CIHR Canadian Institutes of Health Research
IRSC Instituts de recherche en santé du Canada
Submitted by CIHR
Déposé par les IRSC

Biochem J. Author manuscript; available in PMC 2016 September 16.

Published in final edited form as:
Biochem J. 2011 August 15; 438(1): 143–153. doi:10.1042/BJ20110448.

OEP61 is a chaperone receptor at the plastid outer envelope

Ottilie vonLoeffelholz[*,1], **Verena Kriechbaumer**[*,1], **Richard A. Ewan**[†], **Rafal Jonczyk**[*], **Susann Lehmann**[*], **Jason C. Young**[‡], and **Ben M. Abell**[*,2]

[*]Biomedical Research Centre, Sheffield Hallam University, Sheffield S1 1WB, U.K

[†]Faculty of Life Sciences, University of Manchester, Manchester M13 9PL, U.K

[‡]Department of Biochemistry, McGill University, Montreal H3G 0B1, Canada

Abstract

Chloroplast precursor proteins encoded in the nucleus depend on their targeting sequences for delivery to chloroplasts. There exist different routes to the chloroplast outer envelope, but a common theme is the involvement of molecular chaperones. Hsp90 (heat-shock protein 90) delivers precursors via its receptor Toc64, which transfers precursors to the core translocase in the outer envelope. In the present paper, we identify an uncharacterized protein in *Arabidopsis thaliana* OEP61 which shares common features with Toc64, and potentially provides an alternative route to the chloroplasts. Sequence analysis indicates that OEP61 possesses a clamp-type TPR (tetratricopeptide repeat) domain capable of binding molecular chaperones, and a C-terminal TMD (transmembrane domain). Phylogenetic comparisons show sequence similarities between the TPR domain of OEP61 and those of the Toc64 family. Expression of mRNA and protein was detected in all plant tissues, and localization at the chloroplast outer envelope was demonstrated by a combination of microscopy and *in vitro* import assays. Binding assays show that OEP61 interacts specifically with Hsp70 (heat-shock protein 70) via its TPR clamp domain. Furthermore, OEP61 selectively recognizes chloroplast precursors via their targeting sequences, and a soluble form of OEP61 inhibits chloroplast targeting. We therefore propose that OEP61 is a novel chaperone receptor at the chloroplast outer envelope, mediating Hsp70-dependent protein targeting to chloroplasts.

Keywords

chaperone receptor; heat-shock protein 70 (Hsp70); OEP61; plastid; protein targeting; tail-anchored (TA) protein

[2]To whom correspondence should be addressed (B.Abell@shu.ac.uk).
[1]These authors contributed equally to this work.

AUTHOR CONTRIBUTION

Ottilie von Loeffelholz, Verena Kriechbaumer, Rafal Jonczyk and Susann Lehmann conducted the experiments. Richard Ewans conducted the initial bioinformatics analysis to identify OEP61. Ottilie von Loeffelholz, Verena Kriechbaumer, Jason Young and Ben Abell were involved in the experimental design and data analysis. Ottilie von Loeffelholz, Verena Kriechbaumer, Jason Young and Ben Abell were involved in preparing the manuscript prior to submission.

CIHR Author Manuscript
CIHR Author Manuscript
CIHR Author Manuscript

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

INTRODUCTION

Organelles require proteins that are encoded by nuclear DNA, necessitating protein delivery from the cytosol. Generally, this targeting process is promoted by cytosolic factors, which increase specificity and efficiency, and the best understood processes are initiated by N-terminal targeting signals on the precursor protein [1]. Targeting signals can also be provided by internal TMDs (transmembrane domains), which are more hydrophobic than N-terminal targeting sequences. Precursor proteins with either type of targeting signal can be bound by molecular chaperones to promote their delivery to the organellar membranes.

Although the Hsp (heat-shock protein) 70 and Hsp90 chaperones assist protein folding, they can also work with other cytosolic factors to promote organellar targeting. A general mechanism of the chaperones is to protect hydrophobic parts of precursors so that they are maintained in an unfolded targeting compatible state. Hsp70 is widely involved in targeting processes, and it often functions as part of a complex. For example, the targeting of chloroplast proteins bearing an N-terminal targeting sequence is promoted by Hsp70 in combination with a 14-3-3 protein, which is known as the guidance complex [2]. Other proteins bound for the chloroplast are assisted by Hsp90 [3], or by AKR2A (*Arabidopsis* ankyrin-repeat protein) [4,5]. Similar targeting mechanisms exist for mitochondrial protein targeting, which in the case of inner membrane proteins is promoted by Hsp70 and Hsp90 [6–8], and the Hsp70-regulatory cochaperone Hsp40 [9]. Disruption of Hsp70 or Hsp90 activity by specific inhibitors or mutation of a chaperone-binding site reduces targeting of the precursors to mitochondria. Also, the mitochondrial targeting of some matrix proteins are assisted by the peptidyl-prolyl isomerase AIP (aryl hydrocarbon receptor-interacting protein)/XAP2 (X-associated protein 2) [10]. Although many proteins destined for the ER (endoplasmic reticulum) membrane are targeted cotranslationally by SRPs (signal recognition particles; reviewed in [11]), post-translational targeting of TA (tail-anchored) membrane proteins can be mediated by Hsp70 and Hsp40 [12,13], as well as the ASNA1/TRC40 (transmembrane domain recognition complex of 40 kDa)/Get3 (guided entry of TA proteins 3) targeting factor (reviewed in [14]). The post-translational targeting of some yeast ER proteins is also promoted by Hsp70 and Hsp40 [15].

Chaperones are able to deliver precursors to organelles via membrane-bound chaperone receptors. These receptors interact with chaperones via their ‘TPR clamp’ (TPR is tetratricopeptide repeat) domain, comprising three TPR motifs to form a peptide-binding groove of seven α-helices [16]. Specific interaction occurs with the highly conserved C-terminal ends of Hsp70 or Hsp90, which contain carboxyl groups capable of forming ionic bonds with the amino groups within the TPR domain [16,17]. Binding of Hsp70 requires at least a heptapeptide (PTIEEVD), whereas the last five amino acids (MEEVD) of Hsp90 are essential and sufficient for binding [16–18]

At least one TPR-containing receptor can be found at each organelle [19], suggesting a universal role for chaperone receptors in protein targeting. The most prominent examples for chaperone receptors are Tom70 (translocase of the mitochondrial outer membrane 70) at human and yeast mitochondria [7], and Toc64 at plant chloroplasts [3]. Both receptors are proposed to mediate early recognition of chaperone-bound precursor proteins via their TPR

CIHR Author Manuscript
CIHR Author Manuscript
CIHR Author Manuscript

domain. Targeting information is recognized by a separate domain on Tom70 before transfer of the precursor complex to the translocase [7] or, in the case of Toc64, by other receptors that are in closer proximity to the translocase [3]. When Tom70 is knocked out in combination with another mitochondrial receptor Tom20, yeast cells are not viable, even though neither single knockout shows a lethal phenotypic defect [20]. This indicates a functional overlap between Tom70 and Tom20, and may explain the difficulties in confirming a phenotypic effect of Toc64 knockout in *Arabidopsis thaliana* [3,21,22]. In support of this notion, Toc64 was found to interact only with precursors bound to Hsp90 and not to bind the guidance complex containing Hsp70 [3]. Therefore other chaperone receptors may exist at the chloroplast to accept precursors bound to Hsp70.

To identify other potential chaperone receptors we performed a database search for proteins containing a clamp-type TPR domain and a TMD. This resulted in the identification of an uncharacterized protein in *A. thaliana* termed OEP61, which has a TPR clamp domain at its N-terminus and a single TMD at its C-terminus. We show that OEP61 is expressed throughout the mature plant, and localizes to the outer envelope of chloroplasts. OEP61 specifically binds Hsp70, and can selectively recognize precursors destined for the chloroplast. Furthermore, the soluble portion of OEP61 is able to inhibit the chloroplast targeting of precursors. Therefore we propose that OEP61 is a novel chaperone receptor involved in the targeting of chloroplast precursors from the cytosol.

EXPERIMENTAL

Identification of OEP61

Alignment of known TPR clamp domains was used to generate semi-stringent motifs consisting of [K-(ETNDK)-(KQEIR)-(GA)-(NT)-(DEVKT)-(AYFCL)-(YF)] for clamp 1 and [K-(AG)-(YFL)-(YFT)-R-(KR)-(GA)-(AEQK)] for clamp 2, and loose motifs consisting of [(KR)-(ETNDKALQGD)-(LKQEIHSA)-(GA)-(NKT)-(DAELSVNHQKT)-(ACFYLKHQMS)-(YFLV)] for clamp 1 and [K (AGVC)-(YFL)-(AYFTSN)-(RQ)-(IKRQL)-(GAS)-(NATEQKLCS)] for clamp 2. These motifs were used to scan the protein databases Swiss-Prot, TrEMBL and TrEMBL new [23].

