Functional studies of the PreP peptidasome in
Arabidopsis thaliana

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Abstract

Two independent endosymbiotic events gave rise to mitochondria and chloroplasts. Despite the fact that both organelles have their own small genome the majority of organellar proteins are encoded in the nucleus, synthesized in the cytosol and imported into the organelles. The targeting information for most organellar proteins is located in an N-terminal extension called a targeting peptide. Targeting peptides are cleaved off after import by organellar processing peptidases. The cleaved targeting peptides are toxic to organellar functions and are degraded by the PreP peptidasome, the metalloendopeptidase which is the main topic of this thesis.

We have overexpressed, purified and determined the first structure of a plant mitochondrial targeting peptide, the F1β presequence from *Nicotiana plumbaginifolia*, by NMR in a membrane mimetic environment. The structure showed that the targeting peptide formed two helices separated by an unstructured domain. The N-terminal helix being amphipatic. The F1β targeting peptide has been used as a model substrate for the mitochondrial and chloroplast PreP peptidasome.

In *Arabidopsis thaliana* the PreP peptidasome is present as two isoforms, *At*PreP1 and *At*PreP2. We have shown that both forms are expressed and dually targeted to mitochondria and chloroplasts. Both *At*PreP1 and *At*PreP2 degrade targeting peptides and other non-related unstructured peptides up to 65 amino acid residues. Substrate specificity studies showed that both PreP isoforms have a preference for positively charged amino acid residues in the P1′ position and small uncharged residues in the P1 position. Mapping of cleavage sites revealed unique cleavage sites for both isoforms.

We have generated and characterized both single and double *At*PreP1 and *At*PreP2 knockouts in *A. thaliana*. *At*PreP1 was shown to be the major isoform. The double knockout exhibited a chlorotic phenotype with altered mitochondrial and chloroplast morphology. Furthermore, mitochondria were partially uncoupled. Throughout the development there was a slower growth rate and 40% lower biomass production. These results show that the PreP peptidasome is important for efficient organellar functions and normal plant development.
Publications in the thesis


Additional publications


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<tr>
<th>Abbreviation</th>
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<tr>
<td>AAA</td>
<td>ATPases associated with various cellular activities</td>
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<td>ABAD</td>
<td>Amyloid-β binding alcohol dehydrogenase</td>
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<td>Aβ</td>
<td>Amyloid beta</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>Hsp</td>
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<td>IM</td>
<td>Inner mitochondrial membrane</td>
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<td>IMP</td>
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<td>IMS</td>
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<td>MA</td>
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<td>MIP</td>
<td>Mitochondrial intermediate peptidase</td>
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<td>MOP112</td>
<td>Mitochondrial oligo peptidase of 112 kD</td>
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<td>MPP</td>
<td>Mitochondrial processing peptidase</td>
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<td>OM</td>
<td>Outer mitochondrial membrane</td>
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<td>PAM</td>
<td>Protein import associated motor</td>
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<td>PreP</td>
<td>Presequence protease</td>
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<td>SAM</td>
<td>Sorting and assembly machinery</td>
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<td>SPP</td>
<td>Stromal processing peptidase</td>
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<td>TIC</td>
<td>Translocase of the inner chloroplast envelope</td>
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<td>TIM</td>
<td>Translocase of the inner mitochondrial membrane</td>
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<td>TOB</td>
<td>Topogenesis of β-barrel proteins</td>
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<td>TOC</td>
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<td>TOM</td>
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<td>TPR</td>
<td>Tetratrico peptide repeat</td>
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Mitochondria and chloroplasts are two amazing organelles. They reside inside living eukaryote cells performing a vast array of functions. In many ways they perform opposite functions. One produces oxygen so that the other can respire. Yet they are a part of an intricate network adding to the complexities of life. We would not exist if it weren’t for the two of them.

The word mitochondrion comes from the Greek word ‘mitos’- a thread and ‘chondrion’- a grain. In 1949 the primary function of the mitochondrion was discovered by Kennedy and Lehninger, who showed that it is the site of oxidative metabolism (Kennedy and Lehninger, 1950).

The word chloroplast is also of Greek origin, ‘chloro’- green and ‘plast’- entity. This is appropriate given the appearance of chloroplasts. Having plastids or chloroplasts with the ability to harvest the power of light, is what separates the plant kingdom from the animal kingdom. The chloroplasts use light energy to build high energy compounds and in the process they produce oxygen by splitting water (Lopez-Juez and Pyke, 2005).

Origin and evolution

In 1970 Lynn Margulis proposed the endosymbiotic theory (Margulis, 1970). Although it was initially controversial it is now the most widely accepted theory explaining the origin of mitochondria. Several mitochondrial features support this theory. First of all, mitochondria are not synthesized by the cell, but multiply through division. Secondly, mitochondria contain their own genome and a transcription and translation system that is very similar to what is found in bacteria. Thirdly, many mitochondrial proteins group together with α-proteobacteria (Searcy, 2003). The closest now living relative of the mitochondria is the intracellular parasite Rikettsia prowazekii (Lang et al., 1999; Andersson et al., 2003). The presence of cardiolipin has also been used as an argument for the prokaryotic origin of mitochondria. However, the function of cardiolipin in prokaryotes and in mitochondria seems to have changed drastically during evolution (Schlame, 2007).

According to genome data the first mitochondrion is thought to have been an α-proteobacteria engulfed by a primitive anaerobic cell in a single event. Sequencing mitochondrial genomes has revealed a streamlining process whereby most genes encoding mitochondrial proteins have been transferred to the nucleus (For review see Gray et al., 2001).
Similar to mitochondria, the chloroplast is also the result of a single endosymbiotic event. This event took place approximately one billion years ago when an early eukaryote, containing mitochondria engulfed and formed an endosymbiotic relationship with a cyanobacterium. This was the first photosynthetic eukaryote and it is the progenitor of all eukaryotic photosynthetic organisms including plants. By the time these primitive photosynthetic organisms started to colonize land half a billion years ago the organelle we now call chloroplast had already evolved (For review see Lopez-Juez and Pyke, 2005). However, not all photosynthetic eukaryotes were formed by a prokaryote being engulfed by a eukaryote. Some arose by a eukaryote engulfing a photosynthetic eukaryote. For example the diatoms (by engulfing a red algae) arose by a secondary endosymbiotic event as well as apicomplexa (Stoebe and Maier, 2002; Zauner et al., 2006).

Chloroplasts like mitochondria contain their own genome and a functional transcription and translation machinery. Although the chloroplast contains its own genome the majority of the genes originally in the early endosymbiont have been transferred to the nucleus (Stegemann et al., 2003).

Mitochondrial genome

Mitochondrial genomes or mtDNA are remnants of the early endosymbiont genome. During evolution most of the mitochondrial genes have been lost or transferred to the nucleus. The mitochondrial genomes now contain on average 40-50 genes (Barr et al., 2005). However, there are large variations between different mitochondrial genomes. The mitochondrial genome of *Plasmodium reickenowi* (malaria parasite) contains 5699 base pairs encoding 3 proteins in comparison to the largest known so far, the 569630 bp genome of *Zea mays* (maize) encoding 33 proteins, 3 ribosomal RNAs, and 21 tRNAs. The human mitochondrial genome is 16.6 kbp encoding 13 polypeptides, 2 rRNAs and 22 tRNAs (Conway et al., 2000; Clifton et al., 2004; Taylor and Turnbull, 2005). The number of sequenced mitochondrial genomes is staggering; today 1320 mitochondrial genomes have been sequenced and recorded according to the organelle genome resource at NCBI (http://www.ncbi.nlm.nih.gov/genomes/ORGANELLES/organelles.html).

Mitochondrial genomes have been reduced significantly compared to the ancestral α-proteobacteria. *Rickettsia* has 836 genes, however, being an intracellular obligate parasite it is probably a poor example to compare with. A free living α-proteobacteria such as *Caulobacter* contains over 3600 protein encoding genes. This means that the reduction can be up to 1000 fold.

Interestingly, there seems to be some selectivity regarding which proteins have been transferred or lost from the mitochondrial DNA. In over 100 different animal mitochondrial genomes sequenced the majority contains the same 13 genes of the respiratory chain namely the cytochrome c oxidase.
subunits I, II and III, the ATP synthase subunits 6 and 8, the cytochrome b apoenzyme and the NADH dehydrogenase subunits 1-6, 4L (Boore, 1999; Adams and Palmer, 2003).

Organization and function of mitochondria

Mitochondria are organelles of varying shape, size and number, especially if compared between species. Plant mitochondria are in general spherical, whereas yeast mitochondria are arranged in tubular networks mixed with small spherical mitochondria. Additionally, tobacco protoplasts can contain up to 600 mitochondria compared to 5-10 in bakers yeast (Logan, 2006).

Mitochondria are enclosed by an outer mitochondrial membrane (OM) and an inner mitochondrial membrane (IM), separating the intermembrane space (IMS) from the mitochondrial matrix (MA). The inner membrane is highly convoluted, increasing the surface area, forming folds called cristae.

The energy required for producing ATP predominantly comes from the oxidation of reduced compounds such as NADH and FADH$_2$ generated in the citric acid cycle and during β-oxidation of fatty acids. NADH and FADH$_2$ release electrons to the respiratory complexes of the IM. The flow of electrons in the respiratory chain is coupled to unidirectional transport of protons from the MA into the IMS. The respiratory chain has historically been considered to consist of five multisubunit complexes, including the ATP synthase (complex I-V) (for review see Fernie et al., 2004), however, reports suggest that the mitochondrial oxidative phosphorylation system forms super complexes in the mitochondrial membrane (for review see Boekema and Braun, 2007). Complex I, III and IV catalyze translocation of protons to the IMS. The redox reactions in the electron transport chain of the IM ends with the reduction of oxygen to water by cytochrome c oxidase, complex IV. The IM is impermeable to hydrophilic compounds and protons are unable to diffuse back across the membrane (for review see Fernie et al., 2004). As a result an electrochemical gradient is formed. Like a turbine the ATP synthase (complex V) harvests the energy stored in the electrochemical gradient by channeling the protons back into the MA, the energy released is used to phosphorylate ADP to produce ATP. ATP is a phosphate group donor that carries energy between different metabolic pathways. It is not without reason mitochondria are referred to as power plants of the cell (for review see Fernie et al., 2004).

However, mitochondria have more functions than just producing ATP. Other important processes involving mitochondria range from the biosynthesis of amino acids, vitamins and some fatty acids to photo-respiration (in plants) and programmed cell death (Logan, 2006).
Chloroplast genome

The chloroplast genome is also of prokaryotic origin and a result of a single endosymbiotic event, where a cyanobacterium became involved in an endosymbiotic relationship with an early eukaryotic cell.

