



## LETTER TO THE EDITOR

# Protein Import Motors in Chloroplasts: On the Role of Chaperones<sup>[OPEN]</sup>

A recent article in *The Plant Cell* reported the identification of a Ycf2 (hypothetical chloroplast open reading frame2)-FtsHi (filamentous temperature sensitive inactive)-NAD<sup>+</sup> malate dehydrogenase (MDH) complex and proposed it to be the motor for protein translocation into the chloroplast (Kikuchi et al., 2018; highlighted by Hermann, 2018). As authors of a number of articles providing extensive biochemical and molecular genetic data in support of the prevailing model for the chloroplast import motor, we found the consideration of the existing body of literature on this subject to be potentially misleading and sometimes inaccurate in these articles. Our aim in this letter is to present our view that the chloroplast import motor almost certainly also includes the stromal chaperones Hsp93, cpHsp70, and Hsp90C previously implicated in the literature. Our intent is not to dismiss the findings of Nakai and colleagues but to emphasize the need for all investigators in the field to include a balanced consideration of the classical and more recent data when considering the chloroplast protein import motor(s).

Translocation of proteins across the chloroplast envelope requires ATP hydrolysis inside the chloroplast (Theg et al., 1989). Three stromal chaperone ATPases—Hsp93, cpHsp70, and Hsp90C—play an important role in this transport process, supported by decades of research. Kikuchi et al. (2018) suggested that the motor proteins that drive protein import into chloroplasts, however, remain unknown because most previous studies considered the identities of the motors “in the context of Tic110 and Tic40,” and in their hands, neither Tic110 nor Tic40 could be confirmed to interact with the Tic20/Tic56/Tic100/Tic214 (Ycf1) complex they identified. It should be noted that several publications from independent groups

have raised questions regarding the role of the Ycf1 complex in protein import (de Vries et al., 2015; Köhler et al., 2015, 2016; Agne et al., 2017; Bölder and Soll, 2017), and several reviews have proposed alternative functions for the Ycf1 complex based on the available data (Paila et al., 2015; Sjuets et al., 2017; Bölder, 2018). The Ycf2 complex was identified in part by its association with the Ycf1 complex. It is therefore perhaps premature to replace classical and long-established models with new models presenting Ycf2 as the import motor—even more so because over 30 years of published work from multiple laboratories support the direct roles of Tic110, Tic40, Hsp93, and cpHsp70 as components of import complexes and implicate Hsp90C as an additional component of the import motor (see below). Kikuchi et al. (2018) wrote that in their hands “no significant specific associations were observed between the translocating preprotein and stromal Hsp70, Hsp93.” However, it is too early to dismiss the large body of prior work as incorrect. The inability to detect Tic110, Hsp93, Hsp90C, and cpHsp70, for example, could be accounted for by the biochemical fractionation methods used during purification. It is also important to note that this group did not specifically investigate the roles of Tic110, Hsp93, cpHsp70, or Hsp90C in their previous publications. Therefore, their studies do not address the validity of the past work on Tic110 and the three chaperone motor proteins.

Kikuchi et al. (2018) wrote that the Ycf2-FtsHi motor complex is “unique to the green lineage of photosynthetic eukaryotes” but did not mention that the six proteins they have identified as the motor (i.e., Ycf2, FtsHi1, FtsHi2, FtsHi4, FtsHi5, and FtsH12) are not present in most monocots, including maize (*Zea mays*) and rice (*Oryza sativa*; Huang et al., 2013), as is the case for the three proteins the same research group added to the Tic20 channel complex a few years ago

(Tic214/Ycf1, Tic100, and Tic56; Kikuchi et al., 2013). This scenario is in contrast to Tic110, Hsp93, cpHsp70, and Hsp90C, which are universally conserved in all branches of the plant lineage. It is puzzling why, when all other translocon components in the outer membrane, the intermembrane space, and the thylakoid are strictly conserved between all branches of the plant lineage (Shi and Theg, 2013; Chen et al., 2018), most monocots would abandon the Ycf2-FtsHi-MDH motor and the Ycf1 channel complex to evolve different systems for translocation across the inner membrane. In fact, the absence of the Ycf2-FtsHi-MDH motor in monocots requires an alternative motor, and this role by all estimation could be filled by the universally conserved Tic110, Hsp93, cpHsp70, and Hsp90C systems.