DNA constructs

The coding sequence of *A. thaliana* OEP61 (clone pda11784 from RIKEN) was inserted into the pSPUTK construct (Stratagene). Sequences encoding truncated versions of *A. thaliana* OEP61 were cloned into the pET-16b contstruct (Novagen) after amplification by PCR using the following primers: 5′-ATATCTCGAGTTTAACGGGTTAATGGATCC-3′ and 5′-ATATAGATCTTTATTTTCCGAACAACCACTTC-3′ for OEP61-TM (amino acids 1–534); 5′-ATATAGATCTTTATTTTCCGAACAACCACTTC-3′ and 5′-ATTAAACATATGGAGACAATTGCCGATGTG-3′ for L (amino acids 214–534); and 5′-ATTAAACTATGTATCAGATCAATGCAGCTC-3′ and 5′-TTAAATTGGATCCTATGCCTTGCCAGGTCC-3′ for TPR (amino acids 103–213). The R185A mutation was introduced into OEP61-TM by PCR corresponding to the mutation used in Tom70 (R192A for human Tom70 and R171A for yeast Tom70; [7]) using the following primers: 5′-

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

GGAATGTCAAAGCCCTATACGCAAGGGGTCAAGCTTACAGA-3′ and 5′-TCTGTAAGCTTGACCCCTTGCGTATAGGGCTTTGACATTCC-3′. The clone for TPR1 of HopTPR1 (human Hop) is described in [7]. Plasmid DNA for standard curves in qRT-PCR (quantitative real-time PCR) of OEP61 was inserted into the pSPUTK construct with the following primers: 5′-ATATAGATCTACCATGTTTAACGGGTTAATG-3′ and 5′-ATATAGATCTCTAGTTTCCAATATAGCC-3′, and plasmid DNA for the endogenous control was donated by Dr Erich Glawischnig (Technische Universität München, Germany) [24]. Plasmids for the pull down of precursor proteins were obtained from TAIR (http://www.arabidopsis.org/), which was also used to assign subcellular localization. A plasmid for Lhcb1 (clone AB80 from pea; [25]) was kindly donated by Professor Colin Robinson (University of Warwick, Coventry, U.K.). For the removal of targeting sequences, PCR templates were generated to delete residues 268–297 of Toc33, and to delete residues 1–54 of pSSU.

Plant material

A. thaliana Col-0 (Nottingham *Arabidopsis* Stock Centre, Nottingham, U.K.) plants were grown on soil with 14 h light of 100 μmol/m^{-2} per s at 21°C with 60% humidity. Adult plant tissue was harvested after 8 weeks.

Transient expression system

Four-week-old tobacco plants (*Nicotiana tabacum* SR1 cv Petit Havana) were inoculated via their lower epidermal surface with *Agrobacterium* strain GV3101 (pMP90) bearing an expression vector, using a method described previously [26].

Confocal imaging

A Zeiss LSM 510 laser-scanning microscope was used with a 63× oil-immersion objective (Zeiss), as described previously [27]. ImageJ (http://rsbweb.nih.gov/ij/) software was used to test for co-localization. Pixels of the red and green channels of 8-bit images are classed as co-localized if their intensities are higher than the threshold of their channels (default settings 50) and if the ratio of their intensity is higher than the ratio setting value (default settings 50%).

qRT-PCR

Total RNA was extracted from 50–100 mg of plant tissue using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. After treatment with DNAse (New England Biolabs), cDNA was synthesized using random primers (Invitrogen) and M-MuLV Reverse Transcriptase (New England Biolabs) according to the manufacturer's instructions. qRT-PCR was performed with the standard curve experiment on the StepOne Real-Time PCR System (Applied Biosystems) using Sybr Green PCR master mix and the following intron spanning primer sets for OEP61 and actin: 5′-CTGGAAAGTTC-TGATTGCTTC-3′ and 5′-CATCAAGAGGTGTGGTGATTG-3′ for OEP61; and 5′-TGGAACTGGAATGGTTAAGGCTGG-3′ and 5′-TCTCCAGAGTCGAGCACAATACCG-3′ for actin. PCR products were analysed on

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

agarose gels after amplification, and their sequences determined to confirm the products. Quantifications of OEP61 mRNA in different tissues were calculated in relation to actin.

Immunoblotting

Total protein was extracted from plant tissue by grinding frozen plant material in extraction buffer [50 mM Tris/HCl (pH 8.0), 100 mM KOAc (potassium acetate), 1 mM EDTA, 1 mM DTT (dithiothreitol), 20% glycerol, 1% Triton X-100, 1% SDS and 1% plant protease inhibitor (Sigma)]. The insoluble parts were separated from the extract by centrifugation (1600 ***g*** at 4°C for 10 min). Protein concentrations were measured with detergent-compatible Bradford Ultra (Expedeon) reagent using BSA as a standard, and equal amounts of total protein were used for immunoprecipitation. Prior to immunoblotting, OEP61 was immunoprecipitated from total protein extracts. Samples were precleared by adding four volumes of TXIP buffer [10 mM Tris/HCl (pH 7.5), 140 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 2 mM PMSF] and 0.1 vol pansorbin (Calbiochem) and incubated at 4°C for 30 min. The mixture was centrifuged at 14 300 ***g*** for 10 min at 4°C to pellet the pansorbin, and the supernatant was incubated with anti-OEP61 IgG at a 1:1000 dilution, followed by overnight incubation at 4°C. Protein A–Sepharose beads (1:100 dilution; Sigma) were added and incubated for 1.5 h at 4°C. The beads were washed four times with 1 ml of TXIP buffer, denaturing loading buffer was added and the eluted proteins were separated by SDS/PAGE (12% gel). For immunoblotting, the proteins were transferred from SDS/PAGE gels on to PVDF membranes (Millipore) according to the manufacturer's instructions. Primary antibodies were used at a 1:10 000 dilution for the anti-(human Hsp70) IgG (Stratagene) and a 1:1000 dilution for the anti-OEP61 IgG serum (Eurogentec). Anti-OEP61 has been optimized for buffer pH and dilution and evaluated against recombinant Toc64, OEP61, BSA protein and pre-immune serum, as well as small, large and final bleed were tested in pull-down experiments with radiolabelled OEP61 (Supplementary Figure S2 at http://www.BiochemJ.org/bj/438/bj4380143add.htm). The secondary goat anti-rabbit IgG labelled with red-fluorescent Alexa Fluor® 594 dye (Invitrogen) or green-fluorescent IRDye 800CW respectively was used at a dilution of 1:3000 and signals were detected using the ODYSSEY Infrared imaging system (LI-COR Biosciences).

For immunoblotting of OEP61 in *A. thaliana*, Col-0 plants were used to prepare chloroplasts as described for pea plants used in competitive targeting assays [27]. Thermolysin treatment was performed with half of the chloroplasts using 40 units/ml thermolysin for 5 min at 30°C. Chloroplast fractions (30 μl) with and without thermolysin treatment and the supernatant fraction from the initial chloroplast fractionation were immunoblotted using anti-OEP61 IgG.

Transcription and translation

Proteins of interest were fused to the pSPUTK SP6-promoter via overlapping extension PCR [28]. Transcriptions were performed with 15 μg of PCR fusion product and SP6-RNA polymerase (New England Biolabs) according to the manufacturer's instructions. Protein translations were performed in WGE (wheat germ extract; Promega) according to the manufacturer's instructions using Easy Tag Express 35S (PerkinElmer).

CIHR Author Manuscript

CIHR Author Manuscript

CIHR Author Manuscript

Binding experiments

Proteins were expressed in T7 Express *I[q] Escherichia coli* cells (New England Biolabs), and purified by chromatography on Ni-NTA (Ni^{2+}-nitriloacetate) agarose (Promega). Pull-down experiments with recombinant protein or *in vitro* translated protein, were performed as described previously [7] with the following changes: 1 μM His-tagged protein was bound in buffer CG [100 mM KOAc, 20 mM Hepes/KOH (pH 7.5) and 5 mM $MgOAc_2$ (magnesium acetate)] containing 2 mg/ml ovalbumin and 0.1% Triton X-100. Incubation of the matrix-bound recombinant proteins occurred in buffer CG containing 2 mg/ml ovalbumin, 0.1% Triton X-100 and 25% WGE. The decapeptide GAGPKIEEVD mimicking the C-terminus of plant Hsp70 was used as a competitive inhibitor at a final concentration of 500 μM. Buffer CG containing only 0.1% Triton X-100 was used for washing steps. Radiolabelled precursor proteins were synthesised by *in vitro* translation in WGE (Promega), treated with 1 unit/ml apyrase (New England Biolabs) for 5 min on ice and subjected to the same binding assay.

Competitive targeting assay

The competitive targeting assay for OEP61 was performed according to a method described in [27]. Briefly, chloroplasts from pea leaves and mitochondria from maize coleoptiles were incubated with prespun *in vitro* translated protein and incubated at 30°C for 20 min. Organelles were repurified by two successive centrifugation steps (3000 ***g*** at 4°C for 2 min then 3000 ***g*** at 4°C for 20 min) and a wash with 0.1 M Na_2CO_3. Both fractions, and an import sample treated with 40 units/ml thermolysin for 5 min at 30°C, were analysed by SDS/PAGE (12% gel) and Cyclon Phosphor Screen (Packard). ER import was performed co-translationally for 15 min with 50 A_{280} units/ml dog microsomes. Microsomes were pelleted in a TLA100.2 fixed angle rotor (Beckman) and 70 000 rev./min for 45 min and proteins were visualized by SDS/PAGE (12% gel) and Cyclon Phosphor Screen (Packard). For inhibition experiments, 6 μg of OEP61-TM, PEX19 or HopTPR1 were included in the import incubation.

Phylogenetic analysis

The protein sequence of the TPR domain from OEP61 was compared with proteins in public databases in three ways: (i) using BLAST against a non-redundant protein database; (ii) using BLAST against the non-redundant protein database with the organisms restricted to *A. thaliana*; and (iii) compared with TPR domains from *A. thaliana* homologues of known chaperone receptors and Hop (Sti1). Homologues of *A. thaliana* chaperone-binding TPR domains were manually selected using PROSITE (http://www.expasy.org/prosite/). Similar sequences to the TPR domain of OEP61 were identified using BLAST. Duplicates were omitted and protein sequences were aligned using the ClustalW program (Align-m; http://www.ebi.ac.uk/Tools/msa/clustalw2/) using default settings changing the output format setting to PHYLIP (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Unrooted trees were generated with PHYLIP on the Web (http://bioweb2.pasteur.fr/phylogeny/intro-en.html) using the programs protdist, seqboot (included in protdist, using 100 replicates), neighbour, consense (included in neighbour, using 100 replicates) and drawtree. Bootstrap values and names were inserted manually.

