Molecular chaperones involved in preprotein targeting to plant organelles

Dissertation

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Summary

The majority of chloroplast and mitochondrial proteins is encoded in the nucleus and has to be imported post-translationally into the appropriate organelle. After translation in the cytosol molecular chaperones like HSP70, HSP90 and 14-3-3 interact with preproteins to maintain them in an inactive but import competent state and to guide them to the organellar receptors.

In this work a large subset of chloroplast preproteins was analyzed using co-immunoprecipitation experiments which highlighted the general role of preprotein complexes involving HSP70, HSP90/HSP70 or 14-3-3/HSP70. Moreover, the relative abundance of identified HSP90-preprotein complexes enables to integrate chloroplast preproteins, as a diverse protein family, into the list of HSP90 client proteins. HSP90 binding studies using chimeric protein constructs could demonstrate that HSP90 binding requires the full-length protein, therefore suggesting that rather a structural feature in the preprotein is responsible for HSP90 binding. Furthermore, additional HSP90-preprotein complex components were identified which comprise the HSP90 co-chaperones FKBP73, HOP and AHA1. Apart of HSP90 also 14-3-3 was shown to interact with chloroplast preproteins. A combinatorial approach including experimental binding tests and *in silico* data concerning 14-3-3 binding sites revealed that 14-3-3 binding occurs more frequently on preproteins which contain more than one 14-3-3 binding motif in the transit peptide.

The second part of the work focused on preprotein transport to mitochondria. Since in plants preprotein targeting is more complex due to the presence of chloroplasts and mitochondria the question arose whether mitochondrial preproteins use the same cytosolic chaperone machinery as chloroplast preproteins. Experimental data could demonstrate binding of either HSP70 or 14-3-3/HSP70 to mitochondrial preproteins. Due to the frequently observed 14-3-3 binding, phosphorylation of mitochondrial preproteins and its influence on preprotein import behavior was investigated. Phosphorylation could not only be shown to accelerate import into mitochondria, but the data obtained also demonstrate that dephosphorylation of the preprotein is necessary before being efficiently imported. However, no preprotein could be identified in complex with HSP90, not even hydrophobic carrier proteins, which were shown to bind HSP90 in the mammalian system.

Zusammenfassung

Die Mehrheit aller plastidären und mitochondriellen Proteine sind im Kerngenom der Pflanzenzelle kodiert und müssen posttranslational in die entsprechenden Organellen importiert werden. Molekulare Chaperone wie HSP70, HSP90 und 14-3-3 binden hierbei an die frisch im Cytosol translatierten Vorstufenproteine um sie in einem ungefalteten aber importkompetenten Zustand zu erhalten sowie sie zu den Importrezeptoren der Organellen zu leiten.

In dieser Arbeit wurde mit Hilfe von Co-Immunopräzipitations-Experimenten eine große Anzahl plastidärer Vorstufenproteine untersucht. Dabei konnte gezeigt werden, dass Präproteine im Cytosol generell Komplexe mit HSP70, 14-3-3/HSP70 oder HSP90/HSP70 bilden. Plastidäre Vorstufenproteine konnten daher als diverse Proteinfamilie in die HSP90 Substratliste integriert werden. HSP90 Bindestudien mit chimären Proteinen zeigten, dass HSP90 das Vollängenpräprotein für eine Interaktion benötigt, was zu der Annahme führte, dass die HSP90 Bindung aufgrund struktureller Gegebenheiten des Präproteins erfolgt. Weiterhin wurden die HSP90 Co-Chaperone FKBP73, HOP und AHA1 als neue Komponenten des HSP90-Präproteinkomplexes identifiziert. Neben HSP90 spielt auch 14-3-3 eine Rolle beim Präproteintransport. Experimentelle Untersuchungen kombiniert mit *in silico* Anwendungen konnten zeigen, dass eine 14-3-3 Bindung an Präproteine mit mehr als einem 14-3-3 Bindemotif im Transitpeptid vermehrt auftritt.

Der zweite Teil dieser Arbeit konzentriert sich auf Präproteintransport zu Mitochondrien. Da Proteinsortierung und Transport in Pflanzen aufgrund der Präsenz von Mitochondrium und Chloroplast weitaus komplexer als in anderen Systemen ist, wurde untersucht ob sich die cytosolische Chaperon-Maschinerie für die verschiedenen Präproteine unterscheidet. Experimentelle Analysen konnten sowohl HSP70 als auch 14-3-3/HSP70 Bindungen an mitochondrielle Präproteine identifizieren. Aufgrund des häufig beobachteten Auftretens einer Interaktion zwischen Präproteinen mit 14-3-3 wurden sowohl das Phosphorylierungsverhalten mitochondrieller Präproteine sowie dessen Einfluss auf den Präproteinimport in Mitochondrien untersucht. Hierbei konnte gezeigt werden, dass phosphorylierte Präproteine effizienter importiert werden, die Proteine jedoch vor dem Import wieder dephosphoryliert werden müssen. Im Gegensatz zu 14-3-3 konnte keine Bindung mit HSP90 an mitochondrielle Präproteine gezeigt werden, obwohl unter den

verwendeten Präproteinen Homologe zu tierischen Proteinen enthalten waren, die nachweislich mit HSP90 assoziieren.

Table of contents

Abbreviations

AHA	activator of HSP90 ATPase
AKR2A	ankyrin rich protein 2A
AMP-PNP	Adenylyl-imidodiphosphate
At	Arabidopsis thaliana
ATP	Adenosintriphosphate
FKBP	FK506 binding protein
flp	full-length protein
GFP	green fluorescent protein
Gm	Glycine max
h	human
HIP	HSP70 interacting protein
HOP	HSP70/HSP90 organizing protein
HSC	Heat shock cognate
HSP	heat shock protein
ie	inner envelope
IMS	intermembrane space
kDa	kilodalton
LHC II	light harvesting complex II
m	mature protein
MIA	mitochondrial IMS import and assembly machinery
MPP	matrix processing peptidase
MSF	mitochondrial stimulation factor
OXA1	oxidase assembly
p	preprotein
Ps	Pisum sativum
PAGE	polyacrylamide gel electrophoresis
PAM	presequence-associated motor
PS	preimmunserum
SAM	sorting and assembly machinery
Sc	Saccharomyces cerevisiae
SDS	Sodium dodecyl sulfate
SPP	stromal processing peptidase
Ta	Triticum aestivum (wheat)
TCA	Trichloro acetic acid
TIC	translocon of the chloroplast inner envelope
TIM	translocase of inner membrane of mitochondria
TOC	translocon of the outer chloroplast envelope
TOM	translocase of outer membrane of mitochondria
tp	transit peptide
TPR	Tetratricopeptide repeat
Zm	Zea mays

1 Introduction

1.1 Endosymbiosis

Both organelles, mitochondria and chloroplasts, derived from endosymbiotic events which have taken place more than 1.5 billion years ago. Initially, an α-proteobacterium was engulfed by an anaerobic host cell developing during time to present-day mitochondria. In a second endosymbiotic process an ancient cyanobacterium was integrated into this mitochondria containing cell which evolved towards the chloroplast (Gould et al, 2008; Martin & Herrmann, 1998). Both endosymbiotic events were accompanied by a rearrangement of the genetical material of the organelles in order to facilitate cellular regulation of this more complex cell. As a consequence, more than 95% of chloroplast and mitochondrial genes were transferred to the nucleus resulting in loss of autonomy of the organelles (Bock & Timmis, 2008; Leister, 2003; Schleiff & Becker, 2011). Therefore specialized translocation machineries had to be established to reimport nuclear-encoded proteins into the appropriate organelle. Furthermore, organelle-specific signal sequences were added to these proteins, in order to ensure import of the required proteins into the appropriate organelle.

1.2 Chloroplast and mitochondrial signal sequences

Most chloroplast and mitochondrial proteins are synthesized as preproteins on cytosolic ribosomes, containing either an internal or a cleavable N-terminal signal sequence, termed transit peptide for chloroplast proteins or presequence for mitochondrial proteins. After successful import of the preprotein into the organelle the cleavable N-terminal targeting signal is removed by specific peptidases located in the stroma or matrix of the organelle. Comparison of chloroplast and mitochondrial N-terminal sequences in plants disclosed a close similarity of amino acid composition. Both sequence types possess an overall positive charge (Arg, Lys) and are rich in hydrophobic (Ala, Leu, Phe, Val) and hydroxylated (Ser, Thr) amino acids, whereas acidic amino acids are underrepresented. Nevertheless, the number of serine residues is even higher in chloroplast presequences compared to mitochondrial presequences, which possess instead a higher content of arginine residues (Peeters

& Small, 2001). In addition, the average size of chloroplast targeting sequences is slightly larger, although long and short targeting signals are present for both types of preproteins (Zhang & Glaser, 2002). A further difference between the targeting signals of chloroplasts and mitochondria is of structural character. Mitochondrial presequences are able to form amphiphilic α-helices thus facilitating mitochondrial receptor recognition, whereas chloroplast transit peptides are predicted to form a perfect random coil (Von Heijne, 1986; von Heijne & Nishikawa, 1991).

1.3 Protein import into chloroplasts

Most chloroplast preproteins, synthesized in the cytosol, are directed to the chloroplast surface and imported into the organelle via the 'general import pathway' which includes the TOC (translocon of the outer chloroplast envelope) and TIC (translocon of the chloroplast inner envelope) machinery. The import process consumes energy which is provided by ATP and GTP hydrolysis. After entering the stroma the stromal processing peptidase (SPP) cleaves off the transit peptide and the protein is folded or further directed to the thylakoids (for review see Schleiff & Becker, 2011; Schwenkert et al, 2011; Stengel et al, 2007). Outer envelope proteins, lacking a cleavable transit peptide, are described to be spontaneously inserted into the membrane (Schleiff & Klosgen, 2001) while few other chloroplast proteins seem to use alternative import routes like yet unidentified translocation channels or transport via the endoplasmic reticulum and the Golgi apparatus (see review Jarvis, 2008).

1.3.1 The TOC complex

The TOC core complex is composed of the pore forming TOC75 import channel as well as the receptor proteins TOC34 and TOC159 (see review Schwenkert et al, 2011). The latter proteins are related GTPases, exposing their GTPase domains into the cytosol and enabling recognition and specific binding to transit peptides (Smith et al, 2004; Sveshnikova et al, 2000). Whereas TOC34 function seems to be restricted to preprotein binding, TOC159 facilitates entering of the preprotein into the TOC75 import channel. Both GTPases are regulated via phosphorylation and dimerization (Aronsson et al, 2007; Oreb et al, 2011). Another protein, TOC64, is dynamically and loosely associated to the TOC core complex. TOC64 contains a TPR

(tetratricopeptide) domain facing the cytosol. These repetitive domain structures are known to mediate protein-protein interactions and the assembly of multi-protein complexes (D'Andrea & Regan, 2003). Previous studies demonstrated that preproteins associated to the chaperone heat shock protein 90 (HSP90) bind to TOC64 via the interaction of HSP90 with the TPR domain of the receptor protein (Qbadou et al, 2006; Sohrt & Soll, 2000) (Fig. 1).

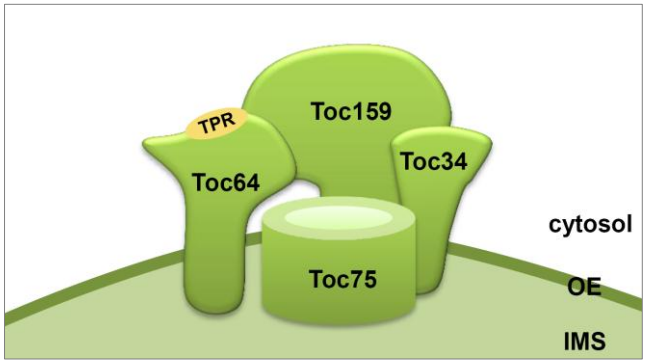


Figure 1: Composition of the TOC complex. The TOC core complex consists of the deeply embedded TOC75 translocation channel and the two receptor proteins TOC34 and TOC159, both possessing a cytosolic exposed GTPase domain. A further receptor protein TOC64 is loosely associated with the core complex, containing a TPR domain facing the cytosol.

1.3.2 Preprotein targeting to the TOC complex

Chloroplast preproteins have to be kept in an import competent state before entering the organelle. In the past decade, different chaperones were identified to interact with chloroplast preproteins in the cytosol. Binding of the heat shock protein 70 (HSP70) to unfolded preproteins prevents their aggregation in the cytosol (Mihara & Omura, 1996; Rial et al, 2000; Zhang & Glaser, 2002). Next to HSP70, other chaperones are able to bind to preproteins which use the TOC and TIC import machinery. The so called ‘guidance complex’, with an estimated size of 220 kDa, involves in addition to HSP70 and the preprotein, the chaperone 14-3-3 probably as a dimer (Fig. 2). Former studies observed that binding of this chaperone occurs at a phosphorylated 14-3-3 binding site within the transit peptide of the small subunit of rubisco (pSSU) and the oxygen evolving protein 23 (pOE23) (May & Soll, 2000; Waegemann & Soll, 1996). Although binding of 14-3-3 to the preprotein is not essential for the import process, import rates were increased 4-fold in 14-3-3-preprotein complexes (May & Soll, 2000). The kinase responsible for preprotein phosphorylation belongs to a

protein family of dual-specificity kinases which have been isolated from *Arabidopsis* (Martin et al, 2006). Recently, it could be shown that the kinase is important during organelle biogenesis when large amounts of proteins are strongly required for proper development of the plant (Lamberti et al, 2011b). Phosphorylated preproteins which are associated with the 14-3-3 chaperone are targeted to the import receptor TOC34 but have to be dephosphorylated before being imported via the general import pathway into the chloroplast (May & Soll, 2000; Waegemann & Soll, 1996).

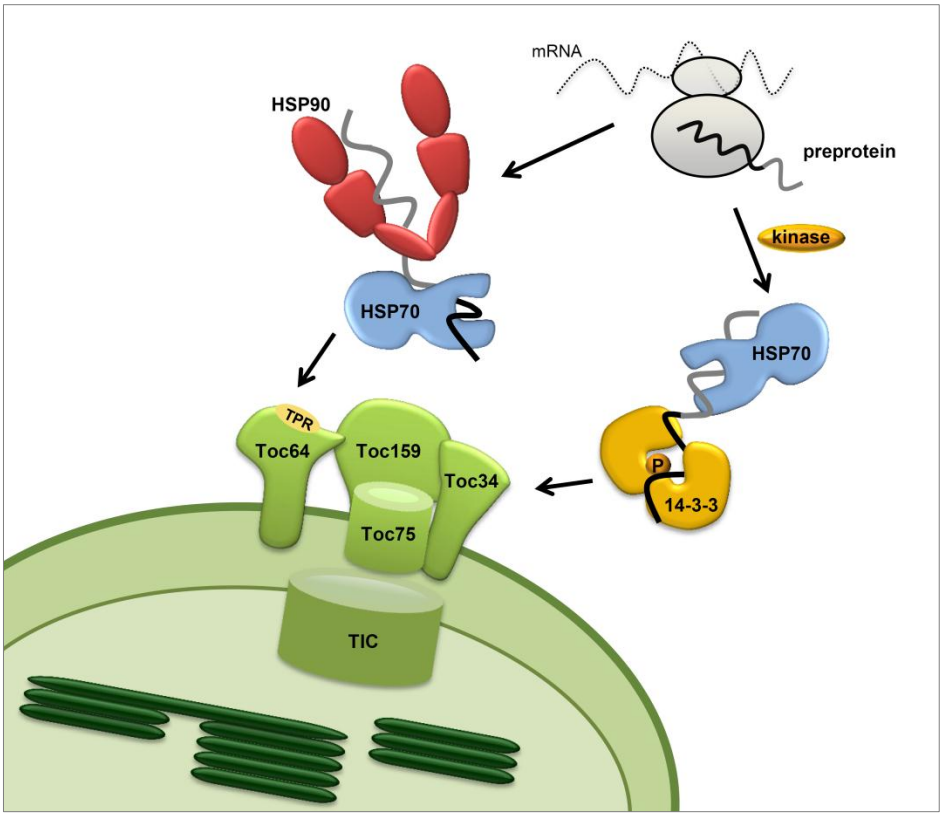


Figure 2: Cytosolic factors involved in preprotein targeting to chloroplasts. Nuclear-encoded chloroplast proteins are synthesized in the cytosol. HSP70 is binding to newly translated preproteins to prevent protein aggregation. Further chaperones assist in targeting of preproteins to the chloroplast membrane. Phosphorylation of transit peptides by a plant specific kinase leads to binding of 14-3-3. The so called 'guidance complex' is directed to the Toc34 receptor. Other preproteins are associated with HSP90 and targeted to Toc64, which serves as a docking protein for HSP90. Preproteins associated with HSP90 and TOC64 are released in a further step to allow import into the chloroplast.

Apart from 14-3-3, another chaperone, HSP90 was found to bind to the preproteins of plastocyanin (pPC1), oxygen evolving protein 33 (pOE33) and nucleotide transport protein 1 (pNTT1), resulting in a higher molecular weight complex of 300-440 kDa. HSP90 is not only binding to preproteins to prevent their aggregation in the cytosol but further serves as a docking protein for the TOC64 receptor (Fig. 2) (Qbadou et al,

2007; Qbadou et al, 2006). Therefore, a more distinct role for HSP90 in the targeting process of preproteins to the envelope membrane was postulated. However, further investigations demonstrated that HSP90 binding to preproteins is not essential, since *Arabidopsis* and *Physcomitrella* mutant plants lacking the TOC64 receptor do not show any defect in protein import (Aronsson et al, 2007; Rosenbaum Hofmann & Theg, 2005). These findings assume that the HSP90 associated preproteins pPC1, pOE23 and pNTT1 do not strongly require TOC64 as receptor protein. Nevertheless, interaction with the receptor might support import of HSP90 bound preproteins under certain developmental conditions. Lately, another chaperone, the small heat shock protein 17 (HSP17) was found to be involved in targeting of outer envelope proteins to the chloroplast surface together with the ankyrin rich protein A (AKR2A) (Bae et al, 2008; Kim et al, 2011; Zhang et al, 2010a).

1.4 Protein import into mitochondria

Mitochondrial preproteins are mainly imported post-translationally into the organelle using the TOM (translocase of outer membrane of mitochondria) complex, which serves as an entry gate for the organelle. Beside this translocation machinery further sorting occurs by different complexes located in the outer and inner membrane of mitochondria. Matrix proteins containing a cleavable targeting signal are imported via the TIM23/PAM complex (TIM: translocase of inner membrane of mitochondria; PAM: presequence-associated motor). After successful import the matrix processing peptidase (MPP) removes the cleavable targeting signal and the protein is folded. Inner membrane proteins are inserted via the OXA1 (oxidase assembly) or the TIM22 complex, whereas outer membrane proteins use the SAM complex (sorting and assembly machinery). Proteins destined for the intermembrane space (IMS) are assembled by the MIA complex (mitochondrial IMS import and assembly machinery). Import of most mitochondrial proteins is not only dependent on ATP hydrolysis but also requires the membrane potential (for review see Schleiff & Becker, 2011; Schmidt et al, 2010).

1.4.1 The TOM complex

The TOM complex is well investigated in model systems such as *Saccharomyces cerevisiae*. The complex comprises seven different subunits: TOM40, TOM22,

TOM20, TOM70, and the small TOM proteins TOM5, TOM6 and TOM7 (Fig. 3A). The import pore TOM40 is deeply embedded into the membrane and almost all preproteins are translocated via this channel protein. The central import receptor TOM22 is in a stable complex with the TOM40 import pore, which recognizes positively charged residues of the presequence (Schmidt et al, 2010; van Wilpe et al, 1999). Nevertheless, NMR studies revealed that mitochondrial presequences which form amphiphilic α-helices associate directly with TOM20 by binding to a hydrophobic pocket of the receptor protein (Saitoh et al, 2007). The third receptor protein TOM70 contains a cytosolic exposed TPR domain, which enables binding of HSC70 (heat shock cognate 70) and HSP90, both able to interact with preproteins (Fan et al, 2006; Young et al, 2003). Previously, it was shown that the TPR domain of TOM70 is further recognized by the cytosolic part of TOM20. Both proteins seem to interact until preproteins are bound to these receptors, indicating a regulatory function of the receptor-receptor interaction (Fan et al, 2011). The small TOM proteins TOM6 and TOM7 serve presumably as assembly and dissociation factor, respectively, whereas TOM5 mediates transitory contact with preproteins (Ryan et al, 2000; Sollner et al, 1989; van Wilpe et al, 1999).

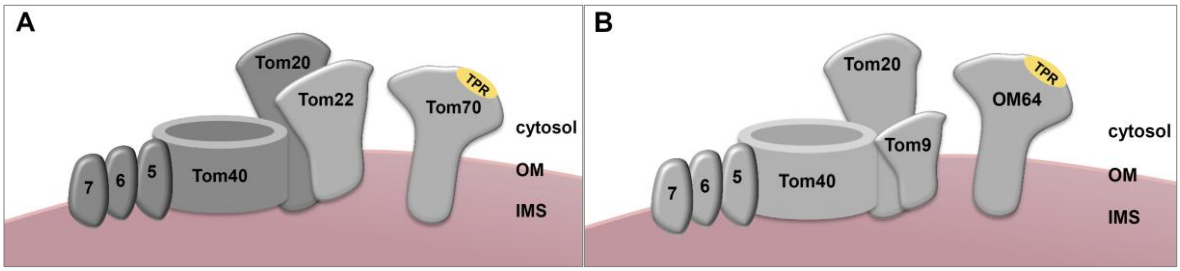


Figure 3: Composition of the TOM complex in different organisms. The TOM complex of mammals, yeast and plants comprises the TOM40 translocation channel, the small TOM proteins TOM5, TOM6 and TOM7 and the receptor protein TOM20. **(A)** Mammals and yeast possess two additional receptor proteins. TOM22 and TOM70, the latter exposes a TPR domain into the cytosol. **(B)** In plants TOM22 is replaced by TOM9. Also plants contain a TPR-containing receptor, OM64, which shows close similarity to the chloroplast receptor protein TOC64.

In contrast to yeast and mammals, plants lost the TOM70 receptor and the TOM22 receptor is replaced by TOM9, which lacks the cytosolic exposed receptor domain (Fig. 3B) (Carrie et al, 2010). Furthermore, *Arabidopsis* mitochondria contain an outer membrane protein, OM64, with 67% sequence identity to TOC64 in chloroplasts (Chew et al, 2004; Lister et al, 2007). Like TOM70 and TOC64, OM64 also possess a

TPR domain facing the cytosol (Chew et al, 2004). The OM64 receptor could not be found strongly associated with the import apparatus and is therefore thought to be just peripheral and dynamically attached to the TOM complex, as it is the case for TOC64 (Lister et al, 2007). The function of OM64 in mitochondrial protein import was demonstrated recently with *Arabidopsis* OM64 mutants, which showed a 30-40% decrease in import of the preprotein F_AD. Furthermore, direct interaction of F_AD with the receptor protein was proposed since specific inhibition of HSP90 with Geldanamycin in wild type *Arabidopsis* plants did not change F_AD import (Lister et al, 2007).

1.4.2 Preprotein targeting to the TOM complex

During the past years, different cytosolic factors binding to mitochondrial preproteins were identified using animal cytosol as a model system. Mitochondrial preproteins interact with a 70 kDa heat shock protein, HSC70, in order to maintain the preprotein in an import-competent and unfolded state (Mihara & Omura, 1996). Additional factors were observed to form complexes with mitochondrial preproteins including the protein factor MSF (mitochondrial stimulation factor) or HSP90 (Fan et al, 2006; Hachiya et al, 1993). HSP90 specifically interacts with inner membrane carrier proteins, which contain hydrophobic stretches and are in complex with HSC70. So far, three example proteins were described including the mammalian mitochondrial peptide transporter (PT), the adenine nucleotide transporter 2 (ANT) and the phosphate carrier protein (PiC) as well as its fungal homolog the ADP/ATP carrier (AAC). HSP90 as well as HSC70 interact with the TPR domain of the TOM70 receptor via a distinct amino acid motif found in both chaperones (Fan et al, 2006). In addition, co-chaperones of HSP70 including HSP40 related J domain proteins and HIP (HSP70 interacting protein) as well as the HSP90 co-chaperone p23 were identified to be associated with the chaperone-preprotein complex in reticulocyte lysate, next to co-chaperones interacting with both heat shock proteins such as HOP (HSP70/HSP90 organizing protein) and TPR2 (Bhangoo et al, 2007). Since MSF is a 14-3-3 protein it is obvious that preprotein transport to both organelles, chloroplast and mitochondria, might share some features concerning the involvement of cytosolic factors. Therefore the question arose whether cytosolic factors in plants might be different from their mammalian homologs, since plants have to differentiate between

chloroplasts and mitochondria. To date, only HSP70 was described to interact with plant mitochondrial preproteins (Zhang & Glaser, 2002).

1.5 Cytosolic chaperones involved in preprotein import

1.5.1 Heat shock protein 70 (HSP70)

The molecular chaperone HSP70 is the most prominent representative of the heat shock protein family ubiquitously found in pro- and eukaryotes. It plays a crucial role in stress protection and protein folding. HSP70 binds to hydrophobic stretches of nascent polypeptide chains, thus preventing misfolding and aggregation of the newly synthesized proteins (Young, 2010). The capability to bind and hydrolyze ATP enables the chaperone to undergo conformational changes which are necessary to bind and release client proteins (Hartl & Hayer-Hartl, 2009; Young, 2010). Whereas binding of HSP70 to cytosolic proteins leads to correct folding, preproteins destined for chloroplasts and mitochondria are kept in an import competent and at least a partly unfolded state (Hartl et al, 1992). An HSP70 binding motif was identified consisting of five hydrophobic amino acids flanked by positively charged residues on both sides, which is found in most targeting sequences of chloroplast and mitochondrial preproteins (Rudiger et al, 1997; Zhang & Glaser, 2002). Nevertheless, binding of HSP70 to chloroplast preproteins is not restricted to the targeting sequence, since it was also observed in mature proteins (May & Soll, 2000). In *Arabidopsis* 14 HSP70 isoforms exist of which six are predicted to be localized in the cytosol (Lin et al, 2001).