Here, we provide a brief summary of the data supporting each of the previously identified motors (Hsp93, cpHsp70, and Hsp90C) in driving protein import into chloroplasts and a list of references that document these findings (Table 1). None of the conclusions listed in Table 1 rely on the association of these ATPases with Tic110 and Tic40, and for this reason, we have chosen not to list the expansive literature establishing roles for Tic110 and Tic40 in protein import. In a review article (Nakai, 2018), the corresponding author of the Kikuchi et al. (2018) article provided his perspective on shortcomings of the data supporting roles for Hsp93, cpHsp70, and Hsp90C in the import motor. We take this opportunity to revisit this question, and in doing so, hopefully provide a more balanced view of the roles of these three chaperones as components of the import motor.

## Hsp93

Hsp93 (not the Ycf2-FtsHi-MDH complex) was the first “pulling AAA-ATPase motor” identified for chloroplast protein import. Hsp93 belongs to the family of class I (with

**Table 1.** List of Experimental Results Supporting Each Chaperone ATPase as Part of the Chloroplast Protein Import Motor

Major Conclusion	Experimental Approach	Reference	Notes
Hsp93			
Hsp93 is a component of import complexes	Anti-Toc75 antibody coimmunoprecipitated Hsp93	(Nielsen et al., 1997) (Akita et al., 1997)	No cross-linker used, solubilized with 1% decylmaltoside Cross-linked with dithiobis(succinimidylpropionate) (DSP)
	Anti-cpHsc70 antibody coimmunoprecipitated Hsp93, Toc75, and Toc34	(Su and Li, 2010)	Toc75 identified by both immunoblotting and mass spectrometry
	Hsp93 cofractionated with other translocon components, including Toc75, in sucrose density gradients	(Nielsen et al., 1997)	
	Anti-Toc34 antibody coimmunoprecipitated Hsp93, Toc75, and Tic20	(Kouranov et al., 1998)	Untreated chloroplasts or chloroplasts cross-linked in the presence of 400 nM preprotein; solubilized with 1% Triton X-100
Hsp93 is a component of complexes actively engaged in protein import	Bound prRBCS, prLHCP, and prPC, but not outer-membrane OEP14, were coimmunoprecipitated by anti-Hsp93 antibodies	(Nielsen et al., 1997)	No cross-linker used, solubilized with 1% decylmaltoside
	PrRBCS was coimmunoprecipitated by anti-Hsp93 antibodies in import time course	(Chou et al., 2003)	Cross-linked with 0.5 mM DSP and solubilized with Triton X-100
	Translocating prRBCS and prL11 coimmunoprecipitated by anti-Hsp93 antibodies	(Su and Li, 2010)	If preproteins added after chloroplast lysis, no coimmunoprecipitation was observed
	Hsp93 detected in soluble import complexes purified using preprotein-GST fusions and GSH beads	(Rosano et al., 2011)	Recombinant preprotein used is pea ferredoxin-NADP reductase transit peptide fused to GST
	Anti-Hsp93 antibody immunoprecipitated large (>700 kD on SDS-PAGE) cross-linked complex containing preproteins	(Akita et al., 1997)	
<i>hsp93</i> mutant chloroplasts are defective in protein import into chloroplasts	In vitro translated preproteins imported into chloroplasts isolated from mutant and wild-type plants	(Constan et al., 2004) (Kovacheva et al., 2005) (Kovacheva et al., 2007; Flores-Pérez et al., 2016) (Su and Li, 2010)	<i>hsp93V</i> -knockout Arabidopsis plants <i>hsp93V</i> -knockout Arabidopsis plants; both prRBCS (photosynthetic) and prL11 (nonphotosynthetic) preproteins used Double mutants of <i>hsp93V</i> knockout and <i>hsp93III</i> knockdown <i>hsp93V</i> -knockout Arabidopsis plants have an import defect; the <i>cphsc70-1 hsp93V</i> double mutant has a more severe import defect
	Transient expression of preproteins in protoplasts isolated from <i>hsp93V</i> mutant and wild-type plants	(Lee et al., 2018)	
Association of Hsp93 with the inner envelope membrane is important for its functions	Hsp93V-ΔN deletion mutant cannot complement the <i>hsp93V</i> null mutant; AtHsp93V-ΔN has the same ATPase activity but severely reduced membrane and Tic110 association	(Chu and Li, 2012)	Hsp93V-ΔN is an Hsp93V mutant with the N-terminal domain deleted
Hsp93 directly binds transit peptides	<i>Escherichia coli</i> expressed recombinant ClpC2 (i.e. Hsp93III) and ClpD specifically bind preproteins in vitro	(Rosano et al., 2011)	The preprotein used is pea ferredoxin-NADP reductase transit peptide fused to GST
	Transit peptides of translocating preproteins directly cross-linked to Hsp93 at early stages of active import into chloroplasts	(Huang et al., 2016)	Two different preproteins, prRBCS and prTic40, were used

