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Review

ATP-dependent molecular chaperones in plastids — More complex than expected☆

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ABSTRACT

Plastids are a class of essential plant cell organelles comprising photosynthetic chloroplasts of green tissues, starch-storing amyloplasts of roots and tubers or the colorful pigment-storing chromoplasts of petals and fruits. They express a few genes encoded on their organellar genome, called plastome, but import most of their proteins from the cytosol. The import into plastids, the folding of freshly-translated or imported proteins, the degradation or renaturation of denatured and entangled proteins, and the quality-control of newly folded proteins all require the action of molecular chaperones. Members of all four major families of ATP-dependent molecular chaperones (chaperonin/Cpn60, Hsp70, Hsp90 and Hsp100 families) have been identified in plastids from unicellular algae to higher plants. This review aims not only at giving an overview of the most current insights into the general and conserved functions of these plastid chaperones, but also into their specific plastid functions. Given that chloroplasts harbor an extreme environment that cycles between reduced and oxidized states, that has to deal with reactive oxygen species and is highly reactive to environmental and developmental signals, it can be presumed that plastid chaperones have evolved a plethora of specific functions some of which are just about to be discovered. Here, the most urgent questions that remain unsolved are discussed, and guidance for future research on plastid chaperones is given. This article is part of a Special Issue entitled: Chloroplast Biogenesis.

1. Introduction

Plastid biogenesis and function depend on the orchestrated expression of nuclear and plastid encoded proteins. While the autonomous plastid genome encodes only a small subset of plastid proteins, the majority of the ~3000 plastid proteins are encoded by nuclear DNA and imported into the organelle after their synthesis in the cytosol [1,2]. These precursors are then processed and sorted to their final destination within the organelle. Comparable to other cellular compartments, plastids contain a number of conserved factors dedicated to maintain a healthy proteome. Many of these factors belong to the family of molecular chaperones that transiently bind other proteins (termed substrates or clients) to assist their folding to the native state. These comprise chaperones that only bind to misfolded polypeptides to prevent aggregation (e.g. small heat shock proteins), chaperones that recognize misfolded proteins to locally unfold them under ATP consumption to allow their refolding to the native state (Hsp60s and Hsp70s), or chaperones that disentangle protein aggregates (Hsp100s). In addition, molecular chaperones functionally support protein translocation across membranes, promote complex assembly and disassembly, and participate in many other regulatory processes within the cell [3–7]. Since many chaperones were found to accumulate in cells exposed to heat stress these proteins often are termed heat shock proteins (HSPs).

The functions of molecular chaperones and underlying mechanisms are best studied in bacteria and the cytosol/ER of eukaryotic cells. In contrast, comparably little is known about their function in plastids, even though the presence of a unique and highly complex thylakoid membrane compartment in chloroplasts is ultiamtely essential for almost all forms of life on earth. Despite their homology with eubacterial counterparts, plastid chaperones harbor distinct properties indicating their specific adaptation to the folding requirements of the organelle's unique proteome.

In this review we aim at summarizing current knowledge on the composition and function of ATP-dependent molecular chaperone systems in plastids. Thus, we focus on the Cpn60/Cpn10, Hsp70, Hsp90, and Hsp100 classes of molecular chaperones.

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2. Plastid chaperonins

2.1. Architecture and functions of plastid Cpn60s

A general characteristic of chaperonins is their barrel-shaped architecture formed by two stacked oligomeric rings consisting of 60 kDa subunits. Eubacterial (GroEL), plastid (Cpn60) and mitochondrial (Hsp60) representatives are categorized as type I chaperonins. They assemble into heptameric rings and require an oligomeric co-factor forming a lid to encapsulate substrates within a central cavity. This cofactor is GroES in bacteria, Hsp10 in mitochondria, and Cpn10/20 in plastids. The structurally more distinct type II chaperonins are found in archaea (thermosome) and the eukaryotic cytosol (TRiC/CCT) and contain eight subunits per ring with two to eight different isoforms. Here, helical protrusions of each subunit contribute to an in-built lid which substitutes for the function of GroES/Hsp10/Cpn10/20 (reviewed in [8,9]).

Historically, plastid chaperonins were one of the earliest described in literature when they were shown to interact with the newly synthesized large subunit of Rubisco [10]. However, in the following years most knowledge about chaperonin architecture and function was gathered for the bacterial isoform GroEL/ES. In general, all chaperonins have in common that substrate binding and release is fuelled by ATP hydrolysis which drives conformational changes for switching between binding-active and folding-active states of the chaperonin complex. The folding cycle is best understood for the bacterial GroEL/ES system, in which both GroEL heptameric rings act in an asymmetric and anti-cooperative behavior also termed "two-stroke engine" (Fig. 1A). Binding of non-native substrate proteins takes place at the inner wall of the cavity and is mediated through exposed hydrophobic residues at the apical domains (in the *cis* ring). Binding of ATP and GroES to the *cis* ring results in conformational changes of GroEL that enlarge the cavity and change its wall surface from a hydrophobic to a highly hydrophilic, net-negative one. This transition is thought to be an important factor promoting folding. The time required by GroEL to hydrolyse ATP provides about 10 s for substrate folding to take place within the cavity. Binding of ATP and GroES to the opposite *trans* ring results in GroES dissociation from the *cis* ring and subsequent substrate and ADP release (reviewed in [9]). It can be assumed that protein folding by chloroplast chaperonins follows a similar mechanism.

Plastid chaperonins possess an intriguing feature that is not shared by other group I chaperonin family members: different isoforms exist for both Cpn60 and Cpn10 [11,12]. Cpn60s exist as isoforms alpha and beta that share only about 50% amino acid sequence identity. For example, the unicellular green alga *Chlamydomonas reinhardtii* encodes three plastid-targeted Cpn60 members, termed CPN60A, CPN60B1, and CPN60B2 [12] (Table 1). *Arabidopsis thaliana* encodes six plastid members, including three abundant subunits (Cpn60α2, Cpn60β2, and Cpn60β3), and three low abundant ones (Cpn60α1, Cpn60β1, and Cpn60β4). Although the exact subunit composition of formed heptameric rings remains elusive, a number of findings suggest that they are composed of a mixture of alpha and beta subunits (reviewed in [13]) (Fig. 1B). In vitro studies with purified recombinant Cpn60 isoforms from *P. sativum* indicated that Cpn60α needs Cpn60β to assemble into hetero-oligomers. In contrast, purified Cpn60β subunits were able to auto-assemble into functional homo-oligomers. Likewise, *B. napus* Cpn60β expressed in *Escherichia coli* assembled into tetradecameric complexes that were able to fold the large subunit of cyanobacterial Rubisco, while the alpha-isoforms neither assembled into oligomers nor did they show any folding activity [14–16]. Also Cpn60β1, Cpn60β2, and Cpn60β3 from *A. thaliana* were shown to form homo-oligomers. However, despite their close sequence similarity physical properties varied between the individual isoforms regarding their ability to assemble into oligomers, oligomer stability, and preference for co-factors in folding assays. These varying properties were suggested to result from minor amino acid sequence variations within the apical

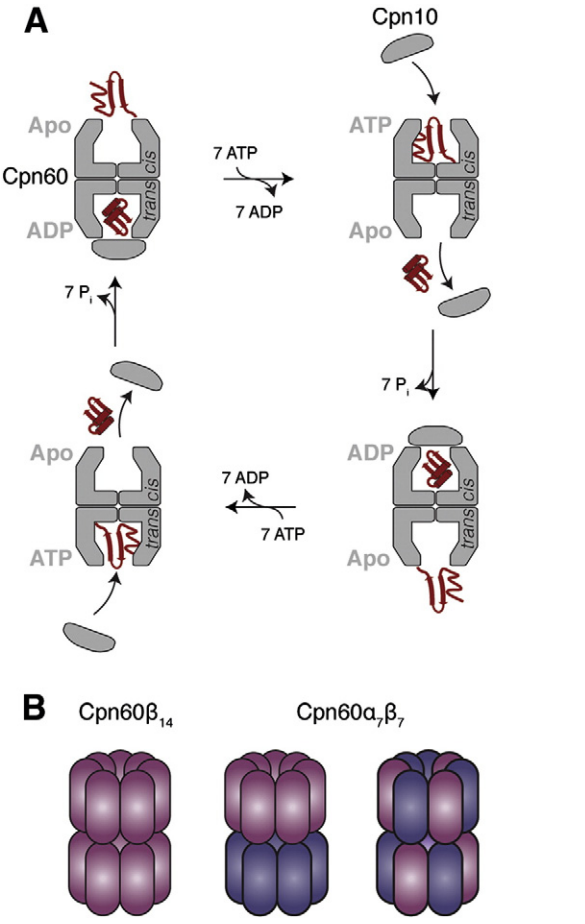

Fig. 1. Schematic folding cycle and composition of plastid chaperonins. A) Folding cycle of plastid chaperonins adopted from that of bacterial GroEL/ES. Unfolded substrates bind to hydrophobic surfaces exposed by the apical domains of Cpn60 subunits in the nucleotide-free *cis*-ring (Apo). Binding of ATP and the Cpn10/Cpn20 oligomer induce conformational changes in the Cpn60 subunits of the *cis*-ring, leading to the exposure of hydrophilic surfaces and to an enlargement of the folding cage. The time needed to hydrolyze the seven ATP molecules in the *cis*-ring provides a period of ~10 s for the substrate to fold in the protected environment of the chaperonin. Binding of ATP and the Cpn10/Cpn20 cofactor to the *trans*-ring causes dissociation of the cofactor from the *cis*-ring and release of substrate and ADP. B) Possible arrangements of Cpn60α (blue) and Cpn60β (violet) within the Cpn60 oligomer. While Cpn60β may assemble into homo-oligomers, Cpn60α requires Cpn60β to form hetero-oligomeric complexes.

domain, which is important for co-chaperonin and substrate binding [17]. In vivo, the composition of Cpn60 oligomers appears to be highly complex. For example, chaperonins were isolated from *A. thaliana* lysates that contained Cpn60α2 together with all four Cpn60β isoforms (50% were Cpn60α2, 15% Cpn60β4, and 35% a mixture of Cpn60β1-3). This particular complex appears specifically dedicated to fold NdhH, a subunit of the chloroplast NADH dehydrogenase-like complex [18].

