Supporting Information

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SI Materials and Methods

Plasmids and DNA Constructs. pETd:preatTic40–3xFLAG–His encoding preatTic40–3xFLAG–His (Fig. 1*A*) was generated by introducing a DNA fragment encoding a 3xFLAG peptide (DYKDHDGDYKDHDIDYKDDDDK) and a six histidine tag in frame with the 3′ coding region of the pET21d:preatTic40 construct (1, 2). pETd:atTic40–3xFLAG encoding preatTic40–3xFLAG was generated by introducing a DNA fragment encoding a 3xFLAG peptide in frame with the coding region of the pET21d:preatTic40 construct. To prepare pET21a:preatSSU–3xFLAG–His encoding preatSSU–3xFLAG–His, pET21a:preatSSU was modified by introducing a DNA fragment encoding a 3xFLAG peptide and a six histidine tag in frame with the 3′ coding region (Fig. 1*A*) (3).

A DNA fragment encoding the precursor to *Arabidopsis thaliana* pyruvate dehydrogenase E1 component subunit alpha, preatE1α, was generated using PCR from a cDNA (At1g01090), with primers that introduced a 5′ NcoI and 3′ EcoRI sites and inserted into the NcoI and EcoRI sites of pET21d to generate pET21d:preatE1α. Plasmids encoding pre-LHCP were a generous gift from Kenneth Cline (University of Florida, Gainesville, FL) (4). Plasmids encoding preatTic20 and preatAPG1 are as described previously (5, 6).

A DNA fragment encoding mature atHsp90C (amino acids 61–780) was generated using PCR from a cDNA for atHsp90C (At2g04030), with primers that introduced 5′ NdeI and 3′ XhoI sites. The fragment was inserted into the NdeI and XhoI sites of pET28a to generate pET28a:His-M-atHsp90C. A DNA fragment encoding mature atcpHsp70-2 (amino acids 79–718) was generated using PCR from a cDNA for atcpHsp70-2 (At5g49910), with primers that introduced 5′ NdeI and 3′ XhoI sites. The fragment was inserted into the NdeI and XhoI sites of pET28a to generate pET28a:His-M-atcpHsp70-2. GST fusion constructs of mature atHsp90C (GST-tev-M-atHsp90C) and mature atTic40 (amino acids 129–371) (GST-tev-M-atTic40) were amplified using PCR with primers that introduced a 5′ XhoI and 3′ XhoI sites. The fragments were inserted into the XhoI site of pGEX-6p-1 (7). pGEX-6p-1 was a generous gift from Daniel N. Hebert (University of Massachusetts, Amherst, MA).

The pEarlygate303:ProAtHSP90C:AtHsp90C construct was generated using the Gateway cloning system (Invitrogen). A DNA fragment consisting of 1185 bp upstream of the ATG start codon and the first 193 bp of the AtHsp90C coding sequence (At2g04030) was amplified from *Arabidopsis* genomic DNA by PCR using primers 1 and 2 (Table S1) and cloned into pENTR/D-TOPO using the pENTR Directional TOPO Cloning kit (Invitrogen) to generate pENTR:ProAtHSP90C. A 2,340-kb cDNA fragment encoding pre-AtHsp90C was amplified by PCR using primers 3 and 4 (Table S1). This product was cloned into the BglI and XhoI sites of pENTR:ProAtHSP90C to generate pENTR:ProAtHSP90C:AtHsp90C. This construct was used in a LR recombination reaction with the pEarlygate303 destination vector (8). The resulting binary vector, pEarlygate303:ProAtHSP90C:AtHsp90C, encodes Basta (glufosinate ammonium) resistance as the plant selectable marker and kanamycin resistance as the bacterial selectable marker.

Protein Expression and Purification. PreatTic40–3xFLAG, preatTic40–3xFLAG–His, and preatSSU–3xFLAG–His for in vitro import experiments were expressed in *Escherichia coli* BL21 (DE3) using 0.4 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) for 3 h at 37 °C. His-tagged preproteins were purified using Ni-NTA matrix (Novagen) under denaturing conditions as described previously (9).

