

## Common functions of the chloroplast and mitochondrial co-chaperones cpDnaJL (CDF1) and mtDnaJ (PAM16) in protein import and ROS scavenging in *Arabidopsis thaliana*

John Gray<sup>a</sup>, Sachin Rustgi<sup>b</sup>, Diter von Wettstein<sup>b</sup>, Christiane Reinbothe<sup>c</sup>, and Steffen Reinbothe<sup>c</sup>

<sup>a</sup>Department of Biological Sciences, University of Toledo, Toledo, OH, USA; <sup>b</sup>Department of Crop and Soil Sciences, School of Molecular Biosciences, Center for Reproductive Biology, Washington State University, Pullman, WA, USA; <sup>c</sup>Biologie Environnementale et systémique (BEeSy), Université Joseph Fourier, Grenoble, France

### ABSTRACT

As semi-autonomous cell organelles that contain only limited coding information in their own DNA, chloroplasts and mitochondria must import the vast majority of their protein constituents from the cytosol. Respective protein import machineries have been identified that mediate the uptake of chloroplast and mitochondrial proteins and interact with molecular chaperones of the HEAT-SHOCK PROTEIN (HSP) 70 family operating as import motors. Recent work identified unexpected new functions of 2 DnaJ co-chaperones in mitochondrial and chloroplast protein translocation and suggest a common mechanism of reactive oxygen species (ROS) scavenging that shall be discussed here.

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CDF1 (cell growth defect factor 1); DnaJ-like co-chaperones (holdases); mitochondria and chloroplasts; organelle biogenesis; PAM (presequence translocase-associated protein import motor); protein translocation; ROS (reactive oxygen species)

### Introduction

Chloroplasts and mitochondria are thought to have arisen from endosymbiosis and as a consequence of mass gene relocation events lost most of their own DNA during evolution.<sup>1</sup> To sustain their various functions both chloroplasts and mitochondria depend on a large number of cytosolic proteins that have to be imported in a developmentally regulated fashion. It was thus far believed that chloroplasts and mitochondria possess unique protein import machineries with little or no common components.<sup>2,3</sup> An exception to the rule is provided by molecular chaperones of the HEAT SHOCK PROTEIN (HSP) 70 family that, in association with other proteins, operate in both organelle types and function as import motors.<sup>4,5</sup> Here we report on a unique class of co-chaperones designated DnaJ and DnaJ-like holdases<sup>6,7</sup> that act as key components in both mitochondrial and chloroplast protein translocation and accomplish unique roles in reactive oxygen species (ROS) scavenging and signaling.

### Two distinct DnaJ-like co-chaperones with conserved structural features operate in chloroplasts and mitochondria

Chloroplasts and mitochondria need to import over 95% of their protein constituents from the cytosol and do so by virtue of specific protein import machineries dubbed translocases of the outer and inner chloroplast envelope membranes (TOC and TIC) versus translocases of the outer and inner mitochondrial membrane (TOM and TIM), respectively.<sup>2,3</sup> To drive the translocation of cytosolic precursor proteins across these multi-protein complexes, chloroplasts and mitochondria make use of import motors that catalyze the unfolding of the precursors in the cytosol, mediate the translocation step and assist in the refolding of the imported proteins inside the organelles. In chloroplasts, the import motor consists of the ATP-driven HEAT-SHOCK PROTEIN 70 (cpHSP70), its membrane anchor proteins TIC40 and TIC110, and a not yet identified nucleotide exchange factor to keep the HSP70 cycle functional.<sup>2,6,7</sup> cpDnaJL as a presumed cpHSP70 co-chaperone is a protein of 258

**CONTACT** Steffen Reinbothe  sreinbot@ujf-grenoble.fr  Biologie Environnementale et systémique (BEeSy), Université Joseph Fourier, LBFA, BP53F, 38041 Grenoble cedex 9, France.

