Characterization of two chloroplast envelope membrane proteins

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Summary

Plastid localized metabolite synthesis (e.g. amino acids, fatty acids or secondary compounds) requires extensive solute exchange across the outer and inner envelope membranes. In this thesis, the topology and function of an outer envelope protein, named OEP7, and of a novel inner envelope protein, named FAX1, were in focus.

OEP7 is a functionally unknown chloroplast outer envelope protein with four homologs in *Arabidopsis*. In the first part of my thesis, the so far unknown topology of At-OEP7.2 was analyzed by proteolysis of chloroplasts and green fluorescence protein (GFP)-fusion labeling in protoplasts, which indicated that OEP7.2 is an outer envelope protein with the N-terminus in the cytosol and the C-terminus in the inter membrane space. Furthermore, *in vivo* targeting of OEP7 deletion constructs fused with GFP in protoplasts suggested that the N-terminal residues 11 to 22 of At-OEP7.2 are necessary and residues 1 to 48 of At-OEP7.2 are sufficient for targeting to the chloroplast outer envelope membrane. I further characterized *oep7.1/oep7.2* double mutants, but although OEP7.2 transcription is strictly regulated in a diurnal rhythm, the OEP7.2 protein amount is stable throughout the diurnal cycle and no growth phenotype of the double mutants was detectable.

FAX1: Because fatty acid synthesis in plants exclusively takes place in plastids, export for further lipid metabolites is required. However, until now few data indicate the mechanism of fatty acid export from plastids. In the second part of my thesis, I selected FAX1 (fatty acid export 1), a novel membrane-spanning protein in *Arabidopsis thaliana*. FAX1 is a plant specific protein and member of the transmembrane 14C (Tmemb_14) family, which is an uncharacterized protein family in eukaryotes and prokaryotes. According to GFP-targeting in protoplasts and immunoblot analysis, FAX1 localizes to the plastid inner envelope membrane. Protease treatment of pea inner envelope vesicels revealed that both N-terminus and C-terminus are in the inter membrane space. *fax1* knockout mutants show a phenotype characterized by reduced biomass, thin inflorescence stems, and strongly impaired male fertility, which was complemented by 35S:: FAX1 expression in the knockout background plants. However, the overexpression of *FAX1* in Col-0 cause increased biomass. Transcriptomic, metabolic and ultrastructural analysis of *FAX1* mutants implies that FAX1 is important for synthesis of secondary metabolites, like cutin and wax (especially ketones), secondary cell walls and pollen exine which all require previous fatty acid export from plastids. Fatty acid analysis indicated that phosphatidylcholine in leaves of *fax1* knockout plants was reduced to 60% of Col-0 wild-type levels.

Zusammenfassung

Die in den Plastiden höherer Pflanzen lokalisierte Biosynthese von Metaboliten (z.B. Aminosäuren, Fettsäuren oder sekundäre Inhaltsstoffe) erfordert einen ausgiebigen Austausch und den Transport gelöster Stoffe über die äußere und innere Hüllmembran. Im Fokus dieser Arbeit standen daher Untersuchungen zu Topologie und Funktion von OEP7 in der äußeren Hüllmembran sowie die Charakterisierung von FAX1, einem bisher nicht bekannten Membranprotein in der inneren Hüllmembran von Chloroplasten.

OEP7 ist ein OEP (*outer envelope protein*) unbekannter Funktion mit vier homologen Proteinen in *Arabidopsis*. In dieser Arbeit wurde die bisher unbekannte Topologie der Isoform At-OEP7.2 über Proteolyse-Experimente an isolierten Chloroplasten und die Signale von GFP-Fusionskonstrukten in Protoplasten aufgeklärt. Die Experimente zeigen, dass OEP7.2 eine α-helicale Membrandomäne in der äußeren Hüllmembran besitzt und der Aminoterminus des Proteins im Cytosol, der Carboxyterminus im Intermembranraum lokalisiert sind. Weitere *in vivo* GFP-Studien mit verkürzten OEP7.2-Peptiden zeigten, dass die N-terminalen Aminosäurereste 11-22 notwendig und die Peptidkette 1-48 hinreichend für die Insertion in die äußere Hüllmembran sind. Weiterhin habe ich im Rahmen meiner Dissertation eine phänotypische Charakterisierung von Doppel-Verlustmutanten der Isoformen *OEP7.1/ OEP7.2* vorgenommen. Obwohl hier der Transkriptgehalt von At-OEP7.2 sehr strikt im Tag/Nacht-Rhythmus reguliert wurde, blieb die Proteinmenge stabil und es konnte kein Wachstumsphänotyp der Doppelmutanten detektiert werden.