Studies with *A. thaliana* mutants of the three abundantly expressed isoforms Cpn60α2, Cpn60β2, and Cpn60β3 indicate an essential function for them in chloroplast biogenesis and development: both *A. thaliana* Cpn60α and Cpn60β isoforms were demonstrated to be required for the proper assembly of the FtsZ plastid division ring [19]. *A. thaliana cpn60α2* single and *cpn60β2*/*cpn60β3* double mutants show impaired plastid division, small growth and an albino phenotype. Interestingly, *cpn60β2* or *cpn60β3* single mutants showed less severe phenotypes, hinting to an overlapping function of these isoforms [19,20]. Cpn60s also appear to play a role in protecting plants during heat stress as indicated by their up-regulation during heat shock [21–25]. In this regard, Cpn60β was shown to protect Rubisco activase from

Table 1
Plastid chaperonins.

Protein type	Proposed function	*A. thaliana* homologs (locus name)[1]	*P. patens* homologs (locus name)	*C. reinhardtii* homologs (locus name)[3]	Localization	Relative protein abundance in *C. reinhardtii*[2]	Mutant phenotypes	References function assignments
α-type Cpn60	Protein folding (e.g. RbcL); plastid division; RNA splicing	Cpn60α1 (At5g18820) Cpn60α2 (At2g28000)	Cpn60α1 (Phpat.024G043800) Cpn60α2 (Phpat.008G059000) Cpn60α3[4] (Phpat.019G064600)	CPN60A (Cre04.g231222)	Stroma	238 ± 16.5	Albino, small growth, severely impaired plant development (*A. thaliana cpn60α2* knockout *schlepperless*)	[10,15,19,20,27,32]
β-type Cpn60	Protein folding (e.g. RbcL, Rubisco activase, NdhH); plastid division	Cpn60β1 (At5g56500) Cpn60β2 (At3g13470) Cpn60β3 (At1g55490) Cpn60β4 (At1g26230)	Cpn60β1 (Phpat.016G017000) Cpn60β2 (Phpat.027G022600) Cpn60β3 (Phpat.006G075500)	CPN60B1 (Cre17.g741450) CPN60B2 (Cre07.g339150)	Stroma	468 ± 45 157 ± 14	Albino, small growth, severely impaired plant development (*A. thaliana cpn60β2/β3* –double knockout) Defective in NDH activity (*A. thaliana cpn60β4* knockout *crr27*)	[10,15,18,19,26,32]
Single-domain Cpn10	Cofactor of Cpn60	Cpn10-1 (At3g60210) Cpn10-2 (At2g44650)	Cpn10-1 (Phpat.001G078400) Cpn10 (Phpat.002G081300)	CPN11 (Cre16.g673729)	Stroma	321 ± 44.5	Wildtype phenotype in *A. thaliana*	[35,37]
Tandem-domain Cpn10	Cofactor of Cpn60; superoxide dismutase activation; ABA signaling	Cpn20 (At5g20720)	Cpn20-1 (Phpat.022G027600) Cpn20-2 (Phpat.019G013500)	CPN20 (Cre08.g358562) CPN23 (Cre12.g505850)	Stroma	199 ± 38.5 279 ± 43	Lethal in *A. thaliana*	[35–37,39]

[1] Protein names are according to the nomenclature proposed by [11]. Some studies use the nomenclature of the TAIR database, which is based on relative expression levels leading to reversed numbering of the pairs Cpn60α1/Cpn60α2 and Cpn60β1/Cpn60β3.

[2] Shown is the rank among 1207 soluble proteins from non-stressed *C. reinhardtii* cells. Protein abundance was determined by intensity based absolute quantification (iBAQ) [199]. The rank variance is derived from three independent biological replicates. The data was taken from [200]. For comparison: Plastocyanin (1 ± 0); rbcL (2 ± 1); RPS18 (10 ± 1); TUB2 (11 ± 3); HSP90A (85 ± 6); FKB12 (86 ± 12); RCA1 (248 ± 24.5); HIS1 (507 ± 24); HSP70G (748 ± 50.5); ATPvH (1002 ± 36.5).

[3] To keep consistent with the nomenclature of *Chlamydomonas* genes [201], CPN60α and β are termed CPN60A and B.

[4] Phylogenetic analysis classified this gene product as Cpn60α. However, no transit peptide sequence was predicted.

thermal denaturation [26]. A non-protein-folding related function of CPN60A was observed in *C. reinhardtii*, where it was found to specifically interact with group II intron RNA, suggesting a specialized role as a general organellar RNA splicing factor [27].

For understanding the unique function of chloroplast chaperonin assemblies, it is essential to reveal their global substrate spectrum. Besides the prominently studied substrate RbcL only few other plastid proteins were identified to associate with the complex, including the coupling factor CF1 [28], ferredoxin-$NADP^+$ reductase [29], phytoene desaturase [30] and the Rieske protein [31]. Notably, several of these substrates require both Hsp70 and Cpn60 to assume the native state. Cpn60 was generally implicated in the folding of newly imported plastid proteins [32].

2.2. Cofactors of plastid chaperonins

Like for Cpn60, various Cpn10 isoforms are found in plastids. Besides the conventional Cpn10 isoforms, plastid Cpn60s were shown to function together with a co-factor of approximately 20 kDa (termed Cpn20), which contains a head-to-tail fusion of two Cpn10 domains, joined by a putative TDDVKD-linker sequence (reviewed in [33]). Both, Cpn10 and Cpn20 have been described to coexist in various plant species ranging from green algae to higher plants: *C. reinhardtii* encodes two plastid Cpn20 homologs (CPN20 and CPN23) and one Cpn10 isoform (CPN11) (Table 1), while *A. thaliana* encodes only one plastid Cpn20 and two Cpn10 isoforms [11,12,34]. Genes encoding Cpn20s are not found in cyanobacteria, indicating that a potential gene fusion event must have occurred in the common ancestor of green algae and higher plants after the endosymbiotic event. For *A. thaliana* it was possible to generate viable single knockout mutants of both *Cpn10* genes, suggesting that the two Cpn10 cofactors can substitute for one another [35]. In contrast, attempts to knockout the *Cpn20* gene failed [35,36].

A better understanding of cofactor complex composition and arrangement is just emerging. It has been reported that recombinant cofactors of *C. reinhardtii* do not form functional homo-oligomers but rather need to assemble as $CPN11_1/CPN20_3$ or $CPN11_1/CPN23_3$ hetero-oligomers. Both assemblies were functional and matched the sevenfold symmetry of the chaperonin complexes with seven Cpn10 domains. Comparable compositions of hetero-oligomeric Cpn10/Cpn20 were observed for *A. thaliana* cofactors [37]. However, also homo-tetrameric Cpn20 complexes were reported to serve as functional Cpn60 cofactor, raising the question how the sevenfold symmetry is accomplished with eight Cpn10 domains [33,34,38]. This might be achieved through proteolytic cleavage of one Cpn20 protein, as seen for the *A. thaliana* $Cpn20_4$ complex [37]. It has been suggested that a varying number of Cpn10/20 components in the hetero-oligomeric cofactor complex might correlate with different binding affinities to the Cpn60 complex and thus provide different folding kinetics for the respective substrates [33]. Together with the diverse Cpn60 subunits, the multiplicity of cofactor oligomers provides a number of combinatorial possibilities, thus facilitating a tremendous flexibility of this system in terms of regulation and specificity.

Recently, Cpn20 of higher plants was postulated to have a second function which is independent of its co-chaperonin role, i.e., in abscisic acid (ABA) signaling and the activation of iron superoxide dismutase (FeSOD) [35,36,39]. Moreover, Cpn20 function might be regulated by calcium since the C-terminus of Cpn20 was shown to bind calmodulin in a calcium-dependent manner [40].

In summary, the many chaperonin complex compositions made possible by the presence of different isoforms might adapt this chaperone to the specific folding requirements in the complex plastid proteome, and moreover might allow it to perform functions not related to protein folding. Therefore, to better understand plastid chaperonins, it will be important to elucidate the subunit compositions realized in chaperonin complexes and their respective substrates.

