Universitetet i Stavanger

DET TEKNISK-NATURVITENSKAPELIGE FAKULTET

MASTEROPPGAVE

Studieprogram/spesialisering: Master's degree in Biological Chemistry	Vår.semesteret, 2014..... Konfidensiell
Forfatter: Edit Schei	…… (signatur forfatter)
Fagansvarlig: Cathrine Lillo Veileder(e): Amr Ramzy Abass Kataya	
Tittel på masteroppgaven: Engelsk tittel: Identification of peroxisomal phosphatase-related proteins in *Arabidopsis thaliana*	
Studiepoeng: 60	
Emneord: Phosphatase Peroxisome PTD PTS	Sidetall: ……69…… + vedlegg/annet: …1… Stavanger, 10/6 2014 dato/år

TABLE OF CONTENT

ACCNOWLEDGEMENTS

I want to thank prof. Cathrine Lillo for giving me the opportunity to work in her lab, and for all the help she has been offering, writing this thesis. Thanks also to Dr Amr R.A. Kataya, for letting me in on his project. All your help, explanations and patience have been priceless, and your enthusiasm is a great inspiration. Thanks to the rest of the lab-members for always being kind and friendly, creating a great working atmosphere, and especially to Maria for always joining when the need for coffee became overwhelming.

I need to thank my family for their patient throughout this project. My children have helped me when the computer programs refuses to co-operate, and have given me space and peace to study. Special thanks goes to my dear husband, Trond, for always being positive and encouraging through all of these five years, never complaining and always being proud of me.

At last I want to dedicate this thesis to my mother, Reidunn Langeland, for showing me that it is never too late to fulfill a dream.

ABSTRACT

Phosphorylation is a key regulatory mechanism in all aspects of plant biology, and protein phosphorylation is the most frequent post-translational protein modification. The complete genomic sequence of the model plant *Arabidopsis thaliana* enables an extensive exploration of the phosphatases encoded by this organism.

The *Arabidopsis* genome contains almost 300 genes that encode proteins containing either Peroxisomal Targeting Signal (PTS) type 1 or type 2. The identification of peroxisomal protein kinases, phosphatases and phosphorylated proteins strongly suggests that plant peroxisomal metabolism is regulated by reversible phosphorylation.

A selection of 9 peroxisome-predicted phosphatase related proteins from *Arabidopsis thaliana* were investigated by *in vivo* subcellular targeting analysis. The purpose was to determine if they did target to peroxisomes. The chosen proteins were Purple Acid phosphatase 7 (PAP7), Mitogen activated kinase phosphatase 1 (MapKP1), Pol-like phosphatase 2 (PLL2), Pol-like phosphatase 3 (PLL3), Trehalose-6-phosphat phosphatase 1 (TPP1-100), 2-phosphoglycolate phosphatase 1 (GLP1), TIP41-like family protein (TIP41), Shewanella-like protein phosphatase 1 (SLP1) and a protein of the Endonuclease/exonuclease/phosphatase family (8030). The first 7 proteins (PAP7, MapKP1, PLL2, PLL3, TPP1-100, TIP41 and GLP1) was predicted to hold a putative peroxisomal targeting signal type 1 (PTS1) at the C-terminal. The SLP1 and 8030 was predicted to contain a putative PTS2 near the N-terminal.

Enhanced Yellow Fluorescence Protein (EYFP) was fused to the putative Peroxisomal Targeting Domain (PTD) of the investigated proteins. The constructs were transiently transformed into epidermal onion cells using a biolistic gene-gun and to *Arabidopsis* mesophyll protoplasts using PEG-transformation. The expression of the construct was examined using inverted fluorescence microscopy. As the PTS1 is the same in PLL2 and PLL3 a total of 8 PTD constructs was made. Out of these 8 PTD constructs, 5 showed to be targeted to peroxisomes. All the peroxisomal PTDs hold a putative PTS1-signal. The proteins with a PTD1 found in peroxisomes were PAP7, MapKP1, PLL2&3, TPP1-100 and TIP41. The last PTS1 predicted protein, as well as the 2 proteins predicted to hold a PTS2, were not found in peroxisomes.

Full-length constructs of 5 of the proteins tagged with EYFP were examined, and 2 were found in peroxisomes. The three proteins found in peroxisomes was Purple acid phosphatase 7 (PAP7) and Trehalose-6-phosphat phosphatase (TPP1-100) .Even though several following-up experiments should be conducted and some full-length phosphatase-constructs were not finished during the time-span of this thesis, the experiments performed during this work shows that phosphatases probably are a part of the regulatory system of peroxisomal metabolism.

ABBREVIATIONS

A	Adenine
Bp	Base pair
BSA	Bovine Serum Albumin
C	Cytosine
CaMV 35S	Cauliflower mosaic virus 35S promoter
CDS	Coding sequence
CFP	Cyan Fluorescence Protein
ER	Endoplasmatic reticulum
EYFP	Enhanced Yellow Fluorescence Protein
G	Guanine
H	Hours
H_2O_2	Hydrogen peroxide
IPTG	Isopropyl-beta-D-thiogalactopyranoside
LB	Luria Broth
MCS	Multiple Cloning Site
Min	minutes
OD	Optical Density
PCR	Polymerase Chain Reaction
PTD	Peroxisome targeting domain
PTS	Peroxisome Targeting Signal
RE	Restriction enzyme
RS	Restriction site
RT – PCR	Reverse Transcriptase Polymerase Chain
S	Reaction
T	Seconds
X-gal	Thymine
YFP	5-bromo-4-chloro-3-indolyl-beta-D-galactoside
	Yellow Fluorescence Protein

1. INTRODUCTION

1.1 PEROXISOMES

Peroxisomes are organelles found in all Eukaryotic cells. They are enclosed by a single membrane and are involved in numerous metabolic reactions. Plant peroxisomes are mainly involved in fatty acid β-oxidation, metabolism of reactive oxygen species and photorespiration. All peroxisomal proteins are encoded by nuclear genes, and have to be imported to the organelle post-translationally (Kaur et al. 2009).

1.1.1 Biogenesis and function

Several theories of peroxisome biogenesis have been proposed, but recent research indicates that two pathways exist. One is the *de novo* formation, where peroxisomal vesicles bud of from a special part of the ER, fuses, and thereby form mature peroxisomes. The other pathway is growth and fission of already existing peroxisomes (Smith and Aitchison 2013).

Several names have been used to describe the organelle. The composition of enzymes differs according to plant tissue and growth conditions. Terms like microbodies, peroxisomes and glyoxysomes are frequently used. It has been shown that the enzymes content inside the organelle is able to transform due to growth conditions (Fukao et al. 2002). To avoid confusion, peroxisomes are now used as the common name (Pracharoenwattana and Smith 2008).

The first peroxisomal task discovered, was the production and degradation of hydrogen peroxide (H_2O_2). Different oxidative reactions performed by peroxisomes result in the formation of this, to the cell, highly toxic compound. The enzyme catalase, which breaks down the H_2O_2 into molecular oxygen and water, are the most abundant enzyme found in the peroxisomes (Kaur et al. 2009).

In plants, the peroxisomes are the only site performing degradation of fatty acids. Fatty acids are imported to peroxisomes, were they are activated to their Coenzyme A-esters, and then enter the fatty acid β-oxidation pathway. This breakdown of fatty acids provides seedlings with essential energy and metabolites for growth into photosynthetic plants. The fatty acid β-oxidation is also important in the generation of several plant hormones, including Jasmonic acid (JA), and also in the remobilization of reserves during senescence (Hu et al. 2012).

The peroxisomes contain enzymes which are participating in the photorespiration process in cooperation with chloroplastic and mitochondrial enzymes. Photorespiration results from the oxygenase reaction, catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) and serves as a carbon recovery system (Maurino and Peterhansel 2010). During the photorespiratory pathway, two transamination reactions are performed in the peroxisomes. In addition large amounts of H_2O_2 is produced and needs to be degraded (Hodges et al. 2013).

1.1.2 Peroxisome Protein import and targeting signals

As all peroxisomal proteins are nuclear encoded and translated in the cytosol, they need to be imported into the organelle. The proteins carry special amino acid residues, which are recognized by transport proteins in the cytoplasm. Different peroxisomal targeting signal exists, and they are called type 1 and 2. Figure 1 shows the prototype of the two targeting signals, with the recognized residues marked in green.

Figure 1: Prototypes of Peroxisomal targeting signal (PTS) type 1 and type 2. Peroxisomal targeting signal residues are marked in green.

Most proteins targeted to the peroxisome, carry a Peroxisomal Prediction Signal type 1 (PTS1) at the C-terminal end. This signal is recognized by a receptor in the cytosol, called PEX5. The PTS1/ PEX5-complex docs by the peroxisomal membrane, this is probably assisted by PEX13 and PEX14. The PTS1 protein is imported into the peroxisome, and the PEX5 returns to the cytoplasm. Peroxisomal proteins containing a Peroxisomal Targeting Signal type 2 (PTS2), located near the N-terminal, are recognized by a protein called PEX7 in the cytoplasm. The PEX7 cannot enter the peroxisome on its own, and binds to PEX5. The PEX5 is then using the PEX13 and PEX14 interaction to transport the PTS2-containing protein into the organelle. A schematic overview of the protein import machinery of the peroxisome is shown in figure 2 (Hu et al. 2012).

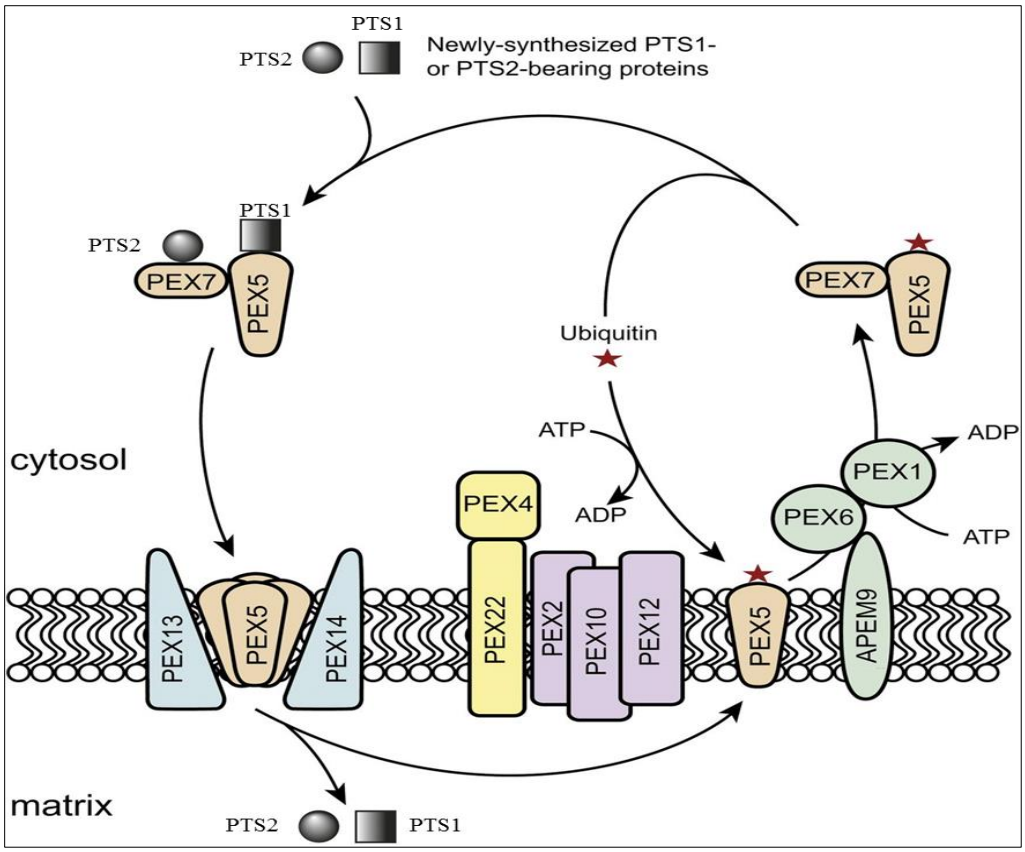


Figure 2: A schematic overview of the protein import system in peroxisomes. Cytosolic PEX5 and PEX7 recognize proteins via binding of PTS1 and PTS2, respectively. Cargo-loaded PEX5 associates with the membrane via interactions with PEX13 and PEX14. PEX7 cannot dock to the membrane on its own and depends on physical interaction with PEX5. PTS1- and PTS2-proteins are released to the matrix, and the receptors are recycled back into the cytosol (Hu et al. 2012).

Apart from a few exceptions, proteins are targeted to the peroxisome matrix by a conserved PTS1 or PTS2. The PTS1 is a Tripeptide found at the C-terminal end of the protein, and the signals can roughly be divided into two groups, namely major (canonical) and minor (noncanonical) PTS1s. The most common PTS1 is serine-lysine-leucine (SKL), but studies have revealed a degeneracy of the motif, and it usually consists of a small uncharged residue, a basic residue and a nonpolar residue (Chowdhary et al. 2012). Several bioinformatic prediction tools have been developed, and together with peroxisomal proteomic analyses, several combinations of the last tree residues have been found. Figure 3 shows combinations of residues at the different positions (Lingner et al. 2011). It has been showed that the properties of residues further upstream, also affects the impact of the targeting signal. Basic residues and proline seemed to enhance targeting efficiency (Reumann 2004).

Pos.	-3	-2	-1
1.	S	K	L
2.	A	R	M
3.	P	S	I
4.	C	N	V
5.	F	L	Y
6.	V	M	F
7.	G	H	
8.	T	G	
9.	L	E	
10.	K	T	
11.	I	F	
12.		P	
13.		Q	
14.		C	
15.		Y	

Figure 3: Amino acid residues predicted to make up different PTS1s.Tripeptide residues previously reported to be present in plant PTS1 tripeptides are shaded in gray. According to experimental data, at least two of the seven residues boxed must be combined with one low-abundance residue to yield functional plant PTS1 tripeptides (Lingner et al. 2011)

Most peroxisomal matrix proteins possess a PTS1 type signal. The PTS1 signal is not cleaved off after the import. Some peroxisomal proteins possess a PTS2. This is a conserved nonapeptid located in the N-terminal part of the protein. The consensus sequence is (R/K)-(L/V/I)-X_5-(H/Q)-(L/A/F), were X could be any amino acid residue (Reumann 2004).

There are also proteins that possess neither PTS1 nor PTS2 signals. Their transport may be based on "piggy-backing," which is translocation into the peroxisomal matrix together withPTS1 containing proteins (Ast et al. 2013).

Examples of dual targeting of proteins have also been shown (Kataya and Reumann 2010).

1.2 PHOSPHATASES

A phosphatase is an enzyme which catalyses a reaction that removes an organic phosphate group from a substrate. This is called dephosphorylation. The process is coupled to the phosphorylation process, which is the addition of a phosphate group to the same substrate by a phosphorylase or a kinase. The phosphatases consist of a large group of different enzymes, some more specific to their substrates than others.

1.2.1 Phosphatase families

The alkaline phosphatase, for example, is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. The enzyme is used as a tool in molecular cloning to remove the phosphate group from the 5'end of linearized DNA to prevent self-ligating.

Acid phosphatases are known to be influenced of the phosphate state in plants. A major group of plant acid phosphatases are the Purple Acid Phosphatases (PAPs). Evolutionarily, PAPs belong to the metallo-phosphoesterase family of proteins, members of which also include phosphoprotein phosphatases, diadenosine tetraphosphatases, exonucleases, 5'-nucleotidases, and other types of phosphomonoesterases (Olczak et al. 2003).

1.2.2 Protein Phosphatases

Essential to the regulation and post-translational modification of proteins are the protein phosphatases. The protein phosphatases remove phosphate groups from specific amino acid residues in proteins, mainly serine, threonine or tyrosine. The enzymes attaching phosphate groups to the same residues are called protein kinases. Protein kinases covalently bind phosphate groups to their target substrate and corresponding protein phosphatases can remove the phosphate group. The alternation of the protein between phosphorylated/dephosphorylated state, work more or less as an "on/off" switch. The phosphorylation regulation is an important part of numerous reactions. Some are cell signaling, were phosphorylation cascades are among the most important and numerous. Others include the change of proteins activity, change of localization in the cell, making the protein interact differently with other proteins ore change the protein turnover (Luan 2003).

Protein phosphatases used to be classified based on their substrate specificity and also on their sensitivity to inhibitors. With the knowledge of sequence similarities and catalytic signatures, the protein phosphatases are now divided into four main classes.

The Phospho Protein Phosphatases (PPP), which are Serine/Threonine-specific protein phosphatases. These are divided into different groups like the PP1, PP2A and PP2B, which consists of different sub-units, all according to the substrate to be dephosphorylated.

The PP1 enzyme is a heterodimeric enzyme, which consists of a catalytic and a regulatory subunit. In Arabidopsis the PP1 genes are named TOPP (Type One Protein Phosphatase), and 9 different catalytic

subunit isoforms are revealed (Farkas et al. 2007) The catalytic sub-units interact with different regulatory units to perform different tasks within the cell. The plant PP1 regulatory proteins interact through a conserved RVxF motif, similar to the consensus motif found in human PP1 interactors (Uhrig et al. 2013)

The PP2A holoenzyme is usually a trimer consisting of a scaffolding A subunit, a catalytic C subunit and a regulatory B subunit. The Arabidopsis encodes 3 scaffolding A subunits, 5 catalytic C subunits and 17 regulatory B subunits. The B subunits are grouped into B, B' and B''. The PP4 and PP6 share sequence similarity to the catalytic subunit of PP2A, and they are suggested to originate from a common ancestor. They all can bind TAP46, which is a substrate of the target of rapamycin (TOR) kinase (Uhrig et al. 2013). The TOR pathway is mentioned in the review of TIP41 in 1.2.2.4.

PP5 is coded by a single gene in Arabidopsis, but alternative splicing gives two different transcripts. The PP7 subfamily is unique to plants. It is probably influenced by Ca^{2+}, because it carries a putative binding motif. It also interact with calmodulin in a similar way to PP2B/calcineurin-like enzymes, which is not identified in plants (Farkas et al. 2007).

The most recent classified PPPs are the protein phosphatases with kelch-repeat domains (PPKL) and the Shewanella-like protein phosphatases (SLP). The SLP phosphatases found in Arabidopsis is completely insensitive to classic small molecule inhibitors. Two groups of SLP are found using phylogenetic analyses. AtSLP1 is predicted to be found in chloroplasts, while AtSLP2 is cytosolic (Uhrig and Moorhead 2011).

The Phospho Metallo Proteins (PPM), which are Serine/Threonine and Mg^{2+} -dependent protein phosphatases. The major class is PP2C. Their catalytic activity requires the divalent cations Mn^{2+} or Mg^{2+}. PP2Cs are monomeric enzymes that exist in all eukaryotes, and are the most abundant phosphatase in plants. PPM phosphatases do not share any sequence homology with PPP phosphatases. However, the protein structures of these two families of phosphatases are relatively similar, indicating the same catalytic mechanisms (Moorhead et al. 2009). In Arabidopsis 76 genes encodes PP2C phosphatases, and these are clustered into different sub-groups based on sequence-similarity. Clusters of the sub-groups are named from A – K. Phosphatases classified as $PP2C_A$ are involved in ABA signaling pathways. Members of clade B have been characterized as regulators of MAPK activities. Arabidopsis has 6 genes encoding this type. Interaction of these phosphatases and Map kinases is observed in cytosol and in the nucleus. The Arabidopsis PP2C family clade C contains the phosphatases POL and PLL (Fuchs et al. 2013).

The Phospho Tyrosine Phosphatase (PTP) is also differentiated into sub-groups. Initially they are divided into Tyrosine specific PTPs, and Dual specificity PTPs (DsPTP). The Tyrosine specific PTPs are further divided into receptor-like or intracellular group. The DsPTPs also dephosphorylates serine/threonine residues. The PTPs are important in animals, but few true PTPs are present in plants (Xu et al. 1998).

The Aspartate-based phosphatases and the Haloacid Dehalogenase (HAD) –family enzymes, which are the most recently discovered groups of protein phosphatases. This superfamily includes enzymes catalyzing carbon or phosphoryl group transfer reactions on a diverse range of substrates, but the phosphatase activity is the most prevalent (Moorhead et al. 2009).

Figure 4 shows the different phosphatase groups and the substrate specifications. The Histidine specific phosphatases are not included in the figure, as they are part of the two-component system occurring in prokaryotes.

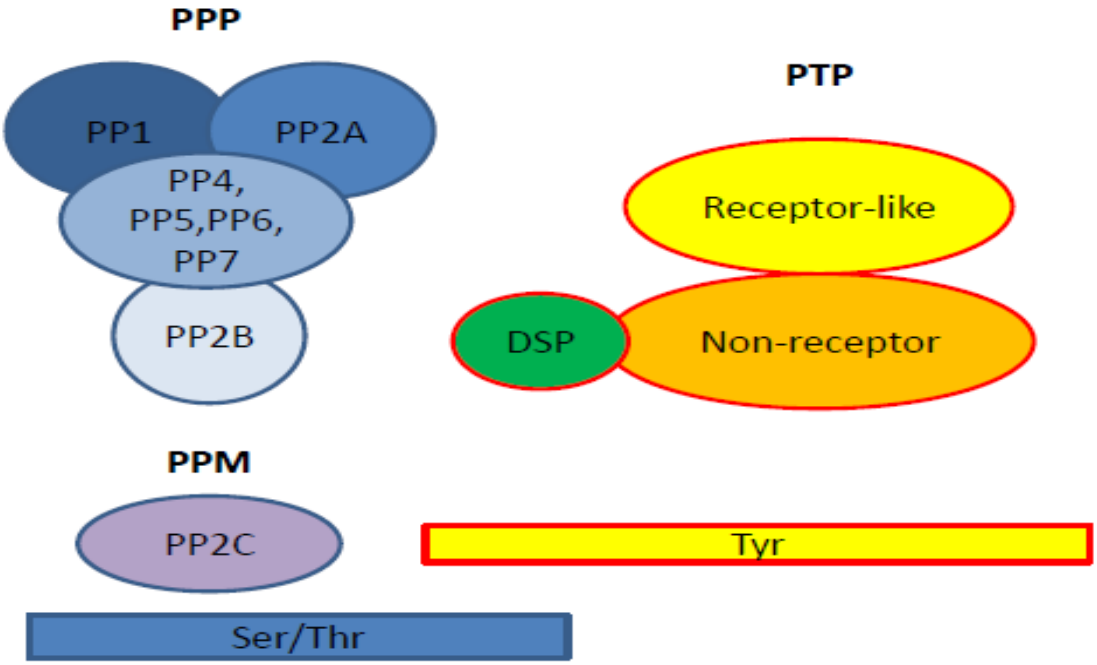


Figure 4: Classification of protein phosphatases according to their substrate specificities. Ser/Thr phosphatases include the PPP and PPM families. The catalytic mechanism of the PTPs are closely related to the DSP, although the DSPs phosphorylate serine and threonine as well as tyrosine (modified figure (Farkas et al. 2007))

1.2.3 Phosphatases investigated in this work

The experiments in this thesis were supposed to reveal peroxisomal location of different phosphatase-related proteins in the model plant *Arabidopsis thaliana*. The different proteins was predicted to contain a putative peroxisomal targeting signal according to the AraPerox database (Reumann et al. 2004) and one protein was added because it was found in peroxisome proteomic studies (Fukao et al. 2002). A short description of the different proteins which were investigated in this work is shown in table 1.

Table 1: Phosphatases investigated in the thesis. (Putative) phosphatase activity described by TAIR (Lamesch et al. 2012)

AGI code	Acronym	PTS signal	(Putative) Kind of phosphatase activity
At2g01880.1	PAP7	AHL>	Acid phosphatase activity, protein serine/threonine phosphatase activity
At3g55270.1	MapKP1	SAL>	Dual specificity phosphatase
At5g02400.1	PLL2	SSM>	Protein phosphatase 2C
At3g09400.1	PLL3	SSM>	Protein phosphatase 2C
At5g10100.1	TPP1-100	PRM>	Catalyze the de-phosphorylation of trehalose-6-phosphate
At5g36700.3	GLP1	TNL>	Phosphoglycolate phosphatase activity, involved in photorespiration
At4g34270.1	TIP41	SKV>	Interacts with TAP42 to negatively regulate TOR signaling pathway. Activates cellular responses to N-starvation
At1g07010.1	SLP1	RRIVAVGDL	Orthologous to the Shewanella cold-active protein-tyrosine phosphatase
At2g48030.1	8030	RISVNHHHL	Endonuclease/exonuclease/phosphatase

On-line databases

To investigate the different phosphatases different tools were used. Descriptions of each protein, including a Protein Coding Gene Model is presented for all phosphatases. The expression levels and patterns are described if they are available. This, as well as The Coding sequence (CDS) from each protein, is obtained from the Arabidopsis Information Resource (TAIR) website, (Lamesch et al. 2012).