His-M-atHsp90C, His-M-psHsp93, His-M-atcphsp70-2, GST-mTic40, and GST-mHsp90 were expressed in *E. coli* BL21 (DE3) using 0.4 mM IPTG for 3 h at 30 °C. His-tagged proteins were purified using Ni-NTA under native conditions at 4 °C. Cells were harvested after induction, and resuspended in buffer A [40 mM Hepes-KOH pH 7.5, 150 mM KCl, 5% (wt/vol) glycerol]. Following disruption, the clarified supernatant was applied to a Ni-NTA matrix. The column was washed with buffer A containing 5 mM imidazole, and proteins were eluted from the column with buffer A containing 200 mM imidazole. Plasmids encoding mature *Pisum sativum* Hsp93 (His-M-psHsp93) were a generous gift from Hsou-min Li (Academia Sinica, Taipei, Taiwan) (10, 11).

GST-fusion proteins were purified using glutathione sepharose 4B (GE Healthcare Life) under native conditions at 4 °C. Cells were harvested after induction and resuspended in buffer B (25 mM Tricine-KOH pH 7.5, 150 mM NaCl). The clarified supernatant was subjected to glutathione sepharose 4B column. The column was washed with buffer B, and proteins were eluted from the column with buffer B containing 100 mM glutathione.

Antibodies. Antisera to Tic110, Toc75, APG1, and OE21 were described previously (12, 13). The antiserum recognizing Hsp90C was raised against amino acids 61–780 of *Arabidopsis* Hsp90C. The antiserum recognizing atTic40 and Hsp93 were raised against full-length *Arabodopsis* Tic40, and amino acids 91–922 of pea Hsp93 (His-M-psHsp93), respectively. For immunoaffinity chromatography, antibodies to Hsp90C and Tic40 were purified by GST fused antigen coupled to sepharose according to the supplier's recommendations (Amersham).

Chloroplast Isolation, Cross-Linking, Immunoprecipitation, or Immunoaffinity Purification. Intact chloroplasts were isolated from 10- to 12-d-old pea seedlings (*P. sativum* var. Green Arrow) as previously described (14). Isolated chloroplasts were resuspended in HS buffer (50 mM Hepes-KOH, pH 7.5, 0.33 M sorbitol) to a concentration equivalent to 1–2 mg chlorophyll per milliliter.

For cross-linking, isolated intact chloroplasts were resuspended in HS buffer to a concentration of 0.5 mg/mL chlorophyll and dithiobis[succinimidyl propionate] (DSP) was added to a final concentration of 1 mM (15). The cross-linking reaction was incubated on ice in the dark for 15 min and quenched by adding glycine to 20 mM and continuing incubation for another 15 min. Cross-linked chloroplasts were reisolated through 35% Percoll silica gel, washed with HS buffer and dissolved with TES buffer (50 mM tricine-KOH pH 7.5, 2 mM EDTA, 150 mM NaCl,) containing 1% Triton X-100 and 0.5% protease inhibitor cocktail (PIC) (Sigma; P9599) at 4 °C for 30 min with constant gentle shaking. After a 30-min centrifugation at 18,000 × *g*, the supernatant was collected and incubated with 20 μg M2 FLAG monoclonal antibody (Sigma) for 2 h.

For immunoprecipitation, the mixture was incubated with 30 μL protein-G agarose (Santa Cruz Biotechnology; sc2002) overnight. The agarose beads were recovered by spinning at 1,000 × *g* for 2 min and washed 10 times with 500 μL TES containing 1% Triton X-100 and 0.1% PIC. Bound proteins were released by incubation with TES containing 1% Triton X-100 and 200 μg/mL FLAG peptide (GenScript; RP10586). Eluates were separated

by SDS/PAGE and analyzed by immunoblotting or directly visualized by silver staining (16).