To date, 122 plastid genomes have been sequenced and recorded according to the organelle genome resource at NCBI (http://www.ncbi.nlm.nih.gov/genomes/ORGANELLES/organelles.html). Similar to mitochondria, chloroplast genomes have also been reduced significantly since the endosymbiotic event. The genes encoding the majority of the chloroplast proteins have been moved to the nucleus. Compared to cyanobacterial genomes which contain between 3168 (Synechocystis sp.) and 7400 (Notoc punctiforme) protein encoding genes, chloroplast genomes are substantially smaller and vary between 58 (Euglena gracilis) and 200 protein coding genes (Porphyra purpurea). The chloroplast genome of the model organism A. thaliana is 154 kbp, encoding 76 proteins and 54 different RNAs (Timmis et al., 2004)

Organization and function of chloroplasts

The chloroplast belongs to a group of organelles termed plastids all of which originate from the original chloroplast. The best known of the different types of plastids, are the colorful chloroplasts. Under the lens of a microscope they are disc shaped organelles of about 5-10 µm in diameter and 0,4-4 µm thick and they are a prominent feature of the green leaf cells. A close examination of chloroplasts reveal an outer membrane enclosing an inner membrane termed, the chloroplast outer envelope, and inner envelope respectively. Enclosed by the inner envelope is the chloroplast stroma. In the stroma there is a third membrane system called the thylakoids. The thylakoids are arranged in a network of small disks forming stacks or grana. What appears to be separate entities is actually a single membrane system enclosing one lumen (For review see Lopez-Juez and Pyke, 2005).

The central components of the thylakoids are the two photosystems (PS1 and PS2), the cytochrome b6f complex and the ATP synthase. It is in the thylakoids that light energy is harvested to generate ATP. The harvested photons provide energy to cause a charge separation within PS2, allowing the oxidation of water into oxygen in the thylakoid lumen. Four electrons and two protons are released in the process. The electrons are transferred via the Q cycle causing more protons to be transferred into the lumen, to cytochrome b6f complex and further to the PS1 complex. The final electron acceptor NADP⁺ is reduced to NADPH. The protons transferred in to the lumen generate a proton gradient that is used to generate ATP in the ATP synthase. The converted and stored energy in the form of ATP and
NADPH is used in CO₂ fixation and it is crucial to the plant metabolism (For review see Krauss, 2003).

Protein targeting to mitochondria and chloroplasts

Most mitochondrial and chloroplastic proteins in a eukaryotic cell are encoded by the nuclear genome and posttranslationally transferred to its final destination. Protein targeting is one of the most important mechanisms for proper organization and function of the cells. To achieve this, distinct targeting mechanisms have evolved for targeting different subsets of proteins into the various cellular compartments. Although many types of targeting signals exist, only the mitochondrial and chloroplastic signals will be described here.

Targeting peptides

Mitochondrial targeting relies on at least two different types of targeting signals. The first and the most common way of targeting mitochondrial proteins is via N-terminal extensions in the mitochondrial precursor proteins called presequences. These harbor all the information necessary for mitochondrial targeting. The second type consists of internal, non-cleavable targeting signals.

The presequence is recognized by receptors on the mitochondrial surface that direct the precursor protein to the mitochondrial translocase complex of the outer membrane (TOM complex) for continued translocation. After completed import the presequence is generally cleaved off for continued maturation of the protein. Some mitochondrial proteins utilizing an N-terminal extension for mitochondrial targeting are also targeted to the chloroplast via the same N-terminal extension. These are called ambiguous targeting peptides and the proteins are referred to as dual targeted proteins (Peeters and Small, 2001).

Presequences are not homogenous. They range in size from 18 to 136 residues in plants with an average of 42, however, most are between 20-60 amino acid residues long. Upon examination of the amino acid distribution and content, some similarities have emerged. A close analysis showed that hydrophobic residues were particularly abundant as well as basic and hydroxylated residues. Acidic residues are almost absent in the mitochondrial presequences (Zhang and Glaser, 2002). However, no distinct motif has been found so far (Bhushan et al., 2006).

One interesting feature among presequences is the ability to form amphipilic α-helices in a membrane mimetic environment. For example, the presequences of pALDH, pMADH and pOCT from rat all contained helical elements in membrane mimetic environments as well as the yeast pF₁β and
pCoxIV presequences (Karslake et al., 1990; Bruch and Hoyt, 1992; MacLachlan et al., 1994; Chupin et al., 1995; De Jongh, 2000).

The importance of the helical elements was clearly demonstrated when the structure of the rat mitochondrial presequence receptor Tom20 (Abe et al., 2000), in a complex with the rat aldehyde dehydrogenase (pALDH) presequence was solved. It clearly demonstrated that the amphiphilic nature of the presequence was important in binding to binding to the rat Tom20 receptor. The amphiphilic helix bound the receptor in an hydrophobic grove, exposing the hydrophilic residues to the aqueous environment. However, none of the presequences mentioned above are plant mitochondrial presequences. Many differences exist between the plant and non-plant mitochondrial import machineries, especially regarding the receptors (see “The translocase of the outer mitochondrial membrane” below). Therefore it was also important to investigate the structural properties of plant mitochondrial presequences.

To this end, we have solved the structure of the first higher plant mitochondrial presequence (Figure 1), the 53 amino acid presequence of the F₁β ATPase from *N. plumbaginifolia* by CD spectroscopy and NMR (Paper I). The F₁β precursor was mutated and overexpressed with a methionine inserted at the processing site. This enabled cleavage of the presequence from the mature region using CNBr, after which the presequence was purified to homogeneity using ion exchange chromatography.

![Figure 1](image.png)

**Figure 1. The three dimensional structure of the F₁β presequence.** A, Ensemble of 24 structures. B, Structure of the N-terminal helix.

Conservation of the native properties of the purified F₁β presequence was confirmed by *in vitro* import and processing experiments. The presequence efficiently inhibited *in vitro* import into potato mitochondria as well as *in vitro* processing by purified MPP/*bc₁* complex of pAOX, pF₁β, pFAD and pHSP70 precursor proteins at micromolar concentrations. Similar to what had been previously seen in yeast (Bruch and Hoyt, 1992) and rat (Karslake et al., 1990), the plant mitochondrial presequence of F₁β also contained helical elements. The structural information obtained by CD spectroscopy measurements in different membrane mimetic environments such as sonium
dodecyl sulfate (SDS), dodecylphosphocholine (DPC) and in acidic phospholipid bicells all indicated helices in the F$_{1}$β presequence. The NMR structure was obtained in SDS micelles and revealed two helices separated by an unstructured domain. The N-terminal helix, which also was amphipathic stretched between Leu5 and Gln15. After the unstructured region, there was a second helix between Lys43 and Tyr53.

The structure of the *A. thaliana* Tom20 receptor was also recently solved (Perry et al., 2006). Mapping of the interactions between a peptide derived from a presequence, the F$_{1}$β(1-14) peptide and the receptor Tom20 revealed that the residues involved in the interactions is located in a hydrophobic groove analogous to what has been seen in rat. Comparisons of the different receptor structures reveal distinct differences suggesting convergent evolution of function.

Chloroplast targeting peptides, also known as transit peptides, show many similarities but also a lot of differences compared to mitochondrial presequences. As for the mitochondrial precursor proteins the majority of chloroplast proteins are posttranslationally imported into the chloroplast, they carry an N-terminal cleavable signal peptide or transit peptide harboring the information guiding the precursor protein to the chloroplast and the transit peptide is generally cleaved off after import.

Transit peptides in general do not form secondary structures although a few reported exceptions exist. For example, the transit peptide of precursor of the small subunit of ribulose 1,5 biphosphate carboxylase-oxygenase (prSSU) forms α-helical structures (Bruce, 2000). However, they vary substantially in length; they are between 13 and 146 amino acid residues long with a mean of 58 residues. As such they are generally longer than mitochondrial presequences (Zhang and Glaser, 2002). Again there are no clear motifs governing targeting. However, transit peptides are enriched in hydroxylated amino acid residues, especially serines and hydrophobic residues and they are usually devoid of basic residues at the very N-terminus. These observations are apparent when examining logos based on the *A thaliana* chloroplast proteome (Bhushan et al., 2006).

**Mitochondrial protein import machinery**

The mitochondrial import machinery (Figure 2) has evolved to recognize and import nuclear encoded proteins since the endosymbiotic event. Some components have an eubacterial origin, whereas several new components have been adapted from the eukaryotic host (Lister et al., 2005).
Chaperone assisted import into mitochondria

Cytosolic factors have been reported to be important in the import of newly synthesized precursor proteins. Both an Hsp70 related protein in yeast and Hsp70 and Hsp90 in a mammalian system have been shown to have a role in import into mitochondria (Murakami et al., 1988; Young et al., 2003). Other components that have been implicated are arylhydrocarbon receptor-interacting protein (AIP) (Yano et al., 2003), presequence binding factor (PBF) (Murakami and Mori, 1990; Murakami et al., 1992), the targeting factor (TF) (Ono and Tuboi, 1990) and the mitochondrial import stimulating factor (MSF) (Hachiya et al., 1993). However, the involvement of these components is not clear since in vivo results are scarce. Independent reports also suggest that some precursor proteins are co-translationally imported into mitochondria (For review see Lithgow, 2000).
The translocase of the outer mitochondrial membrane

A newly synthesized mitochondrial precursor protein has to make contact with the translocase complex of the outer mitochondrial membrane, also known as the TOM complex prior to import. The TOM complex and mitochondrial protein import in general has been most extensively studied in yeast. However, in the recent years a more defined organization of the plant mitochondrial import apparatus has emerged revealing similarities as well as differences between plants and yeast.

The TOM complex in plants was first purified and analyzed in potato tuber mitochondria (Jansch et al., 1998). The complex was resolved into seven different proteins of 70, 36, 23, 9, 8, 7 and 6 kDa. Sequence analysis of the components identified the 36, 23 and 7 kDa proteins as being homologs of yeast Tom40, 20 and 7, respectively. The 70 kDa protein was proposed to be a homolog of Tom70 and two of the small proteins were proposed to correspond to yeast Tom5 and Tom6. In *A. thaliana* six different components have been identified in the TOM complex, namely *At*Tom5, 6, 7, 9, 20 and 40 (Werhahn et al., 2001; Werhahn et al., 2003).

The first interactions between the precursor protein and the TOM complex are with the components functioning as receptors. In *A. thaliana* *At*Tom9 and *At*Tom20 correspond to the yeast and mammalian receptor counterparts Tom22 and Tom20 (Macasev et al., 2000). However, they are not identical. *At*Tom9 displays sequence homology to yeast and mammalian Tom22, but unlike yeast Tom22 *At*Tom9 lacks the acidic receptor domain and does probably not function as a receptor. *At*Tom20 on the other hand does not show any sequence similarity to the yeast or mammalian counterpart. Instead, *At*Tom20 seems to be a result of convergent evolution (Perry et al., 2006). Supporting evidence for a convergent evolution are the structural similarities in the binding of presequences. Both the rat Tom20 and the *At*Tom20 bind presequences via hydrophobic interactions in a hydrophobic groove. Interestingly, *At*Tom20 seems to be a non essential protein (Lister et al., 2007).