All of the phosphatases were investigated to detect conserved domains. The Coding sequence (CDS) from each protein was used as query sequence. Conserved domains are parts of a protein which contains distinct functional units conserved through evolution. Conserved domains serve as building blocks and can be recombined in different arrangements to make proteins with different functions. Conserved domain identifications are made by identifying blocks of amino acids and structural patterns by performing multiple sequence alignments of proteins with known function. A query sequence is then searched against a database to compare patterns. The conserved domain figures shown in this thesis are collected from the Conserved Domain Database (CDD) at The National Center for Biotechnology Information (NCBI) (Marchler-Bauer et al. 2013).

1.2.2.1 At2g01880.1 – Purple Acid Phosphatase 7- PAP7

PAP7 is a 328 amino acid long protein. The gene is coded from the reverse strand, and contains 6 introns. The Protein Coding Gene Model is shown in figure 5. The PAP7 is mainly expressed in seedling roots (Lamesch et al. 2012)

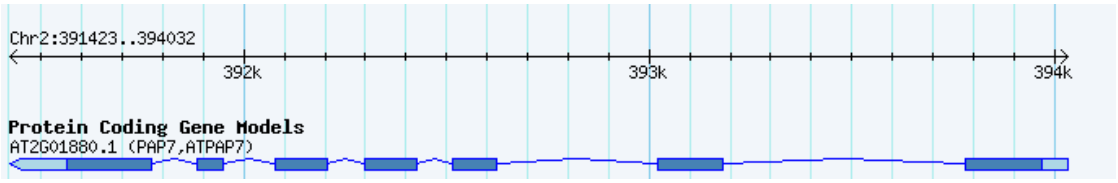


Figure 5: The Protein Coding Gene Model of Purple Acid Phosphatase 7.The solid blue bars show the exons, and the blue line shows the introns (TAIR (Lamesch et al. 2012))

A search for conserved domains, revealed that Purple acid phosphatase 7 (PAP7) belong to the metallophosphatase superfamily, which represent a diverse superfamily of enzymes with a conserved domain containing an active site consisting of two metal ions. The conserved domain is a double beta-sheet sandwich with a di-metal active site made up of residues located at the C-terminal side of the sheets. The protein has similarities to the *Homo sapiens* Acid Phosphatase 5 (ACP5) and related proteins (Marchler-Bauer et al. 2013). The graphical summary of the conserved domains are shown in figure 6. The triangles are pointing at catalytic and metal binding sites.

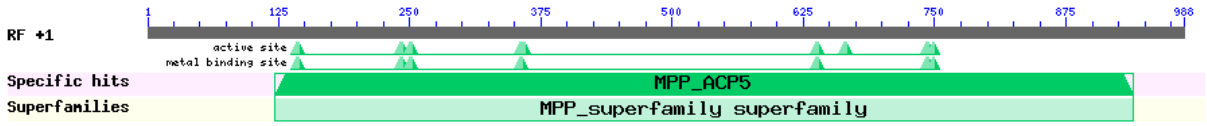


Figure 6: A graphical summary of the conserved domains of PAP7. The grey bar is showing the cDNA of the query sequence (+1nucleotide). The pastel colored bar indicates which superfamily PAP 7 belongs to, and the solid colored bar shows specific hits. The triangles are pointing on the putative active sites (NCBI, CDD (Marchler-Bauer et al. 2013).

The PAP enzymes are characterized by the presence of seven conserved amino acid residues involved in coordinating the di-metal nuclear center in their reactive site. All PAPs are tartrate-resistant. The name Purple Acid Phosphatase refers to the characteristic purple color of a concentrated solution of the protein. Most PAPs are glycoproteins and are targeted to the secretary pathway (Olczak et al. 2003).The Arabidopsis genome contains 29 predicted PAPs. They can be divided into two main groups, the high molecular weight PAPs, which are homodimeric proteins, and the low molecular weight PAPs which are monomeric. PAP7 and PAP8 belong to the low molecular group. Phylogenetic analyzes suggest that these are closer related to the mammalian homologs than the high molecule weight enzymes. (Li et al. 2002).

A search in the InterPro databases suggested a transmembrane helix spanning from amino acid 5 to 24.(Hunter et al. 2012)

The 3 C-terminal amino acids are AHL>, which is a putative peroxisomal targeting signal type 1(Reumann et al. 2004)

1.2.2.2 At5g02400 - POL-like 2 – PLL2 and At3g09400.1 – POL-like 3 – PLL3

Figure 7 shows the protein coding gene models of PLL2 and PLL3. PLL2 is a protein consisting of 674 amino acids, the gene contains 3 introns and is coded from the forward strand. PLL3, on the other hand, is coded from the reverse strand, have 3 introns, and two splice variants exists. The investigated protein is coded from the 1. variant. The PLL3 protein is 658 amino acids long. Both proteins have a protein modification which is N-terminal myristoilation, which is the covalent attachment of a myristoyl group to the N-terminal glycine residue in a protein. TAIR database has no expression annotations regarding these proteins (Lamesch et al. 2012).

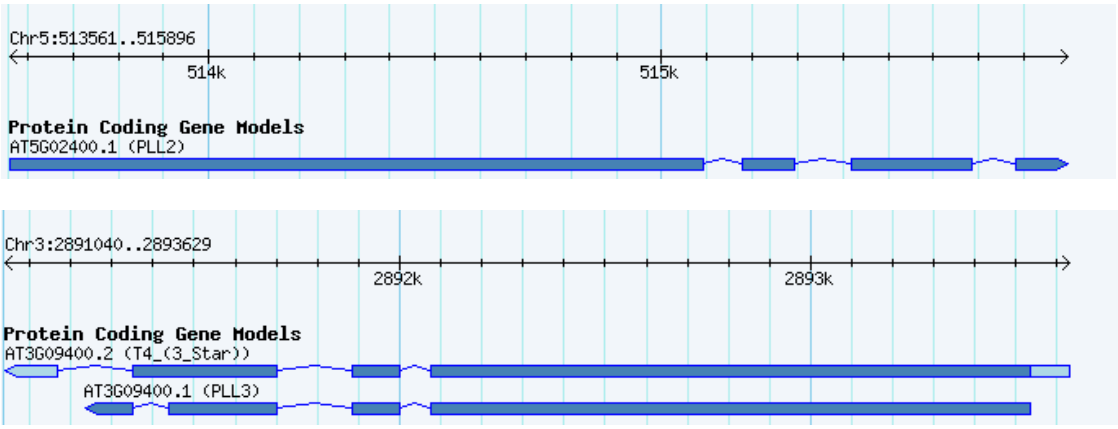


Figure 7: Protein coding models of PLL2 and PLL3.PLL2 is the upper and PLL3 the lower one (TAIR (Lamesch et al. 2012)).

The CDS from both proteins was used to perform a search for conserved domains. Both contained a Serine/Threonine phosphatases, family 2C, catalytic domain, and this was described as a specific hit. The protein architecture and deduced catalytic mechanism of PP2C phosphatases are similar to the PP1, PP2A, PP2B family of protein Ser/Thr phosphatases, but they share no sequence similarity. The conserved domains of PLL2 and PLL3 are shown in figure 8. The active sites are indicated by triangles (Marchler-Bauer et al. 2013).

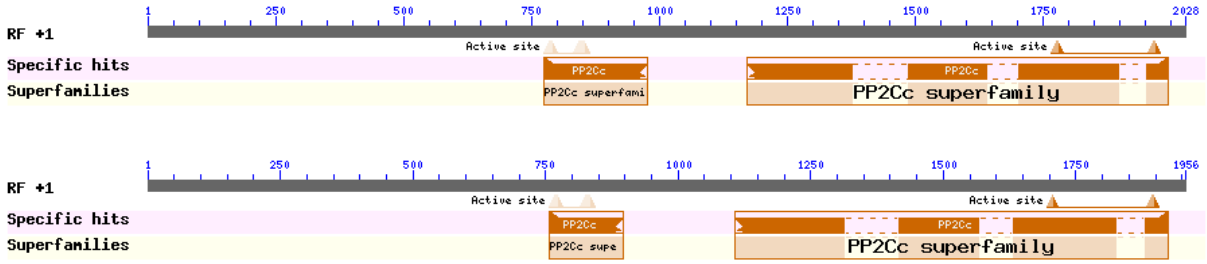


Figure 8: The conserved domains in PLL2 and PLL3. Superfamily and specific hits arePP2Cc, triangles show putative active sites (NCBI, CDD (Marchler-Bauer et al. 2013).

To illustrate the similarity between the two phosphatases, an alignment was made. The amino acid sequences were obtained from NCBI website, aligned using ClustalX (Larkin et al. 2007), and visualized using GhostScriptView (Ghostscript 2005) software. The alignment is shown in figure 9. The N-terminal is highly similar in the last ~150 amino acids.

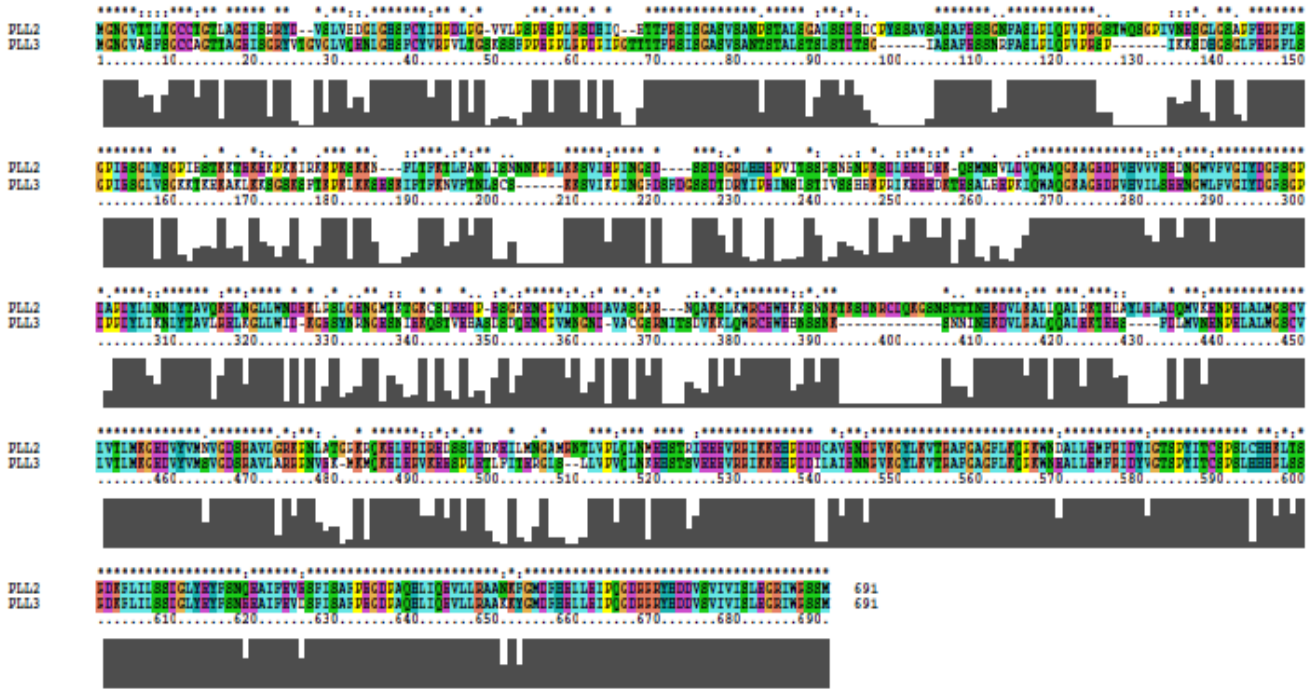


Figure 9: Showing an alignment of PLL2 and PLL. As the grey bars indicate the two phosphatases are highly similar in the last 150 amino acids at the N-terminal. Alignment performed using the ClustalX (Larkin et al. 2007) and GhostScriptView software (Ghostscript 2005).

POL and POL-like (PLL) proteins are a special group of PP2C phosphatases. They have a relatively long insertion in the conserved PP2C area, between catalytic site 3 and 4 according to other PP2Cs. Six Arabidopsis genes share this feature, as well as unique protein structure among phosphatase-encoding genes. The genes are named POL and PLL1 – 5. In an experiment to determine expression levels reverse transcriptase (RT)-PCR analysis was performed using RNA transcripts isolated from various tissues of *Arabidopsis* with gene-specific primers flanking introns. PLL2 was found in all tissues, but at levels barely detectable. PLL3 was not found to be expressed at all. No phenotype was found in knock-out mutant for either *pll2* or *pll3 (*Song and Clark 2005)

The three C-terminal amino acids are SSM> which is a putative peroxisomal targeting signal type 1 (Reumann et al. 2004).

1.2.2.3 At2g48030 - 8030

This protein is 438 amino acids long. The gene is containing 3 introns and is coded from the reverse strand. The Protein Coding Gene Model is shown in figure 10. The protein is expressed in low levels in all tissues, highest expression is found in flowers, especially in early sepals (Lamesch et al. 2012).

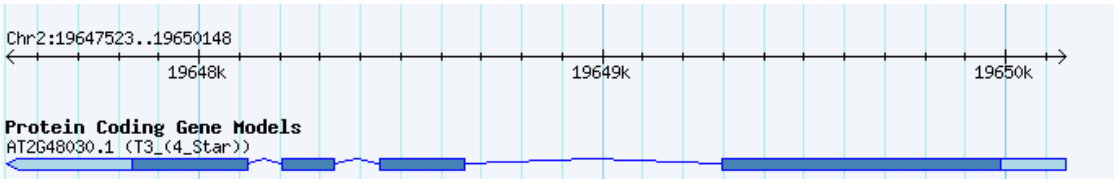


Figure 10: The Protein Coding Gene Model of At2g48030 (TAIR (Lamesch et al. 2012))

The CDS was used to search for conserved domains. A domain belonging to the Exonuclease-Endonuclease-Phosphatase (EEP) domain superfamily was found. This large superfamily includes the catalytic domain of a diverse set of proteins which share a common catalytic mechanism of cleaving phosphodiester bonds. Shown in the graphic summary shown in figure 11, are the putative catalytic, metal binding and phosphate binding sites (Marchler-Bauer et al. 2013).

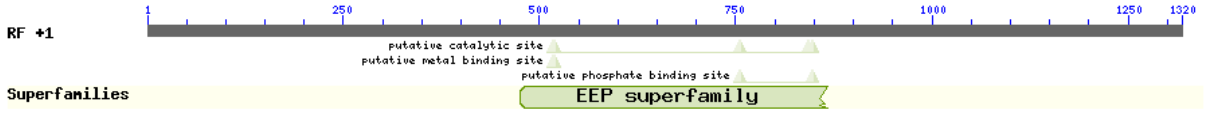


Figure 11: The conserved domains in the At2g48030 (NCBI, CDD (Marchler-Bauer et al. 2013).

The Exonuclease-Endonuclease-Phosphatase domain is found in a large number of proteins including magnesium dependent endonucleases and phosphatases involved in intracellular signaling (Hunter et al. 2012) This protein is described as a DNAse I-like superfamily protein with hydrolase activity. DNase I is a versatile enzyme that nonspecifically cleaves DNA to release 5'-phosphorylated di-, tri-, and oligonucleotide products (Lamesch et al. 2012).

The 8030 contains a putative PTS2 signal in the C-terminal part. The nonapeptid is RISVNHHHL,

corresponding to the RIX_5HI, which is considered to be a major PTS2-signal (Reumann et al. 2004).

1.2.2.4 At4g34270.1 – TIP41-like Family Protein – TIP41

This is a protein consisting of 290 amino acids. The gene contains 7 introns and is coded from the reverse strand. Figure 12 shows the Protein Coding Gene Model. The protein is ubiquity expressed in all developmental stages of the plant, except in mature pollen (Lamesch et al. 2012).

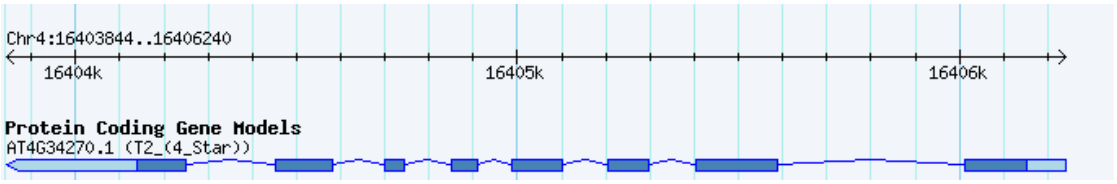


Figure 12: The Protein Coding Gene Model of TIP41-like Family Protein (TAIR (Lamesch et al. 2012)).

The CDS was used for Conserved domains search, and the graphical summary is shown in figure13.The detected superfamily was the TIP41 superfamily, and the specific hit was also TIP41. This domain is described to interact with TAP42 and negatively regulate the TOR signaling pathway, and TOR signaling pathway is activating a cell –growth program in response to nutrients (Marchler-Bauer et al. 2013).

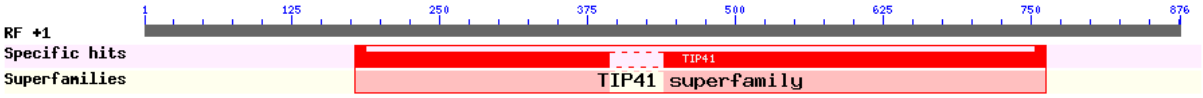


Figure 13: A graphical summary of the conserved domains in TIP41 (NCBI, CDD (Marchler-Bauer et al. 2013).

In *Saccharomyces cerevisiae* TOR (Target of Rapamycin) 1 and 2 are highly conserved Serine/Threonine kinases, and a part of a signaling pathway which activates cell-growth in response to nutrients. The TOR1 and TOR2 kinases control cytoplasmic protein synthesis and degradation through the conserved TAP42 protein. TAP42 inhibits SIT4, which is a type 2 A-related phosphatase, by binding to it. Under adequate nutrient conditions, TOR promotes this association. If nitrogen deprivation occurs, TOR is inactivated, and TAP42 is releasing SIT4. SIT4 dephosphorylates and activates a number of proteins, including several transcription factors. TIP41 binds directly to TAP42, inhibits it, and thereby negatively regulating the TOR pathway. The binding of TIP41 to TAP42 is also regulated by the TOR pathway. Dephosphorylation of TIP41 by SIT4, enhances the association of TIP41 with TAP42. TIP41 thereby seems to be part of a feedback loop in the TOR pathway. (Jacinto et al. 2001) Figure 14 shows the interaction of TIP41 in TOR signaling pathway.

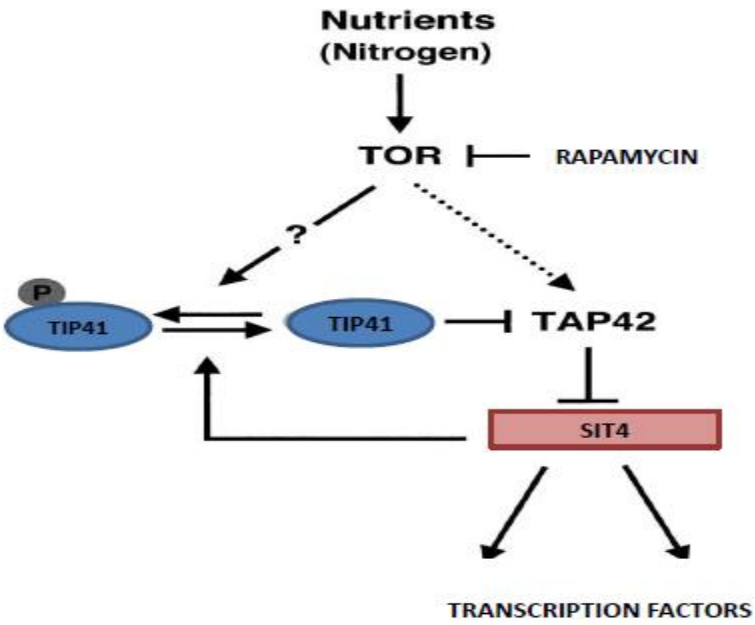


Figure 14: Model of TIP41 Action in the TOR Signaling Pathway. Arrows represent positive regulation, bars represent negative regulation. The dotted arrow between TOR and TAP42 indicates that the phosphorylation of TAP42 by TOR plays a minor role in the regulation of SIT4 (Jacinto et al. 2001).

The Arabidopsis homologue of TAP42 is called TAP46. Tap46 interact with the PP2A and PP2A-like phosphatases PP4 and PP6 (Ahn et al. 2011).

The *tip41* is a constitutive gene, which means it is nearly equally expressed regardless to growth conditions, and is often used as an internal reference gene in gene expression analyses using quantitative reverse transcriptase (qRT)PCR (Chen et al. 2010).

The three C-terminal amino acids are SKV>, a putative peroxisomal targeting signal type 1 (Reumann et al. 2004)

1.2.2.5 At5g10100 - Trehalose-6-Phosphate Phosphatase 1 - TPP1-100

The gene contains 10 introns, and can give 2 splice variants of cDNA. The investigated protein is translated from splice variant 1, which consists of 369 amino acids and is coded by the forward strand. The Protein Coding Gene Model is shown in figure15. The protein is expressed at low level in all tissues, but higher levels are found in roots, plant embryo and guard cells during C globular stage. (Lamesch et al. 2012).

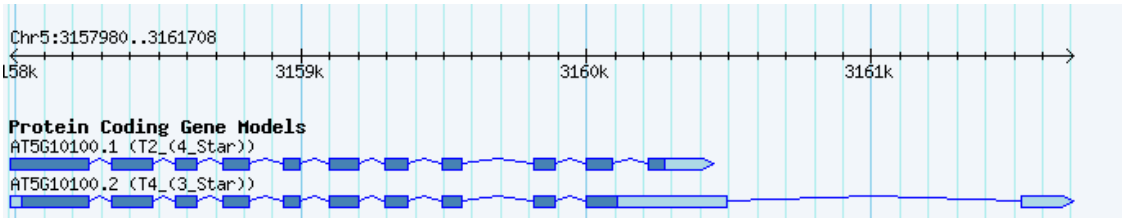


Figure 15: The Protein Coding Gene Model of At5g10100. The investigated protein is the upper one (TAIR (Lamesch et al. 2012)).

The CDS was used to perform a search for conserved domain, and the graphical result is shown in figure 16. A specific hit was found in trehalose-phosphatase characterized in a model which could span more than one domain, and thus not assign to a domain superfamily. The detected superfamily is Haloacid Dehalogenase-like hydrolase, with a specific hit at Trehalose –Phosphatase. These enzymes catalyze the de-phosphorylation of trehalose-6-phosphate to trehalose and orthophosphate (Lamesch et al. 2012).

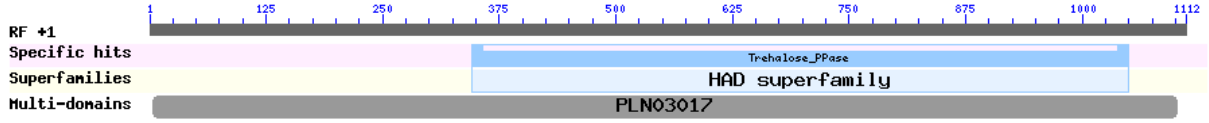


Figure 16: Conserved domains found in Trehalose-6-Phosphate Phosphatase I. The grey bar indicates that the protein is a Trehalose Phosphatase. The specific hit of Trehalose Phosphatase indicated by the solid blue bar, is usually occurring at the C-terminal of Trehalose-6-phosphat synthases as well, probably due to early evolutionary gene duplication. This domain is not active in TPS (NCBI, CDD (Marchler-Bauer et al. 2013).

Trehalose is a non-reducing disaccharide present in all kingdoms, and consists of two molecules of glucose. The building blocks of trehalose are UDP-glucose and glucose-6-phosphate. They are linked by the enzyme trehalose-6-phosphate synthase (TPS), resulting in a molecule of trehalose-6-phosphate (T6P), which is dephosphorylated into trehalose by the enzyme trehalose-6-phosphate phosphatase (TPP)(Goddijn and van Dun 1999). A schematic presentation of the process is shown in figure 17.

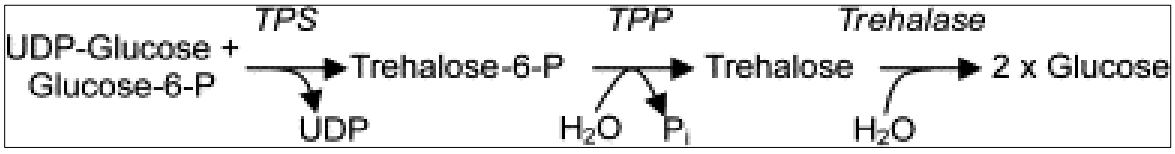


Figure 17: Trehalose synthesis in plants is catalyzed by trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP). Due to the rapid degradation of trehalose by trehalase, trehalose does not accumulate in most plant species(Wingler 2002).

