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ORIGINAL ARTICLE

Eukaryotic Hsp70 chaperones in the intermembrane space of chloroplasts

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Abstract

Main conclusion **Multiple eukaryotic Hsp70 typically localized in the cytoplasm are also distributed to the intermembrane space of chloroplasts and might thereby represent the missing link in energizing protein translocation.**

Protein translocation into organelles is a central cellular process that is tightly regulated. It depends on signals within the preprotein and on molecular machines catalyzing the process. Molecular chaperones participate in transport and translocation of preproteins into organelles to control folding and to provide energy for the individual steps. While most of the processes are explored and the components are identified, the transfer of preproteins into and across the intermembrane space of chloroplasts is not yet understood. The existence of an energy source in this compartment is discussed, because the required transit peptide length for successful translocation into chloroplasts is shorter than that found for mitochondria where energy is provided exclusively by matrix chaperones. Furthermore, a cytosolic-type Hsp70 homologue was proposed as component of the chloroplast translocon in the intermembrane space energizing the initial translocation. The molecular identity of such intermembrane space localized Hsp70 remained unknown, which led to a controversy concerning its existence. We identified multiple cytosolic Hsp70s by mass spectrometry on isolated, thermolysin-treated *Medicago sativa* chloroplasts. The localization of these Hsp70s of *M. sativa* or *Arabidopsis thaliana* in the intermembrane space was confirmed by a self-assembly GFP-based in vivo system. The localization of cytosolic Hsp70s in the stroma of chloroplasts or different mitochondrial compartments could not be observed. Similarly, we could not identify any cytosolic Hsp90 in the intermembrane space of chloroplast. With respect to our results we discuss the possible targeting and function of the Hsp70 found in the intermembrane space.

Keywords Chloroplasts intermembrane space · Hsp70 · Protein targeting · TOC complex

Abbreviations

HSP	Heat shock protein
IMS	Intermembrane space

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Mgd1	Monogalactosyldiacylglycerol synthase 1
Oep21/37	Outer envelope protein of 21/37 kDa
pSSU	Precursor of the small subunit of RUBISCO
SP1	Plastidic type I signal peptidase 1
TIC	Translocon of the inner chloroplast membrane
TIM	Translocon of the inner mitochondrial membane
TOC	Translocon of the outer chloroplast membrane

Introduction

The functional integrity of cellular compartments depends on massive protein transport and translocation by multiple pathways (Schatz and Dobberstein 1996; Wickner and Schekman 2005). Both processes depend on signals often encoded as amino acid extensions or as amino acid motifs within the mature part of the precursor protein (preprotein). For targeting to mitochondria and chloroplasts a multitude of different signals have evolved (Schleiff and Becker 2011), but N-terminal polypeptide extensions are by far the most prominent targeting signals. Although the necessity of specific cytosolic factors for mitochondrial and chloroplast targeting has not been finally documented in vivo, several precursor-targeting routes are proposed. These routes involve the heat shock protein of 70 kDa (Hsp70) and/or the 14-3-3 proteins (May and Soll 2000), the heat shock protein of 90 kDa (Hsp90; Qbadou et al. 2006), the ankyrin repeat protein 2A (Akr2A; Bae et al. 2008) or trafficking via the endoplasmic reticulum (Villarejo et al. 2005). The molecular chaperones are discussed to protect preproteins from misfolding and cytoplasmic aggregation (Jackson-Constan et al. 2001) and to function in the regulation of the cytosolic accumulation of preproteins (Lee et al. 2009; Tillmann et al. 2015). Accordingly, Hsp70 binding sites have been identified in transit peptides as well as in mature domains of many chloroplast proteins (Ivey et al. 2000; Rial et al. 2000; Zhang and Glaser 2002). In turn, Akr2A, 14-3-3 and Hsp90 are discussed to define the targeting pathway to chloroplasts by a yet unknown mechanism.

For selective translocation across the envelope membrane, preproteins are recognized on the plastid surface by the translocon of the outer chloroplast membrane (TOC; e.g. Schleiff and Becker 2011). The TOC_{CORE} complex is composed of the two GTP-regulated receptor proteins Toc34 and Toc159 and the pore-forming Toc75 (Sommer and Schleiff 2009). In addition, preproteins associated with cytosolic Hsp90 chaperones are recognized by a clamp-type tetratricopeptide repeat (TPR)-containing receptor Toc64, which is dynamically associated with the TOC_{CORE} complex (Qbadou et al. 2006).

The transfer of the precursor from the TOC complex to the translocon of the inner chloroplast membrane (TIC; Schleiff and Becker 2011) depends on low ATP concentrations (Olsen and Keegstra 1992; Perry and Keegstra 1994). Currently, at least three proteins are suggested to be involved in the translocation of precursor proteins across the intermembrane space (IMS). Toc12 and Toc64 have been suggested to participate in this process (Becker et al. 2004; Qbadou et al. 2007; Sommer et al. 2013), but their function remains under debate (Rosenbaum Hofmann and Theg 2005; Aronsson et al. 2007; Chiu et al. 2010). In addition, the IMS-localized Tic22 is discussed to be a component of the IMS translocon (Kouranov et al. 1998; Tripp et al. 2012; Rudolf et al. 2013).

The mechanism of preprotein transfer across the IMS is largely unclear. Import experiments using pea chloroplasts or tomato protoplasts studying the importance of the signal length and passenger folding for translocation (Biondo et al. 2010; Ruprecht et al. 2010; Leibovitch et al. 2013) revealed that 60 amino acids in a loose conformation are required at the N-terminus for efficient import into chloroplast. Considering the dimensions of the currently envisioned TOC complex assembly (Sommer et al. 2011) and the presence of an Hsp70 binding side in the N-terminal 10-20 amino acids of the transit peptide (Ivey et al. 2000), the observed requirement of 60 amino acids for efficient translocation is in favor of an additional chaperone association in the IMS. This idea is supported by the observation that the stromal chaperone system does not contribute to unfolding of preproteins at the cytosolic side and translocation (Kovacheva et al. 2007). This additional energy might be provided by an intermembrane localized Hsp70 (imsHsp70) (Marshall et al. 1990; Waegemann and Soll 1991; Becker et al. 2004).

Initially, a eukaryotic Hsp70 chaperone associated with the outer envelope membrane of *Pisum sativum* chloroplasts was observed by immunostaining with antiserum specific for eukaryotic Hsp70s (Marshall et al. 1990; Waegemann and Soll 1991; Ko et al. 1992; Schnell et al. 1994; Becker et al. 2004). This Hsp70 was resistant against protease treatment leading to its assignment as intermembrane space localized. The importance of this imsHsp70 for protein translocation was inferred from its co-purification with arrested preproteins (Waegemann and Soll 1991). In parallel, a eukaryotic Hsp70 associated with the cytosolic side of the outer envelope and with arrested preproteins was described (Com70; Ko et al. 1992). However, all reports have in common that the molecular identity of the Hsp70 remained unknown. Thus, the nature and existence of the imsHsp70 remains under debate, as studies localize the protein alternatively to the cytoplasm or the stroma (e.g. Ko et al. 1992; Ratnayake et al. 2008). The importance of a stromal localized Hsp70 for protein translocation

has been elegantly demonstrated (Shi and Theg 2010; Su and Li 2010; Liu et al. 2014), but this protein is related to the prokaryotic DnaK family (e.g. Ratnayake et al. 2008).

Here, we analyzed the localization of Hsp70 in detail to document that a eukaryotic Hsp70 is associated with the outer envelope and faces the IMS of chloroplasts from pea (*Pisum sativum*) and *M. sativa*. We purified this imsHsp70 and mass spectrometric analysis led to the detection of multiple Hsp70 isoforms. Their localization to the IMS of chloroplasts in vivo was probed using the self-assembly GFP-based system (Cabantous et al. 2005; Gross et al. 2011). Our results indicate that multiple members of the cytosolically localized Hsp70 family, but not of the Hsp90 family of *M. sativa* and *Arabidopsis thaliana,* are co-localized in the IMS of chloroplasts.

Materials and methods

Construct generation

Constructs have been generated as previously described (Gross et al. 2011; Sommer et al. 2011, 2013).Corresponding amplified cDNA-fragments of Hsp70 isoforms of *A. thaliana* and *M. truncatula* were inserted into pAVA vectors containing strand 11 of saGFP at the 3́ position (Gross et al. 2011). Plastidic type I signal peptidase 1 PLSP1, abbreviated in here as SP1; At3G24590) and the monogalactosyldiacylglycerol synthase 1 (MGD1) fused to saGFP1-10 or GFP were generated as described (Bullmann et al. 2010). pML94-GFP (Bionda et al. 2010) was used for the generation of C-terminal fusions of GFP to Hsp70 proteins.

