, peripherally attached chaperone, although the molecular mechanism (i.e. 'trapping' or 'pulling') by which chaperones mediate translocation in these systems is still under debate [10,11,14]. In addition, mitochondrial precursors are also thought to interact with Hsp70 proteins via binding sites in their presequences and with 14-3-3 proteins in a manner analogous to that suggested for chloroplast precursors [20,36,43].

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. In both the ER and mitochondria, Hsp70 proteins are thought to assist in 'pulling in' incoming precursors [10,11,14]. However, most of the evidence for the chloroplast import system currently points to Hsp93, rather than one of the stromal Hsp70s, as accomplishing this task [73–75]. 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 [8]. In ER precursor transport, only one chaperone, BiP or Kar2p, has been found to play a role [12]. In the

mitochondrial import system, chaperones are needed both in the cytoplasm to guide precursors to the outer membrane and in the matrix to drive translocation [13]. Within chloroplast import complexes, however, two additional chaperones have been found in association with the outer envelope membrane [63–66]. 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 at the mitochondrial outer membrane.

Despite the large number of chaperones that have been found to associate with precursors during chloroplast protein import, the functions of these proteins during the import process have not been conclusively established. Currently, it has not even been experimentally determined whether these (or other) chaperones actually mediate the ATP requirements of chloroplast protein import. Consequently, ongoing studies likely will focus less on identification of additional chaperone components and more on a study of their individual functions during import. Although most of the work discussed in this review was done through biochemical studies in pea chloroplasts, efforts in several laboratories are now underway to also address these questions genetically in *Arabidopsis*. In conjunction with continuing investigations on the functions of the non-chaperone import complex components, these studies should continue to provide a clearer model describing the process of precursor protein import into chloroplasts.

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References

[1] X. Chen, D.J. Schnell, Trends Cell Biol. 9 (1999) 222–227.
[2] K. Keegstra, K. Cline, Plant Cell 11 (1999) 557–570.
[3] K. Keegstra, J.E. Froehlich, Curr. Opin. Plant Biol. 2 (1999) 471–476.
[4] T. May, J. Soll, FEBS Lett. 452 (1999) 52–56.
[5] E. Schleiff, J. Soll, Planta 211 (2000) 449–456.
[6] L.J. Olsen, S.M. Theg, B.R. Selman, K. Keegstra, J. Biol. Chem. 264 (1989) 6724–6729.
[7] L.J. Olsen, K. Keegstra, J. Biol. Chem. 267 (1992) 433–439.
[8] S.M. Theg, C. Bauerle, L.J. Olsen, B.R. Selman, K. Keegstra, J. Biol. Chem. 264 (1989) 6730–6736.
[9] S.V. Scott, S.M. Theg, J. Cell Biol. 132 (1996) 63–75.
[10] R.E. Jensen, A.E. Johnson, Curr. Biol. 9 (1999) R779–R782.
[11] M. Pilon, R. Schekman, Cell 97 (1999) 679–682.
[12] T.A. Rapoport, K.E.S. Matlack, K. Plath, B. Misselwitz, O. Staeck, Biol. Chem. 380 (1999) 1143–1150.
[13] J.M. Herrmann, W. Neupert, Curr. Opin. Microbiol. 3 (2000) 210–214.
[14] A. Strub, J.H. Lim, N. Pfanner, W. Voos, Biol. Chem. 381 (2000) 943–949.
[15] G. von Heijne, K. Nishikawa, FEBS Lett. 278 (1991) 1–3.
[16] A. Gragerov, L. Zeng, X. Zhao, W. Burkholder, M.E. Gottesman, J. Mol. Biol. 235 (1994) 848–854.
[17] S. Rüdiger, L. Germeroth, J. Schneider-Mergener, B. Bukau, EMBO J. 16 (1997) 1501–1507.
[18] R.A. Ivey III, C. Subramanian, B.D. Bruce, Plant Physiol. 122 (2000) 1289–1299.
[19] D.V. Rial, A.K. Arakaki, E.A. Ceccarelli, Eur. J. Biochem. 267 (2000) 6239–6248.
[20] X.-P. Zhang, A. Elofsson, D. Andreu, E. Glaser, J. Mol. Biol. 288 (1999) 177–190.
[21] R.A. Ivey III, B.D. Bruce, Cell Stress Chaperones 5 (2000) 62–71.
[22] G. Schatz, B. Dobberstein, Science 271 (1996) 1519–1526.
[23] G. della-Cioppa, S.C. Bauer, B.K. Klein, D.M. Shah, R.T. Fraley, G.M. Kishore, Proc. Natl. Acad. Sci. USA 83 (1986) 6873–6877.
[24] A. Guéra, T. America, M. van Waas, P.J. Weisbeek, Plant Mol. Biol. 23 (1993) 309–324.
[25] T. America, J. Hageman, A. Guéra, F. Rook, K. Archer, K. Keegstra, P. Weisbeek, Plant Mol. Biol. 24 (1994) 283–294.
[26] R.P. Beckmann, L.A. Mizzen, W.J. Welch, Science 248 (1990) 850–854.
[27] R.J. Deshaies, B.D. Koch, M. Werner-Washburne, E.A. Craig, R. Schekman, Nature 332 (1988) 800–805.
[28] C.H. Wu, T. Caspar, J. Browse, S. Lindquist, C. Somerville, Plant Physiol. 88 (1988) 731–740.
[29] R.S. Boston, P.V. Viitanen, E. Vierling, Plant Mol. Biol. 32 (1996) 191–222.
[30] D. Jackson-Constan, K. Keegstra, Plant Physiol. 125 (2001) 1567–1576.
[31] J.A. Miernyk, N.B. Duck, R.G. Shatters Jr., W.R. Folk, Plant Cell 4 (1992) 821–829.
[32] T. May, J. Soll, Plant Cell 12 (2000) 53–63.
[33] K. Waegemann, H. Paulsen, J. Soll, FEBS Lett. 261 (1990) 89–92.
[34] M. Pilon, A.D. de Boer, S.L. Knols, M.H.G.M. Koppelman, R.M. van der Graaf, B. de Kruijff, P.J. Weisbeek, J. Biol. Chem. 265 (1990) 3358–3361.
[35] M. Pilon, B. de Kruijff, P.J. Weisbeek, J. Biol. Chem. 267 (1992) 2548–2556.