(Continued)

**Table 1.** (continued).

Major Conclusion	Experimental Approach	Reference	Notes
Hsp90C			
Hsp90C is a component of complexes containing translocating preproteins	Hsp90C, together with Toc75 and Tic110, specifically copurified with bound preproteins, prRBCS or prTic40	(Inoue et al., 2013)	
Inhibition of Hsp90 ATPase activity reduces protein import	Treatment of isolated chloroplasts with the Hsp90 inhibitor, radicicol, reversibly inhibits ATP-dependent protein import	(Inoue et al., 2013)	
cpHsp70			
Hsp70 binds to chloroplast transit peptides	Hsp70 binds prSSU and prFd transit peptides	(Ivey et al., 2000)	Both DnaK and Hsc70 isoforms of Hsp70 were tested
	Transit peptides containing Hsp70 binding elements were targeted to chloroplasts, whereas those without were not	(Chotewutmontri and Bruce, 2015)	
cpHsp70 is essential in <i>Arabidopsis</i> and <i>Physcomitrella</i>	Targeted knockout of <i>cpHsp70-2</i> in the moss, <i>Physcomitrella</i> , is lethal	(Shi and Theg, 2010)	<i>Physcomitrella</i> has three <i>cpHsp70</i> genes, knockout of <i>cpHsp70-1</i> and <i>cpHsp70-3</i> are without obvious phenotype
	T-DNA insertion knockout of both copies of <i>cpHsp70</i> in <i>Arabidopsis</i> is lethal	(Su and Li, 2010)	Individual knockouts are not lethal
Reduced cpHsp70 slows protein import into chloroplasts	Knockouts of individual <i>cpHsp70</i> genes in <i>Arabidopsis</i> display reduced rates of protein import in young chloroplasts	(Su and Li, 2010)	
	Temperature-sensitive mutants of moss <i>cpHsp70-2</i> display reduced rates of protein import after a heat shock	(Shi and Theg, 2010)	Two different temperature-sensitive mutants behaved similarly
cpHsp70 is a component of import complexes	Anti-Hsp70 antibodies coimmunoprecipitate Hsp93 and Tic40 in moss	(Shi and Theg, 2010)	In CGE knockdown mutant with preproteins added
	Anti-cpHsp70 antibodies coimmunoprecipitate Toc75, Toc34, Tic110, Tic40, and Hsp93 in pea ( <i>Pisum sativum</i> ) chloroplasts	(Su and Li, 2010)	No preprotein addition, indicating that cpHsp70 is preassembled in import complexes
cpHsp70 is in complexes containing translocating preproteins	Anti-cpHsp70 antibodies coimmunoprecipitate translocating preproteins, prSSU and prCGE, in moss	(Shi and Theg, 2010)	In CGE knockdown mutant with preprotein added
	Anti-cpHsp70 antibodies coimmunoprecipitate translocating preproteins, prRBCS and prL11, in pea chloroplasts	(Su and Li, 2010)	
cpHsp70 cochaperones are required for chloroplast protein import	CGE knockdown mutants display reduced rates of chloroplast protein import in moss	(Shi and Theg, 2010)	
Change of cpHsp70 $K_m$ for ATP hydrolysis translates into change of ATP requirement for protein import	Chloroplasts harboring cpHsp70 with a 2.6-fold increased $K_m$ for ATP hydrolysis required 3.1 times as much ATP to import prSSU	(Liu et al., 2014)	Two different cpHsp70 amino acid substitutions behaved similarly