Plant material, protoplast isolation and transformation

Sterile tomato (*Solanum lycopersicum* cv. Moneymaker; Tomato Genetics Resource Center, University of California, Davis, USA) and *Arabidopsis thaliana* (ecotype Columbia; Arabidopsis Biological Resource Center, The Ohio State University, Columbus, OH, USA) plants were grown on gelrite-solidified Murashige and Skoog (MS) medium supplemented with 30 or 20 g/L sucrose, respectively. Tomato and *A. thaliana* leaf mesophyll protoplasts were isolated from 4- to 6 week-old plants and transformed using the polyethylene glycol-mediated transformation protocol (Mishra et al. 2002; Sommer et al. 2011). 10^5 protoplasts were transformed with 20 µg plasmid DNA; if necessary, the final amount of DNA was adjusted by addition of the plasmid pRT-Neo (Tillmann et al. 2015). For saGFP co-expression analysis, 10 µg of plasmid DNA per construct was used. Cells were incubated in light for 12 h at 26 °C.

Microscopic analysis

Intracellular localization of GFP fusion proteins in protoplasts was analyzed by fluorescence microscopy using a Leica SP5 confocal laser scanning microscope with a HCX PL APO lambda blue 63 × NA 1.4 oil objective as in (Bionda et al. 2010). GFP and chlorophyll fluorescence were excited at 488 nm. The emission of GFP and Alexa488 was recorded at 495–540 nm.

Antibodies

Polyclonal antibodies against chloroplast proteins and against the C-terminus of human Hsp70 (αC70) were raised as in (Vojta et al. 2004). The antibody against *Solanum peruvianum* (formerly *Lycopersicon peruvianum*) Hsp70 (α*Lp*Hsp70) was described (Neumann et al. 1987). Monoclonal antibodies against human cytosolic Hsp70 (αSPA820) and spinach Hsc70 (αSPA817) were purchased from Enzo Life Sciences (Lörrach, Germany). The antibody against DnaK of *E. coli* was kindly provided by Prof. Dr. Ulrich Hartl (MPI Martinsried, Germany). Polyclonal αGFP was supplied by Rockland (Limerick, PA, USA). The other used antibodies against Toc64, Toc75, Toc34, Toc159, Tic110, Tic40, Tic22, the outer envelope protein of 21 kDa (Oep21), the outer envelope protein of 37 kDa (Oep37), the large subunit of RUBISCO (LSU), the light harvesting complex protein (LHCP) and Hsp93 have been described (Schleiff et al. 2003a; Becker et al. 2004; Elkehal et al. 2012 and references therein).

Chloroplast and envelope isolation from *P. sativum* and *M. sativa*

Intact chloroplasts from *P. sativum* and *M. sativa* (Weigelt GmbH & Co. E.E. KG, Walluf, Germany) were isolated from 7- to 9 day-old and 15- to 20-day-old seedlings, respectively, grown on vermiculite, according to described procedures (Schleiff et al. 2002; Bionda and Schleiff 2010). Outer and inner envelope membranes from *P. sativum* and *M. sativa* were isolated from plants grown on vermiculite for 9–11 and 16–20 days, respectively (Schleiff et al. 2003b). Mitochondria were isolated as described (Rödiger et al. 2010).

Fractionation, extraction and proteolysis

Cell fractionation was performed by differential centrifugation of cell extracts from 10- to 12-day-old *P. sativum* seedlings (Becker et al. 2004). Subfractionation of chloroplasts was carried out as established for the isolation of envelope vesicles (Schleiff et al. 2003b). Mitochondria were isolated as in Rödiger et al. (2010).

Protein extraction: Chloroplasts (50 µg chlorophyll content) were incubated in (1) 1 M NaCl, (2) 100 mM Na_2CO_3 pH 11.5; (3) in 10 mM Hepes/KOH pH 7.6, 6 M urea for 30 min at 4 °C or (4) 1 % (v/v) Triton X-100, 10 mM Hepes/KOH pH 7.6 for 20 min at RT followed by centrifugation (265,000*g*, 10 min, 4 °C). Extraction of envelope vesicles (20 µg protein content) was performed as above by incubation for 20 min at RT.

Hypotonic lysis

Intact chloroplasts were lysed in 20 mM Hepes/KOH pH 7.4 and 5 mM EDTA for 30 min at 4 °C, followed by centrifugation (100,000*g*, 10 min, 4 °C). Soluble fractions were supplemented with 10 % (v/v) glycerol, frozen in liquid N_2, stored at −80 °C and used for the purification of imsHsp70.

Protease treatment

Outer envelope vesicles (20 µg protein) were incubated with indicated amounts of thermolysin in 25 mM Hepes/KOH pH 7.6, 150 mM NaCl, 0.5 mM $CaCl_2$ (20 min, 4 °C) in the absence or presence of 1 % (v/v) Triton X-100. The reaction was stopped by addition of 5 mM EDTA and vesicles were recovered by centrifugation (265,000*g*, 10 min, 4 °C). Intact chloroplasts were washed with 25 mM Hepes/KOH pH 7.6, 150 mM NaCl, 0.5 mM $CaCl_2$ and treated as above with 1 µg thermolysin per 1 µg chlorophyll. For mass spectrometry, intact chloroplasts (200 µg of chlorophyll) were incubated in 50 mM Hepes/KOH pH 7.6, 330 mM sorbitol, 0.5 mM $CaCl_2$ and 200 µg thermolysin (20 min, 4 °C). The reaction was stopped by addition of 5 mM EDTA. After incubation for 5 min at 4 °C, intact chloroplasts were purified through 40 % Percoll cushion containing 5 mM EDTA (3025*g*, 10 min, 4 °C), washed and hypotonically lysed.

Protein purification

MonoQ-based fractionation

The soluble fraction of *P. sativum* and *M. sativa* chloroplasts (20 mM Hepes/KOH pH 7.4, 5 mM EDTA, 10 % glycerol) was diluted 1:1 in 20 mM Hepes 7.4 and 200 mM KCl and applied to an anion-exchange column (MonoQ HR 5/5, GE Healthcare) pre-equilibrated with five column volumes of E-buffer (20 mM Hepes/KOH pH 7.4, 100 mM KCl, 2.5 mM EDTA, 5 % (v/v) glycerol at 4 °C. After washing with two column volumes of E-buffer, proteins were eluted using a gradient from 100 mM to 1 M KCl in 20 mM Hepes/KOH pH 7.4, 2.5 mM EDTA, 5 % (v/v) glycerol (twenty column volumes). The elution profile was monitored at 280 nm and the protein content in the fractions analyzed by SDS-PAGE and Western blot.

ATP–affinity chromatography was performed using N6-(6-Amino)hexyl-ATP-Sepharose (Jena Bioscience, Jena, Germany) at 4 °C following the manufactureŕs recommendations. In brief, MonoQ-separated fractions containing imsHsp70 or the soluble extract of hypotonically lysed chloroplasts were incubated with 50 µL of pre-equilibrated ATP-Sepharose matrices (3 h, 4 °C, constant rotation). The flow-through was removed (1000*g*, 1 min) and beads were washed 3× with 10 column volumes of ice-cold wash buffer. Bound proteins were eluted with E-buffer. The protein content of the fractions was analyzed by SDS-PAGE, Western blot and colloidal Coomassie (G-250) staining.

Mass spectrometric analysis of the protein samples and peptide assignment

The excision of proteins from SDS-PAGE, the preparation of the samples and the mass spectrometry analyses were performed as in (Ladig et al. 2011) using a 4800 MALDI TOF/TOF from AB SCIEX. MS/MS data sets were processed using the Data Explorer v4.8 software (Applied Biosystems). The peptide sequence query search of *M. truncatula* database Mt3.5 was carried out for each spot using Mascot v2.2 (Matrix Science Ltd, Elgin, SC, USA). Ion score significance threshold was at a default setting of 0.05.