[36] N. Hachiya, R. Alam, Y. Sakasegawa, M. Sakaguchi, K. Mihara, T. Omura, EMBO J. 12 (1993) 1579–1586.
[37] H. Fu, R.R. Subramanian, S.C. Masters, Annu. Rev. Pharmacol. Toxicol. 40 (2000) 617–647.
[38] H.-J. Chung, P.C. Sehnke, R.J. Ferl, Trends Plant Sci. 4 (1999) 367–371.
[39] T. Jahn, A.T. Fuglsang, A. Olsson, I.M. Brüntrup, D.B. Collinge, D. Volkmann, M. Sommarin, M.G. Palmgren, C. Larsson, Plant Cell 9 (1997) 1805–1814.
[40] E.A. Bihn, A.-L. Paul, S.W. Wang, G.W. Erdos, R.J. Ferl, Plant J. 12 (1997) 1439–1445.
[41] T.D. Bunney, H.S. van Walraven, A.H. de Boer, Proc. Natl. Acad. Sci. USA 98 (2001) 4249–4254.
[42] P.C. Sehnke, R. Henry, K. Cline, R.J. Ferl, Plant Physiol. 122 (2000) 235–241.
[43] T. Komiya, N. Hachiya, M. Sakaguchi, T. Omura, K. Mihara, J. Biol. Chem. 269 (1994) 30893–30897.
[44] N. Hachiya, K. Mihara, K. Suda, M. Horst, G. Schatz, T. Lithgow, Nature 376 (1995) 705–709.
[45] T. Komiya, M. Sakaguchi, K. Mihara, EMBO J. 15 (1996) 399–407.
[46] K. Waegemann, J. Soll, J. Biol. Chem. 271 (1996) 6545–6554.
[47] R. Alam, N. Hachiya, M. Sakaguchi, S. Kawabata, S. Iwanaga, M. Kitajima, K. Mihara, T. Omura, J. Biochem. 116 (1994) 416–425.
[48] G. Creissen, H. Reynolds, Y. Xue, P. Mullineaux, Plant J. 8 (1995) 167–175.
[49] K.-S. Chow, D.P. Singh, J.M. Roper, A.G. Smith, J. Biol. Chem. 272 (1997) 27565–27571.
[50] J. Bauer, K. Chen, A. Hiltbunner, E. Wehrli, M. Eugster, D. Schnell, F. Kessler, Nature 403 (2000) 203–207.
[51] A.J. Caplan, D.M. Cyr, M.G. Douglas, Cell 71 (1992) 1143–1155.
[52] K. Terada, M. Kanazawa, B. Bukau, M. Mori, J. Cell Biol. 139 (1997) 1089–1095.
[53] J.R. Glover, S. Lindquist, Cell 94 (1998) 73–82.
[54] H. Ono, S. Tuboi, J. Biol. Chem. 263 (1988) 3188–3193.
[55] K. Murakami, M. Mori, EMBO J. 9 (1990) 3201–3208.
[56] W. Dowhan, Annu. Rev. Biochem. 66 (1997) 199–232.
[57] M. Bogdanov, W. Dowhan, J. Biol. Chem. 274 (1999) 36827–36830.
[58] R. van't Hof, R.A. Demel, K. Keegstra, B. de Kruijff, FEBS Lett. 291 (1991) 350–354.
[59] R. van't Hof, W. van Klompenburg, M. Pilon, A. Kozubek, G. de Korte-Kool, R.A. Demel, P.J. Weisbeek, B. de Kruijff, J. Biol. Chem. 268 (1993) 4037–4042.
[60] M. Pilon, H. Wienk, W. Sips, M. de Swaaf, I. Talboom, R. van't Hof, G. de Korte-Kool, R. Demel, P. Weisbeek, B. de Kruijff, J. Biol. Chem. 270 (1995) 3882–3893.
[61] P. Pinnaduwage, B.D. Bruce, J. Biol. Chem. 271 (1996) 32907–32915.
[62] K. Inoue, R. Demel, B. de Kruijff, K. Keegstra, Eur. J. Biochem. 268 (2001) 4036–4043.