FAX1: Da die Synthese von Fettsäuren in Pflanzen ausschließlich in Plastiden stattfindet, ist der Export von Fettsäuren und Derivaten aus den Plastiden essentiell für alle weiteren Prozesse des Lipidstoffwechsels. Über den zugrundeliegenden Transportmechanismus ist allerdings noch sehr wenig bekannt. Im zweiten Teil meiner Arbeit habe ich mich daher mit der Charakterisierung des Proteins FAX1 (*fatty acid export 1*) beschäftigt. FAX1 ist Pflanzen-spezifisch und gehört zu den "transmembrane 14C" (Tmemb_14) Proteinen, einer noch nicht näher beschriebenen Familie in Eu- und Prokaryoten. Nach meinen Ergebnissen der *in vivo* Lokalisierung von FAX1-GFP Signalen und einer Immunoblot-Analyse, ist FAX1 ein integrales Membranprotein der inneren Hüllmembran von Chloroplasten. Proteolyse-Experimente an isolierten Vesikeln der inneren Hüllmembran von Chloroplasten aus Erbse zeigten, dass sich Amino- und Carboxy-Terminus des Proteins im Intermembranraum befinden. Verlustmutanten von FAX1 haben im Vergleich zum Wildtyp eine geringere Biomasse, dünnere Stängel und eine stark reduzierte männliche Fruchtbarkeit. Dieser

Phänotyp konnte durch die Expression von FAX1 unter Kontrolle des 35S Promoters im Hintergrund der *knockout* Mutante komplementiert werden, während eine Überexpression von FAX1 zu erhöhter Produktion von Biomasse führte. Analysen auf Transkript- und Metabolit-Ebene sowie der zellulären Ultrastruktur von FAX1 Mutanten zeigten, dass die Funktion von FAX1 wichtig ist für die Synthese von sekundären Inhaltsstoffen wie Kutin und Wachs (insbesondere von Ketonen), dem Aufbau der sekundären Zellwand sowie der Pollen Exine. All diese Prozesse benötigen einen vorangehenden Export von Fettsäuren aus Plastiden. Die Analyse des Fettsäuregehalts zeigte weiterhin, dass Phosphatidylcholin in *fax1* knockout Linien auf 60% des Wildtypniveaus reduziert ist.

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Abbreviations

aa	amino acids
AGI	*Arabidopsis* Genome Initiative
AP	alkaline phosphatase
At	*Arabidopsis thaliana*
ATP	adenosine triphosphate
BCA	bicinchoninic acid
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
Col-0	Columbia 0 ecotype
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
DTT	dithiothreitol
E. coli	*Escherichia coli*
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequence tag
GFP	green fluorescent protein
he	heterozygous
Hepes	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
ho	homozygous
IP	isoelectric point
kDa	kilo Dalton
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	messenger RNA
MS	Murashige and Skoog
MW	molecular weight
OEPs	outer envelope proteins
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
Ps	*Pisum sativum*
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
rpm	revolutions per minute
RT PCR	reverse-transcription polymerase chain reaction
RT	room temp
SD	standard deviation
SDS	sodium dodecyl sulphate
T-DNA	Transfer-DNA
Tic	translocon at the inner envelope of chloroplasts
TILLING	targeting induced local lesions in genomes