Results

A protease-resistant cytosolic Hsp70 chaperone exists in chloroplasts

Early experiments proposed the existence of an imsHsp70 (Marshall et al. 1990; Waegemann and Soll 1991; Ko et al. 1992; Schnell et al. 1994; Becker et al. 2004), while some reports question the IMS localization of an Hsp70 (Ko et al. 1992; Ratnayake et al. 2008). Therefore, we inspected whether the Hsp70 detected in chloroplasts is a cytosolic contamination. At first we compared cell extract, isolated chloroplasts and isolated outer envelopes from pea, all normalized to chlorophyll content based on the previous observation that 0.3 µg of total outer envelope protein correspond to 10 µg of chlorophyll from chloroplasts (Elkehal et al. 2012). However, the cell lysate was loaded according to 25 % of the chloroplast chlorophyll. As expected, the majority of the Hsp70 is localized in the cell lysate (Fig. 1a, panel αLpHsp70, lane 1), but a significant portion of Hsp70 is localized with chloroplasts (Fig. 1a, lane 2) and even with isolated outer envelope (Fig. 1a, lane

4). Remarkably, the Hsp70 within the chloroplasts or the outer envelope is thermolysin resistant (Fig. 1a, lanes 3, 5). The efficiency of the thermolysin treatment was confirmed by the observed protease sensitivity of the two TOC components Toc159 and Toc34 (Fig. 1a, panel αToc159, panel αToc34). In contrast to Hsp70, the exclusively outer envelope localized Toc159, Toc34 and outer envelope protein of 37 kDa are enriched in chloroplasts when compared to cell lysate, because cell lysate only contains 25 % of the chlorophyll content compared to the chloroplast sample (Fig. 1a, panels αToc159, αToc34, αOep37, lane 1 vs lane 2). Moreover, the abundance of all three proteins is comparable between chloroplasts and outer envelope (Fig. 1a, panels αToc159, αToc34, αOep37, lane 2 vs lane 4). This suggests that Hsp70 is only associated with but not inserted into the membrane and thus, a significant portion is lost during envelope purification. Similarly, the abundance of the intermembrane space protein Tic22 or of the stromal small subunit of RUBISCO (SSU) is enriched in chloroplasts (Fig. 1a, panels αTic22, αSSU, lane 1 vs lane 2). They are both protease resistant (Fig. 1a, lane 3), but they are not detectable in outer envelopes (Fig. 1a, panels αTic22, αSSU, lane 4).

To exclude that Hsp70 is simply co-purified because of association with chloroplast, we investigated the distribution of actin. Actin is known to be associated with outer envelope proteins like the chloroplast unusual positioning 1 (CHUP1) (Schmidt von Braun and Schleiff 2008) and even an association with the chloroplast translocon is proposed (Jouhet and Gray 2009). We detected a large amount of actin in the cell lysate (Fig. 1a, panel αActin, lane 1). However, in contrast to Hsp70, we did not detect any actin in association with isolated chloroplasts or outer envelopes (Fig. 1a, lanes 2–5). This supports the specificity of the observed chloroplast localization of the cytosolic Hsp70.

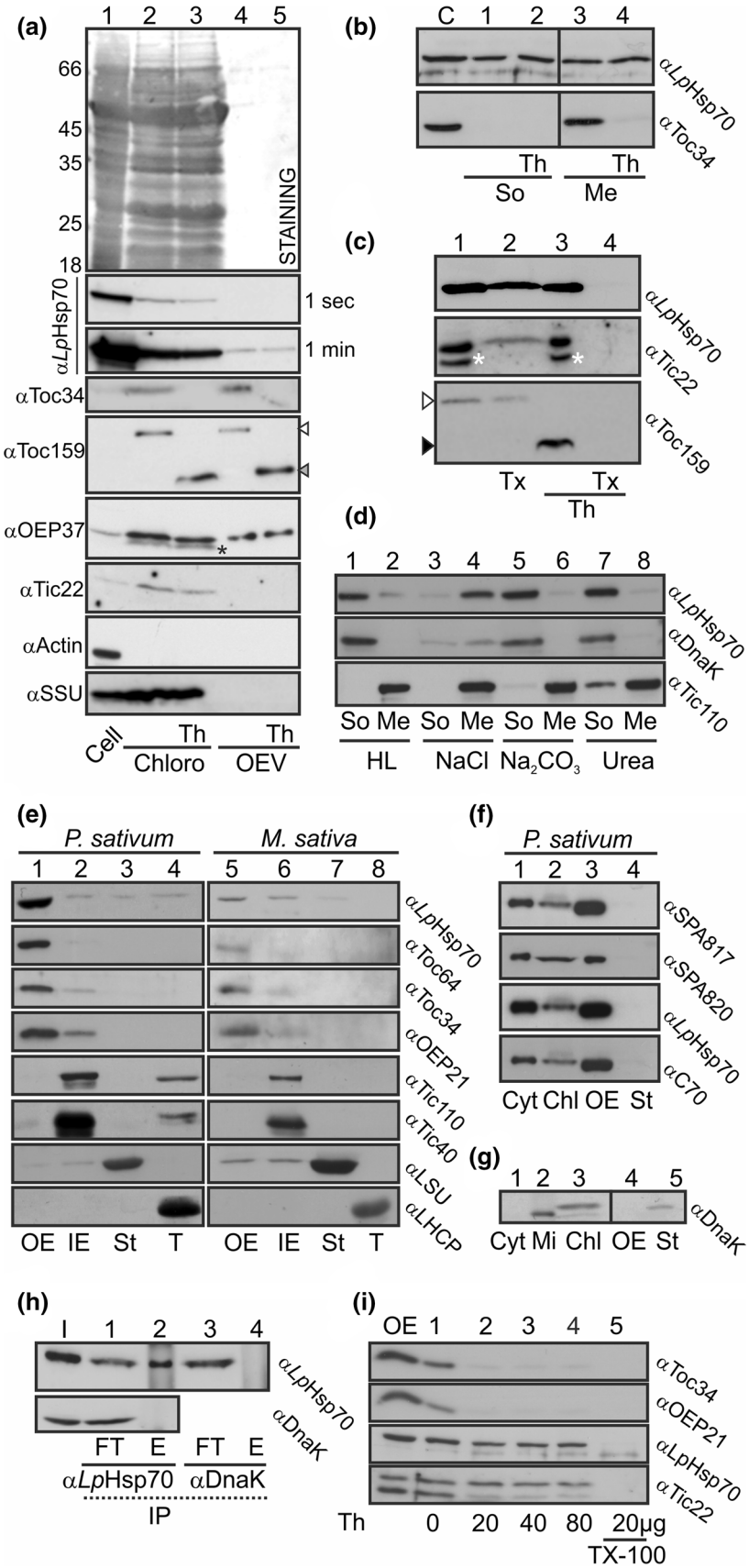
Chloroplasts contain an intermembrane space-localized eukaryotic Hsp70

The initial results suggest that a chaperone known to be localized in the cytosol exists in chloroplasts as well. In addition, the observed outer envelope association suggests a localization of Hsp70 in the intermembrane space. To further support the latter notion, chloroplasts were isolated from pea leaves and treated with thermolysin prior to hypotonic lysis. The cytosolically exposed G-domain of Toc34 was protease sensitive as judged from the Western blot with an antibody specific for this domain (Fig. 1b, lane 4). In contrast, the Hsp70 detected with the antibody against cytosolic Hsp70 from tomato (α*Lp*Hsp70; (Neumann et al. 1987) remained protease-resistant, irrespective of whether the soluble or the membrane-associated form is analyzed (Fig. 1b, lanes 2, 4). To confirm that the Hsp70 is not inherently thermolysin resistant, chloroplasts were incubated with thermolysin in the absence or presence of Triton X-100 (Fig. 1c). After addition of thermolysin, both membrane protected proteins, Tic22 and Hsp70, become protease accessible and are digested (Fig. 1c, lane 4), while Toc159 is accessible without Triton treatment as judged from the occurrence of the 52-kDa fragment (Fig. 1c, lane 3), and fully degraded in the presence of detergent (Fig. 1c, lane 4).

As described before (e.g. Marshall et al. 1990; Waegemann and Soll 1991), we observed a soluble and a membrane-bound Hsp70 fraction. Sodium carbonate or urea treatment released all Hsp70 from the membrane, while Tic110 remained membrane bound (Fig. 1d, lanes 5–8). It is worth mentioning that salt treatment enforced an increased membrane association of Hsp70 and of the stromal prokaryotic-type Hsp70 (DnaK, Fig. 1d, lane 3, 4), which might be related to the association to partially unfolded membrane proteins. The same result is observed for Hsp70 associated with outer envelope vesicles (Fig. S1). Nevertheless, we conclude that the Hsp70 is partially soluble and partially membrane attached, but not membrane embedded. The association of imsHsp70 with the membrane is not nucleotide dependent (Fig. S1).

To evaluate the localization of the Hsp70 in more detail we fractionated chloroplasts from *P. sativum* and *M. sativa* into outer and inner envelope, stroma and thylakoids (Fig. 1e). The fractions were highly enriched, but not entirely free of cross-contaminations as assayed by immunostaining of outer envelope proteins (Toc34, Toc64, outer envelope protein of 21 kDa; Oep21), inner envelope proteins (Tic110, Tic40), the stroma-localized large subunit of RUBISCO (LSU) and the thylakoid-localized light harvesting complex protein (LHCP). The Hsp70 detected with the α*Lp*Hsp70 antibody was most enriched in the outer envelope fraction when compared to inner envelope or stroma of *P. sativum* chloroplasts (Fig. 1e, top panel). We conclude that the membrane-associated Hsp70 is associated with the outer envelope and the soluble pool of Hsp70 is not localized in the stroma.