two ATPase domains, as opposed to class II with only one ATPase domain) AAA+ proteins (ATPases associated with various cellular activities; White and Lauring, 2007; Sauer and Baker, 2011). Members of this family form double-ring hexameric complexes with a central pore. They use the energy of ATP hydrolysis to

create pulses of pulling force (not a Brownian ratchet mechanism as suggested by Hermann [2018]) to actively drive translocation of their substrate proteins through the central pore. Well-known family members include NSF (*N*-ethylmaleimide-Sensitive Factor) for SNARE complex disassembly and p97 (Cdc48)

for pulling proteins out of the endoplasmic reticulum for degradation by the proteasome. Hsp93 was first reported as being present in the chloroplast protein import translocon in 1997 (Akita et al., 1997; Nielsen et al., 1997) and confirmed by at least three other laboratories (Kouranov et al., 1998; Chou et al., 2003; Rosano

et al., 2011). Hsp93 also directly binds transit peptides of translocating preproteins at early stages of the import process (Rosano et al., 2011; Huang et al., 2016). In *Arabidopsis* (*Arabidopsis thaliana*), Hsp93 exists as two isoforms encoded by the genes *HSP93-III* and *HSP93-V*. Hsp93-V is the major functional form, with knockout mutants having pale green leaves and a reduced rate of protein import into chloroplasts. This import-deficient phenotype has been independently observed by at least four laboratories (Constan et al., 2004; Kovacheva et al., 2005, 2007; Su and Li, 2010; Flores-Pérez et al., 2016; Lee et al., 2018), while only one study reported that they observed no import defect (Sjögren et al., 2004). Knockout mutants of *HSP93-III* display no obvious growth or import-deficient phenotype, but knocking out both *HSP93-III* and *HSP93-V* results in embryo lethality (Kovacheva et al., 2007), indicating that the two Hsp93 isoforms play redundant but essential functions.

In addition to reports of Hsp93 functioning as an import motor, Hsp93 has been described to associate with members of the ClpP protease complex and to participate in protein homeostasis through its role in unfolding protein substrates for the ClpP protease complex (Nishimura and van Wijk, 2015). In the review article by Nakai (2018), the author presented three lines of evidence arguing that Hsp93 only functions in protein degradation and not in protein import. The author first cited the work of Moreno et al. (2018) and wrote that RNAi-mediated knockdown of *HSP93* in tobacco (*Nicotiana tabacum*) “causes a significant enrichment, as opposed to a reduction, of a wide variety of imported proteins inside the chloroplasts.” However, although Moreno et al. (2018) showed enrichment of 123 chloroplast proteins, they also showed decreased levels of an additional 153 chloroplast proteins in the *HSP93*-knockdown plants. In fact, Moreno et al. (2018) specifically noted that the vast majority of down-regulated proteins were identified in the proteome of *HSP93*-knockdown plants, as opposed to proteomes of plants with reduced expression of other ClpP complex components, and the authors suggested that this result agreed with the reported function of Hsp93 in protein import.

Second, Nakai (2018) wrote that components of the ClpP complex were recruited to the inner membrane by Hsp93, which associates with the inner membrane via its binding to Tic110 (Sjögren et al., 2014; Flores-Pérez et al., 2016). It was further shown that an *HSP93* mutant with reduced interaction with ClpP components failed to complement the phenotypes of pale green leaves and reduced rates of protein import, suggesting that failure to interact with the ClpP protease was the primary cause of *hsp93* phenotypes (Flores-Pérez et al., 2016). However, it is not clear why reducing the amount of ClpP protease at the envelope would reduce the import rate of the minute amounts of radioactive preproteins used in the import experiments. Moreover, whether the *Hsp93* mutation used in the experiment compromised the ATPase activity of Hsp93 needs to be tested. In any case, these findings do not exclude the possibility that Hsp93 has dual functions in protein import and protein degradation.