Tris	tris(hydroxymethyl) aminomethane
UTR	untranslated region
VDAC	voltage dependent anion channel
w/v	weight per volume
wt	wild-type
x g	times the force of gravity

I. Introduction

Plastids and especially chloroplasts in green leaves are ubiquitous organelles found in plant and algal cells, which are responsible for photosynthesis, as well as synthesis and storage of many molecules and products necessary for plant cell metabolisms (Figure 1). A pair of membranes, named the outer (OE) and inner envelope (IE), surrounds the plastid and thus mediates metabolic communication with the cytosol and other organelles. Therefore, the envelope of plastids contains numerous proteins for transport of metabolites and ions.

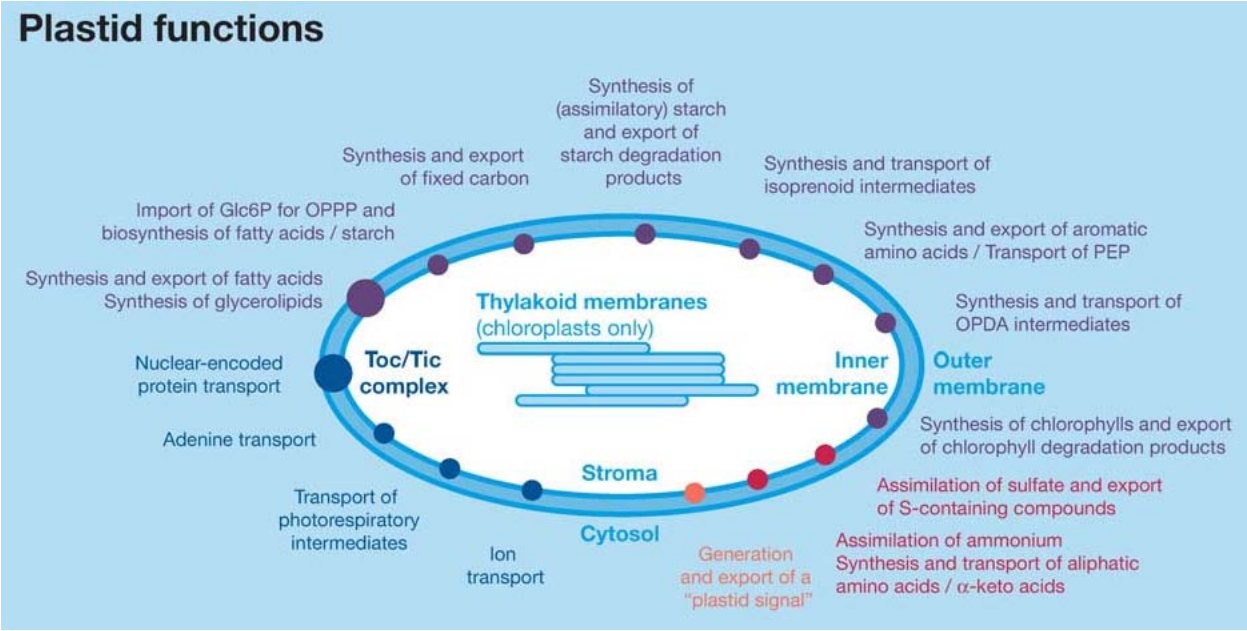

Figure 1: Schematic representation of plastid functions (copied from Weber *et al.*, 2005)
All functions shown in purple, red, and orange also take place in the stroma. Glc6P: glucose 6-phosphate, OPDA: oxophytodienoic acid, OPPP: oxidative pentose phosphate pathway, PEP: phosphoenolpyruvate.

Considering the complexity of plastid metabolite and ion transporters, only known solute transport proteins of the outer envelope (OEP16, OEP21, OEP24 and OEP37), OEP7 (a unknown outer envelope protein), some transporters with well-defined functions in the inner envelope and the mechanism of fatty acid export from plastids are introduced in the following.