[63] K. Ko, O. Bornemisza, L. Kourtz, Z.W. Ko, W.C. Plaxton, A.R. Cashmore, J. Biol. Chem. 267 (1992) 2986–2993.
[64] J.S. Marshall, A.E. DeRocher, K. Keegstra, E. Vierling, Proc. Natl. Acad. Sci. USA 87 (1990) 374–378.
[65] D.J. Schnell, F. Kessler, G. Blobel, Science 266 (1994) 1007–1012.
[66] C. Wu, F.S. Seibert, K. Ko, J. Biol. Chem. 269 (1994) 32264–32271.
[67] L. Kourtz, K. Ko, J. Biol. Chem. 272 (1997) 2808–2813.
[68] K. Cline, M. Werner-Washburne, J. Andrews, K. Keegstra, Plant Physiol. 75 (1984) 675–678.
[69] A. Caliebe, J. Soll, Plant Mol. Biol. 39 (1999) 641–645.
[70] M. Eilers, G. Schatz, Nature 322 (1986) 228–232.
[71] D. Vestweber, G. Schatz, J. Cell Biol. 107 (1988) 2037–2043.
[72] T. Komiya, S. Rospert, C. Koehler, R. Looser, G. Schatz, K. Mihara, EMBO J. 17 (1998) 3886–3898.
[73] M. Akita, E. Nielsen, K. Keegstra, J. Cell Biol. 136 (1997) 983–994.
[74] E. Nielsen, M. Akita, J. Davila-Aponte, K. Keegstra, EMBO J. 16 (1997) 935–946.
[75] A. Kouranov, X. Chen, B. Fuks, D.J. Schnell, J. Cell Biol. 143 (1998) 991–1002.
[76] E.C. Schirmer, J.R. Glover, M.A. Singer, S. Lindquist, Trends Biochem. Sci. 21 (1996) 289–296.
[77] T. Moore, K. Keegstra, Plant Mol. Biol. 21 (1993) 525–537.
[78] E.E. Nielsen, Dissertation, Michigan State University, East Lansing, MI, 1997.
[79] S. Wickner, S. Gottesman, D. Skowyra, J. Hoskins, K. McKenney, M.R. Maurizi, Proc. Natl. Acad. Sci. USA 91 (1994) 12218–12222.
[80] A. Wawrzynow, D. Wojtkowiak, J. Marszalek, B. Banecki, M. Jonsen, B. Graves, C. Georgopoulos, M. Zylicz, EMBO J. 14 (1995) 1867–1877.
[81] E.H. Manting, A.J.M. Driessen, Mol. Microbiol. 37 (2000) 226–238.
[82] T. Stahl, C. Glockmann, J. Soll, L. Heins, J. Biol. Chem. 274 (1999) 37467–37472.
[83] R. Tsugeki, M. Nishimura, FEBS Lett. 320 (1993) 198–202.
[84] J.S. Marshall, K. Keegstra, Plant Physiol. 100 (1992) 1048–1054.
[85] R.J. Ellis, S.M. Hemmingsen, Trends Biochem. Sci. 14 (1989) 339–342.
[86] T.H. Lubben, G.K. Donaldson, P.V. Viitanen, A.A. Gatenby, Plant Cell 1 (1989) 1223–1230.
[87] F. Kessler, G. Blobel, Proc. Natl. Acad. Sci. USA 93 (1996) 7684–7689.
[88] T. Schlicher, J. Soll, Plant Mol. Biol. 33 (1997) 181–185.
[89] K. Waegemann, J. Soll, Plant J. 1 (1991) 149–158.
[90] M.E. Young, K. Keegstra, J.E. Froehlich, Plant Physiol. 121 (1999) 237–243.
[91] K. Chen, X. Chen, D.J. Schnell, Plant Physiol. 122 (2000) 813–822.
[92] B.S. Glick, Cell 80 (1995) 11–14.
[93] S.M. Gisler, E.V. Pierpaoli, P. Christen, J. Mol. Biol. 279 (1998) 833–840.