The eukaryotic nature of the detected Hsp70 protein was supported by immunostaining of cytosolic, chloroplast, outer envelope and stromal fractions with additional antibodies against eukaryotic Hsp70 proteins, namely αC70 (Qbadou et al. 2005) and the commercial αSPA820 and αSPA817, which recognized Hsp70 proteins in all fractions except stroma (Fig. 1f). The stromal Hsp70 is detected only in chloroplast and the stromal fraction (Fig. 1g, lane 5) as determined by immunostaining with antibodies specific for DnaK-like proteins of prokaryotic origin. The protein is distinct from the mitochondrial DnaK-like Hsp70 as judged from migration behavior (Fig. 1g, lane 2 vs. 3).


(a)
1 2 3 4 5
66
45
35
25
18
STAINING
αLpHsp70
1 sec
1 min
αToc34
αToc159
αOEP37
αTic22
αActin
αSSU
Cell
Th
Chloro
Th
OEV
(b)
C 1 2 3 4
αLpHsp70
αToc34
Th
So
Th
Me
(c)
1 2 3 4
αLpHsp70
αTic22
αToc159
Tx
Tx
Th
(d)
1 2 3 4 5 6 7 8
αLpHsp70
αDnaK
αTic110
So Me So Me So Me So Me
HL
NaCl
Na2CO3
Urea
(e)
P. sativum
M. sativa
1 2 3 4 5 6 7 8
αLpHsp70
αToc64
αToc34
αOEP21
αTic110
αTic40
αLSU
αLHCP
OE IE St T OE IE St T
(f)
P. sativum
1 2 3 4
αSPA817
αSPA820
αLpHsp70
αC70
Cyt Chl OE St
(g)
1 2 3 4 5
αDnaK
Cyt Mi Chl OE St
(h)
I 1 2 3 4
αLpHsp70
αDnaK
FT E FT E
αLpHsp70 αDnaK
IP
(i)
OE 1 2 3 4 5
αToc34
αOEP21
αLpHsp70
αTic22
Th 0 20 40 80 20µg
TX-100

◀**Fig. 1** The soluble and membrane-bound form of the chloroplast Hsp70 of eukaryotic origin. **a** Cell lysate (lane *1*; 2.5 µg chlorophyll), chloroplasts (lane *2*; 10 µg chlorophyll) and outer envelope vesicles (OEV, lane *4*; 0.3 µg protein which corresponds to 10 µg chlorophyll; Elkehal et al. 2012) as well as thermolysin-treated chloroplasts (lane *3*; 10 µg chlorophyll) and outer envelope vesicles (OEV, lane 5; 0.3 µg protein) were loaded on SDS-PAGE (*left panel*) and subjected to Western blot with the indicated antibodies (*middle* and *right panel*; *: cross reactivity of the antibody; white triangle: 86 kDa fragment of Toc159; gray triangle: 52 kDa fragment of Toc159). For usage of antibodies against LpHsp70, two blot exposure times are shown. **b** Intact pea chloroplasts (*lane C*, 10 %) were incubated with (lanes *1*, *3*) or without thermolysin (lanes *2*, *4*; Th) followed by re-isolation and hypotonic lysis. 10 % of soluble (lanes *1*, *2*; So) and membrane fractions (lanes 3, 4; Me) were analyzed by Western blot. **c** Intact pea chloroplasts (lane *1*) were incubated with Triton-X100 (lanes *2*, *4*) and thermolysin (lanes *3*, *4*) followed by Western blot with *Lp*Hsp70 (*top*), Tic22 (*middle*) or Toc159 (*bottom*) antibodies. * Cross-reactivity of the antibody, white triangle: 86 kDa fragment, black triangle: 52 kDa fragment of Toc159. **d** Chloroplasts were hypotonically lysed (lanes *1*, *2*, HL) and treated with sodium chloride (lanes *3*, *4*), carbonate (lanes *5*, *6*) or urea (lanes *7*, *8*). Soluble (lanes *1*, *3*, *5*, *7*; So) and membrane (lanes *2*, *4*, *6*, *8*; Me) fractions were separated and 10 % of each fraction was analyzed by Western blot. **e** Outer envelope vesicles (lanes *1*, *5*; OE), IEV (lanes *2*, *6*; IE), stromal (lanes *3*, *7*; St) and thylakoid proteins (lanes *4*, *8*; T) of *P. sativum* (lanes *1–4*) or *M. sativa* (lanes *5–8*) chloroplasts (5 µg protein) were analyzed by Western blot with the antibodies indicated on the right. **f** Cytosolic (lane *1*; 5 µg protein; Cyt), chloroplastic (lane *2*; 10 µg chlorophyll; Chl), outer envelope vesicles (lane *3*; 10 µg protein; OE) and stromal proteins of *P. sativum* (lane *4*; 10 µg protein; St) were immunoblotted with indicated antisera against cytosolic Hsp70. **g** Cytosolic (lane *1*; 5 µg protein; Cyt), mitochondrial (lane *2*; 5 µg protein; Mi), chloroplastic (lane *3*; 10 µg chlorophyll; Chl), outer envelope vesicles (lane *4*; 5 µg protein; OE) and stromal proteins (lane *5*; 5 µg protein; S) were immunostained with αDnaK. **h** The soluble fraction after hypotonic lysis of pea chloroplasts (**c**, 1 % loaded) was incubated with *Lp*Hsp70 (lanes *1*, *2*) or DnaK (lanes *3*, *4*) followed by immunoprecipitation (IP). 2 % of flow through (lanes *1*, *3*; FT) and 10 % of elution fraction (lanes *2*, *4*; **e**) was analyzed by immunostaining with the antibodies indicated on the right. **i** Outer envelope vesicles from *P. sativum* (20 µg protein, OE) were incubated with 0, 20, 40 and 80 µg thermolysin (lanes *1–4*; Th) or with 20 µg thermolysin in the presence of 1 % Triton X-100 (lane *5*). 15 % (Hsp70, Tic22) or 5 % (Toc34, Oep21) of each fraction was analyzed by immunoblotting

We used α*Lp*Hsp70 antibody for immunoprecipitation to further confirm the specificity (Fig. 1h). The soluble fraction after hypotonic lysis of pea chloroplasts was incubated with α*Lp*Hsp70 (Fig. 1h, lane 1, 2) or αDnaK (Fig. 1h, lane 3, 4) followed by immunoprecipitation The results show that the eukaryotic Hsp70 from lysates of pea chloroplasts was only immunoprecipitated by α*Lp*Hsp70 (Fig. 1h, lane 2), but not by αDnaK (Fig. 1h, lane 4), while DnaK cannot be immunoprecipitated by α*Lp*Hsp70 (Fig. 1h, lane 2). Thus, we provide evidence that the antibody used recognizes a protease-resistant (Fig. 1b) eukaryotic Hsp70 (Fig. 1f–h) that is not present in the stroma (Fig. 1e, f).

To further support an IMS localization of the detected Hsp70 we analyzed the protease protection of Toc34, Oep21, Hsp70 and Tic22 in outer envelope vesicles (OEVs; Fig. 1i). Hsp70 and Tic22 are thermolysin resistant, while Toc34 and Oep21 are thermolysin sensitive. However, after membrane disruption by detergent treatment, Hsp70 and Tic22 become thermolysin sensitive (Fig. 1i, lane 5). This result together with the protease resistance in chloroplasts and the identified localization strongly suggests an IMS localization of the Hsp70 protein.

Multiple Hsp70's are identified in the intermembrane space of *M. sativa* chloroplasts

We aimed to identify imsHsp70 from *Medicago* (Fig. 1e) because the genome of *M. truncatula* is sequenced (http://www.medicagohapmap.org/?genome). We treated intact chloroplasts of *Medicago* with thermolysin prior to hypotonic lysis (Fig. 2a). The efficiency and specificity of the protease treatment was confirmed by immunostaining of Toc34, Hsp93 and the large subunit of RUBSCO (LSU; Fig. 2a, lane 1–3). While the Hsp70, the stromal Hsp93 and LSU remained protease resistant, Toc34 was protease sensitive (Fig. 2a, lane 1–3 vs. lane 4–6). The protease was inactivated by chelating of calcium ions with EDTA. Subsequently, we performed the hypotonic swelling to release the content of the stroma and the IMS. This approach was rational as a eukaryotic Hsp70 is only present in the intermembrane space, but not in the stroma (Fig. 1).