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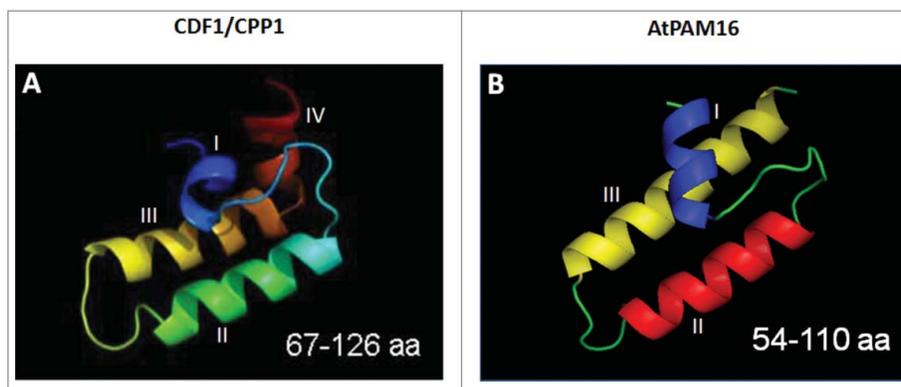
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amino acids encoded by At5g23040 in *Arabidopsis thaliana*.<sup>8</sup> cpDnaJL displays 32.9% sequence homology to the protein encoded by At3g51140 and 22.4% sequence homology to the protein encoded by At2g20920.<sup>9,10</sup> Homologs of cpDnaJL are present in cyanobacteria, green algae, mosses, gymnosperms, and angiosperms,<sup>9,10</sup> suggesting a link to oxygenic photosynthesis. The J-domain of cpDnaJL from *Arabidopsis* and the cyanobacterium *Synechocystis* sp. PCC6803 are distantly related to the J-domain of Tid1, a human mitochondrial homolog of bacterial DnaJ co-chaperones, and other DnaJ proteins (Fig. 1). Lee et al.<sup>9</sup> noted the lack of a highly conserved tripeptide, His-Pro-Asp (HPD) in the J-domain of cpDnaJL, which is normally required for the interaction with HSP70.<sup>6,7</sup> This observation suggests other sequence motives to be involved in CDF1-HSP70 interactions or that the function of cpDnaJL may be fundamentally different from that of canonical DnaJ co-chaperones. Indeed, cpDnaJL was found to accomplish a role as holdase in chloroplasts and etioplasts.<sup>9,10</sup> Holdases are a particular kind of ATP-independent molecular chaperones that bind to protein folding intermediates to prevent their aggregation but without directly refolding them (summarized in refs. 9,10).

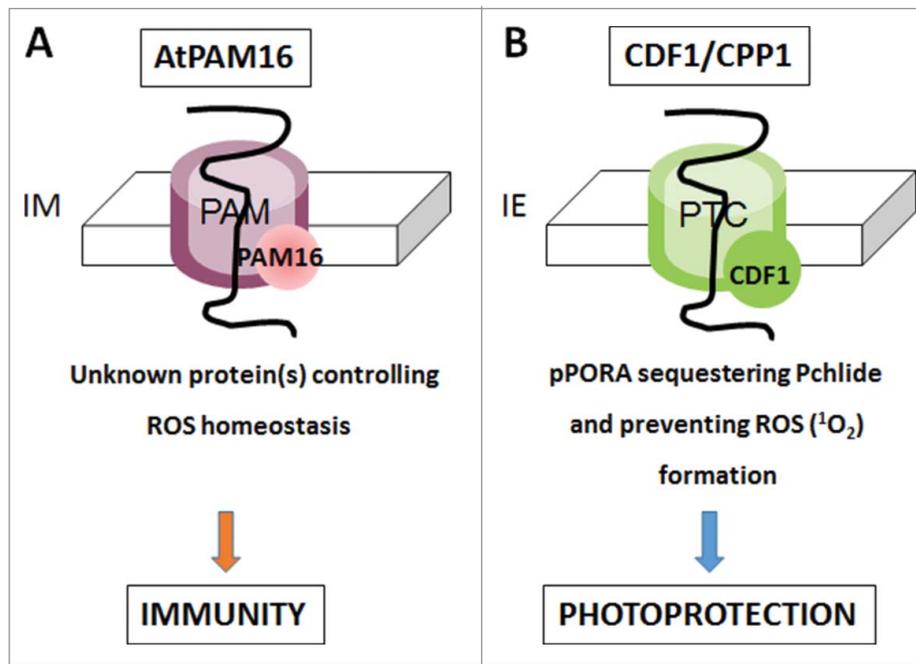
In mitochondria, the import motor needed for the uptake of both presequence-containing and presequence-less cytosolic precursors is composed of mitochondrial HEAT-SHOCK PROTEIN 70 (mtHSP70), its membrane anchor protein TIM44, the homodimeric nucleotide exchange factor GrpE (Mge1), and mtDnaJ (synonymous with PAM16, the presequence translocase-associated motor complex protein of 16 kDa), that forms hetero-dimers with PAM18<sup>11,12</sup> (summarized in Fig. 2A). mtDnaJ is highly conserved and particularly shares  $\approx 85\%$  residue conservation in the J-domain with related proteins (Fig. 1).<sup>13,14</sup>