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

Accession numbers

Locus identifiers and ABRC (*Arabidopsis* Biological Resource Center) clone numbers are given in brackets: OEP61 (At5g21990); Toc33 (At1g02280, 190I17); Toc34 (At5g05000, 167B21); Tic22 (At4g33350, 144A17); At2g17 (At2g17972.1, U50887); ANT-hj (ANT homologue); At5g56450, 285A11); Tom22-I (At1g04070, 136E14); Tim9 (At3g46560, 135L1); Tim10 (At2g29530, U23564); At5g013 (At5g01340, M73L24, mitochondrial substrate carrier); pSSU (At1g67090, U13397, ribulose bisphosphate carboxylase small chain 1A); and Lhcb1 (clone AB80 from pea).

RESULTS

Search for chaperone receptors

To identify potential chaperone receptors a sequence search was designed using the consensus sequence from characterized TPR clamp proteins. Structural alignment was performed for the TPR clamp region of proteins Hop, FKBP (FK506-binding protein) 5, FKBP4, cyclophilin-40, serine/threonine phosphatase 5, cyclophilin seven suppressor, Tom70, Tom34 and Unc-45. This alignment produced degenerate motifs for the two clamp motifs that are required to support chaperone binding, and these motifs were used to scan the protein databases Swiss-Prot, TrEMBL and TrEMBL new [23]; this resulted in the identification of nine novel proteins. For such a protein to act as a receptor, it would also require anchorage to a membrane, so the potential for at least one TMD was used to refine the search. A single uncharacterized protein in *A. thaliana* was found to contain both a TPR clamp domain and a predicted TMD, which we have named OEP61. Figure 1 shows a scheme of the OEP61 structure and the alignment of its TPR domain with other TPR clamp proteins used to generate the consensus sequence. OEP61 homologues can be identified in plants with available sequence data including rice and algae, but homologues could not be identified in other types of organisms. There are high sequence similarities between these homologues in the TPR domain and the C-terminus of the proteins, although the 'linker region' between the TPR clamp and the TMD is much more variable (Supplementary Figure S1 at http://www.BiochemJ.org/bj/438/bj4380143add.htm). This indicates that the function and localization elements are conserved and that OEP61 has the potential to act as a chaperone receptor in plant cells. In line with our classification of OEP61, an independent bioinformatics approach to detect proteins with TPR clamp domains also detects OEP61 (termed AtTPR7) as part of a group of 24 uncharacterized predicted proteins in *A. thaliana* [29].

Phylogenetic analysis of OEP61

To gain insight into the potential function of OEP61, relationships between the TPR domain of OEP61 and other TPR clamp proteins were determined by phylogenetic comparisons of protein sequences. Initially, TPR domains from different organisms were investigated by using BLAST to find similar sequences. The resulting tree contains four functionally distinct families: the TPR domains of PPIases (peptidyl-prolyl *cis–trans*-isomerases), SMAP-1 (stromal membrane-associated protein 1) (Unc-45), Tom34 and unknown protein products (Supplementary Figure S2A). Tom70 and Hop are more distantly related, and hence are not included. The PPIase group is the largest group in the tree and includes cyclophilins and

CIHR Author Manuscript

CIHR Author Manuscript

CIHR Author Manuscript

FKBPs, of which FKBP52, FKBP38 and cyclophillin40 are known to bind Hsp90 [30]. Hsp90 binding has also been shown for the C-terminal TPR domain of Tom34 [31], which is a chaperone receptor at the mitochondrial outer membrane in deuterostomia [19,32]. OEP61 is in a branch together with unknown plant proteins, which show high sequence similarities to the full sequence of OEP61, indicating that OEP61 is part of a novel uncharacterized protein family in plants.

When the analysis was restricted to relationships between OEP61 and TPR clamp proteins in *A. thaliana* a similar pattern emerges (Supplementary Figure S2B). Four families are represented in the tree: Toc64, Hsp70-interacting proteins, TPR domain-containing DnaJs and PPIases. OEP61 is most similar to the TPR domains of PPIases, but is not located inside this group, suggesting that OEP61 is not directly related to any characterized TPR clamp proteins.

To focus on the relationships of the TPR domain, sequences from *A. thaliana* homologues of known chaperone receptors were collected manually and used for phylogenetic analysis (Supplementary Figure S2C). The resulting phylogenetic tree divides the TPR domains into four distinct groups: the three different TPR domains of Hop build a branch each and the fourth branch is built by the TPR domains of three different Toc64 homologues, Toc64-III, Toc64-V and an *A. thaliana* homologue of the human fatty acid amide hydrolase (F13N6) [33], and MGLN6 a fourth uncharacterized protein, which contains a TPR domain with high similarity to the TPR domain of Toc64. OEP61 is part of the Toc64 branch, showing that it is more similar to Toc64 than to any of the Hop TPR domains, although the TPR domains of Toc64 [3] and its mitochondrial counterpart, mtOM64 [34], are more closely related to each other than to the TPR domain of OEP61.

OEP61 is expressed in all tissues

To test the distribution of OEP61 in mature *A. thaliana* plants, the expression levels of OEP61 in different tissues were measured using qRT-PCR and immunoblotting (Figure 2). OEP61 mRNA could be detected in all tissues, indicating that OEP61 is ubiquitously expressed, although levels of OEP61 varied from 25% to 190% compared with actin (Figure 2A). The highest transcription levels were detected in leaf tissues, and the lowest in roots. These results differ slightly from the expression pattern derived for Toc64, which has similar mRNA levels in buds, rosette leaves and roots [21].

Since regulation at the post-transcriptional level may occur (see [35] for an example), immunoblots using an antibody raised and tested (Supplementary Figure S3 at http://www.BiochemJ.org/bj/438/bj4380143add.htm) against recombinant OEP61 were performed (Figure 2B). To reduce interference from abundant proteins such as Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase), OEP61 protein was immunoprecipitated prior to immunoblotting. OEP61 was detected in all tissues tested, suggesting a general role for OEP61. Higher OEP61 protein levels were found in green tissue including the leaves and buds, which correlates with the transcript analysis. The highest protein level was detected in young leaves, suggesting that OEP61 plays an important role during leaf growth.

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

OEP61 is localized at the chloroplast outer envelope

The restriction of OEP61 homologues to plants suggested that OEP61 has a plant specific function and may be localized to the plastids. According to sequence analysis OEP61 lacks an N-terminal cleavable signal peptide (ChloroP: no chloroplast transit peptide detected, score = 0.457, http://www.cbs.dtu.dk/services/ChloroP/; SignalP-HMM signal peptide probability = 0.00, http://www.cbs.dtu.dk/services/SignalP/) and has a single TMD of 22 amino acids at the C-terminus predicted by TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) [36]. These features define OEP61 as a TA protein, a class of membrane proteins that depend on their TMD and flanking sequences for targeting and membrane insertion [37]. TA proteins are localized to most internal membranes of cells, including ER, mitochondria and chloroplasts. It is possible to predict localization in plant cells from the sequence of the tail anchor, although this is not entirely reliable, and is particularly difficult for plastidial proteins [27]. The sequence analysis resulted in a predicted localization in the chloroplast, so this was tested by transient expression of YFP (yellow fluorescent protein)–OEP61 in tobacco leaves (Figure 3A). Here, the fluorescence of YFP–OEP61 co-localizes with the chlorophyll autofluorescent (indicated with arrows) and GFP (green fluorescence protein)-tagged Toc33 (Supplementary Figure S4 at http://www.BiochemJ.org/bj/438/bj4380143add.htm), showed localization of OEP61 to chloroplasts, whereas no overlap in fluorescence could be observed with proteins localized to other organelles, including ER and mitochondria (Supplementary Figure S4). To investigate targeting without the potential interference of tags, OEP61 was synthesized by *in vitro* translation and incubated with purified organelles, as previously performed to analyse the subcellular distribution of TA proteins [27]. In the presence of both chloroplasts and mitochondria, the majority of OEP61 associates with the chloroplast fraction (Figure 3B). No change in molecular mass is detected, confirming the predicted lack of a cleavable targeting sequence. Washes of the organelles included sodium carbonate, providing strong evidence that OEP61 is inserted into the membrane of the outer envelope. Furthermore, OEP61 was unable to insert into ER membranes (results not shown). Therefore the *in vitro* assays provide further support for an exclusive localization of OEP61 to chloroplasts.

Since OEP61 is predicted to be a TA protein, the N-terminus of the protein was expected to be exposed to the cytosol, thereby maintaining a membrane topology similar to the chloroplast TA proteins Toc33 and Toc34 [38]. Thus topology was tested by addition of the protease thermolysin after incubation of OEP61 with chloroplasts, and comparisons were made with Tic22 (intermembrane space protein) and Lhcb1 (thylakoid protein) (Figure 3C). Protease addition resulted in digestion of OEP61 (Figure 3C, lane 2), indicating that most of the protein is outside the chloroplast. Tic22 and Lhcb1 remain intact (Figure 3C, lanes 4 and 6), confirming that the chloroplasts were impermeable to protease. The tail-anchor of OEP61 is too small to be detected efficiently, although we can infer that the TMD is inserted into the outer envelope due to its resistance to sodium carbonate washes, which were included in the fractionation of chloroplasts from mitochondria. A comparable response with protease treatment is observed for the plastidial TA protein Toc33 (Supplementary Figure S5 at http://www.BiochemJ.org/bj/438/bj4380143add.htm). The topology of OEP61 in its native environment was confirmed by its protease sensitivity in *A. thaliana* chloroplasts (Figure 3D). We conclude that OEP61 is a TA protein localized exclusively to chloroplasts. Its

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

topology results in the TPR domain facing the cytosol, consistent with a role as a chaperone receptor.