Trehalose is known to be a protection against various stresses in bacteria, insects and fungi. In most of the higher plants, however, the level of trehalose is barely detectable. Transgenic plants have been made in order to investigate the role of the trehalose metabolism in plants. It seems that the T6P is necessary in plant development, but high levels inhibits growth (Schluepmann et al. 2003). T6P is probably a signaling molecule in the sugar metabolism in plants. Studies indicate that some pathogens are able to manipulate the plant trehalose metabolism to their favor. The *Arabidopsis* genome contains 10 genes with homology to TPP. (O'Hara et al. 2013).

The three C-terminal amino acids are PRM>, a putative peroxisomal targeting signal type 1 (Reumann et al. 2004).

1.2.2.6 At1g07010.1 - Shewanella-like Protein Phosphatase 1 – SLP1

The gene can give 3 different splice-variants. The one used in this thesis is At1g07010.1. It contains 8 introns. The protein is 389 amino acids long and coded by the forward strand. The Protein Coding Gene Model is shown in figure 18. The protein is more or less ubiquity expressed at low levels except in roots, where it is almost absent. In cauline leaves, cotyledon and flower sepals it shows quite high expression levels (Lamesch et al. 2012).

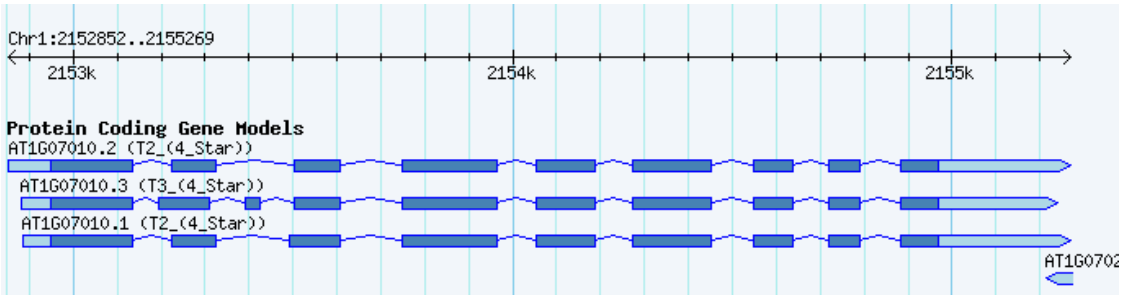


Figure 18: The Protein Coding Gene Model of At1g07010.1. The investigated protein is the lower one (TAIR (Lamesch et al. 2012)).

The CDS Was used in a Conserved Domain search, and the graphical summary is shown in figure 19.The protein is predicted to belong to the metallophosphatase superfamily, as it contains the metallophosphatase domain. This is a diverse superfamily of enzymes, with a conserved domain containing an active site were two metal ions are coordinated with octahedral geometry by a cage of histidine, aspartate, and asparagine residues. The specific hit of the protein is Shewanella-like phosphatases (SLP), which are proteins orthologous to the Shewanella cold-active protein-tyrosine phosphatase (Marchler-Bauer et al. 2013).

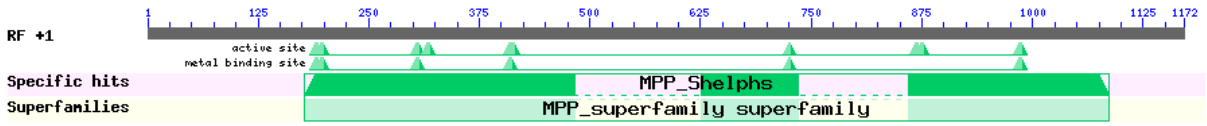


Figure 19: The conserved domains found in At1g07010.1. The triangles are pointing on metal binding- and active sites in the protein (NCBI, CDD (Marchler-Bauer et al. 2013).

In *Arabidopsis thaliana*, SLP are present in two isoforms, and At1g07010 is isoform 1. The SLP phosphatases are members of the PPP family, although they have unique features regarding other PPP family phosphatases in the matter that they are insensitive to okadaic acid and microcystein and no regulatory or scaffolding subunits are known (Uhrig et al. 2013).

SLP1 is described to hold a chloroplastic targeting signal(Kutuzov and Andreeva 2012) but since the protein was detected in a peroxisome proteomic study (Fukao et al. 2002) it was included in these investigations. It contains a putative PTS2 signal in the N-terminal region, which are the amino acids RRIVAVGDL.

1.2.2.7 At5g36700.3 - 2-Phosphoglycolate Phosphatase 1 – GLP1

The At5g36700 can be alternatively spliced to give 4 different cDNA products. The investigated protein in the thesis is splice variant 3. The gene contains 9 introns and is coded from the reverse strand. The protein is 332 amino acids long. Figure 20 shows the Protein Coding Gene Models. The expression is mainly in cotyledons, rosette and cauline leaves. In seeds, siliques and roots it is barely expressed (Lamesch et al. 2012).

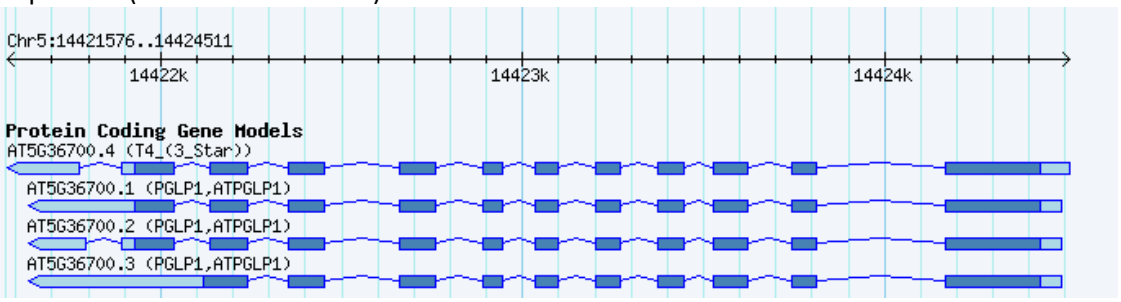


Figure 20: The Protein Coding Gene Models of At5g36700. The protein investigated in the thesis is splice variant 3, which is the lower one (TAIR (Lamesch et al. 2012)).

The CDS from the protein was used to perform a conserved domain search. Phosphoglycolate phosphatase was found as a specific hit. This is classified as a model that can span more than one domain, and is not classified as a superfamily. The superfamily found in the protein is Haloacid Dehalogenase-like hydrolases (HAD). This is a diverse superfamily, but all of them use a nucleophilic aspartate in their phosphoryl transfer reaction. Additionally all members possess a highly conserved alpha/beta core domain. The specific hit is also Haloacid Dehalogenase-like hydrolases. Motifs are marked as triangles in the graphic summary shown in figure 21. Motif I contains an Asp, which is the nucleophile attacking the substrate phosphate group, and Motif II contains a conserved Ser or Thr, found in phosphatases of HAD superfamily, and important for catalytic functions (Marchler-Bauer et al. 2013).

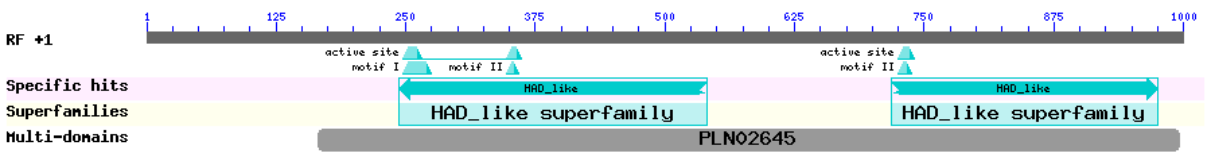


Figure 21: The conserved domains of the At5g36700.3 protein. The grey bar represents a specific hit at Phosphoglycolate phosphatase, which can span more than one domain. The triangles are showing active sites and specific motifs, were motif I contains an Asp, and motif II contains a Ser or Thr (NCBI, CDD (Marchler-Bauer et al. 2013).

During photosynthesis, chloroplasts produce large amounts of 2-Phosphoglycolate (2PG) by the oxygenase reaction of RuBisCO. 2PG is converted to Calvin Cycle metabolites during photorespiration. The first step in this reaction is the dephosphorylation of 2PG by the PGLP1. In Arabidopsis 13 putative PGLP genes are detected. At5g36700 has been established to be encoding the photorespiratory PGLP, which is found in chloroplasts (Schwarte and Bauwe 2007).

The C-terminal amino acids of the splice variant 3 of At5g36700 are TNL> which is considered a putative peroxisomal targeting signal, type 1 (Lingner et al. 2011).

1.2.2.8 At3g55270.1 - MAP kinase phosphatase 1 - MapKP1

This protein consists of 784 amino acids. The gene is coded from the forward strand and has 3 introns. Only one splice variant is known. The Protein Coding Gene Model is shown in figure 22.The protein is ubiquity expressed in low amounts, except in mature pollen, in which it is not expressed at all. The highest expression is found in senescing leaves and dry seeds (Lamesch et al. 2012).

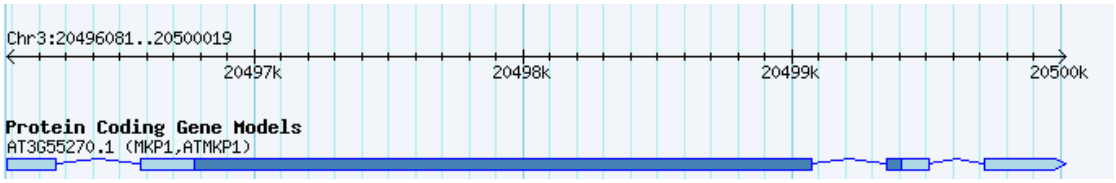


Figure 22: The Protein Coding Gene Model of At3g55270.1 (TAIR (Lamesch et al. 2012)).

The CDS were used in a search to reveal conserved domains. The protein holds a domain belonging to the Dual specificity phosphatases (DSP), and this is also referred to as a specific hit. DSPs are Ser/Thr and Tyr protein phosphatases. The structure is similar to tyrosine-specific phosphatases but the cleft in the active site is not as deep. These domains are colored in blue in figure 23, which are showing the conserve domains of the MapKP1. The yellow bar is indicating an Actin depolymerization factor/cofilin-like domain. These are present in a family of essential eukaryotic actin regulatory proteins, which enhance the turnover rate of actin, and interact with actin monomers and actin filaments. The last conserved domain, indicated by the pink bar is a Myc target protein 1.This family of proteins is regulated by the c-Myc oncoprotein. It regulates the expression of several other c-Myc target genes. The latter two hits are not specific (Marchler-Bauer et al. 2013).

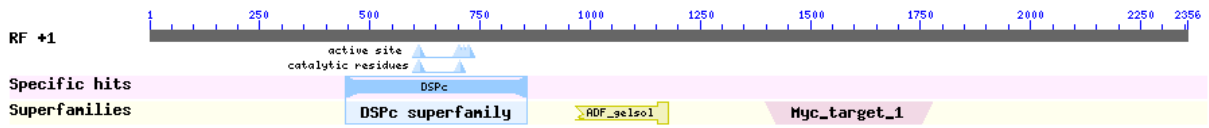


Figure 23: Conserved domains in the MapKP1 protein. The blue bars are referring to a DSP-domain, and triangles are pointing on active sites and the catalytic residues. The yellow bar indicates similarities to an Actin depolymerization factor/cofilin-like domain, and the pink bar to a Myc target protein 1 (NCBI, CDD (Marchler-Bauer et al. 2013).

In *Arabidopsis* 20 genes are encoding MAP kinases, but only 5 genes are putative Map Kinase Phosphatases. It is suggested that the MKPs therefore is involved in signal integration in plants by coordinated regulation of several MAPKs. Findings indicate interactions with MKP3, 4 and 6. MapKP1 is shown to be a positive regulator due to genotoxic stress, but seems to be a negative regulator of salt stress (Ulm et al. 2002). The loss of MAP kinase phosphatase 1 in the mutant *mkp1* results in hypersensitivity to acute UV-B stress, but without impairing UV-B acclimation. The MapKP1-interacting proteins MPK3 and MPK6 are activated by UV-B stress. MapKP1-regulated stress signaling results when UV-B protection and repair are insufficient and damage occurs. The combined activity of these two mechanisms is crucial to UV-B tolerance in plants.(González Besteiro et al. 2011)

MAP kinase signaling cascade has been found to be activated as a response to pathogen-associated molecular patterns (PAMPs), and is probably a part of the plants early innate immunity responses (Asai et al. 2002).

The three C-terminal amino acids are SAL>, a putative peroxisomal targeting signal type 1 (Reumann et al. 2004)

2 MATERIALS AND METHODS

2.1 Cloning Of Putative PTS-Containing Phosphatases

2.1.1 Polymerase Chain Reaction (PCR)

Different enzymes and various programs are used in order to receive the best possible results for the different tasks.

2.1.1.1 Expand High Fidelity^{PLUS}PCR (EHF-PCR)

For amplifying the gene of interest and to supply restriction sites prior to cloning, Expand High Fidelity^{PLUS}PCR System from Roche was used. This system depends upon an enzyme blend of *Taq* DNA polymerase and a thermo stable proofreading protein without polymerase activity. The *Taq* polymerase is a thermo stable DNA polymerase isolated from *Thermus aquaticus.* This DNA polymerase makes DNA products that have Adenine-overhangs at their 3' ends. One of the drawbacks of *Taq* DNA polymerase is its relatively low replication fidelity. It lacks a 3' to 5' exonuclease proofreading activity, but in the EHF^{PLUS} system this problem is solved by adding a special proofreading protein which greatly improve the replication accuracy. This improves the chances to get a target DNA without mutations.

Procedure:

The tubes are mixed on ice

Reagent	Volume	Final concentration
Water, PCR-grade	Up to 50µl	
Expand HiFi^{PLUS}Reaction buffer(5x)	10 µl	1x
dNTP (10mM)	1 µl	0.2 mM
Forward primer (10µM)	2 µl	0.4 µM
Reverse primer	2 µl	0.4 µM
Template DNA	Variable	5-500 ng genomic DNA 100pg – 10 ng plasmid DNA
Expand HiFi^{PLUS}Enzyme Blend (5 U/µl)	0.5 µl	2.5 U
Total volume	50 µl	

The mixture is gently vortexed and spun in a centrifuge for 5 seconds. The PCR is preceded immediately.

PCR-program:

Program	Cycles	Time	Temp
Initial denaturation	1	2 min	94 °C
Denaturation		30 s	94 °C
Annealing	34	30 s	60 °C
Elongation		2 min	72 °C
Final elongation	1	7 min	72 °C
Cooling		∞	12 °C

If constructs are very long, the elongation time is expanded (~ 1 min/kb)

2.1.1.2 Colony-PCR

To check if transformed bacteria have received the desired insert in the vector, colony PCR is performed. Primers which anneals to the vector, on each side of the target, are used. Bacterial colonies appearing on selective media after transformation are used as template. By separating the PCR reaction using agarose gel electrophorese and compare the length of the products it is possible to determine if an insert of the desired length is there. In Colony PCR *Taq* DNA polymerase from Invitrogen is used.

Procedure:

The tubes are mixed on ice

Reagent	Volume	Final concentration
Water, PCR-grade	Up to 20µl	
10xPCR-buffer (-$MgCl_2$)	2 µl	1x
dNTP(10mM)	0.4 µl	0.2 mM
Forward primer (10µM)	1 µl	0.5 µM
Reverse primer	1 µl	0.5 µM
$MgCl_2$ (50 mM)	0.6 µl	0.25 µM
Taq DNA polymerase	0.1 µl	2.5 U
Total volume	20 µl	

Template is added by using a sterile pipette-tip touching a bacterial colony and transfer to tube by pipetting carefully up and down

The mixture is gently vortexed and spun in a centrifuge for 5 s. The PCR is preceded immediately.

PCR-program:

Program	Cycles	Time	Temp
Initial denaturation	1	5 min	95 °C
Denaturation		30 s	94 °C
Annealing	34	30 s	60 °C
Elongation		2 min	72 °C
Final elongation	1	10 min	72 °C
Cooling		∞	12 °C

The bacterial colonies which are investigated are transferred to a new LB + ampicillin containing dish and incubated at 37°C for approximately 5 h and then stored at 4°C.

2.1.1.3 Reverse Transciptase (RT) PCR

This method allows the making of cDNA from RNA. RNA from different tissues and development stages are isolated. By the use of the enzyme reverse transcriptase and primers, first-strand cDNA can be synthesized. Specific primers and agarose gel electrophoresis is used to determine the transcript of interest.

The SuperScript™III Reverse Transciptase from Invitrogen is used.

Procedure:

- Two PCR-tubes are prepared

Tube 1

Reagent	Volume	Final concentration (when mixed)
Primer	2 µl	1 µM
RNA	variable	Up to 500 ng
dNTP	1 µl	0.5 mM
Water PCR-grade	Up to 10 µl	

Tube 2

Reagent	Volume	Final concentration (when mixed)
First Strand Buffer (5x)	4 µl	1x
DTT (0.1 M)	1µl	5mM
RNaseOUT™ (40 U/µl)	1µl	2 U
SuperScript™III RT (200 U/µl)	1 µl	10 U
Water PCR-grade	Up to 10 µl	

- Tube 1is heated in PCR machine, 65°C for 5 min. and is put on ice for 1 min.
- The content of tube 2 is mixed into tube 1.
- The tube is incubated for 1 h at 55 °C in the PCR machine and the reaction is inactivated by heating to 70°C for 15 min.

The cDNA can be used directly as template in an ordinary EHF – PCR as described in 2.1.1.1.

2.1.2 Cloning Vectors

Initially there were three vectors used in these experiments.

2.1.2.1 The pGEM®-T Easy vector

The pGEM®-T Easy vector system (Promega, Madison, WI, USA) was used as an intermediate vector for the full phosphatase-constructs. This vector is a linearized vector with a single 3´-terminal thymidine at both ends. The T-overhangs at the insertion site greatly improve the ligation efficiency as it prevents self-ligation. PCR products generated by certain thermo-stable polymerases (including the Expand High Fidelity^{PLUS}Enzyme Blend) gets an A-overhang and can be ligated directly into the pGEM®-T Easy vector without restriction enzyme digestion. The vector carries the lacZα sequence containing the MCS and can be used in blue/white screening. Figure 24 shows the pGEM®T Easy vector map.

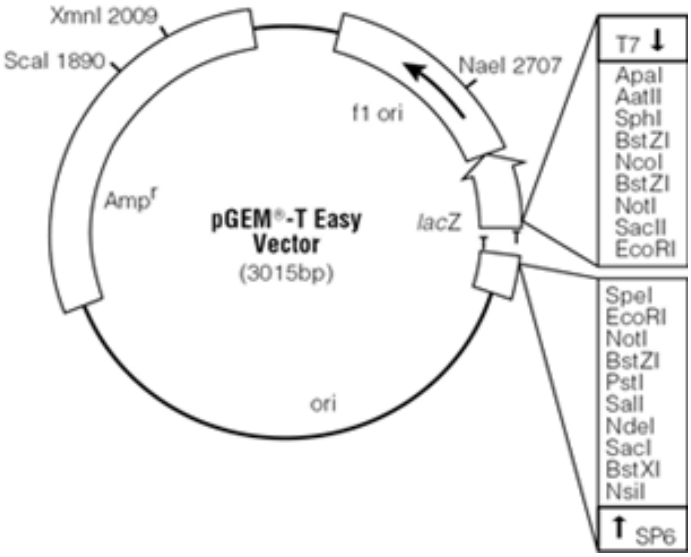


Figure 24: the pGEM®T Easy vector map. The map shows the single T-overhangs and the lacZα sequence with the MCS. The map also shows the different restriction enzymes recognition sites used for further sub-cloning of the insert into other vectors.

2.1.2.2 The pWEN18 vector

The pWEN18 vector was meant to be used to express the phosphatases containing a putative PTS2 domain. The vector contains an EYFP insert with a stop codon, and since the PTS2 signals are situated at the proteins N-terminal end, a stop codon in the EYFP-end is required. The CaMV 35S promoter ensures expression of an insert. The pWEN18 vector was previously provided from Simon Møller group and used by Polina Matre in previous research(Matre 2009) Figure 25 shows the pWEN18 vector map.

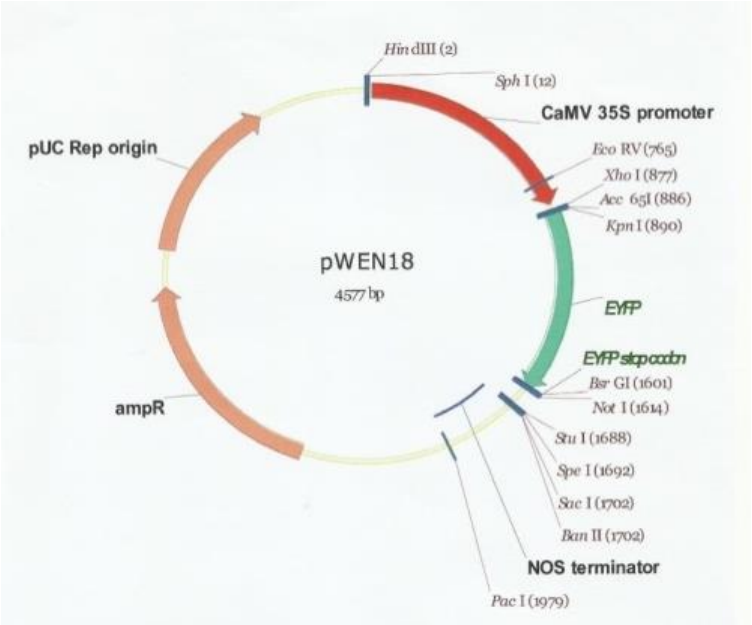


Figure 25: The pWEN18 vector map showing the EYFP with stop-codon. All the restriction enzymes recognition sites are also shown. The EYFP is already put in at the MCS and this gives sites both in front of the EYFP as well in the back.

2.1.2.3 The pCAT-YFP vector

The pCAT-YFP vector, originally used and modified from Fulda group (Fulda et al. 2002)and kindly provided from Sigrun Reumann group. This vector was used for the PTS1 predicted phosphatases. Some constructs were first cloned into the pGEM®-T Easy vector, and subcloned into pCAT-YFP. The pCAT-YFP vector was also used to make EYFP-PTD constructs. These were ligated into a pCAT vector, which was made by removing the EYFP from the original pCAT-YFP vector using restriction enzymes. The vector contains a double CaMV 35S promoter to ensure high expression. The EYFP in this vector does not have a stop-codon. This makes it ideal for cloning of PTS1 containing proteins, as the PTS1 signal is located at the C-terminus of the protein. For the PTS2 signal phosphatases to be sub-cloned into this vector, special primers with stop-codon had to be made. Figure 26 shows the pCAT-YFP vector map.

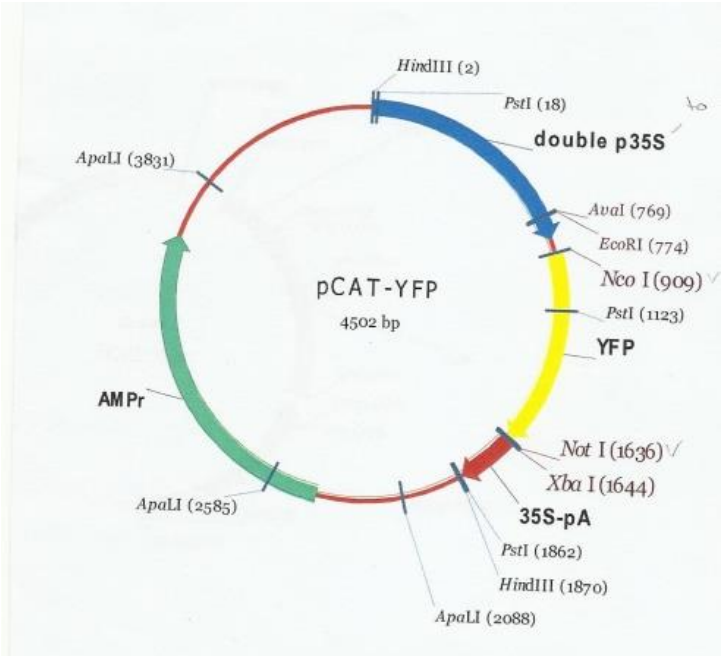


Figure 26: The pCAT-YFP vector map. The EYFP insert has no stop-codon. There are restriction enzyme recognition sites in front and in the back of the YFP.

2.1.3 Reporter Genes and Selectable Markers

In cloning, selectable markers and reporter genes are extensively used. Most vectors contain a gene coding for resistance to an antibiotic. By growing the transformed cells on media added the antibiotic, only cells which have taken up the plasmid are able to grow.

2.1.3.1 Blue/white screening

The lacZ-gene of the lac operon encodes the protein β-galctosidase. The competent *E.coli* cells used for transformation usually has a deletion in a part of the gene coding for one of the four subunits making up the tetramer of the active protein. Some plasmids contain the lacZα sequence containing a MCS, and can be used for blue/white screening. The gene will be disrupted if an insert is taken up by the plasmid, but if the plasmid is self-ligating, the subunit is available. By growing the transformed cells on media containing IPTG (isopropyl-beta-D-thiogalactopyranoside), which is a synthetic analog of galactose and induces the expression, and X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactoside), which is an inert chromogenic substrate for β-galctosidase, the bacterial colonies with insert in their plasmid will be white while the colonies without insert in the plasmid will turn out blue.