The soluble proteins were fractionated by anion exchange chromatography (Fig. 2b; S2). We obtained fractions where the eukaryotic Hsp70 is enriched (Fig. S2). These fractions were pooled and proteins separated by SDS-PAGE (Fig. 2c, d). The protein pattern was comparable between non-treated (Fig. 2c, panels 1, 2) and thermolysin-treated (Fig. 2d, panel 1) samples, and the migration of the prokaryotic and eukaryotic chaperones was confirmed by Western blot (Fig. 2c, panels 3, 4; Fig. 2d, panel 2). The protein bands indicated (Fig. 2c, panel 2; Fig. 2d, panel 1) were analyzed by mass spectrometry (Table S1). The protein bands migrating at higher molecular weight were not analyzed as the identification of the Hsp70 was in focus of our approach. As expected from the Western blot, the first protein band could clearly be assigned to DnaK-like chaperones, while the third band contained eukaryotic Hsp70 proteins.

We noticed a contamination of band 3 by the stromal Xaa-Pro aminopeptidase 1 (e.g. Rutschow et al. 2008), the protein which is migrating as band 4 (Table S1). Thus, to further purify the imsHsp70, the soluble fraction of chloroplasts after hypotonic lysis (Fig. 2e, panel 3) or the

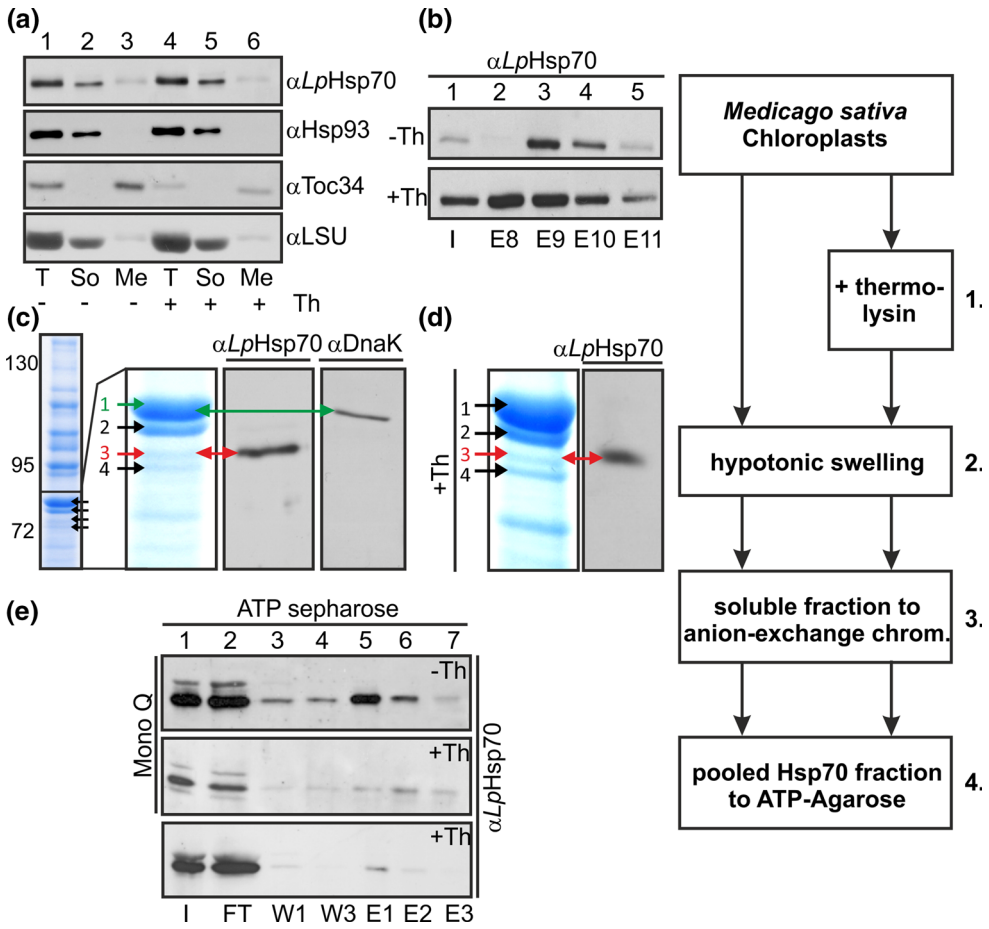


Fig. 2 Isolation of the imsHsp70 from thermolysin-treated *M. sativa* chloroplasts. On the right a scheme of the Hsp70 isolation protocol is shown. **a** Isolated chloroplasts (8 mg chlorophyll) were mock-treated (lanes *1–3*, −Th) or treated with 8 mg thermolysin (lanes *4–6*, +Th), followed by re-isolation and lysis. Mock-treated and thermolysin-treated total chloroplasts (lanes *1*, *4*; T), soluble (lanes *2*, *5*; So) and membrane fractions (lanes *3*, *6*; Me) were analyzed by Western blot with indicated antibodies. Note: in contrast to previous reports Hsp93 (e.g. Chu and Li 2012; Sjögren et al. 2014) was not isolated with membrane fraction under the conditions described in "Materials and methods". **b** The soluble fraction of mock-treated (*top panel*; −Th) and thermolysin-treated chloroplasts (*bottom panel*; +Th) was separated on a MonoQ anion-exchange column. Protein content of 0.5 % of input (I, lane 1) and 4 % of fractions E8-E11 (lanes 2–5) were immunostained with α*Lp*Hsp70. **c** Fractions E9 and E10 from several independent isolations (**b**, −Th) were pooled and concentrated. Samples were separated by SDS-PAGE and either immunostained (2.5 %) with α*Lp*Hsp70 and αDnaK or stained with colloidal Coomassie. Protein bands marked 1–4 were excised and analyzed by mass spectrometry (see Table S1). Numbers on the left indicate the size in kDa. **d** Fractions E8-E10 from multiple independent isolations (**b**, +Th) were subjected to SDS-PAGE, Coomassie-stained or immunostained (2.5 %) with α*Lp*Hsp70. Protein bands marked 1–4 were excised and analyzed by mass spectrometry. **e** Fractions E8-E10 from (**b**) were pooled and incubated with ATP-Sepharose matrices. Bottom panel shows ATP affinity purification of thermolysin-treated chloroplasts without separation by anion exchange chromatography. 2.5 % of input (I), 5 % of flow through (FT), 10 % of wash (W1, W3) and 10 % of elution fractions (E1-E3) was immunoblotted with α*Lp*Hsp70

Hsp70-containing fractions obtained by anion exchange chromatography (Fig. 2e, panel 1, 2) were incubated with ATP-Sepharose. A significant portion of the Hsp70 was bound to the ATP-column (Fig. 2e, lane 5-7) and subsequently analyzed by mass spectrometry (Table S1). This purification enabled the isolation of fractions exclusively containing eukaryotic Hsp70 proteins. Analyzing the eukaryotic Hsp70-containing fractions of the three independent approaches (Fig. 2e), several cytosolic Hsp70 isoforms were identified (Table S1). Although Hsp70 proteins share in general high sequence conservation, the detection of the different isoforms is supported by the identification of isoform-specific peptides (Table 1, Table S1, Fig. S3).

Expression of *M. truncatula* Hsp70 in protoplasts shows intermembrane space localization

The localization of *M. truncatula* cytosolic Hsp70 proteins (Fig. S4) was investigated in vivo using a transient expression system in tomato protoplasts. The localization of cytosolic Hsp70s was analyzed in *A. thaliana* protoplasts

Table 1 Hsp70 peptides identified with significant ion score

Hit[a]	Peptide sequence	Ion score	Miss
Multiple	GEGPAIGIDLGTTYSCVGVWQHDR	32	0
	TTPSYVAFTDSER	133	0
	NAVVTVPAYFNDSQR	104	0
	DAGVIAGLNVLR	7	0
	IINEPTAAAIAYGLDKK	97	1
	MVNHFVQEFK	53	0
	ARFEELNMDLFR	61	1
	FEELNMDLFR	35	0
	EQVFSTYSDNQPGVLIQVYEGER	188	0
	NALENYAYNMR	80	0
Medtr7g024580	NSLENYAYNMR	50	0
Medtr7g024390	NTIKDEKIGSK	4	2
Medtr7g118170	EQVFSTYSDNQPGVLIQVFEGER	105	0
Medtr4g130540	FELTGIPPAPR	9	0
	NSIENYAYNMR	43	0

[a] The protein identified by the peptide (column 1), the peptide sequence identified (column 2), the ion score (column 3), and the number of missed cleavages (column 4) is listed. Peptide positioning is indicated in Figure S3

as well to generalize the findings. To judge the different possible intracellular localizations, the self-assembling GFP system (Cabantous et al. 2005) established for the analysis of protein localization was employed (Machettira et al. 2011). In the saGFP system, the eleven-stranded β-barrel of GFP is split into saGFP1-10, comprising β-strands 1-10 ($G_{S1\text{-}10}$), and the very short saGFP11 fragment, containing only β-strand 11 of GFP (G_{S11}). Fluorescent GFP assembles only when the two fragments are located in the same cellular sub-compartment (Gross et al. 2011).