The TPR domain of OEP61 binds specifically to Hsp70

The presence of a TPR clamp domain indicated that OEP61 would bind to Hsp70 and/or Hsp90. To investigate this potential interaction, purified recombinant OEP61 lacking its TMD (OEP61-TM; Figure 4A) was incubated in WGE and bound proteins were co-precipitated. Compared with a mock incubation (Figure 4B, lane 1), an additional protein band appeared at approximately 70 kDa (Figure 4B) and was shown via Western blotting to be Hsp70 (Supplementary Figure S6 at http://www.BiochemJ.org/bj/438/bj4380143add.htm), suggesting that OEP61 binds to Hsp70. To test whether this interaction was mediated by the TPR clamp domain, we tested the effect of the mutation R185A in OEP61-TM, which is equivalent to the R192A mutation in human Tom70 known to disrupt binding to molecular chaperones [7]. This mutation abolished binding to the 70 kDa band, showing that OEP61 binds to Hsp70 using its TPR clamp domain. Another band of approximately 25 kDa was pulled down by both forms of OEP61-TM, which indicates an interaction that is not dependent on the TPR clamp. The identity of this protein is not known.

The binding of OEP61 to Hsp70 suggests that there is a specific recognition of Hsp70 and an absence of Hsp90 binding. This was tested by incubating His-tagged OEP61 with purified recombinant *A. thaliana* Hsp70 (At5g02500) [39] and the major constitutive Hsp90, termed Hsp81 (At5g56030) [40]. Figure 4(C) shows that His–OEP61 is able to bind Hsp70 efficiently, but does not bind Hsp81. This contrasts with the ability of the His-tagged TPR2A domain of Hop (His–Hop2A) to bind Hsp81 and not Hsp70. Since Hsp70 and Hsp81 were purified using His tags, we verified that their tags had been efficiently removed and did not contribute to their own pull down by incubating Hsp70 and Hsp81 with beads alone (Figure 4C, lanes 7–10). We conclude that OEP61 is able to discriminate between Hsp70 and Hsp81.

To investigate the nature of the interaction between OEP61 and Hsp70 we performed *in vitro* binding assays in wheat germ lysate with various domains (Figure 4A): OEP61TPR is the TPR domain alone, and OEP61L is the 34 kDa linker sequence between the TPR domain and the tail anchor. The N-terminal TPR domain of human Hop (HopTPR1) is known to bind the C-terminal end of Hsp70 specifically [17], and was therefore used as positive control for Hsp70 binding. A strong Hsp70 signal was detected by immunoblot analysis when a TPR domain was present (Figure 4D, lanes 2, 3 and 5). OEP61-TM (Figure 4D, lane 3) has a stronger affinity to Hsp70 than HopTPR1 (Figure 4D, lane 2), which could be due to sequence dissimilarities between human and plant Hsp70. Comparison between the binding capacities of OEP61TPR and OEP61-TM show that the TPR domain binds Hsp70 less efficiently than the complete cytosolic domain of OEP61 (Figure 4D, 47% in lane 5 compared with 100% in lane 3). This suggests that the long linker region between the TPR domain of OEP61 and the TMD is necessary to maintain the structural stability of a functional TPR domain. Hsp70 binding by OEP61L and OEP61R185A (Figure 4D lanes 4 and lane respectively) does not exceed background levels of binding obtained by beads alone (Figure 4D, lane 1), indicating that the binding of OEP61-TM to Hsp70 is mediated by the

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

well-characterized interaction between the TPR clamp and the C-terminus of Hsp70, with no direct contribution from the linker region.

Addition of a peptide representing the C-terminus of the plant Hsp70 strongly inhibited binding to Hsp70 (Figure 4D, lanes 8 and 9), with a similar effect on both OEP61 and HopTPR1. This further supports the conclusion that the interaction between OEP61 and Hsp70 is mediated via the TPR clamp domain. Hsp70 binding by the TPR domain of OEP61 alone is not inhibited by the peptide, suggesting that this interaction occurs due to the chaperone activity of Hsp70 acting on misfolded OEP61; this provides further evidence that the linker domain provides stability to the TPR domain.

OEP61 associates selectively with chloroplast precursors

Localization of OEP61 at the outer chloroplast envelope and interaction of its cytosolic TPR domain with Hsp70 indicates that OEP61 is a plastidial chaperone receptor. To investigate whether it plays a role in protein targeting analogous to Toc64, the interaction of OEP61 with cytosolic precursor complexes was investigated. Precursor proteins were synthesized by *in vitro* translation in WGE, and chaperone binding to the precursor proteins was stabilized by incubation with apyrase. These complexes were tested for their ability to bind OEP61-TM and its inactive form OEP61R185A (Figure 5A). The chloroplast precursors of Toc33, Toc34 and Lhcb1 bind specifically to the native form of OEP61. In contrast, the mitochondrial precursors Tom22-1, ANT-h, and Tim9 and Tim10 do not bind to OEP61. Pull down by both the native and mutated forms of OEP61 is observed for the chloroplast protein Tic22, and the mitochondrial protein At5g013, indicating an interaction that is independent of the TPR clamp. One chloroplast precursor, At2g17, does not interact with OEP61. Taken together, the emerging pattern is that OEP61 is able to interact specifically with some chloroplast precursors, but not with mitochondrial precursors. This pattern holds for different types of precursors, including those in the outer membranes and those translocated to different internal compartments. The requirement for an intact TPR clamp domain suggests that the interaction with precursors is mediated via Hsp70 in the lysate. To determine whether OEP61 binds the targeting sequence of precursors, the C-terminal tail anchor of Toc33 was deleted, and the N-terminal targeting sequence of pSSU was deleted. Both of these deletions abolished binding to OEP61 (Figure 5B). Therefore these results support our general proposal that OEP61 is involved in protein targeting to chloroplasts.

If OEP61 co-ordinates the transfer of precursors to the translocase of the chloroplast outer envelope, we reasoned that a soluble form of OEP61 lacking its membrane anchor (OEP61-TM) would inhibit targeting. Therefore the effect of OEP61-TM on the targeting of Toc33 to chloroplasts was tested by *in vitro* assay. Toc33 targeting was severely impaired by OEP61-TM (Figure 6A, lanes 1 and 2), but was unaffected by the addition of the peroxisomal targeting factor PEX19 (Figure 6A, lanes 3 and 4). No mistargeting to ER occurred (Figure 6A, lanes 5 and 6). The targeting of a mitochondrial protein At3g58840 to mitochondria was unaffected by OEP61-TM (Figure 6A, translation lane 7; lanes 10 and 11), and no mistargeting to chloroplasts was observed (Figure 6A, lanes 8 and 9). Similarly, targeting of the ER protein Sec61β to ER microsomes was unaffected by OEP61-TM (Figure 6A, lanes 12 and 13). These results show that OEP61-TM selectively inhibits chloroplast targeting,

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

without affecting precursors destined for mitochondria or the ER. This is consistent with OEP61 functioning to select precursors for delivery to the chloroplast translocase.

The inhibition of chloroplast targeting by OEP61 could have arisen primarily due to its ability to bind Hsp70, so we have also tested the ability of HopTPR1 to inhibit chloroplast targeting. Figure 6(B) shows that the targeting of Toc33, Lhcb1 and pSSU to chloroplasts is severely impaired by OEP61, but is not significantly affected by HopTPR1. Therefore we conclude that some of the capacity for OEP61 to recognize precursors occurs independently of Hsp70.

DISCUSSION

The need to deliver cytosolic proteins to different organelles has resulted in numerous pathways that use various targeting factors and their cognate membrane-bound receptors. Since chaperones are found to bind many precursor proteins, the importance of chaperone receptors is gaining prominence. Chaperone receptors have been identified at the outer membranes of every organelle [19], and have been shown to increase the efficiency of protein targeting [3,7]. To understand their role in the fidelity of targeting, and gain a systematic understanding, a more complete knowledge of chaperone receptors is necessary. In the present study we identify OEP61 as a novel chaperone receptor at the chloroplast outer envelope.

In the present study we show that OEP61 is part of an uncharacterized protein family expressed ubiquitously in the plant. It inserts into the chloroplast outer envelope via its tail anchor, exposing the N-terminal TPR clamp domain to the cytosol. *In vitro* experiments show that the TPR clamp domain of OEP61 interacts specifically with Hsp70, and together with the linker domain facilitates selective recognition of chloroplast precursor complexes. Non-anchored OEP61 interferes with chloroplast targeting. Thus we propose that OEP61 is involved in protein targeting to chloroplasts, in a manner analogous to the Hsp90-mediated recognition of precursors by Toc64.

OEP61 is expressed throughout the plant, which supports a constitutive role for OEP61 in protein targeting to the chloroplast. The expression pattern is similar for transcripts and protein, with greater levels in green tissues, which is consistent with the localization of OEP61 to chloroplasts. However, detection of OEP61 in root tissues shows that OEP61 is also likely to reside in root plastids. There are no major differences in distribution compared with Toc64, which is also found throughout the plant [21]. The elevated levels of OEP61 in young leaves indicate a role in chloroplast biogenesis since this is dependent on high levels of protein import. Overall, it is clear that OEP61 exists in all tissues at substantial levels, and is likely to function in all plastids.

A crucial feature of OEP61 to support its potential function in protein targeting is the C-terminal TMD, which ensures a cytosolic disposition of the TPR clamp domain. This domain structure is classed as TA, and these proteins invariably possess a topology with the N-terminus in the cytosol [37]. TA proteins are involved in many protein targeting processes such as Toc33 and Toc34 in the chloroplast translocase [41], Tom5, Tom6 and Tom7 in the

CIHR Author Manuscript

CIHR Author Manuscript

CIHR Author Manuscript

mitochondrial translocase [42], and Sec61β, Sec61γ and RAMP4 in the ER translocase [11,43]. Therefore the TA protein classification of OEP61 is consistent with a role in delivering proteins to the chloroplast translocase.

