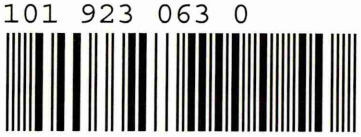

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TPRc1: A novel chaperone receptor at the chloroplast outer membrane

Clara Ottilie Freiin Loeffelholz von Colberg

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Abstract

The correct targeting of proteins to their organelles is crucial for the organisation and viability of a cell. Although mitochondria and chloroplasts have their own genome, over 90% of their protein content is encoded by nuclear DNA and needs to be imported from the cytosol after translation. In this case a cytosolic preprotein-complex is recognized by receptors, which are localized at the outer membrane of the organelle.

Cytosolic chaperones like the heat shock protein 70 (Hsc70) and 90 (Hsp90) have been found to be part of the preprotein-complex to keep the proteins in an unfolded, targeting compatible state. The general import receptors, Tom70 at the outer membrane of mitochondria, and Toc64 at the chloroplast envelope, have been shown to bind these chaperones via a tetratricopeptide repeat (TPR) domain i.e. are chaperone receptors.

A search for membrane proteins containing a TPR domain resulted in an uncharacterised protein from *Arabidopsis thaliana* named TPRc1. Its sequence includes an N-terminal TPR domain and a C-terminal membrane anchor suggesting that TPRc1 is a chaperone receptor.

In this work, phylogenetic comparison of the TPR domain from TPRc1 showed that TPRc1 is most closely related to uncharacterised plant proteins. Comparison of the TPR domain from TPRc1 to *Arabidopsis* proteins resulted in a close similarity between the TPR domain of TPRc1 and the Hsp90 binding TPR domains of PPIases. A comparison with TPR domains from other chaperone receptors showed that the TPR domain of TPRc1 is most closely related to the TPR domain of Toc64. According to quantitative real time RNA analysis and western blotting TPRc1 is expressed in all tissues, but highest protein levels can be detected in buds, flowers, siliques and roots. Evidence from confocal microscopy and targeting assays supports localisation to the chloroplast envelope, with the N-terminus including the TPR domain, facing the cytosol. Pulldown assays suggest that the TPR domain of TPRc1 is able to pull down Hsc70 via interaction with the C-terminal end of Hsc70, and that TPRc1 is able to interact specifically with chloroplast precursor complexes. Crosslinking and immunoprecipitation of radiolabeled, into chloroplasts imported TPRc1 resulted in no adducts, suggesting that TPRc1 is isolated in the membrane. Phenotyping of knockout mutants has not been possible so far, since a T-DNA insertion line with an insertion inside an exon of the TPRc1 gene is not available.

Taken together, we propose TPRc1 to be a novel Hsc70 binding plant chaperone receptor, which is involved into preprotein targeting to chloroplast similar to Toc64.

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The objectives of this work were to characterise the novel protein TPRc1 in terms of abundance, membrane- and cytosolic interaction partners and to analyse the phenotype of TPRc1 depleted plants. I confirm that the experiments and written work have been done by myself. Materials and results, which were not obtained by myself, are acknowledged inside the text. Received help in any kind is addressed in the Acknowledgements.

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The aim of this project is to characterise TPRc1 in terms of abundance, localisation, interaction and function. TPRc1 is predicted to have a TPR domain, which is able to bind the C-terminus of cytosolic chaperones, and a C-terminal membrane anchor. Because TPRc1 homologues are only found in plants, the protein might be located at chloroplasts. Based on this work, TPRc1 is proposed to be involved in targeting of preproteins from the cytosol to the chloroplast envelope.

Most of the proteins are encoded by nuclear DNA and synthesized in the cytosol. Thus, they must be targeted from there to their destination. The organisation of protein targeting in eukaryotic cells is complex and needs to be strictly regulated, since incorrect protein targeting can be the reason for severe diseases such as Alzheimer's or Parkinson's disease caused by protein aggregation or mislocalisation (Offe *et al*, 2006). Additionally, inclusion of protein targeting into biotechnological methods could improve or facilitate the production of molecules through biochemical reactions, e.g. the production of plastic precursors in plants (van Beilen *et al*, 2008). Thus the understanding of protein targeting is an important approach to find new possibilities to prevent and cure localisation-dependent diseases and to find new approaches for biotechnology.

Every organelle possesses its own translocon, which is responsible for recognition and insertion or translocation of the precursor protein into the organelle (summarised by Figure 1). Therefore, the proteins to be targeted themselves or a carrier protein, which interacts with them, are recognized by a receptor localised at the organelle membrane facing the cytosol. The receptor is itself in close proximity to a translocation pore, which allows the protein to pass across the membrane barrier. The main translocation complexes facing the cytosol have been named dependent on their organelle translocase of the outer membrane (TOM) at mitochondria, translocase of the outer chloroplast envelope (TOC), secretory complex Sec61 (Sec61) at the endoplasmic reticulum (ER) and peroxins (PEX) at peroxisomes.

The best understood protein targeting pathway is the cotranslational binding of an N-terminal signal sequence of the precursor by the signal recognition particle (SRP), which recruits the whole translation machinery to the translocation complex at the ER membrane, where the protein is directly

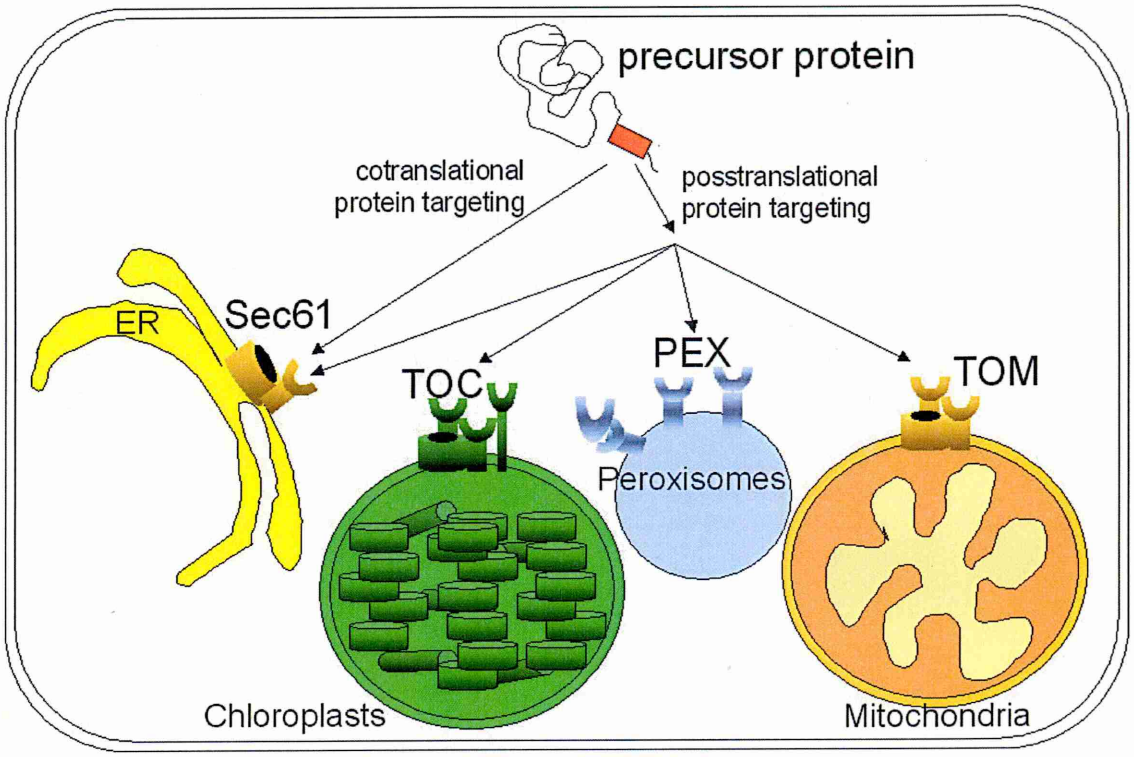


Figure 1: Schematic demonstration of protein targeting. With the exception of cotranslational protein targeting to the ER, precursor proteins are targeted posttranslationally to their organelles. The signal sequence can be N-or C- terminal and is highlighted by a red box in the precursor sequence. Each organelle contains a translocation complex, which recognizes signal peptides specifically. The translocation complexes are called translocase of the outer membrane (TOM) at mitochondria, translocase of the outer chloroplast envelope (TOC), secretory complex Sec61 (Sec61) at the endoplasmic reticulum (ER) and peroxins (PEX) at peroxisomes.

translated into the ER. Beside the cotranslational targeting of proteins to the ER, there exist multiple pathways for protein recognition through an organelle specific signal peptide and posttranslational targeting to its organelle. Additionally some signal sequences are signal anchors, which insert into the membrane of the target organelles.

1.1. Signals for protein targeting and their recognition

Organelles need proteins that are encoded by nuclear DNA and these proteins require targeting from the cytosol. Therefore the identity of precursor proteins from different organelles needs to be differentiated by the receptors at the outer membrane of the organelles specifically. This is mediated by the recognition of a specific signal peptide of the precursor protein by the receptor.

Organelle	Targeting signal	Sequence/ properties	Location (C/ N-terminal /internal)	Receptor (R) / cytosolic recognition (C)
Endoplasmic reticulum	Co-trans-lational signal	positive, hydrophobic, polar	N-terminal	SRP receptor/SRP
	unknown	unknown	unknown	Sec72/Hsp70
	Tail anchor	positive + long/ negative + short/ slightly positive + short	C-terminal	Get1 + Get2/Get3 Also: SRP receptor/SRP Unknown/Hsp70 + Hsp40
Mitochondria	Internal signal	hydrophobic	Internal	Tom70 (mtOM64?), Tom22/Hsp70 and Hsp90
	N-terminal signal	positive + hydrophobic	N-terminal	Tom70, Tom20, Tom22/MSF
	unknown	unknown	unknown	Tom34/Hsp90
	Tail anchor	positive + short	C-terminal	Unknown
Chloroplasts	chloroplast signal peptide	positive + serine/threonine rich	N-terminal	Toc34,Toc159/Hsp70, 14-3-3
	unknown	unknown	unknown	Toc64,Toc34/Hsp90
	Tail anchor	unknown	C-terminal	Unknown
Peroxisomes	PTS1	SKL	C-terminal	Pex5/Pex5
	PTS2	(R/K)/(L/V/I)X_5 (H(Q))(L/A)	N-terminal	Pex7/Pex7
	mPMP	hydrophobic	?	Pex3/Pex19

Table 1: The chemical properties of signal sequences and their recognition by receptors of their target organelles(Chewawiwat *et al*, 1999; Hachiya *et al*, 1993; Holroyd *et al*, 2001; Izard *et al*, 1996; Kalies *et al*, 1998; Koehler, 2004; Lister *et al*, 2007; Platta *et al*, 2007; Ponting, 2000; Young *et al*, 2003; Abell *et al*, 2007; Abell *et al*, 2004; Becker *et al*, 2004; Borgese *et al*, 2007; Fujiki *et al*, 2006; May *et al*, 2000; Qbadou *et al*, 2006; Rabu C et al, 2008; Schuldiner *et al*, 2008; Soll, 2002).

The translocation signal is highly conserved and is often cleaved off during translocation (Agarraberes *et al*, 2001). Table 1 summarises the properties of the known signal peptides.

1.1.1. Protein targeting to the endoplasmic reticulum (ER)

Many secretory proteins, plasma membrane proteins, proteins of the ER, and many other proteins of eukaryotic cells are transported cotanslationally to the ER (Rapoport, 2007). Here, an N-terminal signal sequence is recognized by the signal recognition particle (SRP) during translation in a GTP dependent manner. The signal sequence consists of a short positively charged region followed by a highly hydrophobic core region and a more polar carboxyl terminus (Izard *et al*, 1996). After recognition by SRP the translation machinery is recruited to the ER membrane by the interaction of SRP with the membrane anchored SRP receptor. SRP is released upon GTP hydrolysis by the SRP receptor and the ribosome translates the protein directly into the ER through the translocation channel formed by the Sec61 core complex, which consists of Sec61α, Sec61β and Sec61γ (Bernstein, 1998; Kalies *et al*, 1998). However, there exists at least one other pathway for protein targeting into the ER in yeast, which is posttranslational. For this pathway a heptameric complex formed out of the Sec61 complex and the Sec62-Sec63 complex is required. The Sec62-Sec63 complex consists of four polypeptides: Sec62, Sec63, Sec71 and Sec72 (High, 1995; Kalies *et al*, 1998). Sec62 contains a DnaJ-like domain and is believed to interact with the yeast Hsp70 (Kalies *et al*, 1998). This targeting receptor complex is best defined in yeast (High, 1995), but homologues of Sec62p and Sec63p can also be found in dog pancreas microsomes (Tyedmers *et al*, 2000) giving evidence, that at least part of this complex is generally abundant in eukaryotes.

1.1.2. Protein targeting to mitochondria

Although mitochondria have their own genome, 99% of mitochondrial proteins are encoded by nuclear DNA (Rehling *et al*, 2004), synthesised in the cytosol and imported after translation. The signal sequence of these proteins is hydrophobic, in some cases additionally positively charged and can be N-terminal or internal (Koehler, 2004). The mitochondrial import stimulating factor (MSF), Hsp90 and Hsc70 can bind to the precursor protein in the cytosol and are able to interact with the mitochondrial translocase (Hachiya *et al*, 1993; Zara *et al*, 2009). MSF has been shown to interact with mitochondrial targeting signal dependent on ATP (Hachiya *et al*, 1993; Komiya *et al*, 1994). The mitochondrial outer membrane receptors Tom70 and Tom37 are proposed to interact with the precursor-MSF complex (Hachiya *et al*, 1993). Hsc70 and Hsp90 in mammals and Hsc70 in yeast have been shown to interact with Tom70 indicating a recognition of the cytosolic chaperone-precursor complex by Tom70 (Young *et al*, 2003).

The translocase of the outer membrane (TOM) is composed out of three receptors, Tom20, Tom22 and Tom70, and one general import pore, built out of two Tom40 proteins and stabilised by the small proteins called Tom5, Tom6 and Tom7 (Neupert *et al*, 2007). Tom20 recognizes mainly N-terminal signals while Tom70 is the receptor for precursor proteins with an internal signal (Brix *et al*, 1999; Koehler, 2004). Tom22 is believed to be the organizer of the TOM complex as it is the convergence point for precursor proteins which initially bind to either Tom70 or Tom20 (Koehler, 2004). Plants do not have Tom70, but possess two other mitochondrial receptors, which may replace Tom70 in plants: the outer mitochondrial membrane protein of 64 kD (mtOM64) and METAXIN (Chew *et al*, 2004; Lister *et al*, 2007). For mtOM64 it could be shown, that it interacts with a variety of precursor proteins in yeast two hybrid assays, but a depletion of mtOM64 resulted in no difference from the wild type (Lister *et al*, 2007). The depletion of METAXIN resulted in a lower protein level of membrane proteins, indicating a role in targeting of membrane proteins to mitochondria (Lister *et al*, 2007). However, the exact role for both proteins in the targeting process of mitochondrial precursors is not known.

1.1.3. Protein targeting to peroxisomes

Peroxisomes have a single membrane and do not contain DNA. The import of proteins into peroxisomes occurs either cotranslationally, as they are believed to bud from the ER in vesicle like manner induced by the peroxins (Pex) Pex3, Pex16 and Pex19, or posttranslationally (Platta *et al*, 2007). Import of freshly synthesized proteins can occur via a translocation machinery at the membrane or via dynamic receptors, which are located in the cytosol. Here, the receptor recognizes the precursor in the cytosol and guides it to the docking site at the peroxisomal membrane, which is composed out of Pex13, Pex14 and Pex17 (Holroyd *et al*, 2001; Platta *et al*, 2007). The complex is then disassembled to release the cargo and the receptor returns to the cytosol (Platta *et al*, 2007). This pathway is mediated by the receptors Pex5 and Pex7, which bind to the C-terminal signal sequence SKL (peroxisomal targeting signal 1 (PTS1)) and the N-terminal signal sequence (R/K)/(L/V/I)X_5(H(Q))(L/A) (peroxisomal targeting signal 2 (PTS2)), respectively (Holroyd *et al*, 2001; Platta *et al*, 2007). A translocation channel in the membrane of peroxisomes has not yet been characterised.

Pex19 and Pex3 belong to another protein targeting system to peroxisomes. Pex19 is a 33 kDa cytosolic protein (Matsuzono *et al*, 2006). Pex19 contains an N-terminal flexible region, which is able to interact with Pex3 (42 kDa), a C-terminal rigid domain, which binds multiple peroxisomal proteins in a chaperone like manner and ends with a CAA-X farnesylation site (Fujiki *et al*, 2006; Matsuzono *et al*, 2006). Pex3 is located at the peroxisomal outer membrane and is thought to be the docking site for Pex19 at the peroxisomal membrane. Hettema et al. (2000) show that peroxisomal membrane proteins are mislocated in Pex3 and Pex19 depleted yeast cells (Hettema *et al*, 2000). Thus the Pex19/Pex3 system might be a peroxisomal chaperone receptor system, which mediates protein insertion into the peroxisomal membrane.

1.1.4. Protein targeting to chloroplasts

Chloroplasts import more than 95% of their proteins posttranslationally from the cytosol (Soll, 2002). Most chloroplast precursors have an amino terminal presequence, which is positively charged and rich in serine and threonine residues. The presequence signals targeting to the chloroplast membrane and is cleaved during translocation (Soll, 2002). 14-3-3 proteins together with Hsp70 have been found to build a guidance complex, which binds to the phospho-peptide motif in the presequence upon phosphorylation and to accelerate preprotein translocation by three to four times (May *et al*, 2000). Transfer of the precursor from the guidance complex to the Toc complex requires ATP (May *et al*, 2000). However, the removal of the phosphorylation site in the presequence, which interrupts the binding of the14-3-3 protein to the precursor had no effect on targeting of several precursors (Nakrieko *et al*, 2004) *in vivo*, suggesting little relevance for the guidance complex. Additionally, an interaction of Hsp90 with the chloroplast precursor was proposed, as Hsp90 can be bound by Toc64, which located at the outer envelope (Qbadou *et al*, 2006).

The translocon at the outer envelope of chloroplasts (Toc) complex consists of four main subunits: The receptors Toc159, Toc34 and Toc64 and the translocon channel, which is formed by 16 α-strands of Toc75 (Becker *et al*, 2004; Soll, 2002). Toc34, Toc159 and Toc75 build the core of the preprotein translocon (Wallas *et al*, 2003). Toc34 and Toc159 exist in a stoichiometry of 4-5:1 in the Toc complex (Schleiff *et al*, 2003) and are believed to be the main receptors of the chloroplasts outer envelope. Kinetic studies on Toc34 and Toc159 and their stoichiometric ratio suggest that Toc34 recognizes the signal peptide of the precursor protein, which is then handed over to the central catalytic translocation motor Toc159 (Becker *et al*, 2004). Toc64 has been found to be a transient component of the Toc complex through interaction with Toc34 (Qbadou *et al*, 2006; Schleiff *et al*, 2003). In *Arabidopsis*, there are four homologues for Toc75, two homologues for Toc34, four homologues for Toc159 and one homologue for Toc64, which is localised at the chloroplast outer envelope (Aronsson *et al*, 2007; Kalanon *et al*, 2008; Soll, 2002). Depletion of Toc159 and Toc33 but not of Toc64 results in a strong phenotype or no viability of the plant, suggesting that Toc159 and Toc34 are the main receptors of the

Toc complex (Aronsson *et al*, 2007; Bauer *et al*, 2000; Jarvis *et al*, 1998; Rosenbaum Hofmann *et al*, 2005).

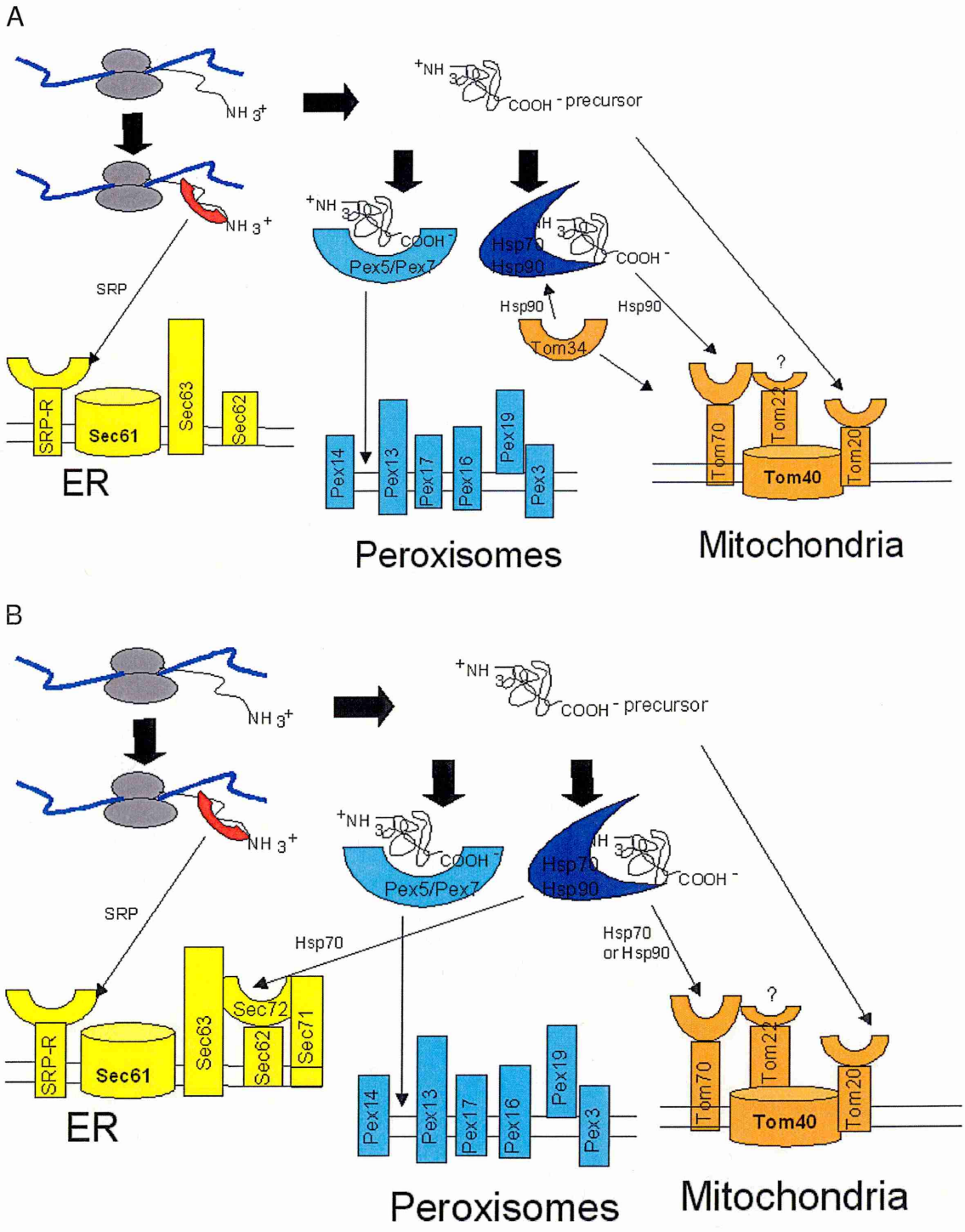


Figure 1.1A and B: Chaperone receptors in protein targeting in animal and fungal cells.
A: Tom34 has been found as an additional chaperone receptor at mitochondria in mammalian cells.
B: Chaperone receptors in protein targeting in fugal cells. Fungi have receptors, which are very similar to the ones found in animals, but have an additional chaperone receptor (Sec72) involved in posttrans-lational targeting at the ER membrane.

Figures 1.1A-C summarise the main receptors involved in protein targeting and the currently known chaperone receptors in animals (Figure 1.1A), fungi (Figure 1.1B) and plants (Figure 1.1C). All organisms use the co-translational targeting mechanism mediated by the SRP and the Sec61 translocon. Fungi have an additional chaperone receptor (Sec72) involved in posttrans-lational targeting, at the ER membrane. Mitochondrial protein trans-location is in all organisms mediated by the pore building Tom40 and the receptor Tom20. Fungi and animals have Tom70 and plants mtOM64 as a chaperone receptor at mitochondria. Tom34 has been found as an additional chaperone receptor at mitochondria in mammals. Import into peroxisomes is believed to be directly mediated by the cytosolic receptors Pex5 and Pex7, which are able to cycle between the cytosol and the membrane and release their cargo upon interaction with the docking complex (Pex13, Pex14 and Pex17). Translocation into chloroplasts of plants is mediated by the core translocon: the channel (Toc75) and the receptors Toc34 and Toc159. The receptor Toc64 is an additional chaperone receptor at the chloroplast envelope.

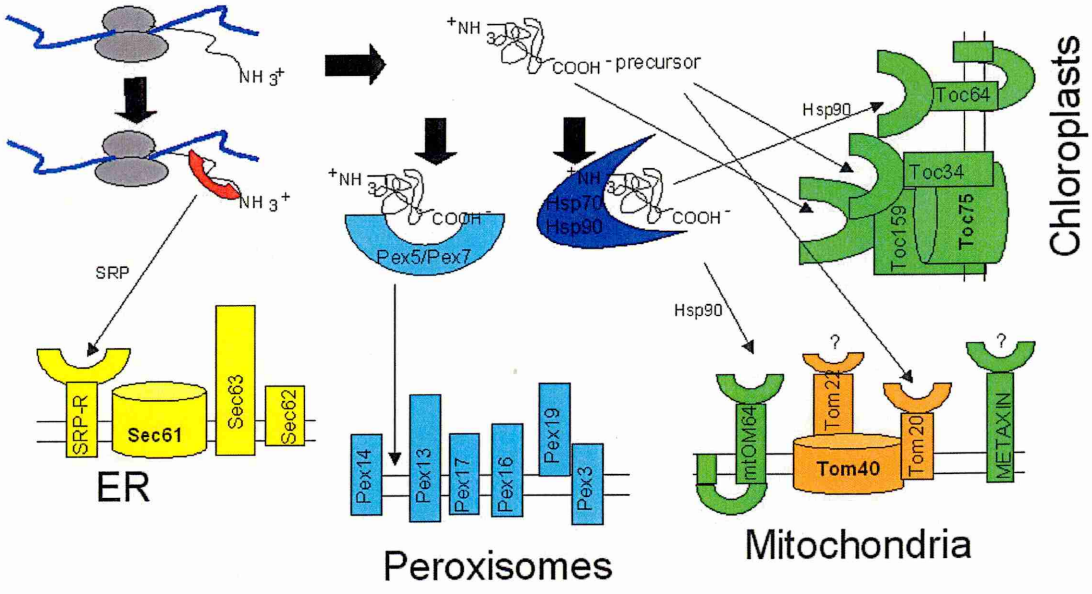


Figure 1.1C: Chaperone receptors in protein targeting in plant cells.
Plants do not have a homologue for Tom70. Here, the Toc64 homologue mtOM64 is currently believed to fulfil the function of Tom70 at mitochondria. Translocation into chloroplasts of plants is mediated by the core translocon: the channel (Toc75) and the receptors Toc34 and Toc159. The receptor Toc64 is an additional chaperone receptor at the chloroplast envelope.

1.1.5. Tail anchor dependent protein targeting

Nuclear encoded membrane proteins have one or more transmembrane domains and can be signal anchored or tail anchored. Their membrane insertion signal does not share a sequence similarity, but is rather determined by the nature of the transmembrane domain, which encloses all targeting information (Abell *et al*, 2003; Rapoport, 2007; Walther *et al*, 2008), and its flanking regions (Borgese *et al*, 2003).

The SNARE proteins of the Vamp/synaptobrevin and syntaxin families, small components of the translocon in the endoplasmic reticulum membrane, several of the Tom and Toc translocase components and TPRc1 (the protein of interest in this work) are tail anchored (TA) proteins. TA proteins are a special class of membrane proteins, which share the topological property of a single, C-terminal transmembrane domain and a lack of an N-terminal signal peptide. Thus, TA proteins require another complex to be targeted to their organelles. The single transmembrane (TMD) domain close to their C-terminus is responsible for their anchoring in the outer membrane of their target organelle as well as for the specificity for the membrane in which they are inserted (Borgese *et al*, 2007; Wattenberg *et al*, 2001). The mechanism by which TA proteins are targeted to their membrane is not completely understood. It has been found that the length and hydrophobicity of the transmembrane domain as well as the charge of flanking residues play a role in specificity for a particular membrane (Borgese *et al*, 2007; Ceppi *et al*, 2005) and that the composition of the target membrane such as its cholesterol content is a criterion for membrane insertion (Brambillasca *et al*, 2005).

The insertion of TA proteins into the ER membrane seems to be dependent on not only a single pathway: As the SRP is involved in the GTP dependent targeting of synaptobrevin2 (Syb2) and Sec61ß into the ER, cytochrome b5 (Cyt5) is completely independent on SRP (Abell *et al*, 2004). Furthermore, the association of TA proteins with Sec62, Sec63 and Sec61 could be shown (Abell *et al*, 2004). This suggests that there exists another pathway of TA-insertion, which uses the same translocation pore as SRP dependent insertion. Indeed, insertion of TA proteins into the ER membrane could as well be observed without SRP (Abell *et al*, 2007). Additionally, there exist at least two more SRP independent pathways for ER TA proteins, which

are ATP dependent and insensitive to a Sec61 knockout (Rabu *et al*, 2007; Rabu *et al*, 2008; Stefanovic *et al*, 2007): One pathway is dependent on Hsp70/Hsp40, which bind to the tail anchor and have been shown to facilitate TA-targeting into the ER membrane (Abell *et al*, 2007). This implies the existence of a receptor complex. However, this complex has not yet been identified. The second pathway is mediated by the recognition of the TMD of the TA protein by Get3 (TRC 40/Asna-1), which targets the TA protein to the ER membrane upon recruitment of the receptor complex Get1/Get2 in yeast (Schuldiner *et al*, 2008). Get3 has been shown to be the homologue of the mammalian Asna-1 (TRC 40) (Schuldiner *et al*, 2008). Thus, both pathways are likely to be conserved. Each tail anchor has been shown to be specifically dependent on Hsp70/Hsp40, Asna-1 or SRP. Here, the binding of Asna-1 or SRP is more likely for TMDs with a higher hydrophobicity (Abell *et al*, 2004; Abell *et al*, 2007; Rabu *et al*, 2008).

For mitochondria targeted TA proteins it could be shown that targeting is independent of the Tom complex and functions without the binding of cytosolic signal-specific targeting factors (Setoguchi *et al*, 2006). One possibility is that cytosolic chaperones were shown to be as well required in TA protein targeting to mitochondria (Setoguchi *et al*, 2006).

Peroxisomal membrane protein (PMP) targeting might be mediated by the Pex19/Pex3 system (Fujiki *et al*, 2006).

The chloroplast TA protein Toc34 was proposed to insert over the Toc75 pore into the chloroplast outer envelope (Soll, 2002). However, the targeting mechanism, cytosolic factors and possible receptors are not known for chloroplast TA proteins.

1.2. Chaperones involved in protein targeting

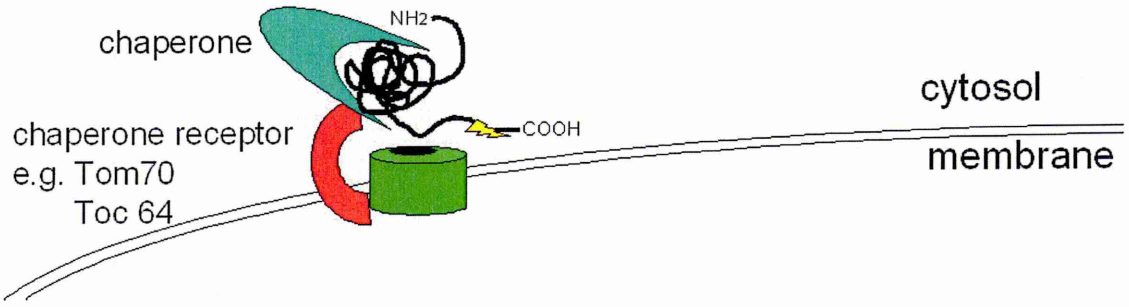


Figure1.2: Proposed principle for targeting of preprotein chaperone complexes to organellar membranes by chaperone receptors. A nascent protein is recognized by cytosolic molecular chaperones such as Hsp70 or Hsp90. The C-terminal end of chaperones is recognized by TPR domain containing receptors at the outer membrane of organelles. This may be a way to import proteins into organelles or in membranes.

Newly translated proteins are often in a complex with cytosolic molecular chaperones (Wickner *et al*, 2005) such as Hsc70 or Hsp90 to prevent aggregation and to achieve correct folding. Additionally, efficient protein targeting to organelles requires receptors at the translocation complexes, which do not only recognize the precursor protein but also proteins, which build a cytosolic complex with the precursor. One class of membrane bound receptors is able to bind the C-terminal end of Hsp70 or Hsp90 specifically. These receptors are called chaperone receptors. Chaperone receptors have commonly at least one transmembrane domain and one or more cytosolic tetratricopeptide repeat (TPR) domain(s) building a clamp, which binds to the C-terminal end of chaperones. Interestingly, each organelle has at least one TPR containing receptor (Schlegel *et al*, 2007). This and the presence of cytosolic chaperones in precursor complexes suggest that chaperone receptors might have an important role in protein targeting. Figure 1.2 shows schematically the proposed involvement of chaperone receptors in protein targeting.

The tetratricopeptide repeat (TPR) motif is a 34 amino acid repeat. The TPR domain contains three TPR motifs. It is composed from three α-helix bundles (I, II and III, also called TPR motif) with two helices (a and b) each and ends with a solvation helix at the C-terminus (Schlegel *et al*, 2007) (Figure 1.2.1). These seven helices together build a groove, which can embrace a peptide and thus mediate interaction with other proteins.

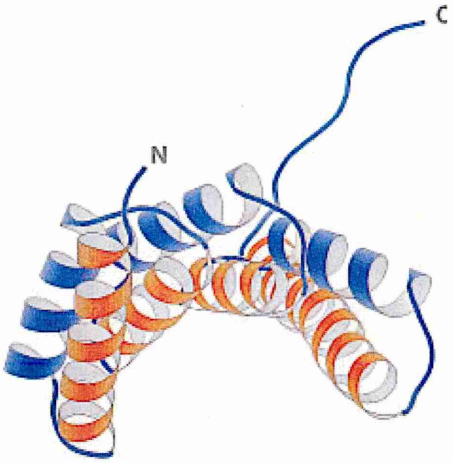


Figure 1.2.1 (Blatch *et al*, 1999): Seven helices form a TPR domain containing three TPR motifs with two helices, a (red) and b (blue), and a C-terminal solvation helix (red)

In the first instance the TPR domain functions generally in protein-protein interactions. Proteins containing TPR domains are involved in several cellular mechanisms such as cell cycle, splicing, transcription, neurotransmitter release, phosphate turnover, signal transduction and chaperone-binding regulation (Blatch *et al*, 1999). Eight amino acids at positions 4 (W/L/F), 7(L/I/M), 8(G/A/S), 11((Y/L/F), 20(A/S/E), 24(F/Y/L), 27(A/S/L), and 32(P/K/E) are highly conserved and thus the criterion for a TPR motif in the primary structure. Functionally different TPR motifs share the amino acids at position 8(G/A/S), 20(A/S/E), 24(F/Y/L) and 27(A/S/L) (Blatch *et al*, 1999). Because of their conservation these amino acids are believed to be responsible for substrate specificity in protein - protein interaction mediated by the TPR domain.

1.2.2. The TPR domain in chaperone-binding

Some TPR domains interact specifically with the highly conserved C-terminal end of the Hsp70 and Hsp90. Both chaperones have the C-terminal end EEVD as a general anchor residue (Brinker *et al*, 2002) in common and differ in the upstream amino acids.

An interesting TPR-containing protein is the Hsp70 and Hsp90 organising protein (Hop), which contains three TPR domains, TPR1, TPR2A and TPR2B. TPR1 is known to bind the C-terminal end of Hsp70 and TPR2A binds the C-terminal end of Hsp90. The function of TPR2B is unknown. The ability of Hop to bind both, Hsp70 and Hsp90 makes it to a model TPR-containing protein for studying chaperone - TPR domain binding.

Scheufler et al. (2000) examined chaperone binding by TPR1 and TPR2A with isothermal titration calorimetry (ITC) and determination of the crystal structures of these domains: Peptides with the size ranging from the last 4 (EEVD) to 12 amino acids of Hsp70 (GSGSGPTIEEVD) and Hsp90 (GDDDTSRMEEVD), and Hsp70 and Hsp90 fragments lacking the chaperone domain (C70 and C90) were titrated to TPR1 and TPR2A. The resulting dissociation constants of the complexes were measured in an isothermal titration calorimeter to test for binding and specificity of the heat shock proteins by the TPR domains of Hop. For TPR2A the pentapeptide MEEVD of Hsp90 is essential and sufficient for binding (Brinker *et al*, 2002; Scheufler *et al*, 2000; Wu *et al*, 2001) while TPR1 binding requires at least a heptapeptide (PTIEEVD) (Brinker *et al*, 2002; Scheufler *et al*, 2000; Wu *et al*, 2001). Furthermore, TPR2A is able to bind C70 with a low affinity while TPR1 does not bind C90 at all. This indicates that the binding of Hsp70 is much more specific than the binding of Hsp90 (Odunuga *et al*, 2003; Scheufler *et al*, 2000). According to the crystal structures derived from TPR2A and TPR1, which were co-crystallised together with the appropriate heptapeptide, the importance for binding of the single amino acids in the chaperone C-terminus seems to differ for the two TPR domains as well. While the amino acids D 0, V -1, I -4 and P -6 (**P**T**I**EEV**D**) are involved in the contact to TPR1, all the last five amino acids with exception of E -2 (**MEEVD**) are close enough to TPR2A for interaction. Thus, in both TPR domains from Hop the last four amino acids of the chaperones are not sufficient for specific binding and the

binding of Hsp70 by TPR1 is more specific than the binding of Hsp90 by TPR2A.