Yeast also have another receptor, namely Tom70, which appears to be absent in *A. thaliana*. Tom70 is the receptor responsible for the recognition of proteins with internal targeting signals such as the carrier proteins (Sollner et al., 1990; Steger et al., 1990; Brix et al., 1997). Considering the clear evidence for the presence of a distinct carrier protein import pathway, it is a surprise that no component corresponding to Tom70 has been identified in plants (Lister et al., 2002). One possible carrier protein receptor in plants is OM64. OM64 presents sequence similarities to the Toc64 chloroplast import receptor and is anchored to the OM. It also possesses receptor like features.
Recently it was shown to interact with and influence the import of several precursor proteins (Chew et al., 2004; Lister et al., 2007). Another protein with receptor like abilities in plants is metaxin. It is a distant homolog to mammalian metaxin (Armstrong et al., 1997) and Sam37 in yeast (Wiedemann et al., 2003). Metaxin is not directly associated with the translocation complex but it can influence import and interact with a number of different precursor proteins (Lister et al., 2007).

Additionally the frequent gene duplication events in Arabidopsis have increased the complexity of the A. thaliana genome and increased the opportunities for functional specialization. Many components in the A. thaliana mitochondrial import machinery (as well as other proteins) are present as several isoforms. Usually one of the isoforms is expressed to a higher extent compared to the others (Lister et al., 2004). In A. thaliana, AtTom20 is encoded by four distinct genes (tom20-1, -2, -3 and -4) of which all are expressed, although at very different levels (Werhahn et al., 2001; Lister et al., 2007). Knockouts of different AtTom20 isoforms in A. thaliana affected import of different precursor proteins differentially, indicating subfunctionalisation (Lister et al., 2007). These results suggest a complex and dynamic system for recognition and import of precursor proteins in plants.

After the initial recognition by the receptors the precursor proteins are transferred and translocated through the general import pore Tom40 (Becker et al., 2005). Tom40 and its associated components are in general conserved compared to fungi and mammalian (Werhahn et al., 2001).

**Protein insertion into the outer mitochondrial membrane**

A protein complex has been identified that is responsible for the insertion of β-barrel proteins into the outer membrane. The complex has two names, TOB (topogenesis of β-barrel proteins) (Paschen et al., 2003) and SAM (sorting and assembly machinery) (Kozjak et al., 2003), I will use the SAM terminology. In yeast the complex consists of at least 5 different components including the two essential components Sam50 (Kozjak et al., 2003) and Sam35 (Milenkovic et al., 2004). The additional subunits named Sam37 (Wiedemann et al., 2003), Mdm10 (Meisinger et al., 2004) and Mim1 (Becker et al., 2008) are also components of the complex but they are not essential. Sam37 seems to be important for efficient release of assembled proteins in the SAM complex.

Many of the proteins in the SAM complex are unique to yeast, for example plants have the Sam50 and a protein similar to Sam37 namely metaxin but not the other subunits Sam35, Mim1 and Mdm10 (Lister et al., 2004; Lister et al., 2007).

Insertion of precursor proteins into the OM requires the precursor to first be imported via the TOM complex, into the IMS, where the precursor
proteins interact with the small TIMs (se below) acting as chaperones. The precursor proteins are then transferred to the SAM complex, folded and inserted into the membrane (Meisinger et al., 2006).

The insertion of α-helical transmembrane proteins in the outer mitochondrial membrane is not as well understood as the insertion of β-barrel proteins. Several models have been proposed that implicate Tom40 in insertion, via lateral opening into the membrane or via some other mechanism requiring larger assemblies of the Tom40. (Rapaport, 2005). Since Tom40 is a β-barrel, it is unlikely that a lateral transfer of α-helical transmembrane segments can occur as it would destabilize the structure (Becker et al., 2005). However, the situation is still unclear. Recent data even suggest that Tom40 is not necessary for the actual insertion process of at least some proteins. Tom70 has been shown to be required but not Tom40 for the insertion of an outer membrane protein called peripheral benzodiazepine receptor (Otera et al., 2007). Additionally the SAM machinery has been reported to be required for the insertion of several α-helical transmembrane proteins into the OM (Stojanovski et al., 2007).

The intermembrane space

The IMS is the compartment separating the mitochondrial OM from the IM. Two different ways of targeting proteins to the IMS have been identified in yeast so far (Herrmann and Kohl, 2007). Some proteins targeted to the IMS have a bipartite targeting signal. It consists of an ordinary presequence followed by a hydrophobic stretch halting the matrix targeting and enabling the lateral transfer into the IM. In the IM the precursor is cleaved at the IMS side of the IM by the inner membrane peptididase 1 (IMP1), thereby releasing the mature part into the IMS. Some proteins of the IMS utilize the conservative sorting mechanism i.e. initial translocation into the matrix for further export to IMS. For example cytochrome c1 and cytochrome b2 have been proposed to utilize this pathway (Gruhler et al., 1995; Haucke et al., 1997; Baumann et al., 2002).

Smaller proteins imported into the IMS do not contain any ordinary targeting signals, but usually carry conserved twin cysteine motifs such as CX3C or CX9C, which is utilized in a disulfide relay system. In general the proteins imported via the disulfide relay system are so small that, in the unfolded state, they are proposed to be capable of diffusing through the TOM complex and upon folding in the IMS they are unable to diffuse back. The folding process requires two proteins, namely MIA40 and ERV1. It appears as the oxidized form of MIA40 binds the precursor with reduced cysteines and upon “reshuffling” of the cysteine bonds, the precursor is released in an oxidized state carrying internal disulfide bridges. MIA40 is
released in a reduced inactive state and reoxidized by ERV1 (Herrmann and Kohl, 2007).

Other yeast IMS proteins involved in the import process are also present in plants. Namely, Tim8, 9, 10 and 13 (as well as MIA40 and ERV1) (Lister et al., 2002; Lutz et al., 2003; Lister et al., 2004). The small Tims form two complexes, Tim8p/Tim13p and the Tim9p/Tim10p complex. The small TIM complex is a hexamer consisting of three protein subunits of each small Tim protein. All small TIMs contain four conserved cysteines forming a Zn$^{2+}$ binding motif. The small TIM complexes relay precursor proteins from the TOM complex to the TIM22 complex and act as chaperones during import of the carrier proteins. It has also been shown that the binding is highly specific indicating a function in the alignment and transfer of the precursor for efficient and precise delivery to the TIM22 complex, and thereby contributing to the correct insertion of the precursor protein into the membrane (Davis et al., 2007).

The translocases of the inner mitochondrial membrane

In the mitochondrial IM there are at least three complexes responsible for translocation across the IM to the MA and insertion of proteins into the IM. The two major complexes are the TIM17:23 and TIM22 complexes. The third is the OXA1 complex (For reviews see Glaser and Whelan, 2007; Neupert and Herrmann, 2007).

The TIM17:23 complex is responsible for the import of all proteins carrying a presequence into the MA and also for the import and insertion of the IM proteins carrying a presequence and a hydrophobic insertion signal, known as a bipartite targeting peptide. Proteins inserted into the inner membrane via TIM17:23 belong to the so called stop-transfer pathway. Most of the research on the TIM17:23 complex has been done in yeast.

In plants, most the major components in the TIM17:23 complex have been identified experimentally or by genetic analysis. The components found so far are AtTim17, 21, 23 and AtTim50. Tim23 is the actual channel through which the precursor proteins are translocated. It is interesting to note that there are significant differences between the yeast Tim23 and AtTim23. Yeast Tim23 contains an extension that is exposed to the IMS which mediates the cation based selectivity of the translocase. In plants this domain is missing and AtTim23 cannot complement yeast Tim23 mutants. At least one of the AtTim17 isoforms is connected to the OM and it can only complement a yeast Tim17 knockout when the extension to the OM is removed. It is possible that AtTim17 links the AtTIM17:23 complex to the TOM complex in plants (Murcha et al., 2005). Tim50 is also an interesting protein, which has been suggested to act as a receptor for the incoming precursor protein. More recently it has been shown to regulate the permeability of the IM by maintaining a closed Tim23. Upon interaction with a presequence,
Tim50 allows the TIM17:23 complex to open and thereby import the protein (Mokranjac et al., 2003; Meinecke et al., 2006).

Connected to the TIM17:23 complex is also the presequence translocase associated motor (PAM). There are two requirements for import into the MA, first of all a membrane potential is required. Secondly, the process is ATP dependent. The initial translocation through the TIM17:23 complex requires the membrane potential and the continued translocation is achieved by the active translocation by the PAM complex. In yeast the PAM complex consists of mtHsp70, Tim44, Mge1, Pam18, and Pam16, Pam17 and Zim17. MtHsp70 is the ATPase performing the motor function by binding and translocating the precursor protein. In the process ATP is hydrolyzed. Pam18 contains a J domain which stimulates the ATPase activity of the mtHsp70. Tim44 connects the PAM complex to the TIM17:23 complex. In A. thaliana, only one protein is missing according to the MPriC database of Mitochondrial Protein Import Components, (http://www.plantenergy.uwa.edu.au/applications/mpric/index.php), namely Pam17 which is supposed to regulate the interactions between Pam16 and Pam18 and thereby stabilize the entire complex (van der Laan et al., 2005).

Tim22 is an essential protein in yeast and it inserts proteins of the carrier pathway into the IM (Kovermann et al., 2002). Until recently no ortholog of yeast Tim22 had been found in A. thaliana even though strong evidence for the carrier pathway existed in plants. Recently two genes were been found in A. thaliana capable of complementing a Tim22 knockout in yeast showing that Tim22 indeed is present in A. thaliana (Murcha et al., 2007).

Oxa1 (Bonnefoy et al., 1994) is a component involved in the insertion of inner membrane proteins that are either imported into the MA prior to insertion or inner membrane proteins encoded in the mitochondria (Hell et al., 1997; Hell et al., 1998; Hell et al., 2001; Baumann et al., 2002). This pathway is called the conservative pathway due to the directionality of insertion from the MA, as it reflects the prokaryotic origin. Nuclear encoded proteins inserted into the inner membrane via the conservative pathway usually have transmembrane segments containing prolines (Meier et al., 2005). Additionally they are also in general less hydrophobic than the transmembrane segments of proteins in the stop transfer pathway. This probably prevents them from arresting in the TIM17:23 complex. In A. thaliana AtOXA1, a mitochondrial localized homolog of OXA1 is capable of complementing yeast OXA1 (Sakamoto et al., 2000).