## Roles of cpDnaJL and mtDnaJ (PAM16) in protein import

Chloroplasts and mitochondria import both presequence-containing and presequence-less proteins from the cytosol. Import of either type of cytosolic precursor into mitochondria is driven by the  $\Delta\psi$  and ATP.<sup>3</sup> Hereby, the TIM23 subcomplex in the mitochondrial inner membrane interacts with the presequence translocase-associated motor complex including mtDnaJ.<sup>11,12</sup> In chloroplasts, the situation is different. cpDnaJL does not seem to participate in overall import of presequence-containing or presequence-less cytosolic precursors; instead it accomplishes a specific role. cpDnaJL is part of a unique protein import site through which NADPH: protochlorophyllide oxidoreductase (POR) A is specifically imported<sup>10</sup> (Fig. 2B). Uptake of the pPORA into the plastids is unique in requiring protochlorophyllide (Pchlde) as import trigger.<sup>14-17</sup> Time courses over import revealed that the pPORA sequentially binds the tetrapyrrole pigment Pchlde<sup>10</sup> and thereby lowers its interactions with molecular oxygen provoking the generation of singlet oxygen as cytotoxin and cell death inducer.<sup>18,19</sup> The interaction between the translocating pPORA polypeptide chain and Pchlde is enabled by the binding of cpDnaJL that operates independently of cpHSP70 and keeps the pPORA in a state ready to bind Pchlde.<sup>9,10</sup> Once pPORA's transit sequence is removed by the stromal processing peptidase, the mature pPORA loaded with Pchlde interacts with PORB and assembles into larger light-harvesting complexes in the prolamellar body of etiolated plants.<sup>20,21</sup> Lack of cpDnaJL, as found after RNA interference-induced gene silencing (*RNAi*), impaired the establishment of these complexes (Fig. 3) and led to overaccumulation of free Pchlde acting as photosensitizer for singlet oxygen formation during



**Figure 1.** Structural models of cpDnaJL (CDF1)(A) and mtDnaJ (PAM16)(B). The 3D-modeling was performed using I-TASSER, an online protein structure and function prediction tool,<sup>30</sup> with the J-domain of Tid1 as template.<sup>31</sup> Despite the limited amino acid sequence identity of cpDnaJL (CDF1)(A) and mtDnaJ (PAM16), the topology of their J-domains is quite similar.



**Figure 2.** Model on the roles of cpDnaJL (CDF1) and mtDnaJ (PAM16) in chloroplasts (A) and mitochondria (B). A, mtDnaJ (PAM16) is supposed to regulate the translocation of hypothetical protein(s) with functions in ROS scavenging and/or signaling across the inner mitochondrial membrane (IM). As a consequence, ROS production would be kept low and plant immunity be inhibited. (B), cpDnaJL regulates the import of PORA through the Pchlido-dependent translocaon (PTC), spanning both the outer and inner plastid envelope (IE) membrane. Hereby, cpDnaJL's function is that of a holdase permitting the binding and sequestration of Pchlido in a protein-bound and thus non-hazardous form, conferring photoprotection onto etiolated seedlings during greening.