1.5.2 HSP90 and its co-chaperones

The heat shock protein 90, present in all bacteria and eukaryotes, is involved in the maturation, activation and maintenance of a diverse but specific set of client proteins (Prodromou, 2011). In bacteria, the protein is only expressed under stress condition, whereas in eukaryotes different isoforms exist which can either be stress induced or constitutively expressed. The active protein functions as a flexible dimer. Each monomer is composed of three different domains: an N-terminal ATPase domain, a middle domain and a C-terminal dimerization domain. The N-terminal domain displays a low intrinsic ATPase activity which enables the formation of different conformational states during nucleotide exchange. Besides other functions, HSP90 is

well investigated during maturation of steroid hormone receptors in mammals and yeast. A so called 'HSP90 cycle' was proposed including further co-chaperones or co-factors (Fig. 4) (Bracher & Hartl, 2006; Wandinger et al, 2008). Many of these factors contain TPR motifs which are recognized by the very C-terminus of HSP90 consisting of the amino acids MEEVD (Wandinger et al, 2008).

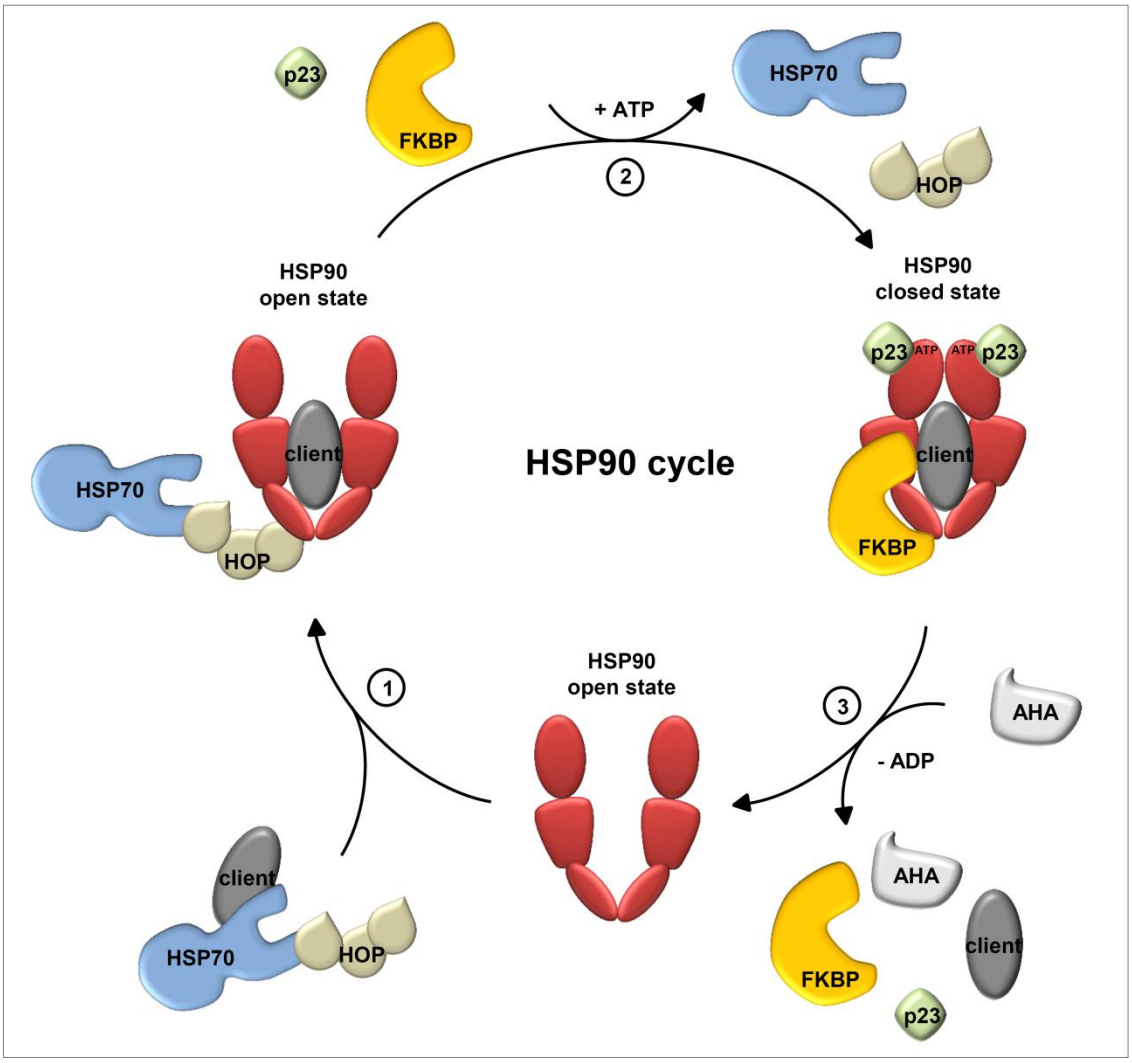


Figure 4: Dynamics of HSP90 and its co-chaperones (according to Bracher & Hartl, 2006). A client protein bound by HSP70 is transferred to the open conformation of a HSP90 dimer by the adaptor protein HOP (1). Binding of ATP to HSP90 leads to a closed HSP90 conformation, which is stabilized by the co-chaperones p23 and FKBP (2). Association of the co-chaperone AHA enhances the intrinsic ATPase activity of HSP90, resulting in an open conformation of HSP90 and the release of all co-chaperones and the mature client protein (3).

Initially, a client protein is handed over from HSP70 to a HSP90 dimer (in an open conformation). The transfer is mediated by the TPR-containing adaptor protein HOP which recognizes both chaperones specifically and simultaneously (Brinker et al, 2002). Upon ATP binding a conformational change takes place leading to a closed

conformation of HSP90 in which the client protein can be modified. Stabilization of the complex is achieved by the association of additional co-chaperones, p23 and a TPR-containing immunophilin (FKBP). A further conformational change is induced by binding of the co-chaperone AHA to the middle domain of HSP90. This co-chaperone strongly enhances the ATPase activity of HSP90, leading to an open conformation of the HSP90 dimer and to the release of the client protein (Bracher & Hartl, 2006). In contrast to HSP70, no binding motif for HSP90 could be identified so far.

In *Arabidopsis* four cytosolic isoforms are present with a sequence identity of 85-96% (Krishna & Gloor, 2001). HSP90.1 is expressed under stress conditions, whereas the other three isoforms are constitutively expressed. Recently three different isoforms of the chaperone were identified in wheat enabling further characterization (Wang et al, 2011).

1.5.3 14-3-3

14-3-3 proteins are a highly conserved protein family, ubiquitously found in eukaryotes, with a molecular mass of about 30 kDa. The protein is involved in different cellular processes such as cell cycle control, multiple signal transduction pathways or targeting of preproteins to organelles (Aducci et al, 2002; May & Soll, 2000). The active protein forms homo- or heterodimers and interacts in general with phosphorylated proteins possessing 14-3-3 binding motifs. So far, three types of 14-3-3 binding motifs were identified: 1) R.[^P]([ST])[^P]P, 2) R..[^P]([ST])[IVLM]. and 3) [RHK][STALV].([ST]).[PESRDIF], all containing a phosphorylated serine or threonine residue (see http://elm.eu.org/). However, exceptions were observed in which substrate binding was independent of phosphorylation (Chevalier et al, 2009). In addition, binding affinity of 14-3-3 was shown to be 30-fold higher in phosphopeptides containing two 14-3-3 binding sites (Yaffe et al, 1997). In *Arabidopsis*, 15 different isoforms exist in the cytosol, and interestingly, some of them were also detected in mitochondria and chloroplast even though they do not contain targeting signals for these organelles (Aducci et al, 2002; Bunney et al, 2001).

1.6 Aim of this work

The major aim of this work was to investigate the role of cytosolic factors during preprotein transport to chloroplasts and plant mitochondria.

It should be analyzed whether binding of chloroplast preproteins to different cytosolic chaperones like 14-3-3 and HSP90 is restricted to few example proteins or whether chaperone binding is a more general phenomenon during preprotein import into chloroplasts. Furthermore, properties of preproteins with different chaperone binding capacity should be characterized using bioinformatical tools in order to determine distinct protein features pointing to a prediction of chaperone preference. HSP90 binding mechanisms should be closer examined experimentally using chimeric preprotein constructs to find a certain binding area for the chaperone. A further aim was to identify novel components associated within the HSP90-preprotein complexes, since the previously determined complex size of about 300-440 kDa indicated the involvement of additional factors.

To date, cytosolic factors playing a role during mitochondrial preprotein delivery in plants have not been analyzed. Hence, identification of chaperone binding factors of plants as well as comparison of those with factors found in fungi or mammalian systems was a challenging question. Mitochondrial preproteins should also be examined in respect of possible phosphorylation events within the presequence and its consequences in import behavior.

2 Material and methods

2.1 Material

2.1.1 Chemicals and membranes

All used chemicals were received in p.a. quality from the following manufacturers: Applichem (Darmstadt), Fluka (Steinheim), GE Healthcare (Freiburg), Invitrogen (Karlsruhe), Merck (Taufkirchen) or Sigma (Taufkirchen). The PVDF transfer membrane for western blot was used form Zefa (Harthausen), the blotting paper from Macherey-Nagel (Düren).

2.1.2 Molecular weight and DNA electrophorese size marker

For determination of protein sizes marker the “MW-SDS-70L” (Sigma) was used resulting in the following sizes: 66, 45, 36, 29, 24, 20 and 14 kDa. For size determination of electrophoretical separated DNA fragments a λ-DNA, which was digested by EcoRI and HindIII was used leading to the fragments of 21 226, 5 148, 4 973, 4 268, 3 530, 2 027, 1 904, 1 584, 1 375, 947, 831 and 564bp.

2.1.3 Enzymes and Kits

All commonly used enzymes like DNA or RNA polymerases, ligases, restriction enzymes, RNAse inhibitors and other enzymes were obtained from the following companies: Eppendorf (Hamburg), Genecraft (Köln), Fermentas (St. Leon-Rot) Promega (Madison, USA) and New England Biolabs (Frankfurt). Cellulase Onozuka R10 and Macerozym R10 were obtained from Promega (Madison, USA) and Yakult Hosha, respectively.

DNA fragments from agarose gels or PCR fragments were purified using the NucleoSpin® Extract II Kit from Macherey-Nagel (Düren). Plasmid purification from *E.coli* was accomplished via either Qiaprep® Spin Miniprep Kit, NucleoBond® PC100 Midi Kit or Plasmid Maxi Kit (Qiagen, Hilden).

2.1.4 *In vitro* translation system

Translation of radiolabeled, recombinant proteins was done in cell free translation systems. Either homemade wheat germ lysate or wheat germ lysate and reticulocyte lysate from Promega were used.

2.1.5 Chromatography media and columns

Co-immunoprecipitation was performed using Protein Sepharose A CL-4B (GE Healthcare). For protein purification or pull down assays of proteins containing a histidine-tag, Ni-NTA beads (GE Healthcare) were used. Antibody purification was done using CNBR^{-} Sepharose 4B (GE Healthcare). Further purification methods were performed with the following columns: anion exchange column Q FF 1ml, hydrophobic interaction column HiTrap Phenyl HP 1ml and size exclusion chromatography columns Superdex 200, Superdex 75 (GE Healthcare) as well as TSKgel SW or TSKgel SWXL from Tosoh Bioscience (Stuttgart) . The latter two were used for separation of protein complexes out of wheat germ lysate. For wheat germ preparation and buffer exchange PD10 columns (GE Healthcare) were used. Buffer exchange for translated proteins was carried out with Micro Bio-Spin® chromatography columns (Bio-Rad).

2.1.6 Plant material and growth

Pea (*Pisum sativum*) seeds of the sort “Salamanca” were grown under long day conditions (14 h light/10 h dark). *Arabidopsis thaliana*, ecotype Columbia, wild type plants were cultivated under long day condition (16 h light, 21°C; 8 h dark, 16°C). Wheat (*Triticum aestivum*) plants were grown under greenhouse conditions.

2.1.7 Bacteria strains

For plasmid propagation *E.coli* TOP10 cells (Invitrogen, Karlsruhe) were used. Overexpression of proteins was performed using either *E.coli* BL21(DE3)pLysS (Novagen, Madison, USA) or RIPL (BL21-CodonPlus(DE3)-RIPL strain) cells.

2.1.8 Vectors and clones

All vectors and clones are listed in Table 1 comprising in addition the corresponding accession number and purpose. Constructs clones in pDEST14 and pGEM3zf+ are gifts provided by J. Whelan.

Table 1: List of all used clones, their accession numbers and purpose

Gen	Vector	Accession	Purpose	Restriction sites for cloning	Lin.	Loc.
14-3-3	pET21d+	P.s.	O			c
AHA1	PCR Blunt	T.a.		no restriction site		c
AHA1	pET21d+	T.a.	O	NheI/XhoI		c

Gen	Vector	Accession	Purpose	Restriction sites for cloning	Lin.	Loc.
AHA1	pENTR-D-TOPO	AT3G12050				c
AHA1	p2GWF7	AT3G12050	GFP			c
FKBP73	PCR Blunt	T.a.		NheI/NotI		c
FKBP73	pET21d+	T.a.	O	NheI/NotI		c
HOP	PCR Blunt	T.a.		no ATG, no stop		c
HOP	pET21d+	T.a.	O	NheI/XhoI		c
HOP1	pDONR207	AT1G62740				c
HOP1	pB7FWG2	AT1G62740	GFP			c
HOP2	pDONR207	AT1G12270				c
HOP2	pB7FWG2	AT1G12270	GFP			c
HOP3	pDONR207	AT4G12400				c
HOP3	pB7FWG2	AT4G12400	GFP			c
HSP70	pET21d+	T.a.	O	NheI/EcoRI		c
HSP90	pET21d+	T.a.	O	NheI/NotI		c
p23	PCR Blunt	T.a.		NheI/XhoI		c
p23	pET21d+	T.a.	O	NheI/XhoI		c
p23-1	pENTR-D-TOPO	AT4G02450				c
p23-1	pB7FWG2	AT4G02450	GFP			c
p23-2	pDONR207	AT3G03773				c
p23-2	pB7FWG2	AT3G03773	GFP			c
p6PGDH	pSP65	AT1G17650	T		XbaI	ch
pAGT2	pF3A	AT4G39660	T	SgfI/PmeI	XbaI	m
pAPE1	pF3A	AT5G38660	T	SgfI/PmeI	XbaI	ch
pAPE1mSSU	pF3A	A.t.	T	SgfI/PmeI	XbaI	ch
pAPO1	pF3A	AT1G64810	T	SgfI/PmeI	XbaI	ch
pAtpD	pF3A	AT4G09650	T	SgfI/PmeI	EcoRI	ch
pAtpDmSSU	pF3A	A.t.	T	SgfI/PmeI	EcoRI	ch
pATPR	pF3A	AT4G21210	T	SgfI/PmeI	XbaI	ch
pCAB1	pF3A	AT1G29930	T	SgfI/PmeI	XbaI	ch
pCAO	pF3A	AT1G44446	T	SgfI/PmeI	SalI	ch
pClp2	pF3A	AT1G12410	T	SgfI/PmeI	XbaI	ch
pClpP	pF3A	AT1G11750	T	SgfI/PmeI	EcoRI	ch
pDAG	pDEST14	AT1G32580	T			m
pDHAR3	pF3A	AT5G16710	T	SgfI/PmeI	SalI	ch
pDHARmSSU	pF3A	A.t.	T	SgfI/PmeI	SalI	ch
pDIC	pF3A	AT2G22500	T	SgfI/PmeI	XbaI	m
pDPE1	pF3A	AT5G64860	T	SgfI/PmeI	SalI	ch
pDSP4	pF3A	AT3G52180	T	SgfI/PmeI	XbaI	ch
pDTC	pF3A	AT5G19760	T	SgfI/PmeI	XbaI	m
pEGY1	pF3A	AT5G35220	T	SgfI/PmeI	XbaI	ch
pEMB1241	pF3A	AT5G17710	T	SgfI/PmeI	SalI	ch
pFd2	pSP65	AT1G60950	T		EcoRI	ch
pFer1	pF3A	AT5G01600	T	SgfI/PmeI	XbaI	ch

Gen	Vector	Accession	Purpose	Restriction sites for cloning	Lin.	Loc.
pFNRL1	pSP65	AT5G66190	T		SalI	ch
pFOLT1	pF3A	AT5G66380	T	SgfI/PmeI	XbaI	ch
pFtsZ	pF3A	AT5G55280	T	SgfI/PmeI	HindIII	ch
pGAPB	pF3A	AT1G42970	T	SgfI/PmeI	XbaI	ch
pGPS1	pF3A	AT4G36810	T	SgfI/PmeI	XbaI	ch
pGPT1	pF3A	AT5G54800	T	SgfI/PmeI	XbaI	ch
pGS2	pF3A	AT5G35630	T	SgfI/PmeI	XbaI	ch
pHCF101	pF3A	AT3G24430	T	SgfI/PmeI	XbaI	ch
pHO1	pF3A	AT2G26670	T	SgfI/PmeI	XbaI	ch
pIM	pF3A	AT4G22260	T	SgfI/PmeI	SalI	ch
pIVD	pF3A	AT3G45300	T	SgfI/PmeI	SalI	m
pK15M2	pDEST14	AT3G15000	T			m
pLHCA5	pF3A	AT1G45474	T	SgfI/PmeI	XbaI	ch
pNdhM	pF3A	AT4G37925	T	SgfI/PmeI	XbaI	ch
pNFU2	pF3A	AT5G49940	T	SgfI/PmeI	XbaI	ch
pNFU4	pF3A	AT3G20970	T	SgfI/PmeI	XbaI	m
pNFU4	pET21a+	AT3G20970	O			m
pNFU4 S10/12A	pF3A	AT3G20970	T	SgfI/PmeI	XbaI	
pNFU4 S10/12D	pF3A	AT3G20970	T	SgfI/PmeI	XbaI	
pNFU4mSSU	pET21a+	A.t. / N.t.	O			m
pPAC	pF3A	AT2G48120	T	SgfI/PmeI	SalI	ch
pPAC	pET21d+	AT2G48120	O			ch
pPC1	pF3A	AT1G76100	T	SgfI/PmeI	XbaI	ch
pPC1	pET21d+	AT1G76100	O	NheI/XhoI		ch
pPC1mSSU	pF3A	A.t.	T	SgfI/PmeI	XbaI	ch
pPetC	pF3A	AT4G03280	T	SgfI/PmeI	SalI	ch
pPGRL1.2	pF3A	AT4G11960	T	SgfI/PmeI	EcoRI	ch
pPORA	pF3A	AT4G27440	T	SgfI/PmeI	XbaI	ch
pPOT	pF3A	AT5G13400	T	SgfI/PmeI	XbaI	ch
pPPT1	pF3A	AT5G33320	T	SgfI/PmeI	XbaI	ch
pPRAT4	pGEM3zf+	AT3G25120	T			m
pPsaE1	pF3A	AT4G28750	T	SgfI/PmeI	XbaI	ch
pPsaK	pF3A	AT1G30380	T	SgfI/PmeI	XbaI	ch
pPsb29	pF3A	AT2G20890	T	SgfI/PmeI	SalI	ch
pPsbS	pF3A	AT1G44575	T	SgfI/PmeI	XbaI	ch
pPsbT	pF3A	AT3G21055	T	SgfI/PmeI	XbaI	ch
pPsbX	pF3A	AT2G06520	T	SgfI/PmeI	XbaI	ch
pRPL28	pF3A	AT2G33450	T	SgfI/PmeI	XbaI	ch
pSMTR	pF3A	AT2G30200	T	SgfI/PmeI	XbaI	ch
pSSU	pF3A	AAA34116	T	SgfI/PmeI	XbaI	ch
pSSU	pET21d+	N.t.	O			ch
pSSUmAPE1	pF3A	A.t.	T	SgfI/PmeI	XbaI	ch
pSSUmAtpD	pF3A	A.t.	T	SgfI/PmeI	EcoRI	ch

Gen	Vector	Accession	Purpose	Restriction sites for cloning	Lin.	Loc.
pSSUmDHAR3	pF3A	A.t.	T	SgfI/PmeI	SalI	ch
pSSUmPC1	pF3A	A.t.	T	SgfI/PmeI	XbaI	ch
pTIM17-1	pGEM 3zf+	AT1G20350	T			m
pTIM22-2	pGEM3zf+	AT3G10110	T			m
ROF1	pDONR207	AT3G25230				c
ROF1	pB7FWG2	AT5G48570	GFP			c
ROF2	pDONR207	AT3G25230				c
ROF2	pB7FWG2	AT5G48570	GFP			c

lin.: enzymes used for linearization; loc.: localization; A.t.: *Arabidopsis thaliana*; N.t.: *Nicotiana tabacum*; P.s.: *Pisum sativum*; T.a.: *Triticum aestivum*; T: *in vitro* transcription/translation; O: overexpression; GFP: *in vivo* GFP-localization

2.1.9 Oligonucleotides

All used oligonucleotides were ordered at Metabion (Martinsried) and are listed in Table 2.

Table 2: Oligonucleotides used for cloning

Primer	5'-3' oligonucleotide sequence	purpose
AGT2-Flexi-for	GAT CGC GAT CGC CAT GGC GTT ACA AAG GCA AC	pF3A
AGT2-Flexi-rev	CTG AGT TTA AAC TCA CAA CCT GGA GAT GGA	pF3A
AHA1 NheI noATG for	CGA TGC TAG CGC GAA GTT CGG CGA GGG CG	pCR blunt/pET21d+
AHA1 TOPO ATG for	CAC CAT GGC AAA GTT CGG TGA AG	pENTR-D-TOPO
AHA1 TOPO no Stop rev	TAT TCC AAA TCC GAA AAC TG	pENTR-D-TOPO
AHA1 XhoI noSTOP rev	CGA TCT CGA GGA CCC CAA AAC CGA ACA CTG	pCR blunt/pET21d+
APE1-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG AGA GGT AGA AGA TAC AG	pF3A
APE1-Flexi-for	GTG CGC GAT CGC CAT GGG ATC TAT AAC G	pF3A
APO1-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG TGC GAC CAT GTC	pF3A
APO1-Flexi-for	GAT CGC GAT CGC CAT GTT GCA GGA ATC TGG GAA AGT AAT C	pF3A
AtpD--Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG AGT AGC TAA TTG AAT CTC AC	pF3A
AtpD-Flexi-for	GGC CGC GAT CGC CATGGC GTC TCT TCA ACA AAC	pF3A
ATPR1-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG GTA GCT TTT AGA GAT GCG AG	pF3A
ATPR1-Flexi-for	GTG CGC GAT CGC CAT GGC TTT GCT CTC G	pF3A
CLP2-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG GCC TAG CCC TGC GCT TTC GTC	pF3A
CLP2-Flexi-for	GTG CGC GAT CGC CAT GGC GGT CTC GTT TAA TAC	pF3A
ClpP-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG GTA TTC TGT TTC	pF3A
ClpP-Flexi-for	GTG CGC GAT CGC CAT GGC GGG TTT A	pF3A

Primer	5‘-3‘ oligonucleotide sequence	purpose
DHAR3-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG ACC CAT AAC CTT TGG XXC	pF3A
DHAR3-Flexi-for	GTG CGC GAT CGC CAT GAT AAG XCT TAG GTT TC	pF3A
DIC1-Flexi-for	GTG CGC GAT CGC CAT GGG TCT AAA GGG TTT TGC TG	pF3A
DIC1-Flexi-rev	CTG AGT TTA AAC AAA GTC ATA GTC CTT GAA C	pF3A
DPE1-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG AAG CCG TCC GTA CAA TGA	pF3A
DPE1-Flexi-for	GTG CGC GAT CGC CAT GTC GAT TCT ACT TAG GCC GTC	pF3A
DSP4-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG AAC TTC TGC CTC AGA AC	pF3A
DSP4-Flexi-for	GTG CGC GAT CGC CAT GAA TTG TCT TCA GAA TC	pF3A
DTC-Flexi-for	GTG CGC GAT CGC CAT GGC GGA AGA GAA GAA AGC TC	pF3A
DTC-Flexi-rev	CTG AGT TTA AAC CAT ACC AAT CTT CTT TTG	pF3A
EGY1-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG AAA TGT GGT TAC AAG CCC TAT G	pF3A
EGY1-Flexi-for	GTG CGC GAT CGC CAT GGG GAC TCT CAC CAG CGT C	pF3A
EMB1241-Flexi-for	GTG CGC GAT CGC CAT GGC CGG TCT ACT CAA AAC GCC GTC	pF3A
EMB1241-Flexi-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG AGA TGA AGA TGA TTC TTC	pF3A
FKBP-tricitum-NotI rev	CGA TGC GGC CGC AGC TTT GCT TTC TTC TGC	pCR blunt/pET21d+
FKBP-triticum-no ATG for	CGA TGC TAG CGA CGA CGA CTT CGA CAT	pCR blunt/pET21d+
FOLT1-Flexi-for	GAT CGC GAT CGC CAT GGC GGC GTC GTG GCA GTG	pF3A
FOLT1-Flexi-rev	CTG AGT TTA AAC CTA ATC TTT TGT TGT TGG	pF3A
FtsZ-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG GAA GAA AAG TCT	pF3A
FtsZ-Flexi-for	GTG CGC GAT CGC CAT GGC GAT AAT TC	pF3A
GapB-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG GTC ATA GAC TTT GCA TTC CTC	pF3A
GapB-Flexi-for	GTG CGC GAT CGC CAT GGC CAX ACA TGC AGC TCT C	pF3A
GPS1-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG GTT CTG TCT ATA GGC AAT G	pF3A
GPS1-Flexi-for	GTG CGC GAT CGC CAT GGC TTC AGT GAC TC	pF3A
GPT1-Flexi-for	GAT CGC GAT CGC CAT GGT TTT ATC GGT GAA GC	pF3A
GPT1-Flexi-rev	CTG AGT TTA AAC TCA GAG CTT TGC CTG GGA ATA C	pF3A
GS2-Flexi-for	GTG CGC GAT CGC CAT GGC TCA GAT CTT AGC AGC TTC	pF3A
GS2-Flexi-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG AAC ATT CAA AGA AAG CTT TTG	pF3A
HCF101-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG GAC TTC GAC TG	pF3A
HCF101-Flexi-for	GTG CGC GAT CGC CAT GCC GCT TCT T	pF3A
HO-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG GGA CAA TAT GAG ACG	pF3A
HO-Flexi-for	GTG CGC GAT CGC CAT GGC GTA TTT AGC TC	pF3A