For mass spectrometry, chloroplasts equivalent to 3 mg chlorophyll were incubated with urea-denatured recombinant atTic40–3xFLAG (200 nM) under protein import conditions and subsequently immunoprecipitated with M2 FLAG monoclonal antibody as described above. The immunoprecipitate was resolved by SDS/PAGE and mass spectrometry was performed by the Center for Advanced Proteomics Research at the University of Medicine and Dentistry of New Jersey.

For immunoaffinity chromatography under native conditions, the chloroplasts or total membranes were solubilized in TES buffer containing 1% Triton X-100 and 0.5% PIC at 4 °C for 20 min with constant gentle shaking. The extract was clarified by centrifugation at 18,000 × *g* for 30 min to remove insoluble aggregates. The supernatant was applied to IgG-sepharose from preimmune sera and sequentially to anti-Hsp90C IgG-sepharose, anti-Tic110 IgG-sepharose, or anti-Tic40 IgG-sepharose (1 mL of packed matrix containing 1 mg of bound IgG). The sepharose was washed with 10 volumes of TES buffer containing 1% Triton X-100 and 0.1% PIC and eluted with 0.2 M glycine, pH 2.5–3 containing 1% (wt/vol) Triton X-100. The proteins in each fraction were precipitated with 10% trichloroacetic acid and analyzed by SDS/PAGE and immunoblotting with antisera to the indicated proteins.

In Vitro Translation and in Vitro Import Assays. All [^{35}S]methionine-labeled in vitro translation products were generated in a coupled transcription–translation system containing reticulocyte lysate according to the manufacturer's instructions (Promega) with the addition of RNase inhibitor. The translated proteins were used directly for chloroplast import assays.

Chloroplast import reactions were performed using [^{35}S]methione-labeled preproteins and chloroplasts corresponding to 20 μg of chlorophyll in a total volume of 100 μL of import buffer (330 mM sorbitol, 50 mM Hepes-KOH, pH 7.5, 25 mM KOAc, and 5 mM MgOAc) for 20 min at 26 °C in the presence of 1–5 mM ATP as described previously (17). For import reactions in the presence of inhibitor, chloroplasts were incubated with radicicol at the indicated concentration for 5 min in the dark before adding in vitro translated preproteins. All samples including the control contained a final concentration of 1.4% (vol/vol) ethanol after the addition of radicicol, which was dissolved in 70% of ethanol.

Protease treatments were performed by adding HS buffer containing 100 μg/mL thermolysin and 1 mM $CaCl_2$ on ice for 30 min, followed by quenching with 20 mM EDTA. The chloroplasts were reisolated through 35% Percoll and washed with ice-cold HS buffer containing 10 mM EDTA.

All samples were resolved by SDS/PAGE and analyzed by phosphor imaging (Fuji Fla-5000 phosphorimager). Equivalent amounts of chloroplasts based on chlorophyll content were loaded in all lanes. MultiGauge V2.02 software was used for quantification. Where necessary, radioactivity from in vitro translation products was normalized to reflect differing number of Met residues.

In Vitro Early Import Intermediate and Import Assays with Purified Recombinant Preproteins. For generating early import intermediate with *E. coli* expressed preproteins, chloroplasts were depleted of internal ATP by incubation for 30 min in the dark in the presence of 400 nM nigericin (18). Energy-depleted chloroplasts corresponding to 40 μg of chlorophyll and purified recombinant pre-SSU–3xFLAG–His were incubated in the presence of 0.1 mM GTP and ATP for 5 min at 26 °C as described previously (19). Chloroplasts were recovered by isolation over a 35% Percoll cushion and washed once with HS buffer.

For import of bound early import intermediates, chloroplasts corresponding to 100 μg from the early import intermediate reactions were washed once and resuspended in HS buffer. Chloroplasts were divided into equivalent samples and preprotein import was initiated with the addition of 1 or 5 mM ATP in the presence or absence of radicicol at 26 °C for 20 min. All samples were resolved by SDS/PAGE and analyzed by immunoblotting with anti-FLAG. ImageJ software was used for quantification.