3. Plastid Hsp70s and their co-chaperones

Hsp70s are highly conserved molecular chaperones composed of an N-terminal nucleotide binding domain (NBD) and a C-terminal substrate binding domain (SBD) that are allosterically coupled [41]. With their SBD Hsp70s interact with hydrophobic sequences flanked by basic residues, a motif statistically occurring every 36 amino acids in every protein [42]. The affinity of the SBD for substrates is determined by the type of nucleotide bound to the NBD: while the ATP-state is characterized by a low affinity for substrates, the ADP-state shows high affinity for substrates [43]. Hsp70 binding to substrates may shield exposed hydrophobic regions to prevent protein aggregation [44]. However, Hsp70 binding may also lead to a local unfolding of bound substrate proteins, thus allowing them to undergo a conformational change [5]. These functions are fundamental for the many processes Hsp70s are involved in, comprising folding of de novo synthesized and misfolded proteins, (dis)assembly of protein complexes, or protein translocation through biological membranes [45–47].

Hsp70s can be divided into Hsp110-type and DnaK-type Hsp70s. Hsp110-type Hsp70s are localized to cytosol (Hsp110) and ER (Grp170) and were shown to cooperate with DnaK-type Hsp70s in solubilizing and reactivating substrate proteins from various protein aggregates [48–50]. DnaK-type Hsp70s can be subdivided into those which are regulated by GrpE-type nucleotide exchange factors (NEF) (the main bacterial and organellar Hsp70s), those regulated by BAG-1 (Hsp70s in the eukaryotic cytosol), and those that do not need any NEF (specialized bacterial and organellar Hsp70s) [51]. In addition to NEFs, Hsp70s function in concert with J-domain co-chaperones [52]. J-domain proteins (JDPs) stimulate the ATPase activity of their Hsp70 partner and deliver specific substrates to its SBD [53,54]. Thus, J-domain proteins mediate substrate specificity and thereby the function of their Hsp70 partner. Moreover, Hsp70s may cooperate with other chaperone systems, e.g. with ClpBs/small HSPs in protein disaggregation and refolding [55], with GroEL/Hsp60 in protein folding [44], or with the Hsp90 system in protein folding and signal transduction [56]. Although almost all of these mechanistical insights were obtained on Hsp70s from bacteria, yeast and mammals they are, for all we know, valid also for plant Hsp70s.

3.1. True plastid Hsp70s appear to exist only in the stroma

Hsp70s have been proposed to exist in four different plastid compartments: the outer envelope [57], the intermembrane space [58–60], the stroma [58,61,62], and the thylakoid lumen [63]. The following evidence argues against the existence of an intermembrane space (ims) Hsp70: (i) imsHsp70 has only been detected immunologically [58–60] and of the three putative chloroplast targeted homologs of *A. thaliana* [64] two are imported into the stroma while the third is not imported in vitro [65,66]. Hence, a gene encoding imsHsp70 is elusive. (ii) The JDP Toc12, suggested to cooperate with imsHsp70 in protein import [60], is in fact a stromal protein [67,68]. Also evidence against the existence of Hsp70s in the thylakoid lumen is increasing: (i) although some proteomics studies have detected Hsp70s in the thylakoid lumen [69,70], another one has not [71]. (ii) Similar to imsHsp70, genes containing Hsp70s with bipartite transit sequences targeting them to stroma and thylakoid lumen are elusive. (iii) The existence of an ATP/ADP carrier in the thylakoid membranes [72] has been questioned recently [73] and without ATP in the lumen ATP-dependent chaperones are meaningless. Hence, as a small subfraction of stromal Hsp70 was shown to be firmly associated with (thylakoid) membranes [62,74,75], Hsp70s detected in the ims and thylakoid lumen preparations are very likely contaminations from stromal Hsp70s.

Hsp70s detected at the outer envelope apparently are eukaryotic-type, cytosolic Hsp70s with a subfraction of them engaged in delivering preproteins to the outer envelope translocon [76,77]. By the unequivocal assignment of genes encoding them, clear evidence also exists for

Table 2
Plastid Hsp70s and co-chaperones.

Protein type	Proposed function	*A. thaliana* homologs (locus name)	*P. patens* homologs (locus name)	*C. reinhardtii* homologs (locus name)	Localization	Relative protein abundance in *C. reinhardtii*[1]	Mutant phenotypes	References function assignments
Stromal Hsp70s	Protein folding; protein import into chloroplasts; (dis-)assembly of VIPP1 oligomers; protection/ repair of PSII from photoinhibition	cpHsc70-IV/cpHsc70-1/AtHsp70-6 (At4g24280) cpHsc70-V/cpHsc70-2/AtHsp70-7 (At5g49910)	PpHsp70-1 (Phpat.026G035400) PpHsp70-2 (Phpat.004G105700) PpHsp70-3 (Phpat.007G061900)	HSP70B (Cre06.g250100)	Stroma; membrane-associated	114 ± 15	Protein import defects; defects in plastid development; high light sensitivity; reduced PS accumulation; albino; embryo lethal	[66,75,79, 91,93,124, 134,135]
C-terminally truncated putative stromal Hsp70s	–	–	–	HSP70D (Cre12.g535700) HSP70F (Cre09.g412900)	Unknown	n.d. n.d.	–	–
GrpE homologs	Nucleotide exchange factor for stromal Hsp70s; required to support protein import into chloroplasts	Cge1 (At1g36390) Cge2 (At5g17710)	CGE1 (Phpat.012G088500) CGE2 (Phpat.004G013500)	CGE1 (Cre07.g341600)	Stroma; membrane associated	231 ± 25	Protein import defects; defects in plastid development	[74,79,91,94]
	–	–	–	CGE2 (Cre14.g632000)	Unknown	n.d.	–	–
Hsp70 escort proteins	De novo folding of chloroplast Hsp70s	ZR1/ET1 (At1g68730) ZR2 (At5g27280)	ZR1 (Phpat.021G072000) ZR2 (Phpat.005G013800)	HEP2 (Cre17.g707950)	Stroma	n.d.	Defects in plastid development	[84,137]
True DnaJ homologs, conserved in green lineage and cyanobacteria	Protein folding	DJA4 (At3g17830) DJA5 (At4g39960) DJA6 (At2g22360) DJA7 (At1g80030)	Phpat.016G077300 Phpat.005G075100 Phpat.007G015300 Phpat.022G074400 Phpat.006G030200	CDJ1 (Cre12.g507650)	Stroma; membrane associated	905 ± 27.5	–	[91]
JDPs, conserved in green lineage	Delivery of VIPP1 to Hsp70	DJC73 (At5g59610)	Phpat.023G006000 Phpat.023G034200	CDJ2 (Cre07.g316050)	Stroma	n.d.		[93]
JDPs, conserved in green lineage	Redox regulation?; contain bacterial-type ferredoxin domain	DJC77 (At2g42750) DJC76 (At5g23240) DJC82 (At3g05345)	Phpat.003G126400 Phpat.027G004700 Phpat.005G051600 Phpat.024G004200 Phpat.020G080400 Phpat.027G004400	CDJ3 (Cre01.g009900 CDJ4 (Cre02.g104500) CDJ5 (Cre07.g320350)	Stroma; membrane associated	n.d. n.d. n.d.		[102]
JDPs, conserved in green lineage	Unknown; contain TPR domains	DJC31 (At5g12430) DJC62 (At2g41520)	Phpat.009G054100	CDJ6/DNJ26/TPR10 (Cre02.g108800)	Unknown	n.d.	–	–

JDPs, only in land plants	Calvin cycle activity? PSII supercomplex assembly?	DJC23/AtJ11 (At4g36040) DJC24/AtJ41 (At2g17880) DJC66/AtJ38 (At3g13310)	Phpat.003G078300	–	Stroma	–	Reduced levels of Rubisco activase/reduced PSII dimers and supercomplexes/oxidative stress response induced	[68]
JDPs, only in land plants	Calvin cycle activity? PSII supercomplex assembly?	DJC22/AtJ8 (At1g80920)	Phpat.018G010700 Phpat.018G010600 Phpat.008G010600 Phpat.022G076800 Phpat.022G076700 Phpat.004G099300 Phpat.003G140300 Phpat.026G020300 Phpat.004G042200 Phpat.012G060300	–	Stroma	–	Reduced levels of Rubisco activase/reduced PSII dimers and supercomplexes/oxidative stress response induced	[68]
JDPs, only in land plants	Recognition of inactive forms of deoxyxylulose 5-phosphate synthase (DXS) and delivery to Hsp70; Calvin cycle activity? PSII supercomplex assembly?	DJC26/AtJ20 (At4g13830)	Phpat.012G027400 Phpat.004G004700	–	Stroma	–	Reduced DXS activity; increased sensitivity to DXS inhibitors; Reduced levels of Rubisco activase/reduced PSII dimers and supercomplexes/oxidative stress response induced	[68,129]
JDPs, only in land plants	Assembly of NDH subcomplex A; contains transmembrane domain	DJC75/CRRJ/NdhT (At4g09350)	Phpat.016G043900	–	Thylakoid membranes	–	Reduced accumulation of subunits of NDH subcomplex A; no NDH activity	[130]
JDPs, only in land plants	Unknown	DJC72 (At2g18465)	–	–	Unknown	–	–	–
JDPs, only in land plants	Unknown	DJC65 (At1g77930)	Phpat.004G093300	–	Unknown	–	–	–
JDPs, only in land plants; lost in monocots	Unknown	DJC69 (At5g18140)	Phpat.006G011600	–	Unknown	–	–	–

[1] See legend of Table 1.

stromal Hsp70s with an organism-specific number of homologs: while *A. thaliana* encodes two stromal isoforms sharing 90.7% identical residues (cpHsc70-1 and cpHsc70-2) [78], the moss *Physcomitrella patens* harbors three that are to ~80% identical (PpHsp70-1 to 3) [79] (Table 2). *C. reinhardtii* encodes only a single major stromal Hsp70 (HSP70B) [61]. However, *Chlamydomonas* encodes two additional Hsp70s (HSP70D and F) that contain putative chloroplast transit peptides and are most similar to cyanobacterial and plastid Hsp70s. They are at most weakly expressed (based on coverage by shotgun proteomics). As HSP70D and F both have truncated C-termini and thus predicted molecular masses of only ~57 and ~42 kDa, respectively, it is dubious whether they have bona-fide chaperone activities. Stromal Hsp70s generally are derived from cyanobacterial DnaK2 [61,65,66,79–81].