We used different reporters to analyze the plastid localization of cytosolic Hsp70 proteins: the cytosol-localized $G_{S1\text{-}10}$, the IMS-localized monogalactosyl diacylglycerol synthase 1 fused to $G_{S1\text{-}10}$ (MGD1-$G_{S1\text{-}10}$, Benning and Ohta 2005), the IMS-localized processing peptidase 1 fused to $G_{S1\text{-}10}$ (SP1-$G_{S1\text{-}10}$, Inoue et al. 2005) and the stroma-localized small subunit of RUBSICO fused to $G_{S1\text{-}10}$ (pSSU-$G_{S1\text{-}10}$). The expressed constructs did not show fluorescence by themselves (not shown), while fusions of MGD1 and SP1 to full-length GFP yielded a fluorescence signature consistent with the IMS localization (Fig. S5; Gross et al. 2011). The specificity of the two IMS localized saGFP reporters was tested by co-expression with the IMS-localized Tic22 fused to G_{S11}. The co-expression resulted GFP signals in certain regions in case of MGD1 as marker and in ring-like GFP signals in case of SP1, both signals surrounding the auto-fluorescence, which is consistent with the IMS localization of the proteins (Fig. 3a). The difference in fluorescence distribution might result from different expression levels. To confirm that this GFP signal does not occur by trapping a translocation intermediate SP1-$G_{S1\text{-}10}$ was co-expressed with pSSU-G_{S11}, which resulted in no GFP fluorescence. Furthermore, the functionality of pSSU-G_{S11} was confirmed by co-expression with pSSU-$G_{S1\text{-}10}$ resulting in stromal GFP fluorescence (Fig. 3b). Thus, translocation intermediates are not arrested as such that GFP can assemble.

Next we co-expressed the four established markers with a *M. truncatula* Hsp70 fused to G_{S11} (Medtr7g024580-G_{S11}). GFP fluorescence was observed when co-expressing the cytosolic and both IMS marker proteins, but not with the stromal marker (Fig. 3c). The detailed inspection revealed that co-expression of MGD1-$G_{S1\text{-}10}$ with Medtr7g024580-G_{S11} yielded a rim like structure (Fig. 3c, bottom left), while co-expression of SP1-$G_{S1\text{-}10}$ with Medtr7g024580-G_{S11} resulted in GFP-fluorescence in certain areas (Fig. 3c, bottom right). Thus, on the one hand we confirm the cytosolic localization of the Hsp70 and on the other hand we show the GFP assembly with the IMS marker. The differential structuring of the GFP signal with the two different IMS marker proteins might reflect that one is membrane localized (MGD1) and the other (SP1) soluble in the IMS. Thus, it is possible that the latter tends to aggregation leading to the punctured structure. However, the occurrence of the GFP signal demonstrates the IMS localization. At the same time we could not confirm a stromal localization of a eukaryotic Hsp70 (Ratnayake et al. 2008). In addition, the absence of fluorescence when co-expressing Medtr7g024580-G_{S11} and pSSU-$G_{S1\text{-}10}$ confirms that GFP is not assembled by formation of

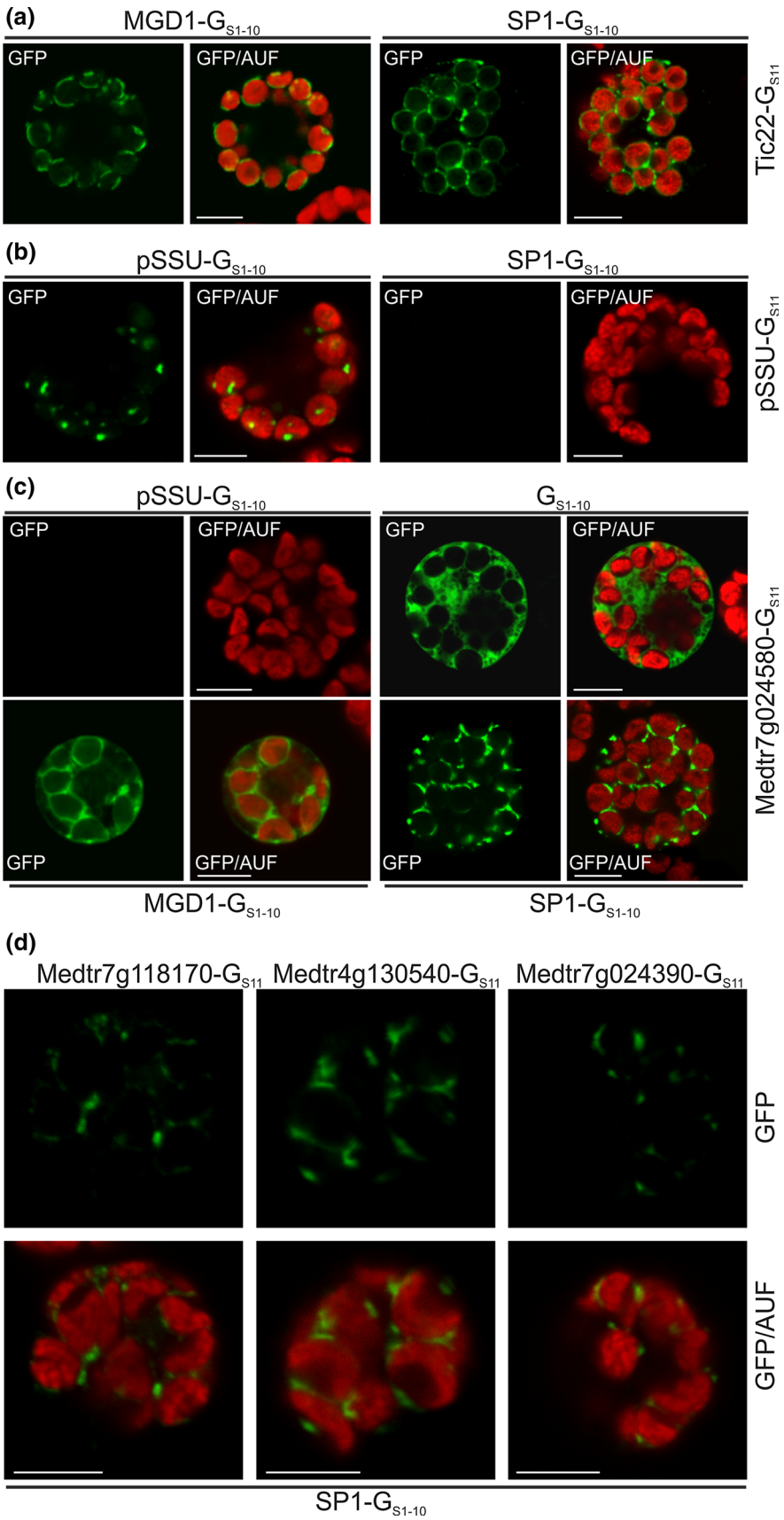


Fig. 3 IMS localization of *Medicago truncatula* Hsp70 proteins in *S. lycopersicum* protoplasts. **a** MGD1-G_{S1-10} (*left*) or SP1-G_{S1-10} (*right*) were co-transformed with Tic22-G_{S11}. **b** pSSU-G_{S1-10} (*left*) or SP1-G_{S1-10} (*right*) were co-transformed with pSSU-G_{S11}. **c** Medtr7g024580-G_{S11} was co-transformed with the indicated constructs in *S. lycopersicum* mesophyll protoplasts. **d** Medtr7g118170-G_{S11}, Medtr4g130540-G_{S11} or Medtr7g024390-G_{S11} is co-expressed with SP1-G_{S1-10}. In a-d The GFP signal (GFP) alone and the overlay with the chlorophyll autofluorescence (GFP/AUF) for a representative protoplast ($n > 100$) from several independent experiments ($n \geq 3$) is shown. All scale bars indicate 10 µm

targeting complexes because pSSU was found to be associated with Hsp70 (May and Soll 2000).