The mitochondrial processing peptidases

The majority of mitochondrial proteins have an N-terminal extension, which has to be proteolytically cleaved for maturation of the protein. Several proteins are involved in the maturation process. The major peptidases
involved are the mitochondrial processing peptidase (MPP), the inner membrane peptidase (IMP) and the mitochondrial intermediate peptidase (MIP).

MPP resides in the MA as a soluble protease in yeast (Yang et al., 1988), whereas in plants it is integrated in the bc1 complex in the IM (Braun et al., 1992; Eriksson et al., 1996). MPP is a heterodimer consisting of one α-subunit responsible for peptide binding and one β-subunit harboring the catalytic activity. The β-subunit contains an essential inverted zinc binding motif (HEXXH) placing it among the metallo proteases (Kitada et al., 1995). In 2001 the structure of MPP of both the active form and a proteolytically inactive mutant was solved. The structures showed the two different substrates co-crystallized with MPP in an extended conformation at the active site. The structure also showed that the active site resides in a central cavity lined with hydrophilic and negatively charged residues. This could facilitate the binding and recognition of the basic presequences of precursor proteins (Taylor et al., 2001).

IMP is involved in the maturation of proteins destined for the IMS (Schneider et al., 1991; Haucke et al., 1997). As previously described, some proteins are sequentially processed by MPP and then by a second protease releasing it into the IMS. IMP catalyses the second processing step at the outer face of the IM. IMP consists of three subunits of which two are catalytic, namely Imp1p and Imp2p, which have different proteolytic specificity (Nunnari et al., 1993; Jan et al., 2000). The third subunit Som1p (Esser et al., 1996) interacts with Imp1p and is necessary for the processing of Mcr1 and Cox2. Som1p can be seen as a regulatory subunit of IMP (Jan et al., 2000).

MIP processes proteins that have been previously processed by MPP in the MA. After the cleavage of the precursor protein by MPP an additional octapeptide is cleaved off by the proteolytic action of MIP. A motif characterizing the cleavage site in the MIP substrates is as follows R-X↓(F/L/I)-X-X-(T/S/G)-X-X-X-X↓, where the first arrow indicates cleavage by MPP and the second by MIP (Isaya et al., 1991). MIP is metal and thiol dependent and has a zinc binding motif HEXXH. It belongs to the family of thimet metallo endopeptidases (Chew et al., 1996). Homologs of MIP are present in all eukaryotes. Saccharomyces cerevisiae CoxIV was shown to be processed by a two step mechanism first by purified MPP/bc1 complex and then by a MA extract from Solana tuberosum, indicating that the same two step processing occurs in plants (A. Ståhl and E. Glaser, unpublished results).
Chloroplast protein import machinery

Chaperone assisted import into chloroplasts

As previously described the majority of the chloroplast proteins are nuclear encoded and carry an N-terminal cleavable extension called a transit peptide harboring the chloroplast targeting signal. After import the transit peptide is proteolytically removed by the stromal processing peptidase (SPP) (Richter and Lamppa, 1998).

Whether chaperones assist import into chloroplasts is a still debated topic. It has been shown that several transit peptides can be phosphorylated in an in vitro assay using cytosolic extracts whereas several mitochondrial control proteins were not phosphorylated, indicating a specific mechanism for phosphorylation of transit peptides (Waegemann and Soll, 1996). Using biochemical approaches a kinase in A. thaliana was identified as the precursor protein phosphorylating kinase (Martin et al., 2006). It was the only serine/threonine kinase in a fraction highly enriched for pSSU phosphorylation activity. The kinase was classified by bioinformatical approaches as a dual Sty kinase being termed Sty8. The phosphorylation of the transit peptide is a requirement for the binding of a so called guidance complex formed by 14-3-3 proteins and cytosolic Hsp70. It was also shown that there was a significant increase in the import efficiency of a phosphorylated transit peptide bound to the guidance complex compared to a nonphosphorylated precursor. This guidance complex is also suggested to be one component helping mitochondria and chloroplasts to discriminate between precursor proteins (May and Soll, 2000). On the other hand, removal of the phosphorylation sites of the transit peptide of the small subunit of ribulose bisphosphate carboxylase/oxygenase did not affect targeting to chloroplasts in vivo nor did it cause mistargeting (Nakrieko et al., 2004), indicating that it is not essential for import but rather may affect import efficiency.

The translocase of the chloroplast outer envelope

The first components of the translocon at the outer envelope membrane of the chloroplast (TOC) were identified when Waegemann and Soll and later Perry and Keegstra managed to purify several components of the TOC complex from pea (Waegemann and Soll, 1991; Perry and Keegstra, 1994).

The core of the TOC complex consists of Toc159, 75 and 34 with a stochiometry of approximately 1: 4: 4-5 forming a complex of approx. 500 kDa. It was also shown that the purified complex could bind the precursor protein of the small subunit of ribulose in a GTP dependent manner (Schleiff et al., 2003). Both Toc159 and Toc34 of the TOC complex are receptors
recognizing the precursor proteins prior to import. They have been shown to have GTP binding domains that accounts for the requirement for GTP for binding of precursor proteins. The current model regarding the recognition and protein import via the TOC complex states that Toc34 initially recognizes the precursor protein, transfers it to the Toc159. Toc159 works as an import motor driven by the hydrolysis of GTP, which causes a conformational change in Toc159 capable of driving the precursor into Toc75. The precursor protein is translocated via Toc75, which probably forms the actual channel through the outer envelope (Schleiff et al., 2003). Toc75 is also the only component which is embryo lethal of the core components (Hust and Gutensohn, 2006).

Additional proteins have been found associated with the TOC complex, Namely Toc64 and Toc12. Toc64 is a chloroplast outer envelope protein of 64 kD. Toc64 has a large polypeptide domain, including three tetratricopeptide repeats (TPR), a typical motif for protein-protein interactions, on the cytosolic surface of the organelle (Sohrnt and Soll, 2000). It was later shown to recognize Hsp90 delivered precursor proteins (Qbadou et al., 2007). However, the functional significance of Toc64 was recently questioned since loss of all the different isoforms of Toc64 in A. thaliana did not produce any phenotype (Aronsson et al., 2007). Toc12 is located on the inner face of the outer envelope and has a C-terminal region forming a J-domain capable of stimulating the ATPase activity of Hsp70 chaperones. It was shown to interact with the IMS localized Hsp70, Toc64 and Tic22. Supposedly Toc12 has a role in the continued translocation of the precursor proteins (Becker et al., 2004).

Evidence also exists for a TOC independent import pathway (Nada and Soll, 2004; Miras et al., 2007). Nada and Soll showed that the 32 kDa chloroplast inner envelope protein (IEP32) was imported into the chloroplast without a cleavable N-terminal targeting peptide. Recognition and translocation of IEP32 did not require the Toc159, Toc75 or Toc34. IEP32 only required ATP for import. Similarly, Miras et al showed that the quinone oxidoreductase (ceQORH), a protein residing in the plastid inner envelope and lacking a cleavable transit peptide could be imported in an ATP dependent manner without using the Toc159 or Toc34 receptors. These results indicate a pathway for inner envelope plastid proteins lacking transit peptides.

The translocase of the chloroplast inner envelope

After translocation through the TOC complex, the precursor protein requires another complex for passage through the inner chloroplast envelope. This complex is called the translocon at the inner envelope membrane of the chloroplast (TIC). Seven components of the TIC complex have been
identified so far, namely Tic110, Tic40, Tic22, Tic20, Tic62, Tic55 and Tic32.

Tic110 is an integral membrane protein containing a hydrophilic C-terminus extending into the stroma. It is anchored in the inner envelope via two membrane-spanning helices in the N-terminus (Jackson et al., 1998). It has been suggested to form the translocation pore in the inner envelope or at least a part of it (Heins et al., 2002), but definitive proof has yet to be presented. Tic110 has also been proposed to play a key role by binding precursor proteins during translocation across the inner envelope and also in the recruitment of chaperones in the stroma. The large stromal domain of Tic110 contains a transit peptide binding site adjacent to its membrane anchor segments. This site is proposed to form the initial binding site for precursor proteins as they emerge from the channel of the TIC translocon and thereby preventing them from slipping back into the IMS (Inaba et al., 2003; Inaba et al., 2005). Additionally, Tic110 has been shown to be an essential protein (Inaba et al., 2005; Kovacheva et al., 2005).

Tic40 interacts with Tic110 and is bound to the inner envelope (Stahl et al., 1999). The role of Tic40 is not completely clear, but it is known to form a complex with Tic110 and Hsp93, where it is proposed to function as a co-chaperone to coordinate the action of Tic110 and Hsp93. Loss of Tic40 significantly reduced import through the TIC complex and the knockout plants were much paler and smaller, presenting a slowed development compared to wild-type plants (Chou et al., 2003).

Tic20 and Tic22 are both associated with the inner envelope, Tic20 is an integral protein while Tic22 is peripheral to the outer face of the inner envelope. The results by Kuranov et al suggest that Tic 22 interact with the precursor proteins just after translocation through the TOC complex, the precursor protein is later transferred to Tic20. Tic20 and Tic22 were also shown to associate with other TOC and TIC components such as Toc34 and Tic110 to form a so called import super complex in the chloroplasts. Suggesting that Tic20 and Tic22 serve as a functional link between the TOC and TIC translocons (Kouranov et al., 1998). Tic62, Tic55 and Tic32 are also components of the Tic translocon (Caliebe et al., 1997; Kuchler et al., 2002; Hormann et al., 2004). Tic32 has been shown to be a NADPH dependent dehydrogenase regulated by calmodulin. Calmodulin affects the dehydrogenase activity of Tic32 in a Ca$^{2+}$ dependent manner and the binding of calmodulin promotes binding of Tic32 to Tic110. In the presence of NADPH the binding of Tic32 is abolished but in the presence of calmodulin the NADPH is displaced. It has been suggested that Tic32 could function as “a switch to differentially integrate redox signals from the inside of the chloroplast with calcium signals from outside the organelle”. Tic32 could thereby regulate the TIC complex via interactions with Tic110 and thereby affecting the activity of the translocon (Chigri et al., 2006). Tic62 is also suggested to be a part of the redox regulated system of the TIC translocon.
Just as Tic32, Tic62 responds in a similar way to NADPH by reducing its interactions with Tic110. Tic62 also interacts with the ferrodoxin NAD(P) oxidoreductase in the stroma (Stengel et al., 2008). Tic55 has been shown to be a Rieske-type iron sulfur protein that interacts with Tic110. It is suggested to function as a redox sensor during pre-protein translocation in chloroplasts (Kuchler et al., 2002). These last three components of the TIC complex suggest a distinct coupling to the redox state of the chloroplast for the regulation of protein import into the chloroplast.