1 Metabolite and ion transport across the plastid envelope membranes

1.1 The plastid outer envelope

OEP16 is the best described outer envelope solute channel with a purely alpha-helical structure (Linke *et al.*, 2004) and with a selectively for amino acid transport (Pohlmeyer *et al.*, 1997). By sequence similarity analysis, three isoforms of OEP16 in *Arabidopsis thaliana*: At-

OEP16.1, At-OEP16.2 and At-OEP16.4 were classified as members of the preprotein and amino acid transporter (PRAT) family (Murcha *et al*., 2007; Pudelski *et al*., 2010). Recent in planta studies revealed that the loss of OEP16, mainly that of OEP16.2, caused metabolic imbalance, in particular that of amino acids during ABA-controlled seed development and early germination (Pudelski *et al*., 2011).

OEP21 is a β-barrel forming protein with eight β-strands and is a rectifying, anion selective channel for phosphorylated carbohydrates and triosephosphate (Bolter *et al*., 1999). There are two ATP binding sites, one internal to the pore and one in the inter membrane space which can bind ATP and triosephosphate, thereby regulating anion selectivity (Hemmler *et al*., 2006).

OEP24 was characterised as a member of β-barrel forming proteins as well (12 β-strands; Pohlmeyer *et al*., 1998; Schleiff *et al.*, 2003). *In vitro* reconstitution of OEP24 in liposomes revealed that it is slightly selective for cations and highly conductive. Furthermore, it is demonstrated that OEP24 carries the fluxes of triose phosphates , sugars, hexose-phosphates, ATP, phosphates, and charged amino acids (Pohlmeyer *et al*., 1998). In yeast, OEP24, which is targeted to the mitochondrial outer membrane, can functionally complement the mitochondrial voltage-dependent anion channel (VDAC; Röhl *et al*., 1999).

In addition, OEP37 represents another β-barrel transmembrane channel with 12 β-strands (Schleiff *et al*., 2003). Until now, the transport function of OEP37 in plants is still unknown since the knockout mutant of OEP37 showed no obvious phenotype (Götze *et al*., 2006)

OEP7 (outer envelope protein of 7 kDa) is an outer envelope protein of chloroplasts, which has only one α-helical transmembrane domain. It was first siolated from spinach chloroplasts and named E6.7 at the chloroplast outer envelope membrane (Salomon *et al*., 1990). Import of [^{35}S]methionine-labeled OEP7 into intact spinach chloroplasts followed by proteolysis has revealed that the positively charged amino acids, flanking the transmembrane domain at the C-terminus are essential to retain the native N_{in}-C_{out} orientation during insertion into chloroplast outer envelope (Schleiff *et al*., 2001). To investigate *in vivo* the targeting mechanism of OEP7 in *Arabidopsis*, OEP7 was expressed as a fusion protein with the green fluorescent protein (GFP) either transiently in protoplasts or stably in transgenic plants (Lee *et al*., 2001). It was shown that the transmembrane domain and its C-terminal neighboring seven-amino acid region of OEP7 were necessary and sufficient for targeting to the chloroplast outer envelope membrane. By an yeast two-hybrid screen to screen for proteins that interact with OEP7, AKR2, an *Arabidopsis* ankyrin repeat protein, was identified to play

an essential role in the biogenesis and membrane insertion of the chloroplast outer envelope proteins (Bae *et al*., 2008). However, few data on the function of OEP7 exist only very recently a homolog of OEP7 in *Suaeda salsa*, named SsOEP8, was indicated to confer oxidative stress tolerance and to induce chloroplast aggregation in transgenic *Arabidopsis* plants (Wang *et al*., 2012). Until now four isoforms of OEP7 in *Arabidopsis* were found.

1.2 The plastid inner envelope

Plastidic phosphate translocators (pPTs) were the first and best characterized transporters in the plastid inner envelope, which functionally act as homodimers (Weber *et al*., 2005; Weber and Linka, 2011). According to the substrate specificity, pPT proteins are classified into three groups: i) the triose phosphate translocators (TPT), ii) glucose 6-phosphate (GPT)/xylulose 5-phosphate (XPT) translocators, and iii) phosphoenolpyruvate translocators (PPT). In green plants, triose phosphates generated by the Calvin-Benson cycle in chloroplasts during the day are exported via TPT in exchange with phosphate. In vascular plants, the main function of TPT is to provide fixed carbon for sucrose synthesis and primary metabolism (for overview see Weber and Linka, 2011).