Primer	5‘-3‘ oligonucleotide sequence	purpose
HOP1 attB ATG for	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CAT GGC G	pDONR207
HOP1 attB no Stop rev	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TTT CAT C	pDONR207
HOP2 attB ATG for	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CAT GGC A	pDONR207
HOP2 attB no Stop rev	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TTT CAT C	pDONR207
HOP3 attB ATG for	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CAT GGC G	pDONR207
HOP3 attB no Stop rev	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC GAA CAC A	pDONR207
HOP-tricitum-no ATG Nhe	CGA TGC TAG CGC CGA CGA GGC GAA GGC	pCR blunt/pET21d+
HOP-tricitum-XhoI rev	CGA TCT CGA GTC TCG TTT GGA CTA TTC CAG	pCR blunt/pET21d+
IM-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG ACT TGT AAT GGA TTT C	pF3A
IM-Flexi-for	GTG CGC GAT CGC CAT GGC GGC GAT TTC AG	pF3A
IVD-Flexi-for	GAT CGC GAT CGC CAT GCA GAG GTT TTT CTC CGC	pF3A
IVD-Flexi-rev	CTG AGT TTA AAC CTA TTC TTC TTT GAA AAG C	pF3A
LHCA5-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG AGA TGT AGA GGT GAA GAG GGT TTG	pF3A
LHCA5-Flexi-for	GTG CGC GAT CGC CAT GGC CGT AGT TTT ACG TG	pF3A
mSSU for	ATG CAG GTG TGG CCA CCA ATT AAC AA	pF3A chimera
NdhM-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG AGC GTC CTC TTG AGG	pF3A
NdhM-Flexi-for	GTG CGC GAT CGC CAT GGT TGC AGC ATT CTC	pF3A
NFU2-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG TAT AAG TTG AAC AGC TG	pF3A
NFU2-Flexi-for	GTG CGC GAT CGC CAT GCA ATT GCT GAC GC	pF3A
NFU4 S10/12A for	CGA ATC GGA GGT CGC AAG GTC GTT TCT G	Mutagenesis
NFU4 S10/12A rev	GAT TGC GAG GCT TGT AAC TGC TCT AGC GCG AAT CGG AGG	Mutagenesis
NFU4 S10/12D rev	CCT CCG ATT CGC GCT AGA GCA GCT ACA AGC CTC GCA A	Mutagenesis
NFU4-Flexi-for	GAT CGC GAT CGC CAT GAA AGG GAT TGC GAG GC	pF3A
NFU4-Flexi-rev	CTG AGT TTA AAC CTA CTC TAC TCT CAT CTC TC	pF3A
p23-1 TOPO ATG for	CAC CAT GAG TCG TCA TCC TGA AG	pENTR-D-TOPO
p23-1 TOPO no Stop rev	CTT GTC TTC CTT AAC AGA TG	pENTR-D-TOPO
p23-2 attB ATG for	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CAT GAG T	pDONR207
p23-2 attB no Stop rev	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC CTT GTT T	pDONR207
p23-tricitum-no ATG Nhe	CGA TGC TAG CAG TCG CCA CCC GAG CAC TAA G	pCR blunt/pET21d+
p23-tricitum-XhoI rev	CGA TCT CGA GTG GCT TTG CTT CTT CAC CAG	pCR blunt/pET21d+
PAC-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG CCA CTT CAA GTT GAG GGC T	pF3A
PAC-Flexi-for	GAT CGC GAT CGC CAT GGC GGC GAC GTC GCT	pF3A
pAPE1(32)mSSU(58)	TTG TTA ATT GGT GGC CAC ACC TGC ATC GAA	pF3A chimera

Primer	5‘-3‘ oligonucleotide sequence	purpose
rev	ACG ACG G	
PC1-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG CTT GAC GGT GAG TTT C	pF3A
PC1-Flexi-for	GAT CGC GAT CGC CAT GGC CGC AAT TAC AT	pF3A
PC1-no ATG NheI for	CGA TGC TAG CGC CGC AAT TAC ATC AGC TAC	pET21d+
PC1-XhoI rev	CGA TCT CGA GCT TGA CGG TGA GTT TCC CAA C	pET21d+
pCAO-Flexi-for	GTG CGC GAT CGC CAT GAA CGC CGC CGT GTT TAG	pF3A
pCAO-His6-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG GCC GGA GAA AGG T	pF3A
pDHAR(42)mSSU(58) rev	TTG TTA ATT GGT GGC CAC ACC TGC ATC ATT GTA ACA A	pF3A chimera
PETC-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG AGA CCA CCA TGG	pF3A
PETC-Flexi-for	GTG CGC GAT CGC CAT GGC GTC CTC ATC CCT TTC C	pF3A
PGRL1.2-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG AGC TTT CCC TCC	pF3A
PGRL1.2-Flexi-for	GTG CGC GAT CGC CAT GGC TTT TAC TC	pF3A
PorA-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG GGC CAA GCC TAC G	pF3A
PorA-Flexi-for	GTG CGC GAT CGC CAT GGC CCT TCA AGC TGC TTC	pF3A
pPC1(66)mSSU(58) rev	TTG TTA ATT GGT GGC CAC ACC TGC ATT AAA ACG ATC G	pF3A chimera
pPsb29(67)mSSU(45) rev	TTG TTA ATT GGT GGC CAC ACC TGC ATT ACA GGA GGC A	pF3A chimera
PPT1-Flexi-for	GAT CGC GAT CGC CAT GCA AAG CTC CGC CGT ATT C	pF3A
PPT1-Flexi-rev	CTG AGT TTA AAC TTA AGC AGT CTT TGG CTT TG	pF3A
PsaE1-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG AGC TGC AAC TTC	pF3A
PsaE1-Flexi-for	GTG CGC GAT CGC CAT GGC GAT GAC G	pF3A
PsaK-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG AAT AGC ACC AAT	pF3A
PsaK-Flexi-for	GTG CGC GAT CGC CAT GGC TAG CAC T	pF3A
Psb29-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG AGA TTT CCG TTC AAC	pF3A
Psb29-Flexi-for	GTG CGC GAT CGC CAT GGC TGC AAC TGC	pF3A
PsbS-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG GCT TTC TTC ACC	pF3A
PsbS-Flexi-for	GTG CGC GAT CGC CGC TCA AAC CAT GCT GC	pF3A
PsbT1-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG GTA GCG GCA GAT	pF3A
PsbT1-Flexi-for	GTG CGC GAT CGC CAT GGC ATC GAT GA	pF3A
PsbX-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG GGT TCT CTT GAC	pF3A
PsbX-Flexi-for	GTG CGC GAT CGC CAT GGC TTC TAC C	pF3A
pSSUmAPE1(33) for	CAA CGG CGG AAG AGT GCA ATG CTC GTC CTC ACA ACC G	pF3A chimera
pSSUmDHAR(43) for	CAA CGG CGG AAG AGT GCA ATG CGC GAC GGC GGC GAG T	pF3A chimera
pSSUmPC1(67) for	CAA CGG CGG AAG AGT GCA ATG CGC TGG AAA	pF3A chimera

Primer	5‘-3‘ oligonucleotide sequence	purpose
	TGC GAT G	
pSSUmPsb29(68) for	CAA CGG CGG AAG AGT GCA ATG CTC AGA GAC AAA GTC G	pF3A chimera
Rof attB ATG for	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CAT GGA A	pDONR207
Rof1 attB for	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CAT GGA T	pDONR207
Rof1 attB no Stop rev	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TTC CTT A	pDONR207
Rof2 attB no Stop rev	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TGC CTT G	pDONR207
RPL28-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG ATT TCT TGC GAA GAT	pF3A
RPL28-Flexi-for	GTG CGC GAT CGC CAT GAC GAC AAT GGC GAC	pF3A
SMTR-Flexi his-rev	CTG AGT TTA AAC GTG GTG GTG GTG GTG GTG AGC ACT GAT GTT TTC GAA AC	pF3A
SMTR-Flexi-for	GTG CGC GAT CGC CAT GCG TTC ACT GCT TCA C	pF3A
SSU Tabak-Flexi-for	GTG CGC GAT CGC CAT GGC TTC CTC AGT TCT TTC	pF3A
SSU Tabak-Flexi-rev	CTG AGT TTA AAC GTA GCT TCT GGC TTG TAG	pF3A
TP SSU Tabak rev	GCA TTG CAC TCT TCC GCC GTT G	pF3A chimera

Standard oligonucleotides for sequencing are listed in Table 3.

Table 3: Oligonucleotides used for sequencing

Primer	5‘-3‘ oligonucleotide sequence
35S-Prom-for	CAA TTT ACT ATT CTA GTC G
35S-Promotor Gateway	GTT CAT TTC ATT TGG AGA
35S-Term-rev	TGC GGA CTC TAG CAT GGC CG
GFP-rev	CTC GCC GGA CAC GCT GAA CTT G
M13 forward20	GTA AAA CGA CGG CCA GT
M13 reverse	GGA AAC AGC TAT GAC CAT G
pDONR207 for	TCG CGT TAA CGC TAG CAT GGA TCT
pDONR207 rev	GTA ACA TCA GAG ATT TTG AGA CAC
pF3A-T7-for	CGA CTC ACT ATA GCA GTG AAG
pF3A-T7-rev	CTC AAG ACC CGT TTA GAG G
pSP65 for	CAC ATA CGA TTT AGG TGA CAC
pSP65 rev	CAG CTA TGA CCA TGA TTA CGC
T7 Prom	TAA TAC GAC TCA CTA TAG G
T7 Term	GCT AGT TAT TGC TCA GCG G

2.1.10 Antisera

Antisera were produced by Biogenes GmbH (Berlin), ordered from different companies or gifts from J. Meurer and A. Breiman as described in Table 4.

Table 4: Antibodies used for co-immunoprecipitation experiments and western blot analysis

Antibody	Clonality	Species	dilution	Source
αHSP70 (3979)	polyclonal	rabbit	1:1000	Laboratory of J. Soll
αHSP90 (4180)	polyclonal	rabbit	1:1000	Laboratory of J. Soll
α14-3-3 (5467)	polyclonal	rabbit	1:1000	Laboratory of J. Soll
αpPC1	polyclonal	rabbit	1:1000	J. Meurer and Laboratory of J. Soll
αFKBP73 (6946)	polyclonal	rabbit	1:1000	A. Breiman and Laboratory of J. Soll
αAHA (7259)	polyclonal	rabbit	1:1000	Laboratory of J. Soll
αp23 (6949)	polyclonal	rabbit	1:1000	Laboratory of J. Soll
αrabbit	peroxidase coupled	goat	1:20 000	Sigma

2.1.11 Accession numbers

Accession numbers of proteins used in experiments are listed in Table 1. Accession numbers of proteins in sequence alignments are as follows: NP001151932 (HOP *Z. mays*), HM998695 (HOP *T.aestivum*), At1g12270 (AtHOP-1), At1g62740 (AtHOP-2), At4g12400 (AtHOP-3), GmHOP (X79770), HsHOP (M86752) and ScHOP (M28486), 3023751 (FKBP73 *T.aestivum*). Accession numbers of proteins used in 14-3-3 motif analysis are listed in appendix 1 and 2.

2.1.12 Specialized laboratory equipment and software

Graphs and figures were illustrated using GraphPad Prism, Adobe Photoshop CS4 and Microsoft Powerpoint. Protein alignments were performed with AlignX/ClustalW (Invitrogen, Karlsruhe). Blast searches and analysis of conserved protein domains were carried out using NCBI Blast (Altschul et al, 1990) and Prosite (Hulo et al, 2006). Protein motif searches were performed using the ELM server (http://elm.eu.org/). Proteins located within mitochondria used for 14-3-3 binding site analysis were selected from PPDB database (http://ppdb.tc.cornell.edu/). Prediction of transit peptides and presequences was performed with ChloroP (Emanuelsson et al, 1999) and TargetP (Emanuelsson et al, 2007).

2.2 Methods

General methods which are not listed in the following part were carried out as described in Sambrook et al. (1989).

2.2.1 DNA-cloning

DNA clones were created using various cloning strategies. PCR was performed either with cDNA or plasmid DNA templates according to (Mullis & Faloona, 1987). For DNA amplification, the proof-reading polymerase Phusion (Finnzymes) was used according to the manufacturer's instructions. Length of elongation as well as annealing temperature during PCR was adapted for each construct. After PCR obtained DNA fragments were excised from 1% agarose gels and purified using the NucleoSpin® Extract II Kit (Macherey-Nagel).

Primers created for cloning DNA into pET or pF3A vectors contained the appropriate restriction sites (see Table 1). After digestion of vectors and PCR products, DNA was once more purified with the NucleoSpin® Extract II Kit followed by ligation of vector and PCR fragment which was carried out for 1 h at room temperature using T4 ligase (Fermentas). Transformation of the generated plasmids was performed in TOP10 cells. Cells were plated onto LB-media containing the appropriate antibiotic and incubated at 37°C over-night. Single colonies were tested for plasmid uptake by PCR using Taq polymerase (Bioron) and primers for vector and insert. Positive colonies were then inoculated over-night in liquid LB-media, plasmids were purified and the full-length insert was sequenced by the "Sequencing Service" of the department of biology I of the LMU Munich, Genomics Service Unit (GSU), using 100 ng plasmid and a suitable vector primer.

Chimeric constructs were generated by overlap PCR, using appropriate oligonucleotides fusing the desired gene fragments.

To introduce point mutations, site directed mutagenesis PCR was performed using the full-length plasmid and suitable primer containing the mutation. Denaturation temperature was increased and elongation time prolonged depending on the plasmid size.

Cloning of DNA fragments by homologous recombination was performed using the GATEWAY-system (Invitrogen) including following vectors: ENTR-D-TOPO,

pDONOR207, pB7FWG2 and p2GWF7. The cloning was performed as described by the manufacturer.

2.2.2 RNA isolation from plants

RNA was extracted out of green tissues of *Arabidopsis* or wheat using the RNeasy® Plant Mini Kit (Qiagen).

2.2.3 cDNA synthesis

Amplification of cDNA out of total RNA was prepared in 10 µl reaction mixtures using 1 µg RNA and the reverse transcriptase MMLV RT (Promega) according to the manufacturer's instructions.

2.2.4 SDS-Polyacrylamide gel

Proteins were separated by SDS polyacrylamide gel electrophorese (SDS-PAGE) using discontinuous gels (Laemmli, 1970) containing a separation gel (10-15% polyacrylamide) and a stacking gel (8% polyacrylamide) in 1x SDS running buffer (25 mM Tris, 1.92 M glycine, 1% SDS). Protein samples were mixed with 1 x loading buffer (4 x loading buffer: 0.25M Tris pH 6.8, 8% SDS, 40% glycerine, 20% ß-mercaptoethanole, 0.016% bromphenol blue) before subjecting onto the gel. After protein separation, gels were either stained with coomassie (50% methanol, 7% acetic acid, 0.18% coomassie brilliant blue R-250), silver (see 3.2.5) or used for western blotting (see 3.2.6).

2.2.5 Silver staining

Proteins were stained according to the method described by Blum et al. (1987). The proteins were fixed to the gel by incubation of the gel for 1 h in fixation solution (50% ethanol, 12% acidic acid, 0.05% formaldehyde). After three washing steps for 30 min in 50% (v/v) ethanol, the gel was pre-impregnated for 2 min in 0.02% (w/v) sodium thiosulfate. Before and after the impregnation step (0.2% silver nitrate, 0.075% formaldehyde) which was performed in darkness for 30 min, the gel was washed with water three times for 30 sec. The gel was then stained with development solution (6% Na_2CO_3, 0.05% formaldehyde, 0.0004% sodium thiosulfate) and the reaction was stopped with stop solution (50% ethanol, 12% acidic acid) when bands became visible.

2.2.6 Semi-dry electro-blot and immunostaining

Proteins separated on polyacrylamide gels were transferred onto a PVDF membrane (pore size 0,2 µm) using a semi-dry blotting apparatus (Kyhse-Andersen, 1984). The activated PVDF membrane (100% methanol) was placed on top of two layers of watman paper soaked with anode I buffer (20% methanol, 300 mM Tris) and two soaked with anode buffer II (20% methanol, 25 mM Tris). The SDS gel was then placed onto the blotting membrane followed by two watman paper soaked with cathode buffer (20% methanol, 40 mM aminocapronic acid). The protein transfer was carried out for 1 h at 0.8 mA/cm^2. After short incubation in 100% methanol the blotted membrane was stained with Ponceau solution (5% (v/v) Acetic acid, 0.3% (w/v) Ponceau S). Protein bands became visible after washing the membrane with water.
Immunostaining with antibodies against distinct proteins was performed as follows: Unspecific binding of the primary antibody was avoided by incubation of the PVDF membrane 30 min with blocking solution (0.75% Tween, 5% skimmed milk in TBS-T) Further the membrane was incubated 1 h with primary antibody. After two washing steps for 10 min with TBS-T buffer (20 mM Tris pH 7.6, 137 mM NaCl, 0.1% Tween), the secondary antibody was added to the membrane and incubated 1 h. Depending on the origin of the primary antibody, the secondary antibody was a horse-raddish-peroxidase-anti-rabbit or horse-raddish-peroxidase-anti-mouse conjugation product. After incubation with the second antibody, the membrane was washed 4 x 10 min with TBS-T buffer and a fluorescence signal was detected in darkness. For this purpose developmental solution I (100 mM Tris pH 8.5, 1% Luminole, 0.44% Coomaric acid) and II (100 mM Tris pH 8.5, 0.18% H_2O_2) were mixed in equal amounts and the solution was added onto the membrane and a x-ray film was placed on the membrane for several minutes depending on the strength of the signal. Oxidation of Luminole triggers the chemo luminescence reaction, while the peroxidase antibody serves as a catalyzer. The chemolumenescence was detected on an x-ray film.

2.2.7 Overexpression of proteins in *E.coli*

Transformed *E.coli* bacteria were grown in LB-medium (1% peptone from casein, 0.5% yeast extract, 1% NaCl) at 37°C to an OD_{600} of 0.6-0.8. Over expression was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside to the cells

and changing to the most suitable temperature. Used *E.coli* strains, temperature and time of the expression are listed in Table 5.

Table 5: List of clones and over-expressing conditions

Clone	Vector	Organism	E.Coli strain	Conditions
14-3-3	pET21d+	P.s.	BL21pLys	18°C over-night
HSP70	pET21d+	T.a.	BL21pLys	18°C over-night
HSP90	pET21d+	T.a.	BL21pLys	18°C over-night
HOP	pET21d+	T.a.	RIPL	30°C 6h
FKBP73	pET21d+	T.a.	RIPL	30°C 3h
p23	pET21d+	T.a.	BL21pLys	30°C 5h
Aha1	pet21d+	T.a.	RIPL	30°C 6h
pPC1	pet21d+	A.t.	RIPL	18°C over-night
pNFU4	pET21a+	A.t.	RIPL	18°C over-night
mNFU4	pET21a+	A.t.	RIPL	18°C over-night
pSSU	pET21d+	N.t.	BL21pLys	18°C over-night
pPAC	pET21d+	A.t.	RIPL	18°C over-night

2.2.8 Protein purification

Pellets from overexpressed 0.5-1 l bacteria culture were resuspended in 25-30 ml Lysis buffer (20 mM Tris pH 7.5, 200 mM NaCl, 20 mM Imidazole) followed by cell disruption using a Microfluidizer (Microfluidics). After 30 min centrifugation at 20 000 x g and 4°C 400 µl Ni-NTA beads were added to the supernatant and the mixture was rotated 30 min at 4°C. The beads were washed with 15 ml Wash buffer (20 mM Tris pH 7.5, 200 mM NaCl, 40 mM Imidazole) and recombinant proteins were eluted in 400 µl fractions with Elution buffer (20 mM Tris pH 7.5, 200 mM NaCl, 300 mM Imidazole).

2.2.9 Antibody production and purification

Polyclonal antibodies were produced by BioGenes (Berlin). Therefore purified recombinant proteins containing a histidine-tag were injected into rabbits and serum containing the antibodies was received after different bleeding times and tested for antibodies specificity.

To avoid false-positive results in co-immunoprecipitation experiments with constructs containing a histidine-tag, all used antibodies were purified. For this purpose 330 mg $CNBR^-$ activated sepharose CL4B (GE Healthcare) was washed 15 min in 30 ml 1 mM HCl. The beads were then mixed with 8 mg L-Poly-Histidine dissolved in 5 ml

Coupling buffer (0.1 M NaHCO3 pH 8.3, 0,5 M NaCl) for 1 h at RT. After washing the beads with 5 ml Coupling buffer remaining active groups were blocked with 5 ml 0.1 M Tris pH 8.0 for 2 h. Beads were washed 3 x with alternating pHs using Buffer 1 (0.5 M NaCl, 0.1 M NaAc pH 4.0) and Buffer 2 (0.5 M NaCl, 0.1 M Tris pH 8.0). In a next steps beads were washed using 10 ml 1 x PBS buffer (10x PBS buffer: 1.4 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 0.18 mM KH_2PO_4mM). To purify antisera against the histidine-tag 1 ml of antisera and 5 ml 1 x PBS were added to the beads and incubated over-night at 4°C. The suspension was then loaded onto an empty column and flow through containing the purified antibody was collected and used for further experiments. Beads were regenerated using 0.1 M Glycine pH 2.4 and stored in 1 x PBS at 4°C.

2.2.10 *In vitro* transcription

In vitro transcription was performed using vectors containing either a T7- or SP6-promotor sequence. The transcription was performed at 37°C for 2 h 20 min in 100 µl aliquots using 1 µg linearized DNA, 10U SP6 or T7-polymerase (Fermentas), 5x transcription buffer, 0.05% BSA, 10 mM DTT, 0.5 mM ACU, 100U Rnasin and 0.375 mM CAP. After 20 min 1.5 mM GTP was added to the pre-capped RNA. If the pF3A vector (Promega) was used, capping was not necessary. Enzymes used for linearization are included in Table 1.

2.2.10 Wheat germ preparation

Fresh wheat germs were floated in a 4:1 mixture of carbon tetrachloride and n-hexane. The floated germs were collected, dried over-night and stored at -80°C until use.

5 g of floated germs were grinded in liquid nitrogen to obtain a fine powder, which was transferred into centrifugal tubes, and mixed with 1-2 ml 2x homogenization buffer (40 mM Hepes pH 7.6, 100 mM KAc, 5 mM MgAc, 2 mM $CaCl_2$, 4 mM DTT, 1 mM benzamidine, 5 mM aminocapronic acid and 0.4 U/ml Rnasin). After two centrifugation steps at 64000 x g for 30 min at 4°C, the supernatant was subjected onto a PD10 column (GE Healthcare) and 1 ml fractions were collected. The fractions were tested for their enzymatic activity by test translations and active fractions were frozen in aliquots in liquid nitrogen and stored at -80°C.

2.2.11 *In vitro* translation

In vitro translation was performed using either homemade wheat germ extract, wheat germ extract (Promega) or reticulocyte lysate (Promega). Proteins were radiolabeled using ^{35}S methionine and cysteine (Perkin Elmer). For each translation the suitable amount of mRNA (1-10%) and potassium (0-100 mM) was adapted. Proteins were translated 40-60 min at 25°C in wheat germ lysate or at 30°C in reticulocyte lysate. Translation in homemade wheat germ was performed using 50% wheat germ lysate, 1 mM amino acids (-methionine, -cysteine), 4% ^{35}S labeled methionine and cysteine,1x buffer A (10x buffer A:140 mM Hepes pH 7.6, 12.5 mM MgAc, 20 mM DTT,+/- 480 mM KAc), 1x buffer B (10x buffer B: 12.5 mM ATP, 12.5 mM GTP, 4 mM Spermidine, 0.25 mM Hepes pH7.6, 160 mM creatine phosphate, 4.5 mg/ml creatine phosphate kinase) and up to 10% mRNA. Translation in wheat germ and reticulocyte lysate from Promega was performed as described in the user instructions.