The TPR domain of OEP61 specifically binds to Hsp70 and interacts preferentially with chloroplast precursors. The simplest scheme for the interaction between OEP61 and chloroplast precursors is sequential binding mediated by Hsp70, in which hydrophobic motifs within the precursor bind to Hsp70, and then the C-terminus of Hsp70 binds OEP61 (Figure 7). Chloroplast precursors are known to bind Hsp70 [2], yet the widespread interactions of Hsp70 with proteins localized throughout the cell implies that a more sophisticated recognition process takes place. This could occur via additional contacts between OEP61 and the precursor, possibly involving the linker region of OEP61. Such an interaction is mediated by additional TPR motifs in Tom70 [44], but in OEP61 no identifiable functional domains other than the TPR clamp can be identified by sequence homology. Nevertheless, the ability of purified OEP61 to distinguish between chloroplast and mitochondrial precursors, and the inability of HopTPR1 to substitute for OEP61 in the inhibition of chloroplast targeting, suggests that OEP61 is able to directly recognize targeting signals. Alternatively, OEP61 might act in concert with additional receptors such as Toc64. A precedent for the co-ordination of multiple receptors is the action of multiple Tom70s in mitochondrial targeting [45], and the identification of large chloroplast precursor complexes suggests that multiple chaperones may be bound to the same precursor [3]. Such a scheme potentially provides great flexibility to the recognition process, and could combine roles for Hsp70 and Hsp90 in the targeting of a single precursor. More complexity is possible if other precursor receptors exist in the outer envelope. One source of such receptors could be other *A. thaliana* TPR clamp proteins, which have been identified by an independent bioinformatics analysis, providing a further 23 uncharacterized genes, some of which generate multiple transcript species by alternative splicing [29].

Another possible mechanism for generating targeting specificity is the specific recognition of individual Hsp70 family members, a concept that is supported by specialization of function of the Hsp70 SSA family in yeast [46]. *A. thaliana* possesses at least 18 distinct Hsp70 proteins [39]. Com70 is an Hsp70 in spinach that binds to chloroplasts and associates with translocating precursors [47]; if a chloroplast-bound Hsp70 exists in *A. thaliana*, OEP61 may co-ordinate its activity. Although such schemes allow a role for chaperone receptors in the specificity of targeting, it is still possible that they simply provide a mechanism for removing chaperones from the precursor to prepare it for recognition by other receptors and for translocation across the membrane. This issue will need to be addressed by manipulating chaperone receptors in plants or in assays with multiple organelles.

Another issue raised by the present study is the functional relationship between OEP61 and Toc64. Toc64 has been shown to interact dynamically with the Toc complex in pea [48], thereby positioning it at the core of targeting and translocation processes, and it has the capability of interacting with precursors bound to Hsp90 [3], yet its depletion in T-DNA (transferred DNA) knockout *A. thaliana* and in *Physcomitrella* does not lead to any major phenotypic effect [21,22]. Potentially, OEP61 binds the same precursors as Toc64, since

mitochondrial precursors are known to form multichaperone complexes that include both Hsp70 and Hsp90 [7], and different chloroplast precursors are known to be bound by Hsp70 or Hsp90 [2,3]. Such an overlap in function may reduce the dependency on Toc64, and thereby explain the difficulty of assessing the functional role of Toc64. Functional redundancy has been observed for the mitochondrial receptors Tom20 and Tom70, in which deletion has a synthetic phenotypic effect [20]. Manipulation of OEP61 and Toc64 in parallel would provide an opportunity to assess the role of both receptors. The spatial relationship between OEP61 and the Toc complex will be an important feature of any co-ordinated action, and remains to be elucidated. The ability of the non-anchored OEP61 to interfere with targeting suggests that the co-ordination with other membrane components is vital for the function of OEP61. Overall, the widespread tissue expression and subcellular co-localization suggests that OEP61 co-exists with Toc64 in the outer envelope of plastids, and together they potentially facilitate the targeting of precursors bound to both Hsp70 and Hsp90.

Several targeting factors are involved in chloroplast localization, and it will be important to understand how they operate alongside the chaperone-mediated system. The guidance complex contains Hsp70, so may interact with OEP61 [2,41]. AKR2A has been characterized as a cytosolic interaction partner of chloroplast membrane proteins [4], and has been shown to be involved in the targeting of Toc33 and Toc34 [5]. AKR2A is therefore expected to compete with Hsp70 for binding to the hydrophobic TMD, and several independent targeting pathways may operate. Determining the preferred targeting pathways for different classes of chloroplast precursors will require a large-scale systematic analysis, and can be expected to show significant redundancy between pathways.

In the present study, we have shown that OEP61 is a novel plastidial chaperone receptor for Hsp70, with the potential to promote the selective targeting of plastidial precursors. Further studies will be required to determine the overall role for OEP61 in protein localization, and its relationship with the Toc complex.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the Arabidopsis Biological Resource Center, RIKEN, Professor Chris Hawes (Oxford Brookes University, Oxford, U.K.), Professor Alison Baker (University of Leeds, Leeds, U.K.), Dr Erich Glawischnig (Technische Universität München, Munich, Germany), Professor Colin Robinson (University of Warwick, Coventry, U.K.) and Peter Cain (University of Warwick, Coventry, U.K.) for supplying plasmids. Thanks to Professor Hawes' group for advice with *in vivo* localisation, and to Dr Robert Mullen (University of Guelph, Guelph, Canada) for discussions on the manuscript.

FUNDING

This work was supported by the BBSRC (Biotechnology and Biological Sciences Research Council Research) [grant number BB/E01559X/1] (to B.M.A.). J.C.Y. holds a Canada Research Chair in Molecular Chaperones, Tier II.

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

CIHR Author Manuscript
CIHR Author Manuscript
CIHR Author Manuscript

Abbreviations used

AKR2A	*Arabidopsis* ankyrin-repeat protein
ER	endoplasmic reticulum
FKBP	FK506-binding protein
GFP	green fluorescent protein
HopTPR1	human Hop
Hsp	heat-shock protein
KOAc	potassium acetate
Ni-NTA	Ni^{2+}-nitriloacetate
PPIase	peptidy-prolyl *cis*–*trans*-isomerase
qRT-PCR	quantitative real-time PCR
SMAP-1	stromal membrane-associated protein 1
TA	tail-anchored
TMD	transmembrane domain
Tom	translocase of the mitochondrial outer membrane
TPR	tetratricopeptide repeat
WGE	wheat germ extract
YFP	yellow fluorescent protein

References

1. Wickner W, Schekman R. Protein translocation across biological membranes. Science. 2005; 310:1452–1456. [PubMed: 16322447]
2. May T, Soll J. 14-3-3 proteins form a guidance complex with chloroplast precursor proteins in plants. Plant Cell. 2000; 12:53–64. [PubMed: 10634907]
3. Qbadou S, Becker T, Mirus O, Tews I, Soll J, Schleiff E. The molecular chaperone Hsp90 delivers precursor proteins to the chloroplast import receptor Toc64. EMBO J. 2006; 25:1836–1847. [PubMed: 16619024]
4. Bae W, Lee YJ, Kim DH, Lee J, Kim S, Sohn EJ, Hwang I. AKR2A-mediated import of chloroplast outer membrane proteins is essential for chloroplast biogenesis. Nat Cell Biol. 2008; 10:220–227. [PubMed: 18193034]
5. Dhanoa PK, Richardson LG, Smith MD, Gidda SK, Henderson MP, Andrews DW, Mullen RT. Distinct pathways mediate the sorting of tail-anchored proteins to the plastid outer envelope. PLoS ONE. 2010; 5:e10098. [PubMed: 20418952]
6. Fan AC, Bhangoo MK, Young JC. Hsp90 functions in the targeting and outer membrane translocation steps of Tom70-mediated mitochondrial import. J Biol Chem. 2006; 281:33313–33324. [PubMed: 16968702]