3.2. Stromal Hsp70s depend on escort proteins

An Hsp70 escort protein (HEP) was first discovered in mitochondria, where it was shown to prevent aggregation of mitochondrial Hsp70s and to maintain them in an active conformation [82]. HEPs are small L-shaped proteins, whose structure is stabilized by a zinc-finger [83]. HEPs were also discovered in chloroplasts with a single homolog identified in *C. reinhardtii* (HEP2) [84], and two in *A. thaliana* (ZR1/ET1 and ZR2) [85] (Table 2). HEP homologs are not found in (cyano)bacteria and therefore must have evolved after endosymbiosis. *C. reinhardtii* HEP2 interacts with stromal HSP70B preferably in the ADP-bound state [84]. Active HSP70B, i.e., HSP70B in a protease-resistant conformation that is capable of interacting with its NEF, could be quantitatively produced in *E. coli* only when co-expressed with HEP2. HEP2 binds to active and inactive HSP70B, but cannot activate inactive forms of HSP70B such as HSP70B expressed in *E. coli* without HEP2. Hep1 was proposed to interact with mitochondrial Hsp70s to maintain them in a functional conformation by preventing their aggregation [82,83,86]. In *C. reinhardtii*, instead of aggregating, stromal HSP70B expressed in the absence of HEP2 assumed a protease-sensitive configuration unable to interact with its NEF [84]. It was suggested that in vivo HEP2 might be required for de novo folding of HSP70B after import into the organelle (Fig. 2). This hypothesis appears supported by the finding that plastid HEP homologs are absent in red algae and glaucophytes, which still encode Hsp70s on their plastid genomes [85]. Also the observation that HSP70B is between 6 and 21 times more abundant than HEP2 appears more in favor of a role of HEP2 in folding/maintenance of HSP70B rather than one as a cofactor during the functional cycle of stromal Hsp70s [84]. Finally, mitochondrial Hep1 was recently shown to support productive de novo folding of the NBD of mitochondrial Hsp70s in vitro [87].

3.3. Stromal Hsp70s require GrpE-type NEFs

An important co-chaperone for stromal Hsp70s is the GrpE-type NEF termed CGE (chloroplast GrpE homolog). Like their stromal Hsp70 partners, also stromal CGEs are of cyanobacterial origin [74]. *A. thaliana*, *P. patens* and *C. reinhardtii* all encode two stromal CGEs [79,88,89] (Table 2). While in moss and in *A. thaliana* both CGEs are true homologs of bacterial GrpE with molecular masses of ~24 kDa, this is not the case for CGE2 from *C. reinhardtii*. The latter is predicted to be a protein of ~152 kDa with the GrpE-domain at the C-terminus and a long N-terminal sequence of yet unknown function.

The biochemically so far best characterized CGE is CGE1 from *C. reinhardtii*, which interacts with chloroplast HSP70B only in its ADP-bound state [90] (Fig. 2). As CGE1 stimulates HSP70B's ATPase activity only if present together with a JDP, the rate-limiting step in HSP70B as in DnaK activity is ATP hydrolysis rather than nucleotide exchange [91,92]. HSP70B and CGE1 constitute about 0.19% and 0.01% of total cell protein, respectively, thus corresponding to a molar ratio of ~6.7:1 [74,93]. CGE1 is only 32% identical to its *E. coli* homolog, GrpE, yet shares a number of important structural features with GrpE [94]. This includes the ability to form dimers and an architecture consisting (from N- to C-terminus) of a paired α-helix, a four-helix-bundle, and a β-sheet domain [95]. Moreover, CGE1 complements the temperature sensitive growth phenotype of an *E. coli grpE* deletion strain and interacts with *E. coli* DnaK [74,94]. Therefore, despite the low sequence conservation and substantial evolutionary distance between CGE1 and bacterial GrpE, the proteins are quite similar at both the structural and functional levels.

An important difference between CGE1 and GrpE relates to their N-termini and dimer formation. The N-terminus of CGE1 contains a coiled-coil motif as opposed to the unstructured N-terminus of GrpE. Deletion analyses revealed that the N-terminal coiled-coil of CGE1 is essential for dimer formation, while dimerization of *E. coli* GrpE is mediated by the four-helix bundle at the posterior part of the molecule [94,96]. Hence, although general structural and functional properties of GrpE and CGE1 appear to be conserved, the proteins have clearly evolved somewhat differently. How dimerization of CGEs from moss and higher plants is realized has not yet been studied.

A peculiar feature of *C. reinhardtii* CGE1 is that it exists as two isoforms, a and b, which differ by an additional valine–glutamine dipeptide at positions 4 and 5 of the mature CGE1b protein absent in CGE1a [74]. This difference is caused by a temperature-dependent alternative splicing of the *CGE1* transcript, with *CGE1b* transcript and protein levels increasing upon heat shock [94]. Curiously, the two isoforms appear to have different affinities for HSP70B: the affinity of CGE1b is about 25% higher than that of CGE1a, indicating that the CGE1 extreme N-terminus plays an important role in determining the affinity of the co-chaperone for HSP70B. However, both isoforms supported the basic ATPase activity and the ability of HSP70B to refold denatured luciferase to exactly the same extent over a wide temperature range [91]. Hence, if the different isoforms affect HSP70B's performance in vivo, the effect is either subtle and therefore was not captured by in vitro assays, or limited to certain physiological conditions or substrates. In any case, as alternative splicing of *CGE* transcripts from moss or higher plants has not been reported yet, it might be a species-specific phenomenon.

3.4. Stromal Hsp70s cooperate with many J-domain co-chaperones

As implicated by their name, JDPs are characterized by the presence of a conserved J domain, containing a characteristic HPD motif which is indispensable for stimulating their HSP70 partner's ATPase activity [52] (Fig. 2). Among the 105 JDPs identified in *A. thaliana* [97], at least 19 are targeted to chloroplasts as shown by in vitro import assays [98]. Based on phylogenetic analysis, these 19 proteins were grouped into 11 clades. Four of these clades are conserved in the green lineage, i.e., from green algae to higher plants [98].

Members of the first of these four conserved clades are true DnaJ homologs, i.e., they contain a J domain, a cysteine-rich region, and a C-terminal domain mediating dimerization. In *C. reinhardtii* this clade contains only a single member (CDJ1 [99]), while it has four members in *A. thaliana* (DJA4-7 [98]) and even five in *P. patens* (Table 2). Interestingly, members of this clade appear to be the only JDPs that are derived from the cyanobacterial endosymbiont. Members of all other clades appear to have evolved after endosymbiosis, as they have no (cyano)bacterial orthologs [98,100]. The second clade conserved in the green lineage contains single members in *A. thaliana* (DJC73) and *C. reinhardtii* (CDJ2), and two members in *P. patens* (Table 2). In addition to the J domain these proteins harbor three regions potentially forming coiled-coils and interact with the vesicle-inducing proteins in plastids 1 (VIPP1) [101]. The third conserved clade is populated with three members each in *C. reinhardtii* (CDJ3-5) and *A. thaliana* (DJC76, 77, and 82), and six in *P. patens* (Table 2). These proteins contain a bacterial ferredoxin domain C-terminally from the J domain, followed by a long region of unknown function [102]. Interestingly, genes encoding proteins with a J domain and a

bacterial ferredoxin domain, but lacking the C-terminal region have also been found in mesophilic *Crenarchaeota* (or *Thaumarchaeota*). Most likely, the genes encoding the chloroplast proteins were transferred to the archaebacteria by horizontal gene transfer [103]. The fourth conserved clade has two members in *A. thaliana* (DJC31 and 62), and single ones each in *C. reinhardtii* (CDJ6 or TPR10) and *P. patens* (Table 2). Members of this clade have the J domain at the C terminus (in all others it is at the N terminus) and contain TPR domains [98].

The remaining seven clades identified in *A. thaliana* are present only in land plants with one clade lost in monocots (containing DJC69 in *A. thaliana*) [98]. In addition to their J domain, all these JDPs harbor distinct conserved domains with unknown function.

In conclusion, loss of all JDPs except for the true DnaJ homologs from the cyanobacterial endosymbiont and evolution of three novel JDP types suggests that the endosymbiotic life style posed new challenges to Hsp70 functions, while ones required in free-living cyanobacteria became dispensable. Moreover, the step from an aquatic life style to one at land appears to have generated further challenges for stromal Hsp70s, as indicated by the duplication of genes encoding stromal Hsp70s and the evolution of novel chloroplast-targeted JDPs.