NMR and CD spectroscopic studies on the tertiary structure of the TPR domain of the Hsp90 binding protein protein phosphatase 5 (PP5), show, that this TPR domain is mainly unfolded at physiological temperatures and undergoes a coupled folding and binding by addition of the C-terminal pentapeptide (MEEVD) of Hsp90 as a ligand (Cliff *et al*, 2005). This effect has only been found with PP5 so far, but may be applicable on TPR domains of other proteins as well.

1.2.3. TPR-containing membrane bound receptors

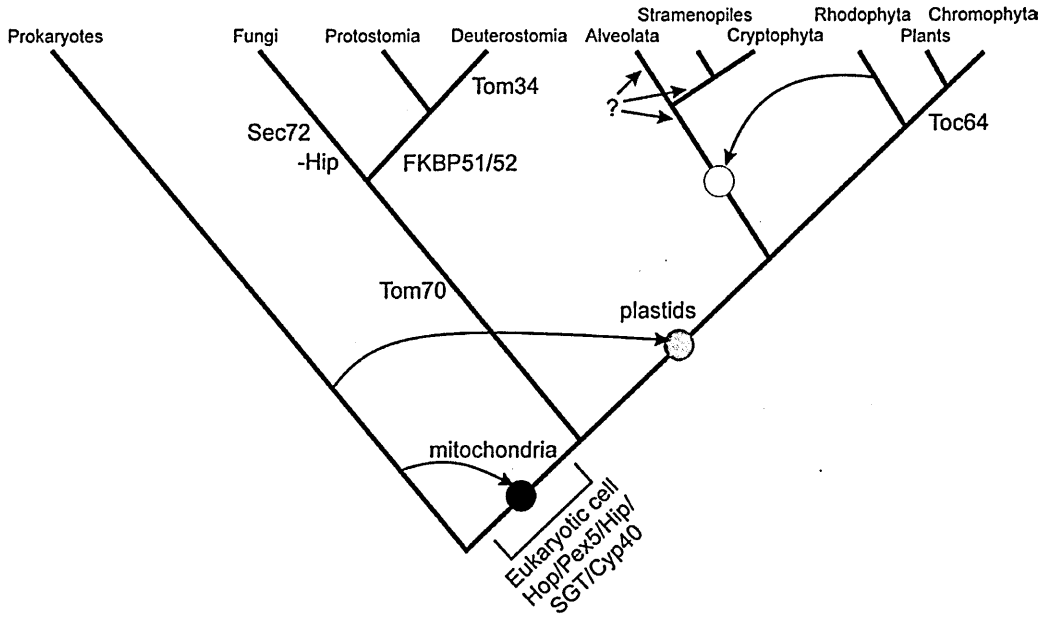


Figure 1.2.3.1.(Schlegel *et al*, 2007): Model of evolutionary development or TPR-containing proteins involved in protein translocation. Tom70 and Toc64 evolved early after separation of the fungi/metazoa group and plants while Tom34 and Sec72 are a late development in deuterostomia and fungi, respectively.

Schlegel et al. (2007) did a phylogenetic comparison of the TPR domain containing receptors, which bind Hsp70 or Hsp90, in eukaryotes to test for evolutionary relationships. The receptors Sec72 at the endoplasmic reticulum, Tom34, mtOM64 and Tom70 at the mitochondria outer membrane, Pex5 at peroxisomes and Toc64 at the chloroplast envelope were considered. Some TPR domains tend to cluster together, which makes a bioinformatical prediction

possible: Sec72 clusters with TPR1 of Hop suggesting a Hsp70 binding. Tom70, Toc64, mtOM64 and the N-terminal TPR domain of Tom34 cluster together suggesting similar function. The C-terminal TPR domain of Tom34 clusters with TPR2A of Hop indicating a possible Hsp90 binding and Pex5 builds its own group. However, the similarity of the TPR domains from different receptors is not very high even if their function is very similar (Figure 1.2.3.1). Thus, it seems that they developed independently at different times and might have a general role in protein targeting, which can be applied to all organelles. The TPR domain of Tom70 seems to have evolved early in the fungi/metazoa group. Sec72 can only be found in fungi and Tom34 only in deuterostomia. Plants seem to have developed one type of Toc64 at chloroplasts (Toc64) and one type at mitochondria (mtOM64), as they are lacking Tom70. Figure 1.1A-C summarises the chaperone receptors involved in protein targeting in animals, fungi and plants. Their biochemical properties will be further discussed.

Tom70

Tom70 is the best characterised chaperone receptor. It is a 617 amino acid long mitochondrial protein with an N-terminal transmembrane domain and a big C-terminal domain facing the cytosol and contains 26 α-helices. Most of these helices build the 11 TPR motifs. The three TPR motifs which are close to the transmembrane domain build a TPR domain similar to Hop, while the remaining TPR motifs are proposed to be responsible for signal peptide binding (Wu Y et al, 2006). Tom70 dimerises in its active form (Figure 1.2.4) (Wu *et al*, 2006; Young *et al*, 2003).

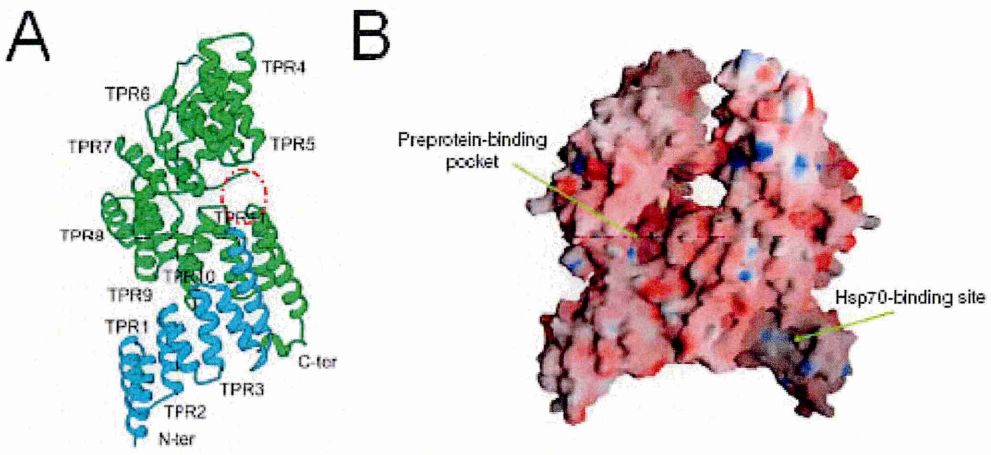


Figure 1.2.4 (Wu *et al*, 2006): Crystal structure of yeast Tom70.
A: Tom70 monomer; the monomer Tom70 contains 11 TPR motifs. The linker region for dimerisation is circled.
B: Tom70 dimer; arrows show the putative peptide binding pocket and the Hsp70/90 binding TPR domain.

Pull down assays of the C-terminal domain of Hsp70 (C70) and Hsp90 (C90) show that the cytosolic domain of mammalian Tom70 binds Hsp90 and Hsp70 specifically and efficiently, while Tom70 with an induced mutation of arginine 192 to alanine (R192A) in the N-terminal, Hop-like TPR domain, which disrupts binding to Hsp90 and Hsp70, does not bind (Young *et al*, 2003). Yeast Tom70 recognizes only Hsp70 but not Hsp90. The inhibition of import of preproteins with C90 in *in vitro* assays with purified mitochondria varies

between preproteins. While the protein import of the mitochondrial phosphate carrier (PiC) and the mitochondrial peptide transporter (PT) are strongly inhibited by C90 or the Hsp90 inhibitor geldanamycin (GA), the Rieske iron-sulfur protein (ISP) is barely inhibited by C90 (Bhangoo *et al*, 2007; Fan *et al*, 2006; Young *et al*, 2003). The simplest explanation for this is that the receptors Tom70 and Tom20 have overlapping roles in protein targeting. Additionally, the C-terminal Bag domain (Cbag), a Hsp70 inhibitor is able to inhibit the import of the ADP/ATP carrier (AAC) and PT (Young *et al*, 2003), which gives evidence for the importance of Hsp70 binding by Tom70 in targeting AAC into mitochondria.

In summary, at least one Tom70 dimer recognizes the internal signal sequence of the preprotein and the C-terminal end of the complexed Hsp70 or Hsp90, and recruits the preprotein-chaperone complex to the translocation pore in mitochondria.

Tom 34

Tom34 occurs only in animals. It has an N-terminal and a C-terminal TPR domain (Schlegel *et al*, 2007), and a hydrophobic region at the N-terminus (Chewawiwat *et al*, 1999). The hydrophobic region seems to be responsible for interaction with the mitochondrial membrane rather than to be a membrane anchor, as it is possible to wash it from purified membrane fractions with a higher salt concentration (Chewawiwat *et al*, 1999). However, it seems to have a role in mitochondrial import, as addition of Tom34 lacking the hydrophobic domain can inhibit the import of precursor proteins, which have an N-terminal transit peptide, into mitochondria (Chewawiwat *et al*, 1999). A faster import of precursors by Tom34 overexpression and inhibition of import by addition of anti-Tom34 antibodies support this hypothesis (Chewawiwat *et al*, 1999). Yeast two hybrid screening with the C-terminal domain of Hsp90 pulled out Tom34 as a putative interaction partner (Young *et al*, 1998). This suggests Tom34 to be a flexible receptor of mitochondria, which assists the recruitment of precursor-chaperone complexes from the cytosol to the mitochondrial membrane.

In contrast, it was found that Tom34 knockout mice are viable under normal conditions (Terada *et al*, 2003), which makes a key role of Tom34 alone doubtful.

Pex5

Pex5 transports most peroxisomal matrix proteins. It contains seven WxxxF/Y motifs, which are believed to be involved in interaction with other peroxisomal membrane proteins and seven TPR motifs in the C-terminal part (Harano *et al*, 2001; Stanley *et al*, 2006) building two TPR domains. As Pex5 shuttles between the cytosol and the membrane (Platta *et al*, 2007), its TPR domain might bind to cytosolic chaperones.

In mammals, two isoforms of Pex5 have been identified: a short form, Pex5pS, which recognizes only PTS1 and a long form, Pex5pL, which can recognize both PTS1 and PTS2. Pex5 associates with Pex13 and Pex14 in the membrane. However, it has been shown, that a Pex5 mutant unable to bind these proteins is still able to transport precursors into peroxisomes and is able to insert into lipid bilayers reversibly *in vitro* (Kerssen *et al*, 2006). This indicates that Pex5 cycles between the cytosol to the membrane to transport freshly synthesized proteins into peroxisomes.

Harano et al. (2001) studied the binding mechanism by which Pex5 binds to its cargo using acyl-CoA oxidase (AOx) as a model for PTS1 containing proteins. According to their results antibodies against the C-terminus of Pex5 and antibodies against Hsp70 can inhibit AOx import. Furthermore, Hsp70 binding was disrupted by addition of ATP. This indicates that the signal sequence of AOx is bound directly by the C-terminus of Pex5 and Hsp70 keeps Pex5 in a binding competent state due to its chaperone activity.

Thus, it can be concluded, that Pex5 binds directly the precursor protein via its TPR domain and is likely not to be a chaperone receptor. Instead the earlier described Pex19/Pex3 system might fulfil the function of a chaperone based targeting pathway to peroxisomes.

Sec72

Sec72 is a 23 kD ER protein with no transmembrane domain in yeast. Because it can be detected at the ER membrane after membrane purification and salt washes, it is believed to have a strong interaction with other ER membrane proteins (Feldheim *et al*, 1994). It has three TPR motifs forming a TPR domain similar to TPR1 of Hop (Ponting, 2000). Sec72 knockout mutants are in contrast to Sec71, Sec62 and Sec63 knockout mutants viable at high and

low temperature. However, an accumulation of ER-targeted proteins like carboxypeptidase Y (CPY) was observed in these mutants, suggesting a role of Sec72 in protein targeting of some ER precursor proteins (Feldheim *et al*, 1994; Green *et al*, 1992). Thus, Sec72 participation in targeting could be the binding of an Hsp70 - precursor complex (Ponting, 2000) and the release of the precursor from Hsp70 to the translocation machinery could then be mediated by the DnaJ domain of Sec71. In contradiction, the final destination of CPY is the vacuole and it contains an N-terminal vacuolar sorting signal, which is cleaved upon arrival in the vacuole (Valls *et al*, 1987). Thus, a targeting via SRP binding is likely for this protein and its aggregation might not be directly related to the Sec72 knockout.

1.2.5. Chaperone receptors in plants

Pex5, Pex19 and Pex3 have been found in plants as well and the plant homologues seem to have a similar function to the one described for the mammalian peroxins. Nito et al (2007) characterised different *Arabidopsis* mutants knocked down for pre-proteins, which are involved in peroxisomal biogenesis. Here, a Pex5 knockdown resulted in mislocation of peroxisomal precursor proteins indicating that plant Pex5 is involved in protein import, and a knockdown of Pex3 as well as Pex19 resulted in a change of peroxisomal morphology, suggesting that Pex3 and Pex19 have a role in correct insertion of peroxisomal membrane proteins. A plant homologue of Sec72 has not yet been found.

Toc64

Sohrt et al. (2000) found a new component of 64 kD in the purified Toc-complex of pea chloroplasts and called it Toc64. Toc64 has a N-terminal transmembrane domain, a short cytosolic domain, two membrane-spanning areas leaving a 30 KD domain, which contains an amidase domain, in the intermembrane space (IMS) and three TPR motifs at the C-terminus, which is facing the cytosol (Qbadou *et al*, 2007).

A. thaliana has three Toc64 isoforms: atToc64-III at the chloroplast membrane, atToc64-V (mtOM64) at the mitochondrial membrane and atToc64-I. AtToc64-I is a cytosolic amidase, and atToc64-III and atToc64-V have a nonsense mutation in their amidase domain (Aronsson *et al*, 2007; Qbadou *et al*, 2006) suggesting another function. Toc64 is only dynamically associated with the Toc core complex and is then in close proximity to Toc34 but not to Toc159 (Qbadou *et al*, 2006; Schleiff *et al*, 2003).

Recombinant Toc64 expressed in *Escherichia coli* is able to prevent import of some preproteins, such as oxygen evolving complex subunit of 33 kDa (pOE33), which is localised in the thylakoid lumen and has N-terminal signal peptides to pass the chloroplast and the thylakoid membrane. This is due to its TPR domain, as Toc64 lacking this domain is not able to inhibit the import (Qbadou *et al*, 2006). This inhibition can be rescued by addition of ATP, which indicates that pOE33 is not directly bound by Toc64, but by an ATP-dependent factor e.g. Hsp70 or Hsp90. Indeed, pOE33 can be co-immuno-precipitated by anti Hsp90 antibodies and the TPR-domain of Toc64 is able to pull down C90 (Qbadou *et al*, 2006), the C-terminal domain of Hsp90, which lacks chaperone activity. ATP induces Toc64 dissociation from the complex, while Toc34 is only able to bind pOE33 after addition of ATP (Qbadou *et al*, 2006). Thus, Toc64 is a receptor at the chloroplast envelope, which recognizes preprotein - chaperone complexes at an early stage and passes the preprotein on to Toc34 in an ATP-dependent manner.

Aronsson et al. (2007) found that Toc64 knockout plants in *A. thaliana* have wild type phenotype. This indicates that there is either an undefined receptor similar to Toc64, or that it has an overlapping function with the other Toc receptors.

mtOM64

There exists no homologue of Tom70 at the mitochondrial outer membrane of plants. Thus, mtOM64 (Toc64-III) is thought to have evolved to replace Tom70 in plants. mtOM64 is an isoform to the chloroplast translocase of the outer membrane 64-III (Toc64-III) in plants. It is N-terminally anchored to the mitochondrial outer membrane and contains three TPR motifs similar to Hop and Tom70 (Lister *et al*, 2007). Blue native polyacrylamide gel electrophoresis

(BN-PAGE) analysis revealed that mtOM64 is not permanently part of the mitochondrial translocation complex (Lister *et al*, 2007).

However, recombinant mtOM64 can inhibit the translocation of some mitochondria targeted proteins e.g. the F_Ad-subunit of mitochondrial ATP-synthase (F_Ad) and pull down assay as well as yeast two hybrid analysis show an interaction of mtOM64 with a variety of mitochondrial precursor proteins (Lister *et al*, 2007). This indicates that mtOM64 is a novel mitochondrial receptor, which might recruit preproteins to the outer membrane of mitochondria in a manner similar Tom70 in plants, since plants lack Tom70.

TPRc1

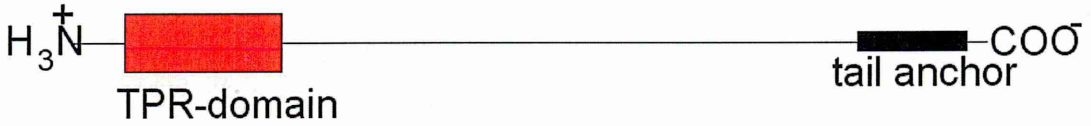


Figure 1.2.5: Systematic structure of TPRc1: TPRc1 was found by bioinformatical search for proteins containing a TPR-domain similar to Hop and a membrane spanning domain.

TPRc1 was pulled out by a bioinformatic search for eukaryotic proteins containing the chaperone binding TPR domain and a transmembrane domain. The criterion for the TPR domain was obtained by structural alignment of known chaperone binding TPR domains from Hop, FK506-binding protein 5, FK506-binding protein 4, cyclophillin-40, serine/threonine phosphatase 5, cyclophillin seven suppressor, Tom70, Tom34 and Unc-45: This alignment resulted in semi stringent motifs consisting out of [K-(ETNDK)-(KQEIR)-(GA)-(NT)-(DEVKT)-(AYFCL)-(YF)] for clamp1 and [K-(AG)-(YFL)-(YFT)-R-(KR)-(GA)-(AEQK)] for clamp 2 and loose motifs consisting out of [(KR)-(ETNDKALQGD)-(LKQEIHSA)-(GA)-(NKT)-(DAELSVNHQKT)-(ACFYLKHQMS)-(YFLV)] for clamp 1 and [K (AGVC)-(YFL)-(AYFTSN)-(RQ)-(IKRQL)-(GAS)-(NATEQKLCS)] for clamp2 (Ewans, unpublished data). A TPR domain has to contain two of the listed motifs to be able to bind chaperones, thus these motifs were used to scan the protein databases Swissprot, TrEMBL and TrEMBL New (Boeckmann *et al*,

2003). Identified proteins were checked to determine whether they contained a transmembrane domain. TPRc1 fulfils these criteria. It has the Swissprot accession number Q8GWM6 and contains a full TPR domain and a tail anchor (Ewans, unpublished data) (Figure 1.2.5). Thus TPRc1 is predicted to be a novel uncharacterised chaperone receptor.

TPRc1 homologues exist only in plants, which suggests that it might be localised in chloroplasts. This is underlined by preliminary data, which show, that recombinant TPRc1 can insert in purified chloroplasts (Abell, unpublished data) and confocal microscopy (Kriechbaumer, unpublished data), which shows, that TPRc1 co-localises with chloroplasts. Additionally, an interaction of TPRc1 with Hsp70 could be shown (clones used here (TPRc1-TM and TPRc1FL) and experimental procedure done by Lehmann, unpublished), which suggests, that TPRc1 is a novel chaperone receptor.

1.3. Aims

The aim of this work is to characterise TPRc1 expression, subcellular localisation and function as well as the cytosolic interaction partners and the interaction partners at the membrane in more detail.

A phylogenetic analysis of the TPR domain of TPRc1 should help to predict its function on the basis of its relation with other chaperone receptors. TPRc1 RNA and protein levels in different tissues were analysed with quantitative RT PCR and western blotting. The preliminary evidence for TPRc1 to bind the C-terminal end of Hsp70 with its TPR domain was tested with pull down assays and the nature of the interaction between the TPR domain of TPRc1 and the chaperones Hsp70 and Hsp90 were analysed. The existence of interaction partners of TPRc1 at the chloroplasts outer envelope should be tested by chemical crosslinking and immunoprecipitation of radiolabeled, into chloroplast imported TPRc1. Finally, phenotyping of TPRc1 depleted plants should explain the broader context of TPRc1 function in cellular processes.

2.1. Media

E. coli were grown at 37°C in 2 YT (171 mM NaCl, 1.6% (w/v) tryptone, 0.5% (w/v) yeast extract, pH ca. 8.0) or Luria broth (LB) (171 mM NaCl, 1% (w/v) tryptone, 0.5% (w/v) yeast extract, pH ca. 8.0) containing the appropriate antibiotic. Solid medium for colony selection contained 1.5% agar.

2.2. Bacterial strains and Plasmids

Table 1 shows the different bacterial strains used. Cloning was generally performed by double digestion of PCR products with restriction endonucleases. These products were then ligated into pET-16b (Novagen) or pIVEX 1.4 (Roche) using appropriate restriction sites. In difficult cases the undigested PCR product was blunt end ligated into pBluescript SK (Stratagene).

Bacterial strain	Genotype	Application
E. coli XL1 Blue	*supE44, hsdR17, recA1, endA1, gyrA46, thi relA1, lac-, lac [F' proAB+ laclq lacZΔM15 Tn10(Tetr)]*; (Bullock et al., 1987)	Standard-cloning
E. coli BL21 (DE3)	hsdS, gal, [λcI, ts857, cnd 1, hsdR17, recA1, endA1, gyrA96, thi1, relA1]; (Studier and Mofat, 1986)	Protein expression
T7 Express I^q Competent *E. coli* (High Efficiency)	MiniF $lacI^q(Cam^R)$ / *fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mcr-73::miniTn10--Tet^S)2 [dcm] R(zgb-210::Tn10--Tet^S) endA1 D(mcrC-mrr)114::IS10*	Protein expression

Table 1: Different bacterial strains and their application. *E. coli* XL1 Blue competent cells were purchased from Promega, T7 Express I^q Competent *E. coli* were bought from New England Biolabs (NEB). Alternatively, *E. coli* XL1blue and *E. coli* BL21 (DE3) competent cells were prepared using the methods below.

2.2.1. Preparation of *E. coli* XL1 blue competent cells

100 ml 2 x YT medium was inoculated with 5 ml overnight culture of *E.coli* XL1 Blue. The cells were grown to an OD_{600} = 0.3 - 0.5, and then were precipitated by centrifugation for 20 min in 4°C with 3000 *g*. Pellet was suspended and incubated in cold TBFI buffer (30 mM KAc, 50 mM MnCl, 100 mM RbCl, 10 mM $CaCl_2$, 15% glycerol, pH5.0 with acetic acid, sterilized by filtration) for 20 min on ice. The cells were pelleted by centrifugation at 4°C for 5 min at 3000 *g*. The pellet was resuspended in 3.6 ml of cold TBFII buffer (10 mM MOPS pH7.0, 10 mM RbCl, 15 mM $CaCl_2$, 15% glycerol, sterilized by filtration), aliquoted and snap frozen in liquid nitrogen. Aliquots were stored at -80°C.

2.2.2. Preparation of *E. coli* BL21 (DE3) $CaCl_2$ competent cells

200ml LB-Medium was inoculated with 1ml overnight culture and grown at 37°C until an OD_{600} = 0.3 - 0.5 was reached. The culture was chilled on ice for 15 min and precipitated by centrifugation at 3000 *g* for 10 min. The pellet was resuspended in 15 ml 1 M $CaCl_2$ and incubated on ice for 15 min. Cells were precipitated at 3000 *g* for 10 min and resolved in 4 ml 1 M $CaCl_2$ containing 15% glycerol. Competent cells were aliquoted to 100 µl and stored at -80°C.

2.3. *E. coli* Transformation (heat shock)

An aliquot of competent cells (25 µl for *E. coli* XL1blue and *E. coli* T7 Express I^q or 100 ml for *E. coli* BL21 (DE3)) was thawed on ice. 100 - 500 ng of plasmid DNA was added, mixed and incubated for 20 min on ice. The transformation mix was then heat shocked at 42°C (45 s for *E. coli* XL1blue, 90 s for $CaCL_2$ competent *E. coli* BL21, 10 s for T7 Express I^q Competent *E. coli*). 300 µl LB medium was added immediately after heat shock and the cells were incubated

at 37°C for 1 h. Transformed Bacteria suspensions were plated on LB plates containing the appropriate antibiotic.

2.4. Polymerase chain reaction (PCR)

PCR was performed with primers ordered from Eurofins MWG Operon, using a Techne model TC-3000 thermocycler. Table 2 gives an overview over

Template	Primer pair	Induced restriction site	Temperature (Melting, Annealing, Polymerisation)	Numbers of cycles	Application
TPRc1 in pSPUTK	TPRc1TPRfor: attaaacatatgtatcagatcaatgcagctc TPRc1TPRrev: ttaaattggatcctatgccttgccaggtcc	NdeI BamHI	95°C, 50°C, 72°C	45	Cloning of the TPR domain of TPRc1 in pET-16b and pIVEX1.4
TPRc1 in pSPUTK	TPRc1a: attaaacatatggagacaattgccgatgtg TPRc1Lrev: Atatagatctttattttccgaacaaccacttc	NdeI BamHI	95°C, 55°C, 72°C	45	Cloning of the Linker region of TPRc1 into pET-16b
TPRc1-TM in pET-16b	R185A: ggaatgtcaaagccctatacgcaaggggtcaagcttacaga R185Aantisense: Tctgtaagcttgacccctgcgtatagggctttgacattcc	None	95°C, 55°C, 68°C	12	Induction of a R185A mutation into the TPRc1-TM clone
TPRc1 in pSPUTK	TPRc1RTfor: ctggaaagttctgattgcttc TPRc1RTrev: Catcaagaggtgtggtgatg	None	95°C, 55°C, 72°C	40	RT-PCR (target)
Actin1 fragment in pGEM	ActinRTfor: tggaactggaatggttaaggctgg ActinRTrev: Tctccagagtcgagcacaataccg	None	95°C, 60°C, 72°C	40	RT-PCR (endo-genous control)

Table 2: Primer sequences and application. Primers were ordered from Eurofins MWG Operon.

the different primer pairs used and their application. PCR products, which were used for cloning, were purified from the components of the PCR reactions with either the PCR purification kit from Qiagen or by precipitation as described by the method below.

2.5. Agarose gel electrophoresis

DNA was subjected to electrophoresis using 0.8 to 1.0% agarose gels containing ethidium bromide at final concentration of 0.5 µg / ml. Gels were cast and run in TAE buffer (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, adjusted to pH7.8 with acetic acid) at 100 Volts using mini-Sub® cells GT (Biorad) . Dependent on the DNA size 100 bp or 1 kb ladder from NEB was used as a size marker.

2.6. Plasmid construction

2.6.1. Plasmid DNA preparation

Small scale plasmid DNA preparation from 1 ml *E. coli* culture was preformed using the Miniprep kit from Qiagen. The yield was between 10 and 15 µg. This method was used to prepare plasmid for transformation and for sequence analysis of ligation products with restriction digest and sequencing.
Large scale plasmid DNA from a 50 ml culture was prepared with the Qiagen Midi prep kit from Qiagen. The yield was between 60 and 100 µg. This method was used to prepare plasmid for cloning.

2.6.2. Nucleic acid precipitation

Nucleic acids were concentrated by precipitation. Therefore, 1/10 volume 3 M NaOAc pH4.8 (pH adjusted with acetic acid) and 2.5 volume EtOH or

isopropanol were added to the nucleic acid solution. The solution was mixed and incubated at -20°C for 20 min - over night followed by centrifugation at 14300 *g* for 10 min. The nucleic acid containing pellet was washed with 100% EtOH, dried and dissolved in dH_2O or TE buffer (10 mM Tris, 1 mM EDTA, pH8.0). DNA concentration was estimated on an agarose gel by comparison to the marker.

2.6.3. Restriction digestion

For cloning, plasmid DNA or PCR products containing restriction sites were digested with restriction enzymes using 1 - 2 U per 1 µg DNA (BglII) or 2 - 4 U per 1 µg DNA (SmaI, BamHI, NdeI) and the appropriate 1 x buffer derived from NEB at 37°C for 1 h. The cloning strategy for the different constructs is summarised in Table 3. For ligation approximately 20 µg of vector and 10 µg of insert were used in the digest. For analytical digestion of ligation products, 1 - 1.5 µg DNA was used. Plasmid DNA for cloning was dephosphorylated with 10 U Antarctic Phosphatase (NEB) in 1 x Antarctic phosphatase reaction buffer at 37°C for 15 min. The dephosphorylation reaction was inactivated by heating up to 65°C for 5 min.

Clone	Vector used for cloning	Restriction sites		Resistance
		Insert	Vector	
TPRc1 TPR in pET-16b	pET-16b	NdeI, BamHI	NdeI, BglII	Ampicillin (0.1 mg / ml)
TPRc1TPR in pIVEX 1.4	pIVEX 1.4	NdeI, BamHI	NdeI, BglII	Ampicillin (0.1 mg / ml)
L in pBluescript SK	pBluescript SK	None	SmaI	Ampicillin (0.1 mg / ml)
TPRc1L in pET-16b	pET-16b	NdeI, BamHI	NdeI, BglII	Ampicillin (0.1 mg / ml)

Table 3: Cloning strategy of different constructs. Selection of positive clones was performed by antibiotic resistance.

2.6.4. Ligation

Digested and dephosphorylated plasmid was mixed with the digested PCR product to be inserted in a ratio between 1 : 3 and 1 : 5 by mass. Vector and insert were ligated for 1 h in a 10 µl reaction containing 1 µl 10 x ligation buffer (NEB), 1 µl 10 x BSA (NEB) and 2 U / µl T4 DNA ligase (NEB). In the case of blunt end ligation the time was exceeded over night. Ligation products were directly transformed into *E. coli* XL1blue.

2.6.5. DNA sequencing

Plasmid DNA in the concentration 1 ng / µl was sent to GATC or EUROFINS MWG OPERON for sequencing using green "Run 24 Barcodes" for a single run. The applied technique is Phred20^{+} and resulted in a typical output length between 600 bp and 1000 bp.

2.7. Protein expression in *E. coli* BL21

500 ml LB medium containing the appropriate antibiotic and 1% glucose was inoculated with 1 ml from a 3 ml over night culture. Cells were grown at 37°C until OD_{600} = 0.4 - 0.6. Cells were heat shocked after addition of 1% ethanol at 42°C for 10 min and chilled on ice for 10 min. Expression was induced with 0.5 - 1 mM IPTG and cells were incubated at room temperature for 3 - 4 h. Table 4 summarises the conditions used for the different expressed plasmids and the protein yield.

Plasmid name	Protein name	size (kDa)	Antibiotic	Heat shock	Induction time/ temperature	Yield per 500 ml induced culture (nmol)
TPRc1-TM in pET-16b	TPRc1-TM	61	Ampicillin (100 µg / ml)	+	3 h / RT	1.2
TPRc1 TPR in pET-16b	TPRc1TPR	18.2	Ampicillin (100 µg / ml)	-	3 - 5 h / RT	6
TPRc1TPR in pIVEX 1.4	TPRc1TPR	18.2	Ampicillin (100 µg / ml)	-	o.n. / RT	N/A (very low)
TPRc1L in pET-16b	TPRc1L	34.6	Ampicillin (100 µg / ml)	+/-	3 h / RT	2
TPRc1R185A in pET-16b	TPRc1R185A	61	Ampicillin (100 µg / ml)	+	3 h / RT	2.4
TPR1 in pPROEXHTa	TPR1	17	Ampicillin (100 µg / ml)	-	4 h / RT	4
TPR2A in pPROEXHTa	TPR2A	16	Ampicillin (100 µg / ml)	-	4 h / RT	7
C70 in pPROEXHTa	C70	26	Ampicillin (100 µg / ml)	-	4 h / RT	1
C90 in pPROEXHTa	C90	20	Ampicillin (100 µg / ml)	-	4 h / RT	3

Table 4: Details to recombinant expression in *E. coli*. The His-tag is approximately 2 kDa of the protein (pET-16b: + 2768.90 Da; pIVEX : + 2825.08 Da). The clones for TPR1, TPR2A, C70 and C90 are kindly provided by J Young (Young, 2003).

2.8. Protein purification

E. coli cells were harvested by centrifugation 4000 *g* for 10 min at 4°C. Pellets were resuspended in 4 ml / g lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazol, pH8.0). 1 mg / ml lysozyme was added and the suspension was incubated for 30 min. Cells were broken by sonication with 25% Amplitude and a 10 sec on an off pulse, and centrifuged at 3000 *g* for 30 min at 4°C. The supernatant was mixed with 1 ml Ni-NTA agarose and incubated for 1 h at 4°C. The protein bound was washed 4 times with 5 ml wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 50 mM imidazol, pH8.0) and eluted 2 - 4 x with 0.5 ml elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 100 mM imidazol, pH8.0). Initially, eluted protein was dialysed against an appropriate buffer (100 mM KOAc, 5 mM $Mg(OAc)_2$, HEPES-KOH pH7.5 for TPRc1 constructs, 50 mM Tris pH8.0, 0.5 mM EDTA, 1 mM DTT for His-tag cleavage from C70 and C90) and stored at -80°C until used. Later, this step was omitted, because the protein lost its function after a short storage time.

2.9. Plant Material

A. thaliana plants (ecotype Col-0) were grown on Chempak multi purpose compost (Garden Direct). Seeds were vernalized for 5 days at 4°C in the dark; growth was continued in a growth chamber (SANYO) at 25°C, 4 Ls and 65% humidity under continuous light. Plant material was harvested from adult plants (ca. 8 weeks old), snap-frozen in liquid nitrogen and stored at -80°C.

2.10. RNA purification

50-100 mg of frozen plant material was ground to powder with a pestle in an eppendorf tube. Total RNA was purified with 1ml TRIZOL® -Reagent (Invitrogen). 1 ml TRIZOL® reagent was added and incubated for 5 min at room temperature. 0.2 ml chloroform was added; tubes were closed and shaken vigorously for 15 s. The mixture was incubated for 3 min at room temperature and centrifuged for 15 min at 12000 *g*. The upper phase was transferred in a fresh tube containing 0.5 ml isopropanol and incubated for 10 min at room temperature. Samples were centrifuged for 10 min at maximum speed. The pellet was washed with 1 ml 100% ethanol, dried for 5 - 10 min and resuspended in 50 µl DEPC treated water. To eliminate genomic DNA contamination the RNA was treated with 4 µl DNAse, 6 µl 10 x DNAse Buffer 37°C for 15 min. The reaction was inactivated by adding 4 µl 25 mM EDTA and heating to 75°C for 10 min.

2.11. cDNA Synthesis

First-strand cDNA synthesis from 0.5 - 5 µl total RNA was performed using random hexamer primers (Invitrogen) and the SuperScript first-strand synthesis system for RT-PCR (Invitrogen) or the M-MuLV Reverse Transcriptase (NEB) according to the manufacturer's instructions. RNA was mixed with 1 µl random primers and 1 µl dNTP mix (10 mM each). Sterile

distilled water was added to 12 µl and the mixture was heated to 65°C for 5 min. After the reaction mix was cooled down on ice 4 µl 5 x first strand Buffer and 1 µl DTT were added and incubated at room temperature for 2 min 1 µl reverse transcriptase was added to each sample and mixed. The reaction was incubated for 10 min at room temperature heated up to 42°C for 50 min and inactivated at 70°C for 15 min.

2.12. Quantitative Real-Time PCR

The cDNAs were diluted 1 : 1 with nuclease-free water. Aliquots of the same cDNA sample were used with all primer sets for real-time PCR. The 20 µl reactions contained 250 nM of each primer, 1 µl of cDNA sample and 1 x SYBR Green PCR master mix (Applied Biosystems). Quantitative real-time PCR was performed on the StepOne Sequence Detection System (Applied Biosystems) in a 48 well reaction plate using the following parameters: 10 min at 95°C and 40 cycles of 95°C for 15 s, 55°C (TPRc1) or 60°C (Actin1; At2g37620) for 30 s and 72°C for 30 s. Each PCR reaction for the standard curve was performed in triplicates using four 1 : 5 dilutions of the standard plasmid. The reactions for the samples were performed in duplicates. Two controls lacking a template were included per run. Specificity of amplification was verified at the end of the PCR run by a melt curve and on an agarose gel. The efficiency of amplification calculated by the software of the PCR machine was always between 90% and 100% (see Appendix).

2.13. Protein extraction from plant material

Frozen plant material was ground in an eppendorf tube with a pestle and suspended with 1 volume of protein-extraction buffer (50 mM Tris (pH8.0), 100 mM KOAc, 1 mM EDTA, 1mM DTT, 20% glycerol, 1% TritonX-100, 1% SDS, 1% plant protease inhibitor (Sigma)) and centrifuged at maximum speed for 10 min. The supernatant was transferred in a fresh eppendorf tube and total protein concentration was estimated by Bradford (Bradford *et al*, 1976). The

maximal possible equal amount of whole protein extract was mixed with 2 x SLB (100 mM Tris-HCl (pH6.8), 4% SDS, 0.2% Bromophenol blue, 20% glycerol, 200 mM DTT). 1 - 4 µg of whole protein extract from each tissue was separated by SDS-PAGE.