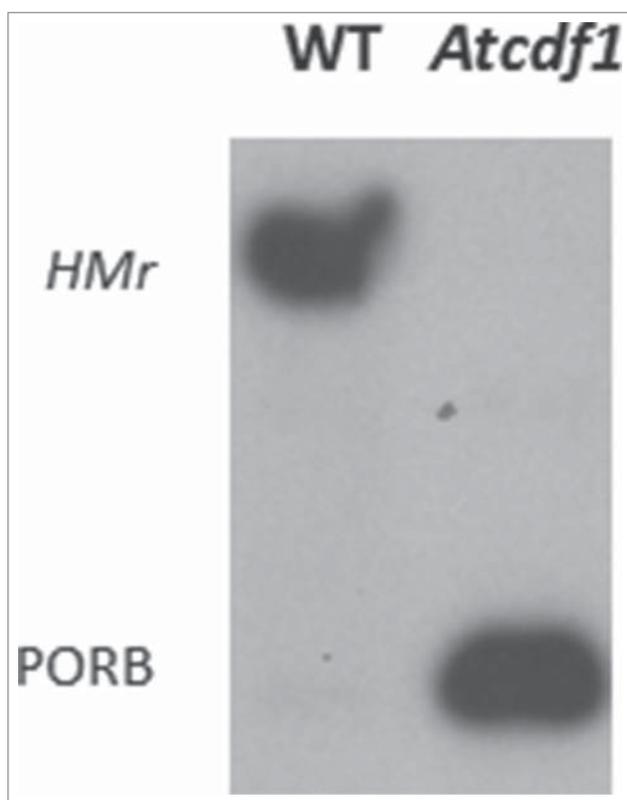
greening.<sup>10</sup> Measurements with the dansyl-based singlet oxygen quencher DanePy confirmed mass generation of ROS in cpDnaJL-depleted *RNAi* seedlings.<sup>10</sup> Accumulation of singlet oxygen and other ROS explains why young-born sprouts rapidly die during greening.<sup>10</sup> Interestingly, similar, essential functions of mtDnaJ in mitochondrial biogenesis and function were deduced from knock-out mutant studies using different PAM16 alleles.<sup>12,13</sup> While single mtDnaJ (PAM16) mutants in *Arabidopsis* had reduced size, double mutants were lethal.<sup>12,13</sup> Thus, a common link of mtDnaJ and cpDnaJL function is suggested that shall be discussed here.

### Common aspects of DnaJ function in mitochondria and chloroplasts

cpDnaJL and mtDnaJ are both essential for organelle biogenesis. In case of cpDnaJL, incapability to sequester Pchlido in a protein-bound form favors generation of singlet oxygen and other ROS that cause seedling lethality during greening.<sup>10</sup> Because cpDnaJL not only binds PORA but also its constitutively expressed counterpart PORB, the growth phenotype also manifests in green plants, as reported by Lee et al.<sup>9</sup> who used virus-induced gene silencing and dexamethasone-induced gene silencing to pinpoint the role of cpDnaJL in green

plants. In case of mtDnaJ, however, lack of an essential protein needed for the import of both presequence-containing and presequence-less cytosolic precursors would lead to depletions in the mitochondrial proteome and pleiotropic defects in mitochondrial activity. Huang et al.,<sup>13</sup> however, proposed a completely different scenario in which mtDnaJ is supposed to play a specific role in the import of a protein regulating plant viability and immunity.