3.5. The stromal Hsp70 system supports protein folding

Although postulated from its homology with the bacterial DnaK chaperone system, an activity in supporting the folding of a denatured protein (luciferase) to its native state was demonstrated only recently for a stromal Hsp70 system, in this case HSP70B–CDJ1–CGE1 from *C. reinhardtii* [91]. Similar to the bacterial system, HSP70B supported folding only when both co-chaperones were present in a proper stoichiometry. Highest folding supportive efficiency was observed when co-chaperones were supplied at ratios close to those found in vivo (HSP70B:CDJ1:CGE1 = 6.7:5.9:1) [91,93,99]. Efficient folding was strictly dependent on the presence of a true DnaJ homolog, JDPs CDJ3/4 were ineffective in promoting folding albeit they stimulated HSP70B's ATPase activity.

A role for stromal Hsp70s in the folding of chloroplast proteins was suggested for the subunits of the coupling factor CF1 [28], ferredoxin-NADP+ reductase [29], the Rieske protein [31], and phytoene desaturase [30]. In all these cases, Hsp70 and chaperonin systems were shown to act coordinately. The identification of a complex formed by stromal HSP70B, CGE1, CDJ1, and HSP90C in *C. reinhardtii* also suggests a cooperation of Hsp70 and Hsp90 systems in chloroplasts [99] (Fig. 2). Possibly, similar to the "foldosome" consisting of components of the Hsp70 and Hsp90 systems in the eukaryotic cytosol [56], the chloroplast equivalent might also be involved in the maturation of specific client proteins involved in signal transduction [104].

3.6. The activity of stromal Hsp70s might be redox regulated

Oxidative stress has been shown to cause multiple protein modifications [105] that lead to the increased expression of molecular chaperones and proteases [106]. However, in yeast and other organisms oxidative stress also results in a dramatic drop in cellular ATP levels, which precludes ATP-dependent folding by molecular chaperones [107,108]. Many proteins become glutathionylated under oxidative stress conditions [109]. Accordingly, mammalian cytosolic Hsc70 in the nucleotide-free state performed significantly better in preventing protein aggregation when it was glutathionylated compared to its unmodified conformation [110]. In the presence of ATP the performance of glutathionylated and unmodified Hsc70 was similar. Recent studies revealed that stromal HSP70B from *C. reinhardtii* is a target for thioredoxin and glutathionylation [109,111]. This suggests that the activity of stromal Hsp70s might be regulated by the redox-state of the chloroplast.

A possible role for stromal Hsp70 in light regulated gene expression was suggested by the finding that the ferredoxin-containing CDJ3 protein appears to be an RNA-binding protein in *C. reinhardtii* [102]. As post-transcriptional regulation of the expression of many chloroplast genes is light-dependent [112], it is possible that CDJ3 and its paralogs represent nuclear-encoded factors that act as redox switches by recruiting stromal HSP70B for the reorganization of regulatory protein

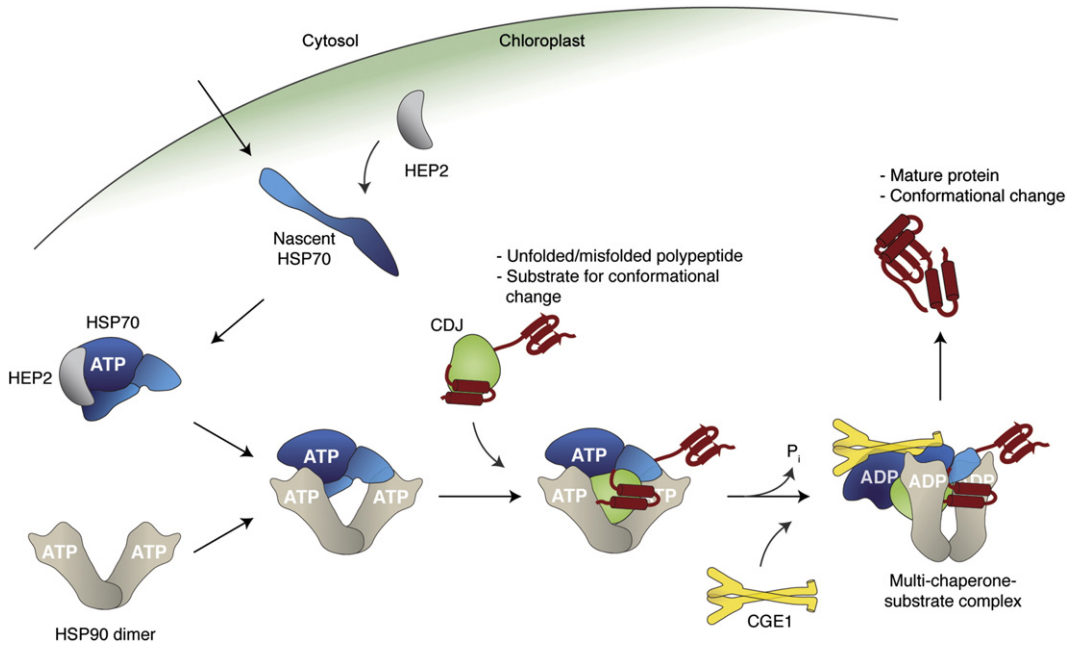

Fig. 2. Formation of the plastid HSP70/HSP90/CDJ/CGE1 multichaperone complex and substrate processing in *C. reinhardtii*. Stromal HSP70B after import into the plastid requires the escort protein HEP2 to attain the functional state. HSP70B appears to form constitutive complexes with dimeric HSP90C. Substrates are delivered to HSP70B by one of at least six chloroplast DnaJ-like (CDJ) co-chaperones. In ATP-driven cycles HSP70B, at least for some substrates supported by HSP90C, induces conformational changes into substrate proteins that support e.g. protein folding or assembly/disassembly of oligomeric complexes.

complexes located on chloroplast transcripts. Chaperone-mediated remodeling of replication for example has been demonstrated in *E. coli*, where DnaK and DnaJ monomerize RepA dimers and dissociate DnaB–helicase–Lambda P complexes to trigger replication of plasmid P1 and lambda phage, respectively [113,114].

3.7. Stromal Hsp70 catalyzes the (dis)assembly of VIPP1 oligomers

The CDJ2 protein was shown to mediate the interaction between stromal HSP70B/CGE1 and VIPP1 in *C. reinhardtii* [101]. VIPP1 dimers form rings of up to 2 MDa [93,115,116]. VIPP1 rings may further assemble into large rod-shaped tubules, whose assembly state is ATP-dependently dynamically altered by the HSP70B–CDJ2–CGE1 chaperones [93,117]. Presumably the chaperones introduce conformational changes into VIPP1 that facilitate oligomer assembly and disassembly (Fig. 2).

C. reinhardtii strains in which VIPP1 was downregulated to ~25% of wild-type levels exhibited several defects in the organization of their thylakoid membranes [118]. Most importantly, levels of PSI, PSII, Cytb_6/f, and ATP synthase were reduced by up to 20% in *vipp1* mutants, while levels of LHCII were increased by ~30%. This came along with aberrant structures resembling prolamellar bodies at positions within the chloroplast where multiple thylakoid membranes emerge. Electron microscopy data suggest that so-called thylakoid centers are located at such positions, which in cyanobacteria are located close to the plasma membrane and contain long tubules as a central component [119–121]. In *C. reinhardtii*, VIPP1 was localized by immunofluorescence to distinct spots within the chloroplast [118]. Interestingly, using YFP fused to the C-termini of stromal cpHsc70-1 and 2, both were also localized to distinct spots within chloroplasts [75]. Thus, it is tempting to speculate that stromal Hsp70s accumulate at regions were VIPP1 oligomers are localized.

It was suggested recently that PSII biogenesis in cyanobacteria occurs at thylakoid centers [122]. As the dimensions of the tubules within thylakoid centers fit exactly those recorded for VIPP1 tubules [93,119,121], it may be possible that both are identical and a reduction in VIPP1 levels thus results in impaired function of thylakoid centers in the biogenesis of PSII, and perhaps also of other thylakoid membrane complexes [118]. In this case, HSP70B–CDJ2–CGE1 – by mediating the dynamic interconversion between VIPP1 monomers and oligomers – would indirectly play a role in the biogenesis of thylakoid membrane complexes. This idea appears supported by the finding that downregulation of the *C. reinhardtii* Alb3.2 protein, which is involved in photosystem assembly, resulted in the upregulation of VIPP1, HSP70B, and CDJ2 [123].

3.8. Stromal Hsp70 is involved in protection/repair of PSII from high-light damage

The *C. reinhardtii* HSP70B gene is strongly induced by (high) light, resulting in an approximately twofold increase in HSP70B protein levels [61,118]. This finding suggested a possible role for this chaperone in processes that help the cell to cope with photodamage. Accordingly, PSII in cells overexpressing HSP70B was less severely damaged and recovered faster after photoinhibition than in wild-type cells, and the opposite was true for cells underexpressing HSP70B [124]. It was hypothesized that HSP70B facilitates the coordinated exchange of damaged D1 protein by de novo-synthesized protein [125]. In support of this idea, HSP70B in the green alga *D. salina* was found to be part of a ~320 kDa complex containing photodamaged D1, D2, and CP47 proteins [126].

VIPP1 was shown to be strongly induced by high light, and the repair of PSII after photoinhibition was retarded in *VIPP1*-RNAi strains [118]. Hence, under high light newly synthesized VIPP1 might form additional thylakoid centers to form sites for PSII repair. Via catalyzing the (dis)assembly of VIPP1 oligomers, HSP70B might be important for the formation of thylakoid centers for PSII repair, thus accounting for its role in mitigating photodamage to PSII [93,121,124].