2.14. SDS Polyacrylamide gel electrophoresis

SDS-PAGE was performed according to the procedure of Laemmli (1970). Separating and stacking gels and running buffer were prepared as described by Sambrook and Russell (2001). The separating and stacking gels were left for polymerization for ca. 40 min. Gels were used immediately or stored up to 7 days in dH_2O containing 0.1% SDS at 4°C. Protein samples were incubated with an equal amount of 2 times concentrated sample buffer (100 mM Tris-HCl (pH6.8), 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM DTT) and incubated at 70°C for 10 min in the case of radioactive and pull down samples or 95°C for 5 min in the case of crude protein extract and recombinant protein samples. Samples were centrifuged prior to loading. SDS gel (10 x 6 x 1. 5 cm) electrophoresis was carried out at 150 volts mini-Protean tetra cells (Bio-Rad), in 1x PAGE-running buffer (25 mM Tris, 250 mM glycine, 0.1% (w/v) SDS). Gels were stained in solution contained 9 parts 50% (w/v) isopropanol, 1 part acetic acid, 125 mg / l each of Coomassie brilliant blue R250 and G250 at room temperature with gentle shaking. Gels were destained in 10% glacial acetic acid. Prestained protein marker or broad range protein marker (NEB) were routinely used as size marker.

2.15. His-tag pull down assay

200 pmol fresh recombinant protein was added to 10 µl Ni-NTA beads, washed 3 times with CG washing buffer (100 mM KOAc, 5 mM $MgOAc_2$, 20 mM HEPES-KOH, pH7.5). CG binding buffer (100 mM KOAc, 5 mM $MgOAc_2$, 10 mM imidazol, 0.5% NP40, 0.5% Tween-20, 1% SDS, 2 mg / ml BSA, 2 mg /. ml ovalbumine, 20 mM HEPES-KOH, pH7.5) was added to a final volume of 500 µl

and the proteins were bound to the beads for 30 min at 4°C. The reaction tubes were incubated on ice for 2 min to let the Ni-NTA beads sediment. The supernatant was replaced with 250 µl fresh CG binding buffer containing 20 µl wheat germ extract (Promega), which was pre-incubated with Ni-NTA beads for 30 min at 4°C and centrifuged at 14300 *g* for 1 min prior to binding. If applicable, an inhibitor peptide with the final concentration of 500 µM was added. After an incubation time of 30 min at 4°C the beads were washed 3 - 4 times with 500 µl CG washing buffer. Therefore, the reaction tubes were incubated on ice until the beads were sedimented, the supernatant was replaced by 500 µl cold CG washing buffer and the content of the tubes was mixed by careful flipping. Proteins were eluted from the beads with 30 µl 2 x SDS loading buffer at 70°C for 10 min. Eluted proteins were separated by SDS - PAGE and analysed by western blotting.

2.16. Western Blotting

Proteins were separated by SDS-PAGE and electro-transferred at 100 V onto a PVDF membrane using a wet-blotter (Bio-Rad) and western transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol). To confirm the transfer of the proteins onto the nitrocellulose membrane, the membrane was stained with Ponceau S (Sigma), photographed and destained in H_2O. The membrane was blocked with 5% milk in TST (6.1 g / l Tris, 8.8 g / l NaCl, 0.5% (v/v) Tween 20, pH8.0) over night. Next, the membrane was incubated with TST buffer and anti-*recombinant* TPRc1 or anti human Hsc70 antibody (Stressgen) (1 : 1000 dilutions or 1 : 20000) for 1 h, and then washed 3 times for 15 min with TST buffer. The secondary antibody, (goat anti-rabbit antibody (Cy5 labelled) IgG (1 : 3000 dilution)) was applied and the membrane was incubated for 1 h in darkness, washed 3 times for 5 min in TST buffer, and scanned with red fluorescence modules using the ODYSSEY Infrared imaging system (LI-COR Biosciences).

2.17. Isolation of pea chloroplast

Young pea leaves (2 - 3 g) from 3 - 6 weeks old plants were harvested, cut into small pieces and put into two 50 ml falcon tubes. After addition of 30 ml ice - cold grinding buffer (2 mM EDTA (pH8.0), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM HEPES (pH7.5), 0.33 M sorbitol, 5.7 mM sodium ascorbate, 2.5 mg / ml BSA) to each tube the suspension was homogenised on level 2 for 30 s at 4°C using the ULTRA -TURRAX® high-performance disperser (IKA®) and the homogenizer attachement S18N-10G (IKA®). The homogenate was filtered through cheese cloth and chloroplasts were precipitated at 2500 *g* for 2 min at 4°C. The supernatant was discarded and the pellet was resolved in the remaining grinding buffer using a fine paint brush. The chloroplast solution was carefully layered onto a Percoll (Fluka) step gradient (1.5 ml 80% Percoll, 5 ml 40% Percoll in grinding buffer) and centrifuged in a swinging bucket (T21) at 3000 *g* for 30 min at 4°C. The lower band between the 80% and the 40% Percoll phase was removed and resuspended in 5 ml grinding buffer. Intact chloroplasts were precipitated by centrifugation at 2500 *g* at 4°C for 2 min. Supernatant was discarded and the pellet was re-dissolved in the remaining liquid. Chloroplasts were stored up to 1 h on ice for further use.

2.18. *In vitro* Translation with wheat germ extract

For in vitro translation of radiolabeled TPRc1 10 µl RNA (0.1 µg / µl) was mixed with 50 µl wheat germ extract (Promega), 80 µM amino acid mix -met, 200 U RNasin plus and 5 µl ^{35}S-met (easytag EXPRESS) in a 100 µl reaction and incubated at 26°C for 30 min. The products were centrifuged at 14300 *g* for 1 min to precipitate any aggregates and stored on ice for further use.

2.19. In vitro import assays into chloroplasts

The import mixture containing 20 µl chloroplasts, 8 µl freshly translated protein and 52 µl TAP (15 mM $MgCl_2$, 15 mM ATP, 0.33 M sorbitol, 8.4 mM methionine, 50 mM HEPES-KOH pH7.5) buffer were incubated at 30°C for 20 min. Pellets were separated from supernatants by centrifugation at 2500 *g* for 2 min and washed with 100 µl 0.1 M Na_2CO_3.

2.20. Chemical crosslinking

Chloroplast pellets were resuspended in LSC-sorbitol buffer (100 mM KOAc, 5 mM $Mg(OAc)_2$, 250 mM sorbitol, 50 mM HEPES-KOH pH7.5). Crosslinker were stored dissolved in DMSO at a concentration of 20 mM at -20°C. Crosslinker were added to a final concentration of 0.5 mM. The reaction mix was incubated at 30°C for 10 min. To quench the reaction, 50 mM final concentration glycine for DSS, 5 mM 2-mercaptoethanol final concentration for BMH or both, 50 mM glycine and 5 mM 2-mercaptoethanol for SMCC were added to the reaction and incubated at 30°C for 10 min. Chloroplasts were spun down at 3000 *g* for 3 min and the pellet was separated from the supernatant. If further steps were applied, the pellet was resuspended in 100 µl LSC-sorbitol.

2.21. Immunoprecipitation

SDS was added to the translated product or the crosslinking products to a final concentration of 1% and mixed. The mixture was heated for 10 min at 70°C and slowly cooled down to 30°C. 4 volumes of TX IP^+ buffer (10 mM Tris-HCl pH7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton x 100, 2 mM PMSF, 2 mM L-methionine) and 0.1 volume pansorbin (Calbiochem) in TX IP buffer (10 mM Tris-HCl pH7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton x 100) were added and incubated at 4°C for 30 min. Pansorbin contains dead *Staphylococcus aureus* cells, which have ProteinA as a part of their cell surface; thus addition of

pansorbin should bind any unspecific binding partners of ProteinA, which might be contained in the protein mixture. The mixture was centrifuged at 14300 *g* for 10 min at 4°C to precipitate the pansorbin and the supernatant was transferred to a fresh eppendorf tube. The cleaned translate was aliquoted. 1 µl antibody was added to each tube and incubated over night at 4°C. 20 µl protein A sepharose sepharose (Sigma) was added and incubated 1.5 h at 4°C. The mixture was washed 4 times with 1 ml TX IP buffer and eluted with 15 µl 2 x SLB at 70°C for 10 min. 10 µl of the supernatant were used for analysis with SDS-PAGE.

2.22. Bioinformatic Tools

Sequence alignments were generally performed with ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Similarity searches were performed with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The localisation of transmembrane domains was determined with DAS (http://www.sbc.su.se/~miklos/DAS/). Protein were analysed on domain structures with Prosite (http://www.expasy.org/prosite/).

2.22.1. Phylogenetic analysis - sequence collection

The tetratricopeptide repeat sequence of TPRc1 was analysed phylogenetically. Similar sequences to the TPR domain of TPRc1 were collected using protein BLAST (blastp) against the non redundant protein sequence (nr) data base (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of all organisms as well as restricted to the organism *A. thaliana*. Additionally, homologue sequences in *A. thaliana* to proteins, which are known to contain a chaperone binding TPR domain were found with the help of BLAST. The location of the TPR domains was detected with Prosite (http://www.expasy.org/prosite/) and the sequence was excised for further analysis.

2.22.2. Phylogenetic analysis -multiple sequence alignment

Single aligning sequences were cut and aligned with ClustalW (Align-m). The output format was changed to Phylip before starting the multiple alignment. The output file was saved as an ".aln" format and uploaded in Phylip on the Expasy tool site (http://bioweb2.pasteur.fr/phylogeny/intro-en.html).

2.22.3 Phylogenetic analysis -Phylip

The alignment file was uploaded with Phylip on the Expasy tool site (http://bioweb2.pasteur.fr/ phylogeny/intro-en.html). The programs protdist, seqboot, neighbour, consense and drawtree were used. Initially the alignment file was opened with protdist, which includes seqboot. Default settings were used, thus amino acid similarity was grouped after George/Hunt/Barker as (N Q D E), (L R H), (F Y W) (C), (M V L I), (G A S T P), distances were estimated with the Jones-Taylor-Thornton matrix, no gamma distribution of rates among positions was done and no weights for sites were used. To generate 100 trees out of the alignment file, the bootstrap-option (seqboot) was selected, using 100 as number of replicates and 11 as odd number. The program gave a protdist outfile, a protdist outtext and a seqboot outfile.

The protdist outfile was further analysed with neighbour, ticking the neighbour option given under the protdist outfile window. Again default settings were used, thus the Neighbour-joining method was the used distance method, the input order was not randomised (J), "print out tree" and "write out trees onto tree files" were used as output options, the outgroup species (O) was 1 and the matrix format "Square". Multiple datasets were analysed (M) using 100 datasets and 11 as an odd number. The consense option was selected. The analysis gave a consense outfile and a consense outtree, a neighbour outfile and a neighbour outtree and a neighbour outtext.

The consense outtree was plotted with drawtree, ticking the drawtree option given under the protdist outfile window. Default settings were used for the number of pixels (500 x 500), Laserjet resolution (300 DPI (3)), Paintbrush (PGX) resolution (VGA 1024 x 768 (3)) and font (Times-Roman). Branch

lengths were used, angle labels were plotted horizontally (L) and in the middle (M), no rotation of tree (R) and no angle of arc for the tree (A) were set. The Horizontal and vertical margins were 1.73 and 2.24, respectively. The relative character of height (C) was set as 1/3. The Equal-Daylight-algorithm was used to iterate to improve the tree (I), no scale of branch length was set (S) and the box "try to avoid label overlap" (D) was ticked.

The program gave a drawtree outtext and a graphic tree file in an postscript format. The tree picture was copied to Microsoft PowerPoint. Bootstrap values from the consense outfile were inserted and the tree shape was modulated manually.

Chapter 3 – Phylogenetic analysis of the TPR domain from TPRc1

To determine relationships between the TPR domain of TPRc1 and other TPR clamp proteins, a comparison of protein sequences was performed. Therefore, the primary protein structure of the TPR domain from TPRc1 was compared with similar sequences by phylogenetic analysis. The protein sequence of the TPR domain was compared to proteins in public databases in three ways: a. using Basic Local Alignment Search Tool (BLAST) against a non redundant protein database, b. using BLAST against the non redundant protein database of *A. thaliana*, c. compared to TPR domains of known chaperone receptors and Hop from *A. thaliana* (see Appendices I-III for protein lists). The resulting sequences were aligned with ClustalW generating a multiple alignment file. The multiple alignment file was imported into Phylip on the web (http://bioweb2.pasteur.fr/phylogeny/intro-en.html) (Felsenstein, 1989). The programs seqboot, protdist, neighbour, consense and drawtree were used to draw the trees. Default settings were used in most cases, if not described otherwise. The multiple alignment file was first submitted to protdist, which estimates distances between the aligned protein sequences. A bootstrap of 100 trees was generated with seqboot, which is build into protdist as an option in this Phylip version. The distances between neighbours were calculated with neighbour allowing 100 trees and the most likely tree was generated with consense. The final tree was plotted with drawtree generating an unrooted tree.

3.1. Phylogeny of sequences derived by BLAST

Figure 3.1 shows the phylogenetic tree, which results from the comparison of the TPRc1 TPR domain to the nonredundant protein collection from NCBI without any restrictions for organisms (for protein list see Appendix I). Four functionally different families group into different branches inside the tree. The bootstrap values, given for the branchings between these families are between 20 and 40. Since the bootstrap values indicate the number of cases between 1 and 100, the sequence relation has the same order as shown in the generated tree, this means a loose order of

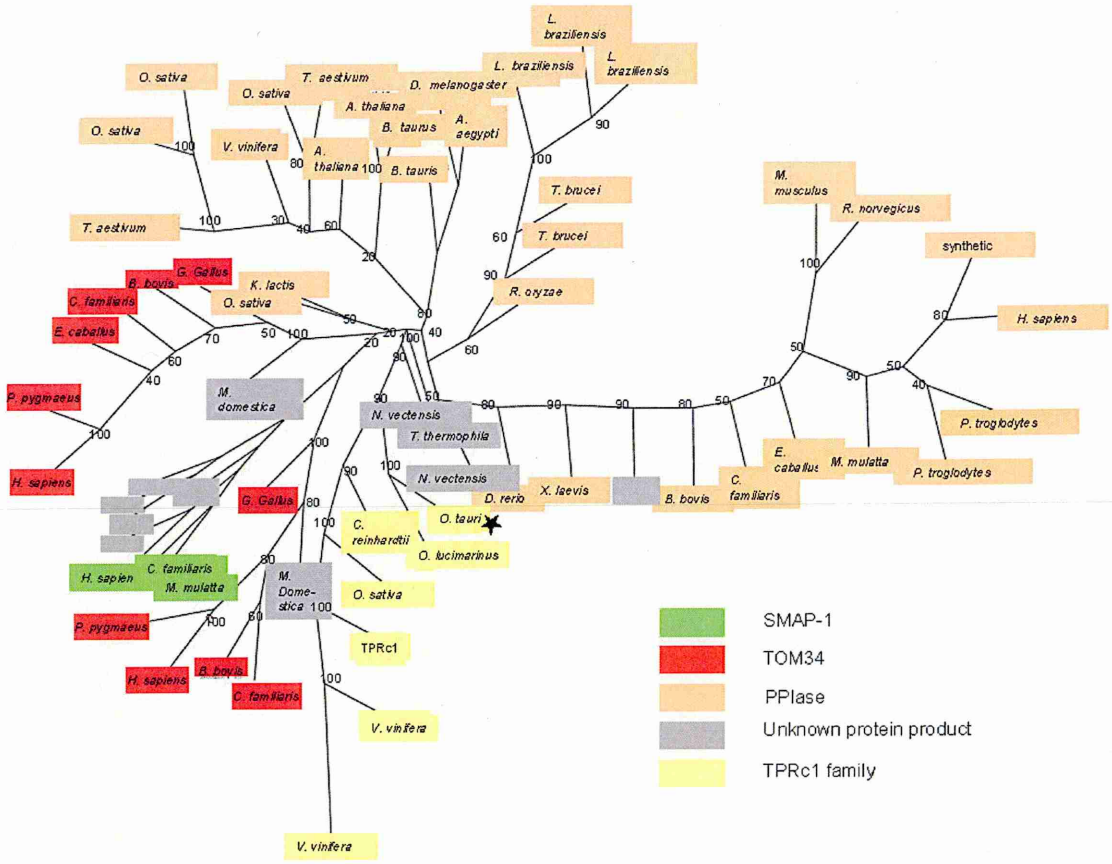


Figure 3.1: The protein sequence of the TPR domain of TPRc1 was compared with the NCBI database for similarity using BLAST. Sequences were aligned with ClustalW and a phylogenetic tree was drawn with Phylip. Bootstrap values defining the likelihood of the order inside the tree are given at the branchings. The TPR domain of TPRc1 has a loose similarity to both TPR domains of the mammalian protein Tom34, an other putative chaperone receptor, and is very close related to the TPR domain of a Hsp90 cochaperone and a predicted chloroplast targeted protein from *Ostreococcus tauri* and *Clamydomonias reinhardti*, respectively. Proteins without defined functions are shown in grey.

relation between the families. The biggest group represented in the tree consists of peptidyl-prolyl *cis/trans* isomerases (PPIases, orange), which can be subdivided into cyclophilins, FK506 binding proteins (FKBPs) and unspecified PPIases (not differentiated in Figure 3.1). Some of the members of the PPIase super-family have a TPR domain, which can bind the C-terminal end of Hsp90 (Kang *et al*, 2008). FKBP51, FKBP52, FKBP38 and cyclophillin40 belong to the group of TPR domain containing PPIases. A notable result is the occurrence of both TPR clamps from Tom34 (red), a chaperone receptor at the mitochondrial outer membrane in deuterostomia (Schlegel *et al*, 2007), indicating a possible similar function of the TPR domain from TPRc1. TPRc1 itself is in a branch

together with unknown plant proteins (yellow). If a BLAST search for the whole sequence of TPRc1 is performed, these proteins would be pulled out in the same order as shown. The close similarity between the TPRc1 TPR domain and the other TPR domains in this branch indicate that TPRc1 is part of a novel, uncharacterised protein family in plants. Two proteins occurring in the TPRc1 branch are especially interesting (marked with an asterix): the predicted Hsp90 cochaperone from *Ostreococcus tauri* indicates that the TPR domain of TPRc1 might interact with Hsp90; and the chloroplast targeted protein from *Clamydomonias reinhardti,* which suggests TPRc1 being localised at chloroplasts.

Drawing a phylogenetic tree with a BLAST output containing a collection of proteins from all organisms will represent the phylogenetic distances between organisms as well as the relationships between types of proteins. For this reason a phylogenetic tree containing the result of a BLAST search of the TPR domain from TPRc1 against only *Arabidopsis* proteins was drawn (Figure 3.2, for protein sequence list see Appendix II). Here, five defined families and three undefined families (grey) are represented in the tree. The PPIase group (B, orange) is most closely related to the TPRc1 branch indicating a late evolution of the TPRc1 TPR domain. The next closest branch (branch E, turquoise) contains two of three TPR regions (DNAJA and DNAJB) of a DNAJ protein. DNAJ is a cochaperone known to interact with Hsp70 due to a conserved J domain (Kelley *et al*, 1998). Additional TPR domains could either recruit other proteins to a Hsp70-DNAJ complex, or if similar to the Hop TPR1 domain, could tighten the interaction to Hsp70 at the C-terminal end. Additionally, in this search two families known to contain TPR domains, which interact with chaperones, the Hsp70 interacting protein family (Hip, branch D, pink) and the Toc64 family (branch C, green). Hip interacts with Hsp70, while Toc64 has a higher affinity to Hsp90 (Qbadou *et al*, 2006). Considering this, it is possible that the TPRc1 TPR domain has chaperone binding properties, but it can not be specified whether it binds the C-terminus of Hsp70 or of Hsp90. PSUOTOC might be a protein fragment of mtOM64. In this tree TPRc1 is shown to group together with a collection of functionally unrelated proteins, such as disease related proteins (DRP and DRP2), transcription factor like proteins (TFR) and calmodulin 8 (CAM8) (branch A, yellow), which do not even contain TPR

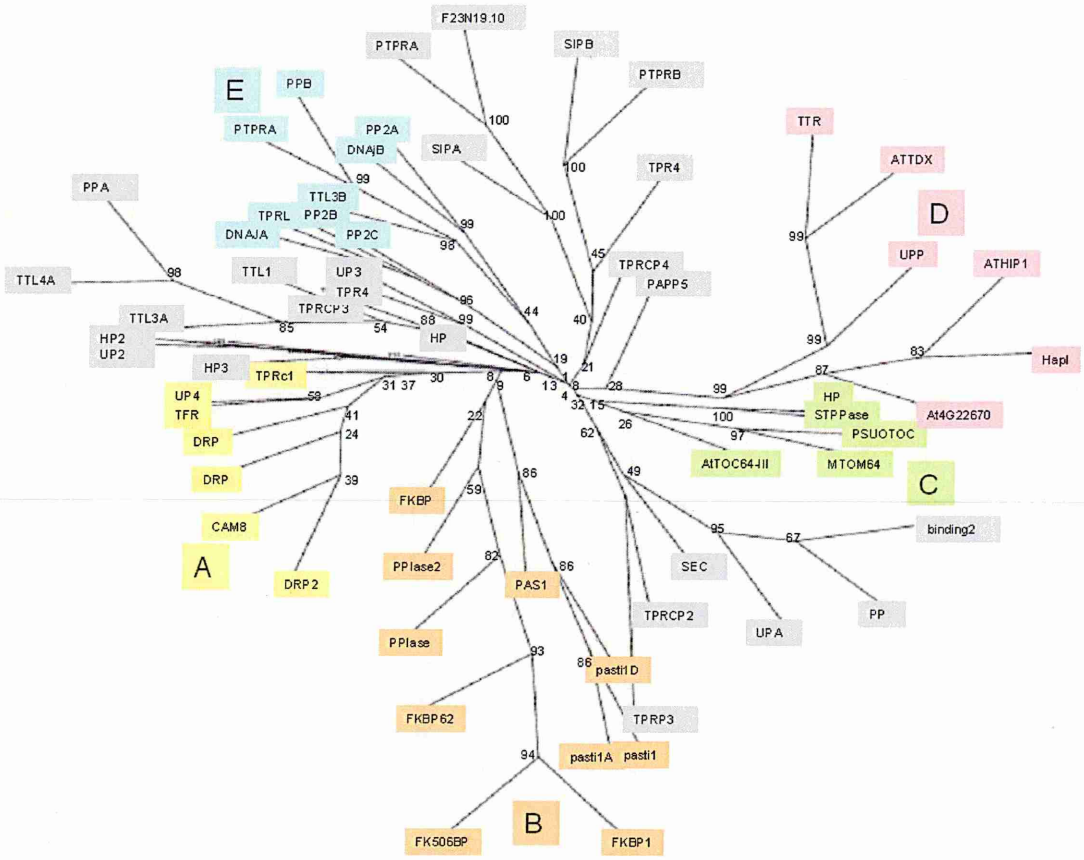


Figure 3.2: Phylogenetic tree of *Arabidopsis* proteins similar to the TPR domain of TPRc1. Sequences resulting from the BLAST search were aligned with ClustalW and a phylogenetic tree was drawn with Phylip. TPRc1 (branch A, yellow) is not closely related to any chaperone receptors, but has a loose similarity to Toc64 (branch C, green). Orange (branch B): PPIase group; pink (branch D): Hsp70 interacting group; blue (branch E): DNAJ group; grey: unspecified.

domains. The reason for this grouping is not a close relation but a varying sequence length pulled out by BLAST and doubled sequences, which both influence the order of the tree. The non redundant protein database from NCBI does partly contain identical protein sequences with different names. As Phylip calculates the maximum likely tree out of a given input, 100% aligning sequences create additional, synthetic branches. As all sequences pulled out by the BLAST search were taken to draw this tree, some sequences submitted are shorter than the sequence of the TPR domain of TPRc1. These short sequences may align better with the TPR domain of TPRc1 than sequences which are actually closer related to TPRc1, but longer. For this reason, e.g. calmodulin 8 is present in the TPRc1 branch although it does not contain a TPR domain. Therefore the duplicated sequences have been removed. Figure 3.3

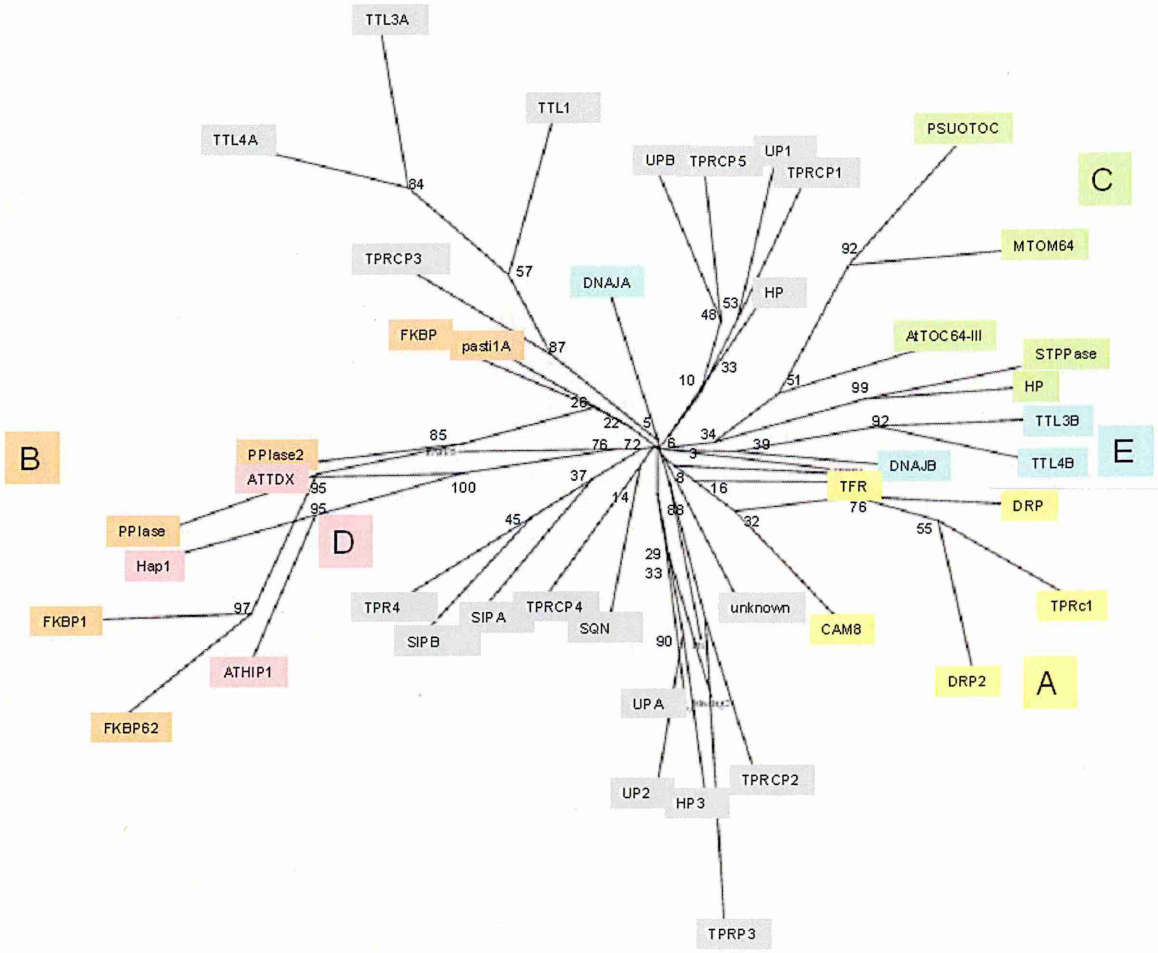


Figure 3.3: Phylogenetic tree of *Arabidopsis* proteins similar to the TPR domain of TPRc1 after removal of duplicated sequences. The only difference in the tree structure is the dispersion of the DNAJ branch.

shows the resulting tree after removal of duplicated sequences. Here, the DNAJ branch is dissolved and spread all over the tree, indicating a low similarity between the two TPR domains from DNAJ. The grouping of the other branches is still the same and the TPRc1 branch is still a collection of proteins with various functions.

The removal of shorter sequences restructures the DNAJ branch (Figure 3.4). The branch containing domains with no relation to each other and TPRc1 is dissolved and TPRc1 does not group together with one distinct family any more. The same conclusion as for the tree in Figure 3.1 can be drawn, as it shows the TPR domain of TPRc1 neither grouping with any known chaperone binding TPR domains, nor grouping together with a domain having a different function. However, the TPRc1 TPR domain is similar to chaperone binding TPR

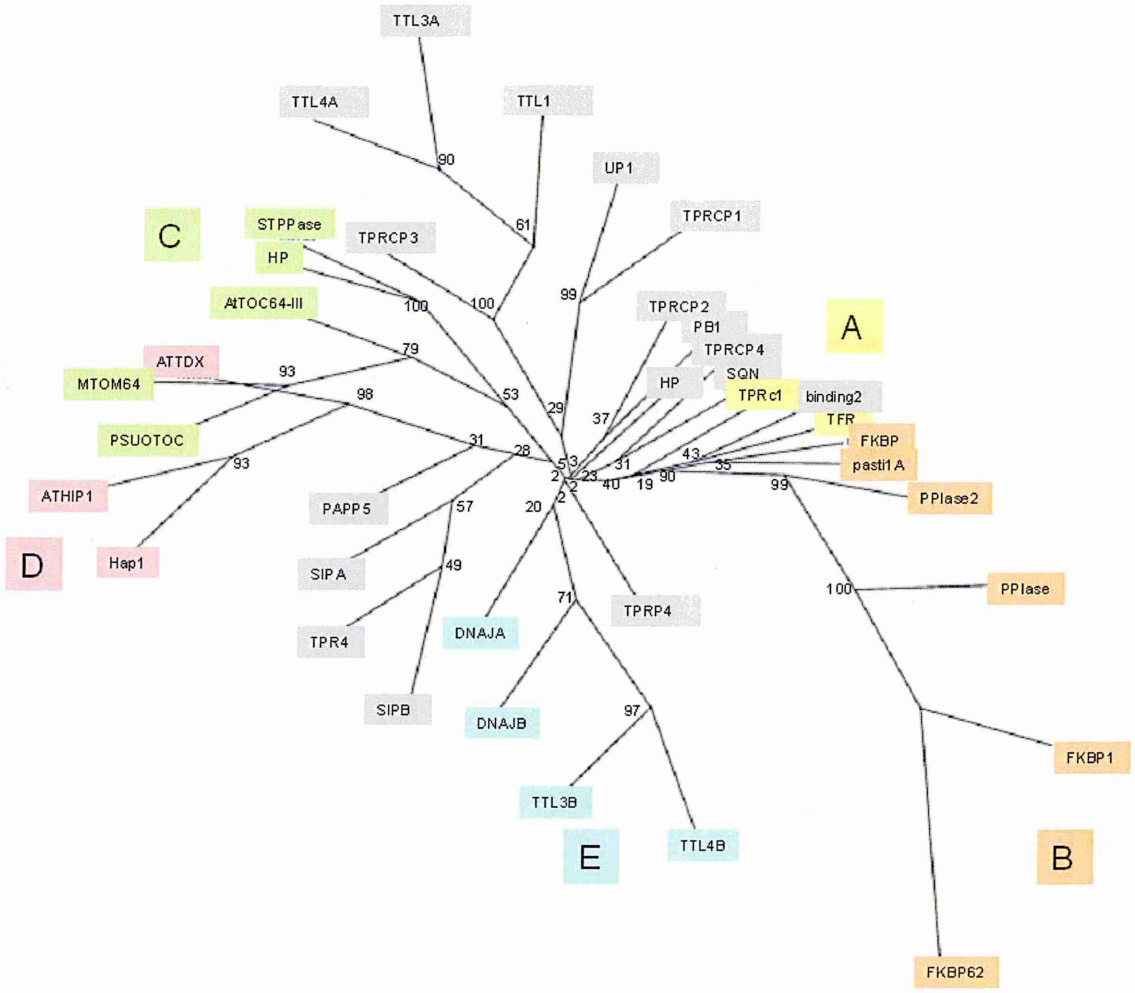


Figure 3.4: Phylogenetic tree of *Arabidopsis* proteins similar to the TPR domain of TPRc1 after removal of duplicated sequences and short sequences. The TPRc1 branch is dissolved, showing, that the TPR domain of TPRc1 does not belong to any of the groups.

domains, which is indicated by the far related Hip (pink) and Toc64 (green) branch. The similarity to both, Hsp70 interacting and Hsp90 interacting TPR domains does not give evidence, whether TPRc1 prefers one to the other.

3.2. Phylogeny of TPR containing receptors

To focus on the relationships between TPR domain, sequences of known chaperone receptors were collected manually and used for phylogenetic analysis (Figure 3.5, for protein sequence list see Appendix III). The proteins were selected according to annotations on the TAIR web site or by Blast search

of known proteins for homologues in *A. thaliana*. The TPR domains were identified with Prosite (http://www.expasy.ch/prosite/) and excised. The resulting phylogenetic tree divides the TPR domains into four distinct groups: The three different TPR domains of Hop build a branch each and the fourth branch is built by the Toc64 TPR domains. Surprisingly, there is a close relationship between the Pex5 TPR domain and the Hsp70 binding TPR domain Hop1, as it is known not to interact with chaperones but directly with the peroxisomal signal sequence PTS1, which differs in its chemical properties from the C-terminal end of chaperones (Harano *et al*, 2001; Schlegel *et al*, 2007). TPRc1 is a part of the Toc64 branch, showing that it is more similar to Toc64 than to any of the Hop TPR domains. In the Toc64 branch chloroplast receptor ATTOC64-III and mtOM64 (ATTOC64-V) are shown to be very closely related. TPRc1 clusters more with the Toc64 homologues, which are involved in other cellular processes than protein targeting. F13N6 is a fatty acid amide hydrolase (Shrestha *et al*, 2003) and the function of MGL6 is unknown. The Toc64 branch is situated closer to TPR2A (Hop2A), the Hsp90 binding TPR domain of Hop.

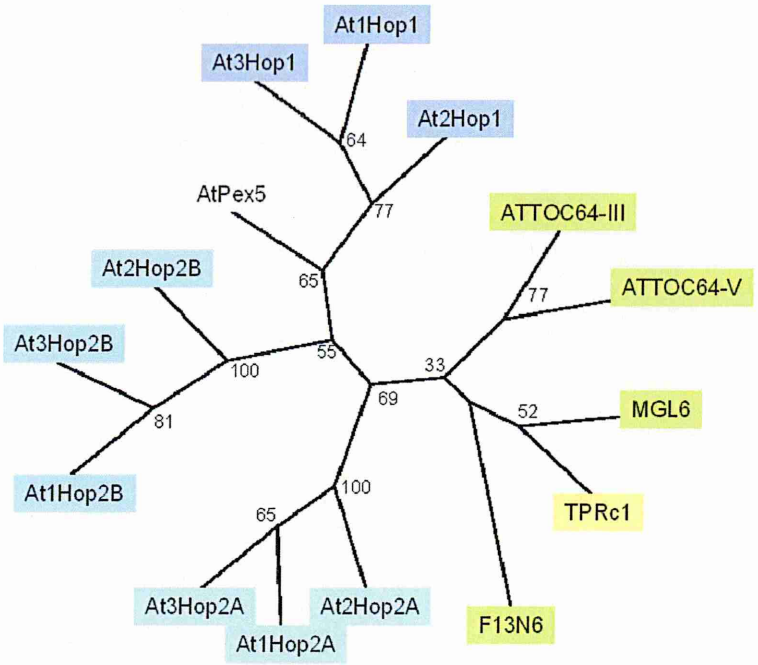


Figure 3.5: Phylogenetic tree of chaperone binding TPR domains from *A. thaliana*. TPRc1 groups together with Toc64, indicating a similar function to the characterised receptor at the chloroplast outer membrane.

3.3. Summary of Phylogeny

Summarised, the phylogenetic analysis of the TPRc1 TPR domain shows that TPRc1 is not a member of any known family: comparison to the TPR domains from a protein collection shows that proteins closely related to TPRc1 are not characterised and exclusively found in plants. A similarity between the Hsp90 binding TPR domains of Toc64 and Tom34 as well as the Hsp70 binding TPR domain of Hip exists. This strengthens the hypothesis that TPRc1 can bind chaperones. Comparison to the TPR domains from *A. thaliana* groups TPRc1 in close proximity with PPIases. The TPR domains of the PPIases cyclophilin40, FKBP52 and FKBP51 have been shown to build a complex with the C-terminal end of Hsp90 (Carrello *et al*, 2004; Chen *et al*, 1999). Comparison of these showed that the PPIases bind Hsp90 with a higher affinity than Hop (Chen *et al*, 1999). Thus, the close relation of the TPRc1 TPR domain to the PPIase TPR domain may indicate an interaction with Hsp90. The TPR domain of cyclophilin40 has been shown to be able to interact with Hsp70 as well, but only if the regions flanking the TPR domain are not deleted (Carrello *et al*, 2004). Thus, an interaction of the pulled out TPR domains from the PPIases with Hsp70 can not be excluded. This data supports the hypothesis that TPRc1 interacts with chaperones through its TPR domain. Whether TPRc1 binds Hsp70 or Hsp90, is however not predictable from this data.

Chapter 4 – Expression level of TPRc1 in different tissues

If TPRc1 has a general role in the process of protein targeting, it should be continuously expressed in all tissues. Therefore the abundance of its mRNA and its protein level in different tissues was tested with quantitative real time PCR and western blotting, respectively. Adult plants were harvested and separated into flowers, buds, stems, cauline leaves, rosette leaves, roots and silliques. The different tissues were used for both experiments.