2.1.3.2 Yellow Fluorescence Protein

YFP is a genetic mutant of the Green Fluorescence Protein originating from the jellyfish *Aequorea victoria.* The protein is excitated by light of a certain wave-length (514 nm) and emits light of a lower wave-length (527 nm). This emittion of light can be detected in a fluorescence microscope. For biotechnical use the Enhanced YFP is mostly used. The gene of interest is fused to the EYFP-gene by cloning. In this thesis phosphatases containing Peroxisomal Targeting Signals (PTS) type 1 and 2 were fused to EYFP. By using fluorescence microscopy, subcellular location of the phosphatases could be determined.

2.1.3.3 Fluorescence organelle markers

Vectors expressing reporter fused proteins were used. PW99 is a modification of the pWEN-18 vector where the YFP is replaced with red fluorescence protein. The PTS1 signal SKL> is fused to the 3' end of the mRFP (RFP-SKL) The protein is imported to the peroxisomal matrix (Matre 2009). Also a glyoxysomal malate dehydrogenase- cyan fluorescence protein (gMDH-CFP), which is a modified pCAT-CFP vector containing the PTS2 signal from glyoxysomal malate dehydrogenase, were used to label peroxisomes (Kim and Smith 1994). An Orange fluorescence protein fused to an ER targeting signal (OFP-ER) was also used (Frank et al. 2008). The markers were provided by Dr. A. Kataya.

2.1.4 Cloning and sub-cloning

2.1.4.1 Determination of DNA concentration using the NanoDrop 2000.

As nucleotides absorb light in the UV spectrum, the concentration of DNA in a sample solution can be measured using a spectrophotometer. Absorption maximum of DNA is 260 nm. By using NanoDrop 2000, both concentration and purity are measured quickly and easily. The purity is calculated using the ratio of absorbance at 260 and 280 nm. NanoDrop 2000 can also measure concentrations of RNA and nucleic acids.

Procedure:

• NanoDrop 2000 spectrophotometer is switched on and wanted settings are plotted using the software provided.

• Pedestal is applied with 2 µl water and dried with lens paper.

•The spectrophotometer is calibrated by running a blank, which is 1 µl of the liquid your DNA sample is dissolved in. The foot of the pedestal is wiped with lens paper between each measurement.

• Sample solution (1µl) is pipetted on the pedestal and the concentration is measured.

• If desired, the concentration can be measured 3 times for each sample and the average is used as a starting point.

• After the last measurement 2 µl water is applied and the pedestal is wiped off.

2.1.4.2 Agarose gel electrophoresis

Agarose gel electrophoresis is a method for separating DNA fragments by size. A current is sent through the gel, and the negatively charged DNA moves towards the positive electrode. Small fragments will move faster than large fragments. By comparing with a standard, it is possible to calculate the size of the fragments. A dye which intercalate in the DNA is added, and the DNA can be made visible by means of UV light. Ethidium bromide was mainly used to this purpose, but it is very toxic and can be replaced by GelRed™ Nucleic Acid Gel Stain. Samples are added GelRed™ and loading buffer. The loading buffer contains glycerol and makes the solution heavy so that the sample sinks down when filled in the well. In addition, it includes blue dye which makes it possible to see how far the samples have moved in the gel.

Procedure for making 1% agarose gel:

- 0.5 g agarose is added 50 ml 1xTAE and heated approximately 1 min in the microwave oven until the agarose is dissolved.
- The liquid is poured gently into a leveled gel-frame with combs. Agarose gel solidifies on the bench (about 20 min).
- The gel is transferred to an electrophoresis-tub, which is filled with 1x TAE and the combs are removed.
- The first well is filled with a DNA molecular weight standard (Hyper Ladder I from Bio Life) and GelRed™. The samples mixed with loading buffer and GelRed™ are filled in consecutive wells.
- An electric field is applied for an amount of time (mostly 80 V/ 40 min)
- The bands are made visible using an UV- transilluminator.

Most pictures are taken using a High Performance Ultraviolet Transilluminator from UVP. Some pictures are taken using Image Quant. Gel bands are cut using low intensity setting at a Dual-Intensity Transilluminator from UVP.

2.1.4.3 Gel extraction

The GenElute™Gel Extraction Kit from Sigma-Aldrich is used. This kit is design for purification of DNA fragments or plasmids from agarose gels in the range from 50 bp up to 10 Kb. The technology is based on lysis of the cells, and DNA-binding to a silica-membrane at low pH. Impurities are washed through the column, and finally the DNA is eluted using pure water or elution solution.

Procedure:

- DNA-bands cut from an agarose-gel are weight and solubilized in 3 x solubilization buffer using heat (50-60°C/10 min). The solution should be yellow.
- Columns are prepared by adding Column Preparation Solution, spun for 1 min at 16000 x g and discard of flow-through.
- The samples are added 1 x isopropanol, transferred to columns and spun for 1 min, discard flow-through.
- Wash solution is added, the columns are centrifuged again and flow-through removed.

- The tubes are centrifuged again to remove left-over washing solution, and the columns are placed into a marked collection tube.
- Elute with pure water, heated to 50°C (in these experiments 20 µL were used), incubate 1 min and spin for 1 min.

2.1.4.4 DNA digestion using restriction enzymes

For digestion of DNA in order to clone or subclone, restriction enzymes are used. These are endonucleases from bacteria, and the enzymes recognize palindromic DNA sequences where they cut the double stranded DNA. The recognition site is usually from 4 to 8 bp long. For cloning, restriction enzymes which give an overhang called "sticky ends" are preferred, as they prevent self-ligation. Double digestion, with the use of two different restriction enzymes facilitates orientation of insert. Using the same restriction enzymes cutting your vector and insert, is leaving both with ends that will fit together and can be joined assisted by the enzyme DNA ligase. Restriction enzymes are delivered with an optimized buffer system. If two enzymes are to be used, the company web-site offers a buffer –optimizing finder for the double digestion. In these experiments the restriction systems from New England Biolabs (NEB) was used, except for the XbaI and NcoI which was from Sigma. The buffer system recommended from NEB was used in all digestion reactions, even if the enzymes delivered from Sigma was used. Table 3 show the restriction enzymes used during this thesis.

Procedure:

- 2 µl NEBuffer
- 2 µl BSA (if required)
- 1 ng of DNA
- 0.5 µl of each restriction enzyme
- Water to 20 µl

Incubate 1 to 3 h at 37°C.

Table 3: Restriction enzymes used in the experiments.

Name	Restriction site	Buffer	BSA	Source
NcoI	5'...C↓CATGG...3'	NEBuffer 3	No	*Nocardia corallina*
	3'...GGTAC↑C...5'			
XbaI	5'...T↓CTAGA..3'	NEBuffer 4	Yes	*Xanthomonas badrii*
	3'...AGATC↑T...5'			
NotI	5'...GC↓GGCCGC...3'	NEBuffer 3	Yes	*Nocardia otitidis-caviarum*
	3'...CGCCGG↑CG...5'			
SacI	5'...GAGCT↓C...3'	NEBuffer 1	Yes	*Streptomyces achromogenes*
	3'...C↑TCGAG...5'			
SacII	5'...CCGC↓GG...3'	NEBuffer 4	No	*Streptomyces achromogenes*
	3'...GG↑CGCC...5'			
XhoI	5'...C↓TCGAG...3'	NEBuffer 4	Yes	*Xanthomonas holcicola*
	3'...GAGCT↑C...5'			
KpnI	5'...GGTAC↓C...3'	NEBuffer 4	Yes	*Klebsiella pneumoniae OK8*
	3'...C↑CATGG...5'			
Acc65I	5'...G↓GTACC...3'	NEBuffer 3	Yes	*Acinetobacter calcoaceticus 65*
	3'...CCATG↑G...5'			

2.1.4.5 PCR – Clean-up

In order to get rid of excess nucleotides, enzyme and impurities after double digestion the Wizard® SV Gel and PCR Clean-Up System kit from Promega was used. The cleanup was performed according to protocol.

- Equal volume of Membrane Binding Solution is added to the PCR amplification
- The prepared PCR product is transferred to the Minicolumn assembly, incubated at room temperature for 1 minute, and centrifuged at 16,000 × g for 1 minute. Flowthrough is discarded.
- Wash solution (700 µl) is added, the columns are centrifuged again and flow-through removed.
- Wash solution (500 µl) is added, the columns are centrifuged again and flow-through removed.
- The tubes are centrifuged again to remove left-over washing solution, and the columns are placed into a marked collection tube.
- Elute with pure water (20 µl in these experiments), incubate 1 min and spin for 1 min.

2.1.4.6 Ligation.

In ligation a DNA ligase enzyme catalyzes the joining of two DNA strands. A covalent phosphodiester bond is made between the 5'phosphat- and the 3' hydroxyl end. This enables the joining of a DNA construct and a vector after restriction digestion of vector and DNA fragment using the same restriction enzymes. The ligation reactions require ATP which is provided in the ligase buffer. In these experiments T4-ligase system from Promega was used. The PTD-1-YFP constructs from MapKP1 and PLL2&3 are exceptions. They were made using the T4-ligase system from Invitrogen, because the one from Promega was not available at the time.

Procedure:

Concentration of vector and insert are determined and the amounts are calculated using the following equation:

$$\frac{ng\ of\ vector\ x\ kb\ of\ insert}{kb\ size\ of\ vector} \times molar\ ratio\ of\ \frac{insert}{vector}$$

Experience has shown that it can be beneficial to increase the amount of insert according to the equation.

- Insert
- Vector
- 2 µl Promega T4 ligase buffer (10x)
- 1 µl Promega T4DNA ligase enzyme
- Water to 20 µl

In the set up using Invitrogen buffer and enzyme, the amount of buffer was 4 µl, as the buffer concentration was 5x.

2.1.4.7 RNA-isolation

The RNeasy®Plant Mini Kit from Qiagen was used according to protocol. The optional on-column DNase digestion was performed.

Procedure:

All the tools which are used to handle the samples are cooled using liquid nitrogen. N_2 (l) is added to a sterile mortar and the plant material is homogenized in this using a cold pestle. The frozen, homogenized material is transferred to an eppendorf-tube, and kept in the liquid nitrogen or frozen at – 80°C until purification.

- β-Mercaptoethanol (β-ME), 10 µl per 1 ml of RLT-buffer is added inside the fume hood.
- The frozen, homogenized plant tissue is transferred to an RNase-free,N_2(l)-cooled, 2 ml micro centrifuge tube and the amount is determined (<100 mg).
- Add 450 µl RLT-buffer and vortex vigorously.
- The lysate is transferred to a QIAshredder spin column, and centrifuged for 2 min at full speed. The supernatant is carefully transferred into a new micro centrifuge tube without disturbing the pellet at the bottom.
- Add 0.5 volume of ethanol (96-100%) to the cleared lysate and mix carefully by pipetting.
- The sample is transformed to an RNeasy spin column, centrifuged at 8000 x g for 15 s. The flow-through is discarded.
- To remove all DNA from the samples, DNase On-Column digestion is performed using DNase I. 10 µl of Stock solution of DNase I is added to 70 µl of Buffer RDD. The tube is gently inverted to mix.

- The spin column membrane is washed by adding 350 µl Buffer RW1 and spin at 800 x g for 15 s. The flow-through is discarded.
- The DNase I incubation mix (80 µl) is added directly to the column membrane, and incubated in RT for 15 min.
- Add 350 µl Buffer RW1 to the column. Centrifuge for 15 s at 8000 x g. Discard flow-through.
- The membrane is washed by adding 500 µl of Buffer RPE. Centrifuge for 15 s at 8000 x g and discard flow-through.
- Another 500 µl of Buffer RPE is added. The column is centrifuged at 800 x g for 2 min.
- The column is put into a new tube and centrifuged again for 1 min at full speed.
- The column is placed into a marked eppendorf-tube and 30 – 50 µl of RNase free water is added to elute the RNA. Centrifuged at 8000 x g for 1 min.
- The elution step can be repeated.

2.1.5 Transformation

Competent cells are used. The term "competent cells" means that cells are prepared to take up extracellular DNA, like a plasmid. In a few bacterial species competence are naturally occurring, but *E. coli* cells must be made competent, e.g. by treatment with high concentration of calcium ions and cooling. Usually a stock of competent cells is made, aliquoted and stored at -80°C. In the transformations performed in these experiments the JM 109 strain of *E.coli* from Promega is used. This strain has proved to be easy to make competent and has given good transforming results previously in the lab. The strain is deficient in β-galactosidase activity due to deletions in both genomic and episomal copies of the lacZ gene and can be used for blue/white screening.

2.1.5.1 Making competent cells

Procedure:

- JM 109 *E.coli* cells from stock are streaked out on a LB-agar plate without ampicillin. The plate is incubated overnight at 37°C.
- One colony is transferred to a 5 ml tube of LB-medium without ampicillin and incubated at 37°C overnight, shaking.
- 1.5 ml of overnight culture is transferred to a sterile Erlenmeyer flask containing 150 ml of LB-broth without ampicillin. The flask is incubated at 37°C, shaking, until OD between 0.5 and 0.7 is reached.
- When the wanted OD is reached, the flask is chilled on ice for 30 min. The cells are collected by centrifugation (2500 rpm/25 min/ 4°C)
- The cells are carefully suspended in 10 ml ice cold TSS-solution
- 100 ml is aliquoted in Eppendorf tubes and put into liquid nitrogen.
- Stored at -80°C

2.1.5.2 Transformation

If using frozen bacteria, they should be thawed on ice. The competent bacterial cells are added plasmids and placed on ice. The plasmid will lie on the outside of the cell's plasma membrane. By subjecting the cells to heat shock, the membrane will temporarily become permeable and the bacteria can take up the plasmid. The cells are put back on ice and the membranes reestablishes. Plasmids include a selection marker such that cells which have taken it up can be identified. Mostly a gene that confers resistance to an antibiotic is used. By transferring the transformed bacteria to a culture medium containing these antibiotics, only bacterial cells that have taken up a plasmid will grow.

Procedure:

- Competent JM109 *E.coli* cells are thawed on ice
- DNA (usually a plasmid) is added
- Incubated on ice for 30 min
- The samples are heat shocked at 42°C for 50 s
- Put back on ice for 2 min.
- Add 0.5 ml LB broth
- Incubate at 37°C for 1 h, shaking
- Plate at LB agar plates containing antibiotics
- Incubate at 37 °C, overnight.

2.1.5.3 Blue/white screening

This is performed when using the pGEM®-T Easy vector in the first step of cloning the full-length EYFP-phosphatase fusion proteins.

Procedure:

- Solid LB-agar-plates with ampicillin are added 10 µl IPTG (100 mM) and 20 µl X-gal (50 mM) which are spread on the plate with a sterile spatula
- The transformed cells are spread on the plate and incubated overnight at 37°C.

The white colonies that appear on the plate are investigated by colony PCR to see if the insert is there. The blue colonies do not contain the insert of interest.

2.1.5.4 Overnight cultures

In order to get a high amount of a plasmid of interest, overnight cultures of the plasmid-containing bacteria and plasmid isolation are performed.

Procedure:

- 5 ml LB-broth + ampicillin in a 15 ml tube
- One colony is touched using a sterile pipette-tip and put into the tube
- The tube is incubated at 37°C overnight, shaking (250 rpm)

2.1.5.5 Plasmid isolation (Mini-prep)

The Gene-elute Plasmid Miniprep kit from Sigma Aldrich is used according to protocol. Recombinant bacteria, which are grown overnight in LB-broth containing a selective antibiotic, are harvested by centrifugation. The bacterial pellet is resuspended and lysed under alkaline conditions. The plasmid is bound to a silica membrane under high salt condition. Washing steps remove impurities, and the plasmid is eluted from the silica membrane by addition of pure water.

Procedure:

First a bacterial pellet is made by pipetting 1.4 ml of bacterial suspension into eppendorf tubes and centrifuge at ≥ 12000 x g for 1 min. The supernatant is discarded and the procedure repeated 2 times. All of the supernatant should be carefully removed the last time, leaving a bacterial pellet.

- Suspension solution (200 µl) is added and the pellet is resuspended by vortexing and/or pipetting up and down
- Lysis solution (200 µl) is added and the tube is carefully inverted. The solution should be transparent, and the lysis solution should not work for more than 5 min
- Neutralizing solution (350 µl) is added and the tube is inverted 4 to 5 times. The tubes are then centrifuged at ≥ 12000 x g for 10 min
- A mini-column is prepared by putting it in a collection tube, add 500 µl of column preparation solution and centrifuge at 12000 x g for 1min.
- The lysate is transferred to the column and spun for 1 min. Flow-through is discarded
- Washing solution (750µl) is added and column is spun again. Flow-through is discarded
- The column is spun again to get rid of all washing solution. The column is transferred to an empty, marked eppendorf tube.
- The column is eluted with Bio grade Water (50 – 100 µl) and spun for 1 min. The plasmid is then in the flow-through.

The plasmid concentration can be measured using Nano-drop. The plasmid can be stored at -20°C.

2.2 Transient transformations and microscopy investigations of putative PTS-containing phosphatases

2.2.1 DNA precipitation for transformation of onion epidermal cells

2.2.1.1 Preparation of gold particles:

- Gold-particles (50 mg) are suspended in 1 ml EtOH (99.5 %)
- The sample is vortexed thoroughly for 2 -3 min.
- The gold particles are sedimented by centrifugation for 3 s at 8000 x g and the supernatant is removed.
- The previous washing steps are repeated twice.
- After the last washing step, the gold particles are resuspended in 1 ml water and vortexed as described.
- The sample is centrifuged (3 s/8000 x g) and the supernatant removed.
- The particles are resuspended in 1 ml water and aliquoted in 50 µl aliquots while vortexing.
- Can be stored at – 20°C.

2.2.1. 2 DNA precipitation onto gold particles:

The precipitation is performed on ice.

- A 50 µl aliquot of washed gold particles per precipitation reaction are thawed on ice or used directly after preparation.
- The following components are added one after the other and the particles are vortexed thoroughly for 1 -2 min after the addition of each compound

Reagent	Amount	Final concentration
Plasmid DNA	Up to 20 µl	About 40 ng/µl
$CaCl_2$ (2.5M)	50 µl	1 M
Sperimidine (0.4 M)	5 µl	10 mM

- The DNA is precipitated onto the particles by centrifugation for 3 s at 8000 x g and removing of the supernatant
- The particles are resuspended in 250 µl of EtOH (99.5 %) by vortexing and scraping the walls of the tube using a pipette tip. The DNA coated particles are then sedimented by centrifugation and removal of the supernatant.
- The washing step is repeated twice.
- The particles are finally resuspended in 55 µl of EtOH by vortexing
- Can be stored at – 20°C.

2.2.2 Cell bombardment.

The BioRad PDS1000/He Biolistic Gene Gun is used. The gold suspension, called the microcarrier, is pipetted onto a disc, called a macrocarrier. The system uses high pressure helium gas, which is stopped by a rupture disc. The rupture disc bursts at a certain pressure. The macrocarrier and the target onion cells are placed in a vacuum chamber. At a certain vacuum, the helium gas is released, the rupture disc bursts, and the pressure forces the DNA coated gold particles to fly through a stopping screen. This screen scatters the gold particles over the target onion cells, and hopefully some will penetrate the nucleus to transform the cell. Figure 27 shows a picture of the gene gun and of the principles of the system.

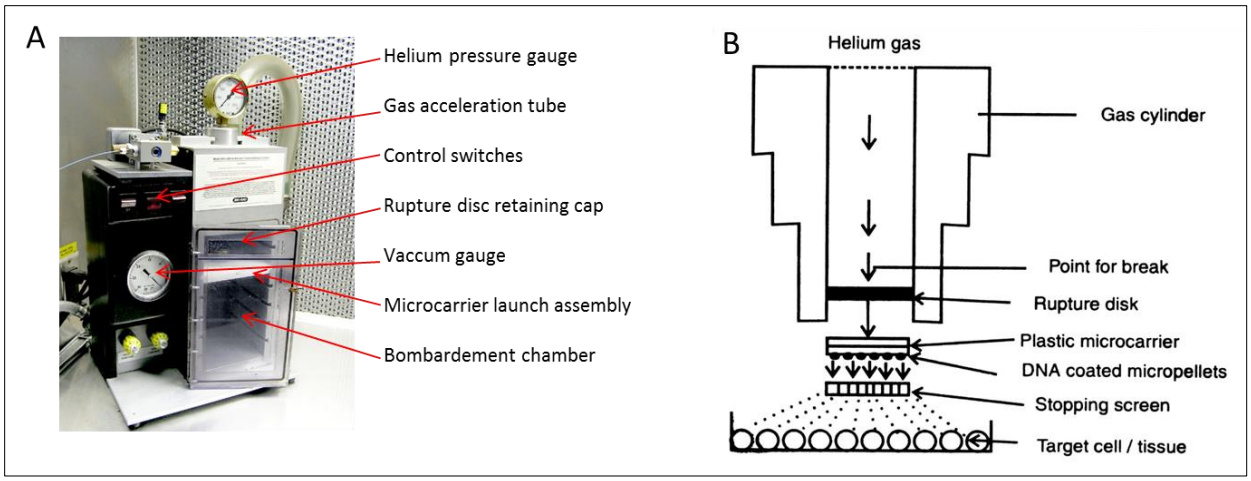


Figure 27: A shows a picture of the BioRad PDS1000/He Biolistic Gene Gun. B shows a schematic figure of the principle of biolistic cell bombardment (Modified figures from http://www.eplantscience.com/index/biotechnology/genes_genetic_engineering/techniques_of_genetic_engineering/biotech_particle_bombardment.php)

2.2.2.1 Preparation of onion samples:

- Tissue paper is wetted and placed in petri dishes.
- An onion is cut in quarters and the inner- and outermost leaves are removed
- Cut chunks of about 2 x 3 cm and place on tissue with inside (epidermal layer) facing upwards

2.2.2.2 Cell bombardment:

- Vortex the DNA-coated gold particles thoroughly and load 5 – 8 µl onto the macrocarrier which is placed in the holder. The particles are spread over about 1 cm^2 and the EtOH should be left to evaporate
- Gene gun chamber is washed with 70% EtOH. The rupture disc is briefly rinsed in ethanol before assembling into the retaining cap.
- Start the vacuum pump. Helium bottle is opened, and the pressure is adjusted to 1400 PSI (about 300 PSI above the breaking point of the rupture disc). Turn on the Gene Gun.
- The sterilized rupture disc is loaded into the retaining cap, and the retaining cap is secured to the end of the gas acceleration tube and tightened with the torque wrench.
- Load a stopping screen and the macrocarrier holder (DNA facing down) into the microcarrier launch assembly.
- The microcarrier launch assembly is placed on the top shelf in the bombardment chamber, the target onion sample is placed in the dish holder on shelf 3.

- The chamber door is closed and vacuum is applied by pressing the middle button to upper position (“Vac”). When the vacuum has dropped to about 280 inches Hg, the vacuum is held by switching the position of the button to the lower position (“Hold”) and the right button (“Fire”) is pressed at the same time. The “Fire” button is released when the rupture disc bursts, and the middle button are held in the middle position (“Vent”) to remove the vacuum.
- The onion samples are incubated at room temperature in the dark for 15 – 24 h.

2.2.3 Isolation of *Arabidopsis* mesophyll protoplasts and DNA – PEG - calcium transfection

The isolation of *Arabidopsis* mesophyll protoplasts and DNA – PEG -calcium transfection was performed according to the Sang-Dong Yoo protocol (Yoo et al. 2007)with some modifications.

2.2.3.1 Protoplast isolation

Day 1:

- Enzyme solution is prepared or thawed.

Enzyme solution (10 ml) Sterile filtered

Reagent	Content	Final concentration
Cellulase (*Trichoderma viride*, SIGMA) (6 U/mg)	15 mg	9 mM
Pectinase (*Rhizopus sp.* SIGMA) (448 U/g)	10 mg	0.45 M
Mannitol (0.5M)	8 ml	0.4 M
KCl (1M)	0.2 ml	20 mM
MES (0.5M)	0.5 ml	250 mM
dH_2O	0.8 ml	
$CaCl_2$ (0.2M)	*0.5 ml*	*10 mM*
BSA (SIGMA)(99 %)	*10 mg*	*0.1 M*

- Leaves from 4 – 5 weeks old *Arabidopsis* plants are harvested using forceps and a razor blade. The leaves are cut in narrow stripes from the middle nerve.
- The leaves are put in a petri dish containing 10 ml of enzyme solution. Both sides of the leaf are dipped in the solution, and left to incubate with upper side up. The dish is filled with one layer (12-18) of leaves.
- incubated overnight in room temperature in the dark.

Day 2:

- Solutions are prepared or brought to room temperature.