2.2.12 Detection of radiolabeled proteins

To visualize radiolabelled proteins, an x-ray film or a reusable screen was placed on dried gels or blotted membranes. Detection occurred by developing the x-ray film or with the help of the Typhoon scanner (GE Healthcare).

2.2.13 Quantification of radiolabelled bands

Quantification was done using the AIDA image analyzer software.

2.2.14 Size exclusion chromatography

Radiolabelled proteins translated in wheat germ lysate were centrifuged twice at 390000 x g for 10 min at 4°C and 10 mM ADP was added to the supernatant to stabilize the preprotein complex. Postribosomal supernatant was then loaded onto a size exclusion chromatography. Flow rate and fraction size were adjusted to the used column. G3000SWXL (Tosoh Bioscience) was run at 0.8 ml/min in NaP_i buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7.0). The samples were collected in 0.25 ml fractions. G3000SW (Tosoh Bioscience) was run at 4 ml/min. Samples were collected in 2.5 ml fractions. For standard curve, marker proteins (aldolase, ferritin, conalbumin, ovalbumin, thyroglobin) were used (GE Healthcare). These experiments have been performed by Regina Schweiger. Eluted fractions were precipitated with trichloro acetic acid (TCA) and then subjected onto a SDS-gel.

2.2.16 Chaperone assay

Postribosomal supernatant of 150 µg wheat germ lysate was incubated with 30 µg recombinant chaperone and 400 µl 1 x PBS (10x PBS buffer: 1.4 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 0.18 mM KH_2PO_4mM) for 1 h at room temperature in a turning wheel. After adding 20 µg Ni-NTA sepharose (GE Healthcare) incubation was prolonged for additional 30 minutes. Protein mixture was washed 4 x with 1 x PBS containing 50 mM Imidazole at 1000 x g, 1 min at 4°C. Proteins were then eluted in 15 µl 1x PBS containing 300 mM Imidazole using Micro Bio-Spin® columns (Bio-Rad) to get rid of the beads.

2.2.17 Co-immunoprecipitation

Binding of antibodies to a protein A sepharose CL-4B matrix (GE Healthcare) was performed using same amounts of both components (each 10 µl) mixed with 400 µl 1x PBS (10x PBS buffer: 1.4 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 0.18 mM KH_2PO_4mM) buffer. After 1 h incubation at room temperature on a turning wheel the beads were washed twice with 400 µl 1x PBS buffer and stored until usage. Freshly translated proteins (40-60 min, 25°C for wheat germ, and 30°C for reticulocyte lysate) were centrifuged 10 min at 40000 g and 4°C and 10 mM ADP was added to stabilize chaperone binding. The translation product was loaded onto the beads containing the antibodies and incubated 30 min at 4°C on a turning wheel. The beads were washed 3-6 times with 800 µl 1x PBS. Elution of the proteins was performed with loading buffer using Micro Bio-Spin® columns (Bio-Rad) to get rid of the beads.

2.2.18 Crosslinking antibodies to Protein A sepharose

Binding of antibodies to a protein A sepharose CL-4B matrix (GE Healthcare) was performed as described in 3.2.17. Beads were centrifuged 1 min at 4000 x g at 4°C, supernatant was discarded and antibody-bound beads were incubated again 1 h in 1 ml DMP buffer (0.02 M DMP, 0.2 M Triethanolamine pH 8.2). After the crosslinking reaction, chemical crosslinker was washed away by incubating the beads additional 2 h in 1 ml Triethanolamine buffer (0.2 M Triethanolamine pH 8.2) and replacing buffer solution once after 5 min. Sample was washed 3 x with 1 x PBS and beads were stored at 4°C over-night.

2.2.19 Mass spectrometric analysis

Mass spectrometric analyses were either performed at the Protein Analysis Unit of the Adolf-Butenandt-Institute (Ludwig-Maximilians-Universität, Munich, Germany) or at the mass spectrometry service of the Department Biologie I (Ludwig-Maximilians-Universität, Munich, Germany).

2.2.20 Chloroplast isolation

Chloroplasts were isolated out of 8 days old wheat (*Triticum aestivum*). Stems were carefully mixed in Isolation buffer (330 mM Sorbitol, 20 mM MOPS, 13 mM Tris pH 7.6, 3 mM $MgCl_2$, 0.1% BSA), filtered through one layer miracloth and centrifuged at 1500 x g, 1 min at 4°C. The obtained pellet was resuspended with a wet brush and loaded onto a Percoll density gradient (40% and 80% Percoll). Intact chloroplasts were separated and washed twice with wash buffer (330 mM Sorbitol, 25 mM HEPES pH7.6).

2.2.21 Mitochondria isolation

Pea mitochondria were purified out of 14-21 days old pea seedlings (*Pisum sativum*). Peas were mixed strongly (4 x 4 sec) in Grinding Media (0.3 M sucrose, 25 mM $Na_4P_2O_7$, 2 mM EDTA, 10 mM KH_2PO_4, 1% PVP-40, 1% BSA, 20 mM Ascorbic acid, pH 7.5) and filtered through one layer miracloth. After centrifugation of the suspension at 2500 x g, 5 min at 4°C the supernatant was again centrifuged at 17500 x g, 20 min at 4°C. The pellet was resuspended with a wet brush, washed with washing buffer (0.9 M sucrose, 30 mM TES, 0.2% BSA, pH 7.5) and centrifuged at 2500 x g, 5 min at 4°C and the supernatant was then centrifuged again at 17500 x g, 20 min at 4°C. The pellet was resuspended with a wet brush and subjected onto a Percoll step gradient consisting of three layers containing 60%, 28% and 21% Percoll solution. Centrifugation was carried out at 26200 x g, 50 min at 4°C with slow deceleration. Mitochondria accumulated at the border between 60% and 28% Percoll were icollected and a further washing step was performed to get rid of the remaining Percoll solution (17500 x g, 20 min, 4°C).

2.2.22 Import into plant mitochondria

Protein import into pea mitochondria was performed with preproteins translated in reticulocyte lysate. Up to 5 µl of translation product was added to 90 µl Import mix (0.3 M sucrose, 50 mM KCl, 10 mM MOPS pH 7.4, 5 mM KH_2PO_4, 0.1% BSA, 1 mM

$MgCl_2$, 1 mM methionine, 0.2 mM ADP, 0.75 mM ATP, 5 mM succinate, 5 mM DTT) and 50 µg freshly isolated mitochondria. Import reaction was carried out at 25°C in time course up to 20 min. Samples were centrifuged after the import reaction 5 min at 16000 x g at 4°C and supernatant was removed. The pellet was resuspended in SDS-loading buffer, followed by cooking the samples before subjecting to SDS-gels. Radiolabelled bands were visualized by autoradiography. For import reactions with dephosphorylated preproteins, containing a phosphatase in the translation product, binding of the preprotein to mitochondria was carried out 8 min on ice before the import reaction was started to prevent dephosphorylation of TOM complex components before import takes place.

2.2.23 Dephosphorylation of radiolabelled preproteins

10 µl freshly translated preprotein was mixed with 3.2 U CIAP (Fermentas) and 2 µl CIAP buffer in a total volume of 20 µl and incubated 30 min at 37°C. A control reaction was performed containing 10 µl of the same translated protein, 2 µl CIAP buffer and 1x PhosStop (Roche) and stored 30 min on ice. After incubation 3.2 U of CIAP were added to the control reaction and both samples were subsequently purified using Micro Bio-Spin® 6 chromatography columns (Bio-Rad).

2.2.24 Phosphorylation assay

Each 50 µl sample contained 3 µg recombinant substrate protein mixed with either 1.5 µl wheat germ lysate (Promega), 2 µl reticulocyte lysate or 30 µl fresh *Arabidopsis* lysate in a Phosphorylation buffer (20 mM Tris pH 7.5, 5 mM $MgCl_2$, 0.5 mM $MnCl_2$) and the presence of 3 µCi ^{32}P-ATP and 2.5 µM ATP. The phosphorylation reaction was carried out 10 min at 23°C and stopped by adding 10 µl of 5 x SDS-loading buffer. 15 µl was loaded onto a SDS-gel and phosphorylated proteins were detected by autoradiography.

2.2.25 Isolation and transient transformation of *Arabidopsis* protoplast

Mesophyll protoplasts were made out of 3-4 week old *Arabidopsis* wild type plants. Leaves were cut into small pieces and incubated in the dark for 90 min at 40 rpm in 10 ml of an enzymatic solution (1% Cellulase R10, 0.3 % Macerozyme R10, 40 mM Mannitol, 20 mM KCl, 20 mM MES pH5.7, 10 mM $CaCl_2$, 0.1 % BSA). Protoplasts were released by shaking 1 min at 80 rpm and filtered through one layer miracloth to remove leave fragments. The flow through was centrifuged 2 min at 100 x g (slow

deceleration) and the pellet carefully resuspended in 500 µl MMg buffer (400 mM Mannitol, 15 mM $MgCl_2$, 4 mM MES pH 5.7, Osm 540) and subsequentially loaded onto a gradient made of 9 ml MSC buffer (10 mM MES, 20 mM $MgCl_2$, 1.2 % sucrose, pH 5.8, Osm 550) and 2 ml MMg buffer to separate broken from intact protoplasts. Centrifugation was carried out for 10 min at 80 x g (slow deceleration) and intact protoplasts were washed once with 5 ml W5 buffer 150 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES pH 5.7, Osm 550-580) (centrifugation 2 min at 100 x g and slow deceleration) and then resuspended in MMg buffer. For transient transformation 20 µg plasmid DNA was added to 100 µl protoplast solution and carefully mixed with 110 µl PEG solution (40 % PEG 4000 (Fluka), 200 mM Mannitol, 100 mM $Ca(NO_3)_2$. After 15 min incubation in the dark, the reaction mixture was diluted with 500 µl W5 buffer and centrifuged for 1 min at 100 x g. Protoplasts were resuspended in 1 ml MMg buffer and incubated 12-24 h in the dark at 25°C. GFP signals and autofluorescence of chloroplasts were visualized by TCS-SP5 confocal laser scanning microscope and LAS AF Light software (Leica).

3 Results

3.1 Association of chaperones with chloroplast preproteins in the cytosol

3.1.1 Classification of preproteins binding to cytosolic chaperones

At the start of this research only a few chloroplast preproteins were described to bind to 14-3-3 chaperones and just three were found to interact with HSP90 in the cytosol (May & Soll, 2000; Qbadou et al, 2006). Since the low number of protein examples does not allow any clear statement about the generality of chaperone association with preproteins, chaperone binding to preproteins was investigated with a larger set of chloroplast preproteins (45 preproteins).

Nuclear-encoded chloroplast proteins were selected to cover a representative mixture concerning different protein properties including localization within the organelle, length of preproteins and transit peptides as well as function of the mature protein (Table 5).

Table 5: Summary and description of all analyzed chloroplast preproteins

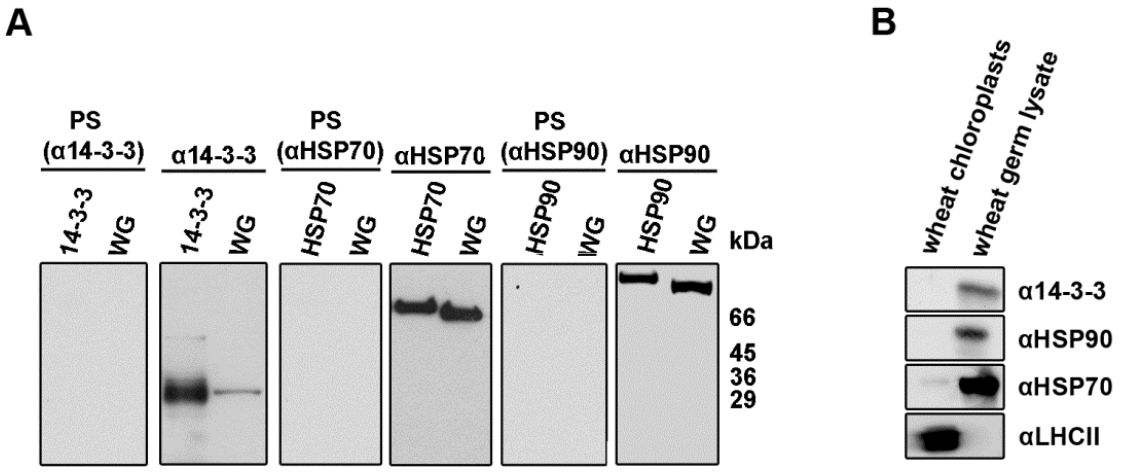
Protein/ Abbreviation	Associated chaperone	Localization	Length of TP/ preprotein	Function	Reference
pAPE1	HSP90	t	32/286	acclimation of photosynthesis to environment	Sun et al, 2009
pAtpD	HSP90	t	48/234	CF1-ATP synthase subunit	Friso et al, 2004
pClpP	HSP90	s	51/271	proteolytic subunit of Clp-type serine protease	Shanklin et al, 1995
pDHAR3	HSP90	s	42/258	dehydroascorbate reductase	Sun et al., 2009
pEMB1241	HSP90	s	64/324	GrpE-like co-chaperone	Sun et al., 2009
pGAPB	HSP90	s	45/447	glyceraldehyde 3-phosphate dehydrogenase	Sun et al., 2009
pGPT1	HSP70	ie	64/388	glucose-6-phosphate/phosphate translocator	Knappe et al, 2003
pHO1	HSP90	s	54/282	heme oxygenase	Muramoto et al, 1999
pNFU2	HSP90	s	16/235	iron sulfur cluster assembly	Leon et al, 2003
pPAC	HSP90	s	22/313	chloroplast development	Meurer et al, 1998
pPC1	HSP90	l	66/171	plastocyanin (photosyntetic electron transport)	Peltier et al, 2002
pPOT	HSP90	ie	38/624	proton-dependent oligopeptide transporter	Prediction TargetP
pPPT1	HSP70	ie	30/408	phosphoenolpyruvate/phosphate translocator	Fischer et al, 1997
pPsaE1	HSP90	t	44/143	photosystem I subunit	Friso et al., 2004
pPsb29	HSP90	s	67/300	thylakoid formation	Wang et al, 2004
pATPR	14-3-3	s	86/403	phosphoprotein phosphatase/ protein kinase	Sun et al., 2009
pCAO	14-3-3	t	36/536	chlorophyllid a oxygenase	Reinbothe et al, 2005
pDPE1	14-3-3	s	45/576	4-alpha-glucanotransferase	Stettler et al, 2009
pGPS1	14-3-3	s	56/371	geranyl diphosphate synthase	Sun et al., 2009

Protein/ Abbreviation	Associated chaperone	Localization	Length of TP/ preprotein	Function	Reference
pGS2	14-3-3	s	45/430	glutamin synthetase	Bartsch et al, 2008
pIM	14-3-3	t	56/351	terminal oxidase	Carol et al, 1999
pNdhM	14-3-3	t	21/217	NDH complex subunit	Sun et al., 2009
pPetC	14-3-3	t	50/229	$cytb_6f$ complex subunit	Friso et al., 2004
pPGRL1.2	14-3-3	t	49/313	required for cyclic electron flow in PSI	DalCorso et al, 2008
pPORA	14-3-3	s	43/401	NADPH-protochlorophyllide oxidoreductase	Sun et al., 2009
pSSU	14-3-3	s	57/180	ribulose 1,5 bisphosphate carboxylase subunit	Klein & Salvucci, 1992
p6PGDH	HSP70	s	44/358	glyoxylate reductase	Simpson et al, 2008
pAPO1	HSP70	s	47/436	accumulation of photosystem I	Amann et al, 2004
pCAB1	HSP70	t	23/267	light harvesting complex of PS II	Sun et al., 2009
pClp2	HSP70	s	54/279	Clp protease subunit	Sun et al., 2009
pDSP4	HSP70	s	54/379	tyrosine protein phosphatase	Kerk et al, 2006
pEGY1	HSP70	t	18/548	membrane-associated metalloprotease	Chen et al, 2005
pFd2	HSP70	s	52/148	ferredoxin	Hanke et al, 2004
pFer1	HSP70	s	47/255	putative ferritin 1	Sun et al., 2009
pFNRL1	HSP70	s	64/360	ferredoxin-NADP(+)-oxidoreductase	Lintala et al, 2007
pFOLT1	HSP70	ie	11/308	folate transporter	Bedhomme et al, 2005
pFtsZ	HSP70	s	90/433	chloroplast division	El-Kafafi el et al, 2008
pHCF101	HSP70	s	64/532	iron sulfur cluster assembly	Lezhneva et al, 2004
pLHCA5	HSP70	t	42/256	light harvesting complex protein of PSI	Sun et al., 2009
pPsaK	HSP70	t	32/130	photosystem I subunit	Sun et al., 2009
pPsbS	HSP70	t	59/265	photosystem II subunit	Sun et al., 2009
pPsbT	HSP70	t	69/103	photosystem II subunit	Sun et al., 2009
pPsbX	HSP70	t	58/116	photosystem II subunit	Sun et al., 2009
pRPL28	HSP70	s	97/143	L28-type protein of S50 ribosomal subunit	Sun et al., 2009
pSMTR	HSP70	s	68/393	malonyl-CoA:ACP transacylase	Sun et al., 2009

Abbreviations: ie: inner envelope; l: lumen; s: stroma; t: thylakoid

After *in vitro* transcription of the full-length cDNA, *in vitro* translation was performed using wheat germ lysate as a model cytosol. This cell-free system contains next to ribosomes also the chaperones involved in preprotein targeting (May & Soll, 2000). Radiolabelled preproteins were co-immunoprecipitated with antibodies against HSP90, 14-3-3 or HSP70 directly after translation to maintain the preprotein chaperone complex and preimmunserum (PS) was used as a negative control to detect unspecific binding. Specificity of all used antibodies and the corresponding preimmunsera was confirmed by western blot with wheat germ lysate (Fig. 5A). Since wheat germ lysate might contain proplastids with plastidic chaperones, all antibodies

were further tested for their specificity in recognizing cytosolic chaperones. The antibodies specifically detected the corresponding chaperone only in wheat germ lysate but not in isolated wheat chloroplasts (Fig. 5B). Conversely, chloroplast light harvesting II (LHCII) proteins were only present in wheat chloroplasts and not in



wheat germ lysate.

Figure 5: Specificity of used antibodies. (A) Antisera against 14-3-3, HSP90 and HSP70 as well as the corresponding preimmunsera were tested by western blot for protein recognition in wheat germ lysate. 0.2 µg recombinant proteins as well as 20 µg wheat germ lysate were subjected to a SDS-PAGE, blotted and western blot analysis showed a distinct and specific band corresponding to the chaperone. **(B)** Chaperone antisera recognized only cytosolic 14-3-3, HSP90 and HSP70 chaperones demonstrated by immunoblot analysis with 15 µg of chloroplast and wheat germ proteins. In contrast, chloroplast LHCII proteins were only detected in wheat chloroplasts and not in wheat germ lysate.

After co-immunoprecipitation proteins were subjected to SDS-gel electrophoresis and preproteins were visualized by autoradiography (Fig. 6A-C). Interaction of HSP70 to preproteins was tested merely randomly (data not shown) or if neither 14-3-3 nor HSP90 association to preproteins was detected. Previous studies already demonstrated that HSP70 is found in preprotein complexes which are associated with either 14-3-3 or HSP90 (May & Soll, 2000; Qbadou et al, 2006). Mean values of the quantification were determined from at least two independent experiments (Fig. 6A-C). Values corresponding to the amount of co-immunoprecipitated preprotein in PS were subtracted as background from those measured for HSP90, 14-3-3 and HSP70 precipitations in Fig. 6A-C, which were set to 100%, respectively.

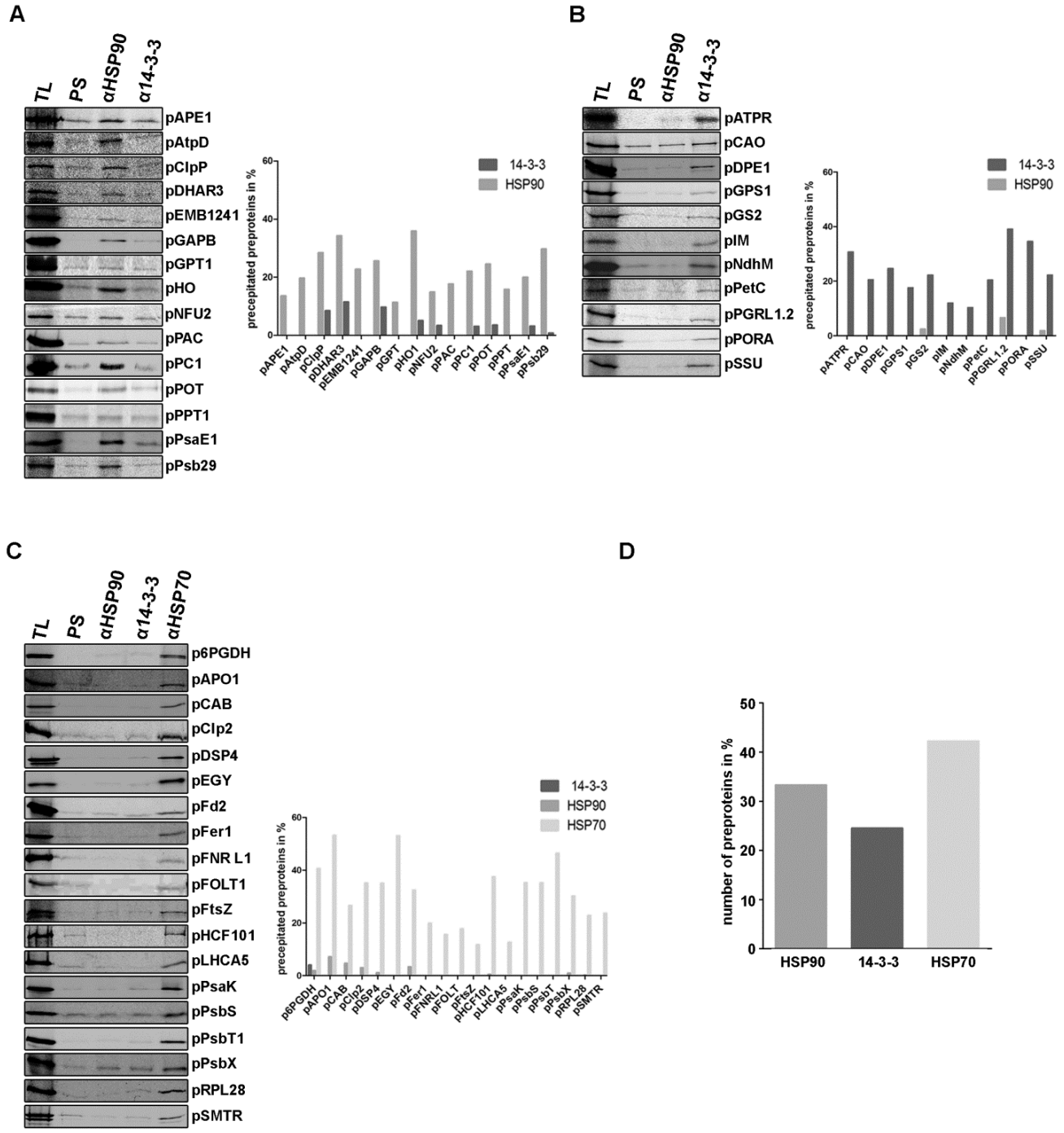


Figure 6: Association of chloroplast preproteins with molecular chaperones HSP90, 14-3-3 or HSP70. (A-C) Radiolabelled preproteins were translated in wheat germ lysate and used for co-immunoprecipitation with antibodies against HSP90, 14-3-3, HSP70 or PS as a control. 5% input of the translation product (TL) were loaded in the first lane (left panel). Quantification was performed for each reaction based on at least two independent experiments. The amount of HSP90 (A), 14-3-3 (B) and HSP70 (C) was set to 100% and PS was subtracted (right panel). **(D)** Preproteins are organized in three subgroups. 33.3% associated with HSP90, 24.5% were bound to 14-3-3 and 42.2% interacted with HSP70.

The data gained from the co-immunoprecipitation experiments show that 33.3% of the tested preproteins are associated with HSP90, 24.5% are binding to 14-3-3 and 42.2% could be detected to interact solely with HSP70 (Fig. 6D).

Moreover, these results clearly demonstrate that chloroplast preprotein complexes involving HSP90, 14-3-3 or HSP70 chaperones are not only restricted to a few proteins but are a common phenomenon in post-translational protein transport.

In order to find any consensus for binding to a distinct chaperone all 45 preproteins were examined closer with respect to different preprotein properties using bioinformatical methods (Table 5). Preproteins of all three chaperone binding subgroups (Fig. 6D) were found to be equally distributed between the three subgroups regarding the localization within the different subcompartments of the chloroplast (ie: inner envelope; l: lumen; s: stroma; t: thylakoid) as well as length of presequence or transit peptide. Likewise protein function cannot serve as prediction for interaction with a distinct chaperone.

In addition to the preprotein properties mentioned above, further analysis was performed regarding amino acid distributions within the experimentally analyzed preproteins. 14-3-3 binding motifs of all proteins were determined using the ELM server as bioinformatical tool (Fig. 7A).