4.1. Quantitative real time PCR

Quantitative real time PCR was performed with the standard curve experiment (Figure 4.1). RNA was prepared from three biological replicates for each tissue and reverse transcribed to cDNA. The cDNA was diluted 1:1 with nuclease free water and used for real time PCR analysis in the StepOne Sequence Detection System from Applied Biosystems. Sybr Green was used as detection method (Figure 4.1A). Actin1 was chosen as endogenous control.

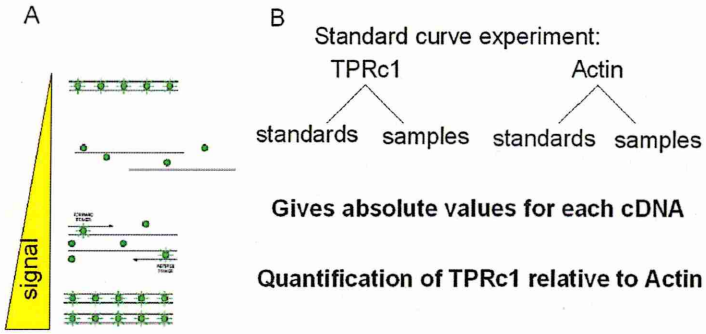


Figure 4.1: Design of the quantitative real time standard curve experiment.
A: Principle of DNA quantification with Sybr Green (taken from the user manual for the StepOne Detection System (Applied Biosystems)). The substance gives a fluorescent signal, when it intercalates within double stranded DNA. Thus, the signal increases after every cycle in an exponential manner.
B: Absolute values of each cDNA were archived with the standard curve experiment. This experiment uses a standard curve measured with known concentration of the target template to define absolute values for each sample. The absolute values for TPRc1 were adjusted to a value relative to Actin1 to make a conclusion for TPRc1 transcription.

Plasmid DNA containing either TPRc1 cDNA or a 435 bp fragment from Actin1 (At2g37620; received from Glawischnig group, (Schuhegger *et al*, 2007)) were used for the standard curves. Each standard curve was drawn with triplicates of four dilution points of the plasmid DNA. The efficiency of amplification was in all experiments greater than 90% (Appendix VI). Thus the possibility that the amplification efficiency has an influence on the measured concentrations is very low. Duplicates for each target cDNA were measured. With this method absolute values of each cDNA were given for TPRc1 and Actin1. To obtain relative values for TPRc1 the absolute values for TPRc1 were divided by the absolute values for Actin1 (explained in Figure 4.1B). The concentration of the plasmids used for the standard dilution series was measured after the experiment with a spectrophotometer (Appendix V).

To verify the specificity of the primers, the size of the PCR products was analysed (example shown in Figure 4.2) on an agarose gel. Only one distinct band at 165 bp for TPRc1 and at 435 bp for Actin1 could be detected in every sample. The faint bands at about 100 bp in the negative controls and samples with little PCR product are primer clouds of unincorporated primers. Additionally, the identity of the PCR products was established by sequencing.

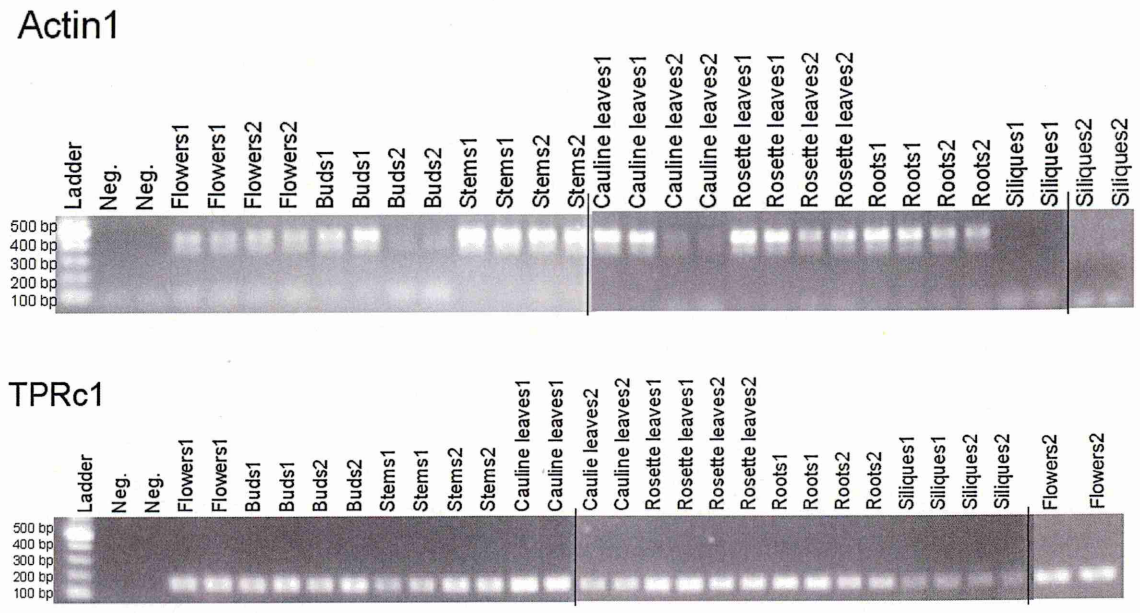


Figure 4.2: The in the real time PCR amplified fragments of TPRc1 and Actin1 cDNA were run on an agarose gel to check for specificity of the primers. Primer clouds are visible at around 100 bp in samples with little cDNA present.

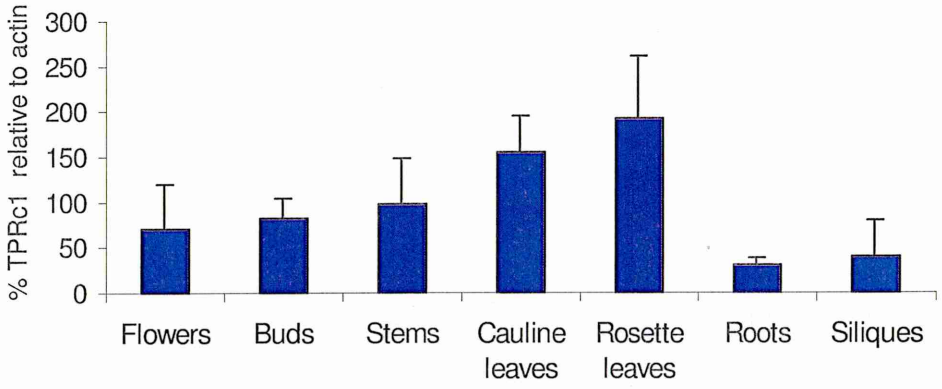


Figure 4.3: Graph of TPRc1 transcription levels relative to Actin1. Shown are means with standard error of the means (SEM). TPRc1 is transcribed in all tissues. The highest transcription levels are in stems, cauline leaves and rosette leaves.

Figure 4.3 shows the transcription levels of TPRc1 relative to Actin1 summarised as means with standard error of the means (SEM). TPRc1 mRNA is present in all tissues. The transcription levels of TPRc1 in the leaf tissues are higher than for Actin1. Highest mRNA levels of TPRc1 were detected in stems, cauline leaves and rosette leaves. The SEM values are between 14% and 55% of the mean values. The highest variation of the mRNA levels in the biological replicates is in flowers, stems and siliques (SEMs are between 30% and 55%). As the duplicates of each cDNA in the experiments did not differ from each other significantly it can be assumed that the differences between the measured transcription levels in these tissues are due to natural variance between the biological replicates.

2.2. Western blotting

To perform western blotting, recombinant TPRc1 lacking the transmembrane domain (TPRc1-TM) and having an N-terminal hexahistidine-tag was expressed in *Escherichia coli* BL21 and purified over a Ni^{2+} colum. This process was repeated and the purified protein was stored at - 80 °C until 1 mg of pure protein was obtained. The pooled protein fractions were separated by sodiumdodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the gel was stained with Coomassie brilliant blue. The TPRc1-TM band at 60 kDa was cut out of the gel and sent to Eurogentech. After generation of the anti-TPRc1 antibody at Eurogentech the antibody was tested for optimal conditions (Figure 4.4). Therefore, whole protein extract from *Arabidopsis* leaves was separated by SDS-PAGE and blotted to a PVDF membrane.

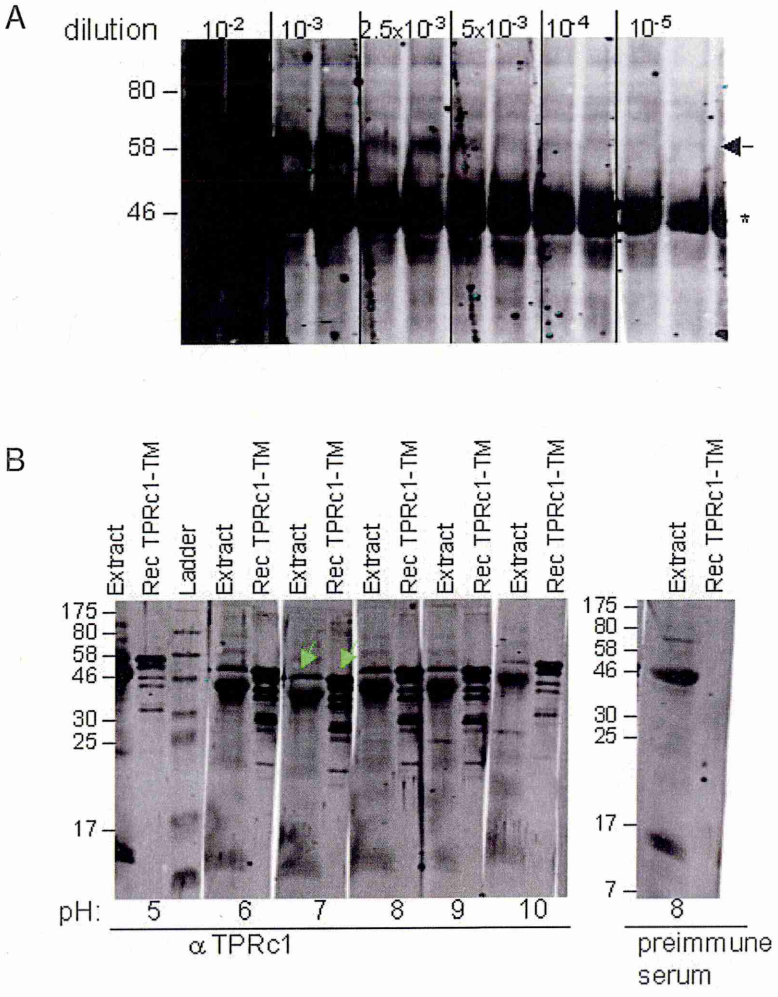


Figure 4.4: Optimisation of conditions for western blotting with the anti TPRc1 antibody.
A: Dilution series of the primary antibody. The optimal dilution of the primary antibody for TPRc1 detection is 1: 1000. Black arrow: TPRc1; *: unspecific band at 46 kDa (large SU of RuBisCO).
B: Immunoblotting of TPRc1 with a pH range between 5 and 10. TPRc1 detection is best at a pH = 7. Detected TPRc1 is highlighted by green arrows.

The membrane was cut into strips and blocked with milk. The strips were incubated with dilutions of the primary antibody between 1:100 and 1:100000. The secondary antibody was diluted 1:10000. Figure 4.4A shows, that a dilution of 1:1000 is best to obtain a strong signal (a black arrow indicated, where the TPRc1 signal is expected). Additionally, the optimal pH for the primary antibody was tested in TST with a pH rage between 5 and 10 (Figure 4.4B) and compared to the preimmune serum. Here, strips containing recombinant TPRc1-TM and whole protein extract from *Arabidopsis* leaves were tested. As shown in Figure 4.4B, the antibody is able to bind the protein in every pH tested. However, the bands at pH7 are brighter and give less background signal (TPRc1 band is indicated by green arrows). Thus, pH7 was used for further experiments. Additionally the detection limit of the antibody was tested (data not shown). The antibody detects recombinant protein greater than 1 pg.

Total protein extract from *A. thaliana* was used for western blotting to test for TPRc1 protein abundance in different tissues. Frozen plant material was ground in extraction buffer. The insoluble parts were separated by centrifugation and the total protein concentration of the supernatants was measured with Bradford reagent at λ = 595 nm. Equal amounts of total protein (1-3 µg) were separated by SDS PAGE and blotted on a PVDF membrane. Recombinant TPRc1-TM was used as a control (not shown). The membrane was incubated with primary and secondary antibody and analysed with the ODYSSEY Infrared imaging system from LI-COR Biosciences.

Figure 4.5 shows the immunoblot against TPRc1 in comparison to the preimmune serum. According to the western blot, full length TPRc1 is

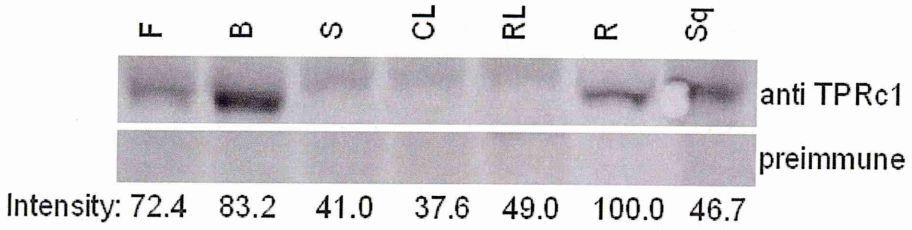


Figure 4.5: TPRc1 is abundant in all tissues. Whole protein extracts from flowers (F), buds (B), stems (S), cauline leaves (CL), rosette leaves (RL), roots (R), and siliques (Sq) were tested on TPRc1 abundance by immunoblotting. TPRc1 can be detected in all tissues. Highest protein levels of TPRc1 are in F, B, R and Sq. The protein is not detected when incubated with preimmune serum. Intensities were calculated in comparison to background seen on the immunoblot with preimmune serum (preimmune).

expressed in all tissues tested. The highest protein levels are in flowers, buds, roots and siliques. As this result is contradictionary to the result obtained by the quantitative RT-PCR, where highest mRNA levels of TPRc1 were detected in stems, cauline leaves and rosette leaves, the possibility of degradation in leave tissues was considered. Therefore, recombinant protein was added to the ground tissue from rosette leaves (RLS) before addition of extraction buffer and immunoblotted (data not shown). Here, the signal for TPRc1 did not become stronger after addition of recombinant TPRc1 in comparison to the same tissue without added protein. Additional bands under 46 kDa were visible in RLS, indicating that TPRc1 may degrade or aggregate in leaves in the conditions used for this experiment. A plant specific protease inhibitor cocktail was already used in the tissue extraction buffer. Thus, there is no simple way to resolve this problem.

2.3. Summary of abundance of TPRc1

Quantitative RT-PCR and western blotting show that TPRc1 is expressed in all tissues. However, the measured RNA and protein levels vary significantly in most tissues. While the quantitative RT-PCR shows highest transcription levels in stems and leaves, highest protein concentrations are in flowers, buds, roots and siliques.

If the mRNA-levels in flowers, buds, roots and siliques are compared to the strength of the respective western blot bands, the ratios of the measured transcription and translation levels seem to correlate. Thus, the translation of TPRc1 from its mRNA seems to be more efficient in these tissues or the TPRc1 protein is degraded fast in stems, cauline leaves and rosette leaves upon cell damage. Another possibility might be an inhibition of TPRc1 translation by RNA interference (RNAi) in these tissues. Nevertheless, TPRc1 protein is present in all tested tissues which indicates, that it might have a general biological function.

TPRc1 is proposed to have a similar function as atToc64-III, the Toc64 isoform that is a part of the translocation complex at the outer envelope of chloroplasts (Sohrt *et al*, 2000). Comparing the quantitative RT-PCR data derived for TPRc1 with the transcription pattern of atToc64-III show, that the amount of the atToc64-III mRNA is equal in roots, buds and rosette leaves (Aronsson *et al*, 2007) while the TPRc1 mRNA level is significantly higher in rosette leaves compared to buds and roots. However, the differences of transcription levels of TPRc1 between the biological replicates were quite high and the western blot shows highest protein levels in buds and roots. Additionally, atToc64-III protein might have another distribution pattern in different tissues than its mRNA. Thus, the possibility that the distribution of TPRc1 and Toc64 is similar inside the plant can not be excluded.

This data gives evidence for expression levels of TPRc1 in different tissues of adult *Arabidopsis* plants. Whether TPRc1 is expressed continuously or only in a certain developmental stage or its expression is dependent on the circadian clock as well as the stability and localisation of the protein inside the cell are however not given by this data.

Chapter 5 – Intracellular localisation of TPRc1

There exist only homologues of TPRc1 in plants. This can be seen in the phylogenetic tree of Figure 5.1, where close related TPR domains are solely from plant proteins, suggesting a role of TPRc1 in a plant specific organelle, such as chloroplasts. Further, the transmembrane domain is at the C-terminus of the protein. Thus it is likely, that TPRc1 is anchored at the membrane and presents the whole N-terminal part of the protein including the TPR domain towards the cytosol. As a consequence, TPRc1 is predicted to be a receptor at the chloroplast outer envelope. This was tested *in vitro* by import assays of TPRc1 into chloroplasts and *in vivo* by transient expression of yellow fluorescent protein - TPRc1 (YFP-TPRc1) in tobacco leaves and confocal laser microscopy.

5.1. Comparison of the C-terminal ends from known tail anchored proteins

The C-terminal end of TPRc1 including the tail anchor was compared to the C-terminus from other TA-proteins, which are known to be targeted to chloroplasts. Therefore, the transmembrane domain (TMD) has been predicted with DAS on the Expasy tool site (Cserzo *et al*, 1997). Sequences including the TMD, 20 amino acids before the TMD and 20 amino acids after the TMD, if the sequence did not finish before, were excised and submitted to ClustalW for multiple sequence alignment (for sequence detail, please see Appendix VI). Aligned C-termini from cytochrome b5, Toc33, Toc34 and TPRc1 are shown in Figure 5.1. As known from other TA-proteins, it can be seen, that there exists

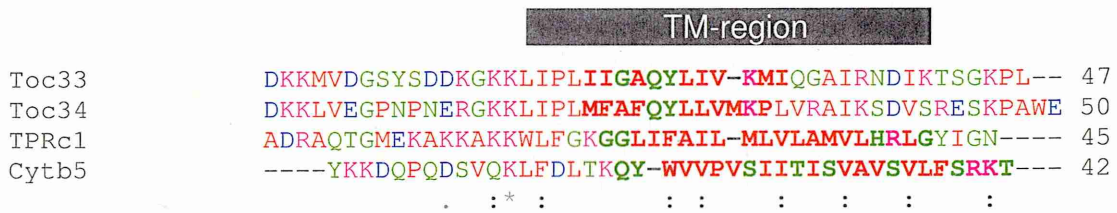


Figure 5.1: Comparison of the transmembrane domains of the until now known TA proteins at the chloroplast outer envelope. The position of the transmembrane domain from Toc33, Toc34, cytochrome b5 (cytb5) and TPRc1 have been defined with DAS (Cserzo *et al*, 1997) and is between 11 and 19 amino acids. Sequences have been cut allowing maximal 20 amino acids flanking the TMD and submitted to ClustalW for multiple alignment.

no sequence similarity between the TMDs, which vary in sequence length and amino acid composition. For this reason the TMD has been called transmembrane region (TM-region) in this Figure. As a consequence of the missing similarity between the amino acid composition and the TMD length it is not possible to compare the amino acids flanking the TMD at the C-terminal side in the alignment form used. When the amino acids flanking the TMD at the N-terminal side are compared, it can be seen, that a lysine is directly before the start of the transmembrane domain in all sequences. Additionally, TPRc1, Toc33 and Toc34 share the pattern of [+ s ++] (pos, small, pos, pos) before the TMD. Cytochrome b5 has two isoforms in *A. thaliana*: one (At5g48810) is targeted to the endoplasmic reticulum (ER) and the other (At1g26340) is targeted to chloroplasts (Maggio *et al*, 2007). It might be possible, that the chloroplast targeted isoform can be targeted to the ER in a small extent, which might explain, why this pattern does not occur for Cyb5. Thus TPRc1 shares some similarity with the flanking region of the TMD from Toc33 and Toc34, which are known to be localised to chloroplasts.

5.2 Confocal laser microscopy

When TPRc1 fused to YFP at the N-terminus (YFP-TPRc1) is overexpressed in tobacco leaves, it localises to chloroplasts (Verena Kriechbaumer, unpublished). Figure 5.2 shows in two independent experiments that the fluorescence of YFP-TPRc1 overlays with the autofluorescence of the chloroplasts (indicated with white arrows). This gives evidence, that TPRc1 is localised in chloroplasts *in vivo*.

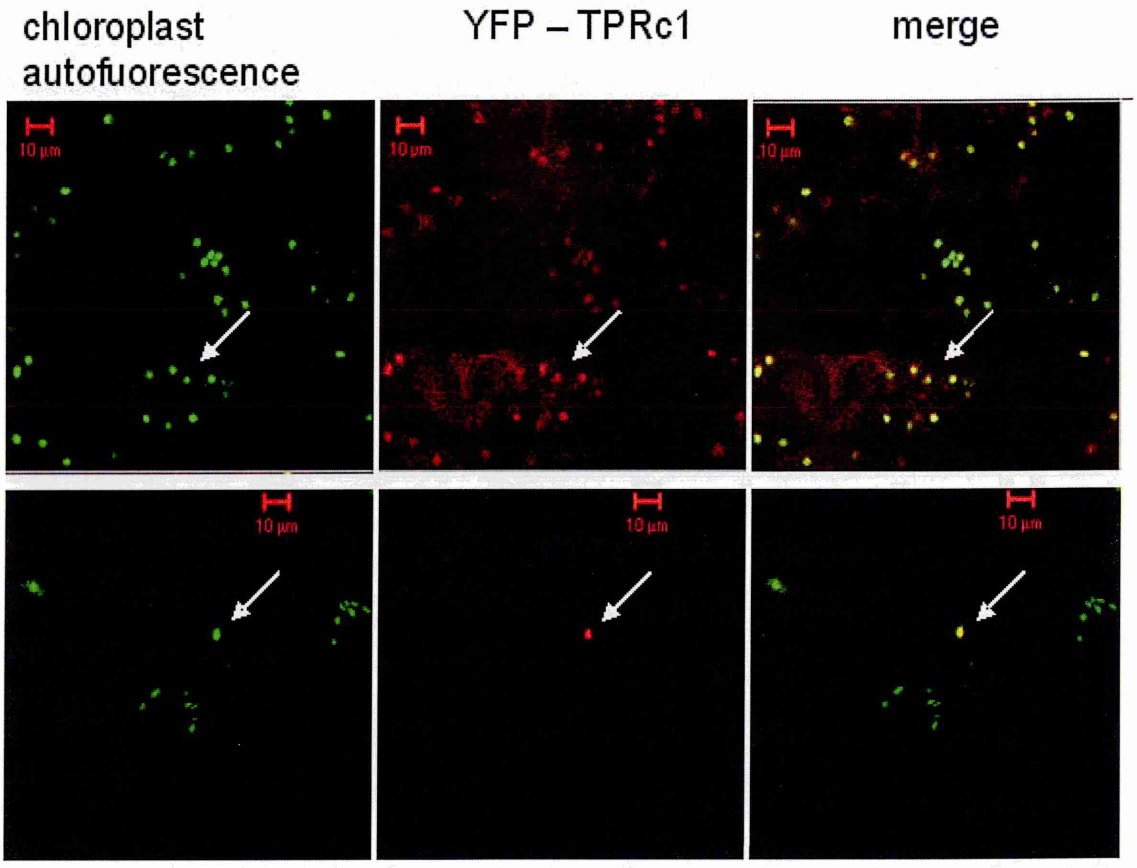


Figure 5.2 (Verena Kriechbaumer, unpublished): YFP-TPRc1 localises in chloroplasts. The autofluorescence of chloroplasts overlays with the signal of YFP-TPRc1 (indicated with white arrows).

5.3. Import of TPRc1 into the chloroplast outer membrane

Import assays into isolated pea chloroplasts were performed to confirm the confocal laser microscopy data and to investigate the topology of TPRc1 at the membrane, thus testing whether TPRc1 is facing the cytosol or inside the intermembrane space (IMS) or whether it is situated inside the chloroplast stroma. Therefore, radiolabeled TPRc1 and Toc33, a Toc34 homologue (Jarvis *et al*, 1998) containing a tail anchor, were translated in rabbit reticulolysate (RRL) and imported into isolated pea chloroplasts. Supernatants (SN) and chloroplast pellets (P) are shown in Figure 5.3A. Here, Toc33 gives a strong signal in the chloroplast pellet. The insertion of TPRc1 into the chloroplasts is not as efficient as the insertion of Toc33: while the signal in the pellet fraction of Toc33 is at least two times stronger than the signal in the supernatant, the signal for TPRc1 is the same in both fractions. The additional band, which is detectable above 220 kDa in the lanes containing TPRc1, indicates aggregation of the protein. Another possibility might be that TPRc1 translation is partly not

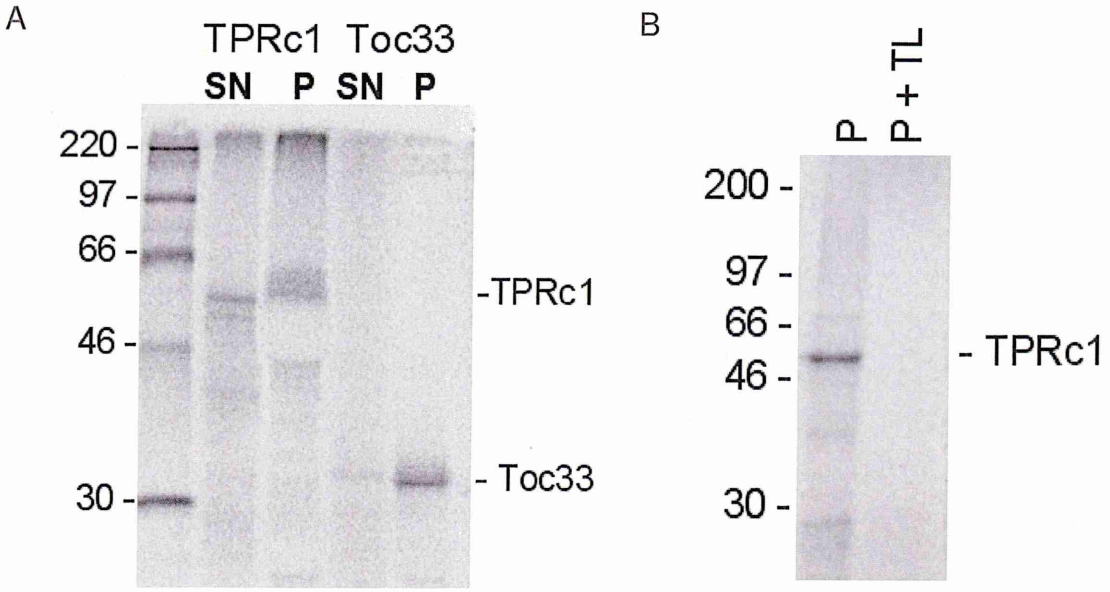


Figure 5.3: *In vitro* studies of TPRc1 topology at the membrane. Marker bands are given in kDa.

A: TPRc1 can be imported into the chloroplast envelope. Radiolabeled TPRc1 was imported into the chloroplast envelope and compared to the import of Toc33.

B (Verena Kriechbaumer, unpublished): Chloroplast pellets were protease treated with thermolysine (+TL) after import of TPRc1. Digestion of the TPRc1 band by the protease indicates, that TPRc1 is facing the cytosol.

complete, resulting in TPRc1 protein missing the tail anchor (e.g. compare to translation product in Figure 7.1). Thus, the comparatively weak import of TPRc1 into chloroplasts may be due to incomplete translation of TPRc1 or aggregation of the translated protein. It can be seen, that the signal of the radiolabeled protein migrates slightly slower in all pellet fractions. The presence of the chloroplast membrane in the pellet fractions seems to interfere with the migration of the proteins in the SDS gel, possibly allowing less negatively charged SDS to bind to the protein, and makes some of the proteins migrating more slowly than the majority does. Another possibility might be the huge protein amount, which can be detected at 46 kDa in western blotting and might hinder TPRc1 to migrate at its normal size at 60 kDa.

Chloroplasts have been treated with thermolysin after TPRc1 import to test membrane topology of TPRc1 (Figure 5.3B; Verena Kriechbaumer, unpublished). Addition of the protease degrades TPRc1. Thus, TPRc1 is facing the cytosol and is not localised inside the chloroplasts. The tail anchor, which should be integrated into the membrane and thus be protected from the protease, is too small to be seen on the gel.

Cell-free targeting assays using a mixture of isolated chloroplasts and mitochondria, in which the majority of TPRc1 is found at chloroplasts, support that TPRc1 localises to chloroplasts (Verena Kriechbaumer, unpublished). Cell-free targeting to ER membranes is very inefficient, and when a glycosylatable sequence is added at the C-terminus no glycosylation is observed, showing that the targeting of TPRc1 to chloroplasts is highly specific (Ben Abell, unpublished).

Taken together, TPRc1 import into chloroplasts and protease treatment suggest that TPRc1 is integrated in the outer membrane of chloroplasts through its tail anchor, and the main part of the protein including the TPR domain is exposed to the cytosol.

5.4. Summary of intracellular localisation of TPRc1

TPRc1 has some similarity to the flanking region before the transmembrane domain of Toc33 and Toc34, which are known to be localised at the chloroplast envelope, and it is found to be inserted into the chloroplast envelope in these experiments. Specificity of TPRc1 for chloroplasts is underlined by confocal laser microscopy and the lack of significant insertion into mitochondrial or microsomal membranes. Its membrane topology is towards the cytosol as it is unprotected from protease treatment. In the case of other TA proteins at the chloroplast envelope, such as Toc34 or OEP7, a small protein fragment protected from the protease could be detected (Qbadou *et al*, 2003; Schleiff *et al*, 2001). In the case of TPRc1, the fragment which would be protected from the protease is too small to be detected in the system used, because the C-terminal end after the transmembrane domain consists only out of four amino acids (YIGN). The shortness and the hydrophobicity of the C-terminal end inside the intermembrane space might result in TPRc1 not being as well anchored at the membrane as Toc33, and thus could explain the relatively poor import of TPRc1 in comparison to Toc33.

Nevertheless, these results show that TPRc1 is localised at the chloroplast envelope and that the protein exposes its TPR domain towards the cytosol making a role of TPRc1 in protein targeting as a receptor possible.

Chapter 6 – Interaction of TPRc1 in the cytosol

It could be shown that TPRc1 is abundant throughout the plant and that it localises to chloroplasts and the TPR domain of TPRc1 is facing the cytosol. According to the phylogenetic prediction of the TPR domain from TPRc1 Hsc70 and / or Hsp90 are proposed to be interaction partners of TPRc1 in the cytosol. This was examined with pull down assays. Therefore, decahistidine-tagged TPRc1 and various truncations were used to pull down Hsc70 or Hsp90 out of wheat germ lysate (WGE). The role of TPRc1 in protein targeting was tested with pull down experiments of precursors in WGE.

6.1. TPRc1 constructs

TRPc1 constructs with different truncations of the protein were cloned into expression vectors for bacterial expression (Figure 6.1A). The appropriate DNA sequence for each construct has been amplified with primers containing restriction sites (for details, see table 1 and 3 in materials and methods). Full length TPRc1 (TPRc1FL) and TPRc1 lacking its transmembrane domain (TPRc1-TM) have been cloned by Susann Lehmann and have been used for further studies. TPRc1-TM was generally preferred for bacterial expression, because the TMD lowers its solubility and thus its yield. The truncated constructs of TPRc1 TPRc1L, which contains the region between the TPR domain and the TMD, and TPRc1TPR, which contains only the TPR domain of TPRc1, as well as TPRc1R185A, which has a point mutation inside the TPR domain, were designed to investigate the function of TPRc1. The amplified DNA sequence of TPRc1TPR was successfully cloned into pET-16b and pIVEX1.4. TPRc1L DNA was inserted into pET-16b. TPRc1R185A was generated by PCR using the site directed mutagenesis kit from Stratagene and TPRc1-TM as a template. For protein expression 500 ml bacterial cell cultures containing the expression vector were grown at 37°C. Expression was induced at room temperature after a heat shock, which should induce chaperone expression in the cells and thus maximise the solubility of the protein. All existing clones contain an N-terminal His-tag. Thus, after protein expression the proteins were His-tag purified with Ni-NTA resin. Figure 6.1B shows eluted fractions of the different constructs of TPRc1 after His-tag purification. His-tag purification

resulted in sufficiently pure protein for the planned applications. Dependent on the expressed construct, yields between 1 nmol and 6 nmol of soluble protein per 500 ml induced culture were obtained. Details for expressions of individual proteins are listed in Table 4.

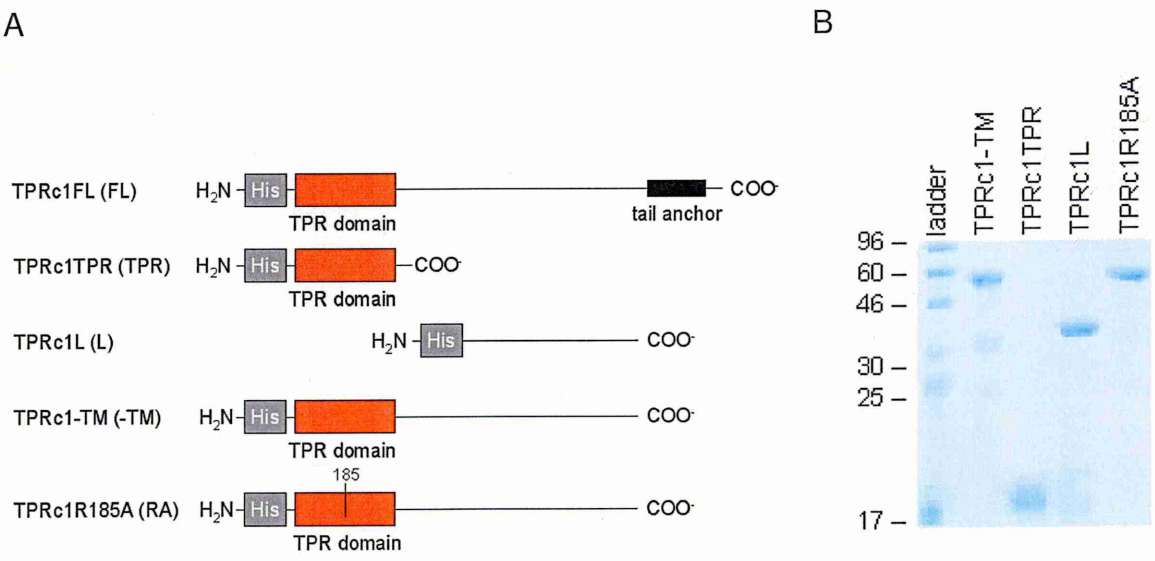


Figure 6.1: Different constructs of TPRc1.
A: Scheme of constructed sequences including full length TPRc1 (TPRc1FL), TPRc1 lacking its transmembrane domain (TPRc1-TM), TPRc1 without TMD and without TPR domain (TPRc1Link), the TPR domain of TPRc1 only (TPRc1TPR) and TPRc1-TM with a R185A mutation inside the TPR domain, which interrupts its function (TPRc1R185A)
B: Eluted fractions of TPRc1-TM, TPRc1-TPR, TPRc1-L and TPRc1-R185A after His-tag purification. Marker bands are given in kDa.

6. 2. TPRc1 pulls Hsc70 out of wheat germ extract

The function of TPRc1 was analysed with His-tag pulldown assays. Susann Lehman showed that TPRc1-TM pulled down a protein at about 70kDa, which was proposed to be Hsc70, from WGE (data not shown). To verify her results obtained by a Coomassie stained SDS gel, the experiment was repeated with additional constructs of TPRc1 and the identity of the pulled down protein was confirmed by immunoblotting against Hsc70 and Hsp90.

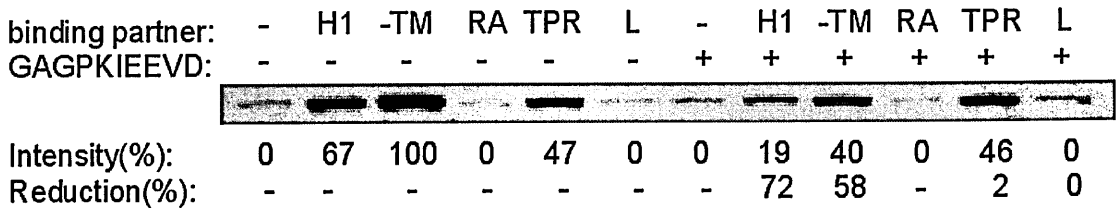


Figure 6.2: The TPR domain of TPRc1 binds to the C-terminal end of Hsc70. Imunnoblot against Hsc70. TPR1 from Hop (H1), TPRc1-TM (-TM), TPRc1TPR (TPR), TPRc1L (L) and TPRc1R185A (R185A) have been expressed in *E. coli* and used for His-tag pulldown from wheat germ extract. An inhibitor peptide GAGPKIEEVD, which mimes the C-terminal end of Hsc70, was added to compete with Hsc70 for binding.

Therefore equal amounts of His-tagged, recombinant proteins were bound to Ni-NTA beads and incubated with WGE. After washing steps, the His-tagged proteins together with interacting proteins were eluted from the beads by denaturation, followed by SDS gel electrophoresis and western blotting with anti human Hsc70 IgG.