The chloroplastic processing peptidases

As in mitochondria, cleavage of the imported precursor is an important process in the chloroplast. The major peptidase responsible for the proteolytic removal of the transit peptide from the precursor protein is the stromal processing peptidase (SPP). SPP was first established as the general processing peptidase in 1997 when Richter and Lamppa overexpressed, purified and *in vitro* tested the processing of eleven different chloroplast precursor proteins. The precursor proteins were shown to be processed in one single proteolytic step by the action of SPP, thereby releasing the transit peptide from the precursor. They could also show that SPP was a metalloprotease containing the zinc binding motif HXXEH. SPP was classified as a member of the pitrilysin metalloproteases placing it among other proteins such as pitrilysin, the insulin degrading enzyme and the mitochondrial processing peptidase (Richter and Lamppa, 1998). Recently, the presequence degrading protease PreP was also added to the pitrilysin family of proteases (Stahl et al., 2002). It was later shown that SPP processes the targeting peptide several times before the release of the generated peptides into the stroma, the release of the mature protein however, was instantaneous (Richter and Lamppa, 1999).

SPP is an essential protein in plants. Even down regulation of SPP can cause severe phenotypes with affected chloroplast biogenesis and development (Zhong et al., 2003). The precursor form of SPP is not proteolytically active. Thus, the SPP precursor is synthesized in an inactive form suggesting that the removal of the transit peptide is essential for the precursor to become active (Richter and Lamppa, 2003). Abolished processing of imported precursor proteins can also cause misfolding and aggregation.
Proteolysis in mitochondria and chloroplasts

Proteolysis plays a central part in life. Both within and outside cells dynamic processes take place where proteases are required. Proteases degrade damaged proteins for the recycling of amino acids, perform limited proteolysis in the maturation process of proteins, degradation during apoptosis and as a regulatory mechanism. Proteases therefore control many aspects of growth and development.

In *A. thaliana* there are at least 723 genes divided in 49 different families coding for potential proteases (Garcia-Lorenzo et al., 2006). The numbers are so comprehensive that it is not possible to describe all families. I will therefore focus on some of the mitochondrial and chloroplastic protease families, divided into two groups, ATP dependent and ATP independent proteases. The processing peptidases have been described previously in the section regarding the protein import machinery and will not be described here.

The ATP dependent proteases

The ATP dependent proteases are dependent on ATP to fulfill their roles. However, hydrolysis of peptide bonds does not require ATP, instead ATP is important for the unfolding of larger proteins to make them accessible for degradation. Proteases usually have their catalytic sites hidden from the general protein moiety to prevent unrestricted proteolysis. The major ATP dependent proteases in mitochondria and chloroplasts are the ClpP, FtsH and Lon proteases (Garcia-Lorenzo et al., 2006).

The Clp family of proteases

The Clp endopeptidase family is encoded by 26 genes in *A. thaliana* and belongs to the S14 Merops family of proteases. Clp proteases are serine proteases dependent on ATP for proteolysis (Garcia-Lorenzo et al., 2006). Indications that plants contained Clp proteases first became apparent in 1990 when Gottesman et al saw genetic evidence for a homolog of *Escherichia coli* ClpA in tomato (Gottesman et al., 1990). The first functional studies in plants came in 1995 when Shanklin et al studied the expression and localization of Clp proteins in *A. thaliana* in...
combination with studies of the proteolytic properties of ClpP from tobacco (Shanklin et al., 1995). It was shown that ClpP and ClpC as well as being constitutively expressed in all tissues were localized to the plastid stroma. Additionally ClpP immunopurified from tobacco extracts hydrolyzed the \textit{E. coli} ClpP substrate N-succinyl-Leu-Tyr-amidomethylcoumarin.

Most of the knowledge about the general features of the Clp proteases comes from what is known about the \textit{E. coli} Clp proteins. In \textit{E. coli} the Clp protease forms a two heptameric rings composed of ClpP subunits stacked on top of each other in a cylindrical shape (Wang et al., 1997). Inside the cylinder there is a large chamber housing 14 catalytic sites (one in each ClpP subunit). Access to the chamber is via two 12 Å pores in the center of the axis of the cylindrical structure. The ClpP subunits in \textit{E. coli} can, \textit{in vitro} efficiently degrade very small peptides \textasciitilde{}5 residues and only very slowly peptides up to 30 residues alone. However, ClpP can not position peptides with hydrophilic or charged residues at the P 1 position at the active site (Thompson and Maurizi, 1994). Degrading larger substrates requires the ATPase components for unfolding and translocation of the substrates into the catalytic chamber. There are also clear structural similarities with the 20S proteasome (Wang et al., 1997). In \textit{E. coli} the ATPase components are the ClpA and ClpX proteins belonging to the HSP100 family of chaperones. ClpA and ClpX form hexameric rings attached to the entrances of the catalytic core, functioning as unfoldases and recognition elements (Sakamoto, 2006).

Plants have a large number of proteins belonging to the Clp family. In \textit{A. thaliana} plastids alone there are at least 15 Clp family proteins. Of these 5 are ClpP serine proteases, 4 are ClpR (ClpP like proteins lacking the catalytic site). There are also the \textit{At}ClpC1, \textit{At}ClpC2 and \textit{At}ClpD that are similar to ClpA in \textit{E. coli}. Three other members of the Clp family with unknown function also exist namely \textit{At}ClpS1, \textit{At}ClpS2 and \textit{At}ClpT. The plastid Clp core is different from what has been seen in \textit{E.coli}. In \textit{Brassicae} the core seems to be composed of a hetero-tetradecamers consisting of different ClpP, ClpR and ClpS subunits and even variations in the composition of the Clp cores based on the type of plastid. In contrast plant mitochondria contain a homo tetradecameric Clp core complex consisting of only ClpP2 subunits, lacking the ClpR and ClpS subunits. ClpX might be present in the mitochondria (Peltier et al., 2004; Adam et al., 2006).

The FtsH family of proteases

The FtsH proteases are membrane bound ATP dependent metalloproteases carrying an AAA domain (ATPases associated with various cellular activities) (Ito and Akiyama, 2005).

Similar to Clp the first discovery of an FtsH protease was made in \textit{E. coli}, that together with what has been discovered in yeast have provided most of
our current knowledge. In 1995 Tomoyasu et al showed that FtsH purified from E. coli was able to degrade the σ-32 protein and that the activity was dependent on both ATP and Zn$^{2+}$ (Tomoyasu et al., 1995). Structurally FtsH forms a ring shaped hexamer anchored in the membrane via two N-terminal transmembrane segments. The protease domain and the AAA domain are located in the E. coli cytoplasm. One of the most interesting features of the FtsH protease is that it can actively dislocate membrane proteins from the membrane in order to degrade them. In general the FtsH proteases are believed to be involved in degradation of short lived regulatory proteins and unassembled subunits in the membrane (Ito and Akiyama, 2005).

The inner membrane of yeast mitochondria harbors two FtsH proteases, the i-AAA protease, facing the IMS, and the m-AAA protease, facing the MA. The i-AAA protease is composed of Yme1 subunits whereas m-AAA proteases are built up of Yta10 and Yta12 subunits (Leonhard et al., 1996).

In A. thaliana 16 genes encode possible FtsH proteases but four lack the catalytic residues. Of these 12 three (AtFtsH3, 4 and 10) are located in the mitochondria and 9 (AtFtsH1, 2, 5-12) in the chloroplast (Sakamoto et al., 2003). AtFtsH11 is slightly special since it has recently been found to be dually targeted to both mitochondria and chloroplasts (Urantowka et al., 2005). Five of the FtsH proteins found in A. thaliana chloroplasts have been implicated in the degradation of photosynthetic proteins during different light conditions (Garcia-Lorenzo et al., 2006).

Most of the FtsH proteins in A. thaliana are present in homologous pairs formed by gene duplications, and within the pairs they are fully redundant. The pairs AtFtsH1/AtFtsH5 and AtFtsH2/AtFtsH8 form complexes by mixing between the pairs, thereby creating an FtsH complex consisting of two AtFtsH1 or AtFtsH5 subunits in complex with four AtFtsH2 or AtFtsH8 subunits. AtFtsH2 and AtFtsH5 are the most abundantly expressed and a knockout of either of the two produces a variegated phenotype. Knockouts of AtFtsH1 and AtFtsH8 presents a wild type phenotype (Adam et al., 2006). In mitochondria AtFtsH4 and AtFtsH11 are similar to Yme1, the i-AAA protease in yeast. However, they were unable to complement a Yme1 deficient strain, suggesting possible divergence in functionality (Urantowka et al., 2005).

**The Lon family of proteases**

The first characterization of the Lon protease was done in E. coli 1981 (Charette et al., 1981) where Lon was demonstrated to be an ATP dependent protease of 94 kDa. Lon performs intracellular proteolysis of abnormal and regulatory proteins as a part of a biological control system affecting processes ranging from membrane fusion to disassembly of protein complexes. Lon is also an AAA protease just like Clp and FtsH.
In *E.coli* two types of Lon exist, namely A and B. Both harbor an AAA-domain and a P-domain containing the active site in the form of a catalytic Ser-Lys dyad. Only LonB has an N-terminal transmembrane domain. Structural data suggests different oligomeric structures of Lon, consisting of four to eight identical subunits. However, recent data suggests a hexameric ring like structure (Rotanova et al., 2006).

In *A. thaliana* 11 genes have been assigned to the Lon family, being named *AtLon1-11*. *AtLon9* and *AtLon10* lack the AAA domain, but they were considered to have similarities significant enough to place them in the same family (Garcia-Lorenzo et al., 2006). However, there are data suggesting that only four Lon proteins are functional in organelar proteolysis in *A. thaliana*. Namely *AtLon1-AtLon4* since they possess the common motifs for Lon proteases (AAA and the proteolytic domain). Localization studies of *A. thaliana* *AtLon1-4* revealed multiple intracellular localizations. *AtLon1* was localized to mitochondria whereas *AtLon2* was localized to the peroxisomes, *AtLon4* has been found to be dually targeted to both mitochondria and chloroplasts. *AtLon3* is suspected to be a pseudogene since it has been impossible to obtain cDNA from it (Ostersetzer et al., 2007).

The ATP independent proteases

The rhomboid family of proteases

Rhomboid proteases are a relatively newly discovered family of proteases. It was not until 2001 that Rhomboid-1 was identified as a protease in *Drosophila*, capable of performing regulated intermembrane proteolysis (RIP). Rhomboid-1 was shown to be a membrane serine protease responsible for the cleavage of the membrane-anchored TGF alpha-like growth factor Spitz in the Golgi. It was also clear that Rhomboid-1 is conserved throughout evolution from archaea to plants and humans (Lee et al., 2001; Urban et al., 2001). The rhomboid family seems to be one of the most conserved membrane protein families known.