Third, it was demonstrated that Hsp93 can be directly cross-linked to transit peptides of different preproteins during early stages of active import (Huang et al., 2016). This finding was criticized by Nakai (2018) as lacking sufficient specificity because, apart from the preprotein-Hsp93 adducts, some un-cross-linked preproteins were also immunoprecipitated by the anti-Hsp93 antibody. Un-cross-linked preproteins were, by far, the dominant radioactive signal in the samples before immunoprecipitation. When the gels were exposed for a longer period to observe weaker cross-linked species, background bands of un-cross-linked preproteins were often observed. This type of result has been seen by many laboratories (Akita et al., 1997; Geissler et al., 2002; Yamamoto et al., 2002; Banerjee et al., 2015; Richardson et al., 2018). When this same cross-linking approach was used in the Kikuchi et al. (2018) study to demonstrate direct cross-linking of FtsHi1 to a preprotein, the region of the gel where the un-cross-linked preproteins would be located was not shown.

### Hsp90C

The evidence for a specific role for Hsp90C is limited to one report (Inoue

et al., 2013). Hsp90C was identified in complexes containing translocating preprotein intermediates in the later stages of protein import, and it was later shown to associate with known TIC complex components, including Tic110, cpHsp70, and Hsp93 (Inoue et al., 2013). Its potential role in import was demonstrated using the reversible Hsp90 ATPase inhibitor, radicicol. Radicicol treatment of isolated chloroplasts did not inhibit binding of model preproteins at the TOC complex, but it did inhibit the ATP-dependent translocation of the polypeptide across the inner membrane (Inoue et al., 2013). These data, in addition to the known interaction of cpHsp70 and Hsp90C in complexes containing additional cochaperones in *Arabidopsis* (Willmund and Schroda, 2005; Willmund et al., 2008), led to the proposal that Hsp90C participates with other stromal chaperones as part of the import motor. Hsp90C is essential for plastid biogenesis, and null mutants are embryo-lethal in *Arabidopsis* (Feng et al., 2014). We do realize that additional molecular genetic data are required to test the role of Hsp90C in import and will need to rely on the identification of specific, viable point mutants that inhibit protein import while maintaining other essential functions of the chaperone in plastid biogenesis.

### cpHsp70

cpHsp70 is a member of the well-known family of 70-kD heat shock proteins (Hsp70) that serve as molecular chaperones. This family, initially postulated to mediate protein folding (Pelham, 1986)—and it certainly does—was recognized early on as having a major role in protein targeting (Chirico et al., 1988; Deshaies et al., 1988). It is now well established that the motor driving the posttranslational import of proteins into mitochondria and the endoplasmic reticulum are *trans*-side Hsp70 chaperones (mtHsp70 and BiP, respectively). Marshall and Keegstra (1992) were the first to detect Hsp70 homologs in chloroplasts, with multiple versions residing in the stroma, the receiving compartment for protein import across the envelope membranes. This led to the prediction that one or multiple stromal Hsp70s, referred to hereafter as cpHsp70, constitute the import motor

coupling ATP hydrolysis to chloroplast protein import (Marshall and Keegstra, 1992). While a motor role was long attributed to Hsp93 (see above), two articles from our laboratories published in 2010 (Shi and Theg, 2010; Su and Li, 2010) and a third published in 2014 (Liu et al., 2014) provided compelling evidence that stromal cpHsp70s contribute significantly to the activity of the import motor. The most relevant experiments are described below and listed in Table 1.

The theoretical underpinnings behind the idea that a chloroplast cpHsp70 participates in powering plastid protein import was provided by the Bruce lab through experiments monitoring the interaction of chloroplast transit peptides with cpHsp70 chaperones. This group not only demonstrated that two well-characterized chloroplast transit peptides had binding sites for both DnaK and Hsp70 (Ivey et al., 2000), they also showed that those transit peptides lacking such binding sites did not target proteins to chloroplasts (Chotewutmontri and Bruce, 2015).