The Hsc70 binding TPR domain from human Hop, TPR1 (H1), was taken as positive control for the pull down of Hsc70. To confirm that beads alone do not bind Hsc70, Ni-NTA only was incubated with wheat germ extract. TPRc1L does not have a TPR domain and should thus not bind Hsp70 and TPRc1R185A has a mutation of the arginine at position 185 to alanine, which has been shown for Tom70 and for Toc64 to interrupt the binding capability of the TPR domain (Young *et al*, 2003; Qbadou *et al*, 2006). Thus, TPRc1R185A and TPRc1L were both used as negative controls. TPRc1TPR and TPRc1-TM were expected to bind Hsc70.

Figure 6.2 shows the immunoblot against Hsc70. Here, it is shown that the TPRc1 constructs containing a functional TPR domain (-TM and TPR) and the positive control, TPR1 from Hop (H1), pull down Hsc70 out of WGE. The binding of Hsc70 is due to interaction with the TPR domain as lanes showing the pull down with the negative controls TPRc1R185A, TPRc1L and Ni-beads only do not contain a higher amount of Hsc70 than Ni-NTA beads only. Numbers from quantification of Hsc70 binding are given in the figure. Hsp90 was not detected by the anti human Hsp90 antibody (data not shown).

The use of recombinant C70 as a competitive inhibitor was found to be not successful in this assay, because C70 seemed to precipitate in the

concentration needed (data not shown). Thus, the design of a peptide to use as a competitive inhibitor was attempted. Scheufler et al. tested the binding affinity of TPR1 to peptides with the size ranging from the last 4 (EEVD) to 12 amino acids of Hsp70 (GSGSGPTIEEVD) in comparison to the recombinant C-terminal domain of Hsc70 (C70) by isothermal titration calorimetry (Scheufler *et al*, 2000). Here, the decapeptide GSGPTIEEVD was found to bind stronger to TPR1 than C70. The C-terminal end of Hsc70 from *A. thaliana* differs in the amino acids at position -6 and -9 from the human one. Thus, the for Hsc70 from *A. thaliana* adequate decapeptide GAGPKIEEVD has been designed to mimic the C-terminal end of plant Hsc70 using the information given by isothermal caliometry studies from Scheufler et al.. Addition of this competitive inhibitor peptide (+ GAGPKIEEVD) decreases the amount of pulled down Hsc70 by TPRc1-TM by 58% and for H1 by 72% (Figure 6.2). This gives evidence that the TPR domain of TPRc1 interacts specifically with the C-terminal end of Hsc70. The lower binding capability and less inhibition for Hsc70 binding by TPRc1TPR (TPR) might reflect a requirement of another part of the protein for efficient folding of an intact domain.

Interaction of TPRc1 with recombinant, human Hsp90 as well as pull down of Hsp90 out of RRL by TPRc1 was tested, but did not show Hsp90 binding by TPRc1-TM (data not shown; done by Verena Kriechbaumer). Thus, these results confirm, that the TPR domain of TPRc1 interacts with the C-terminal end of Hsc70. An interaction with Hsp90 was not possible to demonstrate, but can not be completely excluded, because it is not known whether Hsp90 is present in WGE.

6.3. Pull down of precursor proteins bound to chaperones by TPRc1

Localisation of TPRc1 at the chloroplast envelope and capability to interact with Hsc70 confirms the hypothesis that TPRc1 is a chaperone receptor, which is involved in protein targeting. Therefore, it needed to be tested whether TPRc1 is able to interact with a precursor-chaperone complex. Thus, the capacity of TPRc1 to pull down precursors bound to chaperones was tested with His-tag pull down assays by Verena Kriechbaumer (Figure 6.3, Verena

Kriechbaumer, unpublished). Therefore, chloroplast (Toc33, Lhcb1, and TPRc1) and mitochondrial (Mito3) precursor proteins were translated *in vitro* in WGE and radioactively labelled. To conserve the precursor - chaperone complex the proteins were treated with apyrase, which hydrolyses ATP and blocks ATP-driven release of the precursors from the chaperones (Abell *et al*, 2007). The complex was incubated with recombinant TPRc1-TM bound to Ni-NTA beads (+ TPRc1). Detection of precipitated protein was avoided by a centrifugation step before incubation. The supernatant (S) was separated from the Ni-NTA pellet (P), which contained the complex. The Ni-NTA pellet was washed, the complex was eluted by denaturation and subjected to SDS-PAGE. Ni-NTA beads only (-) and recombinant Pex19 (Pex), which does not have a TPR domain, were used as negative controls. To test precursor-chaperone complex pull down by TPRc1, the precursors of the chloroplast proteins Lhcb1, Toc33 and TPRc1 itself and the precursor of the mitochondrial protein Mito3 were taken.

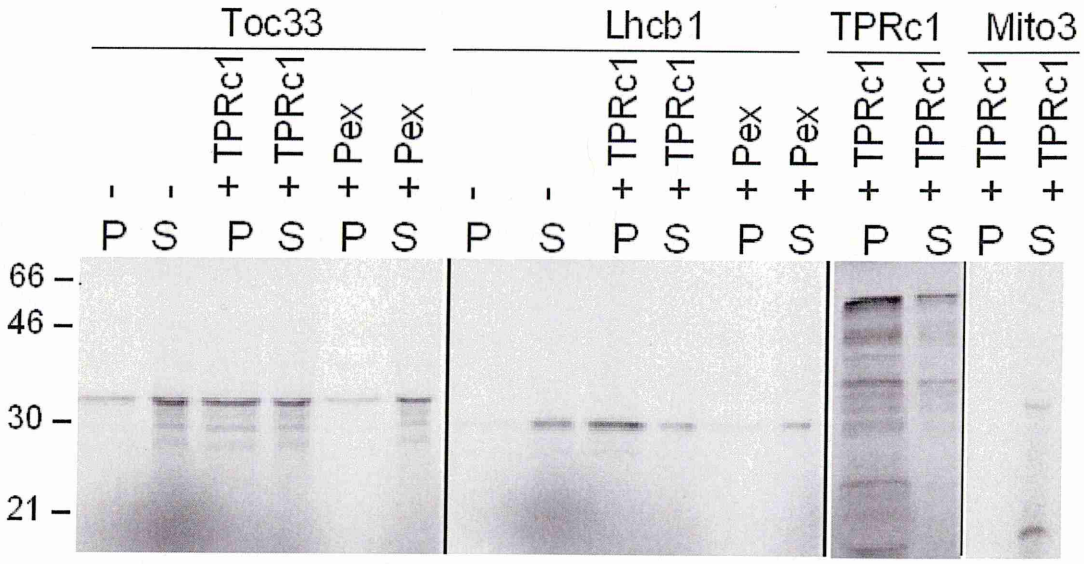


Figure 6.3 (Verena Kriechbaumer, unpublished): Pull down experiments of precursor-chaperone complexes by TPRc1-TM. Radioactive labelled Toc33, Lhcb1, TPRc1 and Mito3 precursors were translated in WGE and incubated with recombinant TPRc1-TM bound to Ni-beads (+ TPRc1). Recombinant Pex19 (Pex) or Ni-NTA beads only were used as negative controls. Shown are pulled down fractions (P) or protein remaining in the supernatant (S). Marker bands are given in kDa.

The precursor-chaperone complex of Lhcb1 remains nearly completely in the supernatant when added to the negative controls, while addition of recombinant TPRc1-TM effects in Lhcb1 being mainly in the pellet. This gives strong

evidence that TPRc1-TM interacts with the precursor-chaperone complex of Lhcb1. Comparison of P-S ratio after addition of recombinant TPRc1-TM with the negative controls shows that the precursor-chaperone complex of Toc33 also interacts with TPRc1. The majority of TPRc1 is as well interacting with recombinant TPRc1-TM. However, in this case it is not clear whether the pull down of TPRc1 is due to dimerisation of the recombinant TPRc1-TM with the radioactive labelled TPRc1 or due to interaction of the recombinant TPRc1-TM with the C-terminus of the chaperone. The precursor-chaperone complex of the mitochondrial protein Mito3 does not interact with recombinant TPRc1-TM, as there is no signal in the pellet for Mito3. This gives evidence that TPRc1 is able to bind chloroplast precursor-chaperone complexes but not mitochondrial precursor-chaperone complexes. The specificity for chloroplast precursor-chaperone complexes indicates that TPRc1 is a receptor involved in protein targeting to chloroplasts and might not only bind to the complex via its TPR domain but as well through another yet unknown domain.

6.4. Summary of interaction of TPRc1 in the cytosol

The described His-tag pulldown assays show, that TPRc1 interacts with the C-terminal end of Hsc70 specifically trough its TPR domain and that TPRc1 is able to pull down chloroplast precursor-chaperone complexes specifically. TPRc1 interaction with Hsp70 C-terminus is proven by the capability of the designed peptide to inhibit binding of Hsp70 competitively. However, pull down assays do not give information about the binding status of the chaperone i.e. whether the N-terminal domain of the chaperone needs to be occupied by a substrate to promote an interaction with TPRc1.

Another interesting question is, whether interaction of TPRc1 with the C-terminal end of Hsp70 has an influence on the chaperone ATPase activity e.g. inhibition of the ATPase activity since it could be shown that Sti1 (yeast Hop) is able to inhibit the ATPase activity of Hsp90 and its displacement by Cpr6, another TPR domain containing protein, reactivates the ATPase activity of Hsp90 (Prodromou *et al*, 1999).

The results from the pull down experiments with the chaperone-precursor complexes indicate that TPRc1 interacts specifically with a chloroplast-precursor, when the precursor form of a chloroplast protein is bound by the chaperone. As this work has been done in the absence of other receptor proteins such as Toc33 / 34 or Toc159, which are known to recognize the N-terminal transit peptide of chloroplast precursors, the specificity must be mediated by TPRc1, but could involve other cytosolic factors. One possibility might be a varying affinity for the Hsc70 isoforms by the different Hsc70 binding TPR domains, assuming that the Hsc70 isoforms bind precursor proteins of different organelles. In this case TPRc1 would specifically bind the Hsc70 isoform, which interacts with chloroplast precursors. Another possible reason for the specificity of TPRc1 is that TPRc1 contains another interacting domain inside the 34 kDa region between the TPR-domain and the tail anchor. This domain could interact with a part of the precursor protein which is exclusively present in chloroplast precursors. It could be shown for Toc64 that the recombinant protein without the TPR domain was able to inhibit precursor import of pSSU into pea chloroplasts, while in the case of pOE33 only the TPR domain of Toc64 was able to inhibit protein import (Qbadou *et al*, 2006). Thus, repeating these experiments with the constructs TPRc1TPR, TPRc1L and

TPRc1R185A might give more information about the reason for the selectivity of TPRc1.

Another question arising is where TPRc1 is located at the chloroplast membrane. Toc33 is a membrane protein of the chloroplast envelope and has been shown to be able to insert into liposomes without a membrane pore (Qbadou *et al*, 2003). Thus the receptor for Toc33 would not necessarily need to be at the chloroplast translocation core complex. Lhcb1, however, is part of the light harvesting complex, which is embedded in the thylakoid membrane, and thus needs to be transported trough the chloroplast envelope via a translocation pore. A receptor, recognizing the precursor of this protein would thus have to be part of the Toc complex. The interaction with one or more components of the Toc complex could be mediated either by the linking region between the TPR domain and the TM domain of TPRc1, or inside the membrane by the TM domain itself.

Toc64 is known to bind Hsp90 (Qbadou *et al*, 2006), and TPRc1 has been shown to interact with Hsc70. A precursor complex with Hsp90 has been shown to exist for pOE33 and Toc64 was shown to be incapable to pull down the guidance complex (Qbadou *et al*, 2006), consisting out of a chloroplast precursor bound by 14-3-3 proteins and Hsp70 (May *et al*, 2000). Thus, there seem to exist multiple pathways for protein targeting to chloroplasts in which TPRc1 and Toc64 may hold different functions.

Chapter 7 – Interaction partners of TPRc1 at the membrane

TPRc1 is abundant in all tissues and is localised to chloroplasts. The TPR domain of TPRc1 is facing the cytosol and has been shown to bind the C-terminus of Hsc70. Additionally, recombinant TPRc1-TM has been shown to be able to interact specifically with chloroplast precursor-chaperone complexes. Thus, TPRc1 has the characteristics of a chaperone receptor similar to Toc64. As a consequence, it could be a part of the translocon of the chloroplast envelope. To investigate this hypothesis, radioactive labelled TPRc1 was inserted into the chloroplast envelope, crosslinked with interacting proteins and immunoprecipitated with antibodies against TPRc1 and members of the Toc complex such as Toc33, Toc75 and Toc159.

7.1. Immunoprecipitation of TPRc1

Radiolabeled TPRc1 should be imported into chloroplasts and crosslinked with its interaction partners. To determine the single interaction partners, the ability of the anti TPRc1 antibody to function in immunoprecipitation needed to be tested. To investigate which fraction of the antibody is best for immuno-precipitation, all fractions received from Eurogentech were tested. Radiolabeled TPRc1 was translated *in vitro* in WGE and denatured with 1% SDS at 70°C. The protein was then incubated with the anti TPRc1 antibody and precipitated

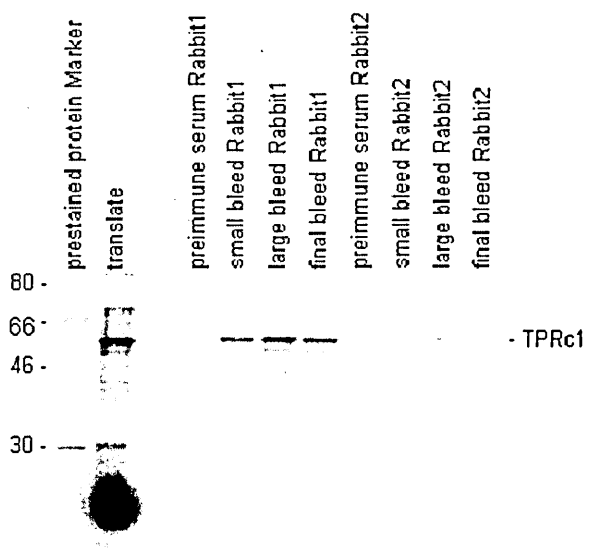


Figure 7.1: Immunoprecipitation of TPRc1 in denaturing conditions. The anti TPRc1 antibody from Eurogentech was tested for specificity in immunoprecipitation on S-^{35}Met radio-labelled in vitro translated TPRc1 in wheat germ extract. Preimmune serum, small, large and final bleeds from two different rabbits (Rabbit1 and Rabbit2) were tested. Marker bands are given in kDa.

by proteinA sepharose. The protein was eluted from the beads by denaturation and separated by SDS-PAGE. The dried protein gel was exposed to a phosphor screen and radio-labelled TPRc1 was visualised with a phosphoimager.

Figure 7.1 shows immunoprecipitated TPRc1 by the different bleeds. Immunoprecipitates with the small bleed, large bleed and final bleed from two different rabbits, Rabbit1 and Rabbit2, are compared to immunoprecipitates with preimmune sera. In both cases the preimmune serum does not interact with the protein. In the case of Rabbit1, all bleeds immunoprecipitate TPRc1 efficiently, since a very strong TPRc1 band can be seen in these fractions (between 10% and 25% of the input). The antibody from Rabbit2 is however far weaker and less sensitive: very faint bands of TPRc1 are visible for large and final bleeds from Rabbit2 and the small bleed from Rabbit2 does not precipitate TPRc1. As the large bleed from Rabbit1 binds most strongly to TPRc1 this bleed was taken for further experiments.

7.2. Cross linking and immunoprecipitation of TPRc1

After verification of the antibody to be suitable for immunoprecipitation, crosslinking of TPRc1 with other membrane proteins at the chloroplast outer envelope followed by immunoprecipitation was performed. Therefore, radiolabeled TPRc1 was imported into isolated pea chloroplasts. Supernatants (SN) were separated and the chloroplast pellets (P) were chemically crosslinked. The chloroplast pellets were denaturated after crosslinking and immunoprecipitated with antibodies against TPRc1, Toc33, Toc75 and Toc159.

Therefore, the efficiency of the crosslinkers bismaleimidohexane (BMH), Disuccinimidyl suberate (DSS) and succinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate (SMCC) was tested. BMH cross-links thiol groups from cysteines, DSS cross-links primary amino groups of lysine, arginine and accessible N-termini of proteins and SMCC cross-links a primary amino groups with a thiol groups. Thus, it was tested, which of the different crosslinkers is most efficient for crosslinking of TPRc1. Addition of DMSO only was used as negative control for crosslinking, because the crosslinkers were diluted in DMSO.

Figure 7.2 shows crosslinked supernatants and chloroplast pellets. Surprisingly, the radioactive signal for TPRc1 is in all pellet fractions approximately 10 kDa higher than the expected size (indicated by an asterix). This band at 70 kDa occurs also in the translate fraction and in the supernatant fractions next to the far stronger TPRc1 band at 60 kDa (indicated by a black arrow). This shift could already be observed in the import assays and might be mediated by hydrophobic interaction of the TMD of TPRc1 with membrane molecules, which might result slower migration of the protein inside the gel. Thus, this band might represent solely TPRc1, which migrates more slowly on the gel. As a consequence, no crosslinking adducts can be seen in the gel.

Since the radioactive signal is very weak in the pellet fractions, it is possible that the import of TPRc1 into the chloroplast envelope was not efficient enough for this experiment. The reason may be an insufficient amount of TPRc1 or unfolding of the protein, since the signal of TPRc1 in supernatants and pellets are not as strong as the signal of 10 % fresh translated TPRc1 taken for the assay. As a consequence, potential adducts would be difficult to see.
It is also possible that an insufficient amount of crosslinker was taken for the assay, as there is no crosslinking product bigger than 60 kDa visible in the supernatant fractions as well. The poor import and no crosslinking products of TPRc1 may thus be the reason for no immunoprecipitated TPRc1 by any of the

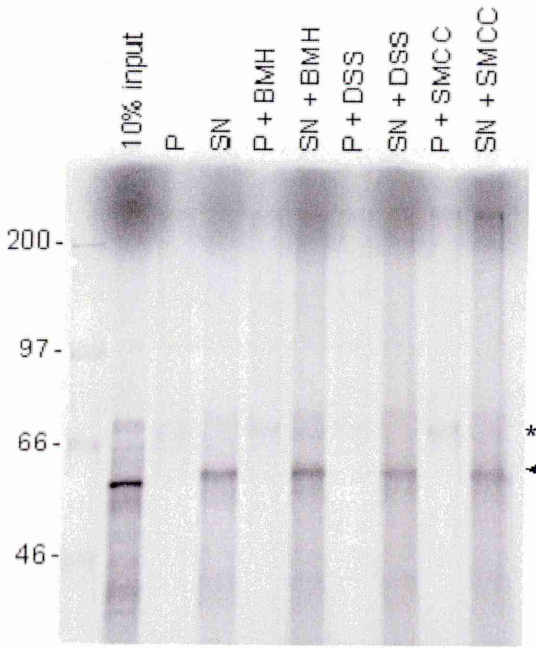


Figure 7.2: Radiolabeled TPRc1 was imported into the chloroplast envelope and crosslinked with BMH, DSS or SMCC. The gel shows 10% input of the fresh translate and supernatants (SN) and chloroplast pellets (P) after crosslinking. Marker bands are given in kDa. TPRc1 migrating at 60 kDA is indicated by a black arrow, putative TPRc1 migrating at ca 70 kDA is indicated by an asterix (*)

antibodies taken (data not shown). It is likely that the amount of protein was under the detection limit after immunoprecipitation. Thus, when the experiment is repeated, a greater amount of material should be used.

7.3. Summary of interaction partners at the membrane

Crosslinking and immunoprecipitation of radiolabeled TPRc1 should give information about TPRc1 being a member of the Toc complex. In this experiment crosslinking products with TPRc1 were not observed. Thus, the current result show the lack of any major crosslinking partner of TPRc1 and suggests, that TPRc1 is isolated in the membrane. However, efficiencies are sometimes low (e.g. (Abell *et al*, 2007)) and crosslinking products might be identified with increased scales, either of the crosslinker or the material used for crosslinking. Thus, this experiment would need to be repeated with more material and after optimising crosslinking conditions.

If it is not possible to get crosslinking adducts with radiolabeled TPRc1, which it is not importing into chloroplasts as well as Toc33 (Figure 5.2A), the experiment should be repeated with radiolabeled Toc33 imported into chloroplasts. To perform this, it would be necessary to test whether TPRc1 is present in pea and whether the anti TPRc1 antibody is able to detect pea TPRc1. Additionally, crosslinking for Toc33 would need to be optimised.
Sohrt et al. (2000) crosslinked the isolated Toc complex from pea chloroplasts. The unspecific crosslinker $CuCl_2$, which oxidises thiol groups, was most efficient in their experiment. Thus, the application of $CuCl_2$ might work better in this experiment.

Failure to obtain crosslinking products with a higher amount of imported protein and more material could be explained by the possibility that TPRc1 is similar to Toc64 and not in all conditions associated with the Toc core complex (Schleiff *et al*, 2003). Thus, it might only associate with the Toc core complex, when a precursor protein is present. Additionally, the existence of several proteins similar to the pore-forming Toc75 (Eckart *et al*, 2002) suggests, that there are several pathways for protein targeting. Thus TPRc1 might be part of another translocation complex than the known Toc complex.

Chapter 8 – Analysis of TPRc1 depleted plants

The functional importance of TPRc1 to the plant was examined with knockout mutations. It is possible to obtain commercially seeds of *Arabidopsis* plants, which have been transformed with an *Agrobacterium* T-DNA and crossed out until only 1-2 T-DNAs are inserted into the *Arabidopsis* genome, from the *Arabidopsis* Information Resource (TAIR). The insertion site of the T-DNA is roughly mapped by TAIR. Therefore, seeds from knockout lines, which have a T-DNA insertion inside the TPRc1 gene, were ordered from TAIR. Homozygous knockout mutants for TPRc1 were identified by genotyping and isolated. The homozygous mutants were grown under continuous light conditions on agar plates and on soil for phenotyping.

8.1. Genotyping TPRc1 mutant lines using PCR

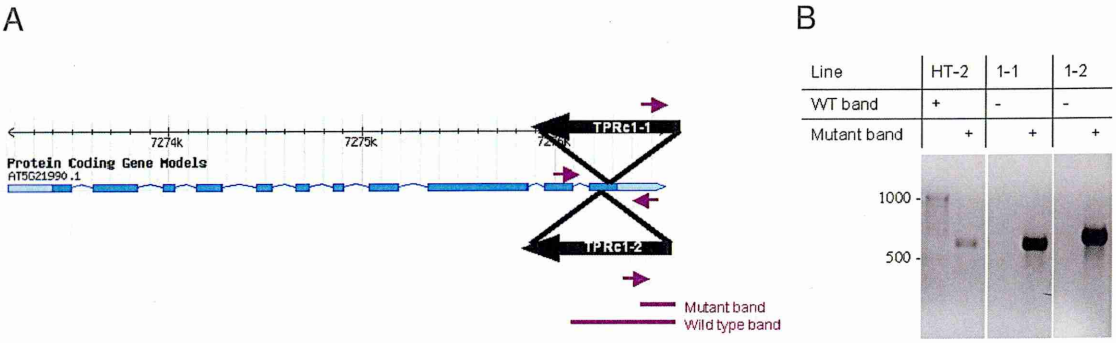


Figure 8.1: Genotyping of Salk lines TPRc1-1 and TPRc1-2.
A: Schematic Figure of the TPRc1 gene. Exons are indicated as thick blue lines. The coding region is shown in a darker blue than the non coding region. Black arrows indicate the insertion of the T-DNA, where it was annotated by TAIR. Violet arrows indicate the primers used for genotyping.
B: Genomic DNA was extracted from mutant lines and analysed for T-DNA insertion by PCR. Products were analysed on an agarose gel. 1-1 and 1-2 are homozygous mutants of TPRc1-1 and TPRc1-2, respectively, and HT-2 heterozygous for TPRc1-2. Marker bands are given in bp.

Two Salk lines with a T-DNA insertion in the last exon of the TPRc1 gene have been ordered from TAIR. These lines have been called TPRc1-1 and TPRc1-2. The theoretical position of the T-DNA insertion inside the TPRc1 gene is described in Figure 8.1A. PCR was used to identify homozygous lines. Primers upstream and downstream of the T-DNA insertion were used to amplify

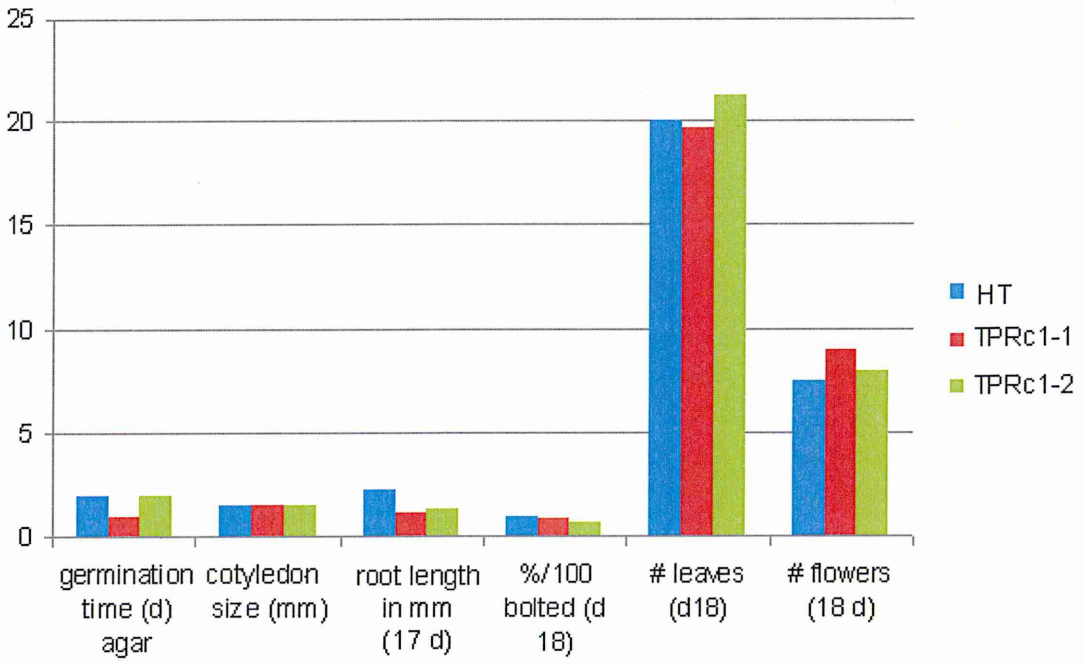
a wild type band and primers annealing inside the T-DNA insertion and downstream of the T-DNA insertion to amplify the mutant band. According to the PCR products TPRc1-1 and TPRc1-2 are homozygous, which is indicated by the lack of a band for wild type primers and a PCR product of the correct size (600 bp) for the mutant primers. The line HT-2 is heterozygous for TPRc1-2 (Figure 8.1B). The isolated wild type seeds for TPRc1-1 were not able to germinate, possibly because of fungal contamination. Thus only the collected seeds from the isolated heterozygous line for TPRc1-2 (HT-2) were used for comparison in phenotyping.

8.2. Phenotyping of mutants

The homozygous mutant lines TPRc1-1 and TPRc1-2 were compared to the heterozygous line HT-2. Therefore, 12 mutant plants of each genotyped line were grown in continuous light conditions on soil at 24°C for phenotyping. Additionally, 10 seeds from each line were sown on half-concentrated MS agar plates and analysed for root length. The mutant plants had no obviously visible phenotype. Thus, the overall shape and time needed for development were observed for phenotyping. Here, germination time, time of bolting, root length, cotyledon size, number of leaves and flowers of the plants were compared. As summarised in Figure 8.2A, there were no significant difference between the mutants and the heterozygous plant, when grown on soil. The roots of 17 to 20 days old mutant plants were significantly shorter than the heterozygous plants, when grown on agar plates (Figure 8.2B).

To confirm the effect of the mutations the mRNA and protein levels of the mutants were tested. Figure 8.3A shows, that the mRNA levels of TPRc1 in cauline leaves and rosette leaves were higher for the homozygous mutant lines TPRc1-1 and TPRc1-2 when compared to the heterozygous line HT-2. Western blotting detects approximately the same protein levels for TPRc1-1, TPRc1-2, the heterozygous HT-2 and in the wild type (Col-0) (Figure 8.3B). Additionally, it

A



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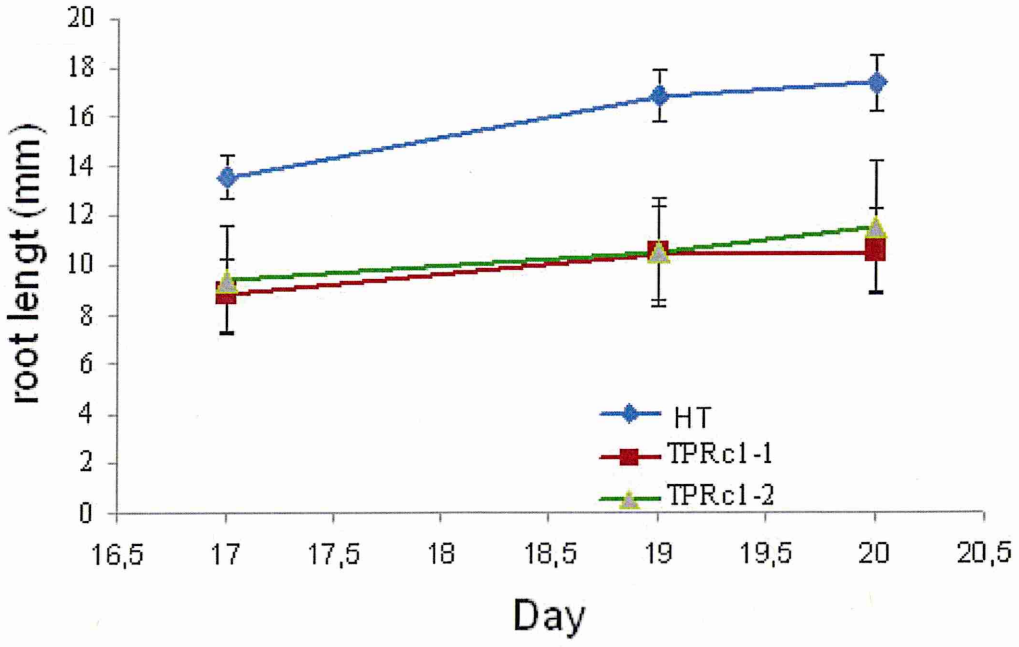
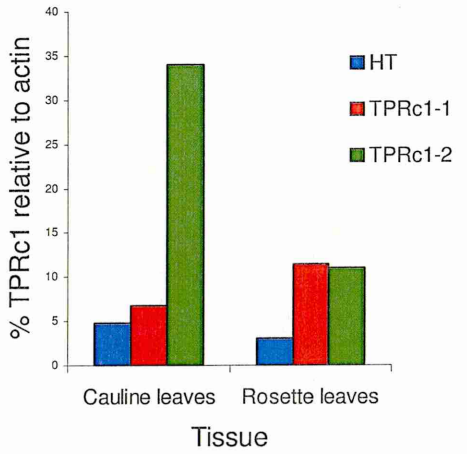


Figure 8.2: Phenotyping of TPRc1-1 and TPRc1-2.

A: TPRc1-1 and TPRc1-2 plants (n = 12) were compared to the heterozygous HT-2 plants (HT).

B: Root length of seedlings (n = 10) grown on ½ MS between day 17 and day 20.

A



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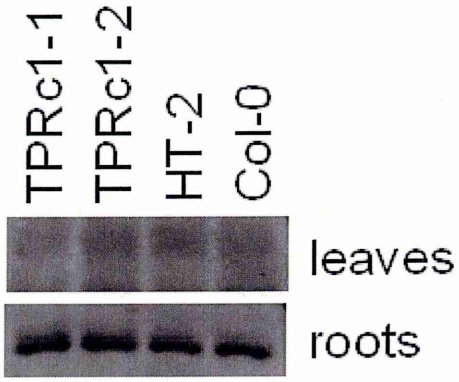

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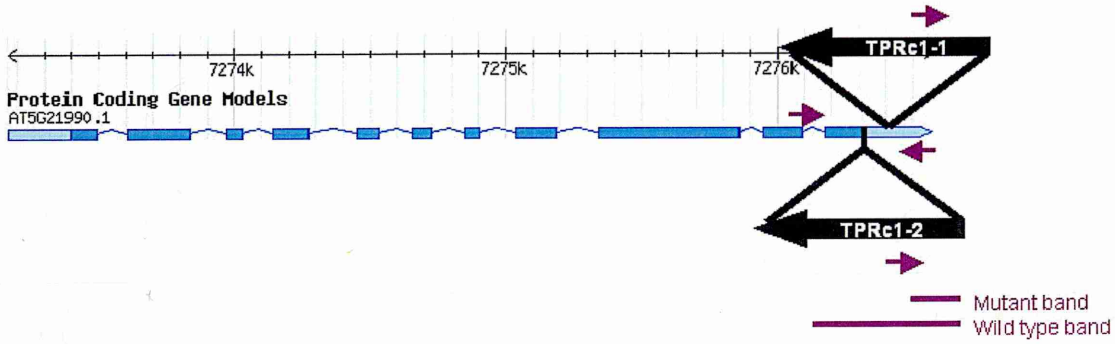

Figure 8.3: Expression of TPRc1 was tested with quantitative RT-PCR and immunoblotting in mutant plants, TPRc1-1 and TPRc1-2, and compared to the heterozygous line.

A: Transcription levels of TPRc1 in cauline (CL) and rosette (RL) leaves of TPRc1-1, TPRc1-2 and HT-2.

B: Immunoblot against TPRc1 from cauline (CL) and rosette (RL) leaves of TPRc1-1, TPRc1-2, HT-2 and the wild type Col-0.

C: Exact positions of the T-DNA insertion in the mutant lines TPRc1-1 and TPRc1-2.

does not seem that the protein is truncated in the mutant lines. This is confirmed by the detected TPRc1 protein in HT-2, as there should be a weaker band in comparison to the wild type or two different sizes of detected TPRc1, if the T-DNA insertion would result in mRNA degradation or the protein would be truncated by T-DNA interruption of the coding region, respectively. Thus, both appear to have normal levels of TPRc1 expression, and are therefore not suitable for functional analysis.

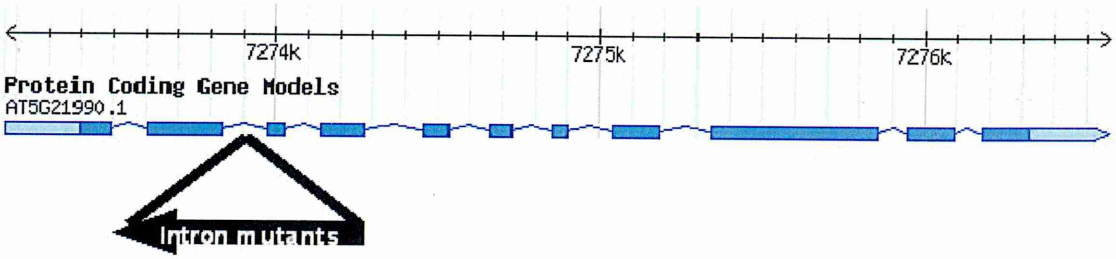

Figure 8.4: Intron mutants available from Salk. T-DNA insertions inside the gene of TPRc1 are located at the second intron without any exception.

PCR products form genotyping were sent for sequencing to determine the exact T-DNA insertion site in the gene. The exact position of the T-DNA insertion in the mutant lines is shown in Figure 8.3C. TPRc1-1 has a T-DNA insertion in the last exon of the gene after the translation stop codon and TPRc1-2 has a T-DNA insertion in the last exon in the translation stop codon. Together with the results obtained with quantitative RT-PCR and western blotting it can be concluded, that TPRc1 is still expressed in the lines TPRc1-1 and TPRc1-2. All remaining Salk lines, which can be ordered from TAIR have a T-DNA insertion in the second intron of the TPRc1 gene (Figure 8.4). As it is rather unlikely that one of these lines is TPRc1 depleted, alternative approaches for analysing TPRc1 knockouts like RNAi are necessary.

8.3. Summary of Analysis of mutant plants

Homozygous mutants from the two knockout lines TPRc1-1 and TPRc1-2 have been successfully isolated and genotyped. Comparison of TPRc1-1 and TPRc1-2 to the heterozygous mutant for TPRc1-2, HT-2, did not show any phenotype on soil. Sequencing of the PCR products from genotyping of the T-DNA insertion lines revealed, that the insertion is for both mutants in 3' untranslated region and western blotting showed no difference in the protein level or size between the mutants and the wild type. The observed difference in the root length between HT-2 and TPRc1-1 and TPRc1-2 on agar plates is thus not the result of a TPRc1 depletion. There exist no other TAIR mutants with a T-DNA insertion inside earlier exons of the TPRc1 gene, which would certainly disrupt the gene resulting in a knockout mutant. Thus, it has not been possible to obtain knockout mutants through TAIR. An alternative approach would be a knockdown of TPRc1 with RNA interference. This may address the question of whether TPRc1 existence is necessary for a healthy plant.

The similarity to Toc64 suggests that TPRc1 would share its role as a chaperone receptor at the chloroplast outer envelope with Toc64. Since Toc64 depleted plants have been shown to have no phenotype in *A. thaliana* as well as in *Physcomitrella pathens* (Aronsson *et al*, 2007; Rosenbaum Hofmann *et al*, 2005), it is possible, that a TPRc1 knockdown alone would not result in any phenotype as well. Thus, presuming, that there do not exist more proteins similar to Toc64 and TPRc1 at the chloroplast outer envelope, a silencing of TPRc1 in Toc64 depleted plants might result in a phenotype, which would give insight into the role of chaperone receptors for protein targeting to chloroplasts.