To support the finding of multiple Hsp70 proteins in the IMS, the other three identified cytosolic Hsp70 isoforms of *M. truncatula* (Table S1) fused to G_{S11} were co-expressed with the IMS marker protein SP1-G_{S1-10}. Similar to Medtr7g024580-G_{S11}, we observed a chloroplasts-surrounding GFP fluorescence for these proteins (Fig. 3d). In contrast, co-expression of the G_{S11} fused Hsp70 homologs with pSSU-G_{S1-10} did not yield a fluorescence signal (not shown). In line with the mass spectrometry findings, the obtained results indicate that a portion of multiple cytosolic Hsp70 chaperones of *M. truncatula* is localized in the IMS of chloroplasts.

Targeting of Hsp70 to the intermembrane space is specific for chloroplasts

The observations presented (Fig. 3) point toward a localization of cytosolic Hsp70 in the IMS. To evaluate whether this is a general concept in plants the cytosolic Hsp70 isoforms 1, 2, 3 and 5 of *A. thaliana* fused to G_{S11} were co-expressed with SP1-G_{S1-10}. We observed a ring-like GFP fluorescence surrounding the chloroplasts for all cases (Fig. 4a; Fig. S6), and fluorescence was specific for chloroplasts as judged by lysis of protoplasts (Fig. 4b). As for Hsp70 from *M. truncatula*, GFP fluorescence was not observed when Hsp70s from *A. thaliana* fused to G_{S11} were

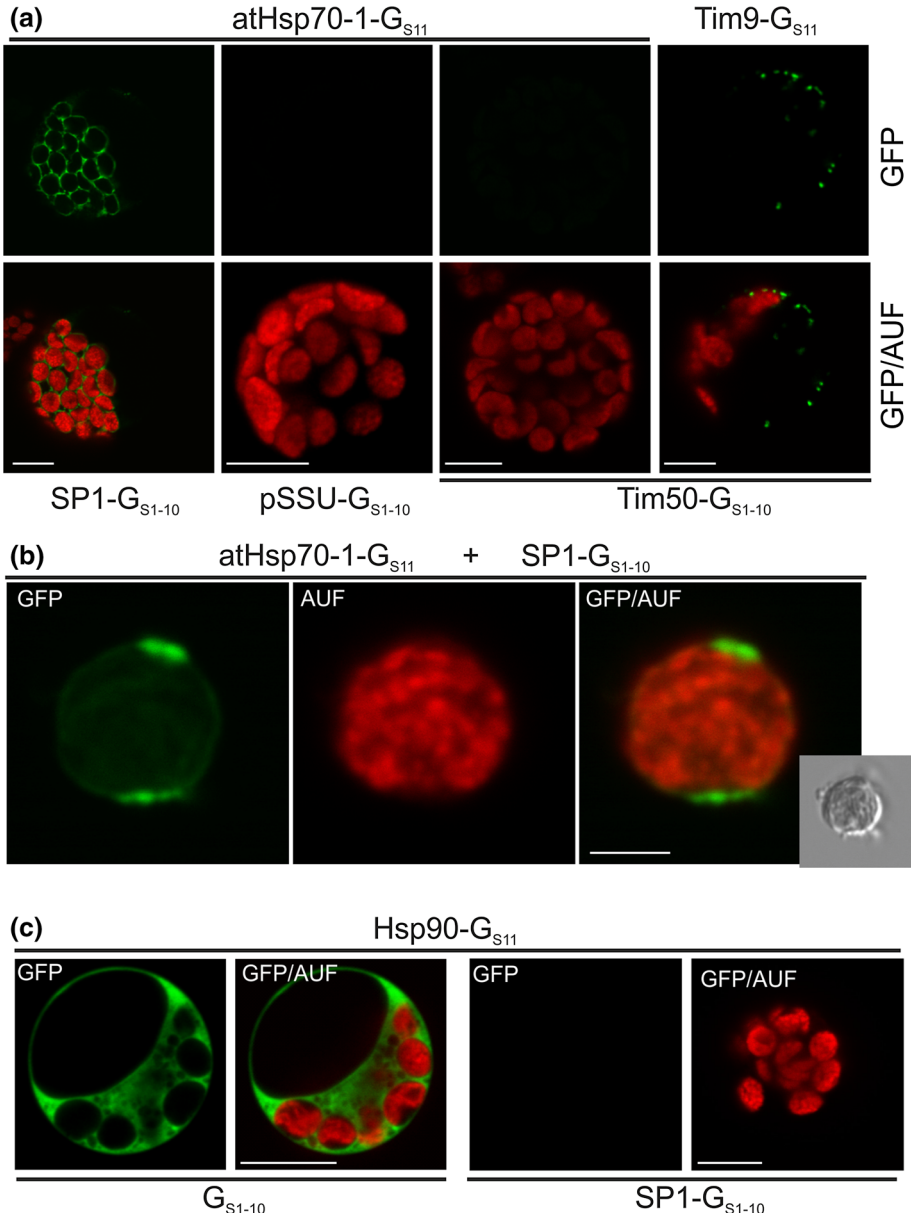


Fig. 4 Specificity of IMS localization of *Arabidopsis thaliana* Hsp70 proteins in *A. thaliana* protoplasts. **a** *A. thaliana* Hsp70 isoform 1 fused to G_{S11} was co-expressed with SP1-G_{S1-10} (*first panel*), pSSU-G_{S1-10} (*second panel*) or Tim50-G_{S1-10} (third panel). As a control, Tim9-G_{S11} was co-expressed with Tim50-G_{S1-10} (*right panel*). The GFP fluorescence (top) and its overlay with chlorophyll auto-fluorescence (GFP/AUF; bottom) of representative protoplasts ($n > 100$) from several independent experiments ($n \geq 3$) are shown. All scale bars indicate 10 µm. **b** *at*Hsp70-1-G_{S11} was co-expressed with SP1-G_{S1-10} in protoplasts, which were subsequently lysed. The GFP fluorescence (*left*) and its overlay with chlorophyll auto-fluorescence (AUF, *middle*) is shown in an overlay (right) for a representative chloroplast ($n > 100$). The image on the right shows the DIC image of the isolated chloroplast. The scale bar indicates 2 µm. **c** Hsp90-G_{S11} was co-expressed with G_{S1-10} (*left*) or SP1-G_{S1-10} (*right*) and GFP fluorescence and its overlay with chlorophyll auto-fluorescence (GFP/AUF) of a representative protoplast is shown ($n > 100$, set of independent experiment $n \geq 3$). All scale bars indicate 10 µm

co-expressed with pSSU-G_{S1-10} (Fig. 4a and data not shown).

Our results so far suggest that targeting of plant cytosolic Hsp70 proteins to the IMS of chloroplasts is a general phenomenon. To confirm that the result is specific for chloroplasts we probed for localization of eukaryotic Hsp70s in the IMS of mitochondria. The G_{S1-10} was targeted to the IMS of mitochondria by a fusion to the IMS-facing region of Tim50 (Fig. 4a). The specificity of this reporter was confirmed by co-expression with the IMS-localized Tim9 fused to G_{S11} (Gross et al. 2011), which resulted in a punctuated pattern of GFP fluorescence indicative of mitochondria (Fig. 4a, right). However, when cytosolic Hsp70 chaperones of *A. thaliana* were co-expressed with Tim50-G_{S1-10} GFP fluorescence could not be observed (Fig. 4a). This suggests that cytosolic Hsp70s are not localized in mitochondrial IMS. Thus, the localization in the chloroplast IMS is specific.

In addition to Hsp70 also Hsp90 was found to be involved in cytosolic preprotein targeting and in the stromal translocation process (Qbadou et al. 2006; Inoue et al. 2013). Therefore, we analyzed whether eukaryotic Hsp90s are localized in the IMS of chloroplasts as well. Co-expression of Hsp90 fused to G_{S11} with G_{S1-10} confirms the cytoplasmic localization of the protein (Fig. 4c). In contrast, co-expression of Hsp90-G_{S11} (Fig. 4c) with SP1-G_{S1-10} did not result in GFP fluorescence. The absence of Hsp90 in the IMS of chloroplasts confirms that only the eukaryotic Hsp70 is localized in the IMS.