3.9. A role for stromal Hsp70 in regulating Calvin cycle activity in land plants?

The AtJ8, AtJ11, and AtJ20 proteins (now termed DJC22, DJC23, and DJC26, respectively) are JDPs present only in land plant chloroplasts [98] (Table 2). AtJ8/DCJ22 was formerly and erroneously also termed Toc12 [60,67]. *atJ8*, *atJ11*, and *atJ20* knock-out mutants each displayed a reduced stability of PSII-LHCII supercomplexes and of PSII dimers in high light [68]. These mutants also showed impaired CO_2 assimilation rates, which correlated with ~20% lower levels of Rubisco activase. Moreover, the mutants appeared to have elicited an oxidative stress response [68]. A possible interpretation for these data might be that the smaller electron sink caused by reduced Calvin cycle activity in the mutants leads to increased water–water cycle activity associated with elevated levels of superoxide and H_2O_2 [127]. However, it appears surprising that although AtJ8, AtJ11, and AtJ20 proteins belong to three different clades with distinct conserved domains in addition to the J domain [98] all are supposed to be involved in the same biological processes. Moreover, the observation that AtJ8 transcript and protein levels are high in dark and decline drastically with increasing light intensities [128] appears inconsistent with a role of this protein in regulating Rubisco activity in the light. Clearly, more work is required to bring these findings into a functional context.

3.10. Stromal Hsp70s in land plants maintain deoxyxylulose 5-phosphate synthase (DXS) in a functional state

AtJ20/DCJ26 was shown to interact with deoxyxylulose 5-phosphate synthase (DXS), which catalyzes the conversion of GAP and pyruvate into deoxyxylulose 5-phosphate [129]. This is the first step of the methylerythritol 4-phosphate (MEP) pathway for the production of isoprenoid precursors. Compounds containing isoprenoids are essential for photosynthesis (such as carotenoids and the side chain of chlorophylls, tocopherols, plastoquinone, and phylloquinone) and for growth regulation (in hormones gibberellin, cytokinin, strigolactone, and abscisic acid). While *atJ20* knock-out mutants showed an increased sensitivity to DXS inhibitors, this was not the case for *atJ8* and *atJ11* mutants, supporting the notion that the latter JDPs are functionally distinct. Apparently, AtJ20/DCJ26 recognizes misfolded DXS and delivers it to stromal Hsp70s for refolding, from where it might also be handed over to proteolytic degradation [129]. As green algae encode DXS but not AtJ20/DCJ26 homologs (Table 2), the intrinsic instability of DXS might have evolved in land plants to serve a regulatory role.

3.11. A role for stromal Hsp70s in NDH complex assembly in land plants

CRRJ/NdhT (DJC75) and CRRL/NdhU contain J domains and are part of the NAD(P)H dehydrogenase (NDH)-PSI complex [130]. Both proteins have transmembrane domains. The J domain of CRRJ contains an intact HPD motif, while that of CRRL does not. Hence, only CRRJ is capable of stimulating Hsp70s ATPase activity. CRRL does not accumulate in *crrj* mutants and levels of NdhH and NdhL are reduced compared to wild type. Only the accumulation of subcomplex A of the NDH complex was affected in the *crrj* mutant, suggesting that CRRJ is required for the assembly of subcomplex A. Neither the accumulation of CRRL, NdhH and NdhL, nor NDH activity was restored when the *crrj* mutant was complemented with a CRRJ variant in which the HPD motif in the J domain was replaced by QPD [130]. This indicates that CRRJ recruits stromal Hsp70 for the biogenesis of NDH subcomplex A. Hence, CRRJ might play a dual role: as a structural component of NDH subcomplex A and, via Hsp70 activity, as an assembly factor for subcomplex A. As green algae lack the NDH complex, they also lack CRRJ and CRRL [130] (Table 2).

3.12. Chloroplast Hsp70s appear to be involved in protein import into plastids

The importance of Hsp70 for plastid protein import becomes more and more evident, although many open questions remain until today [131]. It has been suggested that preproteins are guided to the plastid surface by a guidance complex consisting of 14-3-3 proteins and cytosolic Hsp70 that binds to the transit peptides of the preproteins [132]. However, other studies have shown that the interaction of cytosolic Hsp70 with transit peptides is responsible for the degradation of preproteins rather than for protein import [133]. Until today, the involvement of cytosolic Hsp70 in plastid protein import remains speculative. Moreover, the existence of an imsHsp70 that would account for the observed NTP requirement for protein import in the intermembrane space has been questioned [65,66] (see above).

There is evidence, however, that stromal Hsp70 is actively involved in plastid protein import. In *A. thaliana*, an interaction of stromal cpHsc70 (Heat shock cognate 70) with components of the translocon in the outer/inner chloroplast envelope membrane (TOC/TIC), as well as with importing precursors could be shown [134]. Accordingly, the mutants of both *A. thaliana* isoforms of plastid cpHsc70 show a reduced level of protein import during early development [134]. A similar result was obtained in the moss *P. patens*, where decreased levels of a stromal Hsp70 led to impaired protein import [79]. Like in *A. thaliana*, this stromal Hsp70 was shown to interact with preproteins and TIC components in *P. patens* [79]. It was suggested that stromal Hsp70 might be an 'import motor' that works in parallel to the previously described Tic40–Hsp93 system, an idea which is backed up by the findings that *A. thaliana cphsc70* mutants show additive effects when combined with either *tic40* or *hsp93* mutants [134].

However, the existence of two parallel import motor systems has been questioned recently [135]. In *P. patens*, it has been shown that for both photosynthetic and non-photosynthetic precursors the ATP requirement for their import is determined by the affinity of stromal Hsp70 for ATP [135]. Replacing Hsp70-2 with variants containing single amino acid exchanges that increase their K_m for ATP led to reduced chloroplast import rates and a correspondingly reduced ATP requirement [135]. This argues against the existence of a parallel import motor which should be able to buffer these reductions [135]. It should be noted, however, that there might be species-specific differences in chaperone involvement in protein import, and that different chaperone systems might act as import motors under different conditions or during certain developmental stages.

A role for stromal Hsp70 in protein import is also supported by data obtained on *P. patens cge* mutants. In fact, while knocking out both plastid CGEs is lethal, the *cge1* null/*cge2* knockdown displayed mostly a reduction in plastid protein import [79]. This knockdown leads additionally to an upregulation of stromal Hsp70-2, possibly as part of a compensation reaction [79]. Since the JDP Pam18/Tim14 is essential for mitochondrial protein import by regulating mtHSP70's ATPase activity [136], a role for JDPs in protein import into chloroplast is expected. However, good candidates are yet elusive.

3.13. Plants with reduced levels of stromal Hsp70s are severely impaired in plastid development

cphsc70-1 knock-out mutants in *A. thaliana* display variegated cotyledons, vegetative leaves with irregular margins and small lesions, and show impaired growth rates [66,75]. These phenotypes were much more severe in plants exposed to drought stress. In contrast, knock-out mutants of stromal *cphsc70-2* did not display any visible phenotypes even when exposed to drought stress. *cphsc70-1 cphsc70-2* double knock-out mutants were lethal due to reduced pollen transmission efficiency [66]. Plants in which levels of both stromal Hsc70s were strongly reduced by artificial microRNA or co-suppression were almost completely white [75]. In white tissues and in pale tissues of *cphsc70-1* mutants, no or only small chloroplasts were found that had an altered morphology. The latter contained few, unorganized thylakoid membranes, and levels of photosystem core subunits were strongly reduced [75]. These strong developmental phenotypes are reminiscent of those observed with the maize *etched 1* mutant lacking one of two escort proteins of stromal Hsp70s [137], and also with the moss *cge* mutants [79], i.e., mutants expected to affect the functionality of stromal Hsp70s. It will be interesting to see whether these phenotypes are due to reduced protein import rates [79,134], due to a reduced capability to maintain/generate thylakoid membranes e.g. via VIPP1 [93,101,118,124], the reduced folding of yet unknown substrates, or a combination of these.

4. Plastid Hsp90s

Hsp90 molecular chaperones are highly conserved and ubiquitous. They generally consist of three domains: an N-terminal ATPase domain, a structurally flexible middle domain and a C-terminal domain, which serves for homo-dimerization of two Hsp90 proteins forming a clamp-like complex (reviewed in [138]). Most insights into the mechanisms underlying Hsp90 function have been gained on cytosolic Hsp90s. These undergo dynamic conformational changes during an ATP-driven substrate cycle. In brief, the nucleotide-free Hsp90 dimer is in an open conformation. ATP binding induces association of the N-terminal domains to form a compact conformation in which both Hsp90s are twisted around each other. Subsequent ATP hydrolysis results in an additional conformational shift, and ADP release restores the dimer in the open conformation (reviewed in [139]). Substrate binding occurs in the closed conformation and is mediated by multiple binding sites along the Hsp90 molecule. Cytosolic and other cellular Hsp90s function in concert with a number of cofactors (12 in the cytosol of *S. cerevisiae* cells and at least 17 in the cytosol of mammalian cells) (reviewed in [140]). Cytosolic Hsp90 was shown not to be required for general protein folding, but to play an important role in the maturation of a select group of proteins whose folding is inherently problematic, among them many proteins involved in signal transduction [141]. Hsp90 acts downstream of Hsp40 and Hsp70 [142] and both molecular chaperone systems form a common complex whose formation is promoted by the Hsp70–Hsp90 organizing protein Hop [140,143,144]. During heat stress, Hsp90 appears to stabilize partially unfolded protein conformations, thereby preventing further unfolding and facilitating a more rapid recovery [141]. Studies with diverse model organisms ranging from plants to vertebrates report that Hsp90 acts as a capacitor of genetic variation. Drug induced impairment of Hsp90 function resulted in the occurrence of a variety of phenotypes that depended on the genetic background. Some of the phenotypes became stable and independent of Hsp90 when enriched by selection [145–148].