W5 solution, pH 5-6, (250 ml) Autoclaved

Reagent	Content	Final concentration
NaCl (5 M)	7.25 ml	145 mM
$CaCl_2$ (1 M)	31.25 ml	125 mM
KCl (1 M)	1.25 ml	5 mM
MES (100 mM)	5 ml	2 mM
dH_2O	205.25 ml	
Glucose	225 mg	5 mM

MaMg solution, pH 5.7 (100 ml) Autoclaved

Reagent	Content	Final concentration
Mannitol (0.8 M)	50 ml	0.4 M
$MgCl_2$ (1M)	1.5 ml	15 mM
MES (100mM)	4 ml	4 mM
dH_2O	44.5 ml	

PEG solution, 40%, pH 7-9 (10 ml)

Reagent	Content	Final concentration
PEG 4000 (Merck)	4 g	40 % v/w
dH_2O	6.5 ml	
Mannitol (0.8 M)	2.5 ml	0.2 M
$CaCl_2$ (1M)	1 ml	0.1 M

Gamborg B5-Medium, pH 5.7 (500 m) Sterile filtered

Reagent	Content	Final concentration
Gamborg medium	1.585 g	3.17 g/l
Glucose	45.04 g	0.45 M

- The Petri dish containing enzyme solution and cut leaves is shaken briefly to release the protoplasts.
- The protoplast suspension is filtered through a 75 µm nylon mesh. Rinse dish and filter using 5 ml 0.2 M $CaCl_2$.
- The solution is transferred to a 15 ml Falcon tube and centrifuged for 2 min at 700 rpm.

- The supernatant is discarded and the pellet is resuspended in 10 ml of W5 solution. Incubated on ice for 1h.
- . The protoplasts are counted using a haemocytometer. 10 µl is added and each 4 square is counted as 0.1 µl.
- The protoplasts are centrifuged again for 2 min at 700 rpm. The supernatant is discarded and ice-cold MaMg solution is added, the amount is adjusted to give ~ 1.67 mill cells/ml. (500 000/ 300 µl).

2.2.3.2 DNA – PEG –calcium transfection

- 30 µl plasmid DNA is pipetted into the middle of a small Greiner petri dish and mixed with 300 µl of protoplast solution by pipetting carefully up and down with a 1 ml pipette-tip with a cut end.
- 500 µl PEG solution is added drop by drop from a height of approximately 5 cm.
- Incubate for 30 min
- The solution is diluted step-wise by adding 0.5 ml + 1ml + 1ml +1 ml of W5 solution and transferred to a 10 ml Falcon tube. The petri dish is rinsed using 3 ml of W5 solution
- Centrifuge (2 min/700 rpm) and discard supernatant
- The pellet is resuspended in 1 – 3 ml of B5-Medium, according to amount of protoplasts and incubated in room temperature overnight in the dark.

2.2.4 Determination of subcellular location using Fluorescence microscopy

The principle of fluorescence microscopy is that a special molecule in the specimen to be investigated is excited by light of a certain wave-length. As the excited molecule is returning to its ground state, the absorbed energy is given off as light of another wave-length. By using different filters which allows light of the wanted wave-length to pass and reflect light of other wave-lengths it is possible to detect the molecule of interest.

A Nikon TE 200U inverted fluorescence microscope was used. The microscope was equipped with an Exfo X-Cite 120 fluorescence illumination system and filters for YFP (exciter HQ500/20, emitter S535/30), CFP (exciter D436/20, emitter S480/40), Texas Red® Filter Set for RFP (31004, Chroma Technologies, Rockinghan, VT, USA) and a red chlorophyll autofluorescence filter set (exciter HQ630/40, emitter HQ680/40; Chroma Technologies). Images were captured using a Hamamatsu Orca ER 1394 cooled CCD camera. Volocity II (Improvision) software was used for image processing.

2.2.4.1 Microscopy examination of transient transformed onion epidermal cells

The 10x and 20x lenses were used

- The thin epidermal layer of the onion chunk is removed using tweezers
- The epidermal layer is stretched out on a 76 x 26 mm Microscopy Slide from Thermo Scientific.
- A drop or two of water is added and a 24 x 40 mm cover Slip (Thermo Scientific) is put on top, carefully to avoid air bubbles.
- The slides are put under the microscope, cover slip facing down, and the cells are determined at first using the 10x lens and white light.
- When the cells seem clear, the light is switched to YFP-lenses. Cells are scanned to determine transformed cells, which will emit fluorescence.
- Fluorescent cells are examined using the 20x lens and pictures are captured.

2.2.4.2 Microscopy examination of transient transformed *Arabidopsis* mesophyll protoplasts

The 60x lens is used. A special device for examinating protoplast solution is used. It consists of metal rings holding two circular glass slides separated by a silicon ring together. A picture of the device containing protoplast solution is shown in figure 28.

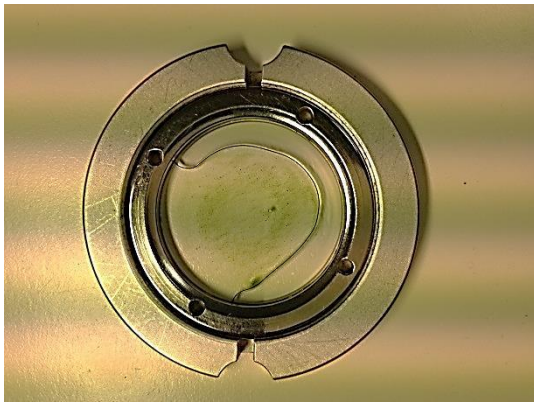


Figure 28: Protoplast microscopy examination device.

- The protoplast-solution is carefully mixed and ~300µl is pipetted to a round microscopy glass.
- The examination device is put together by placing a silicone ring and a new round glass slide on top of it and screw the metal rings together.
- A drop of immersion oil is put on top of the lens. The sample is placed on the oil, and examined to detect transformed cells.

3 RESULTS

3.1 Cloning results

3.1.1 Making competent JM 109 *E.coli* cells.

Competent cells were made following the procedure described in Materials and Methods 2.1.5.1. OD at 600 nm was measured using SmartSpec Plus spectrophotometer. At OD = 0.662 and OD = 0.652 the two beakers were put on ice, and the preparation continued according to the protocol.

Transformation efficiency was estimated by transforming a known amount of plasmid DNA and counting the colonies. Diluted pCAT-YFP vector (1 µl ≈ 0.1 ng/µl DNA) were transformed into 100 µl of competent JM109 *E.coli* cells from the old stock and 100 µl from the new stock following the procedure described in 2.1.5.2.

The plates were incubated at 37°C overnight. The colonies were counted and the transformation was calculated using the equation:

$$\frac{colonies\ on\ plate}{ng\ of\ DNA} * 1000 \frac{ng}{\mu g}$$

Table 4 shows the calculated transformation efficiency.

Table 4: Calculation of transformation efficiency of JM109 competent E.coli cells.

	Colonies on plate	Calc. transformation efficiency
Old stock	1300	1.30E+09
New stock	230	2.30E+08

3.1.2 Peroxisomal Targeting Domains (PTDs)

In order to investigate if the different phosphatases was targeted to peroxisomes, the putative Peroxisome Targeting Domain (PTD) was fused with EYFP and the constructs were used in transformation of onion epidermis cells and *Arabidopsis* mesophyll protoplasts for examination using fluorescence microscopy. Figure 29 shows the principle of the constructs, using the prototype targeting signal residues as an example.

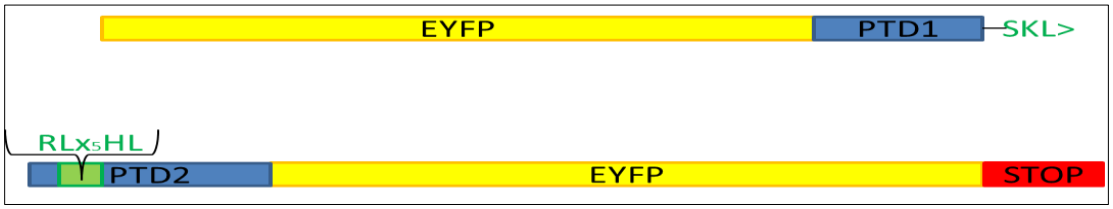


Figure 29: Principle of the EYFP-PTD constructs. The prototype peroxisomal targeting signal residues in green are used as an example.

The peroxisomal targeting domain 1(PTD1) consists of the putative Peroxisomal targeting signal type 1(PTS1) including 10 upstream amino acid residues. The PTD2 is the PTS2 and the surrounding amino acid residues. Table 5 shows the different phosphatases and the putative PTDs.

Table 5: PTD constructs

AGI code	Name	Acronym	Putative PTS
At2g01880.1	Purple acid phosphatase 7	PAP7	AHL>
At3g55270.1	Mitogen activated kinase phosphatase 1	MapKP1	SAL>
At5g02400.1	Pol-like phosphatase 2 & 3	PLL2 & 3	SSM>
At5g10100.1	Trehalose-6- phosphat phosphatase 1	TPP1-100	PRM>
At5g36700.3	2-phosphoglycolate phosphatase 1	GLP1	TNL>
At4g34270.1	TIP41-like family protein	TIP41	SKV>
At1g07010.1	Shewanella-like protein phosphatase 1	SLP1	RRIVAVGDL
At2g48030.1	Endonuclease/exonuclease/phosphatase family	8030	RISVNHHHL

3.1.2.1 EYFP-PTD1 constructs

The EYFP was fused to the N-terminal of the PTD1, leaving the PTS1 with a free C-terminal (see figure 29).

The PTD1 constructs were made using EHF-PCR as described in Materials and methods 2.1.1.1. Forward primer for all constructs was AK88F, holding a NocI restriction site and the start of EYFP (sequence showed in table 6). Reverse primer was the last 10 amino acids from the respective phosphatase, and an XbaI restriction site annealing to the end of the EYFP. The template used was the pCAT-YFP-vector described in Materials and methods 2.1.2.3. The PCR-reaction was checked for inserts of the right size (~700 bp) using 1% agarose gel electrophoresis as described in 2.1.4.2, the gel is shown in figure 29, A. The bands were cut and the constructs cleaned using GenElute™Gel

Extraction Kit from Sigma-Aldrich, as described in 2.1.4.3. Constructs were double digested using the restriction enzymes XbaI and NocI according to description 2.1.4.4, and cleaned using the Wizard® SV Gel and PCR Clean-Up System kit from Promega as described in 2.1.4.5. The pCAT-YFP-vector was also digested, using the same restriction enzymes, in order to remove the YFP. This digestion reaction was gel-extracted to get the pCAT part. Ligation reactions were performed as described in Materials and methods 2.1.4.6. The ligation reactions were transformed into JM109 competent *E.coli* cells according to procedure described in 2.1.5.2. Colony PCR, described in 2.1.1.2, using the insert primers (forward = AK88F, and reverse = specific for each phosphatase) was performed. The agarose-gel in figure 29, B shows the colony-PCR results. Overnight cultures from positive colonies were made like described in 2.1.5.4 and plasmid isolation using the Gene-elute Plasmid Miniprep kit from Sigma Aldrich was performed according to protocol described in .2.1.5.5. . The plasmids were digested using XbaI and NocI and examined by 1% agarose gel electrophoresis to find insert of the right size. The gel is shown in figure 29, C. Plasmids were then sent for sequencing at SeqLab, Göttingen, in order to check that the sequence was right and without mutations.

3.1.2.2 PTD2 –EYFP constructs

The EYFP was fused to the C-terminal of the PTD2, leaving the PTS2 with a free N-terminal (see figure 29).

PTD2 constructs were made in the same way as the PTD1, but forward primers were containing the putative peroxisomal domain and the reverse primer were the same in both constructs (AK95R). The reverse primer had to carry a stop codon, as the EYFP was without this. Figure 29 D, E and F shows gel from EHF-PCR, colony-PCR and after double digestion of the constructs. Primer sequences are shown in table 5. Yellow nucleotides belong to EYFP, light blue are the XbaI RS and the pink shows the NcoI RS. Stop codon is marked in red.

All the PTD constructs were successfully cloned into the pCAT vector.

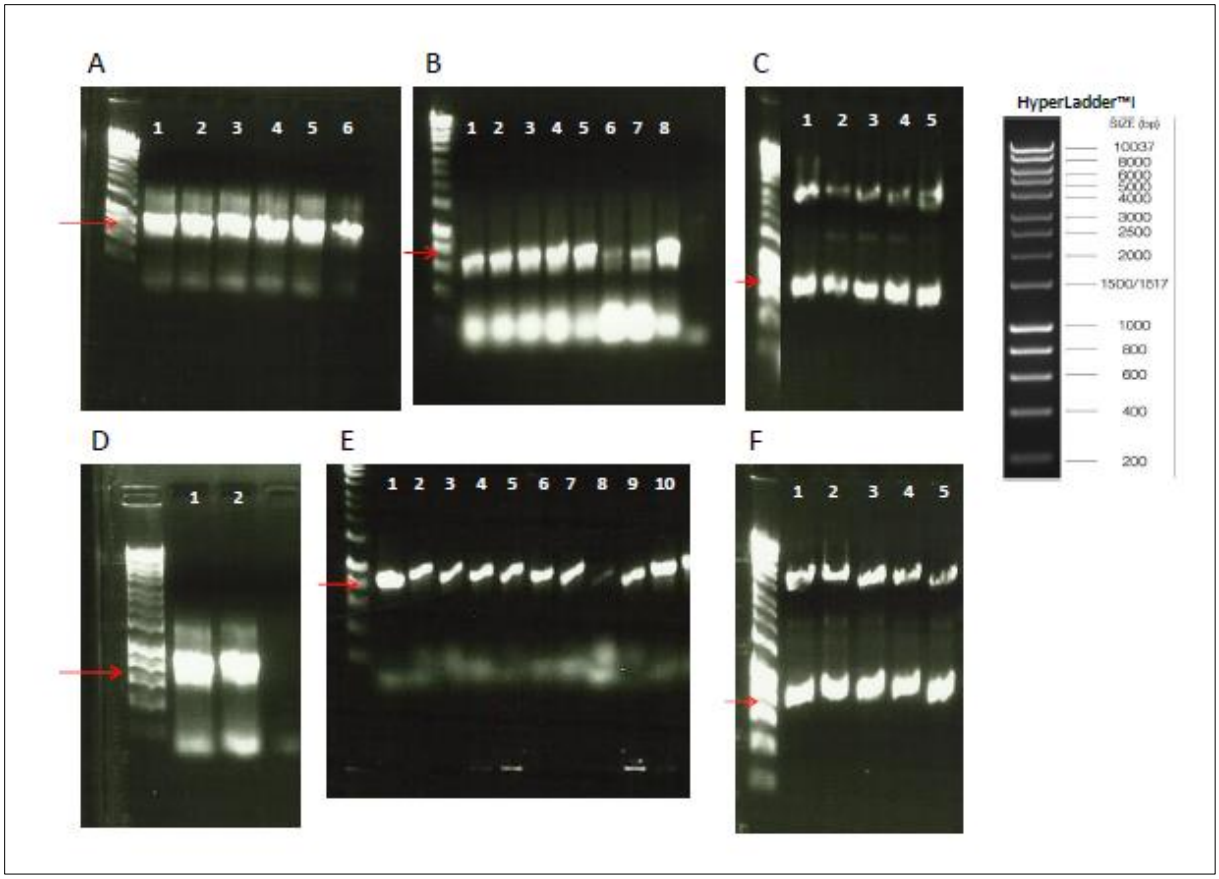


Figure 30: PTD gels. The red arrows are pointing out the approximately size ~700 bp. ***A*** *- EHF-PCR products from PTD1.* ***B*** *- colony-PCR bands from PTD1.* ***C*** *- The digested PTD-containing plasmids.* ***D*** *- The PTD2 templates amplified by EHF-PCR.* ***E*** *- colony-PCR from PTD2.* ***F*** *- digested PTD2-plasmids.*

Table 5: Primer sequences used to make the PTD constructs. Yellow nucleotides = EYFP, blue = XbaI RS and pink = NcoI RS, Red = stop codon.

AK88F	PTD1-forward	CACCATGGCAATGGTGAGCAAGGGCGAGGAG
AK82R	PAP7-PTD	TATGTCTAGAGTCAAAGATGAGCAGATCTTTTGGACAATGAGGACTTGTACAGCTCGTCCATGCC
AK83R	MapKP1-PTD	TATGTCTAGAGTCATAGCGCGCTCAGCAGTGCTAGCAACTCTGTCTTGTACAGCTCGTCCATGCC
AK84R	PLL2 & 3-PTD	TATGTCTAGAGTCACATGGAAGATCTCCAAATTCTTCCTTCAAGCTTGTACAGCTCGTCCATGCC
AK85R	TPP1-100-PTD	TATGTCTAGAGTCACATTCTTGGCTGCATTTGTTTCCATTCCACCTTGTACAGCTCGTCCATGCC
AK86R	GLP1-PTD	TATGTCTAGAGTCACAGATTAGTTATACCCGAGAGGACGAGTAGCTTGTACAGCTCGTCCATGCC
AK87R	TIP41-PTD	TATGTCTAGAGTCAAACTTTACTAGGGATCTTCAGTTTCTGTGTCTTGTACAGCTCGTCCATGCC
AK95R	PTD2-reverse	TATGTCTAGAGTCACTTGTACAGCTCGTCCATGCC
AK96F	PTD2-GLP1	CACCATGGCACGTCGTATCGTTGCAGTTGGAGACCTTCATATGGTGAGCAAGGGCGAG
AK97F	PTD2-8030	CACCATGGCAGCCAGAATCTCCGTCAACCACCACCACCTCTCAATGGTGAGCAAGGGCGAG

3.1.3 Cloning of the full-length phosphatases

The cDNA used as templates were obtained in different ways. Table 6 gives an overview of templates, primer acronyms and restriction sites of the full length phosphatases. Templates named U were ordered from Arabidopsis Biological Resource Center (Ohio, USA) and templates named R were ordered from the RIKEN BioResource Center (Ibaraki, Japan) RNA was isolated from various tissue from WT *Arabidopsis thaliana*, ecotype Columbia.

Table 6: Templates, primer acronyms and restriction enzymes used in order to make full length phosphatase-EYFP fusion constructs.

Acronym	PTS	Template	Size, bp	Primers	Restriction sites
PAP7	AHL>	U84234:BT026449	999	AK27F / AK28R	NotI / SacI
MapKP1	SAL>	R16207:RAFL07-11-L11	2367	AK29F / AK30R	NotI / XbaI
SLP1 -A	RRIVAVGDL	R21723:RAFL04-20-P11	1170	AK31F / AK32R	NotI / SacII
SLP1 -B	RRIVAVGDL	R21723:RAFL04-20-P11	1170	AK33F / AK34R	KpnI / KpnI
PLL3	SSM>	RNA isolated from flowers/leaves	2037	AK35F / AK36R	NotI / SacII
PLL2	SSM>	RNA isolated from flowers/leaves	1965	AK37F / AK36R	NotI / SacII
`8030	RISVNHHHL	U82664:GSLTFB92ZG05	1329	AK38F / AK39R	XhoI / KpnI
TPP1-100	PRM>	RNA isolated from roots/leaves	1112	AK40F / AK41R	NotI / SacI
GLP1	TNL>	U21949	1089	AK78F / AK79R	NotI / XbaI
TIP41	SKV>	U10604	904	AK80F / AK81R	NotI / XbaI

3.1.3.1 PAP7 - At2g01880

A description of the phosphatase is found in 1.2.2.1. The full-length PAP7 construct was already made by Dr Amr Kataya when this thesis started.

3.1.3.2 MapKP1 - At3g55270.1

A description of the phosphatase is found in 1.2.2.8. Two reactions of EHF-PCR were set up as described in materials and methods 2.1.1.1. The R16207 template was used in order to make the fusion construct. Primer sequences are shown in table 6. The PCR result turned out like a smear on 1 % agarose gel. This is shown in figure 31, A. Never the less 20 µl of the sample were ran on gel and a band were cut out in the "right size area" for each sample. The DNA was extracted from the gel following the GenElute™Gel Extraction Kit from Sigma-Aldrich protocol described in 2.1.4.3. One of the gel extracted MapKP1 samples were double digested (see materials and method 2.1.4.4) using XbaI and NotI in order to ligate into the cut pCAT-YFP vector. The other sample was used to ligate into the pGEM®-T Easy vector. Ligation is described in 2.1.4.6. The two ligation reactions were transformed using competent JM 109 *E.coli* cells according to protocol described in 2.1.5.2. The MapKP1/pGEM®-T Easy construct were grown on an IPTG/X-gal containing LB + Ampicillin dish for blue/white screening like described in 2.1.2.1, the MapKP1/pCAT-YFP construct were spread on a LB + ampicillin dish.

Nice colonies appeared on both dishes, and colony-PCR (see materials and methods 2.1.1.2) in order to check for the right insert was performed. It appeared to be a right size insert in the pGEM®-T Easy vector constructs. Figure 31, C shows the agarose gel containing the colony-PCR reactions. Overnight cultures (see materials and methods 2.1.5.5) were made from three MapKP1/pGEM®-T Easy colonies

and from one MapKP1/pCAT-YFP colony in order to isolate plasmids like described in 2.1.5.6. The plasmids were cut using the appropriate restriction enzymes and ran on 1 % agarose gel (80 V for 80 min) in order to check for insert of the right size. The MapKP1/pGEM®-T Easy construct seemed promising. The MapKP1/pCAT-YFP showed something, but it was not obvious. Figure 31, D shows the agarose gel. Both vector-constructs were sent for sequencing. The MapKP1/pCAT-YFP did not contain the right nucleotide sequence. The MapKP1/pGEM-®T Easy construct had the right nucleotides in the beginning and in the end of the construct, but the construct was so long that the middle was not sequenced. Mini-prep of the plasmid was made, cutting using RE NotI and XbaI was performed, followed by Gel Extraction of the insert. After ligation into the pCAT-YFP vector the transformation using JM109 competent *E.coli* cells was performed. Colony-PCR showed insert of the right size. The agarose gel from the colony PCR is shown in figure 31, E. Overnight cultures of 3 colonies were made, followed by plasmid mini-prep and double digestion, all according to protocols. The samples were run on 1% agarose gel, see figure 31, F, insert were localized and the MapKP1/pCAT-YFP (2) were sent for sequencing.

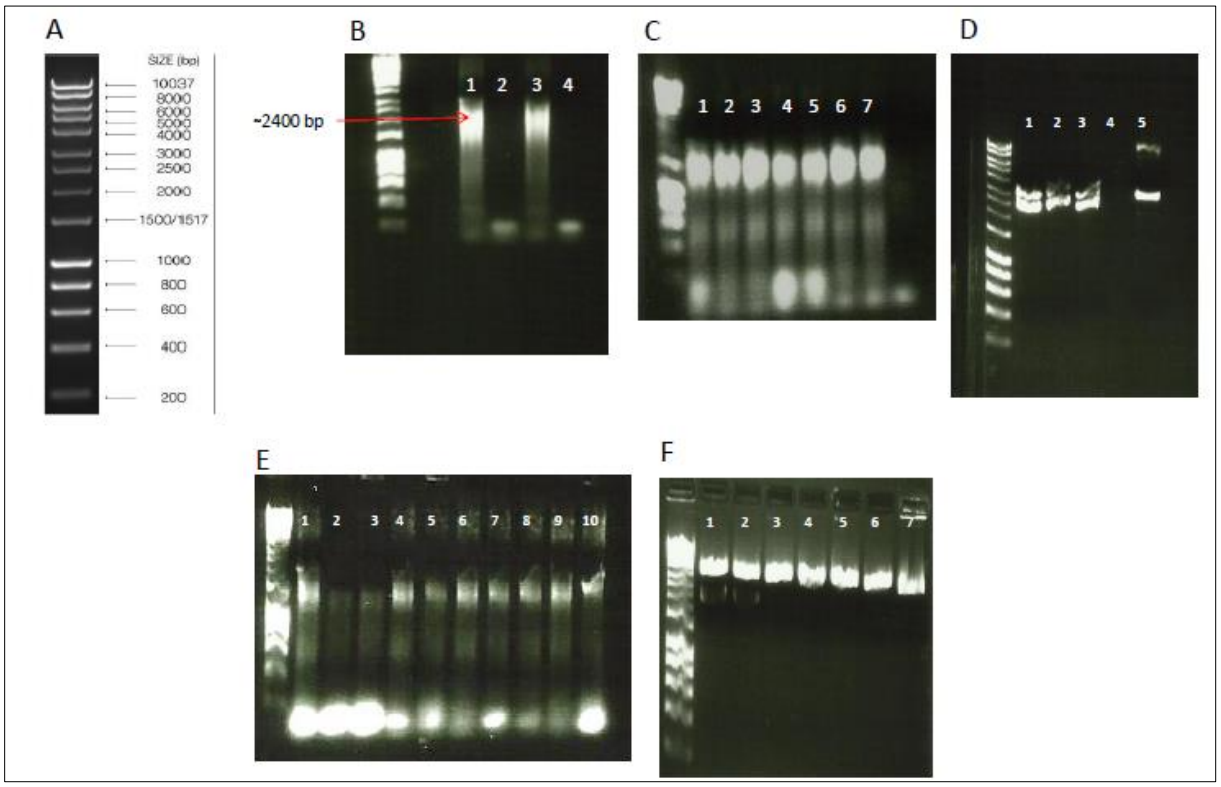


*Figure 31: **A** – HyperLadder™ 1kb (previously HyperLadder I); **B** – MapKP1 EHF-PCR product. A gel band was cut in the «right size area» in the smear, marked in the gel with ~2400 bp; **C** – colony PCR of MapKP1/pGEM®-T Easy using insert primers (AK 29F, AK30R) Plasmid mini-preps were made from colony 5, 6 and 7; **D** – Digestion of MapKP1/pGEM®-T Easy using NcoI and NotI (lane 1-3 and MapKP1/pCAT-YFP (lane 5)). The product shown in lane 1and 5 were sent for sequencing; **E** – colony PCR of MapKP1/pCAT-YFP using primers for plasmid and insert (YFP-Cterm-F,AK30R) Plasmid mini-preps were made from colony 1,4 and 6; **F** – Digestion of MapKP1/pCAT-YFP using XbaI and NotI. Product in lane 2 was sent for sequencing.*

3.1.3.3 SLP1 - At1g07010

This phosphatase which is described in 1.2.2.6 was already cloned into the pCAT-EYFP-M (vector description in 2.1.3.3) and the pWEN18 (vector description in 2.1.3.2) vectors by Dr Amr Kataya. The first construct was called EYFP-SLP1 (A), and hold the EYFP in front of the protein. The latter was called SLP1-EYFP (B), and this construct hold the EYFP at the C-terminal of the protein. The primer sequences are shown in table 6. Targeting to chloroplasts was already described for this phosphatase (Uhrig and Moorhead 2011), but as it was predicted to hold a putative PTS2 (Reumann 2004), it was further investigated in this work.