ATPase Assay. The ATPase assay was based on a coupled enzyme assay with an internal ATP regenerating system (20, 21). Each 20-μL assay contained 40 mM Hepes-KOH pH 7.5, 20 mM KCl, 4 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 0.2 mM NADH (Sigma; N4505), 1 mM phosphoenolpyruvic acid (PEP) (Sigma; P7252), 18–28 units/mL lactic dehydrogenase, and 12–20 units/mL pyruvate kinase mix (Sigma; P0294) and 0.8–1.5 μM of purified His-tagged proteins. A total of 0.4 mM NADH was added to give an initial absorbance of 0.28–0.3 at 340 nm before addition of His-M-atHsp90C, and activity was detected as a decrease in absorbance. Inhibition of ATPase activity by radicicol (Sigma; R2146) was achieved by the addition of radicicol (dissolved in 95% ethanol) to a final concentration of 1, 10, and 100 μM. In control experiments, 0.28% ethanol present alone did not affect the measured ATPase activities. All measurements were made on a NanoDrop 2000c (Thermo Scientific).

***Arabidopsis* Transformation.** *Arabidopsis* (*A. thaliana* Col-0 ecotype) T-DNA line, SALK_120525, was obtained from the *Arabidopsis* Biological Resource Center (ABRC) (www.Arabidopsis.org/abrc/). The SALK_120525 insertion allele was detected using primer sets 5 and 6 specific for the T-DNA insertion in *athsp90c-1* (Table S1).

The pEarlygate303:ProAtHSP90C:AtHsp90C construct was introduced into *A. thaliana* (ecotype Col-0) *hsp90c-1* heterozygous plants by the floral dip method using *Agrobacterium tumefaciens* strain GV3101 (22). For segregation analysis, plants grown on soil were sprayed with a 100 mg/L solution of Basta. Homozygous *hsp90-1/hsp90-1* plants expressing the ProAtHSP90C:AtHsp90C transgene were genotyped using PCR with primer sets for atHsp90C endogenous gene (primers 7 and 8) and cDNA (primers 9 and 10), respectively (Table S1).

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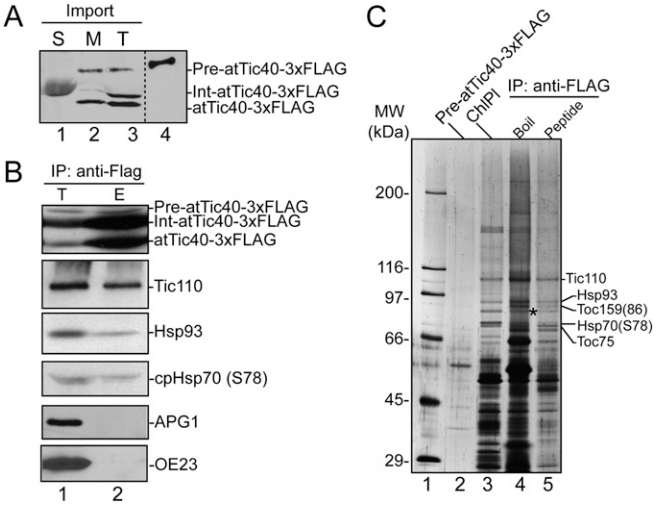