PIC1 (permease in chloroplasts 1) in *Arabidopsis* was identified as an inner envelope protein with four α-helices and is homologous to cyanobacterial permease-like proteins (Duy *et al*., 2007). Transriptomic, metabolic and ultrastructural analysis of both knockout mutants and overexpressors of PIC1 revealed that PIC1 functions in iron transport across the inner envelope of chloroplasts and is crucial for balancing plant iron metabolism in general (Duy *et al*., 2007 and 2011). In a yeast-two-hybrid screen for PIC1 interacting proteins, NiCo, a putative protein, which may function in the transport of nickel and/or cobalt, was identified. Thus, it is implied that PIC1 and NiCo might function together in plastid iron transport (Duy *et al*., 2011).

2 Fatty acid export from plastids

It is known that fatty acid biosynthesis in plants takes place in plastids (Ohlrogge *et al.*, 1979; Buchanan *et al.*, 2002). α-Linolenic acid (18:3 (n-3)) can contribute as much as 90% of the fatty acids in glycolipids in the photosynthetic tissue of some plants. Therefore, 18:3 plants, such as pea and rice, are the one in which glycolipids contain exclusively C18 polyunsaturated fatty acids. And the 16:3 plants, such as spinach, *Arabidopsis* and *Brassica. napus,* contain glycolipids having appreciable amounts of a C16 polyunsaturated fatty acid, localized exclusively in the *sn*-2 position (Buchanan *et al.*, 2002). The beginning of the fatty acid export from plastids is the *de novo* fatty acid synthesis product, the acyl-ACP (acyl

carrier protein; Figure 2). 90% of the acyl-ACP is firstly hydrolyzed to free fatty acids and ACP by acyl-ACP thioesterases, which possibly act at the inner side of the IE or in the stroma (Koo *et al*., 2004). Subsequently, the free fatty acids (FFA) are transported through the IE to the OE. Until now, two hypothesis were suggested for the free fatty acid export through the IE, since no FFA-export related protein was found so far: (i) Some believe that a transporter protein is not necessary for the fatty acid export through the IE (Kamp and Hamilton, 2006; Hamilton, 2007); (ii) Considering the fatty acid transport related proteins in *E. coli*, yeast and animals, and also the ABC transporter PXA1 at the membrane of peroxisomes in *Arabidopsis* (Kunz *et al*., 2009), it is otherwise believed that there should be transport proteins, possibly an ABC transporter, involved in FFA export (Benning, 2009; Li-Beisson *et al*., 2010).