Huang et al.<sup>13</sup> carried out a feed-forward genetic screen for mutants of nucleotide-binding and leucine-rich repeat domain (NB-LRRs) proteins that serve as immune receptors.<sup>22</sup> In a *mutant sncl-enhancing (muse)* screen for such NB-LRRs, a component was identified termed MUSE5 that is an ortholog of mtDnaJ.<sup>13</sup> Interestingly, single mtDnaJ mutants had enhanced resistance against virulent pathogens, suggesting that mtDnaJ may normally operate to sequester a cell death regulator in the mitochondrial compartment. Candidate proteins accomplishing such role could be the heme binding proteins localized in the different mitochondrial inner membrane complexes and being potential ROS generators.<sup>23</sup> If so, this situation would resemble that seen for cpDnaJL that by its activity in the Pchlido-dependent plastid import pathway of PORA lowers the level of potential



**Figure 3.** Lack of POR-containing higher molecular mass (*HMr*) complexes in prolamellar bodies of etiolated *Arabidopsis* seedlings that had been depleted of cpDnaJL (CDF1) by RNAi (*Atcdf1*), as compared to etiolated wild-type (WT) seedlings. Note the complete lack of *HMr* complexes as well as drastically reduced levels of PORB, which cannot stably accumulate in the absence of PORA, in *Atcdf1* plants.

photosensitizers for ROS generation and thereby prevents cell death. Other targets of mtDnaJ could be thus far unknown cell death proteins. Last but not least, mtDnaJ could be involved in controlling the biogenesis and assembly of cytochrome *c* that has a well-established role in apoptotic cell death regulation. *R* protein-mediated immunity in fact involves the release of cytochrome *c* from mitochondria to the cytosol, triggering cell death and boosting ROS production (see. ref. 13; for references). In addition, multiple sources and types of ROS were implicated in the development of the hypersensitive response (HR) to deter pathogens.<sup>24</sup>

Interestingly, another mutant allele of mtDnaJ (*Atpam16-2*, originally named *txr1-1*) was isolated from a forward genetic screen for mutants resistant to the phytotoxin and herbicide thaxtomin A from the potato scab-inducing species *Streptomyces scabies*.<sup>25</sup> This mutant was resistant to exogenously administered thaxtomin A normally provoking severe growth defects.<sup>25</sup> A hypothesis put forth by Huang et al.<sup>13</sup> is that thaxtomin A may be

targeting a mitochondrial matrix protein that, as we propose here, relies on mtDnaJ for import or may bind and inactivate mtDnaJ (AtPAM16) itself. It was further hypothesized that such targeting may serve as a virulence strategy to release a death signal from mitochondria and assists the killing of host cells so that the pathogen can consume the plant's photosynthates.<sup>13</sup>

Another, interesting link between mtDnaJ and ROS is provided by the work of Scheibel et al.<sup>26</sup> who noticed differential effects of thaxtomin on root growth in seedlings undergoing photomorphogenesis or skotomorphogenesis. While root growth was reduced by more than 50% by 100 nM thaxtomin in wild-type seedlings germinating in white light (photomorphogenesis), root growth was unaffected in seedlings developing in darkness (skotomorphogenesis). Because root development in either case was normal in the thaxtomin-resistant mutant (*txr1/Atpam16*), a light-dependent relocation of ROS themselves or ROS-derived signals controlling root growth was anticipated.<sup>26</sup> Alternatively, the insensitivity to the herbicide could be due to altered ROS perception mechanisms rendered inactive by the mutation. Several studies have demonstrated that root meristem growth and differentiation are regulated by ROS production and distribution.<sup>27-29</sup> Thus, failure to correctly localize a protein involved in ROS metabolism would likely affect the root phenotype and may even restore the correct growth pattern. Huang et al.<sup>13</sup> in fact proposed a positive regulatory role of mitochondria during immune responses through ROS generation and that this circuit is counteracted by mtDnaJ. As said before, a possible, mtDnaJ-dependent mechanism could be to control the import of a nuclear-encoded negative regulator of plant immunity that may bind and scavenge ROS. Failure to sequester this factor would lead to enhanced disease susceptibility. If correct, this scenario would resemble the mechanism by which cpDnaJL controls ROS homeostasis in plastids. Proteomics and other approaches are required to define the exact role of mtDnaJ in the import of such immune regulators and/or ROS scavengers and to compare it with that of cpDnaJL.

### Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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