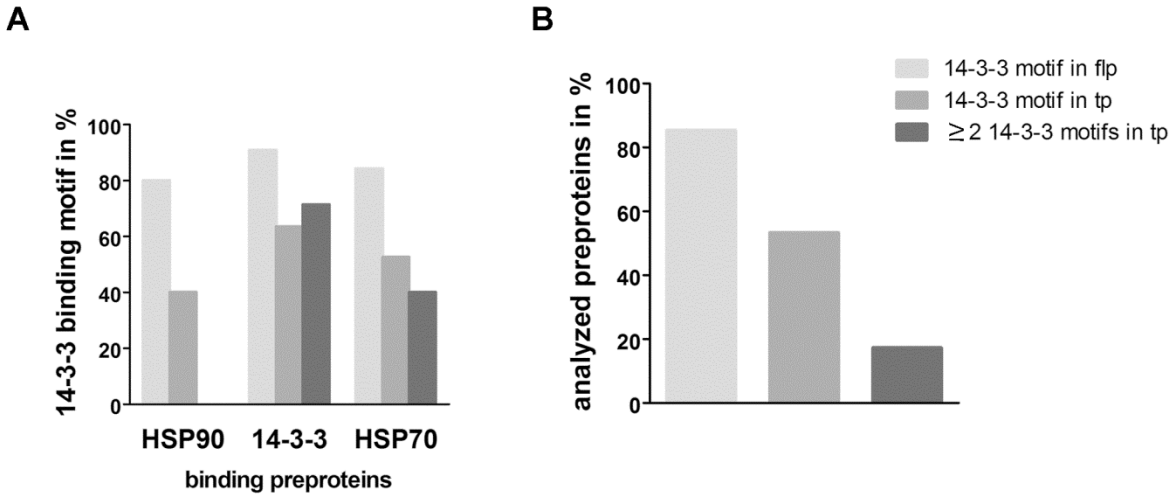


Figure 7: Analysis of 14-3-3 binding motifs in chloroplast preproteins. (A) 14-3-3 binding motifs were found not only in 14-3-3 but also in HSP90 and HSP70 binding full-length proteins (flp). The presence of 14-3-3 binding sites in the transit peptide revealed that most 14-3-3 binding proteins contain more than one 14-3-3 binding motif in the N-terminal part. **(B)** Analysis of 75 chloroplast preproteins showed that more than 85.3% possess a 14-3-3 binding site in the full-length protein, 53.3% contain this motif in the transit peptide and 17.3% possess more than one 14-3-3 binding motif in the transit peptide.

14-3-3 motifs were not only found in 14-3-3 binding preproteins (90.9%) but are also present in HSP90 and HSP70 interacting proteins (80% and 84.2%, respectively) in both, the transit peptide as well as the mature part of the protein. Regarding specifically the transit peptide of all preproteins revealed that 7 of 11 (63.6%) 14-3-3 binding preproteins, 10 of 19 (52.6%) HSP70 associated preproteins and 6 of 15 (40.0%) HSP90 substrates possess a 14-3-3 binding motif in the cleavable N-terminal part of the preprotein.

More intriguing results were obtained by a detailed analysis of the distinct number of 14-3-3 binding motifs within the transit peptides. Indeed, 71.4% of all 14-3-3 binding preproteins possess more than one of those motifs, whereas none of HSP90 associated preproteins and 40% of the identified HSP70 interacting preproteins contain more than one 14-3-3 binding motif. These data are consistent with the observation that additional 14-3-3 binding motifs in one polypeptide enhance 14-3-3 binding affinities (Yaffe et al, 1997).

In a next step, bioinformatical analysis of 14-3-3 patterns of a larger set consisting of 75 chloroplast preproteins was performed. Thereby 14-3-3 binding motifs were detected in 85.3% of the preproteins. 53.3% of the preproteins show at least one motif in the transit peptide, but just 17.3% possess more than one 14-3-3 binding site in the transit peptide (Fig. 7B and appendix 1). Even though three different 14-3-3 binding patterns are known from literature no preference for one of those patterns was observed for 14-3-3 binding preproteins.

3.1.2 Investigation on HSP90 binding to preproteins

In contrast to the specific binding of 14-3-3 to well defined motifs in the transit peptide (May & Soll, 2000 and data not shown), HSP90 binding motifs have not been identified so far. To investigate whether HSP90 binds to preproteins in the transit peptide or the mature part of the protein, four chimeric proteins were constructed consisting of a transit peptide of HSP90 binding preproteins (tpPC1, tpDHAR3, tpAPE1 and tpPsb29) fused to the mature part of SSU (mSSU) which only binds to HSP70 (May & Soll, 2000). The freshly translated chimeric proteins were used for co-immunoprecipitation experiments using antibodies against HSP90, 14-3-3 and HSP70 as before (Fig. 8A and B).

Interestingly, no binding of HSP90 was detected in any of the chimeras, thus indicating that HSP90 binding might occur within the mature part of the protein. To analyze this, the chimeric constructs were reversed - now composed of the 14-3-3 binding transit peptide of SSU (tpSSU) and the mature part of PC1 (mPC1), DHAR3 (mDHAR3), APE1 (mAPE1) and Psb29 (mPsb29) (Fig. 8B). Again no interaction of HSP90 with the chimeric constructs could be observed in co-immunoprecipitation experiments, suggesting that HSP90 binding is not due to a certain amino acid pattern but rather requires structural information from both the transit peptide and the mature part of the protein. In contrast to this, HSP70 binding was detected in all chimeric and authentic proteins (Fig. 8A, B and C) which is in agreement with the finding that HSP70 recognizes exposed hydrophobic stretches of polypeptides.

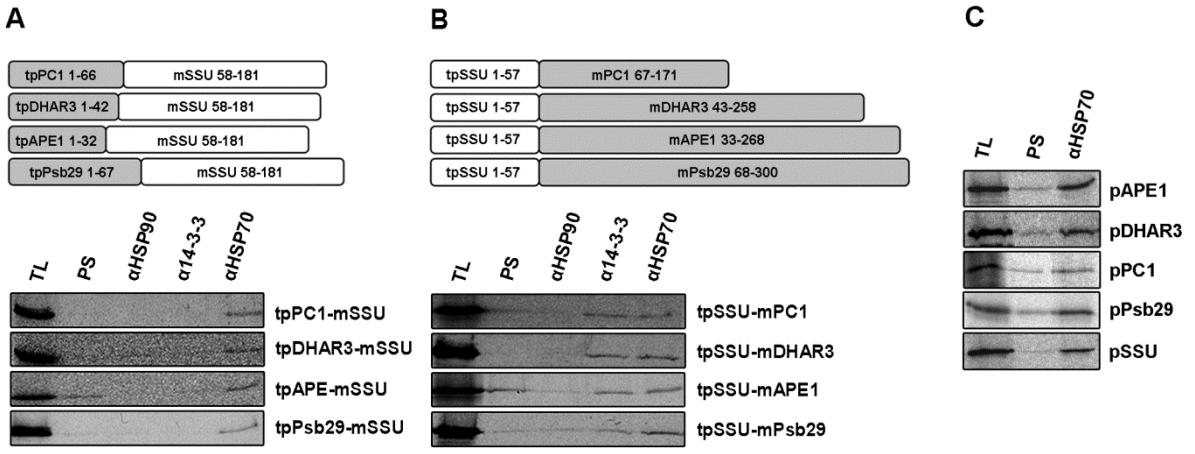


Figure 8: HSP90 binding requires both the transit peptide and the mature part of the preprotein. **(A)** Chimeric proteins composed of the transit peptide of the indicated proteins fused to mSSU (upper panel). All constructs were subjected to co-immunoprecipitation. All chimeras were solely interacting with HSP70. 5% input of the translation product were loaded in lane1 (lower panel). **(B)** Reversed chimeric constructs, in which the transit peptide of SSU (tpSSU) was fused to the mature part of the indicated HSP90 binding proteins (upper panel). All constructs were able to bind 14-3-3 and HSP70, but not HSP90 (lower panel). **(C)** All full length preproteins used for chimeric constructs were found to be associated with HSP70 in co-immunoprecipitation experiment.

3.1.3 Molecular weight of chaperone preprotein complexes

To determine the molecular weight of preprotein chaperone complexes postribosomal supernatant of freshly translated preproteins was fractionated by size exclusion chromatography. All fractions were precipitated with TCA, separated by SDS-PAGE and radiolabelled preproteins were detected by autoradiography. 14-3-3 binding proteins pSSU and pNdhM showed a similar elution pattern accumulating in a molecular weight range of 80-160 kDa (fraction 7-9) (Fig. 9A). These complex sizes

imply the involvement of additional components as already described earlier (May & Soll, 2000). Both preproteins, pSSU (20 kDa) or NdhM (25 kDa), could form a complex with either HSP70 or a 14-3-3 dimer (~60 kDa) alone, but both chaperones could also act simultaneously on the preprotein, since higher molecular weight complexes were also detected to a rare amount (fraction 5-6).

Moreover the preproteins pNFU2, pClpP, pEMB1241 and pPC1, which all interact with HSP90, showed a completely different elution pattern (Fig. 9B). Note that experiments in Fig. 9A and B were performed with different columns. To exclude column dependent differences in the elution pattern some preproteins were subjected to both columns, showing always the same preprotein dependent pattern (data not shown).

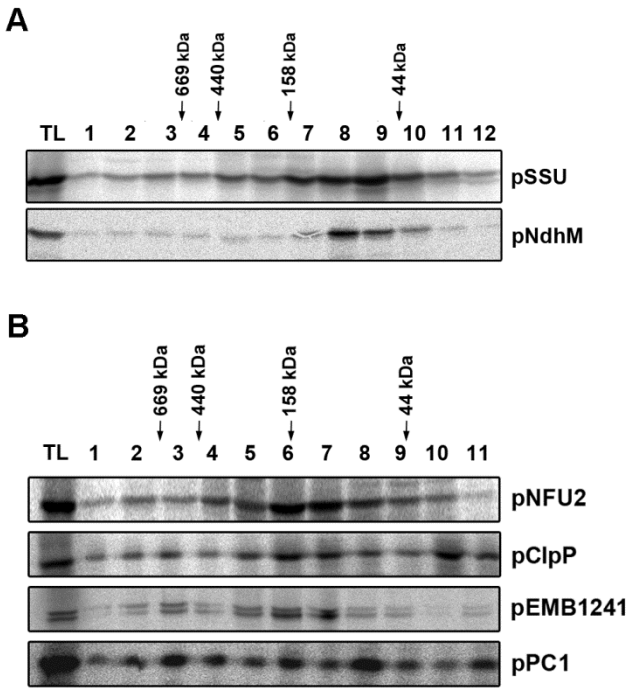


Figure 9: Oligomeric state of preprotein chaperone complexes. Postribosomal supernatant of freshly translated preproteins was subjected onto size exclusion columns. Eluted fractions were precipitated with TCA and separated by SDS-PAGE. Preproteins were detected by autoradiography. Note that different columns were used in A and B. The illustrated experiments have been performed by Regina Schweiger. **(A)** 14-3-3 binding preproteins pSSU and pNdhM were found within a peak fraction corresponding to 80-120 kDa. **(B)** HSP90 binding preproteins pNFU2, pClpP, pEMB1242 and pPC1 were detected in different fractions covering a size of 100-500 kDa.

The radiolabelled HSP90/HSP70 binding preproteins were detected in fractions corresponding to a molecular weight between 100-500 kDa (fraction 3-8). The broad

fractionation range of eluted preprotein strongly suggests that further yet unidentified components might participate in forming a larger oligomeric complex. Dissociated preproteins were observed to accumulate at low molecular weight (fractions 10-11). Both chaperones, HSP70 and HSP90, are known to interact dynamically and yeast and mammalian HSP90 is known to involve additional co-chaperones when bound to a client protein (Bracher & Hartl, 2006; Prodromou, 2011; Wandinger et al, 2008). Sequential binding of such different co-chaperones could explain these changes in the oligomeric states, thus leading to different elution patterns. Association and dissociation of single components of the preprotein chaperone complexes in wheat germ lysate might be regulated in a dynamical process and hence, it is not possible to isolate just one of these intermediate states.

3.1.4 Identification of novel components of HSP90 preprotein complexes

The apparent size of HSP90-preprotein complexes strongly suggested the involvement of further components. To identify those additional interaction partners large scale co-immunoprecipitation (100 µl translation) was performed. The HSP90 binding preprotein pNFU2 and the 14-3-3 binding preprotein pSSU were precipitated with antibodies against HSP90 and 14-3-3, respectively. PS was used as a control. After SDS-PAGE proteins were visualized by silver staining (Fig. 10).

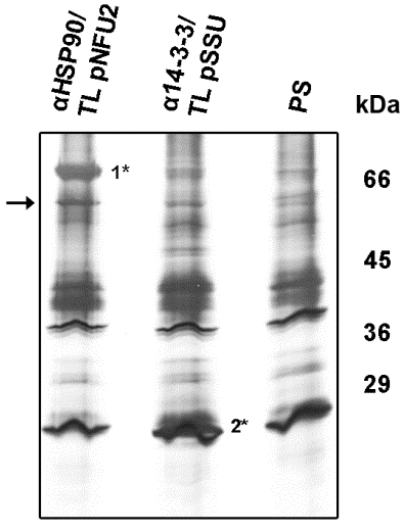


Figure 10: Identification of novel components of the HSP90-preprotein complex. In wheat germ translated pNFU2 and pSSU were co-immunoprecipitated with antibodies against HSP90 and 14-3-3, respectively. PS was used as a control. Proteins were separated by SDS-PAGE followed by silver staining. Precipitated chaperones HSP90 and 14-3-3 are indicated by asterisks 1* and 2*. In both precipitations an additional band at 65 kDa appeared and was further analyzed by mass spectrometry. The analyzed band is indicated by an arrow. In both cases HSP70 was detected. In the sample of HSP90 precipitated preprotein, FKBP73 and a potential HOP protein could further be identified (matching peptides are shown in Figure 11 and 13).

Precipitated HSP90 and 14-3-3 are clearly visible in the corresponding lanes as indicated by 1* and 2*, respectively. Note that preproteins and chaperones are not

present in stoichiometric amounts, since several isoforms of the chaperones are present in wheat germ lysate and not all precipitated proteins are in complex with preproteins.

Beside background bands which are present in all three lanes, an additional band of about 65 kDa appeared as indicated by an arrow, in the HSP90 as well as the 14-3-3 precipitated sample. These bands were further analyzed by mass spectrometry.

In both analyzed bands peptides of matching HSP70 were found, which is in line with earlier findings that preproteins interact in general with HSP70 (May & Soll, 2000; Qbadou et al, 2006 and data not shown). More interestingly, two additional proteins were detected in the same band exclusively in the sample precipitated by HSP90. One of the proteins was the immunophilin FKBP73 (62 kDa) previously characterized in wheat (Blecher et al, 1996). Matching peptides covered 59% of the protein (Fig. 11). FKBP73 belongs to the superfamily of peptidyl-proly *cis/trans* isomerases, which are known to act as co-chaperones for HSP90. The interaction of large immunophilins with HSP90 is mediated by a TPR motif of FKBP and the MEEVD motif of HSP90.

Figure 11: Identification of FKBP73. Matching peptides from mass spectrometry are indicated with bold black lines. The retrieved peptides cover 59% of FKBP73.

Peptides corresponding to the third protein found in the same analyzed band reached a coverage of 23% (peptides are indicated in Figure 13) and aligned with an unknown protein from maize (NP001151932). Protein alignments showed a similarity to HOP proteins of other organisms containing the three typical TPR domains (Fig. 13 and data not shown). HOP proteins behave as co-chaperones facilitating the connection between HSP70 and HSP90 by simultaneous binding of the chaperones to distinct TPR domains of HOP (Smith, 2004).

3.1.4.1 FKBP73 interacts with HSP90 preprotein complexes

To verify the direct interaction of FKBP73 with the HSP90-preprotein complex and to exclude the association of FKBP73 and HSP90 in a different complex further co-immunoprecipitation experiments were performed (Fig. 12A and C). (Pratt et al, 2001).

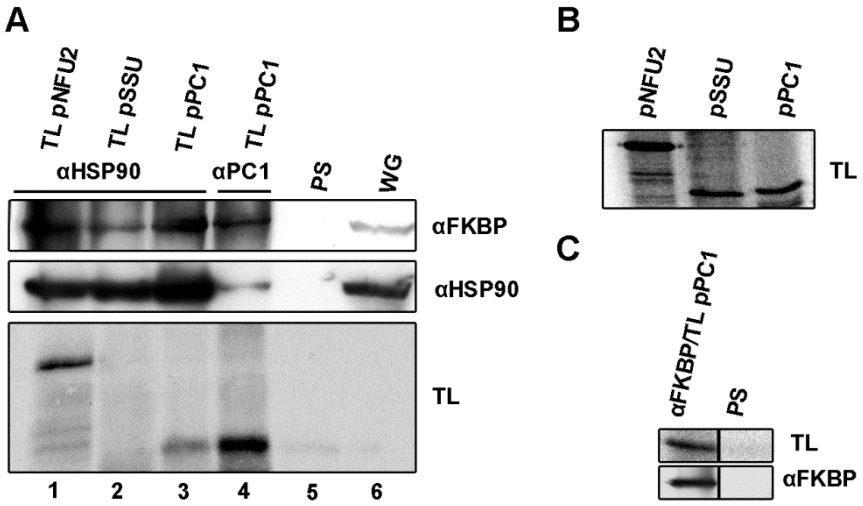


Figure 12: FKBP73 is part of the HSP90-preprotein complex. (A) The direct interaction of FKBP73 with the preprotein-chaperone complex was further confirmed by co-immunoprecipitations. pNFU2, pSSU and pPC1 were co-immunoprecipitated with HSP90 antisera and detected by autoradiography (lower panel, lane1-3). 14-3-3 binding pSSU was not found in the HSP90 complex. Further, pPC1 was precipitated using the corresponding antibody (lower panel, lane 4). PS was used as a control (lower panel, lane 5). Western blot with FKBP73 and HSP90 in these co-immunoprecipitations demonstrates that FKBP73 binds directly to the HSP90-preprotein complex (upper and middle panel, lane 4) but that FKBP73-HSP90 complexes are also present without preprotein in wheat germ lysate in significant lower amount (upper and middle panel, lane 2). 20 µg wheat germ lysate were loaded as a control in lane 6. **(B)** Input of translated preproteins pNFU2, pSSU and pPC1 for co-immunoprecipitations in B was 1%. **(C)** Radiolabelled in wheat germ translated pPC1 was sucessfully co-immunoprecipitated with FKBP antisera but not in the control reaction (upper band). Western blot of this precipitation detected FKBP73 just in the FKBP73 precipitation.

Therefore radiolabelled preprotein pPC1 was immunoprecipitated using antibodies against this protein, followed by western blot analysis with antibodies against HSP90 and FKBP73. Co-immunoprecipitated FKBP73 and HSP90 as well as the radiolabelled protein were clearly visible (Fig. 12A lane 4) but not in the control reaction with PS (Fig. 12A lane5). In a co-immunoprecipitation of radiolabelled pNFU2 and pPC1 (Fig. 12A lane1 and 3) with antibodies against HSP90, the proteins FKBP73 and HSP90 were detected by western blot in higher amounts, which is consistent with the finding that other HSP90 co-chaperone complexes exist in wheat germ lysate. In a mock precipitation with the 14-3-3 binding pSSU and antibodies against HSP90 no preprotein was observed by autoradiography but HSP90 and FKBP73 were detected by western blot analysis (Fig. 12A lane2). Controls for efficient translation are presented in Figure 12B.

An additional proof for direct association of FKBP73 with the HSP90-preprotein complex was performed in co-immunoprecipitation experiments with radiolabelled pPC1 and antibodies against FKBP73. The preprotein was successfully co-precipitated with FKBP73 but not with PS (Fig. 12C).

3.1.4.2 HOP is associated with HSP90 preprotein complexes

To further test whether the peptides detected by mass spectrometry correspond to a HOP protein from wheat (*Triticum aestivum*) the corresponding wheat cDNA was amplified and the protein sequence (TaHOP) was aligned with the maize sequence showing 86.5% identity. The obtained peptides fitted perfectly on the wheat sequence (Fig. 13).

So far, plant HOP was identified in soybean (*Glycine max*) containing three 3-TPR motifs, as known from other organisms (Zhang et al, 2003).The newly identified wheat HOP protein was aligned with a former described soybean HOP (Gm) and non-plant homologs of human (hHOP) and *Saccharomyces cerevisiae* (ScHOP), showing 62.2%, 43.7% and 38.4% sequence identity, respectively. In a blast search against an *Arabidopsis* database three further closely related isoforms were found, recently described as HOP1-3 (Prasad et al, 2010). Sequence identity of the different *Arabidopsis* isoforms was 62.8% and 51.5% when compared to TaHOP (Fig. 13). So far, no client protein of HOP in plants has been identified and the cellular role in plants has not been analyzed yet. Since HOP is described as an adapter protein

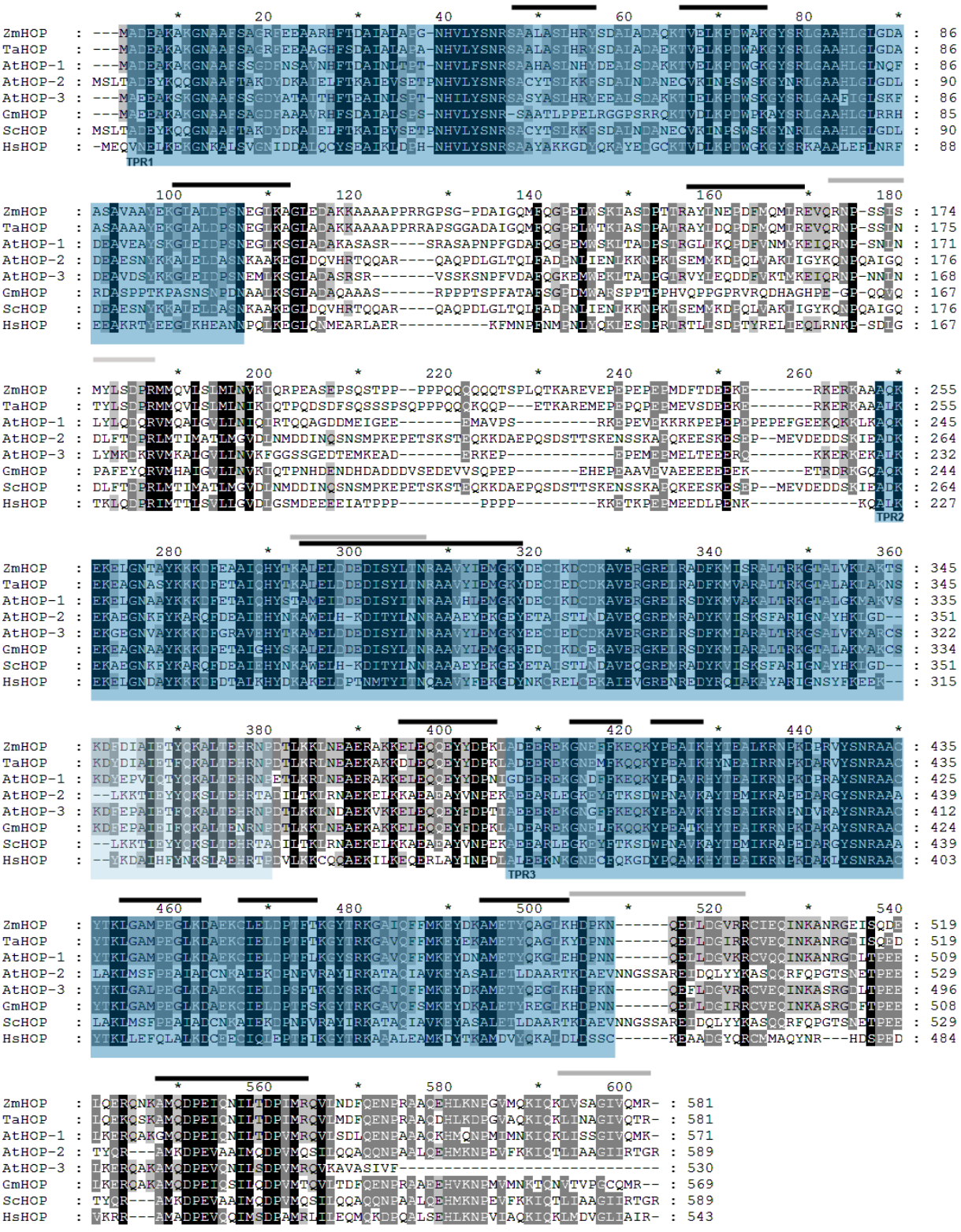


Figure 13: Alignment of HOP proteins from different species. Sequence alignment of HOP proteins of *Z.mays* (ZmHOP) and *T.aestivum* (TaHOP) shows 80% identity. Peptides retrieved from the sequenced band described in Figure 10 are marked with black lines. Grey lines indicate obtained peptides from the co-immunoprecipitation of pPC1 preproteins and pPC1 antisera. Further alignments were performed with HOP proteins from Arabidopsis (AtHOP1-3) and soybean (GmHOP) as plant representatives as well as human (HsHOP) and yeast (ScHOP) homologs. The three typical TPR domains of HOP are indicated in blue.

connecting HSP70 and HSP90 in other organisms, the question arose whether interaction of HOP with the HSP90-preprotein complex was specific for the HSP90-preprotein complex or due to other complexes formed in wheat germ lysate, comprising HOP and HSP90. Therefore, pPC1 was precipitated with pPC1 antisera and after SDS-PAGE and coomassie staining a band corresponding to the molecular weight of HOP was analyzed by mass spectrometry, since no antibody against wheat HOP was available at this time. Indeed, four peptides matching TaHOP were identified with a coverage of 9.9% (peptides are indicated in Figure 13). In a control reaction of 14-3-3 binding pSSU precipitated with pPC1 antisera no HOP peptides were detected.