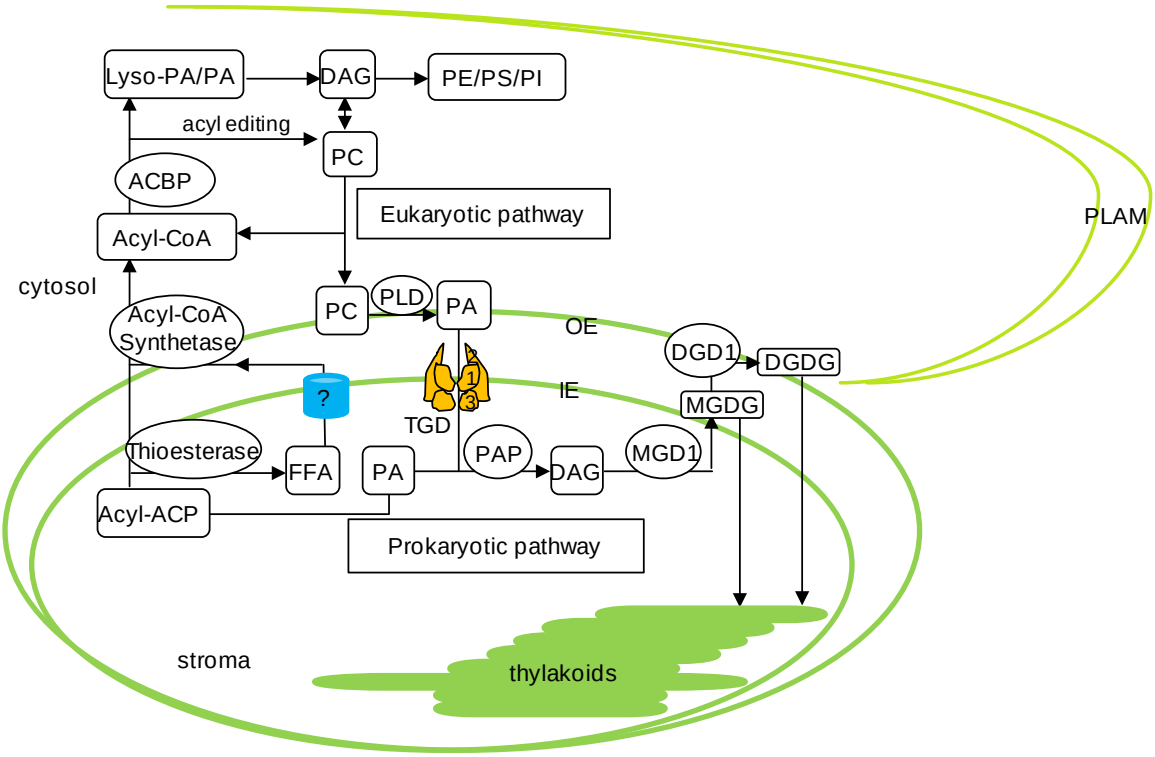

Figure 2 Fatty acid export from plastids and galactolipid biosynthesis in leaves of 16:3 plants
(Modified according to Williams *et al*., 2000; Koo *et al*., 2004; Bate *et al*., 2007; Benning, 2009; Tjellström *et al*., 2012). Left, fatty acid export from plastids: Acyl-ACP is hydrolysed to FFA by thioesterases at the IE. Then FFA is transported to the OE by an unknown fatty acid exporter (?) or by a flip-flop mechanism. Acyl-CoA synthetase at the OE accepts FFA and synthesizes Acyl-CoA, creating an Acyl-CoA pool in the cytosol. Right, galactolipid biosynthesis consists of two pathways: a) the prokaryotic pathway, in the plastid stroma; b) the eukaryotic pathway in the cytosol. Acyl-ACP: Acyl-acyl carrier protein, Acyl-CoA: Acyl-Coenzyme A, FFA: free fatty acids, Thioesterase: Acyl-ACP thioesterase, Synthetase: Acyl-CoA synthetase, ACBP: Acyl-CoA binding protein, PA: Phosphatidic acid, DAG: Diacylglycerol, PC: Phosphatidylcholine, PE: Phosphatidylethanolamine, PS: Phosphatidylserine, PI: Phosphatidylinositol, PLD: Phospholipase D; TGD: TGD complex (PA importer), ?: unknown fatty acid exporter, PAP: PA-phosphatase, DAG: Diacylglycerol, MGDG: Monogalactosyldiacylglycerol, DGDG: Digalactosyldiacylglycerol, MGD1: MGDG synthase, DGD1: DGDG synthase, OE: outer envelope, IE: inner envelope, PLAM: the plastid envelope and ER membrane contact site.