4.1. Functions of plastid Hsp90

Of the seven Hsp90s encoded in *A. thaliana*, only one (AtHsp90-5) is targeted to plastids [149]. *C. reinhardtii* encodes only three Hsp90s, of which also only one (HSP90C) is located to the chloroplast [12] (Table 3). Phylogenetic analysis revealed an interesting aspect concerning the evolution of organellar Hsp90s: While mitochondrial Hsp90s are closely related with bacterial HtpG, plastid Hsp90s are more closely related with members localized in the ER. Thus, plastid Hsp90 may not be derived from its cyanobacterial ancestor but rather by a gene duplication event of ER *HSP90* and subsequent acquisition of a plastid transit sequence [150].

C. reinhardtii HSP90C is a rather abundant and constitutively expressed protein which is localized mainly in the stroma, but a small fraction was also found associated with chloroplast membranes [151]. HSP90C shares common features with Hsp90s from other compartments: it forms dimers and displays a low ATP hydrolysis rate (Km = 48 μM; Kcat = 0.71/min), which is comparable with those of other Hsp90s

Table 3
Plastid Hsp90s.

Protein type	Proposed function	*A. thaliana* homologs (locus name)	*P. patens* homologs	*C. reinhardtii* homologs (locus name)	Localization	Relative protein abundance in *C. reinhardtii* [1]	Mutant phenotypes	References function assignments
Stromal Hsp90	Maturation of proteins involved in signal transduction; VIPP1 oligomer disassembly; protein import	AtHsp90-5 (At2g04030)	Phpat.004G002300 Phpat.012G078000	HSP90C (Cre12.g514850)	Stroma; membrane associated	290 ± 13	Embryo lethal (*A. thaliana* knockout); albino; retarded chloroplast development (*A. thaliana* point mutation *cr88*)	[151,155, 157,158,162]

[1] See legend of Table 1.

[151–153]. While several co-chaperones have been identified that interact with cytosolic Hsp90 and influence its activity, co-chaperones of chloroplast Hsp90s are yet elusive. Cytosolic Hsp90 is recognized by many TPR-domain containing co-chaperones (e.g. Hop or CHIP) at its C-terminal MEEVD acceptor motif [154]. This motif is absent in plastid Hsp90s, however, all chloroplast Hsp90s contain a distinct C-terminal DPW motif, which might potentially serve as orthologous recognition site for co-chaperones [155,156]. Interestingly, similar to its cytosolic homolog, HSP90C in *C. reinhardtii* was shown to form a multi-chaperone complex with stromal HSP70, CDJ1 and CGE1, indicating an orthologous function in protein folding [99,104] (Fig. 3). The latter is indicated also by the inducibility of both chaperone genes by heat stress and light [25,151,155,156]. Moreover, chloroplast Hsp90 and Hsp70 were both found to interact with VIPP1 and postulated to play a role in the disassembly of VIPP1 oligomeric complexes [157,158]. How the interaction between both chaperone systems is realized remains an open question, as purified recombinant HSP70B and HSP90C from *C. reinhardtii* appeared not to interact directly in vitro [151].

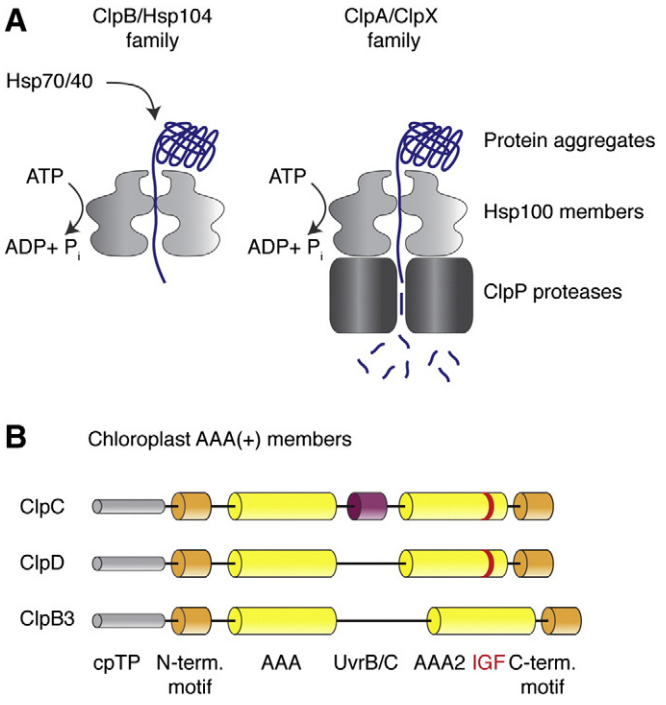

Fig. 3. Schematic structures of plastid Hsp100 family members. A) Hsp100 proteins form hexameric rings (here shown in cross-section, gray) through the pores of which the client proteins (blue) are threaded according to the molecular ratchet model. In contrast to the ClpB/Hsp104 subfamily, members of the ClpA/ClpX subfamily (named according to the bacterial homologs) contain motifs that allow them to interact with the ClpP protease which degrades the client proteins subsequent to their passage through the chaperone's pore. Both types hydrolyze ATP in order to induce the conformational changes that cause the threading. B) *Arabidopsis* chloroplasts contain the Hsp100 family members ClpC1, ClpC2, ClpD and ClpB3. According to the Pfam database [202], all members contain a conserved N-terminal motif (orange) after the chloroplast transit peptide (cpTP, gray) and a common C-terminal motif (orange). Since all chloroplast members are class 1 Hsp100 chaperones they all contain two ATPase domains (AAA and AAA2, yellow) and a variable "middle" domain in between which is longer in ClpB3 than in the other members. ClpC1, ClpC2 and ClpD are homologs of the bacterial ClpA/ClpX subfamily and contain an IGF-motif (red) within the C-terminal part of the second ATPase domain which is necessary for the interaction with ClpP proteases. The function of the UvrB/C motif in the middle domain of ClpC1 and ClpC2 (violet) is unkown.

Cytosolic Hsp90 plays an important role in the maturation of proteins involved in signal transduction [56]. Indications for a similar role of chloroplast Hsp90 came from the analysis of an *A. thaliana* mutant carrying a point mutation in the dimerization domain of this chaperone [155]. This mutant showed a yellow–green phenotype due to retarded development of chloroplasts, particularly in young leaves. In addition, the mutant exhibited reduced light-inducible expression of the *NR2*, *CAB* and *RBCS* genes and retarded deetiolation in red light [155,159]. It was suggested that chloroplast Hsp90 might exhibit a role in the transduction of light signals responsible for the regulation of a distinct set of photosynthesis-related genes. In *C. reinhardtii*, intermediates of chlorophyll biogenesis serve as signaling molecules between the chloroplast and the nucleus that mediate light induced gene expression [160]. Interestingly, in cyanobacteria, HtpG controls the activity of HemE, which is located at the first branching point of the tetrapyrrole biosynthetic pathway. Thus, these findings might point to an orthologous mechanism by which light induction of nuclear genes is influenced by plastid Hsp90 [104,161].

Most recently, stromal Hsp90 was suggested to participate in chloroplast protein import. Co-immunoprecipitation experiments identified the chaperone in complex with import intermediates of nuclear-encoded precursors, and inhibition of Hsp90 by the drug radicicol impaired the translocation of polypeptides at a late stage of import. Furthermore, Hsp90 was found in common complexes with membrane associated cpHsp70, Hsp93 and Tic110 [162]. All homozygous *A. thaliana* T-DNA insertion mutants investigated so far failed to reach the adult stage but were arrested in the heart stage of the embryonic stadium [158]. Studies with co-suppressed chloroplast Hsp90 in *A. thaliana* showed albino phenotypes and reduced expression levels of a number of photosynthesis-related genes, indicating that properly controlled expression of this chaperone is essential for plant development, growth and chloroplast thylakoid formation [158,163]. Whether these phenotypes relate to the roles of chloroplast Hsp90 in VIPP1 oligomer disassembly, protein import, or signal transduction remains to be elucidated.

5. The plastid Hsp100 family of AAA$^+$ ATPases

The Hsp100 family of AAA + ATPases (ATPases associated with various cellular activities) is a conserved protein family that exists in most organisms from bacteria to plants and comprises chaperones that use energy from ATP-hydrolysis to achieve a wide variety of functions such as protein unfolding, untangling of aggregates and protein complex assembly [164]. Hsp100 proteins fall into two classes, with class 1 members having two ATPase domains and class 2 members only one [164]. Plant genomes have members from both classes, but only class

Table 4
Plastid Hsp100s.