Fig. S1. Identification of proteins associated with preatTic40–3xFLAG protein import intermediates. (*A*) Urea-denatured preatTic40–3xFLAG (200 nM) was imported into isolated pea chloroplasts for 30 min under standard conditions. After the import reaction, the chloroplasts (lane 1, T) were lysed by suspension in HM buffer (25 mM Hepes-KOH pH 7.5, 2 mM MgCl2) and separated into membrane (lane 2, M) and soluble (lane 3, S) fractions. Equivalent samples of each fraction (5 μg chlorophyll) were analyzed by SDS/PAGE and immunoblotting with M2 FLAG antibody. Lane 4 contains 10% of the preatTic40–3xFLAG added to the import reaction. The positions of the precursor form (preatTic40–3xFLAG), the intermediate processed form (Int-atTic40–3xFLAG), and mature form (at-Tic40–3xFLAG) of atTic40–3xFLAG are shown (*Right*). (*B*) Immunoprecipitation of preatTic40–3xFLAG protein import intermediates from detergent-solubilized chloroplasts. Chloroplasts from a 30-min preatTic40–3xFLAG import reaction were treated with DSP. Chloroplasts were dissolved in TES buffer containing 1% Triton X-100 and immunoprecipitated with M2 FLAG antibody. Sample of the total chloroplast extract (lane 1, T) corresponding to 5 μg chlorophyll and the immunoprecipitate from chloroplasts corresponding to 100 μg chlorophyll (lane 2, E) were resolved by SDS/PAGE and immunoblotted with antibodies toward the proteins indicated (*Right*). (*C*) Protein profile of immunoprecipitate of preatTic40–3xFLAG protein import intermediates used for mass spectrometry. Chloroplasts from a 30-min preatTic40–3xFLAG import reaction (*A*) were immunoprecipitated with M2 anti-FLAG antibody. Immunoprecipitate (IP: anti-FLAG) was eluted sequentially with FLAG peptide (peptide) or boiling SDS/PAGE sample buffer (boil). Sample of preatTic40–3xFLAG (lane 2, 0.1 ng) and a sample of chloroplasts from the import reaction (ChlPI, lane 3, 0.2 μg chlorophyll) are also shown. Positions of proteins confirmed by mass spectrometry and/or immunoblotting are indicated at *Right*. Asterisk indicates the band identified as Hsp90C by mass spectrometry.

Peptide information

1	RITPLLR
2	FWENFGR
3	FGWSANMER
4	YEFAESTRIK
5	ILEINPDHPIIK
6	VFISDDFDGELFPR
7	EELIDCLGTIAQSGTSK

AtHsp90C; Arabidopsis thaliana Chloroplast Heat Shock Protein 90 (At2g04030)

MAPALSRSLYTSPLTSVPITPVSSRLSHLRSSFLPHGGALRTGVSCSWNLEKRCNRFA
VK^CDAAVAEKETTEEGSGEKFEYQAEVSRLLDLIVHSLYSHKEVFLRELVSNASDALD
KLRFLSVTEPSLLGDGGDLEIRIKPDPDNGTITITDTGIGMTKEELIDCLGTIAQSGTSKFL
KALKENKDLGADNGLIGQFGVGFYSAFLVAEKVVVSTKSPKSDKQYVWESVADSSSYL
IREETDPDNILRRGTQITLYLREDDKYEFAESTRIKNLVKNYSQFVGFPIYTWQEKSRTIE
VEEDEPVKEGEEGEPKKKKTTKTEKYWDWELANETKPLWMRNSKEVEKGEYNEFYK
KAFNEFLDPLAHTHFTTEGEVEFRSILYIPGMGPLNNEDVTNPKTKNIRLYVKRVFISDD
FDGELFPRYLSFVKGVVDSDDLPLNVSREILQESRIVRIMRKRLIRKTFDMIQEISESENK
EDYKKFWENFGRFLKLGCIEDTGNHKRITPLLRFFSSKNEEELTSLDDYIENMGENQKA
IYYLATDSLKSAKSAPFLEKLIQKDIEVLYLVEPIDEVAIQNLQTYKEKKFVDISKEDLELG
DEDEVKDREAKQEFNLLCDWIKQQLGDKVAKVQVSNRLSSSPCVLVSGKFGWSANM
ERLMKAQALGDTSSLEFMRGRRILEINPDHPIIKDLNAACKNAPESTEATRVVDLLYDTA
IISSGFTPDSPAELGNKIYEMMAMAVGGRWGRVEEEEESSTVNEGDDKSGETEVVEP
SEVRAESDPWQD

Fig. S2. Identification of Hsp90C by mass spectrometry. Predicted amino acid sequence of peptides corresponding to *Arabidopsis thaliana* Hsp90C (AT2g04030) obtained by MALDI TOF/TOF mass spectrometry. Predicted amino acid sequence of *A. thaliana* Hsp90C (AT2g04030) with the positions of peptides obtained from mass spectrometry of the 83-kDa protein band from the preatTic40–3xFLAG immunoprecipitate (Fig. S1*C*) indicated in gray.