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

7. Young JC, Hoogenraad NJ, Hartl FU. Molecular chaperones Hsp90 and Hsp70 deliver preproteins to the mitochondrial import receptor Tom70. Cell. 2003; 112:41–50. [PubMed: 12526792]
8. Zara V, Ferramosca A, Robitaille-Foucher P, Palmieri F, Young JC. Mitochondrial carrier protein biogenesis: role of the chaperones Hsc70 and Hsp90. Biochem J. 2009; 419:369–375. [PubMed: 19143589]
9. Bhangoo MK, Tzankov S, Fan AC, Dejgaard K, Thomas DY, Young JC. Multiple 40-kDa heat-shock protein chaperones function in Tom70-dependent mitochondrial import. Mol Biol Cell. 2007; 18:3414–3428. [PubMed: 17596514]
10. Yano M, Terada K, Mori M. AIP is a mitochondrial import mediator that binds to both import receptor Tom20 and preproteins. J Cell Biol. 2003; 163:45–56. [PubMed: 14557246]
11. Rapoport TA. Protein translocation across the eukaryotic endoplasmic reticulum and bacterial plasma membranes. Nature. 2007; 450:663–669. [PubMed: 18046402]
12. Abell BM, Rabu C, Leznicki P, Young JC, High S. Post-translational integration of tail-anchored proteins is facilitated by defined molecular chaperones. J Cell Sci. 2007; 120:1743–1751. [PubMed: 17456552]
13. Rabu C, Wipf P, Brodsky JL, High S. A precursor-specific role for Hsp40/Hsc70 during tail-anchored protein integration at the endoplasmic reticulum. J Biol Chem. 2008; 283:27504–27513. [PubMed: 18667436]
14. Abell BM, Mullen RT. Tail-anchored membrane proteins: exploring the complex diversity of tail-anchored-protein targeting in plant cells. Plant Cell Rep. 2010; 30:137–151. [PubMed: 20878326]
15. Ngosuwan J, Wang NM, Fung KL, Chirico WJ. Roles of cytosolic Hsp70 and Hsp40 molecular chaperones in post-translational translocation of presecretory proteins into the endoplasmic reticulum. J Biol Chem. 2003; 278:7034–7042. [PubMed: 12493732]
16. Brinker A, Scheufler C, Von Der Mulbe F, Fleckenstein B, Herrmann C, Jung G, Moarefi I, Hartl FU. Ligand discrimination by TPR domains. Relevance and selectivity of EEVD-recognition in Hsp70 x Hop x Hsp90 complexes. J Biol Chem. 2002; 277:19265–19275. [PubMed: 11877417]
17. Scheufler C, Brinker A, Bourenkov G, Pegoraro S, Moroder L, Bartunik H, Hartl FU, Moarefi I. Structure of TPR domain–peptide complexes: critical elements in the assembly of the Hsp70–Hsp90 multichaperone machine. Cell. 2000; 101:199–210. [PubMed: 10786835]
18. Wu SJ, Liu FH, Hu SM, Wang C. Different combinations of the heat-shock cognate protein 70 (hsc70) C-terminal functional groups are utilized to interact with distinct tetratricopeptide repeat-containing proteins. Biochem J. 2001; 359:419–426. [PubMed: 11583590]
19. Schlegel T, Mirus O, von Haeseler A, Schleiff E. The tetratricopeptide repeats of receptors involved in protein translocation across membranes. Mol Biol Evol. 2007; 24:2763–2774. [PubMed: 17905998]
20. Ramage L, Junne T, Hahne K, Lithgow T, Schatz G. Functional cooperation of mitochondrial protein import receptors in yeast. EMBO J. 1993; 12:4115–4123. [PubMed: 8223428]
21. Aronsson H, Boij P, Patel R, Wardle A, Topel M, Jarvis P. Toc64/OEP64 is not essential for the efficient import of proteins into chloroplasts in *Arabidopsis thaliana*. Plant J. 2007; 52:53–68. [PubMed: 17655652]
22. Rosenbaum Hofmann N, Theg SM. Toc64 is not required for import of proteins into chloroplasts in the moss *Physcomitrella patens*. Plant J. 2005; 43:675–687. [PubMed: 16115065]
23. Boeckmann B, Bairoch A, Apweiler R, Blatter MC, Estreicher A, Gasteiger E, Martin MJ, Michoud K, O'Donovan C, Phan I, et al. The SWISS-PROT protein knowledgebase and its supplement TrEMBL in 2003. Nucleic Acids Res. 2003; 31:365–370. [PubMed: 12520024]
24. Schuhegger R, Rauhut T, Glawischnig E. Regulatory variability of camalexin biosynthesis. J Plant Physiol. 2007; 164:636–644. [PubMed: 16769150]
25. Cashmore AR. Structure and expression of a pea nuclear gene encoding a chlorophyll a/b-binding polypeptide. Proc Natl Acad Sci USA. 1984; 81:2960–2964. [PubMed: 16593461]
26. Batoko H, Zheng HQ, Hawes C, Moore I. A rab1 GTPase is required for transport between the endoplasmic reticulum and golgi apparatus and for normal golgi movement in plants. Plant Cell. 2000; 12:2201–2218. [PubMed: 11090219]

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

27. Kriechbaumer V, Shaw R, Mukherjee J, Bowsher CG, Harrison AM, Abell BM. Subcellular distribution of tail–anchored proteins in *Arabidopsis*. Traffic. 2009; 10:1753–1764. [PubMed: 19843281]
28. Urban A, Neukirchen S, Jaeger KE. A rapid and efficient method for site-directed mutagenesis using one-step overlap extension PCR. Nucleic Acids Res. 1997; 25:2227–2228. [PubMed: 9153325]
29. Prasad BD, Goel S, Krishna P. *In silico* identification of carboxylate clamp type tetratricopeptide repeat proteins in Arabidopsis and rice as putative cochaperones of Hsp90/Hsp70. PLoS ONE. 2010; 5:e12761. [PubMed: 20856808]
30. Sinars CR, Cheung-Flynn J, Rimerman RA, Scammell JG, Smith DF, Clardy J. Structure of the large FK506-binding protein FKBP51, an Hsp90-binding protein and a component of steroid receptor complexes. Proc Natl Acad Sci USA. 2003; 100:868–873. [PubMed: 12538866]
31. Young JC, Obermann WM, Hartl FU. Specific binding of tetratricopeptide repeat proteins to the C-terminal 12-kDa domain of hsp90. J Biol Chem. 1998; 273:18007–18010. [PubMed: 9660753]
32. Chewawiwat N, Yano M, Terada K, Hoogenraad NJ, Mori M. Characterization of the novel mitochondrial protein import component, Tom34, in mammalian cells. J Biochem. 1999; 125:721–727. [PubMed: 10101285]
33. Pollmann S, Neu D, Lehmann T, Berkowitz O, Schafer T, Weiler EW. Subcellular localization and tissue specific expression of amidase 1 from *Arabidopsis thaliana*. Planta. 2006; 224:1241–1253. [PubMed: 16738862]
34. Lister R, Carrie C, Duncan O, Ho LH, Howell KA, Murcha MW, Whelan J. Functional definition of outer membrane proteins involved in preprotein import into mitochondria. Plant Cell. 2007; 19:3739–3759. [PubMed: 17981999]
35. Carthew RW, Sontheimer EJ. Origins and mechanisms of miRNAs and siRNAs. Cell. 2009; 136:642–655. [PubMed: 19239886]
36. Krogh A, Larsson B, von Heijne G, Sonnhammer EL. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol. 2001; 305:567–580. [PubMed: 11152613]
37. Rabu C, Schmid V, Schwappach B, High S. Biogenesis of tail-anchored proteins: the beginning for the end? J Cell Sci. 2009; 122:3605–3612. [PubMed: 19812306]
38. Qbadou S, Tien R, Soll J, Schleiff E. Membrane insertion of the chloroplast outer envelope protein, Toc34: constrains for insertion and topology. J Cell Sci. 2003; 116:837–846. [PubMed: 12571281]
39. Lin BL, Wang JS, Liu HC, Chen RW, Meyer Y, Barakat A, Delseny M. Genomic analysis of the Hsp70 superfamily in *Arabidopsis thaliana*. Cell Stress Chaperones. 2001; 6:201–208. [PubMed: 11599561]
40. Sangster TA, Queitsch C. The HSP90 chaperone complex, an emerging force in plant development and phenotypic plasticity. Curr Opin Plant Biol. 2005; 8:86–92. [PubMed: 15653405]
41. Agne B, Kessler F. Protein transport in organelles: the Toc complex way of preprotein import. FEBS J. 2009; 276:1156–1165. [PubMed: 19187236]
42. Beilharz T, Egan B, Silver PA, Hofmann K, Lithgow T. Bipartite signals mediate subcellular targeting of tail-anchored membrane proteins in *Saccharomyces cerevisiae*. J Biol Chem. 2003; 278:8219–8223. [PubMed: 12514182]
43. Pool MR. A transmembrane segment inside the ribosome exit tunnel triggers RAMP4 recruitment to the Sec61p translocase. J Cell Biol. 2009; 185:889–902. [PubMed: 19468070]
44. Brix J, Ziegler GA, Dietmeier K, Schneider-Mergener J, Schulz GE, Pfanner N. The mitochondrial import receptor Tom70: identification of a 25 kDa core domain with a specific binding site for preproteins. J Mol Biol. 2000; 303:479–488. [PubMed: 11054285]
45. Wiedemann N, Pfanner N, Ryan MT. The three modules of ADP/ATP carrier cooperate in receptor recruitment and translocation into mitochondria. EMBO J. 2001; 20:951–960. [PubMed: 11230119]
46. Sharma D, Martineau CN, Le Dall MT, Reidy M, Masison DC, Kabani M. Function of SSA subfamily of Hsp70 within and across species varies widely in complementing *Saccharomyces cerevisiae* cell growth and prion propagation. PLoS ONE. 2009; 4:e6644. [PubMed: 19680550]

CIHR Author Manuscript
CIHR Author Manuscript
CIHR Author Manuscript

47. Kourtz L, Ko K. The early stage of chloroplast protein import involves Com70. J Biol Chem. 1997; 272:2808–2813. [PubMed: 9006921]

48. Sohrt K, Soll J. Toc64, a new component of the protein translocon of chloroplasts. J Cell Biol. 2000; 148:1213–1221. [PubMed: 10725334]

CIHR Author Manuscript
CIHR Author Manuscript
CIHR Author Manuscript

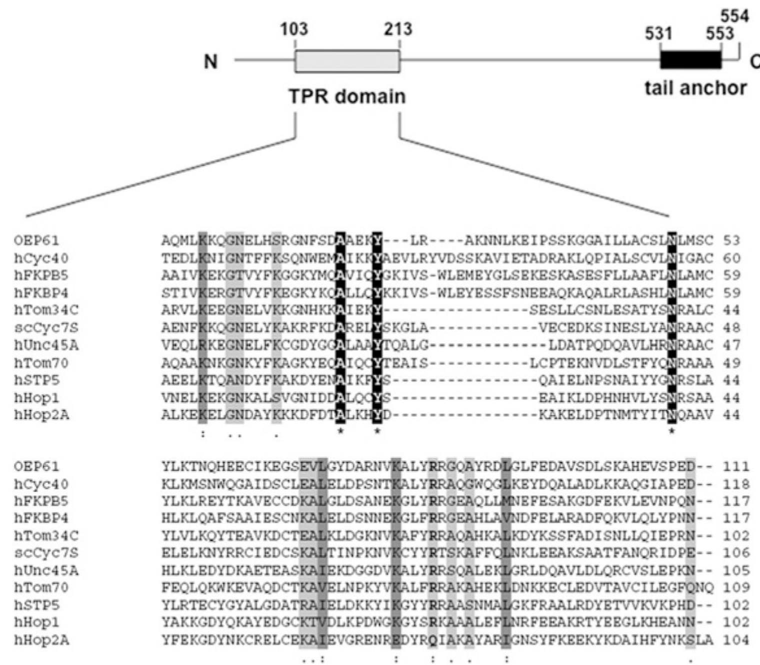

Figure 1. OEP61 relationships with other TPR clamp proteins

Schematic structure of OEP61 (Swiss-Prot accession number Q8GWM6). The N-terminal TPR domain is defined by PROSITE and a C-terminal TMD is predicted by SMART between amino acids 531 and 553. The alignment file shows the TPR domain of OEP61 with the known chaperone binding TPR clamp domains from Hop (hHop1 and hHop2A; P31948), FKBP5 (hFKBP5; Q13451), FKBP4 (hFKBP4; Q02790), cyclophilin-40 (hCyc40; Q08752), serine/threonine phosphatase 5 (hSTP5; P53041), cyclophilin seven suppressor (scCyc7s; A6ZL97), Tom70 (hTom70; O94826), Tom34 (hTom34C; Q15785) and Unc-45 (hUNC45A; Q9H3U1).