3.1.4.3 Involvement of the co-chaperones p23 and AHA1

Further investigations concerning the composition of the HSP90-preprotein complex were focused on the possible involvement of additional HSP90 co-chaperones. So far, the HSP90 cycle co-chaperones p23 and AHA1 were not detected in HSP90-preprotein complexes. Hence, the question was addressed whether these two co-chaperones are part of the HSP90-preprotein complexes.

In other organisms p23 has a stabilizing function on HSP90 complexes by binding to the closed state of HSP90 (Felts & Toft, 2003). In contrast to this, p23 of wheat has recently been described to interact with HSP90 in wheat germ, but was not able to stabilize glucocorticoid receptor-HSP90 complexes in this environment (Zhang et al, 2010b).

In contrast to the stabilizing function of p23, AHA1 was reported to bind to the middle domain of HSP90 in yeast and clearly enhances the intrinsic ATPase activity of the chaperone, which leads to a rapid release of the client protein (Panaretou et al, 2002). In plants, nothing is known about the function of AHA1. Protein alignments could identify a plant homolog in *Arabidopsis* (Meyer et al, 2004) and wheat, showing 20.5% and 20.7% sequence identity respectively, to the yeast AHA1 protein (ScAHA1) (Fig. 14). The AHA1 proteins found in *Arabidopsis* (AtAHA1) and wheat (TaAHA1) display 50.1% sequence identity.

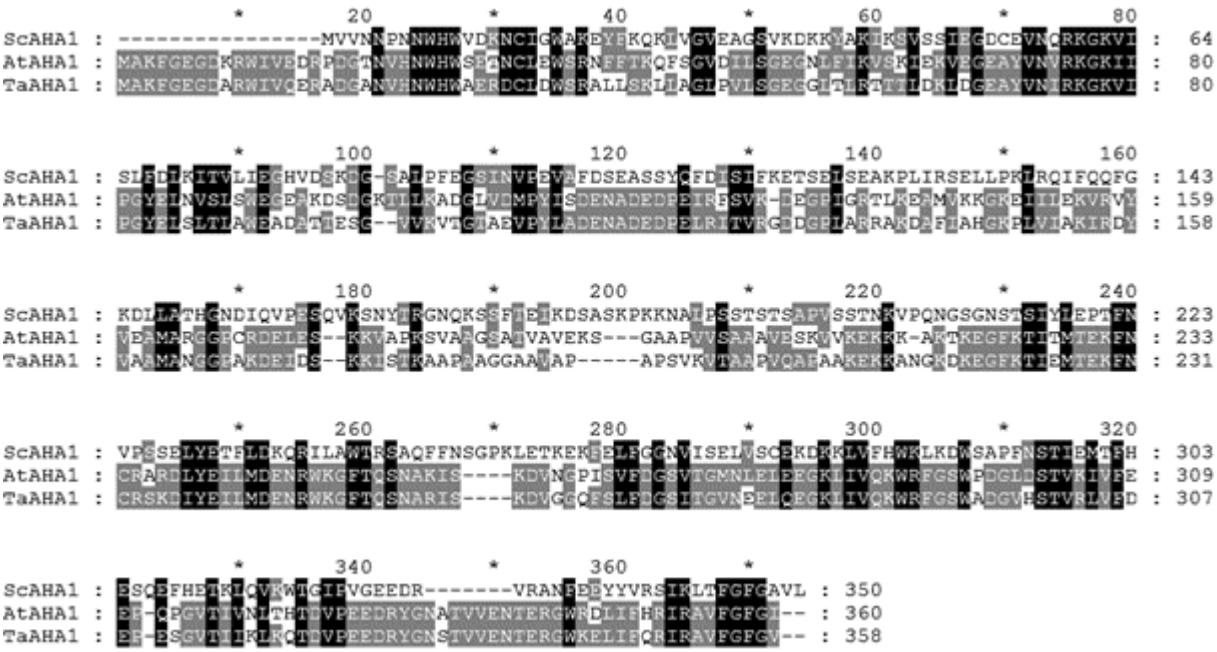


Figure 14: Alignment of AHA proteins from *Saccharomyces cerevisiae* (*Sc*AHA1), *Arabidopsis thaliana* (*At*AHA1) and *Triticum aestivum* (*Ta*AHA1).

To investigate whether the plant AHA1 and p23 proteins are involved in HSP90 hetero-complexes formed in wheat germ lysate, purified HSP90 containing a histidine-tag was incubated with wheat germ lysate. The recombinant chaperone was then re-purified using Ni-NTA beads and subjected to SDS-PAGE. Detection of both potential HSP90 co-chaperones was successfully achieved by immunoblot analysis with antisera directed against AHA1 and p23, demonstrating the interaction of the co-chaperones with HSP90 in wheat germ lysate. A control reaction was performed without recombinant protein to assure specificity of the experiment. 0.5% input were loaded in lane1 (Fig. 15A, left panel). The inverse experiment incubating recombinant AHA1 and p23 in wheat germ lysate followed by immunoblot with HSP90 antisera could only identify AHA1 in complex with HSP90 but not p23 (Fig. 15A, right panel). The recombinant p23 protein used in the described experiment contained a C-terminal histidine-tag possibly interfering with HSP90-complex formation which was also reported for mammalian p23 proteins (personal communication with Brian Freeman).

In order to analyze the possible participation of AHA1 and p23 in HSP90-preprotein complexes co-immunoprecipitation experiments were performed. Firstly, generated antibodies were tested for their ability to immunoprecipitate the corresponding proteins out of wheat germ lysate (Fig. 15B). Antisera raised against both co-

chaperones were cross-linked to protein A sepharose to avoid unspecific reactions in immunoblot analysis. After incubation of the antibodies with wheat germ lysate precipitated proteins were loaded onto SDS-PAGE and immunoblot was performed using AHA1 and p23 antisera. A control reaction was performed using preimmunserum. Both antisera were effectively precipitating the proteins out of wheat germ lysate. As a next step co-immunoprecipitation experiments using radiolabelled pPC1 as a HSP90 client were performed, in which the interaction of the preprotein with HSP90 and AHA1 antisera were demonstrated, but p23 binding could not be detected (Fig. 15C). Since p23 was described to bind to the ATP bound state of HSP90 a co-immunoprecipitation experiment was performed in which AMP-PNP as a non-hydrolysable ATP analogue was added to keep the complex in a closed conformation. Also, these experimental conditions could not identify an interaction of p23 with the HSP90 preprotein complex (Fig. 15D).

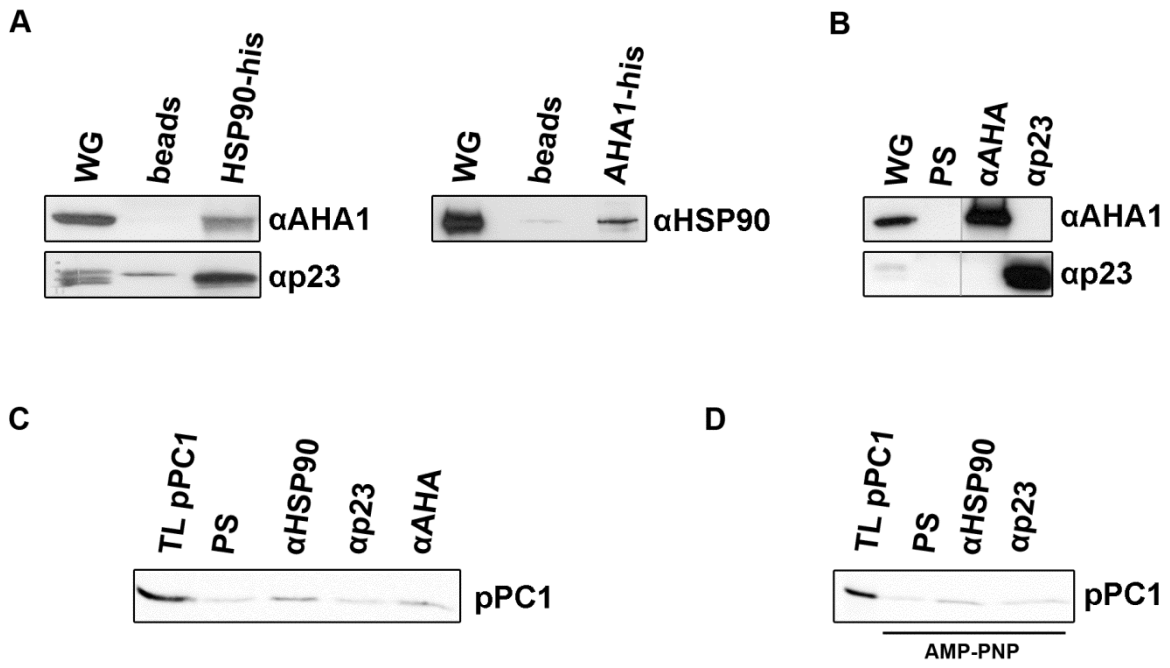


Figure 15: Analysis of wheat AHA1 and p23 in HSP90 complexes in wheat germ lysate. (A) Pull-down of recombinant HSP90 incubated with wheat germ lysate showed interaction of HSP90 with AHA1 and p23 in western blot. HSP90-AHA1 association was also demonstrated by AHA1 pull-down. 0.5% input were loaded in the first lane in (A) and (B). **(B)** Antibodies of AHA1 and p23 are able to immunoprecipitate the corresponding co-chaperones out of wheat germ lysate. **(C)** Co-immunoprecipitation of HSP90 binding pPC1 detected binding of AHA1 but not p23 in the preprotein-HSP90 complex. 5% input were loaded in the first lane of (C) and (D). **(D)** Addition of 1 mM AMP-PMP to the translation mix did not lead to binding of p23 to the HSP90 preprotein complex in co-immunoprecipitation experiments.

3.1.4.4 Localization of *Arabidopsis* HSP90 co-chaperones

In *Arabidopsis* three isoforms of HOP, two isoforms of p23 and ROF, the *Arabidopsis* FKBP51/52 orthologs, and just one isoform of AHA are present. Recently, different localizations for the HOP isoforms were predicted with HOP1 in the cytosol and nucleus, HOP2 in the plasma membrane and HOP3 in mitochondria, plastids and the nucleus (Prasad et al, 2010).

In order to verify these data as well as to determine the proper localization of the other co-chaperones *in vivo* localization experiments were performed. For this purpose co-chaperones fused to GFP were expressed in *Arabidopsis* protoplasts under the control of a 35S promotor and fluorescence signals were detected by confocal laser scanning microscopy (Fig. 16).

The data clearly show GFP-fluorescence signals of all co-chaperones and their isoforms within the cytosol, including also HOP2 and 3. Localization of the co-chaperones within the chloroplasts could be excluded since no overlapping signal was retrieved.

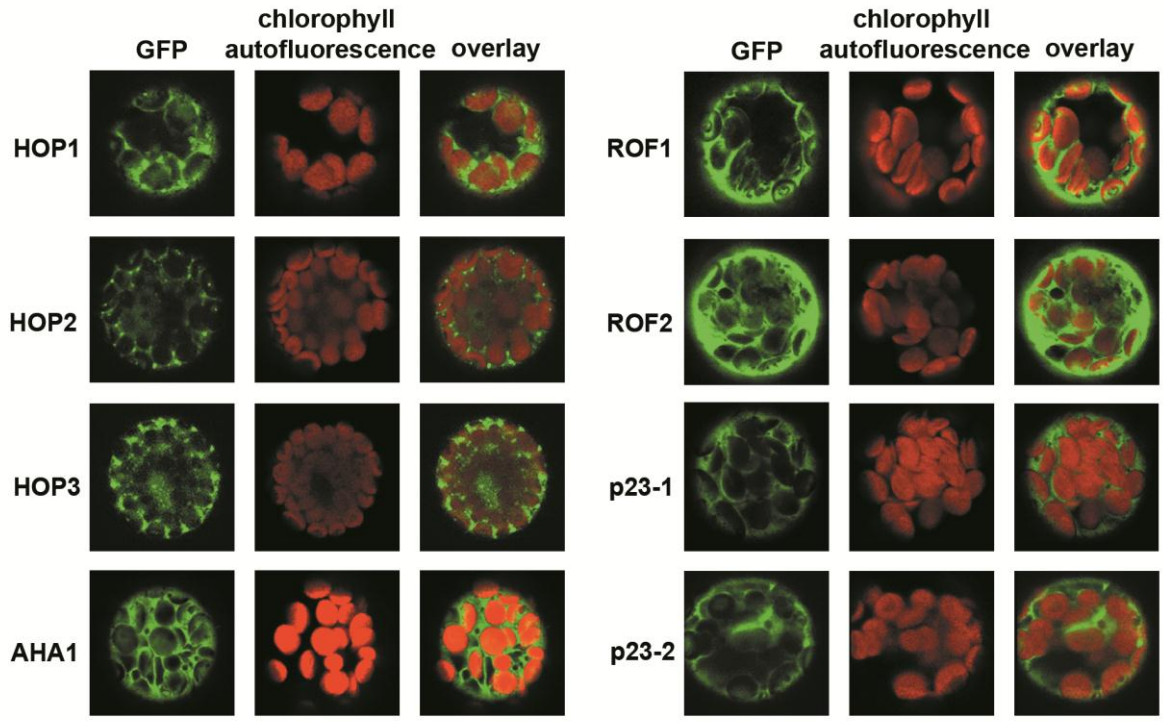


Figure 16: GFP-localization of *Arabidopsis* HSP90 co-chaperones. All *Arabidopsis* isoforms of HOP (HOP1-3), p23 (p23-1 and p23-2), ROF (ROF1 and ROF2) as well as AHA1 were fused N-terminally to GFP and expressed under the control of the 35S promotor. All HSP90 co-chaperones were localized in the cytosol of *Arabidopsis* protoplasts. Left: GFP expression, middle: chlorophyll auto fluorescence, right: overlay.

3.2 Chaperone-mediated targeting of preproteins to plant mitochondria

3.2.1 Interaction of mitochondrial preproteins with chaperones in the cytosol

Similar to preproteins which are directed to the chloroplast also yeast and mammalian mitochondrial preproteins engage molecular chaperones for protection and guidance to the organellar surface. Interestingly, the non-plant cells are using an analogue chaperone system, involving HSP70, the 14-3-3 protein MSF or HSP90 (Fan et al, 2006; Hachiya et al, 1993; Mihara & Omura, 1996). Therefore, the question arose whether distinct guiding mechanisms are used in plant cells to distinguish between the endosymbiotic organelles.

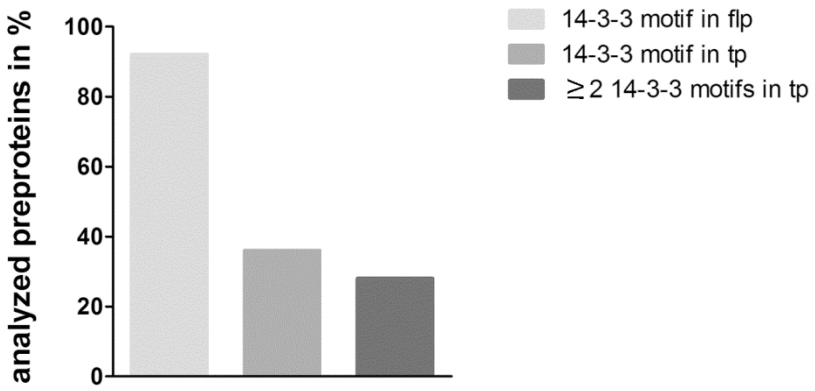


Figure 17: Analysis of 14-3-3 binding sites in mitochondrial preproteins. 92% of a total number of 75 preproteins tested showed 14-3-3 binding motifs in the full-length protein while only 36% were found in presequences or the N-terminal part of the preproteins. More than one 14-3-3 binding sites within the presequence were identified for 28% of the preproteins analyzed.

A first bioinformatical approach analyzing 14-3-3 binding sites in a set of 75 mitochondrial preproteins revealed that 92% contain a 14-3-3 binding site in the full-length preprotein and 36% possess these motifs within the presequence or the N-terminal part. Beside this 28% of all analyzed preproteins contain more than one 14-3-3 binding sites in the presequence (Fig. 17 and appendix 2). These data resemble to those obtained for chloroplast preproteins, suggesting that 14-3-3 chaperones might bind also to mitochondrial preproteins.

To investigate whether 14-3-3 chaperones are binding to mitochondrial preproteins cDNAs encoding for ten mitochondrial preproteins from *Arabidopsis* were transcribed and translated in a wheat germ lysate followed by co-immunoprecipitation with

antibodies against HSP70, HSP90 and 14-3-3. PS was used as a control. The experimental procedure as well as the quantification of the obtained results was performed as described before (3.1). Protein features of all analyzed preproteins comprising length of presequence and full-length protein as well as protein function, localization and predicted 14-3-3 motifs are listed in Table 7.

Table 7: Summary and description of all analyzed mitochondrial preproteins

Protein/ Abbreviation	Associated chaperone	Localization	14-3-3 motif	Length of TP/ preprotein	Function	Reference
pAGT2	14-3-3	m/p	ps/mp	12/ 476	alanine-glyoxylate aminotransferase	Carrie et al, 2009
pDAG	HSP70	m	mp	57/ 229	putative plastid developement protein	Heazlewood & Millar, 2007
pIVD	HSP70	m	ps/mp	29/ 409	isovaleryl-CoA dehydrogenase	Daschner et al, 1999
pK15M2	HSP70	m	ps	56/ 395	putative cobalt ion binding protein	Heazlewood et al, 2004
pNFU4	14-3-3	m	ps	54/283	iron-sulfur cluster assembly	Leon et al, 2003
pPRAT4	14-3-3	m (IM)	-	38/ 624	putative inner membrane translocase component	Murcha et al, 2007
pTIM17-1	14-3-3	m (IM)	mp	218	mitochondrial inner membrane translocase component	Murcha et al, 2007
pTIM22-2	14-3-3	m (IM)	mp	18/ 173	mitochondrial inner membrane translocase component	Elo et al, 2003
pDTC	HSP70	m (IM)	mp	298	mitochondrial dicarboxylate/tricarboxylate carrier	Picault et al, 2002
pDIC	HSP70	m (IM)	mp	313	mitochondrial dicarboxylate carrier	Heazlewood & Millar, 2007

m: mitochondria; p: peroxisome; IM: inner membrane protein; mp: mature protein; ps: presequence

Co-immunoprecipitation experiments detected complexes formed of the preprotein together with 14-3-3 and HSP70 or with HSP70 alone (Fig. 18A and B).

One half of the analyzed preproteins was found to bind to 14-3-3 and HSP70 while the other half was associated only with HSP70. Surprisingly, interaction of preproteins with HSP90 was not detected, although mitochondrial preproteins encoding inner membrane carrier in mammals were shown to bind preferentially to HSP90 (Fan et al, 2006). Since the analyzed preproteins included plant representatives of the inner membrane carrier family (DIC and DTC), as well as further hydrophobic transmembrane proteins (PRAT4, TIM17-1 and TIM22-2), it seems that either the experimental conditions did not favor HSP90-preprotein complex formation or that HSP90 interaction with preproteins does not exist or only for a very limited number of specialized preproteins.

In contrast, 5 of 10 analyzed mitochondrial preproteins were found in complex with 14-3-3. pNFU4 and pAGT2 were both predicted to bind 14-3-3 in the presequence,

whereas Tim17-1 and TIM22-2 possess such a binding site in the mature part. Further, 14-3-3 binding pPRAT4 does neither contain a 14-3-3 binding site in the presequence nor in the mature protein part. Therefore it was not clear whether 14-3-3 binds solely to the presequence or can also associated with the mature part of the preprotein.

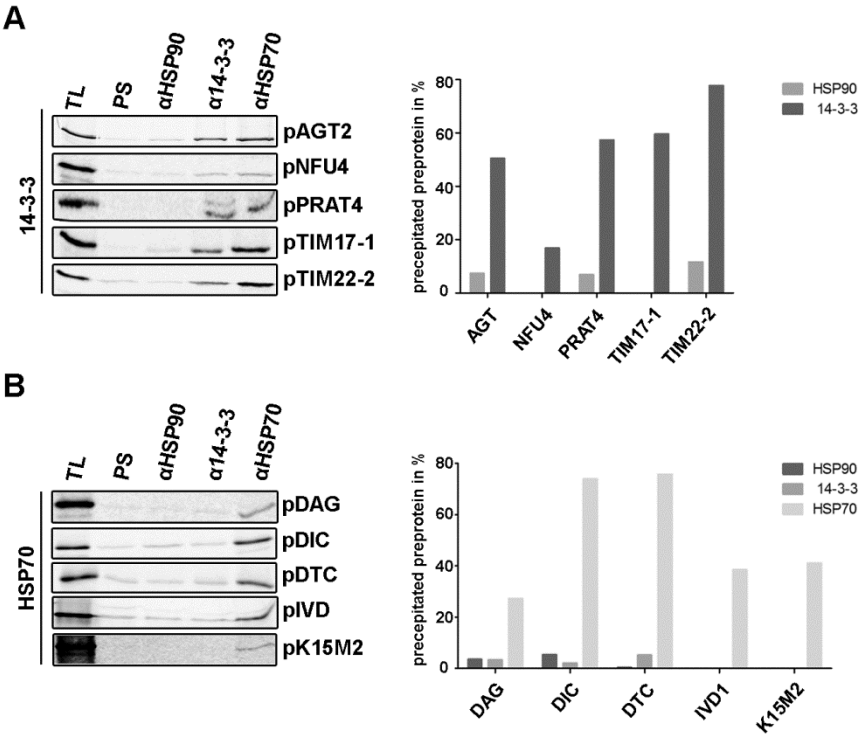


Figure 18: Interaction of mitochondrial preproteins with cytosolic chaperones. (A and B) Radiolabelled preproteins were translated in wheat germ lysate followed by co-immunoprecipitation with antibodies against HSP90, 14-3-3, HSP70 and PS as a control. 5% input of the translation product were loaded in the first lane (left panel). Quantification of at least two independent experiments was performed as described in 3.1.1 (right panel). 50% of the analyzed preproteins were associated with 14-3-3 and HSP70, while the other half was found to bind only to HSP70.

3.2.2 Phosphorylation of mitochondrial 14-3-3 binding preproteins

14-3-3 proteins bind preferentially to phosphorylated 14-3-3 binding sites (Chevalier et al, 2009). Therefore, the observation that half of all experimentally analyzed preproteins destined for mitochondria were bound to 14-3-3 proteins in wheat germ lysate led to the assumption that those proteins might also be phosphorylated in the presequence. To analyze this in more detail phosphorylation assays with recombinant preproteins were performed in wheat germ and reticulocyte lysate (Fig. 19A and B). Next to mitochondrial pNFU4, a chimeric construct composed of the

presequence of NFU4 and the mature part of SSU (pNFU4mSSU) were analyzed in order to investigate whether phosphorylation of pNFU4 occurs in the presequence. Thereby mSSU was used as a carrier protein, since previous studies could show that the mature form of SSU is not phosphorylated (Waegemann & Soll, 1996). Control reactions were performed using the chloroplast preproteins pSSU and pPAC, which were found to be only phosphorylated in plant lysates (Lamberti et al, 2011b; Martin et al, 2006). A further control reaction was performed without recombinant protein.

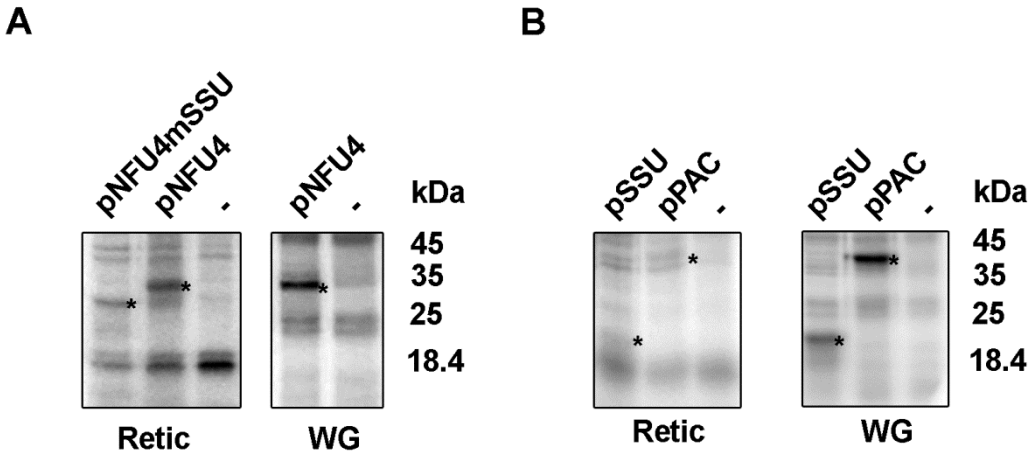


Figure 19: Phosporylation of mitochondrial preproteins. (A) Reticulocyte and/or wheat germ lysate were incubated with 3 µg recombinant pNFU4mSSU or pNFU4 in the presence of radiolabelled ATP. A control reaction was performed without recombinant protein. Phosphorylation of pNFU4mSSU and pNFU4 was visualized by autoradiography. **(B)** Phosphorylation assays of chloroplast preproteins pSSU and pPAC demonstrated phosphorylation of both preproteins only in wheat germ lysate.

After the phosphorylation assay SDS-PAGE was performed and ^{32}P which was incorporated into the recombinant proteins was visualized by autoradiography. Phosphorylation of pNFU4 was detected in wheat germ lysate as well as in reticulocyte lysate. This is in striking contrast to the chloroplast preproteins pSSU and pPAC, which are solely phosphorylated in wheat germ lysate. Therefore, these observations suggest that the kinase responsible for mitochondrial preprotein phosphorylation is not plant specific. In addition, pNFU4mSSU phosphorylation could be detected in reticulocyte lysates demonstrating that pNFU4 is phosphorylated in the presequence (Fig. 19). The phosphorylated band of pNFU4mSSU was weaker than that observed in the full-length pNFU4, indicating that NFU4 might also be phosphorylated in the mature protein part.