Chapter 9 – Discussion and Conclusion

Chaperones have been found to be part of the cytosolic precursor complex of organellar proteins, which are encoded by nuclear DNA (Abell *et al*, 2007; May *et al*, 2004; Young *et al*, 2003). Additionally, chaperone receptors recognising the chaperones of this complex can be found at each organelle (Schlegel *et al*, 2007). This suggests a role of chaperone receptors in protein targeting. The typical properties of chaperone receptors are a cytosolic chaperone binding TPR domain and a membrane association, which can be either mediated through one or more transmembrane domains or through a strong interaction with a membrane protein. For example Toc64 is anchored in the membrane of the chloroplast outer envelope trough transmembrane spans and Sec72 is associated with the ER membrane through interaction with Sec71. The binding of Hsp70/Hsp40 to the transmembrane domain of ER TA proteins (Abell *et al*, 2007; Rabu *et al*, 2008) and the absence of Sec72 in mammals and plants suggests that there may be an alternative chaperone receptor existing at the mammalian and plant ER membrane.

Searching for a membrane protein, which contains a TPR domain, TPRc1 was identified by bioinformatics as a TPR domain containing TA protein from *A. thaliana*. Thus, the aim of this study was to characterise this novel protein in terms of expression, localisation and topology, function and contribution in protein targeting.

Quantitative RT-PCR and western blotting revealed that TPRc1 is expressed in all tissues. Highest protein levels were detected in buds, flowers, siliques and roots. Its tail anchor was observed to insert into the chloroplast outer envelope, exposing the main part of the protein to the cytosol, including the TPR domain. Phylogeny of the TPR domain of TPRc1 predicts an interaction with Hsp90, and experimental data show that the TPR domain of TPRc1 pulls down Hsc70 from WGE due to interaction with the C-terminus of Hsc70. Additionally, TPRc1 is capable of specifically binding chloroplast precursor complexes in WGE. Thus, experimental evidence suggests, that TPRc1 has a similar role as Toc64 in protein targeting to chloroplasts. The identification of interaction partners of TPRc1 at the membrane with crosslinking and immunoprecipitation has not been successful, giving current evidence for TPRc1 being isolated at the membrane. Phenotyping of TPRc1 depleted plants has not been possible so far, because of the lack of available seeds from TAIR with a T-DNA insertion inside an exon of the TPRc1 gene.

9.1. TPRc1 is part of a novel protein family in plants

Phylogenetic comparison of the TPR domain of TPRc1 reveals only close relationships with TPR domains of other plant proteins and a similarity search for whole protein sequence of TPRc1 results in the same proteins, which all contain a tail anchor and a TPR region or a TPR domain. Thus TPRc1 is present in higher plants like rice and grape but also in the moss *Physcomitrella patens* and the green algae *Ostreococcus* and *C. reinhardtii*. In comparison to TPRc1 Toc64 has not been found in *C. reinhardtii* so far and contains still an active amidase domain in *P. patens* and *Ostreococcus* (Kalanon *et al*, 2008), indicating another function of Toc64 in these organisms. The fact that the Toc components Toc34 and Toc159 have several homologues in *A. thaliana* and *P. patens* but only single homologues in *C. reinhardtii* and *Ostreococcus* (Kalanon *et al*, 2008) suggests that they build the original Toc complex. If TPRc1 is involved in protein targeting, it seems to be a more archetypical chaperone receptor than Toc64 and TPRc1 might be associated with the archetypal Toc complex. Additionally, TPRc1 is detected in all tissues by quantitative RT-PCR and immunoblotting. Thus, its abundance in all tissues and the presence of homologues in a wide range of different other plants justifies the hypothesis for TPRc1 to have a general role in the cellular organisation of plants.

9.2. TPRc1 and atToc34 have similar expression levels

TPRc1 is localised at the chloroplast outer envelope. This can be demonstrated by co-fluorescence of YFP-TPRc1 with chlorophyll measured by confocal laser microscopy and also in *in vitro* import assays of TPRc1 to mitochondria, the endoplasmic reticulum and chloroplasts, where TPRc1 inserts preferentially into chloroplasts. Its localisation is at the chloroplast envelope, because it is unprotected from protease treatment after the import similar to Toc33 and Toc34 (Gutensohn *et al*, 2000; Qbadou *et al*, 2003; Schleiff *et al*, 2001). Thus, it is surprising, that the highest protein level of TPRc1 is in roots, flowers, buds and siliques and not in the green tissues, while the other chaperone receptor at the chloroplast outer envelope, Toc64-III, was shown to be expressed in equal amounts in roots, flowers and leaves (Aronsson *et al*,

2007) or to have higher expression levels in leaves, than in roots (Vojta *et al*, 2004). Gutensohn et al. (2000) measured the mRNA levels of the two Toc34 homologues in *Arabidopsis*, atToc33 and atToc34. Here, it is shown, that atToc34 is similarly distributed as the TPRc1 protein and the protein and mRNA levels for atToc33 and atToc34 in rosette leaves of adult plants is very low (Gutensohn *et al*, 2000). In the case of the different distributions of atToc33 and atToc34 the opinion differs, whether they bind different classes of chloroplast precursor proteins and are thus both important all over the plant (Gutensohn *et al*, 2000; Jelic *et al*, 2003) or the Toc complex might contain different receptors dependent on the tissue (Yu *et al*, 2001). A varying specificity of precursor import into root and leave plastids (Yan *et al*, 2006) suggests that the composition of the import machinery changes in different tissues. Thus, this might be an explanation for the existence of TPRc1 and Toc64-III at the chloroplasts outer envelope. Here, Toc64-III might be associated with atToc33, and TPRc1 might interact with atToc34, since their expression pattern is similar. Interestingly, Toc64-V, which is localised at mitochondria has a similar distribution as TPRc1 (Aronsson *et al*, 2007).

9.3. TPRc1 does not associate with the Toc-core complex

An interaction of pea Toc64 with the Toc complex could be shown by the analysis of crosslinking products of proteins inside the outer envelope from pea chloroplasts (Sohrt *et al*, 2000). Here it was tested whether TPRc1 can be crosslinked with members of the Toc-core complex. Therefore, radiolabeled, to chloroplasts imported TPRc1 was crosslinked and resulting adducts should be immunoprecipitated with antibodies against members of the Toc core complex. Results presented here, however, showed that crosslinking of TPRc1 did not yield any adducts with TPRc1. Thus, TPRc1 adducts could not be immunoprecipitated, and no evidence exists that TPRc1 interacts with the Toc complex. This may reflect a lack of association with other proteins, or associations may be below the detection limit of experiments at this scale. Alternatively, import of another member of the Toc complex followed by crosslinking and immunoprecipitation with the anti TPRc1 antibody would be another way to test whether TPRc1 is a member of the Toc complex. A further

failure of crosslinking TPRc1 to any interaction partner at the chloroplast outer envelope would suggest that TPRc1 might only transiently associate with the Toc core complex upon ligand binding (Figure 9.3. C) as proposed for Toc64 (Schleiff *et al*, 2003). Alternatively, TPRc1 may be isolated in the membrane (Figure 9.3. A).

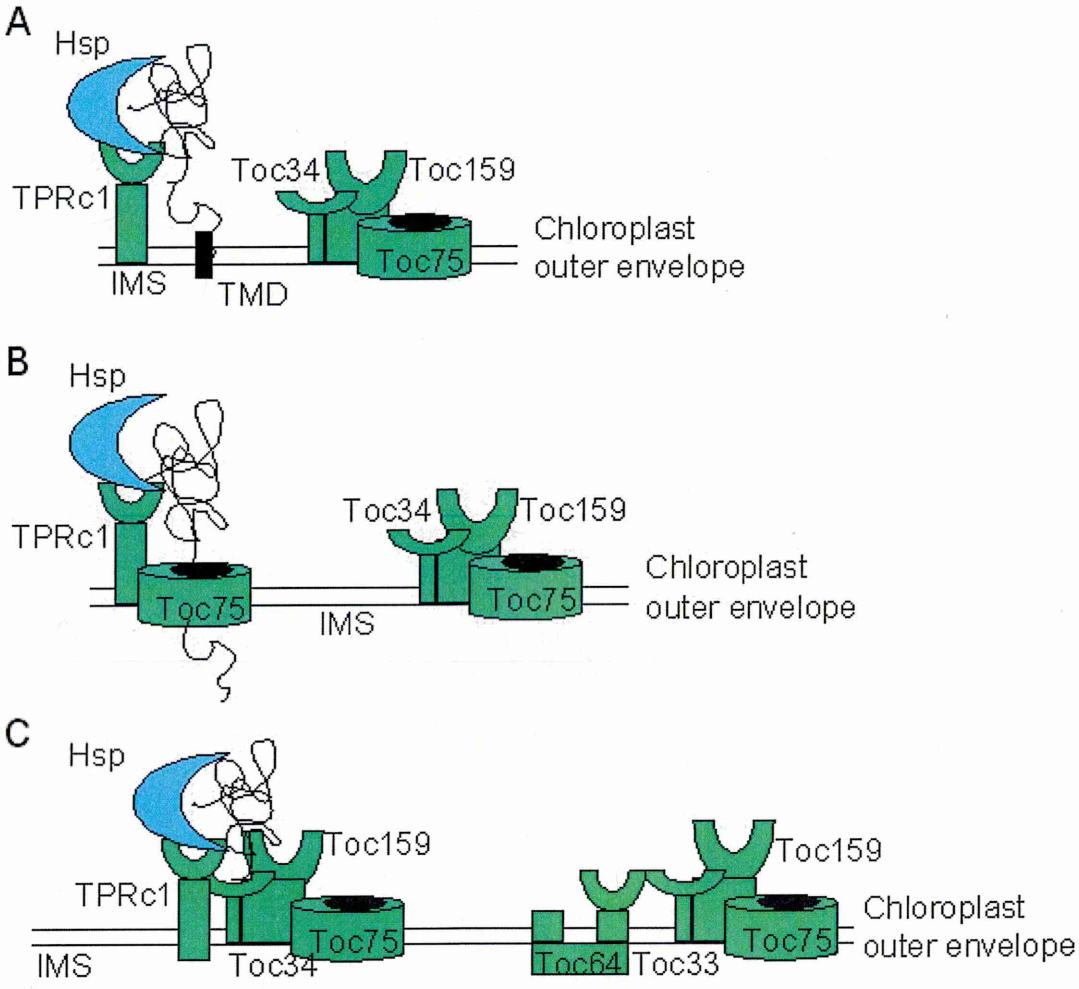

Figure 9.3: Possible ways for TPRc1 to function as a receptor:

A: TPRc1 is permanently isolated at the membrane. Here it could function as a receptor for outer membrane proteins only, which insert spontaneously into the membrane, when they are in close proximity.

B: TPRc1 is a receptor of an autonomous translocation complex

C: TPRc1 and Toc64 are receptors, which associate with the Toc complex upon ligand binding. AtToc33 is the interaction partner of atToc64-III and atToc34 is the interaction partner of TPRc1. (IMS: intermembrane space; Hsp: heat shock protein)

A greater amount of monomeric Toc75 and Toc34 has been found to be in non photosynthetic chloroplasts from roots, where highest TPRc1 levels are

detected (Kikuchi *et al*, 2006). Thus, it is possible, that an independent Toc75-TPRc1 complex exists (Figure 9.3. B), as speculated for Toc64 (Qbadou *et al*, 2006; Schleiff *et al*, 2003).

9.4. The TPR domain of TPRc1 binds the C-terminus of Hsc70

The localisation of TPRc1 at the chloroplast outer envelope exposing the soluble part of the protein including the TPR domain of TPRc1 towards the cytosol suggests a role of TPRc1 as a chaperone receptor in protein targeting of chloroplast precursors.

Hsp90 alone, and a guidance complex consisting out of a 14-3-3 protein and a Hsc70 isoform, are until now the only known cytosolic components, thought to assist precursor docking to the Toc complex (May *et al*, 2000; Qbadou *et al*, 2006; reviewed in Agne *et al*, 2009). Thus, the involvement of a receptor containing a chaperone binding TPR domain, which assists as a first loose binding partner for the precursor at the Toc complex, is possible. Additionally, evidence for the TPR domain of TPRc1 to bind preferably the C-terminus of Hsc70 or Hsp90 might allow a conclusion on its substrate specificity dependent on its interaction partners in the cytosol.

Undirected phylogenetic comparisons of the TPR domain from TPRc1 with TPR domains from other proteins shows that the TPR domain from TPRc1 is part of an unknown protein family, which is similar to the chaperone binding TPR domains of PPIases and loosely related to the TPR domains of Tom34, Toc64 and Hip. The TPR domain of PPIases, Tom34 and Toc64 are known to prefer binding of Hsp90 (Carrello *et al*, 2004; Chen *et al*, 1999; Chewawiwat *et al*, 1999; Qbadou *et al*, 2006; Young *et al*, 2003) while the TPR domain of human Hip is known to bind Hsc70 (Young *et al*, 2004). Assuming that the binding properties of Hip have the same specificity for Hsc70 in *Arabidopsis*, it can be concluded, that both kinds of chaperone binding TPR domains occur in the tree. Thus, undirected comparison of the TPR domain from TPRc1 shows a phylogenetic relation with both types of chaperone binding TPR domains and the TPR domain of TPRc1 is the closest related to the Hsp90 binding TPR domains from PPIases (Kang *et al*, 2008). Since the relation is based on

comparison of the primary protein structure, whether TPR domain of TPRc1 binds Hsp90 or Hsc70 can not be clearly predicted, but Hsp90 binding is more likely from these comparisons. Additionally, selective comparison of TPRc1 with the three TPR domains from Hop and the TPR domains from the chaperone receptors Toc64-III and Toc64-V indicates that the TPRc1 TPR domain is more similar to the Toc64 TPR domain than to any of the well characterised Hop TPR domains. The relation of the TPR domain from TPRc1 is equal to mtOM64 and Toc64 in the phylogenetic tree. This indicates that TPRc1 and Toc64-III both interact with Hsp90. As a contradiction, experimental evidence suggests an interaction of the TPR domain from TPRc1 with the C-terminal end of Hsc70, but interaction with Hsp90 was not observed.

TPR domains, which are similar to TPR2A, have next to their binding of Hsp90 a loose affinity to Hsc70 (Scheufler *et al*, 2000). This was also observed for Toc64-III, which is more similar to TPR2A than to TPR1 (Qbadou *et al*, 2006). The similarity between the TPR domains from Toc64-III and Toc64-V is 65.7% and the similarity of the TPR domain from TPRc1 to the Toc64-III and -V TPR domains is between 15% and 21.7%, respectively (Appendix III). This similarity is not sufficient to prove that TPRc1 binds also Hsp90. However, to confirm that TPRc1 only interacts with Hsc70, it needs to be verified that it is possible to detect plant Hsp90 with the anti human Hsp90 antibody used. Since a detection of Hsp90 bound by the positive control TPR2A failed, it might be possible that plant Hsp90 is either not recognized by the anti human Hsp90 antibody or that Hsp90 is missing in WGE. On the other hand, it could be shown that Hsp90 can be pulled down by TPR2A but not by TPRc1-TM from RRL (Verena Kriechbaumer and Susann Lehmann, unpublished data) suggesting that TPRc1 does not bind Hsp90 in WGE.

9.5. Hsc70 is likely to play an important role in targeting to chloroplasts

A knockdown of all cytosolic Hsp90 homologues in *Arabidopsis* has no influence on the phenotype of the plant (Sangster *et al*, 2007). Thus it is rather unlikely, that Hsp90 is essential for protein targeting to chloroplasts. As a consequence the guidance complex consisting out of a 14-3-3 protein and Hsc70 might have a more important role than the alternative Hsp90 assisted targeting pathway. Additionally, it has been shown that Hsc70 is very likely to be involved in various cytosolic complexes of precursor proteins (Abell *et al*, 2007; May *et al*, 2000). This correlates with the experimental evidence that TPRc1 pulls down Hsc70. In *Arabidopsis* the Hsp70 homologue atHsp70-1 (At5g02500) is the most likely candidate for a cytosolic complex, as it is constitutively expressed and relatively abundant (Lin *et al*, 2001).

9.6. Chaperones and their receptor might mediate specificity in protein targeting

A differentiation in the recognition of the presequence of chloroplast precursors by atToc33 or atToc34 can not be determined through the analysis of precursor levels in atToc33 depleted plants, because the difference in the signal sequences is not part of the binding site of atToc33 or atToc34 (Vojta *et al*, 2004). The down-regulated proteins are enriched in hydroxylated amino acids at the beginning of the sequence followed by hydrophobic residues, approximately 10 amino acids long (Vojta *et al*, 2004). It has been shown, that chaperones bind to the hydrophobic TMD of TA proteins (Abell *et al*, 2007). This 10 amino acids long feature in the signal sequence shows a feature similar to a tail anchor, which needs to be exposed to the cytosol for recognition and may easily aggregate if a binding partner does not increase its soluability. Thus, this hydrophobic part could be a binding site for Hsc70 or Hsp90. Hence, the differentiation could be mediated through the presence of a chaperone receptor, such as Toc64 or TPRc1, as an interaction partner of Toc33 or Toc34. Additionally, it was shown that Toc64 is incapable of binding to the guidance complex (Qbadou *et al*, 2006). Thus, it might be possible, that TPRc1 and Toc64 are both present as chaperone receptors at the chloroplast outer

envelope and have different affinities for the precursor complexes: while TPRc1 might prefer the guidance complexed precursors, Toc64 might bind the precursors, which are bound by Hsp90 (Qbadou *et al*, 2006). Figure 9.6 presents the proposed function of TPRc1 and Toc64 in early precursor protein recognition.

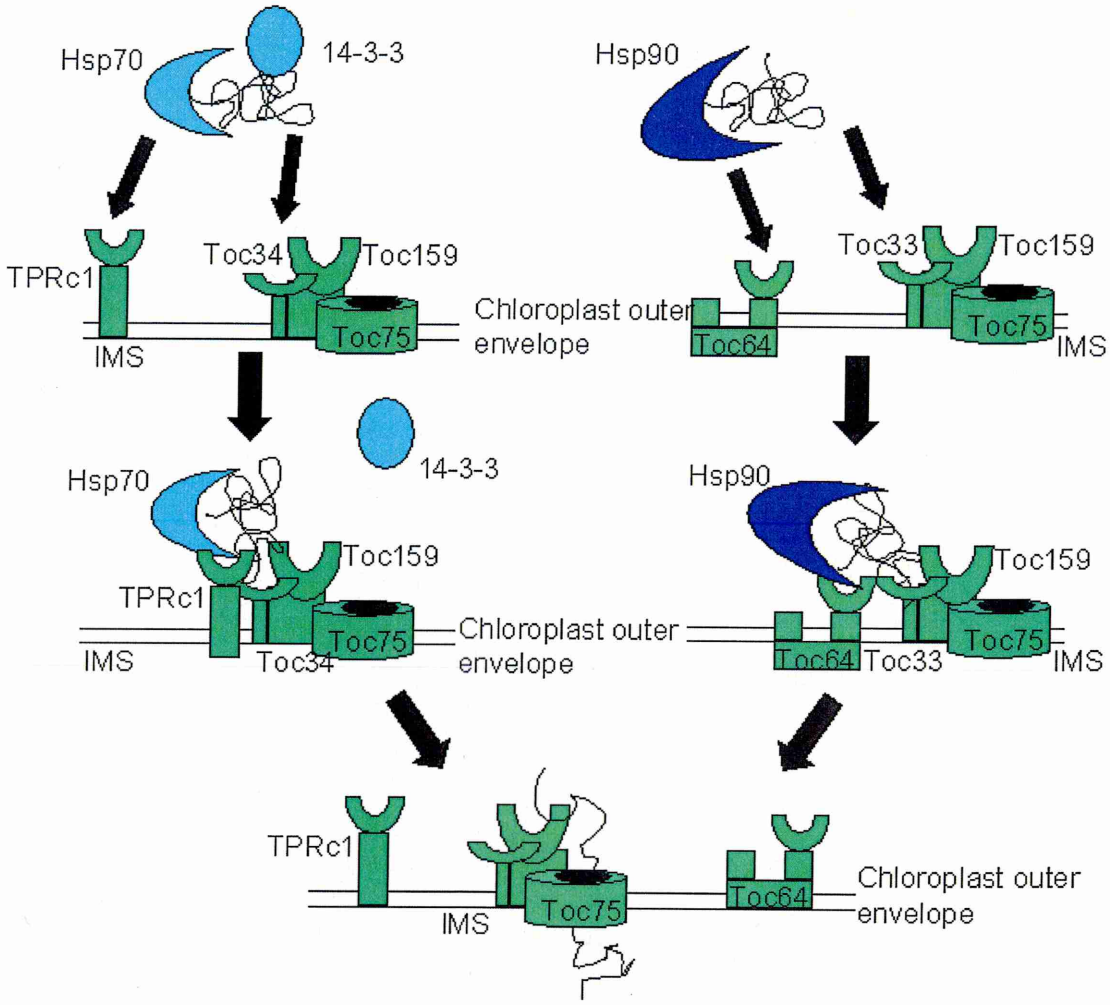

Figure 9.6: Proposed scheme of TPRc1 and atToc64 action: TPRc1 and atToc64 (Toc64) might be involved in the early recognition of the guidance complex and Hsp90-chloroplast precursor complex, respectively. Binding of the chaperone by the chaperone receptor might be followed by binding of signal sequence of the precursor by atToc33 (Toc33) or atToc34 (Toc34) and release of the 14-3-3 protein. The precursor is then handed over to Toc159, which initiates translocation through the Toc75 complex into the IMS. Release of the chaperone from the precursor might then be followed by dislocation of the chaperone receptor from the core-complex.

9.7. TPRc1 might not only interact Hsc70

Here, it is shown that TPRc1 is capable of pulling down radiolabeled, *in vitro* translated chloroplast precursors. The pull down of the precursor complex is only possible when the translation products are treated with apyrase, which hydrolyses ATP and thus stabilises the binding of chaperones to the precursor. Since Hsc70 but not Hsp90 is present in the guidance complex, and TPRc1 has been shown to interact with Hsc70, TPRc1 is a possible candidate for an interaction partner of the guidance complex. Additionally, it can be shown that the interaction with the chloroplast precursor complexes is specific, as the mitochondrial precursor complex (Mito3) is not pulled down by TPRc1-TM. This suggests, that TPRc1 does not only interact with the chaperone but also with the precursor protein or a cytosolic protein involved in targeting to chloroplasts. 14-3-3 proteins as part of the guidance complex are the only cytosolic components known to be specifically involved in protein targeting to chloroplasts. Thus, it should be tested whether the precursor-chaperone complexes, which could be pulled down by recombinant TPRc1-TM, contains as well a 14-3-3 protein and whether a 14-3-3 protein is important for interaction of TPRc1-TM with the precursor-complex.

9.8. Possible substrate specificity for chloroplast chaperone receptors

TPRc1 and Toc34 are distributed similarly in the plant and Toc34 was found to be involved in import of precursors, which contain a hydrophobic area before the signal peptide (Vojta *et al*, 2004). It could be shown that Toc34 does not bind to this hydrophobic area (Vojta *et al*, 2004) and hydrophobic areas provide a possible chaperone binding site (Abell *et al*, 2007). Thus, chaperone binding together with a chloroplast specific recognition could mediate the specificity for these precursors in a translocation complex. Therefore it would be also interesting to determine whether TPRc1 binds only the precursor-chaperone complexes containing a precursor with a hydrophobic area inside the signal sequence and not the precursor-chaperone complexes containing a precursor without a hydrophobic area (Vojta *et al*, 2004).

9.9. Possible redundancy between TPRc1 and Toc64

The knockout of Toc64-III as well as the triple knockout of the Toc64 amidase, Toc64-III and Toc64-V did not result in any phenotype in *A thaliana* and *P. patens* (Aronsson *et al*, 2007; Rosenbaum Hofmann *et al*, 2005). This might be due to redundancy between TPRc1 and Toc64 at the chloroplast outer envelope. Thus analysis of TPRc1 depleted plants as well as analysis of a Toc64-III/TPRc1 double knockout would be a possibility to investigate this hypothesis. Additionally, the generation of a TPRc1/Toc64 quadruple knockout would result in a mutant plant, which is depleted for all known chaperone receptors in *A. thaliana*. This could be a possibility to explore the general importance of chaperone receptors in protein targeting. However, genotyping of the lines, which have a T-DNA insertion inside an exon of the TPRc1 gene, revealed no knockout mutants. Thus, the analysis of the phenotype in TPRc1 depleted plants was not possible. As an alternative, a RNA interference based knockdown or in *Arabidopsis* plants induced overexpression of TPRc1-TM, which can not be targeted to chloroplasts due to its lack of a transmembrane domain and should result in a dominant negative effect, could be used. This would as well be an easier approach than to generate a quadruple mutant, which is TPRc1- and Toc64 - depleted, since TPRc1 and Toc64-V are located on the same chromosome and the likelihood of a crossing over event, which would generate a only T-DNA based quadruple knockout, is very low.

9.10. Remaining questions

Chaperones have been found to be present in many cytosolic precursor complexes (Abell *et al*, 2007; Rabu *et al*, 2008; Setoguchi *et al*, 2006; Young *et al*, 2004). Additionally, at least one chaperone receptor can be found at the outer envelope of every organelle (Schlegel *et al*, 2007) and were found to be at least temporary part of the main translocation complexes (High, 1995; Kalies *et al*, 1998; Sohrt *et al*, 2000; Young *et al*, 2003). Thus, it is rather surprising, that chaperone receptors have been in many cases found not to be essential. This could be explained in two different ways:

First, chaperone receptors are involved in an early stage of protein targeting. Here they might be an accelerating factor in recruitment of the precursor to the membrane, which eases protein targeting inside the cell, but is a dispensable mechanism.

Second, chaperone receptors play a central role in protein targeting and their currently found dispensability is only a result of redundancy. TPRc1 and Toc64 being present at the chloroplast outer envelope as possible candidates for a chaperone receptor for protein targeting to chloroplasts supports this hypothesis.

Additionally, a combination of different chaperones binding to the precursors might build the base for specificity. Thus, a broader understanding of TPRc1 would help to explain the role of chaperone receptors on protein targeting.

9.11. Conclusion and future work

Summarised, the obtained experimental results give evidence that TPRc1 is present in all tissues and localised at the chloroplast outer envelope, exposing its TPR domain towards the cytosol. Furthermore, the TPR domain of TPRc1 is able to bind the C-terminal end of Hsc70, and TPRc1 interacts specifically with chloroplast precursor complexes. Thus, based on current topological and functional data TPRc1 is proposed to be a chaperone receptor at the chloroplast outer envelope, which is involved in protein targeting. The existence of interaction partners of TPRc1 at the membrane needs to be further investigated to determine whether TPRc1 is part of a translocation complex. Additionally, the reason for the specificity of TPRc1 for chloroplast precursors bound by chaperones and the identity of this chaperone, as well as the existence of a 14-3-3 protein in this complex needs to be further investigated. Here, the use of TPRc1 truncations and variants might be a first step to explore whether a part other than the TPR domain is also interacting with the precursor complex. Ultimatively, analysis of knockout plants would show whether TPRc1 alone or in combination with another chaperone receptor results in a phenotype similar to Toc33 depleted plants, which have a pale phenotype and a deficiency for precursor import into chloroplasts (Jarvis *et al*, 1998), giving evidence for TPRc1 being essential in *A. thaliana*.

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a.a.	Amino acid
AcOH	Acetic acid
ADP	Adenosindiphosphat
ATP	Adenosintriphosphat
ATTDX	TPR domain containing thioredoxin
B	Buds
BLAST	Basic Local Alignment Search Tool
BMH	Bismaleimidohexane
BP	Binding protein
bp	Base pairs
BSA	Bovine serum albumine
C	Celsius
$CaCl_2$	Calcium chloride
C70	C-terminal domain of Hsp70
C90	C-terminal domain of Hsp90
CAM8	Calmoduline 8
cDNA	complementary DNA
CL	Cauline leaves
Col-0	Columbia 0
Cyp40	Cyclophillin 40
Cytb5	Cytochrome b 5
D	Day
Da	Dalton
DAS	Dense Alignment Surface
DEPC	Diethyl pyrocarbonate
DMSO	Dimethylsulfoxid
DNA	Desoxyribonucleic acid
DSS	Disuccinimidyl suberate
DRP	Disease related protein
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
F	Flowers
Fg	Femtogram
FK-506	Fermentek catalogue number 506
FKBP	FK-506 binding protein
G	Gram
G	Gravity force
H	Hour
H1	TPR1 of Hop
H2A	TPR2A of Hop
H2B	TPR2B of Hop
Hap	Hsp70 assotiated protein
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hip	Hsp70 interacting protein
His-tag	Histidine tag
Hop	Hsp70/Hsp90 organizing protein
HP	Hypothetical protein
Hsc70	Heat shock cognate protein 70

Hsp70	Heat shock protein 70
Hsp90	Heat shock protein 90
HT-2	Heterozygous for TPRc1-2
IgG	Immuneglobuline G
KAc	Potassium acetate
Kda	Kilo Dalton
KOAc	Potassium acetate
KOH	Potassium hydroxide
IPTG	Isopropyl β-D-1-thiogalactopyranoside
l	Liter
LB	Luria broth
Lhcb1	Light harvesting complex b1
MetOH	Methanol
µg	Microgram
Mg	Milligram
Min	Minute(s)
µL	Microliter
Ml	Mililiter
µM	Micromolar
Mm	Milimeter
MM	Millimolar
M	Molar
$MgCl_2$	Magnesium chloride
$Mg(OAc)_2$	Magnesium acetate
MnCl	Manganesium chloride
MOPS	3-(N-morpholino)propanesulfonic acid
MRNA	Messenger RNA
MS	Mushige and Skoog
NaCl	Sodium chloride
NaH_2PO_4	Monosodium phosphate
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
NEB	New England Biolabs
Ni-beads	Nickel-beads
Ni-NTA	Nickel-nitrotriacetic acid
NCBI	National Center for Biotechnology Information
Ni^{2+}	nickel in oxidation state +2
Ng	Nanogram
Nm	Nanometer
NM	Nanomolar
OD	Optical density
OEP	Outer envelope protein
P	Pellet
P	Decapeptide inhibitor
PAGE	Polyacrylamide gel electrophoresis
PAS	Pasticcino
Pasti	Pasticcino
PCR	Polymerase chain reaction
Pg	Picogram
PM	Picomolar
PP	Putative protein
PPIase	Peptidyl prolyl *cis/trans* isomerase

PPSUTOC	Putative subunit of Toc complex
PTPR	Putative Tetratricopeptide repeat containing protein
PVDF	Polyvinylidenfluorid
QRT-PCR	Quantitative real-time PCR
R	Roots
RbCl	Rubidium chloride
RL	Rosette leaves
RLS	Stacked rosette leaves
RNA	Ribonucleic acid
RRNA	Ribosomal RNA
RRL	Rabbit reticulolysate
RT-PCR	Real-time PCR
S	Stems
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SEC	Secret agent
SEM	Standard error of the means
SIP	Stress inducible protein
SMCC	Succinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate
SN	Supernatant
Sq	Siliques
SQN	Cyp40
SRP	Signal recognition particle
STPPase	serine/threonine protein phosphatase-related
TA	Tail anchor(ed)
TAIR	The Arabidopsis Information Resource
T-DNA	Transferred DNA
TFR	Transcription factor related
Tic	Translocalse of the inner chloroplast envelope
Tim	Translocalse of the inner mitochondrial membrane
Toc	Translocalse of the outer chloroplast envelope
TL	Thermolysine
Tom	Translocalse of the outer mitochondrial membrane
TM	Transmembrane
TMD	Transmembrane domain
TPR	Tetratricopeptide repeat
TPRc1FL	Full length TPRc1
TPRc1L	Linking region of TPRc1
TPRc1R185A	TPRc1 with R185A mutation
TPRc1-TM	TPRc1 lacking the TMD
TPRc1TPR	TPR of TPRc1
TPRCP	Tetratricopeptide repeat containing protein
TPRL	Tetratricopeptide repeat like
TPRP	Tetratricopeptide repeat containing protein
Tris	Tris(hydroxymethyl)aminomethane
tRNA	Transmitter RNA
TST	Tris - Salt – Tween
TTL	Tetratricopeptide repeat thioredoxin like
TTR	Tetratricoredoxin
U	Unit(s)
UP	Unknown protein

UPP	Unnamed protein product
WGE	Wheat germ lysate
w/v	Weight per volume
w/v	Weight per weight
YFP	Yellow fluorescent protein
%	Per cent

Appendix I

List of matching sequences from BLAST search against the TPR domain of TPRc1 without restrictions for organisms

Name	Organism	Accession number (BLAST)	Sequence
TPRc1TPR	*Arabidopsis thaliana*	emb|CAC34492.1| dbj|BAC43348.1| ref|NP_680187.2| GENE ID: 832259 AT5G21990	NAAQMLKKQGNELHSRGNFSDAAEKYLR AKNNLKEIPSSKGGAILLACSLNLMSCY LKTNQHEECIKEGSEVLGYDARNVKALYR RGQAYRDLGLFEDAVSDLSKAHEVSPE DETIADVLRDV
Vitis1	*Vitis vinifera* (unnamed protein product)	emb|CAO67415.1|	NAAQMLKKQGNELHNKGKFNEASQKYLL AKKNLTGIPASKGRTLLLACSLNLMSC YLKTKQYDECIQEGTEVLAYDPKNVKALY RRGQAYKELGQLNDAVSDLNKAYGVS PEDETIGEVLRDV
Vitis2	*Vitis vinifera* (hypothetical protein)	emb|CAN64899.1|	NAAQMLKKQGNELHNKGKFNEASQKYLL AKKNLTGIPASKGRTLLLACSLNLMSC YLKTKQYDECIQEGTEVLAYDPKNVKALY RRGQAYKELGQLNDAVSDLNKAYGVS PEDETIGEVLRSL
Oryza1	*Oryza sativa* (hypothetical protein)	ref|NP_001052858.1| emb|CAE02746.2| dbj|BAF14772.1| emb|CAH67576.1| gb|EAY94214.1| gb|EAZ02853.1| ref|NP_001058942.1| dbj|BAC79645.1| dbj|BAF20856.1| GENE ID: 4342455 Os07g0161000 gb|EAZ15931.1| GENE ID: 4335913 Os04g0437500	SGAKMLKQQGNELHRCEQYSEAAAKYKL AKDNLKSIPSQSAHSLQLVCTLNLMACY LKTRNFEECINEGSEVLTYDSSNVKAYYR RGQAYKELGNLEAAVGDLSKAHELSPDD ETIAAVLRD
Chla1	*Chlamydomonas reinhardtii* (predicted chloroplast-targeted protein)	ref|XP_001701985.1| gb|EDP06960.1| GENE ID: 5727502 TPR1	NASNQLKAEGNQLHNRGAFAEAAEKYER AKTNVASMAGKEAADLARACTLNLSSC YLNLKQFSKCLENCNSVLASEPSNLKALY RRGQAYMGTGSWLDASSDLERALKMAK EIDPSQAVPIRD
Ost1	Ostreococcus lucimarinus CCE9901 (TRP-containing protein)	ref|XP_001420268.1| gb|ABO98561.1| GENE ID: 5004336 TPR5a	GSETLKKEGNKLVGEGKHADAVEKYARV KENLKDDVNAAAKTLRLSCMLNMALCF NKIGKHDGAISECTEALELEPRSLKAYYR RGQAYVAKGELEQGVNDLMRANKLSPG D ETVAGEL
Ost2	*Ostreococcus tauri* HSP90 co-chaperone CPR7/Cyclophilin (ISS)	emb|CAL55701.1|	ASEKLKSEGNKLVGEGKHAEAIEKYARVK ANLSEDGSAEAKTLRVSCLLNSALCFNK IGKHGDAISECAAALELEPRSLKAYYRRG QALVAMGDLERGVEDLMRANKLSPGDET V
PPIase	*Rhizopus oryzae* (PPIase D)	P0C1I1|PPID_RHIOR	ATHLKDIGNTYFKKGDHANAAKKYLKAIR YLNEKPAFDENDPKELEGKFAAIKIPC YLNRSMCALKLGEYSECVKVTTTVLEYDS KYLKPTDITKAYFRRGSAKMNTRDFEG AIEDFEKAHEKDPEDAGI
Xenopus	*Xenopus laevis Xenopus tropicalis* (peptidylprolyl isomerase D)	ref|NP_001087854.1| gb|AAH82380.1| GENE ID: 447715 MGC81732 ref|NP_988984.1| gb|AAH61335.1| GENE ID: 394581 ppid	AENVKNIGNNFFKSQNWEMATKKYNKAL RYVESCKDVTGDDNISKLNPIAVSCN LNIAACKLKVSDFRAAIDSCNEALEIDPSH TKALYRRAQGWQGLKDYEQALEDLK KAHELSPDDKAVSSEILR
Danio	*Danio rerio* (peptidylprolyl isomerase D)	ref|NP_001002065.1| gb|AAH71388.1| GENE ID: 415155 ppid	SVAEDLKNIGNNFFKAQNWQSAIKKYSKA LRYLEMCGNIVDDDSSQKKLEPTALS CILNTAACKLKLKLWQEAIESCDEVLELNQ TNTKALFRRAQAWQGLKEFNKAMV DLKKAHEIAPEDKAIGN