Recently five structures have been solved of bacterial rhomboid proteins namely, *E. coli* GlpG in different conformations (Wang et al., 2006; Wu et al., 2006; Ben-Shem et al., 2007; Wang and Ha, 2007) and *Haemophilus influenzae* GlpG (Lemieux et al., 2007). The structures all showed 6 transmembrane helixes arranged in an orderly fashion creating a funnel inside which the catalytic serine and histidines were located on TM helix 4 and 6. In spite of the recent structural information the structures could not answer how the substrate can access the catalytic site, since it is shielded
from the membrane environment where the substrate resides. Two different mechanisms were proposed regarding either the movement of a large loop in the structure called L1 revealing an entrance point between TM1 and TM3 or that the substrate enters between TM5 and TM6. Mutational analysis revealed that substitutions in the L1 loop efficiently abolished the proteolytic activity, whereas the substitutions in the transmembrane helixes produced more random results clearly indicating that the L1 loop probably is covering the entrance point (Reviewed in Lieberman and Wolfe, 2007).

In yeast a mitochondrial rhomboid protease called Pcp1 has been identified as the protease responsible for the processing of Mgm1, involved in mitochondrial membrane remodeling and also for processing of Ccp1. The mammalian homolog PARL was capable of complementing a yeast Pcp1 knockout strain (Esser et al., 2002; McQuibban et al., 2003).

\textit{A. thaliana} contains 15 genes coding for potential rhomboid proteases, however, the experimental data are scarce in plants. Two rhomboids have been localized so far to the Golgi in \textit{A. thaliana} namely \textit{AtRBL1} and \textit{AtRBL2} (Kanaoka et al., 2005). For the other rhomboids in \textit{A. thaliana} only predictions exist regarding localization (Garcia-Lorenzo et al., 2006).

\section*{The Deg family of proteases}

DegP was initially characterized in \textit{E. coli} in 1989 when it was shown to be a protein of 48 kDa required for high temperature growth, hence the initial name HtrA (Lipinska et al., 1989). In 1990 the \textit{E. coli} HtrA (DegP) was purified and characterized as an ATP independent endopeptidase sensitive to diisopropylfluorophosphate, suggesting that DegP is a serine protease (Lipinska et al., 1990). Later experiments showed that DegP was a temperature sensitive serine protease. Structurally DegP forms a hexamer consisting of two trimeric rings of DegP subunits. The proteolytic sites are located in a central cavity that is only accessible laterally. Apparently DegP can exist in two conformations. In the chaperone conformation at low temperature, the protease domain of DegP exists in an inactive state, in which substrate binding in addition to catalysis is abolished. Elevated temperature on the other hand causes a conformational change in DegP allowing proteolysis to take place. The suggested reason for this is that the extensive protein damage which can occur at high temperature makes degradation more efficient than refolding, so at low temperature DegP acts as a chaperone aiding refolding instead of being a protease (Clausen et al., 2002).

\textit{E. coli} and cyanobacteria have three members of the Deg protease family each, in \textit{A. thaliana} there are 16 genes coding for Deg proteases, \textit{AtDeg1-16}. \textit{AtDeg1}, 2, 5 and 8 have all been localized to the chloroplast. All of them except \textit{AtDeg2}, which is located on the stromal side of the thylakoid membrane, are located in the thylakoid lumen. \textit{AtDeg3}, 4, 6, 10, 11, 12, 13
and 15 have all been predicted to be mitochondrial (Garcia-Lorenzo et al., 2006). However, AtDeg15 have also been predicted to be peroxisomal and a AtDeg15 T-DNA insertion line in A. thaliana was unable to process the glyoxysomal malate dehydrogenase precursor to its mature form (Helm et al., 2007). The localization of AtDeg7 is unknown. AtDeg1, and AtDeg2 have both been shown to be involved in repair of photosystem II in A. thaliana. The results correlate well with the increased expression of AtDeg1, AtDeg2 and also AtDeg8 seen during light stress (Haussuhl et al., 2001; Sinvany-Villalobo et al., 2004; Kapri-Pardes et al., 2007). Generation of homozygous AtDeg1 knockout lines was unsuccessful suggesting that AtDeg1 is an essential protein, even reduced levels of AtDeg1 showed a relatively strong phenotype of reduced growth and early flowering in combination with a pale green phenotype (Kapri-Pardes et al., 2007).

PreP

PreP, the presequence protease (Paper II, III and IV) is also an ATP independent protease located into both mitochondria and chloroplasts. It will be described below in detail since it is the main subject of this thesis.

The processing peptidases

The ATP independent mitochondrial and chloroplastic processing peptidases, MPP, MIP, IMP and SPP have previously been described in the section, Protein import machinery.

Additional proteases

SppA is an ATP independent serine protease. In A. thaliana AtSppA displays significant homology with the prokaryotic membrane bound protease SppA, also called protease IV (Lensch et al., 2001). In E. coli SppA has been characterized as a signal peptide peptidase responsible for the degradation of signal peptides cleaved by the leader peptidase (Suzuki et al., 1987). In plants SppA is located in the chloroplast thylakoid membranes and according to protease assays, it is partially exposed to the stroma. The expression of SppA seems to be light dependent. Similar to E. coli SppA, AtSppA forms a complex of about 270 kDa suggesting a homotetrameric complex (Lensch et al., 2001). Recently the structure of the E. coli SppA was solved, showing that SppA in E. coli indeed forms a homotetrameric complex, containing four active sites. The active sites are composed of catalytic Serine-Lysine dyads residing in a “hydrophobic bowl”. A similar structural arrangement is also suggested in plant homologs (Kim et al., 2008).
EGY1 is an ATP independent metalloprotease of 59 kDa containing eight trans-membrane domains. Initially it was identified as an ethylene dependent gravitropism-deficient and yellow green 1 mutant (Hence the name EGY1). Deletion of the EGY1 gene produces a dual phenotype, reduced chlorophyll accumulation and abnormal hypocotyl gravicurvature (sense of gravity and direction of growth). Experiments using EGY1-GFP fusion constructs showed that EGY1 in localized to chloroplasts. EGY1 mutants show reduced grana and poorly developed lamellae networks combined with reduced amounts of light harvesting complexes. The regulation of gravicurvature seems to be directly connected to chloroplast size and development (Chen et al., 2005; Guo et al., 2007).

CND41 was first described as a DNA binding protein of 41 kDa in tobacco chloroplasts (Nakano et al., 1997). A few years later it was established that CND41 possess ATP dependent proteolytic activity, sequence characteristics of an aspartic acid protease as well as a low pH optimum (2-4) (Murakami et al., 2000). Interestingly it was shown that CND41 was inhibited by ATP, suggesting an inhibitory mechanism during normal conditions. This would also be consistent with the pH optimum that is outside the pH range observed under normal conditions. Young and mature plants overexpressing CND41 in tobacco did not present any phenotype. However, senescence was promoted in yellowing leaves suggesting an important role for CND41 in senescence. Indeed CND41 is only significantly present in wild type plants during senescence, suggesting that the overexpressed CND41 is inactive until senescence. During senescence CND41 is activated by a slight processing. It had previously been demonstrated that there was a link between the degradation of rubisco and CND41, which now was strengthened (Kato et al., 2005).

PreP, the organellar peptidasome

The discovery
PreP, the presequence protease was initially identified in the MA prepared from potato tuber mitochondria as a mitochondrial presequence degrading protease, hence the name PreP (Stahl et al., 2002). However, indications for the existence of such a protease came earlier. First of all the fate of presequences was unknown, only one had been assigned a function namely subunit 9 of the cytochrome bc1 complex (Brandt et al., 1993). The absence of short peptides suggested the removal by either the
action of proteases or via some form of export system. This is necessary since accumulation of peptides would probably be detrimental to the mitochondria. Mitochondrial presequences have the capacity to dissipate the membrane potential and to induce mitochondrial permeability transition (Hugosson et al., 1994; Nicolay et al., 1994; Kushnareva et al., 2001). The first experimental evidence for degradation came when rapid degradation of the pF₁β presequence in mitochondrial lysates was observed (Stahl et al., 2000).

The proteolytic activity was mainly restricted to the matrix fraction, making it possible to devise a purification procedure capable of isolating the proteolytic activity to a relatively small number of mitochondrial proteins. Using 2D gel electrophoresis 8 different protein spots were identified but only one matched to a predicted protease(s). Namely a zinc metallo protease (Zn-MP) and a putative zinc metallo protease (Put Zn-Mp) in A. thaliana according to the NCBI database (Stahl et al., 2002). These two highly similar proteases will be referred to as AtPreP1 and AtPreP2, respectively.

Evolution and origin of PreP

The MEROPS database is built upon two different levels of organization, namely family and clan. Peptidases are assigned to a family based on sequence similarity and families thought to be homologous are assigned to clans (Rawlings et al., 2006). PreP is a member of the ME clan, family M16 and subfamily C of peptidases. The ME clan is identified by the presence of an inverted metal binding HXXEH motif and the model peptidase for the M16 family is the E.coli protease pitrilysin. However, the M16 family is further divided into smaller subfamilies. Namely A, B and C. Typical members of the respective families are in A: Pitrilysin and IDE, in B: MPP and SPP and in C: PreP and falcilysin.

Homologs of PreP are present in all kingdoms except among archea (and viruses). The AtPreP ortholog in yeast is called MOP112 (Kambacheld et al., 2005) and in human hPreP (Falkevall et al., 2006) or hMP1 (Mzhavia et al., 1999). Both orthologs have significant similarity to AtPreP1 and AtPreP2. Deletion of MOP112 was shown to influence peptide export from yeast mitochondria. hPreP might have a connection the neurodegenerative disorder known as Alzheimer disease. However, MOP112 is different from the plant and mammalian counterparts since there is a discrepancy in the intracellular localization. MOP112 has been localized to the IMS in yeast (Kambacheld et al., 2005), whereas mammalian (Falkevall et al., 2006) and plant PreP (Berglund et al, unpublished) are located in the matrix.

In most organisms e.g. human, tomato, rice and populus, only one ortholog of PreP is present but in A. thaliana there are two isoforms, which are the result of a recent gene duplication event (paper III and paper IV).
Phylogenetic analysis (Figure 3) of PreP indicates that cyanobacteria do not contain any PreP orthologs, whereas the δ-proteobacteria and green nonsulfur bacteria do. Human, yeast and plant PrePs group together with the δ-proteobacterial and green nonsulfur bacterial homologs, whereas cyanobacterial homologs group closer together with yeast, mammalian and plant MPP. The δ-proteobacteria, are related to the α-proteobacteria, the proposed ancestor of mitochondria and the green non sulfur bacteria. AtPreP1 is 47% identical over 975 residues to a protein in the δ-proteobacteria (Syntrophobacter fumaroxidans) and also to a protein in the green nonsulfur bacteria (Roseiflexus castenholzii). In addition, the closest homologs of PreP in cyanobacteria according to the cyanobacterial genome database (cyanobase) are more closely related to MPP in A. thaliana than to PreP.