3.2.3 Influence of phosphorylated preproteins on preprotein import

Since 14-3-3 binding and phosphorylation of chloroplast preproteins was shown to increase import efficiency into chloroplasts (May & Soll, 2000) the question arose whether 14-3-3 association and phosphorylation of mitochondrial preproteins is also important for mitochondrial preprotein import. Therefore, pNFU4, which was shown to bind 14-3-3 and is phosphorylated also in reticulocyte lysate, was analyzed with focus on protein import. For this purpose serine residues within the 14-3-3 binding site of pNFU4 were mutated to alanine or aspartic acid, resulting in pNFU4 S10/12A and pNFU4 S10/12D, respectively. While the serine to alanine exchange hinders phosphorylation, the serine to aspartic acid exchange mimics a phosphorylated residue due to the size and negative charge of the amino acid. Both constructs enable the analysis of the relevance of phosphorylation concerning 14-3-3 binding and preprotein import.

Co-immunoprecipitation experiments of the mutant proteins demonstrated that binding of 14-3-3 to pNFU4 S10/12A was abolished due to the amino acid exchange, while the pNFU4 S10/12D mutant protein was still able to interact with the chaperone, although slightly weaker than wild type (Fig. 20A).

Furthermore, time dependent import studies were performed with both, wild type and mutant pNFU4 (Fig. 21B, upper part). In comparison to wild type pNFU4, import efficiency of pNFU4 S10/12A was decreased, indicating that phosphorylation might accelerate import into mitochondria (Fig. 20B upper part). Import efficiency of three independent import experiments was quantified. In addition, import studies with the phosphomimicking pNFU S10/12D mutant protein showed a severe inhibition in the import rate suggesting that the negatively charged aspartic acid hinders preprotein import.

To confirm that the observed effect is due to differences in the phosphorylation state, wild type pNFU4 was translated and either retained phosphorylated or dephosphorylated using calf intestine alkaline phosphatase (CIAP) (Fig. 20C). After import of both constructs a strong defect was observed in the import rate of the dephosphorylated protein. The import efficiency was much more affected in the dephosphorylated protein than detected for the pNFU4 S10/12A mutant protein in Fig. 20B, which might be due to further protein phosphorylation sites of pNFU4, possibly within a second 14-3-3 binding site in amino acid position 18-23.

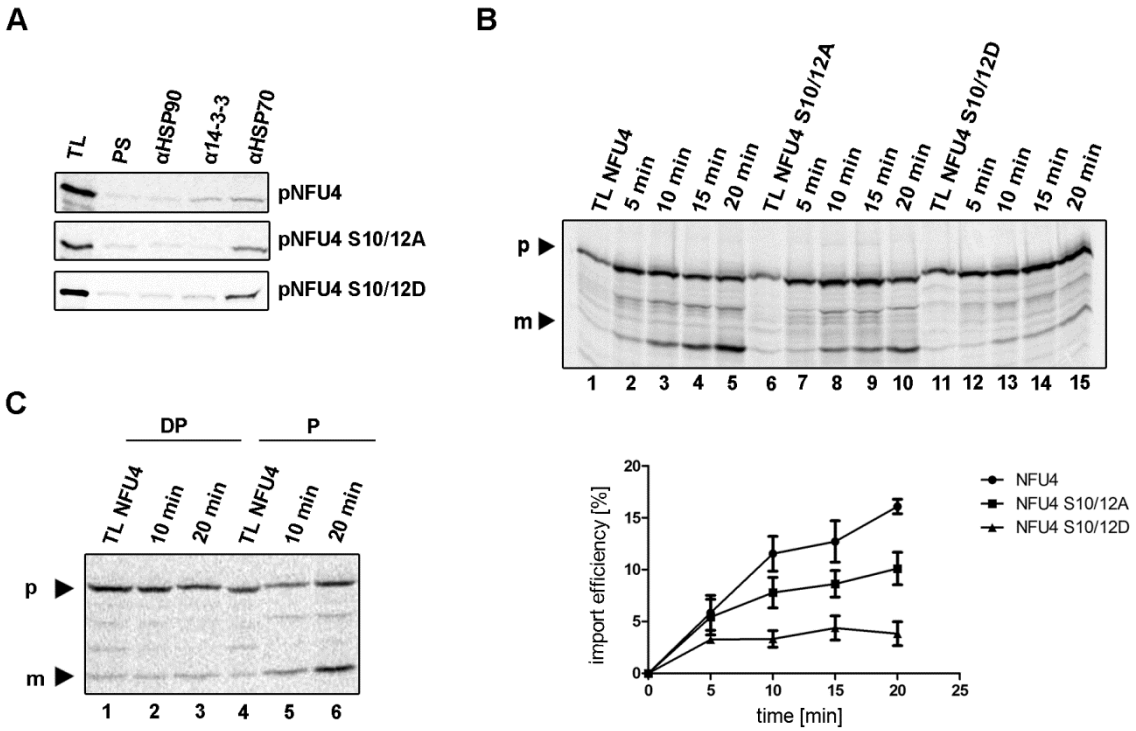


Figure 20: Phosphorylation enhances preprotein import into mitochondria. (A) Co-immunoprecipitation of pNFU4, pNFU4 S10/12A and pNFU4 S10/12D with antibodies against HSP90, 14-3-3 and HSP70. PS was used as a control. 5% input are loaded in the first lane. While pNFU4 S10/12A is not able to bind 14-3-3 anymore, association of this chaperone is still visible in pNFU4 S10/12D and the wild type pNFU4. **(B)** Import of radiolabelled pNFU4, pNFU4 S10/12A and pNFU4 S10/12D into isolated pea mitochondria. 10% input were loaded for each preprotein. Import efficiency was measured in a time course showing a decreased import of pNFU4 S10/12A and a strong inhibition of pNFU4 S10/12D import compared to wild type (upper part). Quantification of three independent import experiments (lower part). **(C)** Import of dephosphorylated pNFU4 in comparison to phosphorylated pNFU4 showed a strong decrease in import efficiency for the dephosphorylated protein.

4 Discussion

4.1 Chaperone-mediated preprotein transport to chloroplast

4.1.1 The role of HSP90 and 14-3-3 in preprotein binding

In the past, interactions of the chaperones HSP90 and 14-3-3 were shown only for a very limited set of chloroplast preproteins (May & Soll, 2000; Qbadou et al, 2006). It remained unclear whether binding of those chaperones is restricted to the small number of tested proteins or if chaperone binding is a general rule in guiding preproteins to the chloroplast receptor proteins TOC34 or TOC64. Therefore a large set consisting of 45 chloroplast preproteins was investigated in respect to their ability to bind to chaperones. The preproteins were selected randomly to cover an extensive spectrum of different protein properties to avoid any biased results. The co-immunoprecipitation experiments demonstrated that 15 preproteins bound to HSP90, 11 interacted with 14-3-3 and 19 were associated only with HSP70 and none of the other chaperones. Nevertheless, it has to be taken into consideration that preproteins which were found to interact solely with HSP70 might form complexes with yet unidentified components which remain to be investigated in future approaches. In addition, it was shown that more than half of the tested preproteins were associated with either HSP90/HSP70 or 14-3-3/HSP70, indicating that binding of those chaperones to chloroplast preproteins is of general importance and not restricted to a few example proteins. To uncover a certain consensus for chaperone binding different protein properties were investigated in more detail. No consistency was detected for binding to a distinct chaperone when analyzing length of preproteins and transit peptides, protein function or organellar localization of the mature protein. Furthermore, 14-3-3 binding motifs were predicted for almost all tested preproteins. Closer examination showed that 63.6% of 14-3-3 binding preproteins, 40.0% of HSP90 associated preproteins and 52.6% of preproteins interacting with HSP70 contained 14-3-3 binding motifs within their transit peptides. Interestingly, 71.4% of the preproteins bound to 14-3-3 had more than one 14-3-3 binding motif within the transit peptide whereas only 40% of HSP70 and none of HSP90 associated preproteins showed such an accumulation of 14-3-3 binding sites in the cleavable N-terminus. In addition to the experimental data further bioinformatical sequence analysis was performed using a set of 75 chloroplast preproteins. The evaluation of these data showed that more than one 14-3-3 binding sites were present in only

17.3% of the transit peptides of the analyzed preproteins while 14-3-3 binding sites were detected in 85.3% of all analyzed full-length proteins. These findings together with the experimental data suggests that accumulation of 14-3-3 binding sites in chloroplast transit peptides indicate a true 14-3-3 interaction with the preprotein, but experimental evidence is still required to confirm each prediction.

The number of preproteins found to interact with HSP90 in the co-immunoprecipitation experiments was intriguing, since also in other organisms the number of clients is rather limited (Taipale et al, 2010). Even with a growing amount of client proteins identified in other organisms during the last years, it was not possible to determine a specific HSP90 binding motif (Taipale et al, 2010; Wandinger et al, 2008). Therefore, binding behavior of preproteins to HSP90 was studied. For this purpose chimeric constructs consisting of the transit peptide of HSP90 binding tpPC1, tpDHAR3, tpAPE and tpPsb29 were fused to the mature form of SSU. No binding of HSP90 could be detected while HSP70 binding was not affected. Also in the reverse experiment no HSP90 was associated with any of the constructs but 14-3-3 was bound to the transit peptide of SSU. In addition, HSP70 association was observed with these constructs. Taken together the data obtained suggest that HSP90 binding requires the entire preprotein, and chaperone binding may occur due to a higher order secondary structure of a partly folded preprotein.

4.1.2 HSP90-preprotein complexes include HSP90 co-chaperones

Until now, knowledge about the function and interaction of cytosolic HSP90 with client proteins in plants is scarce. Knock-down lines of *Arabidopsis* (in which four cytosolic HSP90 isoforms are expressed) or plants treated with HSP90 inhibitors showed a general involvement of the protein in buffering of genetic variation (Queitsch et al, 2002; Sangster et al, 2007; Sangster & Queitsch, 2005). Moreover, the discovery that plant NLR proteins (nucleotide binding and leucine-rich repeat protein) were found to be HSP90 clients demonstrated the involvement of HSP90 in pathogen resistance (Hubert et al, 2003). The HSP90-NLR protein complex interacts with two additional proteins – SGT1 and RAR1- to maintain a signal competent state and possibly to protect the protein from degradation. SGT1, possessing TPR domains is not only associated with HSP90 but also with HSP70 and thus believed to fulfill a similar role to HOP as an adapter protein (Noel et al, 2007; Stuttmann et al,

2008). Protein sequence analysis of both, SGT1 and RAR1, predicted a structural homology to the HSP90 co-chaperone p23 (Hubert et al, 2009; Hubert et al, 2003). It therefore seems that the resistance machinery of plants use specialized co-chaperones for its needs, similar to Cdc37 in mammals, which acts as a specific co-chaperone for kinases associated with HSP90 (Taipale et al, 2010). The HSP90 machinery described in other organisms includes different co-chaperones involved in proper functioning of the chaperone. Even though the list of HSP90 client proteins is still quite small, the role of different co-chaperones is well investigated (Pearl & Prodromou, 2006; Prodromou, 2011; Taipale et al, 2010). In comparison, functional HSP90 machineries in plants were only investigated *in vitro* using human client proteins (Reddy et al, 1998; Zhang et al, 2003). Therefore, research of this work focused on HSP90-preprotein complexes. Earlier studies as well as size exclusion chromatography experiments described in this work could not determine a distinct size of the HSP90-preprotein complex but observed the presence of the complex in a broader molecular weight range from 100-500 kDa, indicating that additional components are involved. Mass spectrometric analysis as well as co-immunoprecipitation experiments with antibodies against the preprotein or co-chaperone identified next to HSP70 further complex components. The immunophilin FKBP73, a HOP-like protein and AHA1 were identified, all of which are known as classical co-chaperones of HSP90 in other organisms. In contrast to the described co-chaperones, interaction of p23 to HSP90-preprotein complexes was not detected under the experimental condition used. Since p23 was reported to bind to the closed state of HSP90 a non-hydrolysable ATP analogue AMP-PNP was used to retain HSP90 in a closed conformation. Even under those conditions interaction of p23 with the HSP90-preprotein complex was not favored. Previous studies with human and yeast p23 proteins showed a stabilizing role for p23 by maintaining HSP90-glucocorticoid receptor complexes which could not be confirmed for p23 in plants when human glucocorticoid receptor proteins were added to wheat germ lysate (Zhang et al, 2010b). Since p23-HSP90 complexes were found to be present in wheat germ lysate it was speculated that the co-chaperone might form complexes only with plant HSP90 clients. This assumption could not be verified under the used experimental conditions, indicating that p23 might act on HSP90 in different cellular processes with a specific subset of HSP90 client proteins.

4.1.3 Model for HSP90-preprotein targeting to the chloroplast

A possible scenario how preproteins might be delivered to the chloroplast surface is proposed in the following paragraph (Fig. 21). HSP70 seems to bind to all chloroplast preproteins (Mihara & Omura, 1996; Rial et al, 2000; Zhang & Glaser, 2002) and chaperone interaction with the preprotein might already take place during or shortly after translation to keep the preprotein in a protected and import competent state (May & Soll, 2000; Qbadou et al, 2006). Preproteins are then escorted to the chloroplast import receptors by guiding complexes involving either HSP70 alone, 14-3-3/HSP70 or HSP90/HSP70 (May & Soll, 2000; Qbadou et al, 2006).

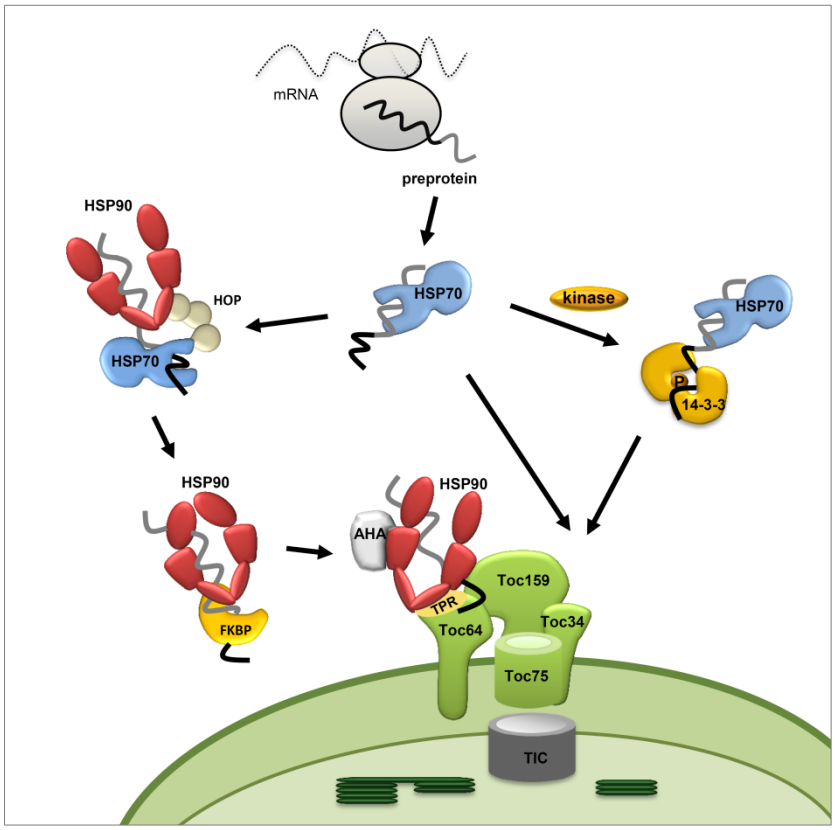


Figure 21: Working model for chaperone-mediated preprotein transport to the chloroplast. Freshly translated preproteins bind to cytosolic chaperones. Next to HSP70 also 14-3-3 or HSP90 can be involved in preprotein complexes. HSP90-preprotein delivery involves thereby co-chaperones including the TPR-containing proteins HOP and FKBP73 as well as AHA1 which binds to the middle domain of HSP90.

HSP90-preprotein sorting might be strongly dependent on the very C-terminal EEVD motif of HSP90 which is able to bind to distinct TPR motifs of other proteins. The participation of HSP90 co-chaperones in HSP90-preprotein complexes led to a working hypothesis in which information of the HSP90 cycle of other model systems

was transferred to HSP90-preprotein targeting (for summary see (Bracher & Hartl, 2006). In a first step HSP90 complexes might be formed via the co-chaperone HOP which serves as a scaffold protein connecting HSP90 with the preprotein loaded HSP70. Thereby both heat shock proteins are able to bind to distinct TPR motifs within HOP. After transfer of the preprotein to HSP90, ATP binding could lead to a conformational change and a closed state of HSP90 in which the TPR-containing immunophilin FKBP73 might replace HOP. The newly formed intermediate complex might stabilize the closed state of HSP90 until docking onto the TPR-containing TOC64 receptor (Qbadou et al, 2006). Even though the TOC64 receptor is not crucial for viability (Aronsson et al, 2007; Rosenbaum Hofmann & Theg, 2005) preprotein delivery via HSP90 might promote enhanced import of some preproteins under certain developmental or stress conditions. Release of the HSP90 bound preprotein might then be favored by the interaction of AHA1, a protein stimulating the ATPase activity of HSP90 which further leads to an opening of the HSP90 dimer. However, the distinct function of each co-chaperone in plants especially in HSP90-preprotein sorting has still to be further elucidated.

4.2 Chaperone-mediated preprotein transport to mitochondria

4.2 1 Mitochondrial preproteins associate with 14-3-3 and HSP70

Association of freshly translated mitochondrial preproteins with cytosolic chaperones in plants has not been extensively investigated until now. Analysis of chaperones involved in preprotein protection and guidance to mitochondria in other model systems showed the participation of HSP70, the 14-3-3 protein MSF or HSP90. Thereby HSP90 was found to interact specifically with hydrophobic proteins as demonstrated with inner membrane carriers (Fan et al, 2006). The chaperones used in mammals and yeast resemble the cytosolic chaperone machinery which is important for preprotein targeting to the chloroplast. To coordinate preprotein sorting in the plant system differentiation between chloroplast and mitochondria is necessary and might require other chaperones for protection and transport.

Prediction of 14-3-3 binding sites, analyzing 75 mitochondrial preproteins with bioinformatical tools, showed a similar distribution of 14-3-3 binding sites in mitochondrial preproteins when compared to 14-3-3 binding motifs in chloroplast presequences. Almost all analyzed preproteins possess a 14-3-3 binding site and

36% of them carry one within the presequence or the very N-terminal part. Accumulation of 14-3-3 binding sites in the N-terminus was detected for 28% of the preproteins. Experimental analysis of 10 mitochondrial preproteins from plants demonstrated indeed a preferential binding to either 14-3-3 together with HSP70 (50%) or HSP70 alone (50%)

Nevertheless, 14-3-3 binding motifs were just rarely detected in presequences of experimental determined 14-3-3 binding preproteins, which might either be due to chaperone binding somewhere else in the mature protein part or might indicate the presence of yet unidentified 14-3-3 binding motifs. In contrast to chloroplast preproteins, a large number of mitochondrial preproteins does not contain a cleavable sorting signal, thus indicating that 14-3-3 binding to mitochondrial preproteins might not always be important in the N-terminal but also in the mature protein part.

Among the analyzed preproteins also hydrophobic inner membrane carrier were present which were expected to interact with HSP90 when compared to mammalian systems. However, experimental data could not verify a clear interaction with HSP90 to the tested mitochondrial plant carrier preproteins. Nevertheless, the presence of HSP90-preprotein complexes *in vivo* cannot be excluded, since the experimental set up might interfere with HSP90-preprotein complex formation.

4.2.2 Phosphorylation of mitochondrial preproteins enhances import efficiency

Phosphorylation of mitochondrial preproteins was recently reported for TOM22 as well as MIM1 (mitochondrial import protein1) in *Saccharomyces cerevisiae* (Schmidt et al, 2011). Both proteins are involved in preprotein import into mitochondria. TOM22 is a component of the TOM core complex which is inserted into the outer mitochondrial membrane after being phosphorylated by the casein kinase 2 (CK2) in the cytosol. Phosphorylated TOM22 associates specifically with the TOM20 receptor, mediated by a phosphorylation-dependent interaction site of TOM20, leading to a stabilized TOM core complex. In contrast, TOM core complex assembly is severely reduced in dephosphorylated mitochondria (Rao et al, 2011). Furthermore TOM20 serves as import receptor for TOM22 and phosphorylation enhances the targeting process (Rao et al, 2011). Also phosphorylation of the MIM1 preprotein by CK2 functions in TOM complex assembly since TOM70 and TOM20 are inserted into the outer membrane using the MIM1 pathway (Rao et al, 2011). Apart from regulative

functions of preprotein phosphorylation for the maintenance of the mitochondrial import apparatus no research was focused on phosphorylation of other preproteins due to the low abundance of serine residues within the presequences of mammals (3%) and *Neurospora crassa* (10%) (Glaser et al, 1998). However, plants exhibit a much higher content of serine residues (17%) and therefore, phosphorylation could be a regulative step in preprotein import into plant mitochondria. The high abundance of preproteins bound to 14-3-3 was also indicating a possible phosphorylation of preproteins targeted to mitochondria since this chaperone binds preferentially to a phosphorylated binding motif (Chevalier et al, 2009). Indeed, phosphorylation could be detected for mitochondrial preproteins not only in wheat germ lysate but also in reticulocyte lysate. This is in striking contrast to chloroplast preprotein phosphorylation, which is dependent on a plant specific kinase suggesting the involvement of a different kinase for mitochondrial preprotein phosphorylation (Lamberti et al, 2011b; Martin et al, 2006). Furthermore, phosphorylation of the presequence of pNFU4 was demonstrated using a chimeric construct consisting of the presequence of pNFU4 and the mature part of the non-phosphorylated SSU. Nevertheless, mature NFU4 might also be phosphorylated, since the phosphorylated band of the full-length preprotein was stronger than that of the presequence of NFU4. Since chloroplast preproteins show an increased import rate into the organelle (May & Soll, 2000) the role of mitochondrial preprotein phosphorylation was further investigated during preprotein import into isolated mitochondria. Preprotein import into mitochondria was shown to be inhibited when proteins are translated in a wheat germ lysate (Dessi et al, 2003). Therefore import experiments were performed using reticulocyte lysate in which pNFU4 phosphorylation was already demonstrated. Phosphorylation mutants were created in which serine residues 10 and 12, within the 14-3-3 binding motif, were either exchanged by the non-phosphorylatable amino acids alanine or aspartic acid, the latter one introducing a negative charge into the presequence, thus leading to a phosphomimicking mutant. Import rates were reduced in pNFU4 S10/12A and strongly inhibited in the pNFU4 S10/12D mutant, clearly demonstrating a regulative effect in preprotein import compared to wild type. The impaired import of the pNFU4 S10/12D mutant suggests that dephosphorylation of the preprotein is necessary after receptor recognition and before import, which would reflect a similar situation like observed for chloroplast preproteins (Lamberti et al, 2011a). Future approaches might further identify the kinase as well as the

phosphatase which play a role in preprotein import into mitochondria. Changes of import behavior of pNFU4 S10/12A simply due to the amino acid exchanges in the presequence could be excluded by additional import studies using dephosphorylated pNFU4 prior to the import reaction. The phosphorylated preprotein was imported more efficiently into mitochondria in comparison to the dephosphorylated preprotein. Nevertheless, it has to be taken into account, that this approach might also dephosphorylate additional possibly phosphorylated amino acids within the preprotein which might also influence the import process. Both *in vitro* import experiments propose a regulative function of preprotein phosphorylation but the obtained data have to be verified using more example proteins.

4.2.3 Model for preprotein targeting to mitochondria in plants

A lot of work has been performed during the last decade in order to investigate preprotein delivery to chloroplasts and mitochondria. Since plants have to deal with both endosymbiotic organelles research was focused on possible differences in the targeting process.

Earlier studies were concentrated on amino acid distributions of N-terminal targeting sequences. Next to a lot of common features only slight differences between both sequence types could be determined. A higher content of arginine residues which introduce a positive charge into the protein was detected in plant mitochondrial presequences (11.3%) in comparison to chloroplast transit peptides (5.8%). Conversely, serine residues were more abundant in chloroplast transit peptides compared to mitochondrial presequences (Peeters & Small, 2001). These data support a possible discrimination between the organelles at the receptor but additional factors might be present at earlier steps during the targeting process.

In this work cytosolic factors could be identified not only in participating in preprotein transport to the chloroplast but were also found to be associated with mitochondrial preproteins. The following paragraph displays a working model of preprotein targeting to mitochondria in plants (Fig. 22).

Next to HSP70, 14-3-3 is binding to mitochondrial preproteins which are phosphorylated possibly in the 14-3-3 binding site. The kinase responsible for preprotein phosphorylation might thereby differ from the dual-specificity kinase which phosphorylates chloroplast preproteins, since phosphorylation is also occurring in reticulocyte lysate. Phosphorylation of the preprotein might further enhance import of

preproteins into the organelle but dephosphorylation seems to be even more indispensable for an efficient import. Preproteins might not only be recognized by the receptor protein TOM20 but also by the TPR-containing receptor OM64. The presence of the latter one suggests an interaction of cytosolic HSP70 or HSP90 with the TPR domain of the receptor protein and preproteins might thus be delivered indirectly by those chaperones to the mitochondria membrane. So far, interactions of HSP90 with mitochondrial preproteins could not be verified, but distinct HSP90 interacting mitochondrial preproteins might be identified in future approaches.