Orni1	*Ornithorhynchus anatinus* (PREDICTED: similar to vomeronasal V1r-type receptor V1re25)	ref|XP_001510363.1| GENE ID: 100079388 LOC100079388	AEDIKNIGNTFFKSQNWELAIKKYSKVLRY VESSKAAAEDTSNLNPVALSCILNIAA CKLKMSNWQGAIESCIEALAIDPSNTKAL YRRAQGWQGIKEYDQALADLKKAQDI TPEDKAIQAETLR
Nema1	Nematostella vectensis (predicted protein)	ref|XP_001633810.1| gb|EDO41747.1| GENE ID: 5513522 NEMVEDRAFT_v1g103092	AEKLKVIGNEQFKQQKYEVAKKKYKKALR YLDEFQNSDMEDNAKKMAAIALPC YLNSAACKLKLAEYPSAIEDCNEALKLDA NSAKALFRRGQANEHMKDYEEAMV DLQKASKLAPGDKGIINEMSKV
Triticum1	Triticum aestivum (peptidylprolyl isomerase)	sp|Q43207|FKB70_WHEAT emb|CAA60505.1| GENE ID: 543314 FKBP70	AAGTKKEEGNALFKSGKYARASKRYEKA AKFIEYDTSFSEDEKKQSKQLKITCNL NNAACKLKLKDYKQAEKLCTKVLELDSRN VKALYRRAQAYTQLADLELAEVDI KKALEIDPENRDV
Equus1	*Equus caballus* (similar to cyclophilin, predicted)	ref|XP_001500384.1| GENE ID: 100061820 LOC100061820	LKNIGNTFFKSQNWEMAIKKYTKVLRYVE CSKAVIEKADGSRLQPVALSCVLNIG ACKLKMSNWQGAIDSCLEALKIDPSNTKA LYRRAQGWQGLKEYDQALADLKKA QEIAPEDKAIQAELLK
Pan1	*Pan troglodytes* (peptidylprolyl isomerase D isoform 1, predicted)	ref|XP_001145554.1| GENE ID: 737256 PPID ref|XP_001145793.1| GENE ID: 737256 PPID	LKNIGNTFFKSQNWEMAIKKYAKVLRYVD SSKAVIETADRAKLQPIALSCVLNIG ACKLKMSNWQGAIDSCLEALEVDPSNTK ALYRRAQGWQGLKEYDQALADLKK AQEIAPEDKAIQAELLK
Macaca1	*Macaca mulatta* *Macaca fascicularis* (similar to peptidylprolyl isomerase D)	ref|XP_001097233.1| GENE ID: 701347 LOC701347 dbj|BAE89174.1| ref|XP_001083759.1| GENE ID: 696657 LOC696657 ref|XP_001094929.1| GENE ID: 705500 LOC705500	LKNIGNTFFKSQNWEMAIKKYAKVLRYVD SSKAVIETADRAKLQPIALSCVLNIG ACKLKMSNWQGAIDSCLEALEIDPSNTKA LYRRAQGWQGLKEYDQALADLKKA QEIAPEDKAIQAELLK
Leish1	*Leishmania major strain Friedlin* (peptidyl-prolyl cis-trans isomerase)	ref|XP_843581.1| gb|AAZ14699.1| GENE ID: 3684757 LmjF35.4770	AGESIRQIGNSHFKNAAYDSAIEKYAKAV RYLNQVENKEGHPEVDEKLIACYN NHAMCAIKLQQWSEARHTASLALGVDAK NAKAFFRRGTAALNAGDADGAVE DLTQAHQIEPENAEITAKLNE
Tryp1	*Trypanosoma cruzi strain CL Brener* (40 kDa cyclophilin)	ref|XP_821542.1| gb|AAQ55216.1| gb|EAN99691.1| GENE ID: 3554440 Tc00.1047053506885.400 ref|XP_806069.1| gb|EAN84218.1| GENE ID: 3535980 Tc00.1047053503687.40	DVGEEIRQIGNKLFKASDFENAIQKYEKAA RFVKTINKTTANDVAVNEKLIACY NNTAACAIKLGQWSEARNAASRVLELDN SNAKALFRRGFASLSAGDSESAVAD FTKAQKLDPDNTEIVTVLQ
Oryza3	*Oryza sativa* (hypothetical)	gb|EAZ07707.1| gb|EAZ43401.1|	KEEGNALFKLGKYVRASKRYEKAAKFIEY DSSFSEDEKKQSKQLKVTCNLNNA ACKLKLKDYKQAEKLCTKVLELDSQNVKA LYRRAQAYMQLADLELAEVDIKK ALEIDPDNRQVLDV
Canis1	Canis *familiaris* (similar to peptidylprolyl isomerase D isoform 1)	ref|XP_532704.1| GENE ID: 475481 PPID	LKNIGNTFFKSQNWEMAIKKYTKVLRYVE SSKAVAEQADRLKLQPMALSCVL NIGACKLKMSNWQGAVDSCLEALEIDPS NTKALYRRAQGWQGLKEYDQALA DLKKAQEIAPEDKAIQAELLK
Equus2	*Equus caballus* (similar to TOM34)	ref|XP_001500576.1| GENE ID: 100070847 LOC100070847	ARVLKEEGNELVKKGNHKKAIEKYSESLS FSNLESATYSNRALCYLVLK QYKEAVKDCTEALRLNAKNVKAFYRRAQ AYKALKDYKSSLADISSLLQIEPKN
Arab2	Arabidopsis thaliana (peptidylprolyl isomerase)	dbj|BAE99990.1|	AAGKKKEEGNVLFKAGKYARASKRYERG VKYIEYDSTFDEEEKKKSKDLKIAC NLNDAACKLKLKDYKEAAKLSTKVLEMDS RNVKAMYRRAHAYLETADLDLA ELDIKKALEIDPDNKEV
Pan3	*Pan troglodytes* (peptidylprolyl isomerase D isoform 2)	ref|XP_001145721.1| GENE ID: 737256 PPID	LKNIGNTFFKSQNWEMAIKKYAKVLRYVD SSKAVIETADRAKLQPIALSCVLNIG ACKLKMSNWQGAIDSCLEALEVDPSNTK ALYRRAQGWQGLKEYDQALADLKK AQEIAPEDK
Arab3	Arabidopsis	ref|NP_199668.1|	AAGKKKEEGNVLFKAGKYARASKRYERG

	thaliana (peptidyl-prolyl cis-trans isomerase, putative / FK506-binding protein, putative)	dbj|BAB10690.1| GENE ID: 834913 AT5G48570	VKYIEYDSTFDEEEKKKSKDLKIACN LNDAACKLKLKDYKEAAKLSTKVLEMDSR NVKAMYRRAHAYLETADLDLAELD IKKALEIDPDNKEV
Bos1	*Bos taurus* (peptidylprolyl isomerase D)	ref|NP_776578.1| sp|P26882|PPID_BOVIN pdb|1IHG|A pdb|1IIP|A dbj|BAA03159.1| gb|AAI13319.1| gb|AAA30484.1| GENE ID: 281420 PPID	LKNIGNTFFKSQNWEMAIKKYTKVLRYVE GSRAAAEDADGAKLQPVALSCVLNIG ACKLKMSDWQGAVDSCLEALEIDPSNTK ALYRRAQGWQGLKEYDQALADLKKA QEIAPEDKAIQAELLK
Gallus1	*Gallus gallus (similar to cyclophilin)*	ref|XP_426283.2| GENE ID: 428725 PPID	LSCVLNIGACKLKLSDWQGAIESCSEALQI DPANTKALYRRAQGWQGIKDLDQA LADLKKAHEIAPEDKAI
Gallus2A Gallus2B	Gallus gallus (hypothetical, TOM34)	ref|XP_417366.2| GENE ID: 419188 TOMM34	AQTLKEEGNKLVKKGNHKKAIEKYSESLK LNQECATYTNRALCYLTLKQHKEA VQDCTEALRLDPKNVKAFYRRAQALKEL KDYKSSIADINSLLKIEPKNTAALRLLQEL SAGDLRRAGNEEFRRGQYGAAAELYSRA LAVLEDAGEAAAEERSVLLANRAAC QLRDGACRGCVADCCSALSLTPFAIKPLL RRAAAYEALESFALAYVDYKTALQV
Vitis3	*Vitis vinifera* (unnamed protein product)	emb|CAO40813.1|	CHLNMAACLIKLKRYEEAIGQCSIVLAEDE NNVKALFRRGKARAELGQTDAARE DFSKARKYAPEDKAISRELR
Mus1	*Mus musculus* (unnamed protein product)	dbj|BAC26192.1| GENE ID: 101869 Unc45a gb|EDL07004.1| GENE ID: 101869 Unc45a dbj|BAC33017.1| dbj|BAC33070.1| GENE ID: 101869 Unc45a sp|Q99KD5|UN45A_MOUSE dbj|BAE39037.1| GENE ID: 101869 Unc45a	SSAEQLRKEGNELFKCGDYEGALTAYTQ ALSLGATPQDQAILHRNRAACHLKLED YSKAESEASKAIGKDGGDVKALYRRSQA LEKLGRLDQAVLDLKRCVSLEPKNKV FQESLRNI
Homo1	*Homo sapiens* (peptidylprolyl isomerase D)	emb|CAG46878.1| gb|AAX36351.1| GENE ID: 5481 PPID ref|NP_005029.1| sp|Q08752|PPID_HUMAN gb|AAA35731.1| dbj|BAA09923.1| gb|AAH30707.1| gb|AAT97986.1| gb|EAX04853.1| gb|ABM82488.1| gb|ABM85675.1| GENE ID: 5481 PPID	LKNIGNTFFKSQNWEMAIKKYAEVLRYVD SSKAVIETADRAKLQPIALSCVLNI GACKLKMSNWQGAIDSCLEALELDPSNT KALYRRAQGWQGLKEYDQALADL KKAQGIAPEDKAIQAELLK
Tryp2	*Trypanosoma brucei TREU927* (peptidyl-prolyl cis-trans isomerase)	ref|XP_827280.1| gb|EAN76950.1| GENE ID: 3660669 Tb09.211.1350	AGEEIRQIGNNLFKGGDYENAMEKYAKVT RYLKAVNKTSANEGTINEMLIACH NNAAASAVKLSRWSDARNAATRVLDIDG SNVKALFRRGTACLGSGDPESAIA DLSKAKALDPQNTEVAAKLQ
Oryza4	*Oryza sativa* (putative 70 kDa peptidylprolyl isomerase)	ref|NP_001062292.1| dbj|BAD11570.1| dbj|BAF24206.1| GENE ID: 4346090 Os08g0525600	KEEGNALFKLGKYVRASKRYEKAAKFIEY DSSFSEDEKKQSKQLKVTCNLNNA ACKLKLKDYKQAEKLCTKVLELDSQNVKA LYRRAQAYMQLADLELAEVDIK KALEIDPDNRDV
Mus2	*Mus musculus* (hypothetical)	ref|XP_001473983.1| GENE ID: 100045251 LOC100045251 dbj|BAE29632.1| dbj|BAE30104.1| GENE ID: 67738 Ppid ref|NP_080628.1| sp|Q9CR16|PPID_MOUSE dbj|BAB22767.1| dbj|BAB29056.1| gb|AAH11499.1|	LKNIGNTFFKSQNWEMAIKKYAKVLRYVD SSKAVIEKADRSRLQPIALSCVLN IGACKLKMSNWQGAIDSCLEALEMDPSN TKALYRKAQGWQGLKEYDQALAD LKKAQEIAPGDKAIQAELLK

		gb|AAH19778.1| dbj|BAC34686.1| GENE ID: 67738 Ppid gb|EDL15462.1|	
Rattus1	*Rattus norvegicus* (peptidylprolyl isomerase D)	ref|NP_001004279.1| sp|Q6DGG0|PPID_RAT gb|AAH76386.1| GENE ID: 361967 Ppid ref|XP_576630.1| ref|XP_001057061.1| gb|EDL90962.1| GENE ID: 501204 RGD1560149_predicted gb|EDM00868.1|	LKNIGNTFFKSQNWEMAIKKYAKVLRYLD SSKAVIEKADVSRLQPIALSCVLNI GACKLKMSNWQGAIDSCLEALEMDPSNT KALYRKAQGWQGLKEYDQALAD LKKAQEIAPGDKAIQAELLK
Kluyv	*Kluyveromyces lactis* (unnamed protein product)	ref|XP_451972.1| emb|CAH02365.1| GENE ID: 2896870 KLLA0B09966g	ATNFKNQGNDLYKGKRFKDARAMYLKAL DVKCDVLSINESLYLNLAACELEI KNYRSCINYCREALKLNAKNVKAFFRIGK AYLELGRFEDSLEAVQVGLAVDPE NGALKSI
Oryza5	*Oryza sativa* (peptidyl-prolyl cis-trans isomerase, FKBP-type)	gb|EAY79939.1| gb|ABA91481.1| gb|EAZ17439.1| gb|EAY82248.1| gb|EAZ19631.1| ref|NP_001066146.1| gb|ABA96472.1| dbj|BAF29165.1| GENE ID: 4351491 Os12g0145500 ref|NP_001065747.1| dbj|BAF27592.1| GENE ID: 4349772 Os11g0148300	AADRRKIEGNEYFKEKKFEEAMQQYEMAI AYMGDDFMFQLFGKYRDMALAV KNPCHLNMAACLIKLKRFDEAIAQCSIVLA EDENNVKALFRRGKARAELGQTE SAREDFLKAKKHSPEDKEIQRELRSL
Leish2	*Leishmania braziliensis* (cyclophilin-40 putative)	ref|XP_001568571.1| emb|CAM43690.1| GENE ID: 5419533 LbrM34_V2.4730	AAEEIRQIGNSHFTSAAFDFAIDKYSKAVR YLNQVENKDAHPEVDKKLIACYN NSAMCAIKLERWSEARQTASLALSVDAK NAKALFRRGMAALSTGDADSAVED LTLAHQTEPENAEIAAKLSE
MonoA MonoB	*Monodelphis domestica* (hypothetical)	ref|XP_001379550.1| GENE ID: 100029920 LOC100029920	ARALKEEGNELVKKGKHKEAVEKYSESLT FSSLESATYTNRALCYLSLKKYKE AVKDCTEALKLDSKNIKAFYRRAQAFKEL EDYQSSLEDVNSLLSIEPENSAATK LRQEV LRVAGNESFRSGQYAEAAELYGRALDAL RETGPANPEEESVLYSNRAACHLKD GNCTHCIKDCSVALSLVPFGIKPLLRRAAA YEALEKYQLAYVD
Arab4	*Arabidopsis thaliana* (ROTAMASE FKBP 1)	dbj|BAB02082.1| gb|AAB82061.1| ref|NP_189160.3| sp|Q38931|FKB70_ARATH gb|AAB82062.1| GENE ID: 822117 ROF1	AASKKKEEGNSKFKGGKYSLASKRYEKA VKFIEYDTSFSEEEKKQAKALKVAC NLNDAACKLKLKDYKQAEKLCTKVLELES TNVKALYRRAQAYMELSDLDLAE FDVKKALEIDPNNREV
Aedes	*Aedes aegypti* (fk506-binding protein)	ref|XP_001654613.1| gb|EAT37524.1| GENE ID: 5573443 AaeL_AAEL010491 gb|ABF18224.1|	AKLFKEKGTGYFKENKFKLALKMYEKSLS FLSSSDSQESKQSQLAVYLNKALC YQKLNDHDEAKDACNEALNIDKKSVKALY RRGQSRLSLGDFEKALEDFNAVR EIEPENK
Bos2A Bos2B	Bos taurus (similar to translocase of outer mitochondrial membrane 34 isoform 3)	ref|XP_870544.2| GENE ID: 508142 LOC508142	ARALKEEGNELVKKGNHKQAIEKYSESL WFSNLESATYSNRALCHLELKQFQE AVKDCTEALRLDGKNVKAFYRRAQAYKA LKDFRSSFADIDSLLQIEPRN SVEELRTTGNQSFRNGQFAEAATLYSRA LRMLQEQGSSDPEKESVLYSNRAACH LKDGNCIDCIKDCTSALALVPFSLKPLLRR ASAYEALEKYPLAYVD
Pongo1	*Pongo pygmaeus* (UNC45 homolog A)	sp|Q5RAP0|UN45A_PONPY emb|CAH91170.1|	SSVEQLRKEGNELFKCGDYGGALAAYTQ ALGLDATPQDQAVLHRNRAACYLK LEDYDKAETEASKAIEKDGGDVKALYRRS QALEKLGRLDQAVLDLQRCVSLEP KNKVFQEALRNI
Oryza6	*Oryza sativa* (hypothetica)	ref|NP_001052535.1| gb|AAD29708.2|AF140495_1 emb|CAE05842.2| dbj|BAF14449.1| gb|EAZ30344.1| GENE ID: 4335556 Os04g0352400	AAAKKKDEGNVWFKMGKYAKASKRYEK AAKYIEYDSSFTDDEKKQSKALKV SCKLNNAACKLKLKEYREAEKLCTKVLEL ESTNVKALYRRTQAYIELADLELA ELDVKKALEIDPDNRDVKMVYK

		gb|EAY93685.1|	
Dros	*Drosophila melanogaster* (FKBP59)	ref|NP_524895.2| sp|Q9VL78|FKB59_DROME gb|AAF52818.1| gb|AAL13958.1| GENE ID: 47762 FKBP59 gb|AAF18387.1|AF163664_1	AKVYKEKGTNYFKKENWALAIKMYTKCK NILPTTVHTNEEVKKIKVATHSNIA LCHQKSNDHFEAKQECNEVLALDKNNVK ALYRRGQCNLTINELEDALEDFQK VIQLEPGNKAAAN
Homo2A Homo2B	*Homo sapiens* (hTOM34p)	gb|AAC64484.1| GENE ID: 10953 TOMM34 dbj|BAD96672.1| GENE ID: 10953 TOMM34 ref|NP_006800.2| ref|XP_001153344.1| ref|XP_514669.2| sp|Q15785|OM34_HUMAN emb|CAB89422.1| gb|AAH01763.1| gb|AAH07423.1| gb|AAH14907.1| emb|CAG33046.1| gb|AAV38811.1| gb|AAV38812.1| gb|AAX41275.1| dbj|BAF32949.1| gb|EAW75886.1| gb|ABM82077.1| gb|ABW03362.1| GENE ID: 10953 TOMM34	ARVLKEEGNELVKKGNHKKAIEKYSESLL CSNLESATYSNRALCYLVLKQYTE AVKDCTEALKLDGKNVKAFYRRAQAHKA LKDYKSSFADISNLLQIEPRN DSVEELRAAGNESFRNGQYAEASALYGR ALRVLQAQGSSDPEEESVLYSNRAA CHWKNGNCRDCIKDCTSALALVPFSIKPL LRRASAYEALEKYPMAYVD
Rattus2	*Rattus norvegicus* (isoform CRA_c)	gb|EDM08630.1| ref|NP_001032736.1| sp|Q32PZ3|UN45A_RAT gb|AAI07920.1| gb|EDM08629.1| GENE ID: 308759 Unc45a	SSAEELRKEGNELFKCGDYEGALTAYTQ ALSLGATPQDQAILHRNRAACHLKL EDYSKAESEASKAIEKDGGDVKALYRRS QALEKLGRLDQAVLDLKRCVSLEPK NKVFQESLRNI
Vitis4	*Vitis vinifera* (unnamed protein product)	emb|CAO15270.1| emb|CAO46497.1|	AAGKKKEEGNVLFKAGKYARASKRYEKA AKYIEYDSSFGEEEKKQAKTLKVT CNLNNAACKLKLKDYKEAEKLCTKVLDIQ SKNVKALYRRAQAYIHLADLDLA EFDIKKALEIDPDNRDV
Canis2	*Canis familiaris* (similar to smooth muscle cell associated protein-1 isoform 1)	ref|XP_545861.2| GENE ID: 488743 UNC45A ref|XP_850021.1| GENE ID: 488743 UNC45A	SAVEQLRKEGNELFKCGDYEGALTVYTQ ALGLGATPQDQAILHRNRAACHLK LEDYDKAETEASKAIEKDGGDVKALYRRS QALEKLGRLDQAVLDLQRCVSLE PKNKVFQEALRNI
Pongo2A Pongo2B	Pongo pygmaeus (translocase of outer mitochondrial membrane 34)	gb|ABO52985.1|	AKVLKEEGNELVKKGNHKKAIEKYSESLL CSNLESATYSNRALCYLVLKQYT EAVKDCTEALKLDGKNVKAFYRRAQAHK ALKDYKSSFADISNLLQIEPRN DCVEELRAAGNESFRNGQYAEASALYGR ALRVLQAQGSSDPEEESVLYSNRA ACHLKDGNCRDCIKDCTSALALVPFSIKPL LRRASAYEALEKYPMAYVDYKT VLQIDDSVTSALEGI
sPPIase	Synthetic construct	gb|AAX43155.1|	LKNIGNTFFKSQNWEMAIKKSAEVLRYVD SSKAVIETADRAKLQPIALSCVLN IGACKLKMSNWQGAIDSCLEALELDPSNT KALYRRAQGWQGLKEYDQALAD LKKAQGIAPEDKAIQAELLK
Triticum2	*Triticum aestivum* (peptidylprolyl isomerase)	emb|CAA68913.1| GENE ID: 543368 FKBP77	AASEKKDEGNAWFKMGKYAKASKRYEK AAKYIEYDSSFSEDEKKQSKAVKIS IKLNNAACKLKLKDYKEAEKICSKVLELES TNVKALYRRAQAYTELVDLELAE LDIKKALEIDPDNREV
Homo3	*Homo sapiens* (SMAP-1)	ref|NP_061141.2| sp|Q9H3U1.1|UN45A_HUMAN dbj|BAB20273.1| gb|AAH06214.1| gb|AAH37992.1| gb|EAX02129.1| gb|ABM83879.1| gb|ABM87199.1| GENE ID: 55898 UNC45A ref|NP_001034764.1| dbj|BAB20266.1| gb|AAH45635.1|	SSVEQLRKEGNELFKCGDYGGALAAYTQ ALGLDATPQDQAVLHRNRAACHLK LEDYDKAETEASKAIEKDGGDVKALYRRS QALEKLGRLDQAVLDLQRCVSLEP KNKVFQEALRNI

		gb|EAX02127.1| dbj|BAF84311.1| GENE ID: 55898 UNC45A	
Leish3	*Leishmania infantum* (cyclophilin-40)	ref|XP_001469283.1| emb|CAM72388.1| GENE ID: 5073380 LinJ35.4880	AGESIRQIGNSHFKNAAYDSAIEKYAKAV RYLNQVENKEVHPEVDEKLIACYNN HAMCAIKLQQWSEARHTASLALSVDAKN AKAFFRRGTAALKAGDADGAVEDL TQAHQIEPENAEITAKLSE
Macaca2	*Macaca mulatta* (smooth muscle cell associated protein-1 isoform 2)	ref|XP_001092829.1| GENE ID: 715579 LOC715579 ref|XP_001092253.1| GENE ID: 715579 LOC715579	SSVEQLRKEGNELFKCGDYEGALGAYTQ ALGLDATPQDQAILHRNRAACHLKL EDYDKAETEASKAIEKDGGDVKALYRRS QALEKLGRLDQAVLDLQRCVSLEPK NKVFQEALRNI
Tetra	*Tetrahymena thermophila SB210* (TPR Domain containing protein)	ref|XP_001016358.1| gb|EAR96113.1| GENE ID: 4507021 TTHERM_00128770	QTIKNQGNEFIKNKEYQNATYKYESGLKTI KNDQNSVFDEVQQSLLNNLSLAYL KNNQFAECIETATEALKSQPSNVKLLYRR AQAYSGTQEYEKAKSDLKEGLKLDP NN
Bos3	*Bos taurus* (hypothetical)	ref|NP_001092542.1| gb|AAI42512.1| GENE ID: 535692 MGC148594	SSVEQLRKDGNELFKCGDYEGALTAYTQ ALGLGATPQDQAILHRNRAACHLKL EDYEKAETEATKAIEKDGGDIKALYRRSQ ALEKLGRLDQAVLDLQRCVSLEPKN KVFQEALRNI
Canis3A Canis3B	Canis familiaris (similar to translocase of outer mitochondrial membrane 34)	ref|XP_534431.1| GENE ID: 477239 TOMM34	ARALKEEGNELVKKGNHKKAIEKYSESLS FSDMESATYSNRALCHLVLKQYKEA VKDCTEALRLDGKNVKAFYRRAQAYKAL KDYKSSFEDISSLLQLEPRN DSVEGLRAAGNQSFRNGQFAEAAGLYSR ALRALQAQGCSNPEEESILFSNRAACH LKDGNCRDCIKDCTSALALIPFSMKPLLR RASAYEALEKYPLAYVDYKTVLQIDD KVASALEGI
Bos4	*Bos taurus* (similar to FKBP51)	ref|XP_615814.3| GENE ID: 535704 LOC535704	AAIVKEKGTVYFKGGKYMQAVIQYGKIVS WLEMEYGLSEKESKASESFLLAAFL NLAMCYLKLREYTKAVECCDKALGLDSA NEKGLYRRGEAQLLMNEFESAKGDF EKVLEVNPQNK
Nema2	*Nematostella vectensis* (predicted)	ref|XP_001633358.1| gb|EDO41295.1| GENE ID: 5513055 NEMVEDRAFT_v1g242802	KEEGNELYVDGKYKDAAEKYAEALGCLE QLSIREKPGDEEWVKLDQMKIPFLLN FSQCKLLLGEYYEVIKHTSTVLEKDKDNV KALFRRAKAHKACWDPEEARSDFKR AAELDP

Appendix II

List of matching sequences from BLAST search against the TPR domain of TPRc1 restricted to *A. thaliana*

>TPRc1 AQMLKKQGNELHSRGNFSDAAEKYLRAKNN LKEIPSSKGGAILLACSLNLMSCYLKTNQHEECIKEGSEVLGYDARNVKALY RRGQAYRDLGLFED AVSDLSKAHEVSPED	At5g21990
>PPIase KEEGNVLFKAGKYARASKRYERGVKYIEYDSTFDEEEKKKSKDLKIACNLND AACKLKLKDYKEAAKLSTKVLEMDSRNVKAMYRRAHAYLETADLDLAELDI KKALEIDPDN	AT5G48570
> TPRCP1 KAEGNKLFVNGLYEEALSKYAFALELVQELPESIELRSICYLNRGVCFLKLGK CEETI KECTKALELNPTYNKALVRRAEAHEKLEHFEDAVTDLKKILELDP	AT4G30480 TPR containing protein
>UP1 KAEGNKLFVNGLYEEALSKYAFALELVQELPESIELRSICYLNRGVCFLKLGK CEETI KECTKALELNPAYNKALVRRAEAHEKLEHFEDAVTDLKKILELDP	unknown accession AY085050.1
> FKBP1 ASKKKEEGNSKFKGGKYSLASKRYEKAVKFIEYDTSFSEEEKKQAKALKVAC NLNDAACKLKLKDYKQAEKLCTKVLELESTNVKALYRRAQAYMELSDLDLA EFDVKKALEIDPNN	AT3G25230
> FK506BP ASKKKEEGNSKFKGGKYSLASKRYEKAVKFIEYDTSFSEEEKKQAKALKVAC NLNDAACKLKLKDYKQAEKLCTKVLELESTNVKALYRRAQAYMELSDLDLA EFDVKKALEIDPNN	accession AB026647.1
> FKBP62 ASKKKEEGNSKFKGGKYSLASKRYEKAVKFVEYDTSFSEEEKKQAKALKVA CNLNDAACKLKLKDYKQAEKLCTKVLELESTNVKALYRRAQAYMELSDLDL AEFDVKKALEIDPNN	accession U49453.1 Arabidopsis thaliana FK506 binding protein FKBP62 (rof1)
>PPIase2 KEEGNLLYKTQKYERAAKKYNKAAECIENGKFEGGDEKQVKALRVSCFLNG AACSLKLK NFLETIVLCSEVLDIEFQNVKALYRRAQSYIEVGDLISAEMDINRALEADPEN	AT1G58450
> FKBP AVKNPCHLNIAACLIKLKRYDEAIGHCNIVLTEEEKNPKALFRRGKAKAELGQ MDSARDDFRKAQKYAPDD	At3g21640
> PAPP5 AEEFKSQANEAFKGHKYSSAIDLYTKAIELNSNNAVYWANRAFAHTKLEEY GSAIQDASKAIEVDSRYSKGYYRRGAAYLAMGKFKDALKDFQQVKRLSPND	AT2G42810
> pasti1A ADKIRSTGNRLFKEGKFELAKAKYEKVLREFNHVNPQDEDEGKIFGDTRNML HLNVAACLLKMGEWRKSIETCNKVLEAKPGHVKGLYRRGMAYIAGGEYDD ARNDFN	pasticcino1A accession U77365.1
> pasti1 ADKIRSTGNRLFKEGKFELAKAKYEKVLREFNHVNPQDEDEGKIFGDTRNML HLNVAACLLKMGEWRKSIETCNKVLEAKPGHVKGLYRRGMAYIAGGEYDD ARNDFN	pasticcino1 accession AL132960.2
> pasti1D ADKIRSTGNRLFKEGKFELAKAKYEKVLREFNHVNPQDEDEGKIFGDTRNML HLNVAACLLKMGEWRKSIETCNKVLEAKPGHVKGLYRRGMAYIAGGEYDD ARNDFN	pasticcino1D accession U77366.1
> PAS1 ADKIRSTGNRLFKEGKFELAKAKYEKVLREFNHVNPQDEDEGKIFGDTRNML HLNVAACLLKMGEWRKSIETCNKVLEAKPGHVKGLYRRGMAYIAGGEYDD ARNDFN	AT3G54010

> TTL3A RTRGNELFSSGRFSEACVAYGDGLKQDDSNSVLYCNRAACWYKLGLWEKSV EDCNHALKSQPSYIKALLRRAASYGKLGRWEDAVKD	TETRATRICOPETIDE-REPEAT THIOREDOXIN-LIKE 3 A + B At2g42580
> TTL3B LKRMGNDMYRRGSFSEALSLYDRAILISPGNAAYRSNRAAALTALRRLGEA VKECLEAVRIDPSYSRAHQRLASLYLRLGEAENA	
> TTL4A RTRGNELFSSGRYSEASVAYGDGLKLDAFNSVLYCNRAACWFKLGMWEKSV DDCNQALRIQPSYTKALLRRAASYGKLGRWEDAVRD	TETRATRICOPETIDE-REPEAT THIOREDOXIN-LIKE 4 A + B AT3G58620
> TTL4B VKKAGNVMYRKGNYAEALALYDRAISLSPENPAYRSNRAAALAASGRLEEA VKECLEAVRCDPSYARAHQRLASLYLRLGEAENA	
>PPA RTRGNELFSSGRYSEASVAYGDGLKLDAFNSVLYCNRAACWFKLGMWEKSV DDCNQALRIQPSYTKALLRRAASYGKLGRWEDAVRD	putative protein A + B accession AL137082.1
>PPB VKKAGNVMYRKGNYAEALALYDRAISLSPENPAYRSNRAAALAASGRLEEA VKECLEAVRCDPSYARAHQRLASLYLRLGEAENA	
>STPPase KEQGNEFFKQKKFNEAIDCYSRSIALSPNAVTYANRAMAYLKIKRYREAEVD CTEALNLDDRYIKAYSRRATARKELGMIKEAKEDAEFALRLEPE	serine/threonine protein phosphatase-related At1g56440
>BP ALVVKLEGNSLFSSGDIAGAAEKYSEALSLCPMRSKKERVVLYSNRAQCHLL LQQPLVAISDATRALCLHNPVNRHAKSLWRRAQAYDMLGLAKESLLD	binding AT5G10200
>SQN VKAHGNEHFKKQDYKMALRKYRKALRYLDICWEKEGIDEETSTALRKTKSQI FTNSAACKLKFGDAKGALLDTEFAMRDEDNNVKALFRQGQAYMALNNVDA AAESLEKALQFEPND	cyp40 At2g15790
>HP KEQGNEFFKQKKFNEAIDCYSRSIALSPNAVTYANRAMAYLKIKRFAHCLFH WFYSFITVTLAEVDCTEALNLDDRYIKAYSRRATARKELGMIKEAKEDAEFAL RLEPE	hypophetical protein accession AC058785.8
>PB1 LKEEGNKLFQKRDYEGAMFRYDKAVKLLPRDHGDVAYLRTSMASCYMQMG LGEYPNAINECNLALEASPRFSKALLKRARCYEALNKLDFAFRDSRVVLNMEP EN	octicosapeptide/Phox/Bem1p AT2G25290
>TPRCP2 ATSAKNKGIDNQRQGQYADAIKWLSWAVILMDRAGDEAGSAEVLSTRASCY KEVGEYKKAVADCTKVLDHDKKNVTILVQRALLYESMEKYKLGAEDLRMV LKIDP	TPR containing protein At3g16760
>TTR AQSEKSKAMEAISDGRFDEAIEHLTKAVMLNPTSAILYATRASVFLKVKKPNA AIRDANVALQFNSDSAKGYKSRGMAKAMLGQWEEAAADL	tetratricoredoxin accession AY084415.1
>ATTDX AQSEKSKAMEAISDGRFDEAIEHLTKAVMLNPTSAILYATRASVFLKVKKPNA AIRDANVALQFNSDSAKGYKSRGMAKAMLGQWEEAAADL	TETRATICOPEPTIDE DOMAIN-CONTAINING THIOREDOXIN AT3G17880
>Hapl AQEAKGKAMEALSEGNFDEAIEHLTRAITLNPTSAIMYGNRASVYIKLKKPNA AIRDANAALEINPDSAKGYKSRGMARAMLGEWAEAAKDLHLASTI	HSP associated protein like accession AL021635.1
>UPP AQSEKSKAMEAISDGRFDEAIEHLTKAVMLNPTSAILYATRASVFLKVKKPNA AIRDANVALQFNSDSAKGYKSRGMAKAMLGQWEEAAADL	unnamed protein product accession AB019230.1
>ATHIP1 AQEAKGKAMEALSEGNFDEAIEHLTRAITLNPTSAIMYGNRASVYIKLKKPNA AIRDANAALEINPDSAKGYKSRGMARAMLGEWAEAAKDL	At4g22670
>AT4G22670 AQEAKGKAMEALSEGNFDEAIEHLTRAITLNPTSAIMYGNRASVYIKLKKPNA AIRDANAALEINPDSAKGYKSRGMARAMLGEWAEAAKDL	
>TPRP3 SCYKEVGEYKKAVADCTKVLDHDKKNVTILVQRALLYESMEKYKLGAEDLR	TPR containing protein AT3G16760

MVLKIDP	
> TPR4 LKEKGNEFFKAGNFLKAAALYTQAIKLDPSNATLYSNRAAAFLSLVKLSKA LADAETTIKLNPQWEKGYFRKGCVLEAMEKYEDALAAFEMALQYNPQ	TPR containing protein AT1G04190
> SEC NLAIIYKQQGNYSDAISCYNEVLRIDPLAADALVNRGNTYKEIGRVTEAIQDY MHAINFRP	transferase, transferring glycosyl groups AT3G04240
> MTOM64 MKEKGNAAYKGKQWNKAVNFYTEAIKLNGANATYYCNRAAAFLELCCFQQ AEQDCTKA MLIDKKNVKAYLRRGTARESLVRYKEAAADFRHALVLEPQN	AT5G09420
> TTL1 RARGNDLYKSERYTEASSAYAEGLRLDPCNAILYCNRAACWFKLGMWERSI EDCNQALRYQPSYTKPLLRRAASNSKMERWGAAVSD	TETRATRICOPETIDE-REPEAT THIOREDOXIN-LIKE 1 At1g53300
> ATTOC64-III NRAAAYLELGGFLQAEEDCTKAITLDKKNVKAYLRRGTAREMLGDCKGAIE DFRYALVLEPNN	AT3G17970
>UP2 CKEEANAYFLFDPEVIKGLYQRGLVYFDVPVYQDDRFKVSK	unknown protein AT1G35220
>TPRL LKAAGNEAFQSGRHTEAVEHYTAALACNVESRPFTAVCFCNRAAAYKALGQ FSD AIADCSLAIALDQNYSKAISRRATLFEMIRDYGQAASDMER	tetratricopeptide repeat like protein accession AJ344539.1
>HP2 CKEEANAYFLFDPEVIKGLYQRGLVYFDVPVYQDDRFKVSK	hypophetical protein accession AC069160.7
>DNAJA LKAAGNEAFQSGRHTEAVEHYTAALACNVESRPFTAVCFCNRAAAYKALGQ FSD AIADCSLAIALDQNYSKAISRRATLFEMIRDYGQAASDMER	DNAJA +B AT5G12430
> DNAJB RGNNAYKIGDLSRAEESYTQGIDSVPRIETSRNCLRALMLCYSNRAATRMALG RMREAIADCTMASSIDSNFLKVQVRAANCYLSLGEIEDA	
>UP3 LKRRGNHCFRSRDFDEALRLYSKALRVAPLDAIDGDKSLLASLFLNRANVLH NLGLLKESLRDCHRALRIDPYYAKAWYRRGKLNTLLGNYKDAFRDIT	unknown protein accession AC051630.4
>PP2A RGNNAYKIGDLSRAEESYTQGIDSVPRIETSRNCLRALMLCYSNRAATRMALG RMREAIADCTMASSIDSNFLKVQVRAANCYLSLGEIEDA	putative protein A + B + C At5g12430
>PP2B LPAAGNEAFQSGRHTEAVEHYTAALACNVESRPFTAVCFCNRAAAYKALGQ FSDAIADCSLAIALDQNYSKAISRRATLFEMIRDYGQAASDMER	
>PP2C KREGNKTLESSIPLAATIRELLRLKVLPSSSMSIALNLHLLFRIQLPAAGNEAFQ SGRHTEAVEHYTAALACNVESRPFTAVCFCNRAAAYKALGQFSDAIADCSLA	
>TPRP4 LKRRGNHCFRSRDFDEALRLYSKALRVAPLDAIDGDKSLLASLFLNRANVLH NLGLLKESLRDCHRALRIDPYYAKAWYRRGKLNTLLGNYKDAFRDIT	TPR containing protein AT1G33400
>HP3 CKEEANAYFLFDPEVIKGLYQRGLVYFDVPVYQD	hypothetical protein accession AC079605.13
>F23N19.10 KAKGNAAFSSGDFNSAVNHFTDAINLTPTNHVLFSNRSAAHASLNHYDEALS DAKKTVELKPDWGKGYSRLGAAHLGLNQFDEAVEAYSKGLEIDP	accession AC007190.4
>binding2 TAYAFQRELESAIADFTKAIQSNPAASEAWKRRGQARAALGEYVEAVEDLTK ALVFEPEHRKAIQELSIGLSIENTIECLYLRGSCYHAVGEYRDAVKDYDATVD V	AT4G37460
>PP TAYAFQRELESAIADFTKAIQSNPAASEAWKRRGQARAALGEYVEAVEDLTK ALVFEPEHRKAIQELSIGLSIENTIECLYLRGSCYHAVGEYRDAVKDYDATVD V	putative protein AT4g37460

>PTPRA KAKGNAAFSSGDFNSAVNHFTDAINLTPTNHVLFSNRSAAHASLNHYDEALS DAKKTVELKPDWGKGYSRLGAAHLGLNQFDEAVEAYSKGLEIDP	putative TPR-repeat protein A + B At1g62740
>PTPRB REKGNDFFKEQKYPDAVRHYTEAIKRNPKD- PRAYSNRAACYTKLGAMPEGLKDAEKCIELDPTFLKGYSRKGAVQFFMKEY DNAMETYQKGLEHDP	
>SIPA KAKGNAAFSSGDFNSAVNHFTDAINLTPTNHVLFSNRSAAHASLNHYDEALS DAKKTVELKPDWGKGYSRLGAAHLGLNQFDEAVEAYSKGLEIDP	stress-inducible protein, putative A + B At1g62740
>SIPB REKGNDFFKEQKYPDAVRHYTEAIKRNPKDPRAYSNRAACYTKLGAMPEGL KDAEKCIELDPTFLKGYSRKGAVQFFMKEYDNAMETYQKGLEHDP	
>UPA TAYAFQRELESAIADFTKAIQSNPAASEAWKRRGQARAALGEYVEAVEDLTK ALVFEP	unknown protein A + B At4g37460
>UPB EHRKAIQELSIGLSIENTIECLYLRGSCYHAVGEYRDAVKDYDATVDV	
>TPRCP3 KGNELFKSGRFQEACAAYGEGLDHDPRNSVLLCNRAACRSKLGQFDKSIEDC TAALSVRPGYGKARLRRADCNAKIEKWELAVGDYEILKKESPED	tetratricopeptide repeat (TPR)-containing protein At5g65160
>UP4 GHHFTVTSRHQDAAREYLEAYKLMPESPLINLCVGAALINLALGFRLKNRHE CLAQGFAFLYNNLRICSNSQEALYNVARAYQHVGLVTLAASYYEKVLAIYEK D	Unknown protein accession AC034257.3
>PSUOTOC MKEKGNAAYKGKQWNKAVNFYTEAIKLNGANATYYCNRAAAFLELCCFQQ AEQDCTKAMLIDKKNVKAYLRRGTARESLVRYKEAAA	putative subunit of TOC complex accession AL391712.1
>TFR GHHFTVTSRHQDAAREYLEAYKLMPESPLINLCVGAALINLALGFRLKNRHE CLAQGFAFLYNNLRICSNSQEALYNVARAYQHVGLVTLAASYYEKVLAIYEK D	transcription factor-related AT1G17680
>TPRCP4 FKEEGNECVRKGKKHYSEAIDCYTKAISQGVLSDSETSILFSNRSHVNLLLGN YRRALTDAEESMRLSPHNVKAVYRAAKASMSLDLLNEAKSYCEKGIENDP	tetratricopeptide repeat (TPR)-containing protein AT1G04130
>CAM8 ITEIDSDSNGTIEFAEFLNLMAKKLQESDAEEELKEAFKVFDKD	(CALMODULIN 8) At4g14640
>unknown GEYLHATGNFELAKEMYQKAIQGVRETKESMCSCNMNLKAVSL	accession AY084796.1
>DRP SIGNLINLQELYLSECSSLVELPSSIGNLINLKTLNLSEC	disease resistance protein accession AC073178.9
>TPRCP5 KAEGNKLFVNGLYEEALSKYAFALELVQELPESIELRSICYLNRGVCFLK	tetratricopeptide repeat (TPR)-containing protein AT4G30480
>DRP2 ELPSFGDAINLQKLLLRYCSNLVELPSSIGNAI	disease resistance protein At5g11250

Appendix III

Sequences of TPR domains from known chaperone receptors in *Arabidopsis*.