Discussion

Multiplicity of Hsp70 proteins in the intermembrane space of chloroplasts—a functional hypothesis

The existence of a eukaryotic-type imsHsp70 was under debate ever since, because the molecular identity has never been described. Some reports suggested the localization of the Hsp70 at the cytosolic site of the outer envelope (Ko and Kourtz 1997) or in the stroma of chloroplasts (Ratnayake et al. 2008). We show that a cytosolic Hsp70 is enriched in the IMS of *P. sativum* and *M. sativa* chloroplasts (Fig. 1), where it exists as a membrane-associated as well as a soluble form (Figs. 1, 2). The partitioning of the Hsp70s between cytoplasm and IMS of chloroplasts (Figs. 3, 4) is specific for cytosolic Hsp70s, as other chaperones (e.g. Hsp90) are not targeted to chloroplasts (Fig. 4). Furthermore, the occurrence of cytosolic Hsp70s in the IMS is specific for chloroplasts, as no co-localization with mitochondrial IMS marker proteins could be demonstrated (Fig. 4). Moreover, the eukaryotic nature of the imsHsp70 is supported by several observations. (1) Four antisera raised against cytosolic Hsp70 proteins recognize the protein (Fig. 1), while (2) the antibody against DnaK does not (Fig. 1). The reported reactivity of SPA820 antibody towards the stroma-localized protein (Ratnayake et al. 2008) could not be confirmed (Fig. 1). (3) Mass spectrometric analysis of soluble proteins from thermolysin-treated chloroplasts revealed the presence of cytosolic Hsp70s (Fig. 2, Table S1). (4) The analysis of the in vivo distribution of the cytosolic Hsp70 proteins strongly suggests an IMS localization (Figs. 3, 4). These results are in line with the detection of different cytosolic Hsp70 isoforms (Hsp70-1, Hsp70-2, Hsp70-3, Hsp70-4 and Hsp70-5; *A. thaliana* nomenclature) in previous proteomic studies of *A. thaliana* (e.g. Kleffmann et al. 2004; Zybailov et al. 2008) and *P. sativum* chloroplasts (Bräutigam et al. 2008). The multiplicity of cytosolic Hsp70s in the IMS of chloroplasts also explains why first attempts to identify a specific protein by MS/MS have failed (Schnell et al. 1994).

The discovery of Hsp70s in the IMS leads to the question concerning their function. On the one hand, Hsp70 might be involved in protein translocation across the IMS. On the other hand, the Hsp70 might function in folding of IMS localized proteins. The importance for protein translocation can be rationalized by the following argumentation. Translocation across both mitochondrial membranes requires at least 80 N-terminal amino acids in a loosely folded conformation, while 60 N-terminal amino acids are just sufficient for translocation across the inner membrane (summarized in Schleiff and Becker 2011). In contrast, chloroplast preproteins are in need of a 60 amino acid loosely folded N-terminus for efficient translocation into chloroplasts (Bionda et al. 2010). One currently discussed TOC complex assembly (Schleiff et al. 2003b; Sommer et al. 2011) consists of a cytosolic exposure of the POTRA domains of Toc75 and of the G-domains of Toc159 and Toc34, the membrane embedded β-barrel of Toc75 and a structurally not yet defined 52 kDa domain of Toc159 in the intermembrane space. At least 30 fully extended amino acids are required to span the *cis* side and the membrane domain of the TOC complex considering an amino acid repeat distance of about 7.0 Å. Moreover, a globally conserved Hsp70 binding side in the N-terminal 10–20 amino acids of transit peptides has been described (Ivey et al. 2000). The estimated length requirement considering a full extension of the peptide of 50 amino acids is a good approximation of the observed 60 amino acid requirement. Thus, comparing the results for mitochondria and chloroplasts it was proposed that the 60 amino acids of chloroplast signal are just long enough to reach into the IMS, but not to cross both membranes and to engage stromal chaperones at once (Bionda et al. 2010; Schleiff and Becker 2011).

The above suggested mechanism would require an initial translocation force provided by a yet unknown factor in the

IMS. This additional energy could also explain the observation that the translocon of chloroplasts provides an initial preprotein unfolding energy for translocation that is higher than the one found for mitochondria (Guéra et al. 1993; Ruprecht et al. 2010; Leibovitch et al. 2013) and translocation is dependent on an energizing step in the IMS (Olsen and Keegstra 1992). A putative candidate for the initial ATP-dependent intraorganellar 'motor' could be the Hsp70 homologue at the *trans* side of the outer envelope (Marshall et al. 1990; Waegemann and Soll 1991; Schnell et al. 1994). In support of the latter, experimental evidence was provided that the Hsp70 is associated with the TOC complex, likely by interaction with Toc64 and Toc12 (Waegemann and Soll 1991; Becker et al. 2004). Moreover, the participation of an imsHsp70 in translocation was concluded from the direct interaction with the precursor of the small subunit of RUBISCO, pSSU (Waegemann and Soll 1991). Finally, a function of imsHsp70 in TOC guided preprotein translocation would be consistent with the quantification of the components of the translocon. It was reported that the outer membrane-associated Hsp70 has an abundance of about 25 % of Toc34 (Vojta et al. 2004) of which four copies exist in the general translocon (Schleiff et al. 2003b). Moreover, the molecular amount of Hsp70 is half of Toc64 (Vojta et al. 2004) discussed as IMS chaperone anchor (Qbadou et al. 2007). Last, the abundance of Hsp70 is about 50 % of that of Tic20 (Vojta et al. 2004) that is currently discussed as translocation channel of the inner membrane (Nakai 2015). Taking into account that not all of the imsHsp70 is bound to the outer membrane (Fig. 1), a sufficient pool of chaperones appears to exist in this compartment.

Taking all lines of evidence together we propose that the imsHsp70 is directly involved in preprotein translocation. However, the Hsp70 might also be involved in protein folding in the IMS. Although the intermembrane space proteome has not yet been analyzed, enzymes involved in lipid synthesis like MGD1 (Miège et al. 1999) or protein targeting like Tic22 (Kouranov et al. 1999) or Plsp1 (Inoue et al. 2005) have been localized within this compartment. For MGD1 and Tic22 it was shown that they utilize the TOC translocon for transport across the membrane (Vojta et al. 2007) and thus reach the intermembrane space as an unfolded polypeptide. The folding pathway of these proteins is not yet known, but might be assisted by the imsHsp70. However, the final reconstruction of function of the Hsp70 in the IMS as well as of the translocation events in the IMS requires additional experimental evidence.

Dual localization of Hsp70 in the cytosol and the intermembrane space of chloroplasts

While cytosolic Hsp70s are highly conserved in their amino acid sequences (e.g. Fig. S3), one eukaryotic-type Hsp70 sequence with a distinct C-terminal amino acid composition, which could serve as signal, was described, namely Com70 (Ko et al. 1992). The sequence alteration is caused by a single deoxythymidine insertion at base pair position 1936. However, this deoxythimidine insertion could not be confirmed by cDNA sequencing of more than ten independent Com70 clones of spinach (not shown). Thus, a cytosolic Hsp70 with distinct amino acid sequence that could serve as targeting signal does not exist, raising the question of how the chloroplast targeting is accomplished. Remarkably, proteomic studies identified that up to 11.4 % of all chloroplast proteins do not contain a classical transit peptide (Armbruster et al. 2009). The translocation of these proteins occurs by an alternative, TOC/TIC-independent pathway as exemplified for the two inner envelope proteins, ceQORH (chloroplast envelope Quinone Oxidoreductase Homologue) and Tic32 (Miras et al. 2002; Nada and Soll 2004). All attempts to perform import experiments with isolated chloroplasts using wheat germ or reticulocyte lysate to in vitro translate Hsp70 did not give a conclusive result. After incubation, only a minor portion of the protein was resistant against protease treatment, while a significant portion of the protein precipitated after Triton X-100 treatment (not shown). This suggests that factors not present in this isolated system might be important for efficient translocation.

In addition, a significant portion of plant proteins is localized in more than one cellular compartment (Carrie et al. 2009). One example for a protein localized in the cytoplasm and an organelle is the antioxidative enzyme Cu,Zn-SOD1 (Cu, Zn-superoxide dismutase 1) and its copper chaperone CCS (copper chaperone for superoxide dismutase). Both lack a mitochondrial targeting signal, but are present in the IMS of mitochondria in *S. cerevisiae* as well (Sturtz et al. 2001). The mitochondrial localization of SOD1 thereby strictly depends on the presence of CCS in the IMS, which was postulated to serve as a *trans* side receptor (Sturtz et al. 2001). A similar trapping mode was postulated for mitochondrial cytochrome *c*, whose accumulation in the IMS is regulated by a peripheral inner membrane cytochrome *c* heme lyase that catalyzes the covalent attachment of the heme cofactor to cytochrome *c* (Mayer et al. 1995). Therefore, Hsp70 is just another example of abundant cytosolic proteins dually targeted to the IMS of organelles. However, the most likely noncanonical mode of dual targeting to the IMS of chloroplasts has to be explored in future.

Author contribution statement ES developed the concept; TB and LG performed the biochemical characterization; TB the HSP localization; DGP, MSL and MK the mass spectrometric analysis; All were involved in data analysis; ES and LG wrote the manuscript; all commented, corrected and approved the manuscript.

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