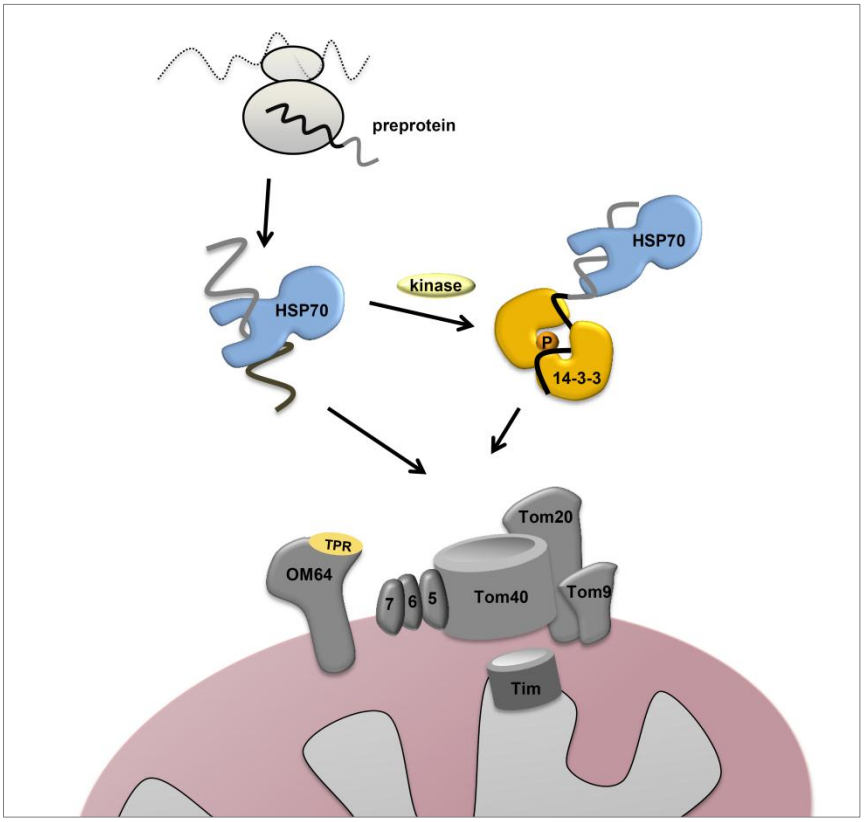


Figure 22: Working model of preprotein transport to mitochondria. Next to HSP70, 14-3-3 chaperones are predominantly bound to mitochondrial preproteins. A cytosolic kinase phosphorylates preproteins before interaction with 14-3-3 occurs. HSP90-preprotein complexes might also exist, but the presence has to be determined in future approaches.

The presence of a similar chaperone machinery for preprotein delivery to chloroplasts and mitochondria might be regulated and part of the differentiation process, since different isoforms of cytosolic chaperones exist (in *Arabidopsis* 4 HSP90 and 15 14-3-3 isoforms). Further approaches to identify specific chaperone isoforms within preprotein complexes outline challenging research projects.

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6 Appendix

6.1 Analysis of 14-3-3 binding sites within 75 chloroplast preproteins

	accession	length TP/ mature protein	14-3-3- motifs position	recognized amino acids	Pattern
1	AT1G02910	92/453	282-287	RLSTNR	3
		92/453	386-391	KAETVS	3
2	AT1G06680	31/263	56-62	RLALTLL	2
		31/263	19-24	RSSSSS	3
		31/263	90-95	KTNTDF	3
3	AT1G08540	39/572	342-348	RLVISIA	2
		39/572	59-64	RLPSFD	3
		39/572	230-235	KTASGI	3
		39/572	239-244	KTGSSP	3
		39/572	271-276	KLLTVR	3
		39/572	398-403	KSLSDQ	3
4	AT1G08550	82/462	371-377	RIEKTVE	2
		82/462	403-409	RTEMTLF	2
5	AT1G11750	51/271	-	-	-
6	AT1G12410	54/279	48-54	RVYKSLQ	2
7	AT1G15820	46/258	-	-	-
8	AT1G17650	44/358	48-54	RVYSSLQ	2
9	AT1G29930	23/267	133-138	KAGSQI	3
10	AT1G30380	32/130	-	-	-
11	AT1G32200	62/459	260-265	R VITDP	1
		62/459	56-62	RGGVSVR	2
		62/459	146-152	RADETVV	2
		62/459	260-265	R VITDP	3
		62/459	297-302	KANTRS	3
12	AT1G42970	45/447	17-22	RLQSKS	3
		45/447	66-71	KAVTTS	3
		45/447	155-160	KVVSNR	3
		45/447	262-267	HSYTGD	3
13	AT1G44446	36/536	19-24	KTRSSF	3
		36/536	118-124	LDKPSSS	2
14	AT1G44575	59/265	-	-	-
15	AT1G45474	42/256	18-24	RDASSVI	2
		42/256	26-32	RRISSVK	2
16	AT1G60950	52/148	40-45	KSGTAR	3
		52/148	92-97	RAGSCS	3
		52/148	102-107	KVVSGS	3
17	AT1G64810	47/436	47-52	RLGTVF	3
		47/436	244-249	RRRTQP	1
18	AT1G76100	66/171	26-31	KTLSTI	3
		66/171	33-38	RSTSAT	3
19	AT2G06520	58/116	52-57	KALTGI	3
20	AT2G20890	67/300	47-52	RSTSKS	3
		67/300	190-195	RAGSKE	3
21	AT2G26670	54/282	84-89	RLHTKD	3
22	AT2G28800	55/462	374-380	RAKRSIA	2
		55/462	46-51	KLFTTS	3
		55/462	152-157	AARQSG	3
23	AT2G30200	68/393	62-68	RSRISMS	2
		68/393	64-70	RISMSVS	2
		68/393	343-348	RQVTSP	1
		68/393	384-389	KSASFE	3
24	AT2G33450	97/143	18-23	KSMTKS	3
25	AT2G48120	22/313	112-117	KLVSSF	3
		22/313	170-175	RVETEI	3
26	AT3G21055	69/103	30-35	RASTSD	3
27	AT3G24430	64/532	85-90	KALSQI	3
		64/532	462-468	RSAQSVD	2
28	AT3G52180	54/379	25-30	HSSSSS	3
		54/379	52-57	HAVSES	3
		54/379	254-260	RKTVTLT	2
		54/379	281-287	RIPLTLD	2
		54/379	351-356	RLSSED	3
29	AT4G03280	50/229	-	-	-
30	AT4G09650	48/234	185-190	RVKTVI	3
31	AT4G11960	49/313	35-40	RSISLR	3
		49/313	40-46	RRRLTLL	2
		49/313	303-308	RLITLP	1/3
32	AT4G17600	39/262	39-44	RASSDS	3
33	AT4G21210	86/403	57-63	RARTLR	3
		86/403	66-71	KLDSTI	3
		86/403	154-159	HLFSGI	3
		86/403	217-222	HLGTNP	3
34	AT4G22260	56/351	16-22	RPLVTLR	3
		56/351	54-60	RVQATIL	3
		56/351	304-310	RACQTLG	3
		56/351	313-319	RSPHSIL	3
35	AT4G27440	43/401	128-134	RAAKSVG	2
36	AT4G28750	44/143	-	-	-
37	AT4G36810	56/371	26-31	KSRSRS	3
		56/371	28-33	RSRSCP	1/3
		56/371	43-49	RSKRTVS	2
		56/371	46-51	RTVSSS	3
		56/371	148-153	HTMSLI	3
38	AT4G37925	21/217	138-143	KVYSYF	3
39	AT5G01600	47/255	-	-	-
40	AT5G16710	42/258	242-247	RAETED	3
41	AT5G17710	64/324	18-23	HAPSVP	3
		64/324	149-154	KVASLS	3

	accession	length TP/ mature protein	14-3-3- motifs position	recognized amino acids	Pattern
		64/324	206-211	RAKSQI	3
42	AT5G20720	50/253	16-22	RSLASLD	2
43	AT5G21930	65/883	39-44	RHCSRP	1
		65/883	666-672	RYSKT VV	2
		65/883	352-358	RVGDSLL	2
		65/883	70-75	KSITSD	3
		65/883	538-543	RLASID	3
		65/883	549-554	KT GTL	3
		65/883	582-587	KTATHP	3
44	AT5G33320	30/408	55-60	RLASSD	3
45	AT5G35220	18/548	81-86	KTATFE	3
		18/548	419-424	HAATVS	3
46	AT5G35630	45/430	94-99	KSRTIE	3
47	AT5G38660	32/286	234-239	KALTLS	3
48	AT5G39830	26/448	-	-	-
49	AT5G42480	67/801	45-50	RLLSDF	3
		67/801	639-644	KSSSSF	3
		67/801	776-781	RTYTTR	3
50	AT5G42650	32/518	25-30	RVLTRP	1/3
		32/518	46-51	RTGSKD	3
		32/518	269-274	HTFSLP	3
51	AT5G46290	46/473	18-23	RLPSNR	3
		46/473	84-89	KLLSGE	3
		46/473	152-157	KLNTID	3
		46/473	267-272	TGMG GL	3
		46/473	384-389	KSTSGI	3
52	AT5G48300	70/520	54-60	RLCKSVV	2
		70/520	31-36	RTLSFS	3
		70/520	70-75	KAVSDS	3
		70/520	277-281	KVDTTI	3
53	AT5G49030	86/1093	64-69	RFSTEP	1
		86/1093	116-121	RANSLT	3
		86/1093	550-555	RTATMD	3
		86/1093	598-603	HVKTKE	3
		86/1093	613-618	HVKSII	3
		86/1093	875-881	RSCQTV L	2
54	AT5G49940	16/235	-	-	-
55	AT5G50210	69/718	39-45	RRIYTIN	2
		69/718	96-101	KSLTEP	3
		69/718	123-128	KTESNR	3
		69/718	225-230	KVPSFE	3

	accession	length TP/ mature protein	14-3-3- motifs position	recognized amino acids	Pattern
		69/718	603-608	KTSSDS	3
56	AT5G51070	89/945	54-59	RFSTTP	1
		89/945	727-733	RRPF TVV	2
		89/945	16-21	RLLSAS	3
		89/945	890-895	R TVTEI	3
57	AT5G51100	46/305	252-257	KLVSWE	3
		46/305	262-267	RLESAI	3
58	AT5G51820	52/623	-	-	-
59	AT5G53280	67/272	28-33	HLISKS	3
60	AT5G53490	33/236	24-30	REPRSLV	2
		33/236	27-33	RSLVTVH	2
61	AT5G54290	21/354	52-57	RLESKS	3
62	AT5G54800	64/388	26-32	RSPVSLS	2
63	AT5G54810	52/470	26-31	HLKSPF	3
		52/470	43-48	RSKSSS	3
		52/470	45-50	KSSSFS	3
		52/470	238-243	HSGTAT	3
		52/470	345-350	HAATLT	3
64	AT5G55280	90/433	53-58	RSDSTR	3
		90/433	58-63	RSKSMR	3
		90/433	424-429	KSSSPR	3
65	AT5G57850	57/373	205-210	KVVTSS	3
66	AT5G58260	45/209	162-167	KVLSKS	3
67	AT5G58330	52/443	10-15	KTTSPF	3
		52/443	118-123	KLASGE	3
68	AT5G61410	45/281	21-27	RPERSLL	2
		86/477	398-404	RAGGTMT	2
		86/477	373-378	KLGSLT	3
69	AT5G64040	81/171	117-122	RAFTVQ	1
70	AT5G64860	45/576	6-11	RPSSSP	1
		45/576	21-26	RLSSPD	3
		45/576	35-40	RNRTKP	1
		45/576	567-573	RDLLSLY	2
71	AT5G66190	64/360	41-47	RRVVSVK	2
72		64/360	117-123	REGQSIG	2
73	AT5G66380	11/308	40-45	RGSSLP	1
		11/308	251-257	RYIDSLH	2
		11/308	202-207	KSESTD	3
74	AT5G67030	59/667	-	-	-
75	AAA34116	57/180	31-34	SAAS	old

6.2 Analysis of 14-3-3 binding sites within 75 mitochondrial preproteins

	accession	length TP/ mature protein	14-3-3- motifs position	Recognized amino acids	Pattern
1	AT1G06570	12/473	352-358	RKRSSIG	2
2	AT1G09210	22/424	114-119	KLLSGD	3
		22/424	173-178	HVYTFI	3
		22/424	326-331	KSGSLF	3
3	AT1G14810	47/375	29-35	RVKMSLQ	2
4	AT1G20350	-/217	196-201	HSESRT	3
5	AT1G32350	41/318	40-45	RLLSSD	3
		41/318	189-194	HLMTFI	3
		41/318	162-267	HLKSLR	3
6	AT1G32580	57/229	16-21	RLISTS	3
		57/229	71-76	KSGSNF	3
7	AT1G47260	43/278	6-12	RAIYTVG	2
		43/278	271-276	KVPSTQ	3
8	AT1G50940	35/363	22-27	RSISIS	3
		35/363	190-195	R STSFP	1
		35/363	33-39	RCISTLI	2
		35/363	252-258	RALKSVE	2
		35/363	190-195	STSFP	3
9	AT1G51390	95/275	60-65	RSSSFP	1
		95/275	6-12	RLLNSLS	2
		95/275	13-19	RHTASVT	2
		95/275	14-19	HTASVT	3
		95/275	60-65	RSSSFP	3
10	AT1G51980	59/503	475-480	KVISKP	2
		59/503	420-425	THLLER	3
11	AT1G55900	25/376	-	-	-
12	AT1G59900	5/389	5-10	RLSSRS	3
		5/389	23-28	RLISTD	3
		5/389	291-296	HSMSDP	3
13	AT1G63940	46/486	380-385	HTDTYD	3
		46/486	466-471	KLASAS	3
		46/486	247-252	RLFT PS	3
14	AT1G79230	58/379	16-21	RLITPS	3
		58/379	134-139	RKTSLP	1
15	AT1G79950	42/1040	844-850	RNGLTIL	2
		42/1040	1029-1035	RKRQSII	2
		42/1040	8-13	RSGSPT	3
		42/1040	778-783	RLVTEQ	3
		42/1040	964-969	KLNTEE	3
		42/1040	72-78	RVIESLQ	2
		42/1040	589-595	RNSMTVW	2
16	AT1G80230	58/171	39-44	RASSIP	1
		58/ 171	9-14	HLKSIS	3
		58/ 171	39-44	RASSIP	3
17	AT2G05710	90/ 990	47-53	RSFGTIS	2
		90/ 990	88-94	RTFSSMA	2
		90/ 990	421-427	RSDETVA	2
		90/ 990	15-20	RAASSR	3
		90/ 990	20-25	RSSSLF	3
		90/ 990	202-207	KLGSDS	3
		90/ 990	935-940	HLPTDI	3
		90/ 990	957-062	KSFTCT	3
18	AT2G14170	32/607	43-48	KSKTKR	3
		32/607	60-65	KLRSSS	3
		32/607	62-67	RSSSST	3
		32/607	584-589	KTVTQQ	3
		25/367	3-9	RQSFSLL	2
		25/367	170-175	K VITKF	3
19	AT2G17270	-/309	138-143	RVQTQP	1
		-/309	138-143	RVQTQP	3
		-/309	287-292	KVLSGF	3
20	AT2G20530	-/286	100-105	R VLTRP	1
		-/286	158-164	RKI LTLR	2
		-/286	100-105	R VLTRP	3
21	AT2G20800	9/582	9-14	RASSLF	3
		9/582	555-560	KLVSWR	3
22	AT2G22500	56/395	159-165	RNYKSVL	2
23	AT2G26080	41/1044	40-46	RYVSSVS	2
		41/1044	71-76	RSISVD	3
		41/1044	269-274	KVVTVD	3
		41/1044	402-407	KATSNI	3
		41/1044	450-455	KLGTAQ	3
24	AT2G33210	32/585	25-30	RLNSTR	3
25	AT2G36070	44/469	87-92	KVRT KQ	3
		44/469	369-374	RA FTSQ	3
26	AT2G44350	6/473	5-10	RSVSAF	3

	accession	length TP/ mature protein	14-3-3- motifs position	Recognized amino acids	Pattern
		6/473	456-461	KSVTMD	3
		6/473	101-106	RGLSIP	1
		6/473	262-268	RLYITIH	2
		6/473	134-139	KVPSKE	3
27	AT2G44520	57/431	-	-	-
		57/431	71-76	KSGSNF	3
28	AT3G01800	64/267	8-13	RALSSR	3
		64/267	181-187	RLVASIP	2
		64/267	110-115	K LRTGR	3
29	AT3G03070	29/110	19-25	RRNFSVA	2
30	AT3G06790	45/244	8-14	RTLSTLL	2
		45/244	214-220	RRRETMQ	2
		45/244	16-21	KTLSSS	3
		45/244	44-49	KVSSSR	3
31	AT3G07480	25/159	7-12	KLSSQI	3
		25/159	70-75	RALTHT	3
32	AT3G08580	-/381	-	-	-
33	AT3G10110	18/173	-	-	-
34	AT3G10690	71/950	26-32	RLSSSLL	2
		71/950	723-728	KLFSGI	3
		71/950	494-499	KASSHS	3
35	AT3G10920	6/231	88-93	KLQ SAI	3
36	AT3G12780	75/481	26-32	RARASLL	2
		75/481	72-78	RGVVSMA	2
		75/ 481	62-67	KVESVR	3
		75/ 481	277-282	EVESLV	3
37	AT3G13860	31/572	7-12	KLSSSI	3
		31/572	19-24	KLVSGR	3
		31/572	88-93	KSISFQ	3
		31/572	385-390	KSTSTF	3
38	AT3G15000	56/395	13-19	RPAKSLS	2
		56/395	4-9	HTISRS	3
39	AT3G19480	38/588	16-21	RLVTTP	1
		38/588	16-21	RLVTTP	3
		38/588	210-215	H VITHD	3
40	AT3G20970	54/283	6-12	RLVTSLS	2
		54/283	18-23	KVVSGT	3
41	AT3G23990	31/577	227-232	RGYTSP	1
		31/577	247-253	RRGISMA	2
42	AT3G25120	-/189	-	-	-
43	AT3G45300	29/409	20-25	RSFSSR	3
		29/409	106-112	RASGS VA	2
		29/409	59-64	KTNSFP	3
		29/409	141-146	KLISGE	3
44	AT3G48000	21/538	4-10	RRVSSLL	2
		21/538	12-17	RSFSAS	3
45	AT3G48850	72/363	191-196	RVQTQP	1
		72/363	29-34	HLLKSQP	3
		72/363	191-196	RVQTQP	3
46	AT3G48870	45/952	611-616	KVSSDE	3
		45/952	729-735	RR PYTLV	2
47	AT3G54110	29/306	-	-	-
48	AT3G60100	25/464	5-10	RSVSAI	3
		25/464	54-59	KLKSEQ	3
		25/464	98-103	RGM SIP	1
		25/464	255-261	RLYVTI H	2
		25/464	131-136	KVPTKE	3
49	AT4G08390	94/372	4-10	RVSLTLN	2
		94/372	47-53	RSTLTLS	2
		94/372	40-45	RSSSSS	3
		94/372	69-74	RLSSSS	3
		94/372	98-103	KSSSSD	3
50	AT4G10750	65/358	55-60	RYSSSP	1
		65/358	15-21	RDVTSLL	2
		65/358	75-81	RSKTSLK	2
		65/358	85-91	RGGETLY	2
		65/358	30-35	KSLSPS	3
		65/358	49-54	KTLTPI	3
51	AT4G11120	63/395	61-66	RSFSSE	3
52	AT4G24190	23/823	723-728	RIASDP	1
		23/823	62-68	RESESMS	2
		23/823	761-767	RIYNSVK	2
		23/823	365-370	HSLSKD	3
		23/823	550-555	KLTSLD	3
		23/823	4-10	RTLVSVL	2
53	AT4G26970	60/995	15-21	RLSSSLS	2
		60/995	93-99	RKYATMA	2
		60/995	426-432	RSDETVS	2
		60/995	10-15	RSASAR	3
		60/995	45-50	RSKSFS	3
		60/995	57-62	RVCSAS	3
54	AT4G31500	15/499	138-143	RVA SFR	3

	accession	length TP/ mature protein	14-3-3- motifs position	Recognized amino acids	Pattern
55	AT4G31810	27/409	8-13	RRVSEP	1
		27/409	236-241	KLLTDD	3
		27/409	345-350	KVVSGD	3
		27/409	399-404	KLPTAQ	3
56	AT4G35650	5/368	3-9	RRSVSIF	2
		5/368	4-9	RSVSIF	3
		5/368	39-44	RT VTLI	3
		5/368	171-176	KVITKF	3
57	AT4G39660	12/476	18-24	RRAISLL	2
		12/476	10-15	RATSDI	3
		12/476	175-180	RLYTGS	3
58	AT5G04130	76/732	35-41	RHSSTLS	2
		76/732	23-28	RLFSSS	3
		76/732	36-41	HSSTLS	3
		76/732	386-392	TLNT LA	2
		76/732	494-499	KSSSLP	3
59	AT5G08300	18/347	7-13	RLIGSLS	2
		18/347	203-208	RSGTLT	3
60	AT5G08690	13/556	5-11	RVLSSLL	2
		13/556	12-17	RSSSGR	3
		13/556	28-33	RLPSPS	3
		13/556	489-494	RF LSQP	1
61	AT5G09590	57/682	463-468	RNTTIP	1
		57/682	642-647	KAVSKI	3
62	AT5G13400	38/624	24-30	RKQLSVF	2
		38/624	362-367	KLCTVT	3
		38/624	562-567	KAATRD	3
63	AT5G13420	61/438	19-24	KSSSSS	3
		61/438	40-45	KLSSSQ	3
		61/438	120-125	KAISTS	3
64	AT5G13450	36/238	25-30	RSKSLF	3
65	AT5G14040	75/375	202-207	RVQTQP	1
		75/375	202-207	RVQTQP	3
66	AT5G14210	23/812	430-435	REKSVP	1
		23/812	115-120	RLTSLR	3
		23/812	459-464	RLISQT	3
		23/812	476-481	RSFSFE	3
		23/812	807-812	KSDTSS	3
		23/812	299-305	RFLFSLP	2
		23/812	139-144	RL NSLE	3
67	AT5G14590	65/484	474-479	KLKTQF	5
		65/484	92-98	RVIWSMI	2
		65/484	212-218	RATDTVI	2
68	AT5G19760	-/298	152-157	HALTRI	3
69	AT5G26780	29/517	7-13	RLSSSVK	2
		29/517	27-33	RFMSSLS	2
70	AT5G42150	63/315	21-27	RLTQSMA	2
		63/315	302-308	RMENTVG	2
		63/315	10-15	RTISSS	3
71	AT5G50920	38/929	30-36	RSRRSVK	2
		38/929	532-537	KAGTLR	3
		38/929	559-564	KAESET	3
		38/929	590-595	KVSTDE	3
		38/929	708-714	TGA GAAC	2
		38/929	332-337	AGGAAG	3
72	AT5G62530	9/556	30-36	RLNHSIP	2
73	AT5G65720	51/453	12-17	RTLTKP	1
		51/453	4-9	KVISAT	3
		51/453	12-17	RTLTKP	3
		51/453	144-149	HVITTQ	3
74	AT5G66760	32/634	40-46	RSSYTIV	2
		32/634	237-243	RSSQTIL	2
		32/634	15-20	KTSSLF	3
		32/634	259-264	HTCTGD	3
		32/634	334-449	RSMTME	3
		32/634	517-523	RTQE TLE	2
75	AT5G66860	76/249	6-12	RGLRSVT	2
		76/249	9-14	RSVTAE	3

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Curriculum Vitae

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Scientific publications and poster presentations

Publications:

Fellerer C, Schweiger R, Schoengruber K, Soll J & Schwenkert S. 2011. **Cytosolic HSP90 Cochaperones HOP and FKBP interact with freshly synthesized chloroplast preproteins of *Arabidopsis.*** Mol Plant. *i*4:1133-1145

Umate P, Fellerer C, Schwenkert S, Zoryan M, Eichacker LA, Sadanandam A, Ohad I, Herrmann RG, Meurer J. 2008. **Impact of PsbTc on forward and back electron flow, assembly, and phosphorylation patterns of photosystem II in tobacco.** Plant Physiology. 148(3):1342-53.

Poster:

Fellerer C, Schweiger R, Schoengruber K, Soll J, Schwenkert S. **Association of HSP90 and its cochaperones with organellar preproteins.** EMBO conference series - the biology of molecular chaperones. 2011. Grundlsee. Austria

Fellerer C, Schoengruber K, Soll J & Schwenkert S. **The Function of HSP90 in Preprotein Sorting.** 5th int. conference on the HSP90 Chaperone Machine. 2010. Les Diablerets. Switzerland

Fellerer C, Schoengruber K, Lamberti G, Schwenkert S, Soll J. **Precursor protein transport in higher plants – from cytosol to chloroplast**. Cellular Protein Transport Conference. 2009. Heidelberg. Germany

Ehrenwörtliche Versicherung

Ich versichere hiermit ehrenwörtlich, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

München, den 27.03.2012

Erklärung

Ich habe zuvor nicht versucht, anderweitig eine Dissertation einzureichen oder mich einer Doktorprüfung zu unterziehen. Die Dissertation wurde keiner weiteren Prüfungskommission weder in Teilen noch als Ganzes vorgelegt.

München, den 27.03.2012