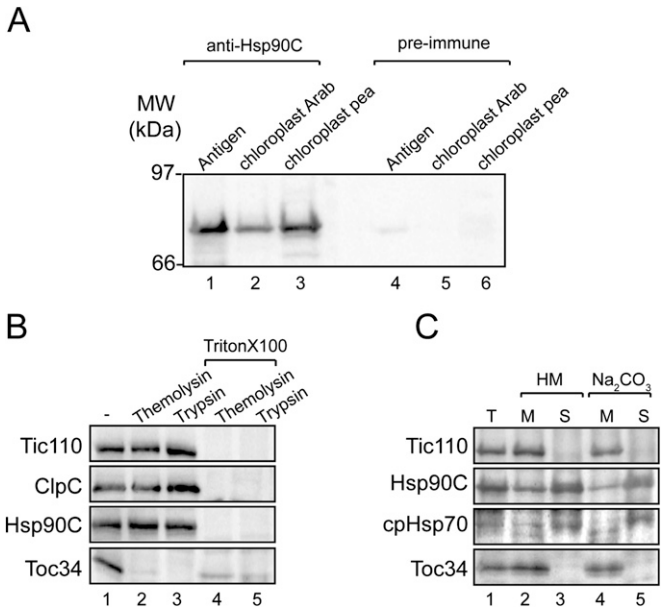

Fig. S3. Characterization of the atHsp90C antibodies. (*A*) *Arabidopsis* Hsp90C antibodies (anti-Hsp90C) or preimmune serum (preimmune) were used to immunoblot total chloroplast extracts (5 μg of chlorophyll) from pea (lanes 3 and 6) or *Arabidopsis* (lanes 2 and 5). A total of 0.05 μg of the *E. coli* expressed *Arabidopsis* Hsp90C antigen was loaded in lanes 1 and 3. (*B*) Hsp90C is localized to the chloroplast stroma. Intact pea chloroplasts were treated with 100 μg/mL thermolysin or 100 μg/mL trypsin in the presence or absence of 1% Triton X-100 as indicated. Reactions were incubated on ice for 30 min, and proteolysis was stopped with 1 mM PMSF, 0.05 mg/mL Nα-Tosyl-L-lysine chloromethyl ketone (TLCK), 0.1 mg/mL soybean trypsin inhibitor, and 2 μg/mL aprotinin (trypsin) (23) or 20 mM EDTA (thermolysin). Chloroplasts were analyzed by immunoblotting with antibodies against various proteins as indicated (*Left*). (*C*) Major fraction of Hsp90C is soluble, with a minor fraction peripherally associated with chloroplast membranes. Chloroplasts (lane 1; T) were lysed by suspension in HM buffer or 0.2 M Na_2CO_3, pH 11.5, and incubated on ice for 30 min as indicated. Membrane (lanes 2 and 4; M) and soluble (lanes 3 and 5; S) were separated by centrifugation at 18,000 × *g* for 1 h at 4 °C (2). Soluble fractions were removed and concentrated by precipitation in 20% trichloroacetic acid. An equivalent sample of each fraction (5 μg chlorophyll) was analyzed by SDS/PAGE and immunoblotting with antibodies against various proteins as indicated (*Left*).