Protein	Gene
>TPRc1 AQMLKKQGNELHSRGNFSDAAEKYLRAKNNLKEIPSSKGGAILLACSLNLMSCYLKTNQ HEECIKEGSEVLGYDARNVKALYRRGQAYRDLGLFEDAVSDLSKAHEVSPED	At5g21990
>ATTOC64-I	AT1G08980
> F13N6 sssekeqgneffkqkkfneaidcysrsialspnavtyanramaylkikryreaevdctealnlddryikaysrratarkelgmik eakedaefalrlepes	AT1G56440
> MGL6 AEVLSTRASCYKEVGEYKKAVADCTKVLDHDKKNVTILVQRALLYESMEKYKLGAEDLR M VLKIDPGN	AT3G16760
>ATTOC64-III AEIAKEKGNQAFKEKLWQKAIGLYSEAIKLSDNNATYYSNRAAAYLELGGFLQAEEDCTK AITLDKKNVKAYLRRGTAREMLGDCKGAIEDFRYALVLEPNN	AT3G17970
> ATTOC64-V SEVMKEKGNAAYKGKQWNKAVNFYTEAIKLNGANATYYCNRAAAFLELCCFQQAEQDC TK AMLIDKKNVKAYLRRGTARESLVRYKEAAADFRHALVLEPQN	AT5G09420
>Pex5 advhivlgvlynlsrefdraitsfqtalqlkpndyslwnklgatqansvqsadaisayqqaldlkpnyvrawanmgisyanqg mykesipyyvralamnpka	AT5G56290
>At1hop1 ADEAKAKGNAAFSSGDFNSAVNHFTDAINLTPTNHVLFSNRSAAHASLNHYDEALSDAK K TVELKPDWGKGYSRLGAAHLGLNQFDEAVEAYSKGLEIDPSN	AT1G62740
>At1hop2A AQKEKELGNAAYKKKDFETAIQHYSTAMEIDDEDISYITNRAAVHLEMGKYDECIKDCDK AVERGRELRSDYKMVAKALTRKGTALGKMAKVSKDYEPVIQTyqkALTEHRNP	
>At1hop2B GDEEREKGNDFFKEQKYPDAVRHYTEAIKRNPKDPRAYSNRAACYTKLGAMPEGLKDA EKCIELDPTFLKGYSRKGAVQFFMKEYDNAMETYQKGLEHDPNN	
>At2hop1 AEEAKSKGNAAFSSGDYATAITHFTEAINLSPTNHILYSNRSASYASLHRYEEALSDAKKT IELKPDWSKGYSRLGAAFIGLSKFDEAVDSYKKGLEIDPSN	AT4G12400
>At2hop2A ALKEKGEGNVAYKKKDFGRAVEHYTKAMELDDEDISYLTNRAAVYLEMGKYEECIEDCD KAVERGRELRSDFKMIARALTRKGSALVKMARCSKDFEPAIETfqkALTEHRNP	
>At2hop2B AEEEREKGNGFFKEQKYPEAVKHYSEAIKRNPNDVRAYSNRAACYTKLGALPEGLKDA EKCIELDPSFTKGYSRKGAIQFFMKEYDKAMETYQEGLKHDPKN	
>At3hop1 AEEAKAKGNAAFSSGDFTTAINHFTEAIALAPTNHVLFSNRSAAHASLHQYAEALSDAKE TIKLKPYWPKGYSRLGAAHLGLNQFELAVTAYKKGLDVDPTN	AT1G12270
>At3hop2A AKKEKELGNAAYKKKDFETAIQHYSTAIEIDDEDISYLTNRAAVYLEMGKYNECIEDCNK AVERGRELRSDYKMVARALTRKGTALTKMAKCSKDYEPAIEAfqkALTEHRNP	

>At3hop2B
GDEEREKGNDFFKEQKYPEAIKHYTEAIKRNPNDHKAYSNRAASYTKLGAMPEGLKDAE
KCIELDPTFSKGYSRKAAVQFFLKEYDNAMETYQAGLEHDPSN

SeqA Name Len(aa) SeqB Name Len(aa) Score

1 TPRc1 111 2 ATTOC64-III 102 17
1 TPRc1 111 3 ATTOC64-V 102 22
1 TPRc1 111 4 F13N6 101 29
1 TPRc1 111 5 MGL6 68 20
2 ATTOC64-III 102 4 F13N6 101 49
2 ATTOC64-III 102 5 MGL6 68 38
3 ATTOC64-V 102 4 F13N6 101 43
3 ATTOC64-V 102 5 MGL6 68 36
4 F13N6 101 5 MGL6 68 26
2 ATTOC64-III 102 3 ATTOC64-V 102 67

CLUSTAL W (1.83) multiple sequence alignment

```
ATTOC64-III AEIAKEKGNQAFKEKLWQKAIGLYS------EAIKLSDNNATYYS---NRAAAYLELGGF 51
ATTOC64-V SEVMKEKGNAAYKGKQWNKAVNFYT------EAIKLNGANATYYC---NRAAAFLELCCF 51
TPRc1 AQMLKKQGNELHSRGNFSDAAEKYLRAKNNLKEIPSSKGGAILLACSLNLMSCYLKTNQH 60
      ::: *::** .. :..* * : * . .* . * :.:*: .

ATTOC64-III LQAEEDCTKAITLDKKNVKAYLRRGTAREMLGDCKGAIEDFRYALVLEPNN 102
ATTOC64-V   QQAEQDCTKAMLIDKKNVKAYLRRGTARESLVRYKEAAADFRHALVLEPQN 102
TPRc1       EECIKEGSEVLGYDARNVKALYRRGQAYRDLGLFEDAVSDLSKAHEVSPED 111
              :. :: ::.: * :**** *** * . * : * *: * :.*::
```

Alignment score between ATTOC64-III and ATTOC64-V: 67/102 *100% = 65.7%

Alignment score between TPRc1 and ATTOC64-III (estimated): (17/102 +17/111)/2 * 100% = 16%

Alignment score between TPRc1 and ATTOC64-V (estimated): (22/102 +22/111)/2 * 100% = 20.7%

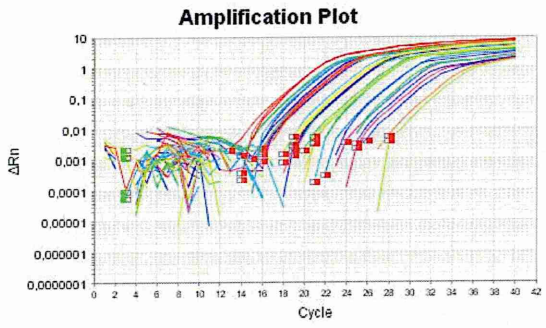
CLUSTAL W (1.83) multiple sequence alignment

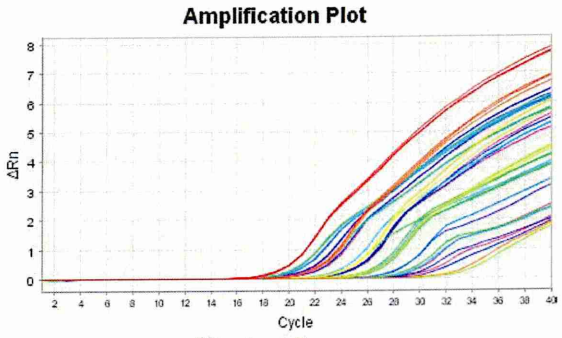
```
ATTOC64-III AEIAKEKGNQAFKEKLWQKAIGLYS------EAIKLSDNNATYYS---NRAAAYLELGGF 51
ATTOC64-V   SEVMKEKGNAAYKGKQWNKAVNFYT------EAIKLNGANATYYC---NRAAAFLELCCF 51
MGL6        ------------------------------------AEVLS---TRASCYKEVGEY 17
F13N6       SSSEKEQGNEFFKQKKFNEAIDCYS------RSIALS-PNAVTYA---NRAMAYLKIKRY 50
TPRc1       AQMLKKQGNELHSRGNFSDAAEKYLRAKNNLKEIPSSKGGAILLACSLNLMSCYLKTNQH 60
                                                          * . . .: : .

ATTOC64-III LQAEEDCTKAITLDKKNVKAYLRRGTAREMLGDCKGAIEDFRYALVLEPNN 102
ATTOC64-V   QQAEQDCTKAMLIDKKNVKAYLRRGTARESLVRYKEAAADFRHALVLEPQN 102
MGL6        KKAVADCTKVLDHDKKNVTILVQRALLYESMEKYKLGAEDLRMVLKIDPGN 68
F13N6       REAEVDCTEALNLDDRYIKAYSRRATARKELGMIKEAKEDAEFALRLEPES 101
TPRc1       EECIKEGSEVLGYDARNVKALYRRGQAYRDLGLFEDAVSDLSKAHEVSPED 111
              :. : ::.: * : :. :*. . : : . * . :.* .
```

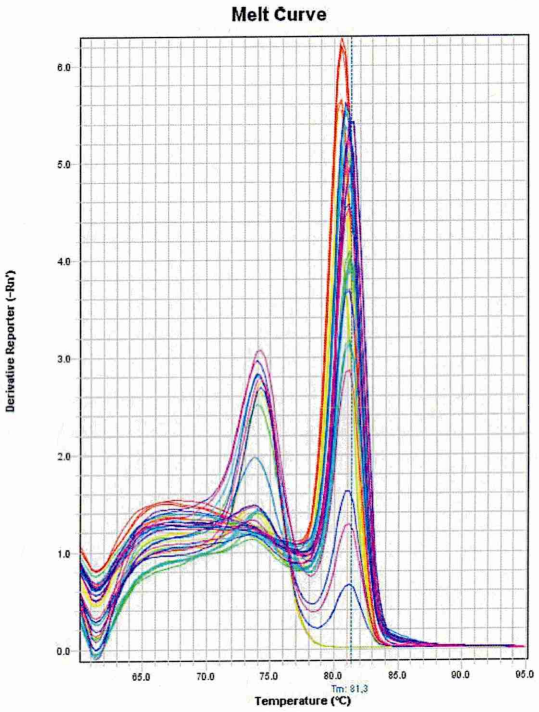
Appendix IV

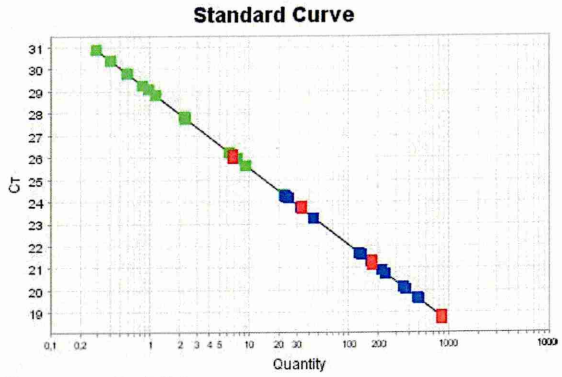
Quantitative RT PCR raw data:

Actin1 22/04/08:



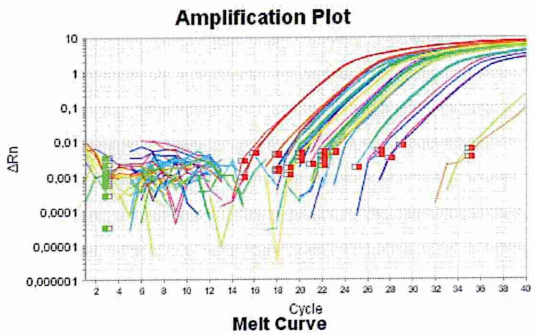


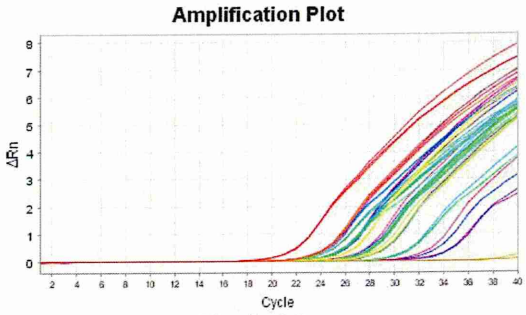


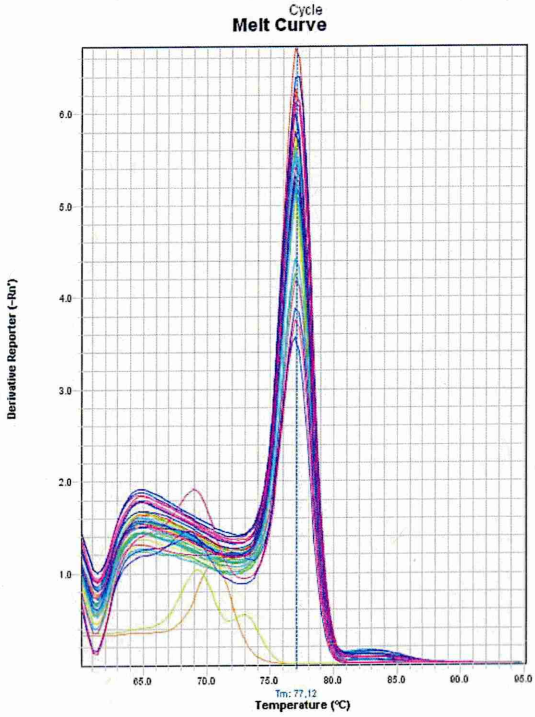


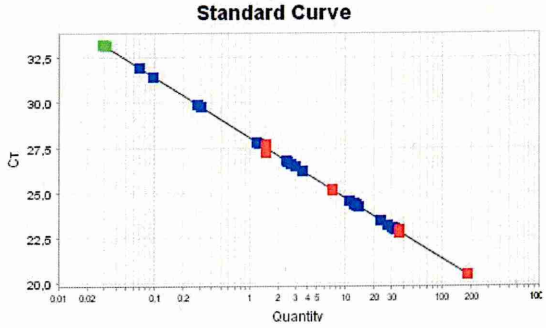
Slope: -3.473
Y-inter: 29,002
R2 : 0.999
EFF% : 94.045

TPRc1 22/04/08:



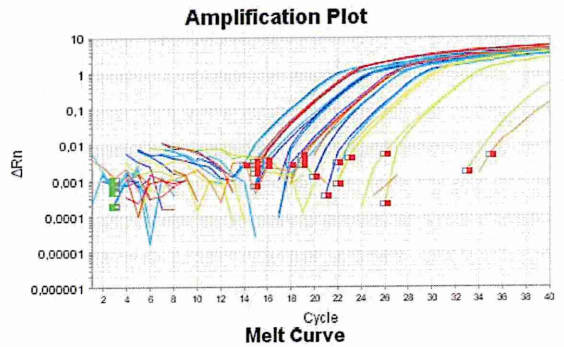


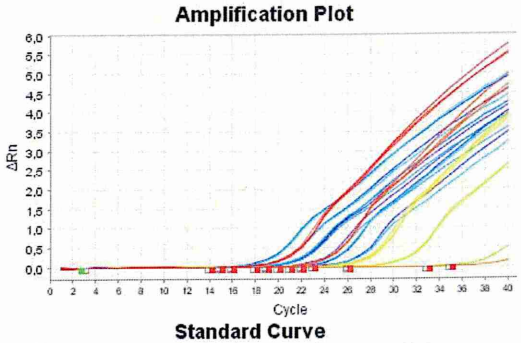


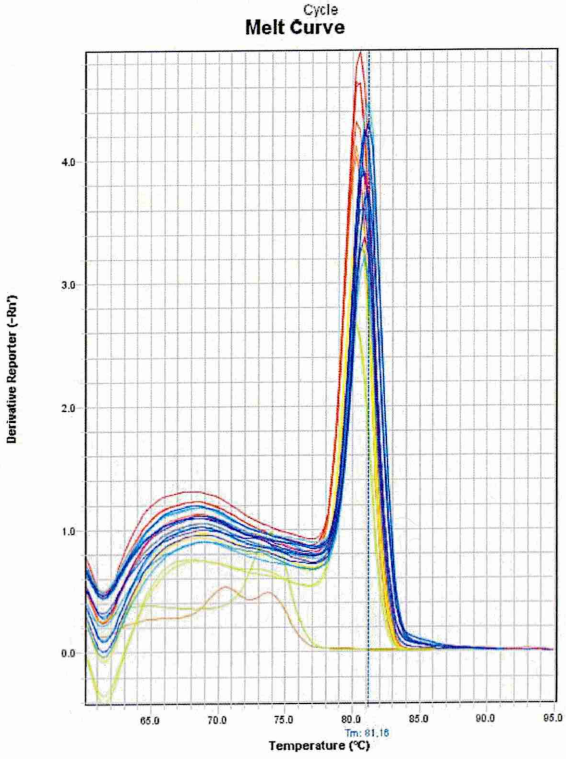


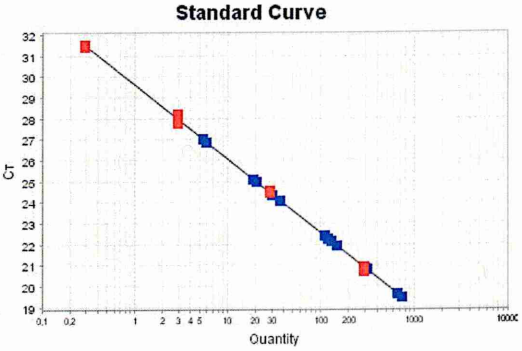
Slope: -3.327
Y-inter: 24,907
R2 : 0.996
EFF% : 99.793

Actin1 30/05/08:



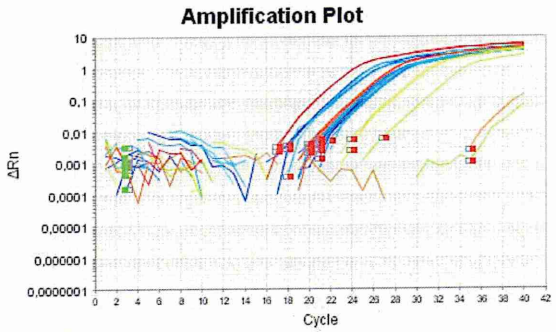


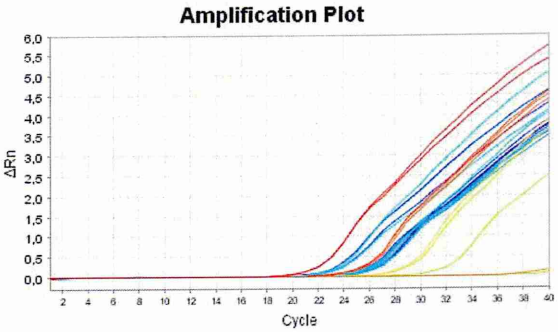


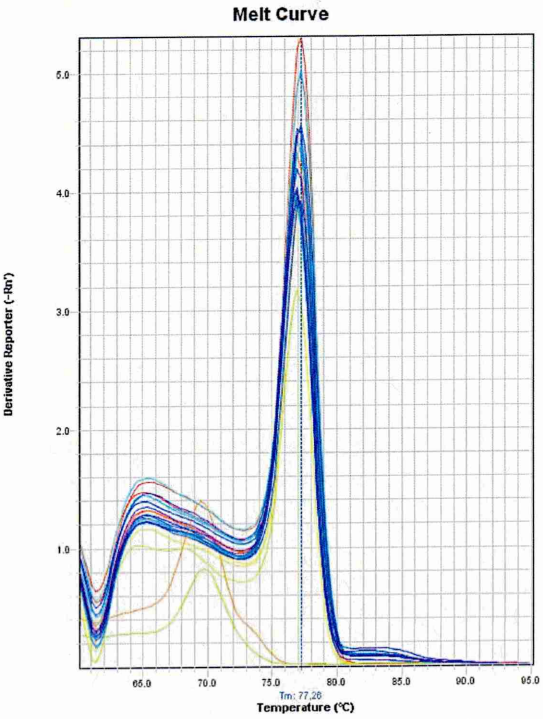


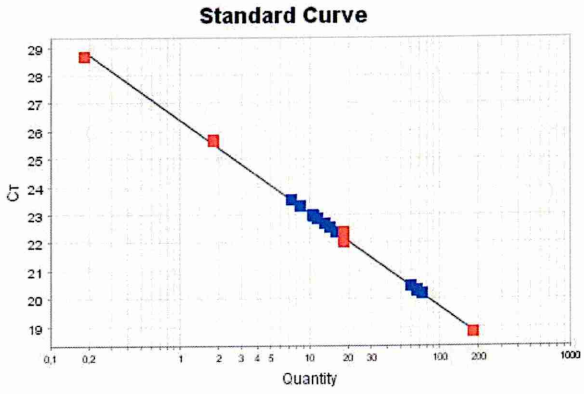
Slope: -3.542
Y-inter: 29,614
R2 : 0.999
EFF% : 91.555

TPRc1 30/05/08:



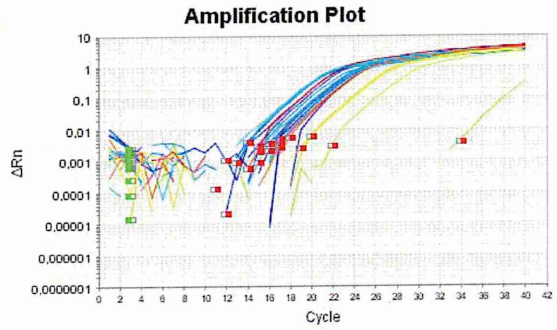


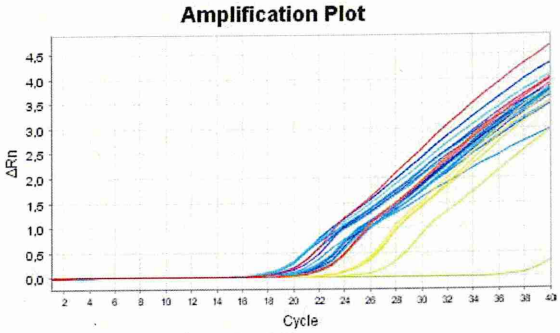


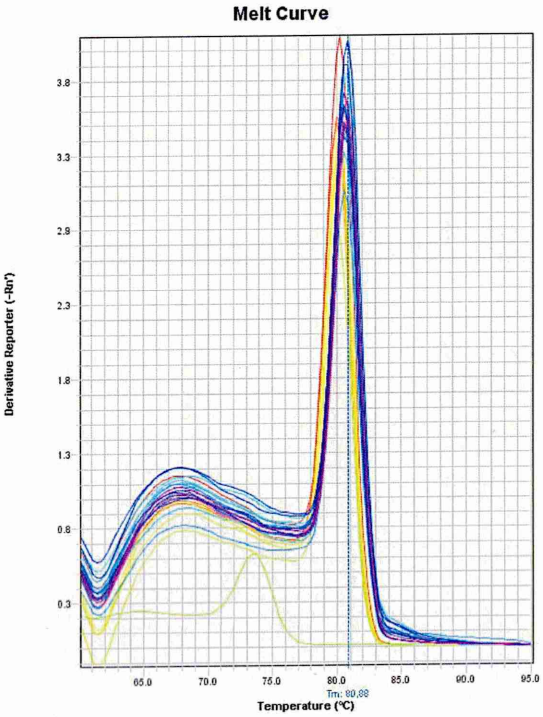


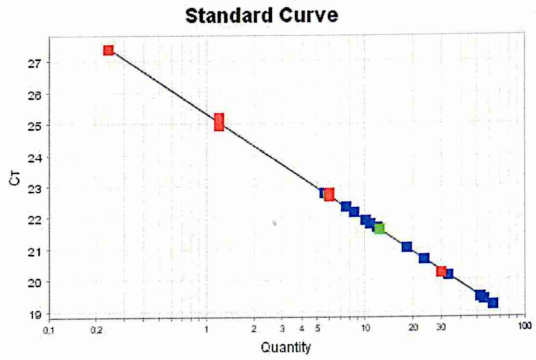
Slope: -3.335
Y-inter: 26,398
R2 : 0.998
EFF% : 99.457

Actin1 09/07/08:



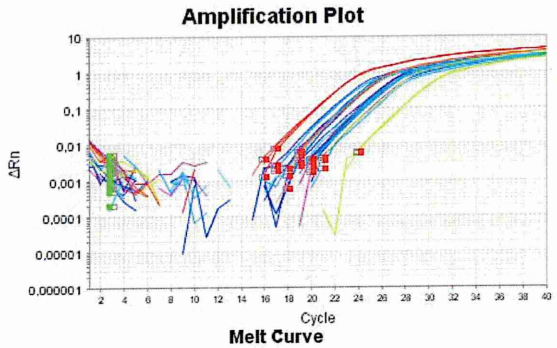


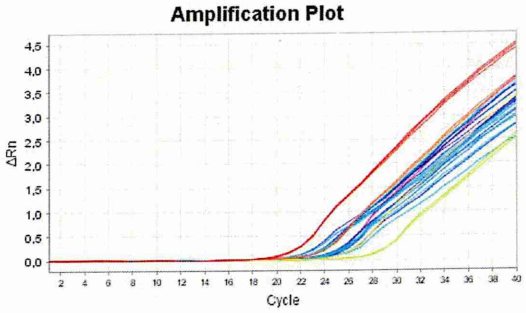


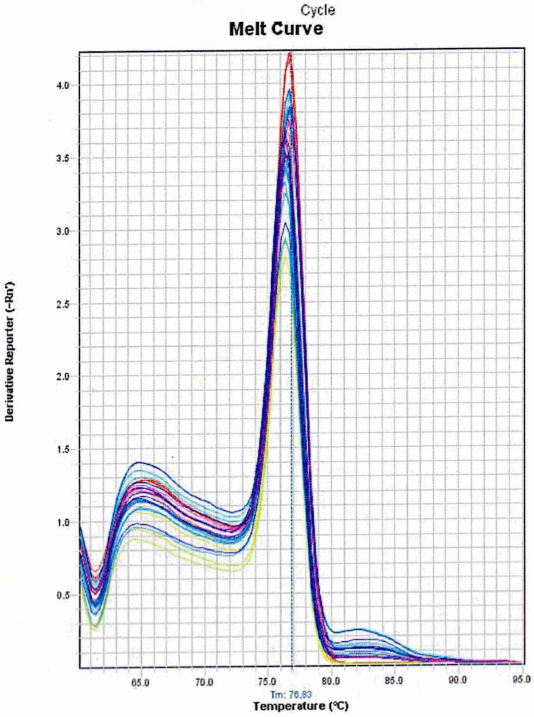


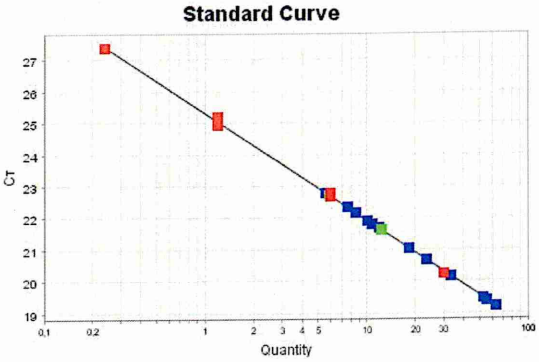
Slope: -3.392
Y-inter: 25,32
R2 : 0.998
EFF% : 97.172

TPRc1 09/07/08:







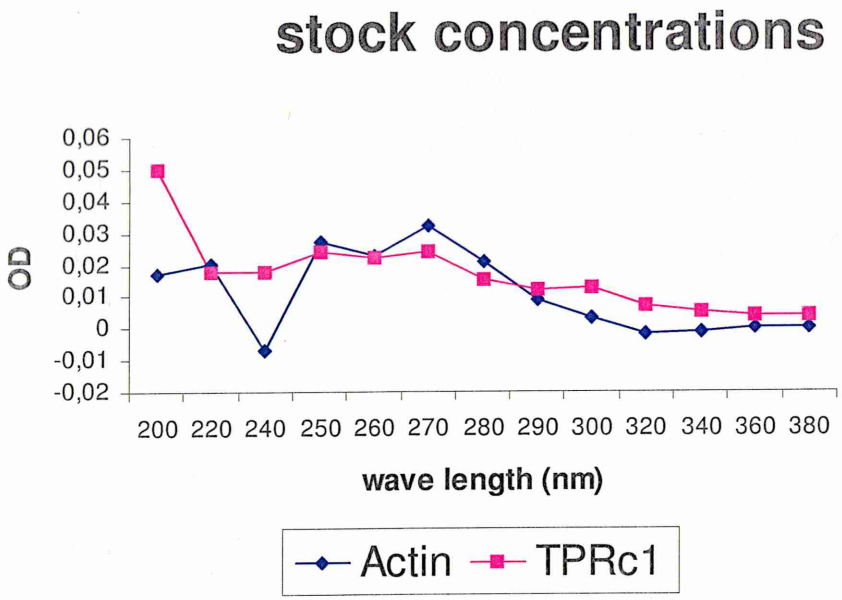


Slope: -3.473
Y-inter: 29,909
R2 : 1
EFF% : 94.056

Appendix V

Quantification of plasmid DNA used for standard curves

λ	Actin1	TPRc1
200	0,017	0,05
220	0,02	0,018
240	-0,007	0,018
250	0,027	0,024
260	0,023	0,022
270	0,032	0,024
280	0,021	0,015
290	0,009	0,012
300	0,003	0,013
320	-0,002	0,007
340	-0,001	0,005
360	0	0,004
380	0	0,004



Plasmid	Purity ($\lambda_{260}/\lambda_{280}$)	DNA concentration	Concentration of DNA fragment (fg) used as highest concentration in standard curve
Actin fragment in pGEM	1,095238	0,023	1165.066
TPRc1 in pSPUTK	1,466667	0,022	161.8502

Appendix VI

Alignment of TMDs from known chloroplast TA proteins

Name	Sequence	TM (derived with DAS)
Toc33	mgslvrewvg fqqfpaatqe klieffgklk qkdmnsmtvl vlgkggvgks stvnsligeq 61 vvrvspfqae glrpvmvsrt mggftiniid tpglveagyv nhqalelikg flvnrtidvl 121 lyvdrldvyr vdeldkqvvi aitqtfgkei wcktllvlth aqfsppdels yetfsskrsd 181 sllktirags kmrkqefeds aiavvyaens grcskndkde kalpngeawi pnlvkaitdv 241 atnqrkaihv dkkmvdgsys ddkgkkllpl iigaqylivk miqgairndi ktsgkpl	262-297
Toc34	maalqtlrew igiqqfppat qskllеilgk ykeedvsslt vlvmgkggvg ksstvnsvig 61 ekaaavstfq seglrptlvs rtrsgftlni idtpgliegg yvndqainii krfllnmtid 121 vllyvdrldv yrvddldrqv vgaitdafgk eiwkksalvl thaqfsppdg lnynhfvskr 181 snallkviqt gaqlkkqdlq gfsipvilve nsgrchknes dekilpcgts wipnlfnkit 241 eisfngnkai hvdkklvegp npnergkkli plmfafqyll vmkplvraik sdvsreskpa 301 welrdsglas rrs	252-288
TPRc1	MFNGLMDPEMIRLAQDQMSRMTPADFARIQQQMMS NPDLMNMATESMKNMRPEDLKQAAE QLKHTRPEDMAEISEKMAKASPEDIAAMRAHADAQFT YQINAAQMLKKQGNELHSRGNFS DAAEKYLRAKNNLKEIPSSKGGAILLACSLNLMSCYLK TNQHEECIKEGSEVLGYDARNV KALYRRGQAYRDLGLFEDAVSDLSKAHEVSPEDETIA DVLRDVKERLAVEGPGKASRGVV IEDITEEKNVTSGENKKPSKEANGHAQGVKTDVDGLQ ALRDNPEAIRTFQNFISKTDPDT LAALSGGKAGDMSPDMFKTASSMIGKMSPEEIQKMV QTASSFKGDNPFAPTAPSTENGFT PTPDMLKLASDMMGKMSPEERERMFNMASSLKANA PASTSYGNAEASEPRESLGASGSSS GNSFVAPRSGFEPSIPSAPPADLQEQMRNQMKDPAM RQMFTSMIKNMNPEMMASMSEQFG MKLSQEDAAKAQQAMASLSPDALEKMMRWADRAQT GMEKAKKAKKWLFGKGGLIFAILML VLAMVLHRLGYIGN	510-154
Cytb5	mptltklysmeeaathnkqddcwvvidgkvydvssymdehpggddvlla vagkdatddfedaghskdarelmekyfigeldesslpeipelkiykkdqpq dsvqklfdltkqywvvpvsiitisvavsvlfsrkt	94-135