3.1.3.4 PLL2 – At5g02400 and PLL3 - At3g09400

An attempt to achieve the PLL2 and PLL3 templates was made using RNA extracted from leaves. The concentration was ~1200 ng/µl. RT-PCR like described in 2.1.1.3 was performed. The reaction was used as a template in an EHF-PCR (see 2.1.1.1) with the primers AK35F and AK36R for PLL3 and AK37F and AK36R for PLL2. The primer sequences are shown in table 6. The standard procedures to make a pGEM®-T Easy construct were followed. The sequencing did not identify the right products, but a genomic PLL3 construct was revealed. Several attempts to make the construct were done, using RNA treated with DNase from mature leaves, old leaves, siliques, flowers and roots as templates. The described procedures were followed, and the resulting pCAT-YFP constructs were sent for sequencing. The PLL2 was not obtained and the PLL3 construct had a deletion in the start codon as well as one in the middle of the sequence.

3.1.3.5 8030 - At2g48030

An attempt to subclone this phosphatase which is described in 1.2.2.3 into the pWEN18-vector, which is further described in 2.1.3.2, had been performed without success. The 8030 insert had however been cloned into a pGEM®-T Easy vector. Description of pGEM®-T Easy is found in 2.1.3.1. This vector was cut using XhoI and KpnI restriction enzymes according to protocol described in 2.1.4.4. The same restriction enzymes were used to cut the pWEN18 vector, and then the two reactions were ligated as described in 2.1.4.6, and transformed into competent JM109 *E.coli* cells like described in 2.1.5.2. Plenty of colonies appeared on the selective media after the transformation, but several colony-PCRs performed like description in 2.1.1.2 showed no insert in the vector.
In order to prevent self-ligation of the vector, the same set-up with restriction enzymes were performed, but the vector-reaction were added Shrimp Alkaline phosphatase (rSAP), which removes the phosphate groups on the 5'end of the opened vector. A setup in which the restriction enzymes were changed to XhoI and Acc 65I was performed at the same time. Ligation and transformation were performed according to protocols. In both experiments transformations of ligation reactions of only the vector was used as controls. There were no colonies on the control-plates, which indicated no self-ligation, but the colony-PCR did still not show any inserts in the colonies growing on the 8030 + SAP/ pWEN18 or the other 8030/ pWEN18 plates. A double digestion of the plasmid was performed, but showed no insert.

It was decided to make new primers in order to be able to use the pCAT-YFP vector instead. The new primer sequences are shown in table 6. The insert was made and digested using SacI and NotI. The insert was ligated into a pCAT-DECR-YFP vector, which had been digested using the same restriction enzymes to linearize it and cut out the DECR-part and leave a pCAT-YFP vector with a stop codon in the end of the YFP. Unfortunately the colonies after this transformation did not hold the insert.

A new stock of the pWEN18 vector which was used successfully in another sub-cloning experiment was used, and the first set-up was tried again. Lots of colonies appeared, but of the 60 colonies investigated, none was carrying the 8030 insert. A web-search indicated that the insert may kill the E. coli JM 109 bacteria. No attempts in finding alternatives were done.

3.1.3.6 TPP1-100 - At5g10100

Description of the protein is found in 1.2.2.5. The cDNA was made from RNA extracted from leaves according to protocol described in 2.1.1.3. The reaction was used as a template in an EHF-PCR (see materials and methods 2.1.1.1) with the primers AK40F and AK41R. The primer sequences are shown in table 6. The construct was ligated into the pGEM®-T Easy vector and transferred to LB plates containing ampicillin and added IPTG and X-gal, in order to perform blue/white screening. Protocols are described in materials and methods 2.1.4.6 and 2.1.5. The plasmids were double digested using NotI and SacII, and ran on 1% agarose-gel. The result is shown in figure 32, B. The constructs seemed to be of the right size (1112 bp) and the product called pGEM®-T Easy-TPP1-100 (1) was sent for sequencing. The sequencing showed the correct construct, and sub-cloning of the insert into the pCAT-YFP vector was performed (see 2.1.4 and 2.1.5). The resulting plasmids were digested using SacI and NotI and investigated on 1% agarose gel. The result is shown in figure 32, C. The product called pCAT-YFP-TPP1-100 (2) was sent for sequencing and confirmed to be right.

3.1.3.7 AtGLP1 - At5g36700.3

Description of the protein is found in 1.2.2.7. The cDNA was purchased from ABRC. The construct was amplified using EHF-PCR like described in 2.1.1.1. The product was examined on 1% agarose gel, like described in 2.1.4.2, and result is shown in figure 32, A. The primer sequences are shown in table 6. The PCR-product was gel-extracted and ligated directly into the pCAT-YFP-vector and transformed according to protocols (see 2.1.4.3, 2.1.4.6 and 2.1.5.2). Double digestion of the plasmid was performed, using NotI and XbaI. The reaction was investigated using a 1% agarose-gel. The result is shown in figure 32, C. The result seemed right, and the plasmid was sent for sequencing. The sequencing revealed a wrong sequence. As initial microscopy experiments using the EYFP-PTD1 construct of this phosphatases PTS1-signal (TNL>) not indicated peroxisomal targeting, a second trial to make the full length construct was not performed

3.1.3.8 TIP41 - At4g34270.1

Description of the protein is found in 1.2.2.4. The cDNA was purchased from ABRC. The primer sequences are shown in table 6. The construct was made using EHF-PCR (see materials and methods 2.1.1.1). The product was examined on 1% agarose gel, like described in 2.1.4.2, and the result is shown in figure 32, A. The PCR-reaction was gel-extracted and ligated directly into the pCAT-YFP-vector (see materials and methods 2.1.4) and transformed into JM109 *E.coli* competent cells according to protocol described in 2.1.5.2.The plasmid was double digested using NotI and XbaI, and the reaction was investigated on 1% agarose-gel. The result is shown in figure 32, C. The construct called pCAT-YFP-TIP41 (2) was sent for sequencing. The sequencing confirmed the right construct.

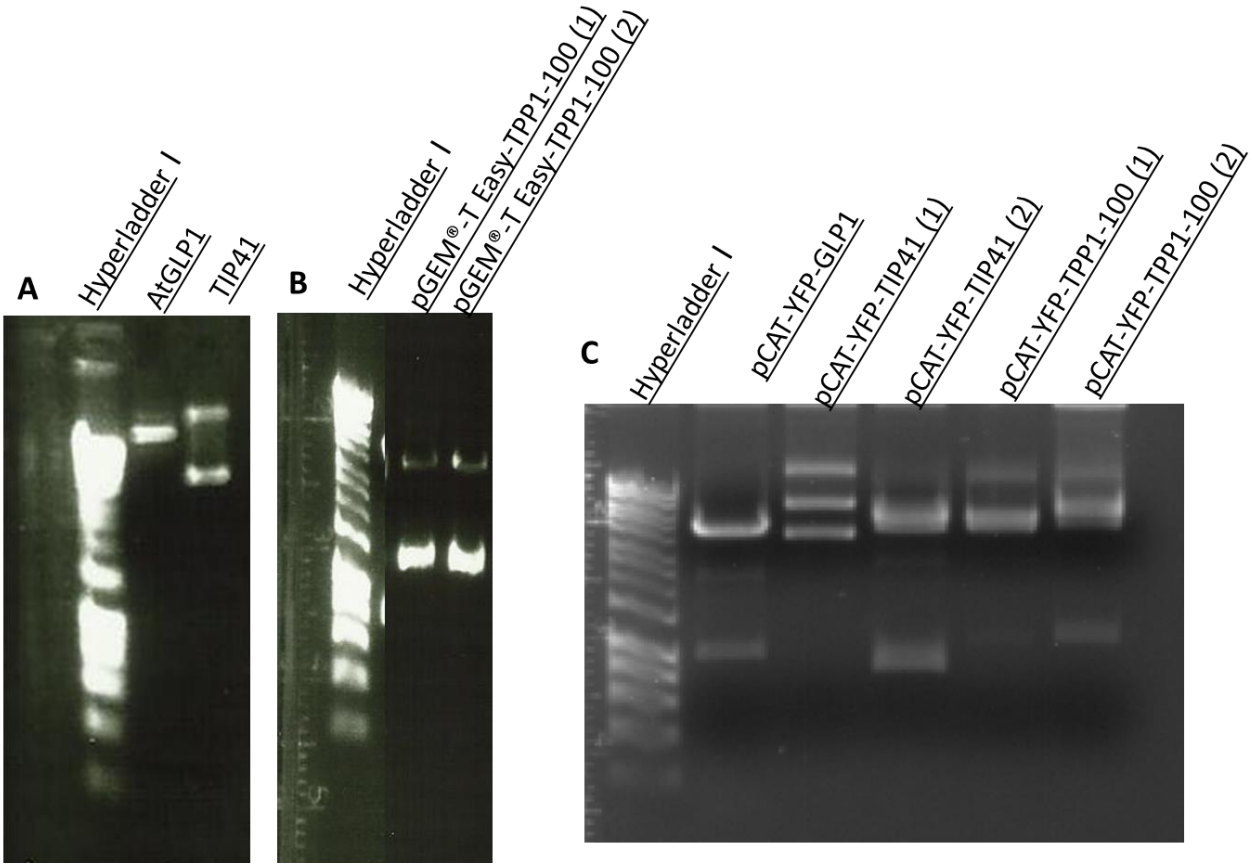


Figure 32: Agarose gel from different stages in the making of full-length constructs. ***A*** *– EHF-PCR results from amplifying of the GLP1(U21949) and TIP41 (U10604)templates.* ***B*** *– From double digestion of pGEM®-T Easy-TPP1-100 plasmids (RE – NotI, SacII).* ***C*** *– From double-digestion of the pCAT-YFP-plasmids. GLP1-digested with NotI, XbaI. TIP41 digested with NotI, SacI. TPP1-100 digested with SacI, NotI.*

Table 6: Primer sequences used to make full-length phosphatase/EYFP constructs

Acronym	Forward primer	Reverse primer
PAP7	AK27F - GCGGCCGCTATGAAGATGCACGTATGTTTTA	AK28R-GAGCTCTTAAAGATGAGCAGATCTTTTG
SLP1	AK31F-GCGGCCGCTATGGCTTCCCTTTACCTCAA AK33F- GGTACCATGGCTTCCCTTTACCTCAA	AK32R- CCGCGGTTAAATGTAATCTGCAACCT AK34R- GGTACCAATGTAATCTGCAACCT
MapKP1	AK29F- GCGGCCGCTATGGTGGGAAGAGAGGATGCG	AK30R - TCTAGAGTTATAGCGCGCTCAGCAGTG
PLL3	AK35F - GCGGCCGCTATGGGAAATGGAGTCGCGAGTTTTA	AK36R - CCGCGGTCACATGGAAGATCTCCAAATTCTT
PLL2	AK37F - GCGGCCGCTATGGGAAATGGAGTCACCACTTTGA	AK36R - CCGCGGTCACATGGAAGATCTCCAAATTCTT
TPP1	AK40F - GCGGCCGCTATGTCAGCTAGTCAAAACAT	AK41R - GAGCTCTCACATTCTTGGCTGCATTT
GLP1	AK78F - ACTGCGGCCGCTATGCTGAGTAGATCAGTTGCT	AK79R - CAAGTCTAGAGTTACAGATTAGTTATACCCG
TIP41	AK80F - ACTGCGGCCGCTATGGAGACGGTGGTCGATAAAGAT	AK81R - CAAGTCTAGAGTTAAACTTTACTAGGGATCT
.8030	AK98F - ATGAGCTCTCATGTTGAACCTCATTGCTTTCCTC	AK99R - ATGCGGCCGCTGCTTTATAGTAGTGTGTCCTCC

3.2 Microscopy results

The microscopy investigations of the different EYFP- constructs were performed using a Nikon TE 200U inverted fluorescence microscope, equipped like described in materials and methods 2.2.4. All experiments were performed >3 times in the onion epidermis cell system and in the *Arabidopsis* mesophyll protoplast system. All investigations which revealed fluorescence appearing in dots were repeated and different organelle markers were included in subsequent experiments.

All images without remarks are taken approximately 24 h after transformation.

NB – because the images turned out to be significantly darker on paper compared to how they appeared on the screen, most images is adjusted to be shown brighter than the originals.

3.2.1 Microscopy investigation of PTD-EYFP constructs

Table 7 presents an overview of the localization investigations of the putative Peroxisomal Targeting Domains (PTDs)

Table 7: Targeting results of PTDs.

Acronym	Type of PTD-construct	Putative PTS	Targeting result
PAP7	EYFP-PTD1	AHL>	Peroxisomes, cytosol, nucleus
MapKP1	EYFP-PTD1	SAL>	peroxisomes
PLL2 & 3	EYFP-PTD1	SSM>	Peroxisomes, cytosol
TPP-1-100	EYFP-PTD1	PRM>	Peroxisomes,
GLP1	EYFP-PTD1	TNL>	cytosol
TIP41	EYFP-PTD1	SKV>	peroxisomes
SLP1	PTD2-EYFP	RRIVAVGDL	cytosol
.8030	PTD2-EYFP	RISVNHHHL	cytosol

The EYFP-PTD1 and PTD2-EYFP constructs confirmed by sequencing was amplified and mini-preps with high concentration were made by using 7.5 – 10 ml of bacterial suspension and perform plasmid isolation according to protocol described in 2.1.5.5. The constructs were precipitated onto gold particles and shot into onion epidermal cells as described in materials and methods 2.2.1. Approximately 5 µg of DNA was used. The next day the microscopy samples were prepared as described in 2.2.4.1.

The first examination of the transient transformed cells showed cells with fluorescence. The EYFP-PTD1 constructs of PAP7, MapKP1, PLL2&3, TPP1-100 and TIP41 were found in dots. The EYFP- PTD1 construct of GLP1 seemed to be cytosolic. Images of onion cells transiently transformed using the different EYFP-PTD1 constructs is shown in figure 33. The EYFP-PTD1 constructs of PAP7 and PLL2 & 3, are also expressed in the cytosol, and the EYFP-PTD1/PAP7 seems to appear inside the nucleus as well.

The PTD2-EYFP constructs of SLP1 and 8030 seemed to remain in the cytosol. The results of microscopy examination of the PTD2-EYFP constructs of SLP1 in onion epidermal cells are shown in figure 34.

The EYFP-PTD1 and PTD2-EYFP constructs were also used to transiently transform *Arabidopsis* mesophyll protoplasts, using PEG transfection. The protoplasts confirmed the pattern already

noticed in the onion epidermal cells. Protoplast transiently transformed to express the PTD2-EYFP construct of 8030 is shown in figure 34.
No organelle markers were included in these samples.

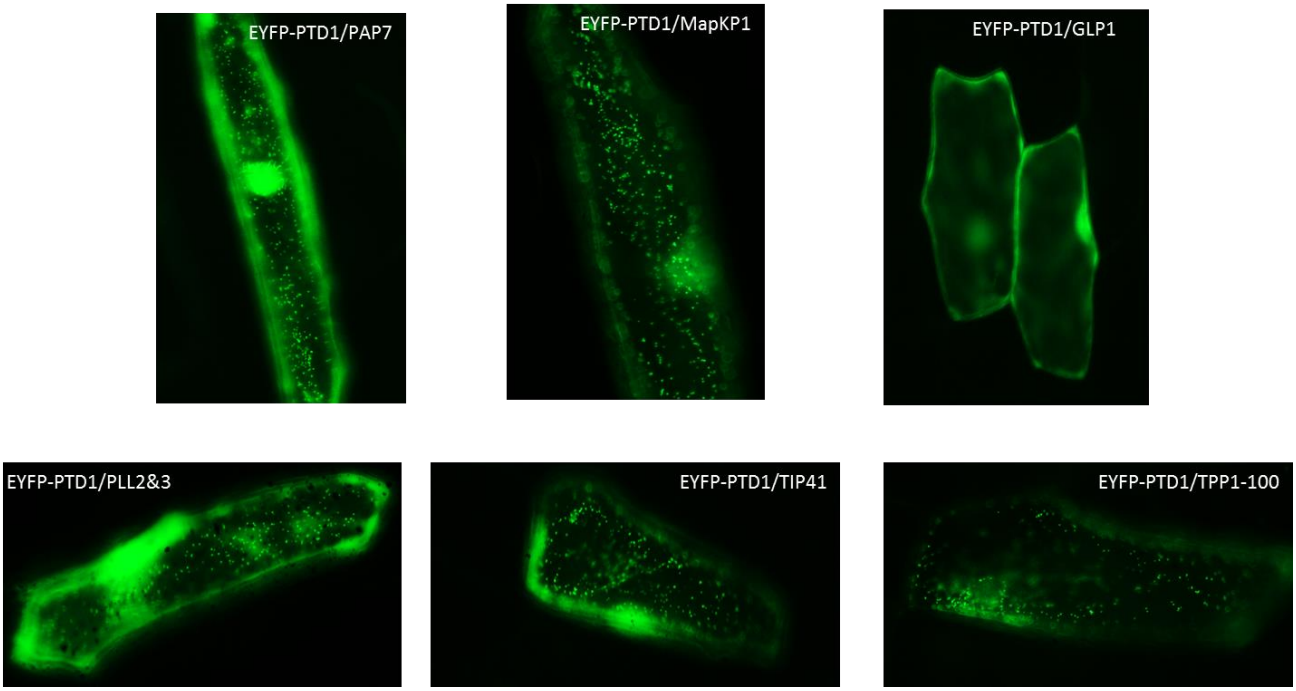


Figure 33: Microscopy images of EYFP-PTD1 constructs shown in onion epidermal cells. The EYFP-PTD1 constructs of PAP7, MapKP1, PLL2&3, TIP41 and TPP1-100 appears in dots, while the EYFP-PTD1 construct of GLP1 seems to be expressed in the cytosol. The images are captured through a 20 x magnifying lens.

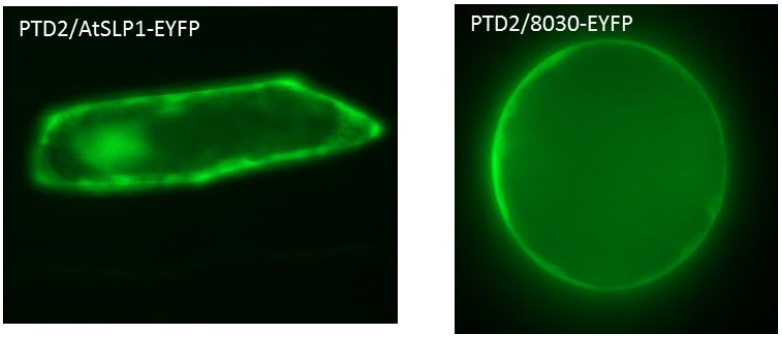


Figure 34: Microscopy images of PTD2-EYFP constructs. The PTD2-EYFP construct of SLP1 is shown in an epidermal onion cell, photographed trough a 20 x magnifying lens, and seems to be cytosolic. The PTD2-EYFP construct of the 8030 is shown in a transiently PEG-transformed protoplast from Arabidopsis, photographed trough a 60 x magnifying lens. The tagged proteins seem to stay in the cytosol.

The microscopy experiments were repeated, and peroxisomal markers were included. Images were captured through a yellow fluorescence filter, and these are called EYFP. These images will show the fluorescence emitted by the EYFP-tagged proteins. The same cells were also photographed through a filter capturing red fluorescence and these images are called RFP. These images will show the fluorescence emitted by the peroxisomal marker. The EYFP and RFP images were also merged to show potential overlapping of dots. The dots observed in the EYFP-PTD1 constructs of PAP7, MapKP1, PLL 2&3, TIP41 and TPP1-100, appeared to be peroxisomal. The EYFP-PTD1/PAP7 seemed

to appear in the nucleus in protoplasts as well, and all of the constructs are also seen in the cytosol. Figure 35 shows protoplasts transiently transformed using EYFP-PTD1 constructs as well as the peroxisomal marker PW99 described in 2.1.2.3. The bright field pictures are included to show that the cells are intact.

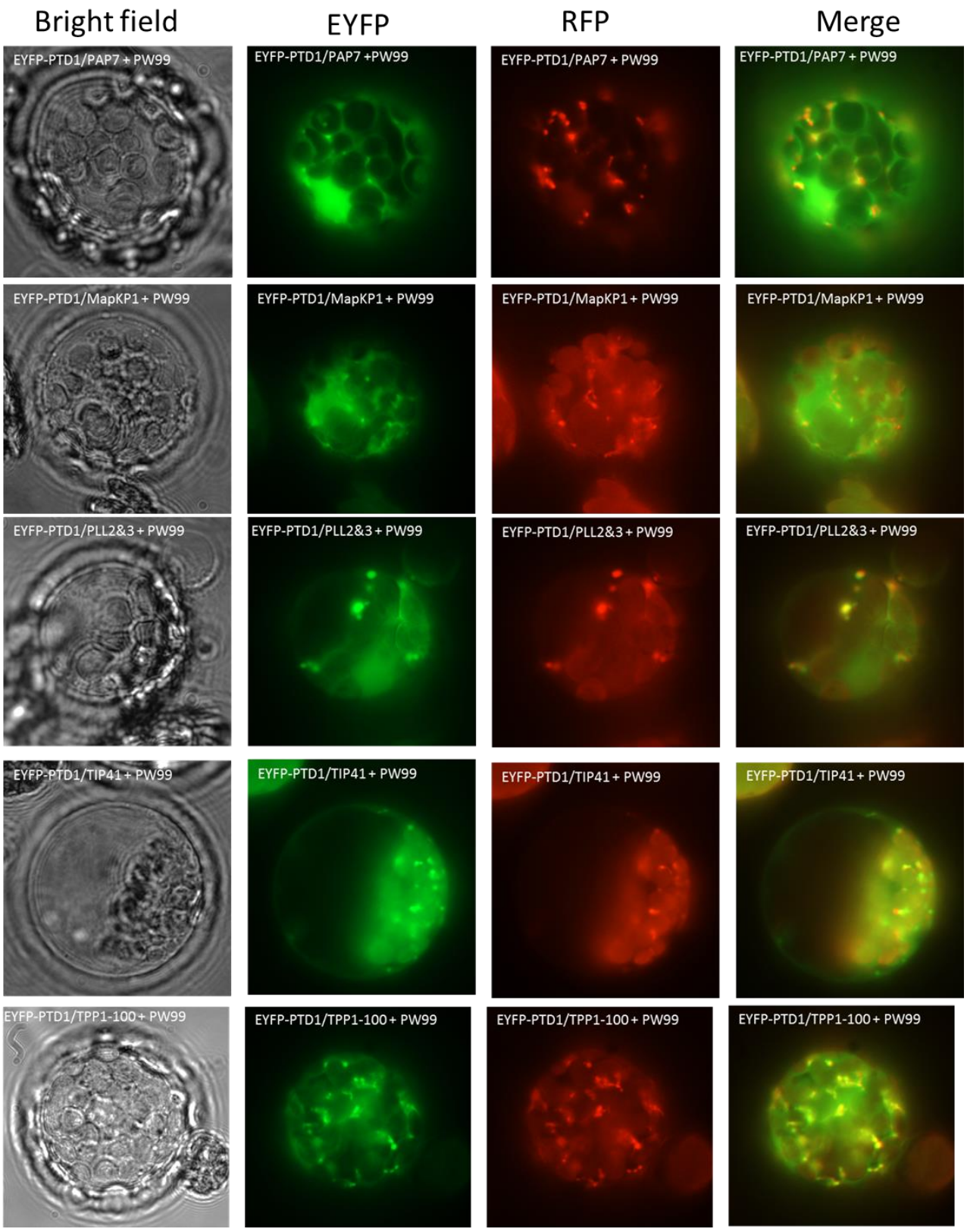


Figure 35: EYFP-PTD1 constructs which were targeted to peroxisomes shown in Arabidopsis mesophyll protoplasts. The EYFP-images (green) are captured through a yellow fluorescence filter and shows the fluorescence emitted from the EYFP-PTD1 fusion construct. The RFP-images (red) are captured through a red fluorescence filter and shows the fluorescence emitted from the peroxisomal marker. The EYFP and RFP-images were merged and the overlapping fluorescence spots appear in yellow. The images are captured through a 60 x magnifying lens.