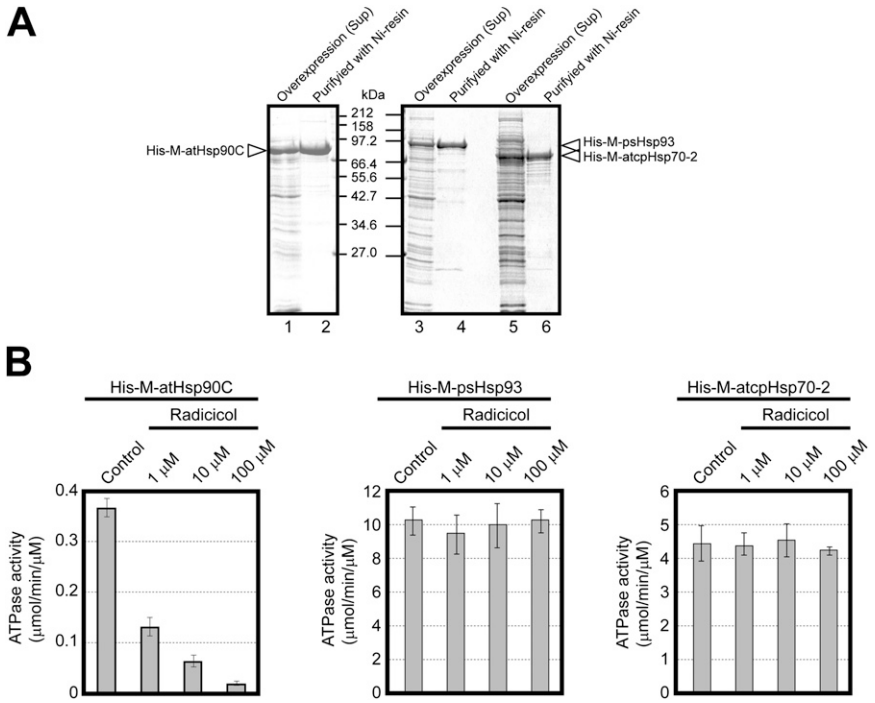

Fig. S4. Effect of radicicol on the ATPase activity of purified atHsp90C, psHsp93, and atcpHsp70-2. (*A*) His-M-Hsp90C, His-M-psHsp93, and His-M-cpHsp70-2 were expressed in *E. coli* (lane 1, 3, and 5) and purified by Ni-NTA chromatography (lane 2, 4, and 6). Sup, supernatant fraction after overexpression. (*B*) Inhibition of His-mHsp90C's ATPase activity by increasing concentrations of radicicol. 0.8–1.5 μM proteins were incubated with 2 mM ATP in the presence of the indicated concentrations of radicicol. ATPase assay was performed based on a regenerating coupled enzyme assay described in *SI Materials and Methods*. Each data bar represents the mean ± SEM ($n = 3$).