Figure 3. Evolutionary analysis of PreP with other homologs. An unrooted phylogenetic tree of PreP1 together with homologs from Genbank and Cyanobase. At, Arabidopsis thaliana; Hs, Homo sapiens; Sc, Sacharomyces cerevisae; Pp, Physcomitrella patens; Re, Roseiflexus castenholzii (NC_009767); Sf, Syntrophobacter fumaroxidans (ABK17348); Te, Thermosyneccoccus elongatus (tll0051); Ss, Synechocystis sp. (sll2008); As, Anabaena sp. (all1021). MPP, mitochondrial processing peptidase; MOP112, mitochondrial oligopeptidase of 112 kDa. Alignment was performed by T-Coffee 5.05. Phylogenetic tree was constructed by using the PHYLIP program package for inferring phylogenies according to: Felsenstein, J. 2005. PHYLIP (Phylogeny Inference Package) version 3.6.
The degree of identity to AtPreP is low, it does not exceed 25% for any of the cyanobacterial homologs. The results indicate a bacterial origin of PreP rather than a cyanobacterial in the eukaryote lineage (Nilsson et al unpublished).

**Dual localization of PreP**

Analysis of the N-terminal portion of AtPreP1 in *A. thaliana* using targeting prediction programs gave an indication that AtPreP1 possibly could be localized to both mitochondria and chloroplasts. As a consequence the subcellular localization and targeting capacity of AtPreP1 was investigated using *in vitro* approaches such as import of radiolabeled constructs into mitochondria and chloroplasts alone or in a dual system (Rudhe et al., 2002) and also by *in vivo* import into tobacco protoplasts, using AtPreP1-GFP fusion constructs. In addition, Western blot analysis was also used to investigate the PreP content in whole mitochondria and chloroplasts, as well as subfractionated mitochondria and chloroplasts (Bhushan et al., 2003).

The results showed that; AtPreP1 was localized to both mitochondria and chloroplasts. Western blot analysis utilizing an antibody that recognizes both AtPreP1 and AtPreP2 could not distinguish which one of the isoforms was actually present where and more importantly if both AtPreP1 and AtPreP2 or only one of them is present *in vivo*. However, it was possible to establish that at least one isoform of PreP was present in the MA as well as in the chloroplast stroma (Bhushan et al., 2003; Moberg et al., 2003).

AtPreP2 was investigated in a similar manner as AtPreP1 (Paper II). AtPreP2 displayed similar results as previously observed for AtPreP1. AtPreP2 was also dually targeted to both mitochondria and chloroplasts. *In vitro* import into mitochondria and chloroplasts using either the single or dual import system as well as *in vivo* methods (GFP-AtPreP2 fusion construct) clearly showed that AtPreP2, just as AtPreP1 had the capacity to be targeted and imported into both mitochondria and chloroplasts. An additional method was used for analyzing the localization of PreP. The protein content of purified chloroplasts was extracted, enzymatically digested with trypsin and the peptide content analyzed using capillary HPLC in combination with ESI-MS/MS. Several unique peptides were identified for both AtPreP1 and AtPreP2, with high scores for AtPreP1. It was also possible to detect transcripts of both AtPreP1 and AtPreP2 which clearly shows that both proteins are expressed in a tissue dependent manner. In conclusion, both AtPreP1 and AtPreP2 are dually targeted to mitochondria and chloroplasts (paper II).
Intracellular function of PreP

Initially PreP was considered as a presequence degrading protease (Stahl et al., 2000; Stahl et al., 2002). However, it did not take long until PreP was shown to be a more general protease (Moberg et al., 2003). PreP could also degrade transit peptides as well as other unstructured peptides (paper II).

In an effort to investigate the similarities and differences between AtPreP1 and AtPreP2 in A. thaliana several studies were undertaken (Paper III). It was clear that AtPreP1 and AtPreP2 were different from each other regarding substrate specificity. Both the degradation patterns obtained for a fluorescent substrate and of the mitochondrial pF1β presequence of Nicotiana plumbaginifolia showed clear differences. Degradation of the fluorescent peptide generated an intermediate fragment almost inaccessible to degradation by AtPreP2, whereas AtPreP1 did not form a similar fragment at all. However, the results from the degradation of the overexpressed presequence were more informative. MALDI-TOF analysis of the degradation products revealed unique fragments. The fragments generated by AtPreP1 were in the range of 10 to 16 residues, whereas the fragments produced by AtPreP2 were ranging from 10 to 24 residues. The fragments made it possible to map the cleavage sites within the presequence for both proteases. Both proteases showed a preference for basic amino acids in the P1’ position and small, uncharged amino acids or serine residues in the P1 position. It was also possible to show that both AtPreP1 and AtPreP2 had unique cleavage sites within the presequence.

Degradation studies of different PreP substrates as well as the results from the fragment analysis above showed that AtPreP1 and AtPreP2 were similar in many respects but also different in some aspects. The C-terminal part of the pF1β presequence was almost inaccessible for degradation by AtPreP2. On the other hand AtPreP1 degraded it efficiently. It was also established that AtPreP1 and AtPreP2 both had a maximum substrate size limit of ~65 residues and that efficiently excluded folded proteins such as insulin. AtPreP1 and AtPreP2 could also degrade substrates unrelated to targeting peptides. These results in combination with phylogenetic analysis indicate that AtPreP1 and AtPreP2 have undergone subfunctionalisation and that AtPreP1 and AtPreP2 are general peptidases responsible for the degradation of an array of different peptides.

The recently solved structure of AtPreP1 (Johnson et al., 2006) supports many of the findings described above and will be discussed in more detail below.
Crystal structure and proteolytic mechanism of PreP

The structure of PreP (Figure 4) was solved from an overexpressed and crystallized inactive $At$PreP1 variant at 2.1 Å (Johnson et al., 2006). It was the first structure of an M16 protease in a closed, substrate bound conformation. Even though the protease was expressed in an inactive conformation and crystallized in absence of any added substrate, the catalytic site presented an electron density corresponding to a six residue peptide.

The structure showed that $At$PreP1 is comprised of four topologically similar domains, which together form two bowl shaped halves connected by a hinge region. The two halves in PreP, form a large internal chamber of about 10 000 Å$^3$ where the active site resides. In the closed conformation the active site is completely shielded from the outside environment and there is no way for a protein or peptide to enter into the closed chamber. This clearly defines the maximum size of substrate that can be degraded, it has to fit into the chamber and the chamber has to close to form the active site. This is in accordance with the results in paper III where a maximum size of substrate was established to be ~65 residues.

The active site is formed by the inverted zinc binding motif HXXEH, where His77 and His81 coordinate the zinc ion and the Glu80 acts as a base catalyst. Additional important residues discovered on the basis of the structure are the Glu177, which is an additional zinc ligand and the two most surprising residues, the Arg848 and Tyr854 from the C-terminal part of $At$PreP1. These residues forms hydrogen bonds with the bound substrate and
thereby probably stabilize the transition state. Indeed both are essential for proteolytic activity as substitution abolishes activity. These results can also help explain why deleting the C-terminal domain of SPP (a member of the M16 protease subfamily B) results in an inactive protease that is still capable of binding a substrate (Richter and Lamppa, 2003). Residues from the C-terminus are vital for activity and the enzyme has to be in a closed conformation to catalyze cleavage.

Another interesting observation was that other metals in addition to zinc are required for proteolytic activity. Crystallization was only possible in the presence of Mg$^{2+}$ and in the structure there are two Mg$^{2+}$ binding sites. One resides between domain 3 and 4, the second is at the surface of the fourth domain. Investigation of the proteolytic activity in the presence or absence of different divalent ions clearly showed that the activity was metal dependent. No activity could be seen in the absence of Mg$^{2+}$ or Ca$^{2+}$ and the activity was restored completely at 10 mM concentration of either ion.

Since the active site resides inside a closed cavity within PreP, motion is required for access to the catalytic sites. This was demonstrated by the introduction of cysteine bridges between the two halves. Under oxidizing conditions a cysteine bridge is formed, which locks the enzyme in a closed conformation. The activity was restored under reducing conditions.

Fortunate events during the crystallization of AtPreP1 led to the co-crystallization of a six residue long peptide in the active site (Johnson et al., 2006). Since the peptide originated from the overexpression in *E. coli*, the bound peptide was a representation of an average peptide and not a single, unique peptide, it was possible to deduce the most probable motif for a substrate. The electron density indicated that LTR with the cleavage occurring between the threonine and arginine is the preferred optimal cleavage motif. These results agree well with the findings in paper III.

**PreP and Alzheimer disease**

Alzheimer disease is a severe neurodegenerative disease associated with neuronal death, dementia and ultimately death. It is also common with mood swings, paranoia and aggression. The neuropathologic features of Alzheimer disease are the formation of amyloid plaques and neurofibrillar tangles. The extracellular plaques are built up of 39-42 residue peptides originating from the amyloid precursor protein (Clark and Karlawish, 2003). Most attention has focused on the extracellular effects of amyloid formation, but recently it has shifted towards intracellular processes involved in the progression of Alzheimer disease. Mitochondria have been shown to be affected early in the progression of the disease with altered metabolism as one of the earliest signs. The amyloid β-protein (Aβ) has been shown to bind the amyloid-β binding alcohol dehydrogenase (ABAD) and induce apoptosis, which can be inhibited by
interfering the interaction of ABAD and Aβ (Lustbader et al., 2004). It has also been shown that the toxic Aβ peptide accumulates in the matrix of brain mitochondria from Alzheimer patients and that accumulation can be seen prior to plaque formation. The accumulation was associated with impaired mitochondrial function (Caspersen et al., 2005).

Recently hPreP was characterized. It was shown to be located to mitochondria and to be capable of degrading several different types of Aβ peptides including Aβ1-40, Aβ1-42 and the Arctic form of Aβ. Immuno-inhibition studies also showed that hPreP was the only protease present in the mammalian mitochondria capable of degrading Aβ peptides. Thus, hPreP might be an important link towards understanding Alzheimer disease (Falkevall et al., 2006).

The PreP double knockout in A. thaliana
In order to further analyze PreP in vivo and the damage caused by targeting peptides to biological membranes, we decided to test the effect of single and double knockouts of AtPreP1 and AtPreP2 (Paper IV). The knockouts were generated in A. thaliana by utilizing T-DNA insertion lines obtained from the SALK collection for AtPreP1/At3g19170 (Salk_048944) and AtPreP2/At1g49630 (Salk_133220).