CIHR Author Manuscript
CIHR Author Manuscript
CIHR Author Manuscript

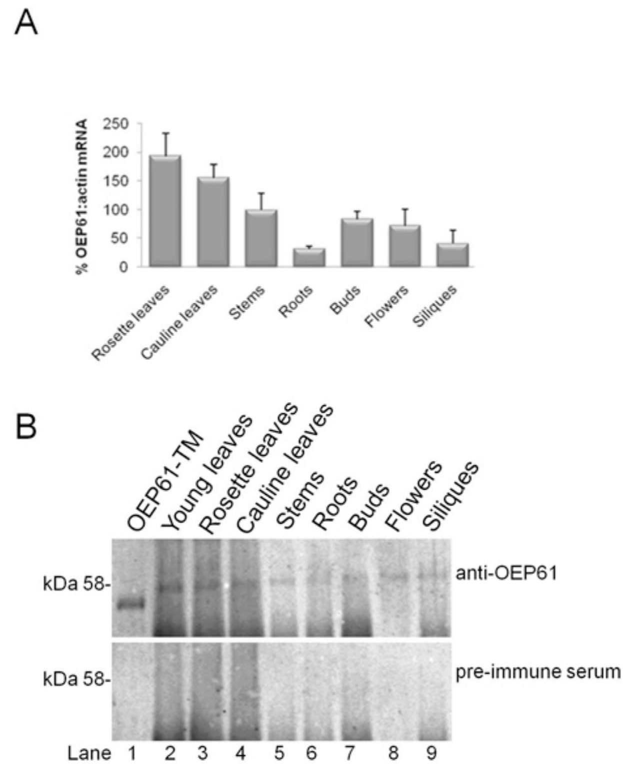

Figure 2. OEP61 is expressed throughout the mature plant

(**A**) RNA levels of OEP61 relative to actin were measured in rosette leaves, cauline leaves, stems, roots, buds, flowers and siliques from adult plants by qRT-PCR. Results are means ± S.E. ($n = 3$). (**B**) Immunoblot against immunoprecipitated OEP61 from young leaves (2 weeks after germination), and rosette leaves, cauline leaves, stems, roots, buds, flowers and siliques from adult plants. Recombinant OEP61 lacking its TMD (OEP61-TM) is used as a comparison.

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

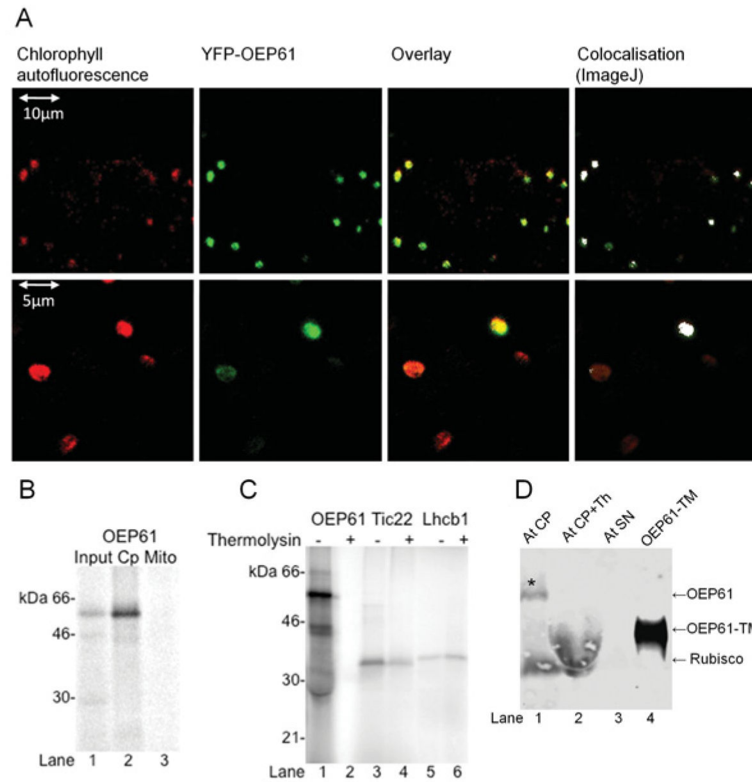

Figure 3. OEP61 is localized to the outer envelope of chloroplasts

(**A**) Confocal microscopy image of the YFP-tagged OEP61 construct transiently expressed in tobacco leaves. Its subcellular localization is overlaid with chlorophyll autofluorescence. Areas of significant co-localization (by ImageJ) are shown in white. (**B**) Competitive targeting assay. OEP61 was synthesized by *in vitro* translation in WGE (lane 1, 10% input) and incubated with purified chloroplasts and mitochondria. The chloroplasts (Cp, lane 2) and mitochondria (Mito, lane 3) were repurified and washed with sodium carbonate before analysis by SDS/PAGE. (**C**) Topology of OEP61 at chloroplasts. Radiolabelled *in vitro* translated protein was incubated with purified pea chloroplasts, which were pelleted and washed with sodium carbonate, followed by thermolysin proteolysis in lanes 2, 4 and 6. Tic22 resides in the intermembrane space and Lhcb1 resides in the thylakoids. Molecular mass markers are shown in kDa on the left-hand side. (**D**) Topology of native OEP61 at chloroplasts. *A. thaliana* chloroplasts were isolated and washed with sodium carbonate prior to treatment of equal quantities with (+ Th) or without thermolysin, and then immunoblotted alongside supernatant (AtSN) and recombinant OEP61-TM.

CIHR Author Manuscript

CIHR Author Manuscript

CIHR Author Manuscript

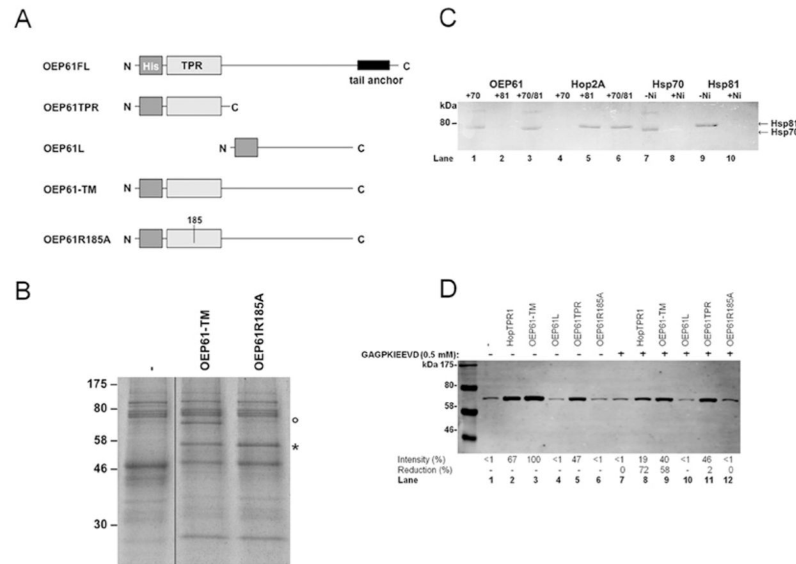

Figure 4. OEP61 interacts specifically with Hsp70 via its TPR clamp domain

(**A**) Variants of OEP61 constructs: OEP61FL (amino acids 1–554; full length), OEP61TPR (amino acids 103–213), OEP61L (amino acids 214–534), OEP61-TM (amino acids 1–534) and OEP61R185A (point mutation of amino acid 185 from arginine to alanine in OEP61-TM). (**B**) OEP61-TM and OEP61R185A were incubated with WGE and isolated by their His-tag. The resulting binding partners were analysed by SDS/PAGE and Coomassie staining. Recombinant OEP61 is indicated by a star; the additional band at 70 kDa is indicated by a circle. (**C**) His–OEP61-TM was incubated with Hsp70 and Hsp81, and a pull down with equal amounts of each recombinant protein was performed. The resulting binding partners were analysed by SDS/PAGE and Coomassie staining. Hsp70 and Hsp81 input are shown as – Ni, and the removal of their His-tags was tested by pull down without His–OEP61 (+ Ni). (**D**) OEP61 variants and the HopTPR1 domain were incubated with WGE and isolated by their His-tag. Binding to Hsp70 was assessed by immunobloting with anti-Hsp70 IgG, and the signals were calculated in relation to the strongest signal, which was set as 100% (lane 3). The effect of binding competition with the peptide GAGPKIEEVD was also tested (lanes 7–12). The molecular mass in kDa is indicated on the left-hand side.