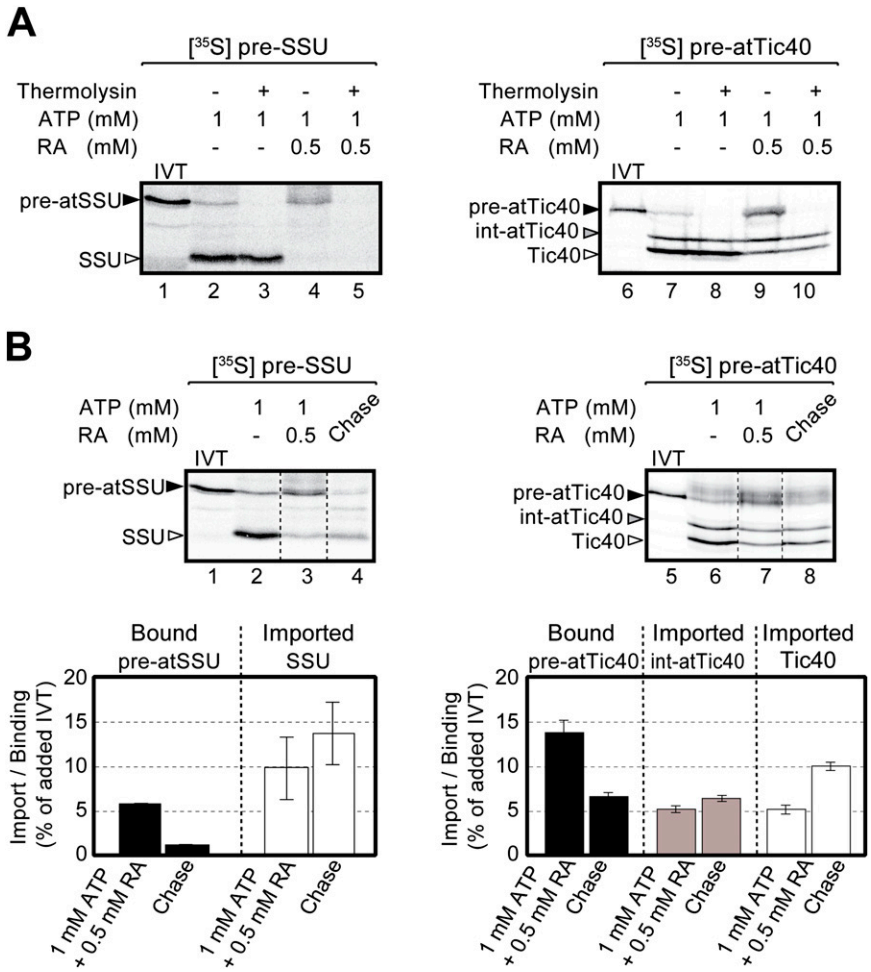

Fig. S5. The protease-sensitivity of pre-SSU and preatTic40 bound to chloroplasts in the presence of radicicol and ATP is consistent with early import intermediates. (*A*) In vitro translated [^{35}S] preatSSU (lanes 1–5) or [^{35}S] preatTic40 (lanes 6–10) were incubated with pea chloroplasts and 1 mM ATP at 26 °C for 20 min in the presence or absence of 0.5 mM radicicol as indicated. After the import reaction, equivalent chloroplast samples were treated in the presence or absence of 100 μg/mL thermolysin on ice for 30 min as indicated. Protease treatments were terminated by the addition of 20 mM EDTA and chloroplasts were resolved by SDS/PAGE and analyzed by phosphor imaging. Lanes 1 and 6 contain 10% of the [^{35}S] preproteins added to each reaction (IVT). (*B*) Isolated pea chloroplasts were incubated with in vitro translated [^{35}S] preatSSU (lanes 2–4) or [^{35}S] preatTic40 (lanes 6–8) and 1 mM ATP at 26 °C for 20 min (lanes 2, 3, 6, and 7) in the presence or absence of 0.5 mM of radicicol as indicated. In lanes 4 and 8, an equivalent sample of chloroplasts to lanes 3 and 7 were washed to remove radicicol and incubated in the presence of 5 mM ATP to promote protein import, respectively (chase). Lanes 1 and 5 contain 10% of the [^{35}S] preprotein added to each reaction (IVT).

Table S1. List of primers

Primers used for making construct for transgenenic plant by using the Gateway system	
Primer 1	CACCATGGAACCATTAGCTTAGG
Primer 2	CTCGAGGAATTCGGTGCCGTTATCAGG
Primer 3	CATGCCATGGCTCCTGCTTTGAGTAG
Primer 4	CCGCTCGAGTCAATCTTGCCAAGGATCACTCT
Primer sets for PCR analysis of genomic DNA from plants	
T-DNA insertion in *athsp90c-1*	
Primer 5	GGTTGTTGTGTCCACCAAAAG
Primer 6	GCGTGGACCGCTTGCTGCAACT
atHsp90C endogenous gene	
Primer 7	AGTGTCTTCTGGTTAATGTATTTC
Primer 8	CATTTGTATCACTAACAGTTGC
atHsp90C cDNA	
Primer 9	CAAGTCTGCCCCTTTCTTGG
Primer 10	AGGCGTCTCGCATATCTCATTA

Underlines indicate the sequence for TOPO cloning in primer 1, restriction enzyme sequence in primer 2, and start codon of atHsp90C in primer 3, respectively.