The phenotype and morphological changes in the knockout plants
It was clear from the initial analysis of the single PreP mutants in A. thaliana that a high degree of complementation existed between AtPreP1 and AtPreP2. Only a very weak pale green phenotype was seen close to the center of the rosette in the AtPreP1 knockout strain whereas the AtPreP2 knockout seemed unaffected. The PreP double knockout presented a stronger phenotype.

The first true leaves of the PreP double knockout were pale green, which was a clear indication of affected chloroplasts. Indeed, measurements of chlorophyll content in wild type and young PreP double knockout seedlings clearly showed a decrease in total chlorophyll compared to the wild type. The double knockout plants became gradually more like wild type plants during development and in adult plants only the center of the rosette was still pale green. Additionally, the double knockouts were also smaller in size than the wild type, whereas the single mutants were comparable to the wild type.

Analysis of AtPreP1 and AtPreP2 expression and AtPreP1 and AtPreP2 protein content in wild type single and double knockouts clearly showed that AtPreP1 is the “master” protease. AtPreP1 is present at much higher levels in
all tissues and all developmental stages with regards to expression and also higher with respect to protein content. This also helps explain why the AtPreP1 knockout could not be fully complemented by AtPreP2. AtPreP2 is probably not expressed to sufficient levels in the AtPreP1 knockout to fully complement the AtPreP1 knockout.

Electron microscopy analysis of the first true leaves also showed that the double knockout and the AtPreP1 knockout chloroplasts contained substantially less starch than wild type. In addition, the double knockout had less stacked grana, thylakoids and almost no starch. There were also changes in the mitochondrial morphology in the double knockout compared to wild type. The sizes of the double knockout mitochondria varied to a great extent and were frequently seen in clusters. Considering the variations in mitochondrial morphology it is not surprising that the double knockout mitochondria were partially uncoupled compared to wild type. No differences could be seen in the respiration of mitochondria prepared from adult plants.

It is clear that PreP is important for maintaining the normal function of mitochondria and chloroplasts in young seedlings and it is possible that the higher respiratory activity of mitochondria in young seedlings, makes them more sensitive to the stress caused by reduced or abolished peptide degradation. The loss of PreP might allow accumulation of undigested peptides in sufficient amounts to interfere with mitochondrial function and/or to cause uncoupling. Uncoupling inevitably leads to energy losses that need to be compensated for. One way is an increased starch consumption to maintain metabolism and respiration (Zeeman et al., 2007). This hypothesis is supported by experimental data showing reduced starch inside AtPreP1 and especially in double knockout chloroplasts.

Peptidolysis in the PreP double knockout

It was also of great interest to investigate how the peptidolytic activity was affected in PreP double knockout mitochondria and chloroplasts. Wild type mitochondria easily degraded the pF1β2-54 presequence that is a model substrate for PreP while there was no activity in the double knockout mitochondria. Clearly, the double knockout mitochondria did not contain any additional protease capable of degrading the pF1β2-54. This was not completely surprising since immunoinhibition of MA extracts have been shown to be completely inhibited by PreP antibodies. However, it was not unlikely that some protease could have been upregulated as a compensatory mechanism upon loss of PreP.

In the chloroplasts the situation was somewhat different. The peptidolytic activity in the chloroplast stroma was completely absent in the PreP double knockout, which is similar to what was seen in the MA fraction.
both wild type and the PreP double knockout there was a minor but significant metal dependent proteolytic activity against the pF1β2.54 presequence present in the chloroplast membrane fractions.

Compensatory mechanisms

The complete loss of proteolytic activity in mitochondria inevitably leads to the conclusion that some other system exists for the removal potentially toxic peptides. In reality there are only two ways of removing peptides: They are either degraded into single amino acids or smaller fragments or exported for degradation in a different location by a different proteolytic system.

Peptide export in mitochondria has previously been studied. The deletion of the PreP homolog MOP112 in *S. cerevisae* caused an increase in peptide export from the MA, presumably via the ABC transporter Mdl1 located in the IM. Furthermore, a deletion of Mdl1 in yeast decreased mitochondrial peptide export without peptide accumulation inside the MA (Young et al., 2001). Similar to what we observed in *A. thaliana*, deletion of MOP112 also impaired respiratory growth in yeast (Kambacheld et al., 2005).

*A. thaliana* encodes at least 120 different ABC transporters involved in various types of transport ranging from ion to peptide transport. The closest homologs of the yeast Mdl1 transporter are the *AtTAP1* and *AtTAP2* proteins, which have been located to small punctuated structures (Rea, unpublished). This indicates the possible existence of a peptide export system in plant mitochondria similar to that found in yeast (Rea, 2007). However, the rescue mechanism in the PreP double knockout (and to some extent the *AtPreP1* knockout plants) is not perfect since seedlings display signs of stress, such as ~40% reduction in growth rate, chlorosis in newly formed leaves and impaired organellar function.

In the chloroplast there was an additional peptidolytic activity present in the membrane fraction. The presence of this other protease(s) in the chloroplast might be sufficient to prevent a more severe phenotype. Additionally, it is also possible that some peptides are exported via ABC transporters.

The experimental data indicate that PreP is most important during high mitochondrial activities both in yeast during respiratory growth and in plants during early development since it is under these conditions the phenotypes and biochemical changes have been observed. A possible explanation is that mitochondria in young seedlings have a higher activity, and turnover of proteins. As a consequence they require more active proteolysis, peptidolysis or peptide export. In the double knockout however, it is possible that peptide accumulation occurs to an extent that affects membrane integrity of mitochondria and chloroplasts. Adult plants do not seem to be greatly
affected by the loss of PreP. However, during conditions where the mitochondrial activity is high such as in young seedlings, its importance is more apparent.
Future perspectives and concluding remarks

In recent years a wealth of data regarding PreP has been accumulated. Intracellular localization, substrate specificity and expression have been investigated. In addition, the 3D structure of AtPreP1 has been solved and the effects of a PreP double knockout in plants have been characterized. Still there are many unanswered questions.

One of the main questions that relate to the present study would be to investigate whether AtPreP1 and AtPreP2 are differentially regulated under different forms of stress such as heat, salt, drought and light as well as to analyze how the single and double knockouts are affected under these conditions. Another question would be why PreP is present at higher levels in flowering tissues than in other tissues? Is it possible that a high protein turnover in flowering tissues requires up-regulation of proteolytic systems including PreP. Furthermore, it has to be determined if the phenotype observed in the PreP double knockout is caused mainly by dysfunction of mitochondria, chloroplasts or both organelles. Present results cannot distinguish if the effect on the chloroplasts is secondary and caused by mitochondrial dysfunction. In order to answer this question, PreP constructs should be designed with specific mitochondrial or chloroplast targeting peptides to enable selective complementation in each compartment. Stable transformation of the double knockout with these constructs should provide the information about which organelle is responsible for the observed effects.

The current hypothesis regarding the rescue mechanisms that help to prevent damage and death caused by peptide accumulation in plant or yeast PreP knockout mitochondria is that peptides might be exported. Increased efforts should be made to characterize the ABC transporters that have capacity to transport peptides across membranes. Preferably in a PreP knockout background. Furthermore, in the course of our studies an enigmatic proteolytic activity was found in the chloroplast membrane that was capable of performing similar function as PreP, i.e. degradation of short unstructured peptides in a metal dependent manner. It would be of great interest to identify this protease to increase our understanding of the proteolytic processes in chloroplasts.

As PreP is present in both mitochondria and chloroplasts it is of high interest to investigate the evolutionary origin of PreP. Sequence analysis suggests a bacterial rather than cyanobacterial origin of PreP. PreP has a dual targeting peptide and it is not known how these peptides interact with organellar receptors. 3D structural data of dual targeting peptides especially in complex with organellar receptors would provide crucial information in our understanding of the sorting process.

The structure of AtPreP1 was solved in a closed conformation. It would be interesting to solve the structure of PreP in an open conformation that
would allow us to draw detailed conclusions about the dimensions for substrate binding and access to the catalytic cavity. The structure of *At*PreP1 also revealed two novel metal binding sites not connected to the active site. One residing between domain 3 and 4 and the other at the surface of the fourth domain (Johnson et al., 2006). *At*PreP1 is dependent on these two metal binding sites since substitution of residues in both sites leads to a reduced proteolytic activity (Bäckman et al. unpublished). Further investigations are required to examine why these metal binding sites are important.

The results obtained in plants as well as in yeast indicate that the mitochondria are dependent on PreP for normal function, especially during developmental stages when a high mitochondrial activity is required. Studying PreP *in vivo*, in mammalian systems could provide new insights into the functions of PreP and its proposed involvement in Alzheimer disease. What lies in the future is hidden until we are there.

"Veritate, scientia, labore"
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References


Bonnefoy N, Chalvet F, Hamel P, Slonimski PP, Dujardin G (1994) OXA1, a Saccharomyces cerevisiae nuclear gene whose sequence is conserved from prokaryotes to eukaryotes controls cytochrome oxidase biogenesis. J Mol Biol 239: 201-212


Braun HP, Emmermann M, Kruft V, Schmitz UK (1992) The general mitochondrial processing peptidase from potato is an integral part of cytochrome c reductase of the respiratory chain. EMBO J 11: 3219-3227


Chupin V, Leenhouts JM, de Kroon AI, de Kruijff B (1995) Cardiolipin modulates the secondary structure of the presequence peptide of cytochrome oxidase subunit IV: a 2D 1H-NMR study. FEBS Lett 373: 239-244


involves the mAAA complex and the putative rhomboid protease Pcp1. J Mol Biol 323: 835-843


PreP forms a unique 10,000 Angstroms\(^3\) chamber for proteolysis. EMBO J 25: 1977-1986


Kushnareva YE, Polster BM, Sokolove PM, Kinnally KW, Fiskum G (2001) Mitochondrial precursor signal peptide induces a unique
permeability transition and release of cytochrome c from liver and brain mitochondria. Arch Biochem Biophys 386: 251-260


May T, Soll J (2000) 14-3-3 proteins form a guidance complex with chloroplast precursor proteins in plants. Plant Cell 12: 53-64


chloroplast and mitochondrial proteases. Plant Physiol 135: 1336-1345


Yang M, Jensen RE, Yaffe MP, Oppliger W, Schatz G (1988) Import of proteins into yeast mitochondria: the purified matrix processing protease contains two subunits which are encoded by the nuclear MAS1 and MAS2 genes. EMBO J 7: 3857-3862
Yano M, Terada K, Mori M (2003) AIP is a mitochondrial import mediator that binds to both import receptor Tom20 and preproteins. J Cell Biol 163: 45-56