CIHR Author Manuscript
CIHR Author Manuscript
CIHR Author Manuscript

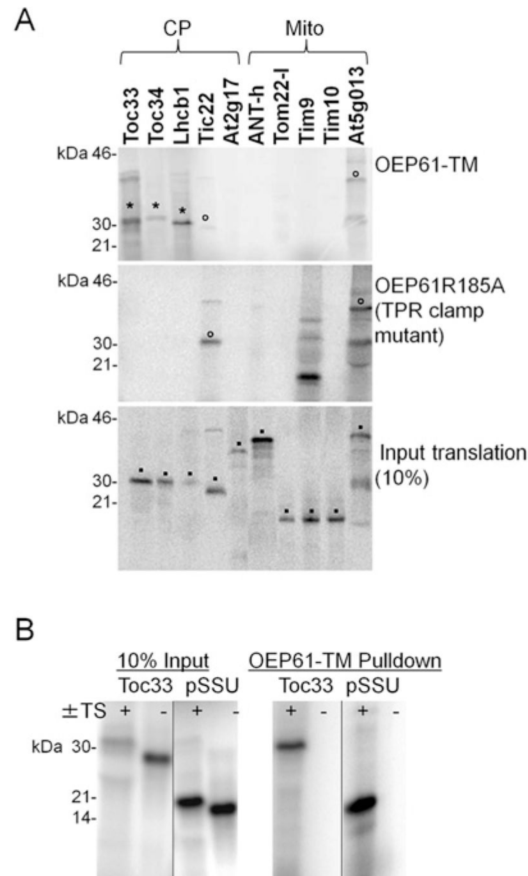

Figure 5. OEP61 associates specifically with precursors destined for the chloroplast via its TPR clamp domain

(**A**) Radiolabelled precursors of Toc33, Toc34, Lhcb1, Tic22, At2g17, ANT homologue (ANT-h), Tom22-I, Tim9, Tim10 and At5g013 were synthesized in WGE, treated with apyrase and then incubated with His-tagged OEP61-TM or the mutated form OEP61R185A. Products bound to His-tagged protein and pulled down with Ni-NTA beads were analysed by SDS/PAGE. Loading was adjusted to correct for the translation efficiency of each protein. Full-length products are indicated by a black square, specific pull downs are indicated by a star and non-specific pull downs are indicated with a circle. Molecular masses are given in kDa on the left-hand side. (**B**) Pull down by OEP61-TM was also performed for Toc33 and pSSU with and without their targeting sequences (TS).

CIHR Author Manuscript
CIHR Author Manuscript
CIHR Author Manuscript

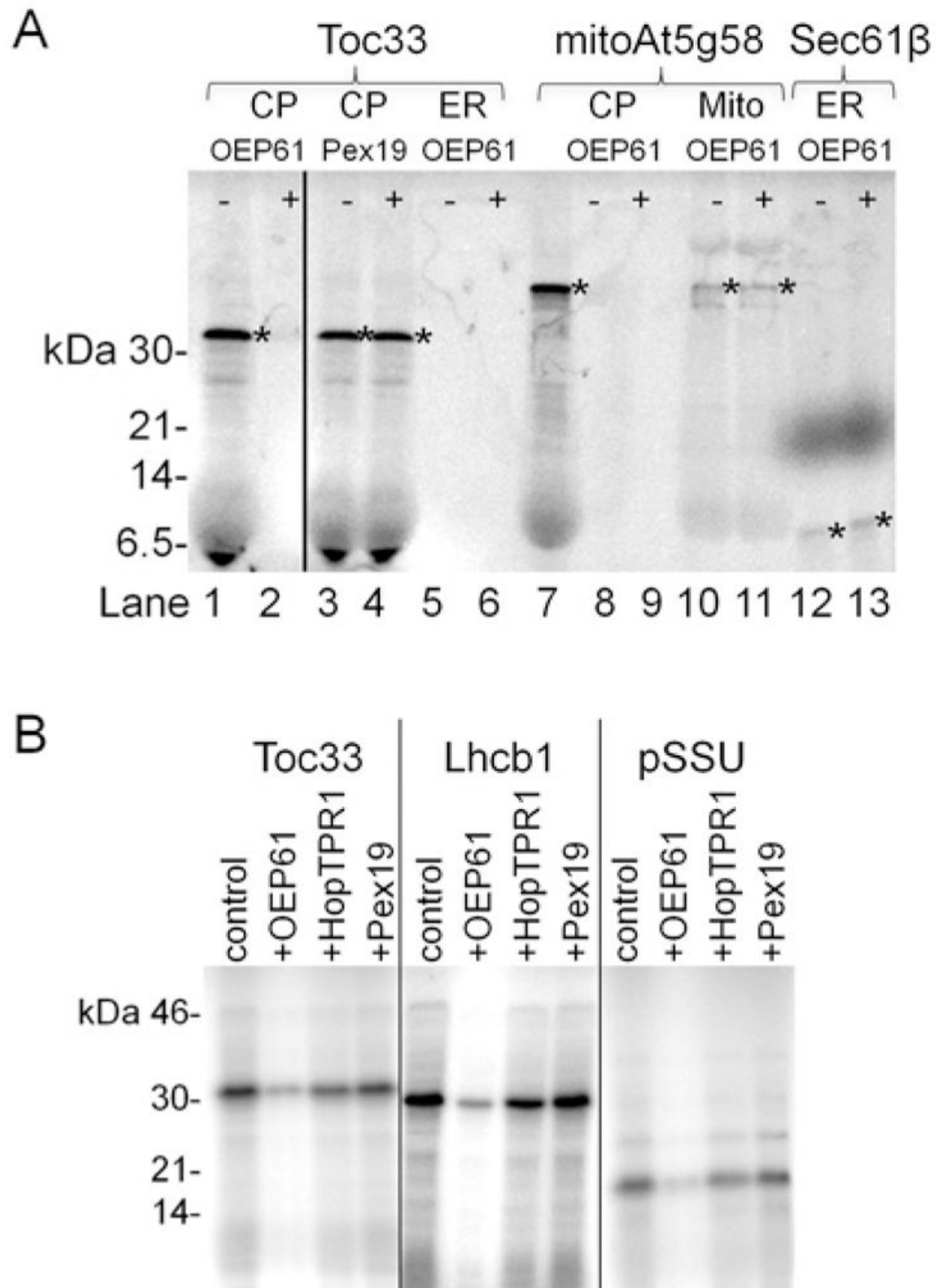

Figure 6. Soluble OEP61 inhibits chloroplast targeting

(**A**) Radiolabelled precursors were synthesized by *in vitro* translation and incubated with chloroplasts or ER membranes (Toc33 and Sec61*β*), or chloroplasts and mitochondria in a competitive assay (mitoAt5g58), in the presence of OEP61-TM or PEX19. The organelles were pelleted and analysed by SDS/PAGE. (**B**) Chloroplast targeting assays were performed for Toc33, Lhcb1 and pSSU in the presence of OEP61-TM, the Hsp70-binding domain of Hop (HopTPR1) or PEX19. Molecular mass markers are shown in kDa on the left-hand side.

CIHR Author Manuscript
CIHR Author Manuscript
CIHR Author Manuscript

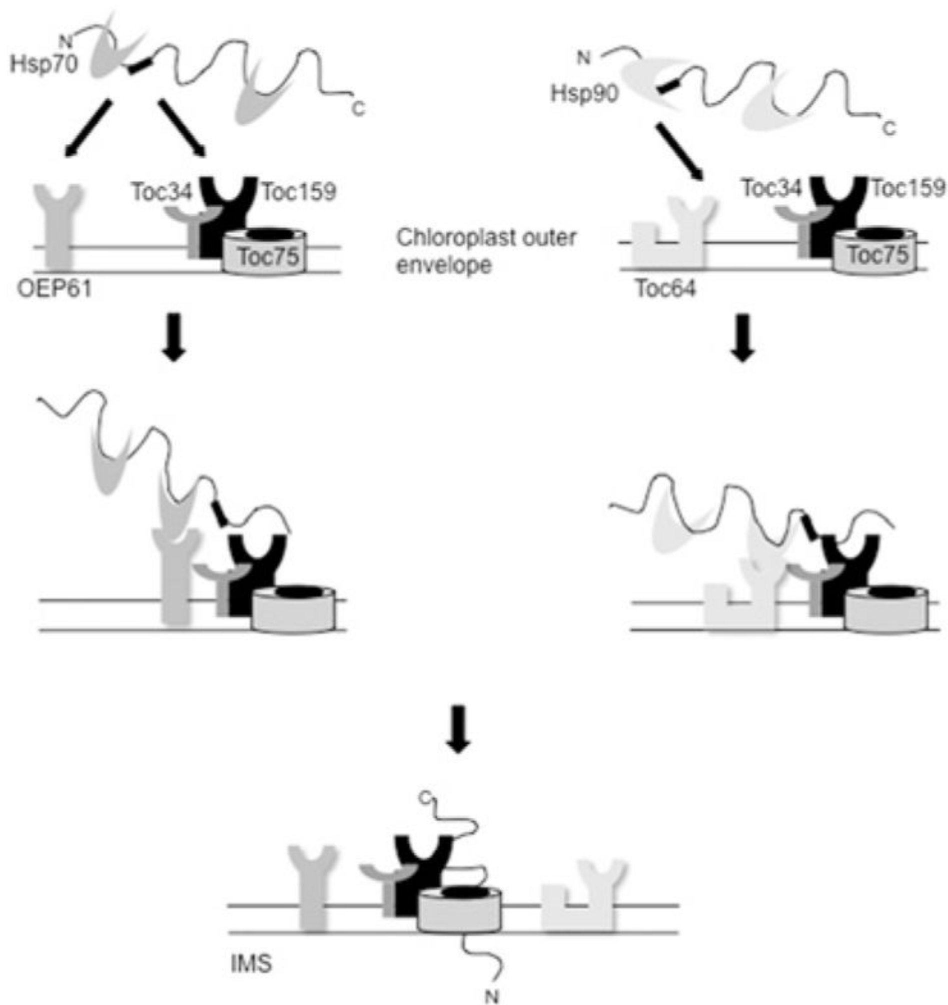

Figure 7. Proposed scheme of OEP61 and Toc64 action

OEP61 may be involved in the early recognition of Hsp70-bound precursors, in parallel with the recognition of Hsp90-bound precursors by Toc64, and it is also possible that precursors may engage both Toc64 and OEP61. Binding of the chaperone by the chaperone receptor would deliver the targeting sequence of the precursor to Toc34 (or Toc33) and promote release of the molecular chaperones. The precursor would then become translocated across the outer envelope using established mechanisms involving Toc159 and the pore formed by Toc75. OEP61 and Toc64 would then dislocate from the core complex.