3.2 2 Microscopy investigation of full-length phosphatase constructs

The microscopy investigation of the EYFP-constructs of the full-length proteins was performed in the same way as the investigation of the EYFP-PTD1 and PTD2-EYFP constructs. First the plasmids were precipitated onto gold particles, shot into onion epidermal cells using a biolistic gene gun and investigated using fluorescence microscopy. The procedures are described in materials and methods 2.2. During repetitive investigations, different organelle markers were included. The organelle markers were chosen according to the findings during the first scans. The same procedure was followed using the *Arabidopsis* mesophyll protoplasts system. In the first experiments the transformation were performed without markers, and thereafter repeated including the appropriate ones. Table 8 shows an overview of the targeting investigations performed using the full-length EYFP-constructs.

Table 8: An overview of targeting experiments of the EYFP constructs of full-length proteins.

Acronym	Type of construct	Putative PTS	Targeting result
PAP7	EYFP-PAP7	AHL>	peroxisomes
MapKP1	EYFP-MapKP1	SAL>	cytosol
PLL2	EYFP-PLL2	SSM>	no full-length made
PLL3	EYFP-PLL3	SSM>	no full-length made
TPP1-100	EYFP-TPP1-100	PRM>	peroxisome, nucleus, nucleolus
GLP1	EYFP-AtGLP1	TNL>	no full-length made
TIP41	EYFP-TIP41	SKV>	nucleus
SLP1-A	EYFP-AtSLP1	RRIVAVGDL	Cytosol, granules
SLP1-B	AtSLP1-EYFP	RRIVAVGDL	chloroplasts
.8030	8030-EYFP	RISVNHHHL	no full-length made

3.2.2.1 EYFP- PAP7

The EYFP fused full-length PAP7 construct was transformed into onion epidermal cells. The first microscopy investigation showed some cells with fluorescence in dots, and some cells where the fluorescence was concentrated to the membrane or cell-wall. Further experiments including a peroxisomal selectable marker revealed the fluorescence to appear mainly in peroxisomes short after the transformation (24h), and secreted into extracellular space or transferred to cell membrane after some time (48h and longer). The peroxisomal marker seemed to be expressed in the same way as the EYFP-PAP7, that is to express fluorescence extracellular or in cell membrane. An ER-marker was included in a transformation in order to figure out if the ER played a distinct role in the distribution of the constructs, but no clear patterns of this was found (results not shown). Figure 36 shows onion epidermal cells expressing yellow fluorescence (green images marked EYFP) from the EYFP-PAP7 construct and red fluorescence expressed from a peroxisomal marker (red images marked RFP). Images captured 24 h after transformation shows the fluorescence mainly in peroxisomes, while the images captured after 7 days shows fluorescence at the edges of the cell, and EYFP-PAP7 seemed to be expressed around or in the nucleus membrane. Peroxisomal marker used in the images

captured after 24 h is gMDH-CFP, a PTS2-CFP construct. The marker used in images captured after 7 days is PW99, a RFP-PTS1 fusion construct. Both markers are described in further details in 2.1.2.3. The cyan fluorescence of the PTS2-CFP marker was converted to red in order to get better merged pictures.

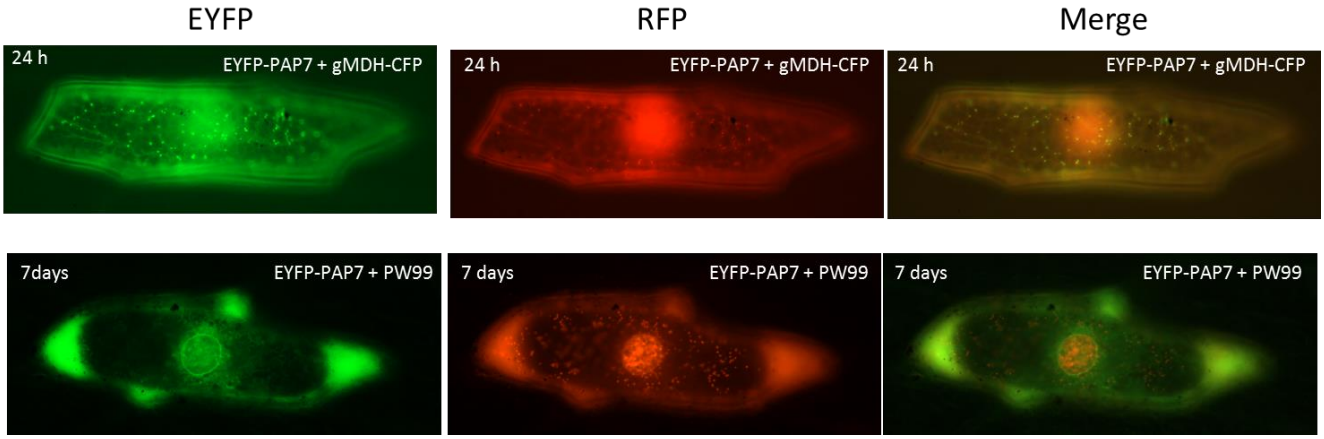


Figure 36: Images showing fluorescence of the EYFP- PAP7 construct. The EYFP-images (green) are captured trough a yellow fluorescence filter and shows the fluorescence emitted from the EYFP-PAP7 fusion construct. The RFP-images (red) are captured trough a red fluorescence filter and shows the fluorescence emitted from the peroxisomal marker. The EYFP and RFP-images were merged and the overlapping fluorescence spots appear as yellow. The images are captured through a 20 x magnifying lens.

The same investigations were repeated using transiently transformed *Arabidopsis* mesophyll protoplasts. The fluorescence of the fusion protein seemed to be in peroxisomes, but also to some extend in or near the cell membrane. The ER-marker was included in protoplast transformations as well, but did not lead to any conclusion (images not shown). Figure 37 shows the fluorescence from the EYFP-PAP7 construct compared to the fluorescence from the peroxisomal marker PW99, a RFP-PTS1 fusion construct described in 2.1.2.3. The EYFP-PAP7 fusion protein seemed to be targeted to peroxisomes in the protoplasts. The EYFP-tagged protein is also expressed in the cytosol to some extent.

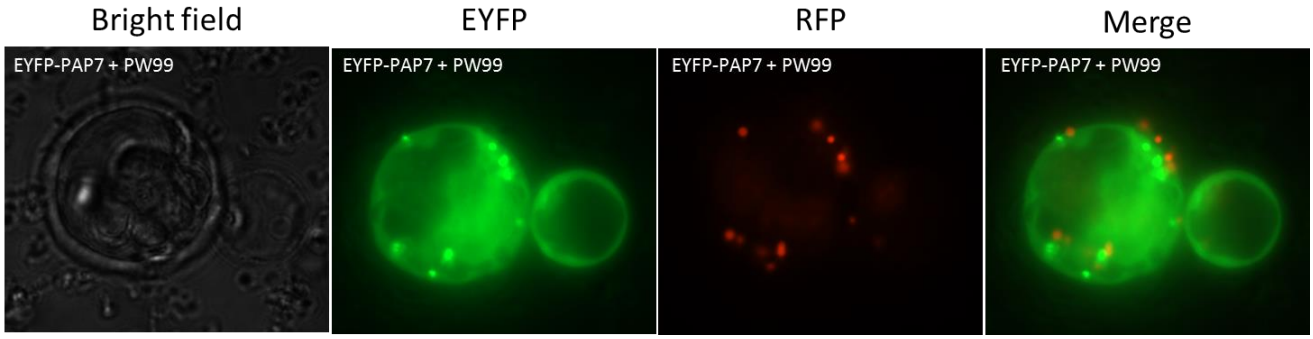


Figure37: Arabidopsis mesophyll protoplasts transiently transformed to express the EYFP-PAP7 construct and RFP from the peroxisomal marker PW99. The EYFP-images (green) are captured trough a yellow fluorescence filter and shows the fluorescence emitted from the EYFP-PAP7 fusion construct. The RFP-images (red) are captured trough a red fluorescence filter and shows the fluorescence emitted from the peroxisomal marker. The EYFP and RFP-images were merged and the overlapping fluorescence spots appear as yellow. The fluorescent dots seem to be in the same places, even if the cell has moved slightly, so the overlap in the merged picture is not exact. The Bright field picture is included to show that the cell is intact.

3.2.2.2 EYFP-MapKP1

The construct of EYFP fused to full-length MapKP1 was transformed into onion epidermal cells. The first investigations indicated the fusion protein mainly to be expressed in the cytosol. Figure 38 shows an epidermal onion cell transiently transformed to express the EYFP-MapKP1 fusion protein. The figure also shows a transiently transformed *Arabidopsis* protoplast expressing the EYFP-MapKP1 after 48h. No markers were included in these experiments.

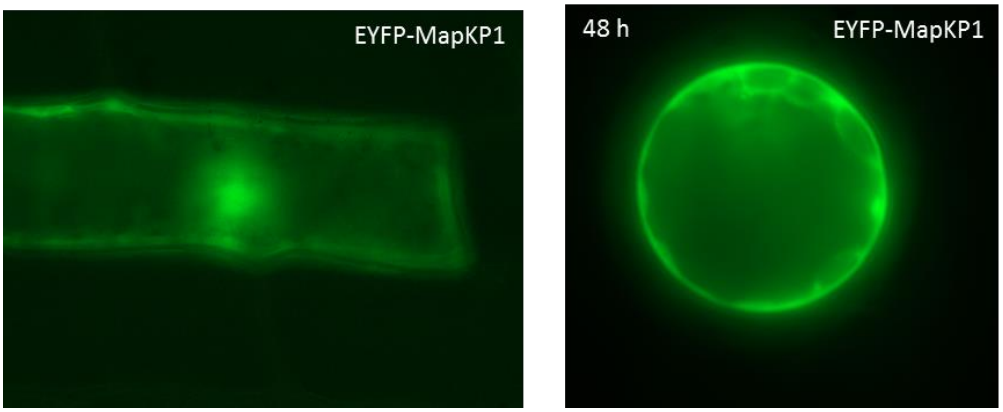


Figure 38: Fluorescence from the EYFP-MapKP1 construct shown in a transformed onion epidermal cell and in Arabidopsis mesophyll protoplasts. The onion cell is photographed through a 20 x magnifying lens, the protoplasts through a 60 x magnifying lens.

The same microscopy investigations were repeated using transiently transformed *Arabidopsis* mesophyll protoplasts. The peroxisomal marker gMDH-CFP, a PTS2-CFP construct was included (see 2.1.2.3). The cyan fluorescence of the PTS2-CFP marker was converted to red. The same pattern as in onion epidermal cells appeared, and the construct was concluded to be cytosolic. Images are shown in figure 39.

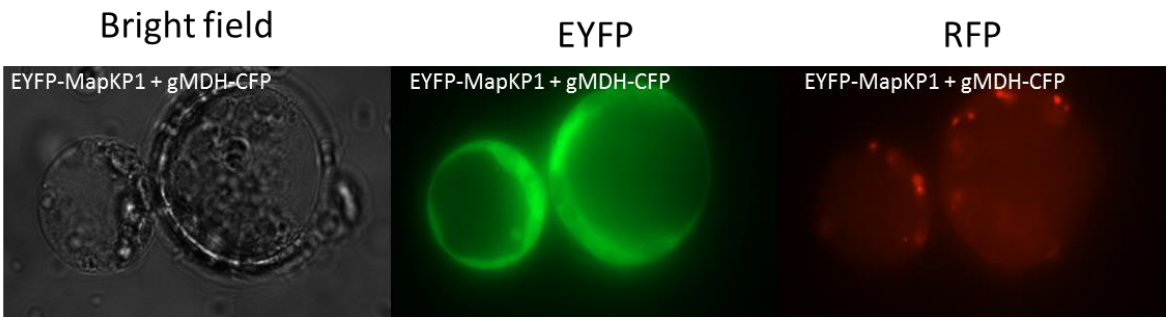


Figure 39: Arabidopsis mesophyll protoplasts transiently transformed to express the EYFP-MapKP1 construct and CFP from the peroxisomal marker gMDH-CFP. The EYFP-images (green) are captured trough a yellow fluorescence filter and shows the fluorescence emitted from the EYFP-MapKP1 fusion construct. The RFP-images (red) are captured trough a red fluorescence filter and shows the fluorescence emitted from the peroxisomal marker. The cyan fluorescence of gMDH-CFP, a PTS2-CFP marker was converted to red. TheEYFP-MapKP1 construct was concluded to be cytosolic. The Bright field picture is included to show that the cell is intact. The images are captured through a 60 x magnifying lens.

3.2.2.3 EYFP-SLP1 (A) and SLP1-EYFP (B)

The construct EYFP-AtSLP1 (A), consisting of EYFP fused to the N-terminal of AtSLP1 was transformed into onion epidermal cells. The first microscopy investigation showed some cells with fluorescence in dots (images not shown). The same investigations were repeated using transiently transformed *Arabidopsis* mesophyll protoplasts. The fluorescence of the fusion protein seemed to be in some dots as well as in the cytosol. Figure 40 shows protoplasts expressing fluorescence from the EYFP-SLP1 construct as well as the peroxisomal marker gMDH-CFP, a PTS2-CFP construct (see 2.1.2.3).The cyan fluorescence of the PTS2-CFP marker was converted to red in the lower images in order to get better merged pictures. The images show the fluorescence from the EYFP-SLP1 (A) construct compared to the fluorescence from the peroxisomal marker. The fluorescence from the dots shown in the lower images does not overlap, and was considered not to be peroxisomes. Any further analyses of the dots were not performed.

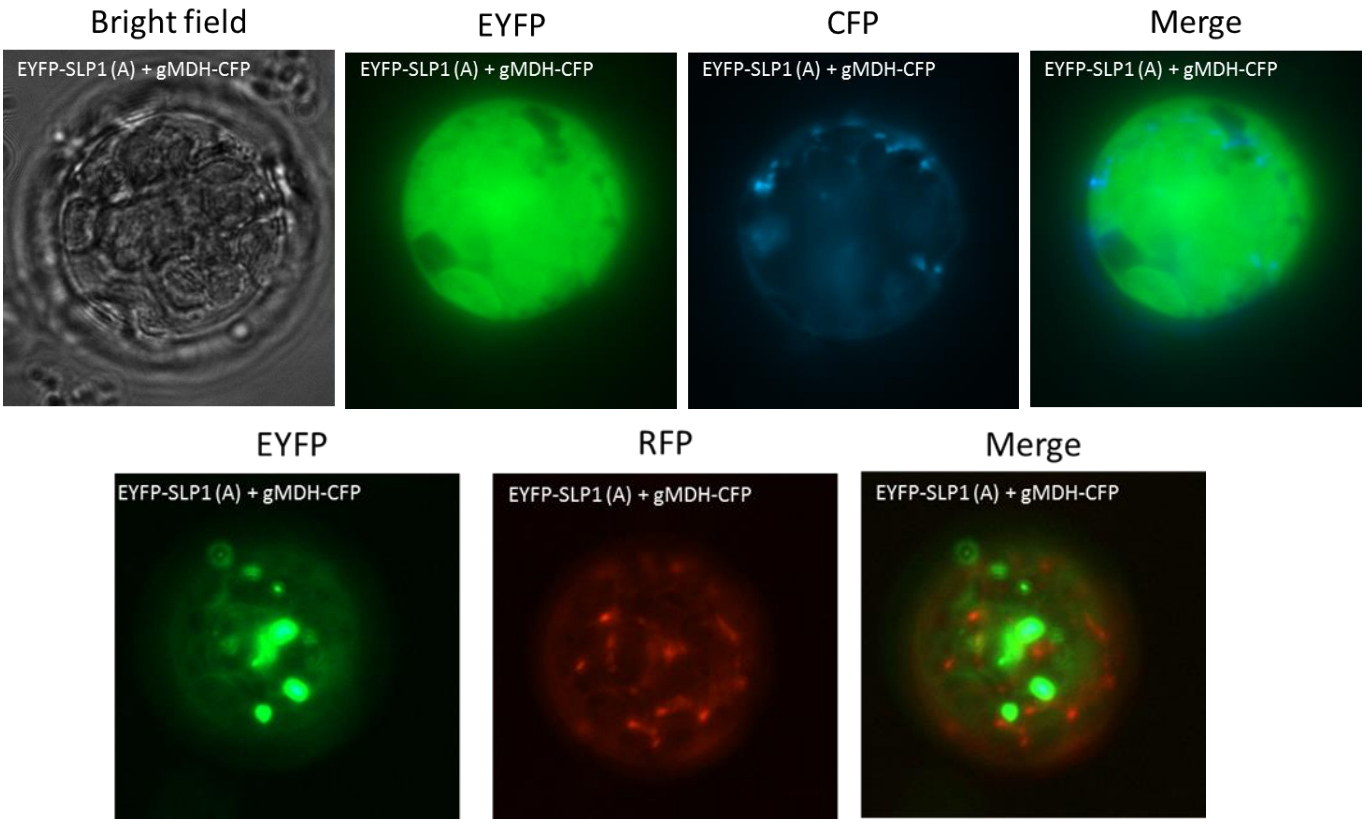


Figure 40: Images of fluorescence originating from the EYFP-SLP1 (A)fusion protein and CFP/RFP from the peroxisomal marker gMDH-CFP. The EYFP-images (green) are captured trough a yellow fluorescence filter and shows the fluorescence emitted from the EYFP-SLP1 (A) fusion construct. The CFP-images (blue) are captured trough a cyan fluorescence filter and shows the fluorescence emitted from the peroxisomal marker. The cyan fluorescence of gMDH-CFP in the lower image, called RFP, was converted to red in order to get better merged pictures. The EYFP-tagged protein seems to be expressed in cytosol and chloroplasts. The lower images shows some clusters of EYFP, but they seems not to be peroxisomes. The Bright field image is added to show the intactness of the cell. The images are captured through a 60 x magnifying lens.

The construct SLP1-EYFP (B), consisting of SLP1 with EYFP fused to its C-terminal, leaving the N-terminal free, was transformed into onion epidermal cells. The first microscopy investigation showed some cells with fluorescence in dots. The dots seemed to be too big to be peroxisomes. Further experiments including the peroxisomal selectable marker PW99 (see 2.1.2.3) revealed the SLP1-EYFP (B) dots not to be peroxisomes. Figure 41 shows the fluorescent dots from the SLP1-EYFP (B) construct and the RFP peroxisomal marker. The merged image shows no overlapping.

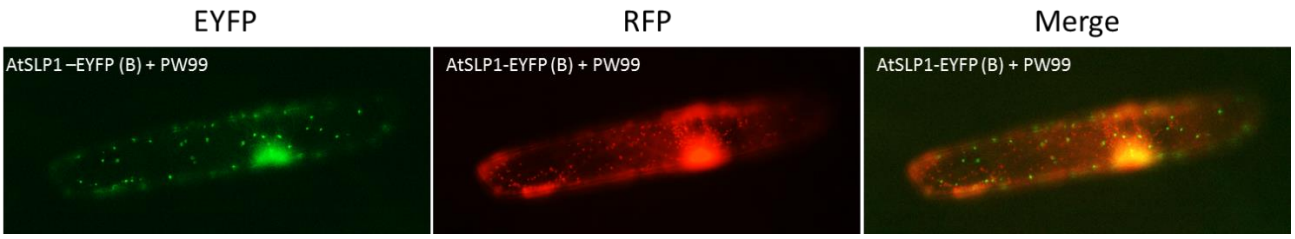


Figure 41: Images of fluorescence originating from the SLP1-EYFP (B) fusion protein, and the RFP peroxisomal marker PW99. The fluorescence originating from the SLP1-EYFP (B) construct does not seem to be peroxisomes, as they are quite big. The Merged image shows no overlapping of the EYFP and the RFP emitting protein constructs. The images are captured through a 20 x magnifying lens.

The same investigations were repeated using transiently transformed *Arabidopsis* mesophyll protoplasts. As the SLP1 was predicted to be targeted to chloroplasts (Uhrig and Moorhead 2011), images using a red chlorophyll auto-fluorescence filter was captured. The fluorescence from the SLP1-EYFP (B) construct was clearly appearing in chloroplasts. Figure 42 shows the images from the protoplast transformation.

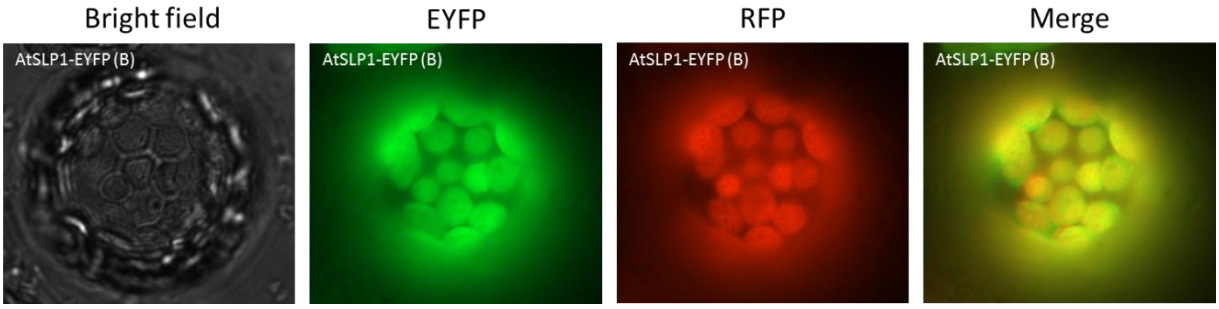


Figure 42: Images of fluorescence originating from the SLP1-EYFP (B) fusion protein captured trough a yellow fluorescence filter (green), and a red chlorophyll autofluorescence filter (red).The two images are merged, and the overlapping fluorescence appears yellow. The fluorescence emitted from the SLP1-EYFP (B) constructs are clearly originating from the chloroplasts. The Bright field image is added to show the intactness of the cell. The images are captured through a 60 x magnifying lens.

3.2.2.4 EYFP-TIP41

The EYFP-TIP41 construct was transformed into onion epidermal cells. The first investigations indicated the fusion protein to be mainly expressed in the nucleus. The same investigations were repeated using transiently transformed *Arabidopsis* mesophyll protoplasts. The same pattern appeared, and the construct was concluded to be mainly expressed in the nucleus. Figure 31 shows a fluorescence onion epidermal cell as well as protoplasts transiently transformed to express the EYFP-TIP41 construct

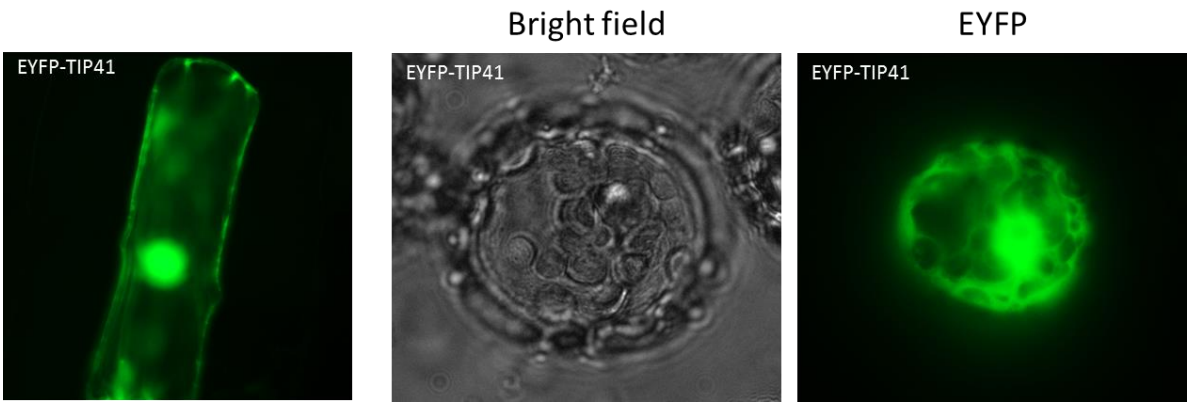


Figure 43: Fluorescence from the EYFP-TIP41 construct shown in an epidermal onion cell and in protoplasts. The construct seems to be concentrated in the nucleus. The onion cell is photographed through a 20 x magnifying lens, the protoplasts through a 60 x magnifying lens.