The exogenous long chain fatty acid transport and trafficking system in *E. coli* consists of three central components, FadL, an outer membrane fatty acid transport protein (Nunn and Simons, 1978; Black *et al*., 1987), FadD, an inner membrane long chain acyl-CoA synthetase (Overath and Raufuss, 1967; Klein *et al*., 1971) and FadR, a long chain acyl-CoA regulator which localizes in the cytosol (DiRusso *et al.*, 1999; DiRusso and Black, 2004). In yeast (*Saccharomyces cerevisiae*), the fatty acid transport system contains two membrane-bound components: i) Fat1p, fatty acid transport protein, which has significant homologies to the mammalian fatty acid transport proteins (FATPs); ii) the very long chain acyl-CoA synthetases, Faa1p or Faa4p, the fatty acyl-CoA synthetases to activate exogenous fatty acids (Faergeman *et al.*, 2001; Zou *et al.*, 2003). Unfortunately, no orthologs of FadL, Fat1p or FATP were identified in plant genomes until now. However, two ABC transporter system related to fatty acid transport provided hints for the fatty acid export function in plastids. The fatty acid transport from ER to plastids in *Arabidopsis* needs an ABC transporter complex which consists of the TGD1, 2 and 3 subunits: a FA-binding protein in the inter membrane space (TGD2), the inner envelope intrinsic permease (TGD1) and the stromal ATP-binding subunit (TGD3). The TGD1,2,3 transporter complex is proposed to accept and transfer the phosphatidic acid from the outer envelope to phosphatidic acid phosphatase (PAP) at the inside of the inner envelope membrane (see Figure 2; Awai *et al*., 2006; Lu *et al*., 2007; Xu *et al*., 2005; Benning, 2009). Another ABC transporter in *Arabidopsis* involved in fatty acid traffic is the peroxisomal ABC-transporter1 (PXA1), which is required for fatty acid respiration via peroxisomal β-oxidation (Zolman *et al*., 2001; Footitt *et al*., 2002; Hayashi *et al*., 2002; Kunz *et al*., 2009).

After the free fatty acid export through the inner envelope, it is widely agreed that acyl-CoA synthetases are involved in the acyl-CoA synthesis at the outer envelope membrane (see Figure 2; Koo *et al*., 2004). It is known that *Arabidopsis* possesses an acyl-activating enzyme superfamily with 63 different genes, including 9 long chain acyl-CoA synthetases (LACS; Shockey *et al*., 2002, 2003). However, no acyl-CoA synthetase was identified to have a role during fatty acid export from plastids. One of the nine LACS, LACS9, was experimentally verified to localize at the envelope of chloroplasts but has no essential synthetase function during fatty acid export since the loss function of LACS9 has no phenotype (Schnurr *et al*., 2002). Therefore, other members of the acyl-activating enzyme superfamily should have an important role in fatty acid export *in vivo*.

Recently, phosphatidylcholine (PC) is predicted to play an important role during newly synthesized acyl chains trafficking from plastids to the endoplasmic reticulum (Tjellström *et*

al., 2012). Here, the eukaryotic pathway of galactolipid biosynthesis connects cytosolic, ER and OE localized PC with the plastid export of free fatty acids (Figure 2). Free fatty acids are transferred to be acyl-CoA by acyl-CoA synthetase in the cytosol. Kinetic labeling experiments with [^{14}C] acetate, [^{14}C] gylcerol, and [^{14}C] carbon dioxide in pea demonstrated that acyl editing is an integral component of eukaryotic glycerolipid synthesis, which means most newly synthesized acyl groups are incorporated directly into PC through an acyl editing mechanism (Bates *et al*., 2007). Furthermore, considering that *in vivo* evidence for acyl-CoA as a carrier of acyl chains from plastid to ER is lacking and according to rapid kinetic labeling experiments Tjellström *et al*. (2012) propose that PC may be central to acyl fluxes that occur between plastids and the ER. It is also suggested that determining the molecular identity and biochemical activity of the proteins catalyzing the initial incorporation of nascent fatty acid into PC will provide an important breakthrough for our current understanding of fatty acid export from plastids (Benning, 2009).

3 Aim of the thesis

The metabolite and ion transport across the outer and inner envelope of plastids is mediated by plastid envelope proteins. OEP7, as the smallest, functionally unknown outer envelope protein, was focused in this work. Considering that the characterization of the OEP7.2 isoform in *Arabidopsis* has never been described and knockout mutants of OEP7.1 in *Arabidopsis* showed no phenotype, one aim of my work was to describe the topology of OEP7.2 and the characterization of *oep7.1/oep7.2* double knockout mutant in *Arabidopsis*.