Protein type	Proposed function	*A. thaliana* homologs (locus name)	*P. patens* homologs	*C. reinhardtii* homologs (locus name)	Localization	Relative protein abundance in *C. reinhardtii*[1]	Mutant phenotypes	References function assignments
Class I B-type Hsp100	Untangling of protein aggregates under stress conditions; proteome remodeling during development	ClpB3 (At5g15450)	Phpat.024G030900	CLPB3 (Cre02.g090850)	Stroma	649 ± 14.5	Pale-green, undeveloped thylakoid membranes (*A. thaliana apg6*); temperature sensitive; seedling lethal	[165,176,177]
Class I C-type Hsp100	Degradation of proteins in leaves under normal conditions; part of the plastid protein import machinery	ClpC1/Hsp93-V (At5g50920)	Phpat.005G085200	CLPC (Cre43.g760497; AV397626)[2]	Stroma; envelope membrane-associated	n.d.	Severely pale-green, stunted growth (*A. thaliana hsp93-V*); wild-type phenotype (*A. thaliana hsp93-III*)	[182,183,185–187,190,191]
		ClpC2/Hsp93-III (At3g48870)	Phpat.006G026200 Phpat.003G070900 Phpat.016G072600 Phpat.025G027200					
Class I D-type Hsp100	Degradation of proteins during specific stress conditions	ClpD (At5g51070)	Phpat.018G053900 Phpat.021G017500	CLPD (Cre10.g465550)	Stroma	715 ± 11.5	Wild-type phenotype	[191,194]

[1] See legend of Table 1.
[2] For *C. reinhardtii* CLPC only a partial genome sequence covering the N-terminus is available; cDNAs exist.

1 members localize to plastids, namely ClpB, ClpC and ClpD proteins. The name Clp refers to the ability of many Hsp100 proteins to associate with the caseino*l*ytic *p*rotease, a ubiquitous serine protease that is known to degrade caseine. *Arabidopsis* plastids contain one ClpB protein called ClpB3, two ClpC proteins (ClpC1 and ClpC2) and one ClpD protein [165–167]. Rice plastids contain the ClpB protein ClpB-c, while two ClpC proteins are predicted to localize to plastids [168]. The *C. reinhardtii* genome contains homologs of all the plastid-localized *A. thaliana* Hsp100 proteins [169] (Table 4).

Upon binding of ATP, members of the Hsp100 protein family can form hexameric rings with a central pore [164]. For the yeast protein Hsp104, a family member which is involved in the untangling of protein aggregates, two models have been suggested that describe its mode of action [170]. In the molecular crowbar model each monomer of the ring binds a different part of the aggregate, and ATP hydrolysis induces a conformational change of the whole ring such that after multiple rounds of binding and ATP hydrolysis the aggregate is ultimately pulled apart [170]. In the molecular ratchet model free N- or C-termini of the entangled proteins bind close to the central pore of the hexameric ring, and multiple rounds of ATP hydrolysis induce a pulling force that threads the protein through the pore and thus releases it from the aggregate [170]. Backsliding is likely prevented by the spatial orientation of special side-chains of the Hsp100 proteins which face the pore [170].

ClpB proteins differ from other class 1 Hsp100 chaperones by a longer middle domain (the part between the two ATPase domains) which seems to be important for their specific function and for the interaction with other chaperones (Fig. 3A) [55,171,172]. The yeast ClpB protein Hsp104 was shown to disassemble heat shock-induced protein aggregates in conjunction with the Hsp70/Hsp40 system in vivo, where Hsp70 binding to the aggregated substrate induces Hsp104 binding and prevents re-aggregation after Hsp104 activity [173,174]. Since all ClpB proteins show a high degree of homology, plastid ClpB proteins may have similar functions to yeast Hsp104 [168,175]. Indeed, the expression of plastid ClpB proteins is induced by heat shock in tomato, and mutants have a temperature-sensitive phenotype [176]. In *A. thaliana*, the expression of plastid ClpB3 is also increased upon heat shock, but mutants have a severe seedling-lethal phenotype which suggests an additional, essential role for ClpB3 in normal development [165,177]. In maize, higher levels of plastid ClpB have been detected in bundle sheath cells compared to mesophyll cells, and a role for plastid ClpB in proteome remodeling during development has been suggested [178]. Thus, plastid ClpB may not only play a role in the disassembly of heat-induced aggregates, but also in crucial steps during development that may require proteome remodeling.

ClpC is a functional homolog of bacterial ClpA, which forms an ATP-dependent serine protease together with ClpP (Fig. 3A). Indeed, plastid ClpC can functionally replace bacterial ClpA, and associates with plastid ClpP in the stroma [179,180]. Unlike other Hsp100 family proteins, ClpC does not accumulate upon heat-shock but instead upon de-etiolation, which suggests a constitutive role in developed leaves [181]. In *A. thaliana*, the two isoforms of plastid ClpC are called ClpC1 and ClpC2 (Table 4). Other sources use the names Hsp93-V and Hsp93-III for the same proteins, formed by the apparent molecular weight of 93 kDa and the corresponding chromosome number of the gene location [182]. ClpC1/Hsp93-V is the major isoform, shows higher expression throughout development, and mutants are chlorotic, small and have a reduced photosynthetic performance [182–184]. While mutants of the minor isoform, ClpC2/Hsp93-III, have a wild-type phenotype, double mutants are embryo-lethal, suggesting that ClpC2/Hsp93-III can replace ClpC1/Hsp93-V to some extent in the *clpc1/hsp93-V* mutant [182,183,185].

In *A. thaliana*, both ClpC proteins have also been associated with plastid protein import. Under limiting ATP supply, ClpC is associated with precursors and components of the TOC/TIC (translocon at the outer/inner chloroplast envelope membrane) complex [186,187]. More specifically, it was shown that importing precursors bind to the

stromal domain of Tic110 which triggers the release of Tic40 from Tic110 and the stimulation of ClpC/Hsp93 ATP-hydrolysis by Tic40 [188]. An increase in the ATP concentration destabilizes the interaction of ClpC/Hsp93 with the translocon components, suggesting that the interaction is transient and dynamic [187]. Moreover, it was shown recently that ClpC/Hsp93 binds to a precursor only when the transit peptide is correctly located at the N-terminus [189]. For these reasons, and since *clpC/hsp93* mutants were shown to have reduced protein import rates by several studies [182,183,190], it was concluded that ClpC/Hsp93 is the component of the protein import motor which uses the energy in form of ATP that is necessary to drive protein import.

However, it was recently shown that plastid cpHsc70 also participates in the propulsion of plastid protein import, and it was suggested that cpHsc70 acts in parallel to the Hsp93–Tic40 system [134]. A novel study that examined the ATP requirement for protein import in more detail came to a different conclusion: in the moss *P. patens* the activity of plastid cpHsc70 correlates with the ATP requirement for protein import, suggesting that all ATP for protein import propulsion is used by plastid cpHsc70 [135]. This challenges the idea that plastid ClpC/Hsp93 functions in protein import propulsion. Interestingly, an independent study showed that the amount of ClpC which is bound to the envelope is exceeded by the amount of ClpP bound to the envelope, suggesting that all envelope-localized ClpC might associate with ClpP [191]. Thus, it was proposed that ClpC/Hsp93 does not function in protein import propulsion but rather acts together with ClpP as a specific, protein import associated protease, presumably in protein import quality control [191]. After these new insights, the role of the envelope-associated Tic110–Tic40–Hsp93 complex needs probably to be re-investigated.

ClpD is a plastid protein with high homology to ClpC but lacks a predicted UvrB domain (Fig. 3B; Table 4). It has been shown to be localized to the stromal fraction only [191,192] and thus is unlikely to participate in any envelope-localized activity similar to ClpC. However, the IGF motif which is necessary for the association of ClpC with the ClpP protease core [193] is also present in ClpD (Fig. 3B), suggesting that ClpD might have a specific, stroma-localized protease function. ClpD was discovered in *A. thaliana* in a screen for proteins which are induced upon dehydration, and it was thus originally named ERD1 (early response to dehydration 1) [194]. Apart from dehydration, the corresponding gene expression was also increased upon dark-induced etiolation and natural senescence [195]. In line with these findings, it was shown that the *ClpD* gene promoter contains a dehydration-responsive and an etiolation-responsive element, and the corresponding transcription factors were identified [196,197]. On the protein level, however, a consensus could not yet been found, and it remains unclear whether ClpD accumulates upon dehydration, etiolation and senescence or rather under different conditions [167,192,198]. Recently, it was shown that the ClpD protein level decreases in *clpC1* mutants, and a structural interaction between the two chaperones was hypothesized [191]. Since the ClpP proteolytic cores exceed both ClpD and ClpC chaperones in number, it was suggested that both ClpC and ClpD might act together with the ClpP protease, albeit with different substrate specificities due to their different N-termini [191].

6. Concluding remarks

The ATP-dependent chaperone systems in plastids (with the exception of plastid Hsp90) are orthologous with the bacterial systems. Therefore it is not surprising that the fundamental mechanisms underlying their functions are largely conserved. However, it turns out that plastid chaperones display several peculiar features, like (i) the distinct subunit composition of (co)chaperonins, (ii) the large diversity of J domain co-chaperones for plastid Hsp70s with ten new types that have evolved in land plant plastids when compared to cyanobacteria, or (iii) the loss of the cyanobacterial HtpG and recruitment of an originally ER-targeted Hsp90 to the plastid. These peculiar features suggest specific adaptations of plastid chaperone systems to the requirements imposed by a proteome that had to cope with changing challenges during plant evolution. To better understand the specific chaperoning requirements of the plastid proteome more research on plastid chaperone systems is required. This research may also reveal novel functional insights into fundamental aspects of molecular chaperones that only become apparent when studying the peculiarities inherent to the plastid systems.

Transparency Document

The Transparency Document associated with this article can be found, in the online version.

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