3.2.2.5 EYFP-TPP1-100

The EYFP-TPP1-100 full-length construct was transformed into onion epidermal cells. The first investigations indicated the fusion protein to be mainly expressed in the nucleus and nucleolus. By increasing the contrast to avoid the bright light from the nucleus, it was possible to see fluorescence in dots as well. Figure 43 shows transformed onion epidermal cells expression the EYFP-TPP1-100 construct. As the paper copy appears darker than the screen image B in Figure 44 is shown in full size in the enclosure.

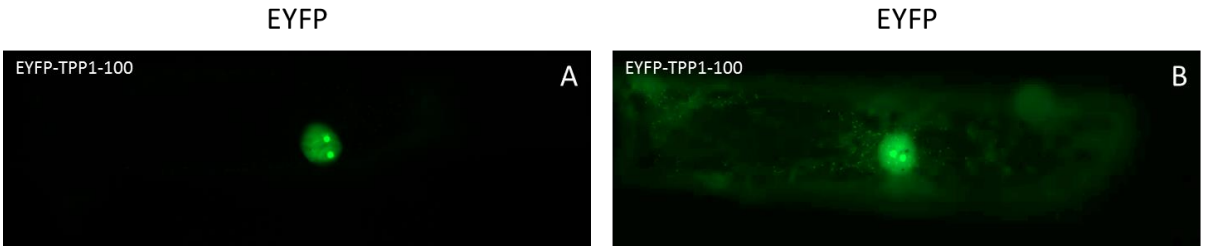


Figure 44: Fluorescence from the EYFP-TPP1-100 construct in onion epidermal onion cells. The nucleoli and the nucleus are emitting bright fluorescence (A). By increasing the contrasts fluorescence dots appeared (B) Images were captured through a 20 x magnifying lens.

The same investigations were repeated using transiently transformed *Arabidopsis* mesophyll protoplasts. The result seemed to be the same. Nucleolus and nucleus fluoresced bright, but dots were detectable when the contrast in the images was increased. Figure 45 shows transiently transformed protoplasts expressing the EYFP-TPP1-100 fusion protein and the peroxisomal marker gMDH-CFP. The cyan fluorescence was converted to red. The overlapping in the merged images

indicates that the fusion protein is expressed in the peroxisomes. The dots seem to appear in the same area, but the cells were slightly moving as the images were captured.

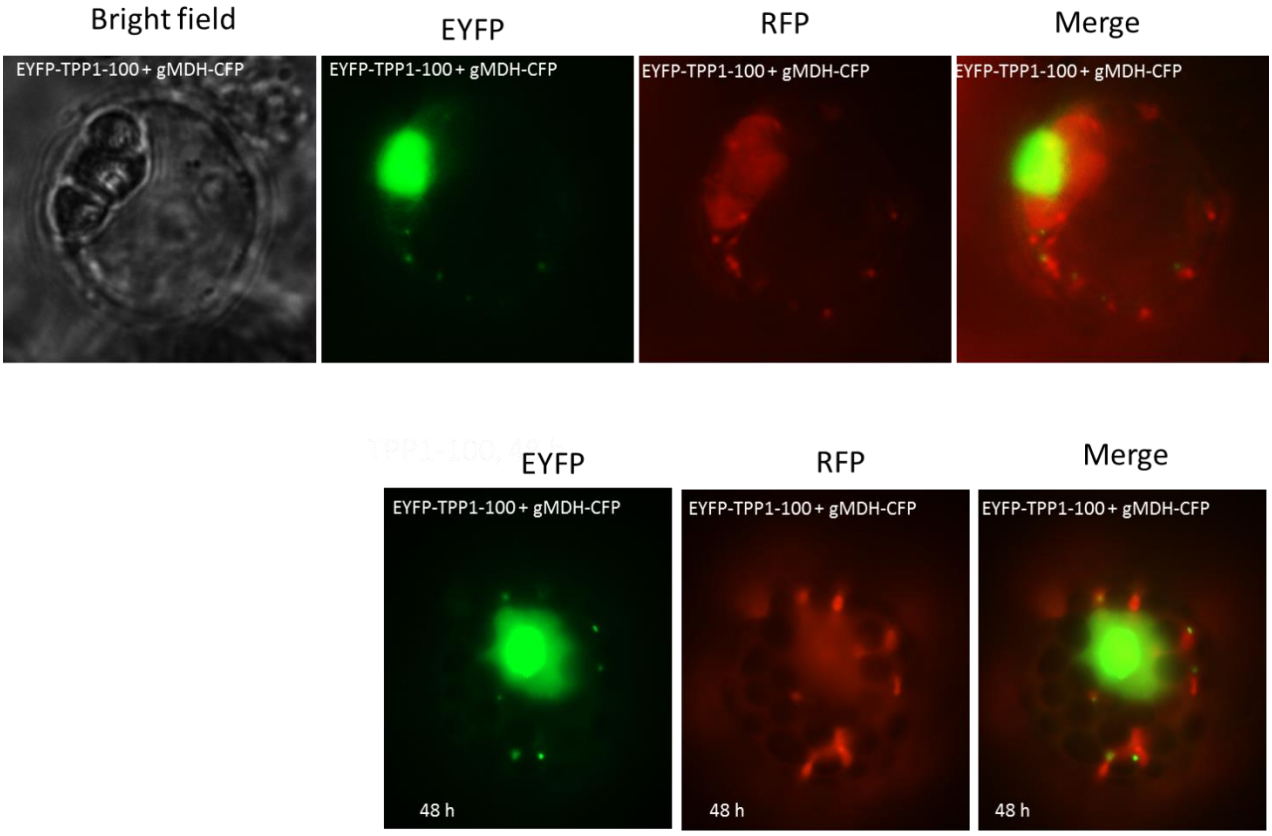


Figure 45: Images of transiently transformed Arabidopsis mesophyll protoplasts expressing the EYFP-TPP1-100 construct. The protoplasts do also express the peroxisomal PTS2-CFP marker gMDH-CFP. The EYFP-images (green) are captured trough a yellow fluorescence filter and shows the fluorescence emitted from the EYFP-TPP1-1001 fusion construct. The RFP-images (red) are captured trough a red fluorescence filter and shows the fluorescence emitted from the peroxisomal marker. The cyan fluorescence was converted to red in order to get better merged pictures. The EYFP-TPP1-100 construct seems to be expressed in nucleolus, nucleus and in peroxisomes. Images captured after 48 h shows the same pattern. The Bright field image is added to show the intactness of the cell. The images are captured through a 60 x magnifying lens.

4 DISCUSSION AND OUTLOOK

It has been reported that the Arabidopsis genome contains almost 300 genes that encode proteins with either PTS1 or PTS2 sequences, and the identification of peroxisomal protein kinases, phosphatases and phosphorylated proteins strongly suggests that plant peroxisomal metabolism is regulated by reversible phosphorylation (Reumann 2011).

The different phosphatase-related proteins investigated was predicted to contain a putative peroxisomal targeting signal according to the AraPerox database (Reumann et al. 2004) and one protein was added because it was found in peroxisome proteomic studies (Fukao et al. 2002).

To investigate if the PTS actually were peroxisomal, a fusion protein containing EYFP and the putative signal was made. The intention was that if the signal actually was found in peroxisomes, the full-length protein has the ability to enter the organelle as well. As shown in the PTD targeting results table 7, 5 out of the 6 EYFP-PTS1 constructs were targeted to peroxisomes, while the 2 putative PTS2-EYFP constructs were found only in cytosol. Even prototypic peroxisomal targeting signal can fail to enter peroxisomes. The amino acid residues in vicinity of a signal contribute to the distribution. Acidic residues are shown to weaken a signal, while basic residues can promote the peroxisomal targeting (Ma and Reumann 2008).

As there are proteins imported into peroxisomes in other ways than by PTS1 and PTS2, it is not accurate to claim the proteins containing a non-targeted domain, to be non-peroxisomal. In this investigation, though, all the proteins were picked on the basis of the putative domain. This makes it likely to assume that the PTS is the reason for the targeting according to these proteins. An exception is the AtSLP1 (At1g07010) which was added to the investigation because it was found in a proteomic analysis of leaf peroxisomal proteins (Fukao et al. 2002).

Not all of the EYFP-fused full length protein constructs were successfully made. As the putative PTS1-signal of AtGLP1 (At5g36700.3) seemed not to be a peroxisomal signal, the construction of the EYFP-AtGLP1 fusion protein was not repeated, when the sequencing showed that coding sequence of the first construct was nonsense. Several unsuccessful attempts to make the 8030 (At2g48030)-EYFP construct was performed. Attempts to clone EYFP-PLL2 (At5g02400.1) and EYFP-PLL3 (At3g09400.1) was repeated continuously throughout the whole time of this work. As bioinformatics studies of these two latter proteins revealed several homologues in other species also containing the PTS1 signal, they are of great interest to investigate. New samples of RNA from roots and leaves of young WT *Arabidopsis* plants have been extracted, hoping that fresher RNA will give better results. Site directed mutagenesis on the EYFP-PLL3-construct described in 3.1.3.4 is to be performed.

Both the transiently transformation of onion epidermal cells, as well as the PEG transformation of *Arabidopsis* mesophyll protoplasts, are well established methods in protein targeting experiments. During the investigations of the different EYFP-constructs, several experiments were performed using both systems. The transformation efficiency varied from experiment to experiment. Another issue was the state of the plants used to extract mesophyll protoplasts. To get the best transiently transformation and expression, quite young plant (~6 weeks) seemed to be preferable.

The investigations of EYFP-PAP7 showed fluorescence in peroxisomes, but also in cell membrane. This latter expression was more pronounced in the epidermal onion cells, suggesting that the protein construct actually could be excreted and located in the extracellular space between cell membrane

and the cell wall. As the cell wall is digested during the extraction of protoplast, extracellular location would not be seen as easily using this system. The investigation of the protein, described in 1.2.2.1 suggested a transmembrane region near the N-terminal (5-24). This could target the protein to membrane as well as peroxisomes. In the examined onion-cells, the fluorescence originating from the peroxisomal marker seemed to follow the same pattern as the EYFP-PAP7 construct. A conclusion if this was a coincidence due to dying cells, or actually indicates that peroxisomes can act as transport and delivery vesicles is impossible to make at this stage. Further investigations on different PAP7 constructs should to be performed.

The microscopy examinations of the other EYFP-constructs of full-length proteins were quite unambiguous. All transformed cells seemed to express the fusion protein in the same way in onion epidermal cells and mesophyll protoplasts.

The AtSLP1 was predicted to contain a PTS2, but the construct with EYFP fused to the putative PTS2-domain was expressed in cytosol. The full-length protein with EYFP fused to the C-terminal (AtSLP1-EYFP (B)), was found in chloroplasts. This location has already been shown (Uhrig and Moorhead 2011).The other construct had the EYFP fused to the N-terminal (EYFP-AtSLP1 (A)). This construct was found in cytosol in some of the transformed cells, and in dots in others. The dots did not appear to be peroxisomes, when compared to a peroxisomal marker. The EYFP-tagged protein could target to another organelle, or the protein could be clustered together like granules. No further investigations to determine the origin of these dots were performed.

The EYFP-TPP1-100 construct produced strong fluorescence from nucleolus and nucleus. The fluorescence from these compartments actually had to be reduced to reveal that also peroxisomes were expressing the fused protein. It seems obvious that the EYFP-TPP1-100 contains at least dual targeting domains.

The EYFP- MapKP1 construct expressed fluorescence in cytosol.

Peroxisomes are involved in several metabolic reactions, and the proteins in the organelles matrix changes due to growth state and conditions (Fukao et al. 2002). Various experiments using plants at different growth states as well as plants stressed by different growing conditions (light, salt, drought etc.) could give other results than the ones using cells from young, healthy plants.

The EYFP-protein-constructs which appeared in peroxisomes in the microscopy investigations should be cloned without the putative targeting amino acids as well. These construct is then supposed to remain in the cytosol, or, if they contain other internal signals, be targeted to other organelles. Products holding the EYFP in the opposite end of the proteins should also be constructed and investigated in the same way, as most targeting signal is near the N-terminus, and they are “masked” in a construct holding the EYFP in front.

Further investigations would probably include characterizations of T-DNA knock-out mutants of the respective phosphatase related proteins.

Even if all constructs were not completed and investigated during this thesis, the experiments performed shows that phosphatases probably are a part of the peroxisomal metabolism.

5 REFERENCES

Ahn CS, Han J-A, Lee H-S, Lee S, Pai H-S (2011) The PP2A regulatory subunit Tap46, a component of the TOR signaling pathway, modulates growth and metabolism in plants. The Plant Cell Online 23: 185-209

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu W-L, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J (2002) MAP kinase signalling cascade in Arabidopsis innate immunity. Nature 415: 977-983
Ast J, Stiebler AC, Freitag J, Bölker M (2013) Dual targeting of peroxisomal proteins. Frontiers in physiology 4

Chen X, Truksa M, Shah S, Weselake RJ (2010) A survey of quantitative real-time polymerase chain reaction internal reference genes for expression studies in< i> Brassica napus</i>. Analytical biochemistry 405: 138-140

Chowdhary G, Kataya AR, Lingner T, Reumann S (2012) Non-canonical peroxisome targeting signals: identification of novel PTS1 tripeptides and characterization of enhancer elements by computational permutation analysis. BMC plant biology 12: 142

Farkas I, Dombradi V, Miskei M, Szabados L, Koncz C (2007) < i> Arabidopsis</i> PPP family of serine/threonine phosphatases. Trends in plant science 12: 169-176

Frank J, Kaulfürst-Soboll H, Rips S, Koiwa H, von Schaewen A (2008) Comparative analyses of Arabidopsis complex glycan1 mutants and genetic interaction with staurosporin and temperature sensitive3a. Plant physiology 148: 1354-1367

Fuchs S, Grill E, Meskiene I, Schweighofer A (2013) Type 2C protein phosphatases in plants. FEBS Journal 280: 681-693

Fukao Y, Hayashi M, Nishimura M (2002) Proteomic analysis of leaf peroxisomal proteins in greening cotyledons of Arabidopsis thaliana. Plant and cell physiology 43: 689-696

Fulda M, Shockey J, Werber M, Wolter FP, Heinz E (2002) Two long-chain acyl-CoA synthetases from Arabidopsis thaliana involved in peroxisomal fatty acid beta-oxidation. The Plant journal : for cell and molecular biology 32: 93-103

Ghostscript G (2005) GSview.

Goddijn OJ, van Dun K (1999) Trehalose metabolism in plants. Trends in plant science 4: 315-319

González Besteiro MA, Bartels S, Albert A, Ulm R (2011) Arabidopsis MAP kinase phosphatase 1 and its target MAP kinases 3 and 6 antagonistically determine UV-B stress tolerance, independent of the UVR8 photoreceptor pathway. The Plant Journal 68: 727-737

Hodges M, Jossier M, Boex-Fontvieille E, Tcherkez G (2013) Protein phosphorylation and photorespiration. Plant Biology 15: 694-706

Hu J, Baker A, Bartel B, Linka N, Mullen RT, Reumann S, Zolman BK (2012) Plant peroxisomes: biogenesis and function. The Plant Cell Online 24: 2279-2303

Hunter S, Jones P, Mitchell A, Apweiler R, Attwood TK, Bateman A, Bernard T, Binns D, Bork P, Burge S (2012) InterPro in 2011: new developments in the family and domain prediction database. Nucleic acids research 40: D306-D312

Jacinto E, Guo B, Arndt KT, Schmelzle T, Hall MN (2001) TIP41 interacts with TAP42 and negatively regulates the TOR signaling pathway. Molecular cell 8: 1017-1026

Kataya AR, Reumann S (2010) Arabidopsis glutathione reductase 1 is dually targeted to peroxisomes and the cytosol. Plant signaling & behavior 5: 171-175

Kaur N, Reumann S, Hu J (2009) Peroxisome biogenesis and function. The Arabidopsis book/American Society of Plant Biologists 7

Kim D-J, Smith SM (1994) Expression of a single gene encoding microbody NAD-malate dehydrogenase during glyoxysome and peroxisome development in cucumber. Plant molecular biology 26: 1833-1841

Kutuzov MA, Andreeva AV (2012) Prediction of biological functions of Shewanella-like protein phosphatases (Shelphs) across different domains of life. Functional & integrative genomics 12: 11-23

Lamesch P, Berardini TZ, Li D, Swarbreck D, Wilks C, Sasidharan R, Muller R, Dreher K, Alexander DL, Garcia-Hernandez M, Karthikeyan AS, Lee CH, Nelson WD, Ploetz L, Singh S, Wensel A, Huala E (2012) The Arabidopsis Information Resource (TAIR): improved gene annotation and new tools. Nucleic acids research 40: D1202-1210

Larkin M, Blackshields G, Brown N, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947-2948

Li D, Zhu H, Liu K, Liu X, Leggewie G, Udvardi M, Wang D (2002) Purple acid phosphatases of Arabidopsis thaliana comparative analysis and differential regulation by phosphate deprivation. Journal of Biological Chemistry 277: 27772-27781

Lingner T, Kataya AR, Antonicelli GE, Benichou A, Nilssen K, Chen XY, Siemsen T, Morgenstern B, Meinicke P, Reumann S (2011) Identification of novel plant peroxisomal targeting signals by a combination of machine learning methods and in vivo subcellular targeting analyses. The Plant cell 23: 1556-1572

Luan S (2003) Protein phosphatases in plants. Annual review of plant biology 54: 63-92

Ma C, Reumann S (2008) Improved prediction of peroxisomal PTS1 proteins from genome sequences based on experimental subcellular targeting analyses as exemplified for protein kinases from Arabidopsis. Journal of experimental botany 59: 3767-3779

Marchler-Bauer A, Zheng C, Chitsaz F, Derbyshire MK, Geer LY, Geer RC, Gonzales NR, Gwadz M, Hurwitz DI, Lanczycki CJ, Lu F, Lu S, Marchler GH, Song JS, Thanki N, Yamashita RA, Zhang D, Bryant SH (2013) CDD: conserved domains and protein three-dimensional structure. Nucleic acids research 41: D348-352

Matre P (2009) Protein phosphatase 2A and regulation of nitrate reductase in plants. UiS, Stavanger

Maurino VG, Peterhansel C (2010) Photorespiration: current status and approaches for metabolic engineering. Current opinion in plant biology 13: 248-255

Moorhead GB, De Wever V, Templeton G, Kerk D (2009) Evolution of protein phosphatases in plants and animals. The Biochemical journal 417: 401-409

O'Hara LE, Paul MJ, Wingler A (2013) How do sugars regulate plant growth and development? New insight into the role of trehalose-6-phosphate. Molecular plant 6: 261-274

Olczak M, Morawiecka B, Watorek W (2003) Plant purple acid phosphatases-genes, structures and biological function. ACTA BIOCHIMICA POLONICA-ENGLISH EDITION- 50: 1245-1256

Pracharoenwattana I, Smith SM (2008) When is a peroxisome not a peroxisome? Trends in plant science 13: 522-525

Reumann S (2004) Specification of the peroxisome targeting signals type 1 and type 2 of plant peroxisomes by bioinformatics analyses. Plant physiology 135: 783-800

Reumann S (2011) Toward a definition of the complete proteome of plant peroxisomes: where experimental proteomics must be complemented by bioinformatics. Proteomics 11: 1764-1779

Reumann S, Ma C, Lemke S, Babujee L (2004) AraPerox. A database of putative Arabidopsis proteins from plant peroxisomes. Plant physiology 136: 2587-2608

Schluepmann H, Pellny T, van Dijken A, Smeekens S, Paul M (2003) Trehalose 6-phosphate is indispensable for carbohydrate utilization and growth in Arabidopsis thaliana. Proceedings of the National Academy of Sciences 100: 6849-6854

Schwarte S, Bauwe H (2007) Identification of the photorespiratory 2-phosphoglycolate phosphatase, PGLP1, in Arabidopsis. Plant physiology 144: 1580-1586

Smith JJ, Aitchison JD (2013) Peroxisomes take shape. Nature Reviews Molecular Cell Biology 14: 803-817

Song SK, Clark SE (2005) POL and related phosphatases are dosage-sensitive regulators of meristem and organ development in Arabidopsis. Developmental biology 285: 272-284

Uhrig RG, Labandera A-M, Moorhead GB (2013) < i> Arabidopsis</i> PPP family of serine/threonine protein phosphatases: many targets but few engines. Trends in plant science 18: 505-513

Uhrig RG, Moorhead GB (2011) Two ancient bacterial-like PPP family phosphatases from Arabidopsis are highly conserved plant proteins that possess unique properties. Plant physiology 157: 1778-1792

Ulm R, Ichimura K, Mizoguchi T, Peck SC, Zhu T, Wang X, Shinozaki K, Paszkowski J (2002) Distinct regulation of salinity and genotoxic stress responses by Arabidopsis MAP kinase phosphatase 1. The EMBO journal 21: 6483-6493

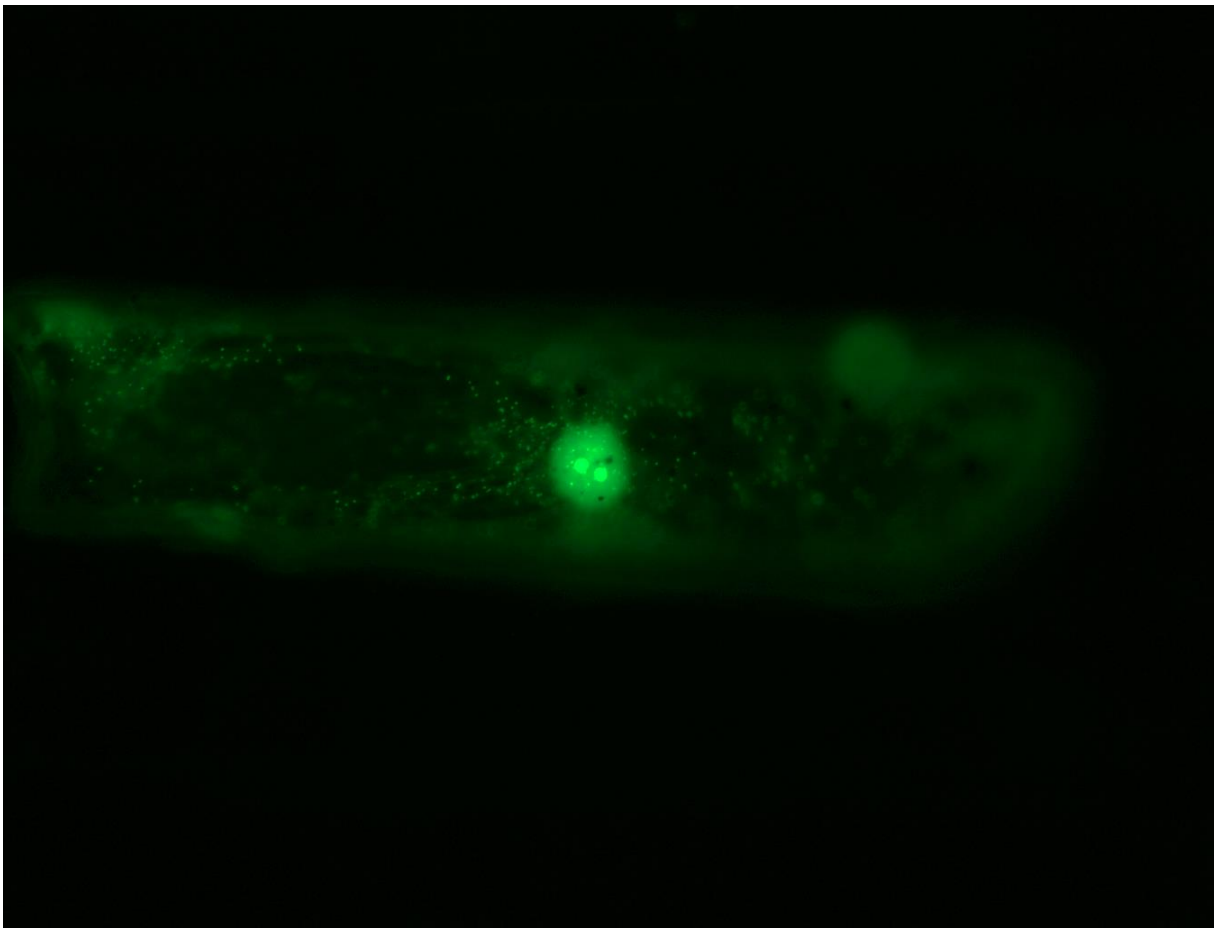
Wingler A (2002) The function of trehalose biosynthesis in plants. Phytochemistry 60: 437-440

Xu Q, Fu H-H, Gupta R, Luan S (1998) Molecular characterization of a tyrosine-specific protein phosphatase encoded by a stress-responsive gene in Arabidopsis. The Plant Cell Online 10: 849-857

Yoo S-D, Cho Y-H, Sheen J (2007) Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nature protocols 2: 1565-1572

ENCLOSURE

A larger copy of image B in figure 44. The EYFP-tagged TPP1-100 is also appearing in dots.



An up-scaled image of figure 44, A. The dots which seems to be peroxisomes are showing when the contrast and brightness is adjusted.