Perhaps the more compelling evidence in favor of cpHsp70 as an import motor comes from experiments from two of our laboratories, performed without knowledge of each other's efforts, with different plant species and published essentially simultaneously. We showed first that relevant genes for cpHsp70 are essential (Shi and Theg, 2010; Su and Li, 2010). While this does not implicate cpHsp70 directly in chloroplast protein trafficking, it is a hallmark of organellar targeting mutants in other systems (Altmann and Westermann, 2005; Zhang and Ren, 2015). We then demonstrated that chloroplasts isolated from knockout mutants of either of the two *Arabidopsis* cpHsp70 isoforms, or from *Physcomitrella* *cpHsp70* temperature-sensitive mutant plants, were compromised in their ability to import proteins. This extended to a knock-down mutant of the cpHsp70 cochaperone CGE, encoding a chloroplast homolog of bacterial GrpE that exhibits Hsp70-specific nucleotide-exchange activity. Significantly, the ATPase activity of the moss cpHsp70-2 showed a strong preference for the moss CGE, demonstrating that the cochaperones targeted their cognate chaperones (Liu et al., 2014). We went on to show that cpHsp70 interacted with known components of the TOC and TIC

complexes and that they immunoprecipitated preproteins in the act of being transported (Shi and Theg, 2010; Su and Li, 2010). Such experiments are the gold standard for identifying protein components involved in organellar protein import. In fact, the same approach contributes significantly to the conclusions of Kikuchi et al. (2018) as well as in the earlier and still controversial article calling into question the composition of the TIC complex (Kikuchi et al., 2013). In light of our experiments, we hold that the statement by Kikuchi et al. (2018) that "no direct physical interaction between the TIC complex and the stromal Hsp70 has so far been observed" is incorrect.

The experiments published by Liu et al. (2014) also provide persuasive evidence that cpHsp70 is a major part of the import motor. In that work, we made two point mutations in the moss cpHsp70-2 predicted to increase the  $K_m$  for ATP hydrolysis (based on knowledge acquired from bovine Hsp70). Our rationale was that decreasing cpHsp70-2 affinity for ATP should translate into an increased requirement for ATP for *in vitro* protein import, and this is exactly what happened. While the  $K_m$  for ATP hydrolysis in the mutant cpHsp70-2s was increased 2.6-fold over that of the wild type, the  $K_m$  for ATP utilization during the protein import reaction increased threefold to fourfold, depending on the preproteins examined. We concluded that while cpHsp70-2 is not necessarily the only ATPase contributing to protein import, it dominates the energetics, and thus must be part of the motor.

## CONCLUDING REMARKS

We believe that it is not possible to explain our experimental results, taken collectively, without invoking a role for the three chaperones (Hsp93, cpHsp70, and Hsp90C) in preprotein translocation across the chloroplast envelope. The sentence quoted above from Kikuchi et al. (2018) ends with "...the idea that the stromal Hsp70 acts as the import motor remains an open question." We submit that there are many interesting open questions concerning the individual roles that these chaperones play in the import motor, but whether they participate at all is not one of them. To this point, Huang et al. (2016)

have attempted to study the different contributions of the chaperones and showed that Hsp93 directly binds to translocating preproteins only at early stages before and during transit peptide processing, while cpHsp70 is associated with preprotein complexes throughout the import process. It is likely that structural data for the complexes and biochemical experiments with reconstituted systems will be required to fully understand the underlying functional mechanisms. However, we can speculate now that initial pulling by Hsp93, accompanied by continuous translocation through Brownian ratchet motions provided by cpHsp70, will be required for efficient preprotein import.

The Ycf2-FtsHi-MDH may indeed perform some motor function in chloroplasts of *Arabidopsis* and tobacco, but this does not exclude Hsp93, cpHsp70, and Hsp90C from being part of an import motor; the extensive experimental evidence we list herein indicates just the opposite. It is unclear why the work on Ycf1/2 and the Hsp93/70/90 chaperone ATPase complexes must be mutually exclusive. We hold that the most productive way forward is to engage in efforts to generate a unifying model that reconciles all of the accumulated data. This will be facilitated by in-depth analysis of the specific relevance and function of each component as well as the methodology used to describe their role along the protein import process.

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