As described above until now transport for fatty acid export proteins from chloroplasts are unknown. Therefore, the second aim of my thesis was to identify and characterise new envelope membrane proteins of plastids. Here the senescence related protein FAX1 at the inner envelope was a potential candidate. The goal of my work thus included the molecular and in planta characterisation of FAX1 involving studies on mutant plants (transcriptomics, metabolomics and ultrastructural analyses). In the long term, my work should further reveal the mechanism of fatty acid trafficking between plastids and ER, and the impact on secondary metabolism in plant development.

II. Materials

1 Chemicals

All chemicals used in this work were purchased in high purity from AppliChem (Darmstadt, Germany), Biomol (Hamburg, Germany), Difco (Detroit, USA), Fluka (Buchs, CH), GibcoBRL (Paisley UK), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Roche (Penzberg, Germany), Sigma-Aldrich (Steinheim, Germany), or Serva (Heidelberg, Germany).

2 Detergents

n-dodecyl-*β*-D-maltoside (DoMa), sodium dodecyl sulphate (SDS), and Triton X-100 (TX-100) were obtained from Roth (Karlsruhe, Germany).

3 Enzymes

Restriction enzymes and DNA-polymerases were purchased from Roche (Penzberg, Germany), MBI Fermentas (St. Leon-Rot, Germany) and New England Biolabs GmbH (Frankfurt am Main, Germany). T4-DNA ligases were obtained from MBI Fermentas (St. Leon-Rot, Germany) and Invitrogen (Karlsruhe, Germany). Reverse Transcriptase was purchased from Promega (Madison, USA). RNase-free DNase I was obtained from Roche (Mannheim, Germany) and RNase from Amersham Biosciences (Uppsala, Sweden). Cellulase R10 and Macerozyme R10 for digestion of the plant cell wall were from Yakult (Tokyo, Japan) and Serva (Heidelberg, Germany). Taq Polymerase was purchased from Diagonal (Münster, Germany), Eppendorf, MBI Fermentas, Clontech (Saint-Germainen-Laye, France), Finnzymes (Espoo, Finland) and Bioron (Ludwigshafen am Rhein, Germany).

4 Kits

RNA extraction from plants was isolated using the “Plant RNAeasy Kit” from Qiagen (Hilden, Germany). The “Plasmid Midi Kit” for high yield DNA purification and the “Nucleospin Extract II Kit” for purification of DNA fragments from agarose gels were purchased from Macherey and Nagel (Düren, Germany). For *in vitro* translation, the “Flexi Rabbit Reticulocyte Lysate System” from Promega (Madison, USA) was used.

5 Molecular weight markers and DNA markers

For agarose-gel electrophoresis, *Pst*I digested Phage DNA (MBI Fermentas) was used as a molecular size marker. For SDS-PAGE and Tricine-SDS-PAGE, the Low Molecular Weight Marker from Sigma-Aldrich (Steinheim, Germany) was used.

6 Antisera

The following antibodies were generated in this work: α-At-OEP7.2 NT (N-terminal region of At-OEP7.2), α-At-OEP7.2 CT (C-terminal region of At-OEP7.2), α-Ps-FAX1-NT (N-terminal region of Ps-FAX1), α-Ps-FAX1-CT (C-terminal region of Ps-FAX1) and α-At-FAX1 (N-terminal region of At-FAX1) (Table 1). All peptides were analyzed, synthesized and used to generate antiserum in rabbit by Pineda (Berlin, Germany). Primary antibodies directed against At-OEP7.1, Ps-LSU, Ps-LHCP, Ps-OEP16.1, Ps-Tic62 and At-PIC1 used in this work were available in the lab.

Table 1: Peptide antibodies used in this work

Antibody	Peptides synthesized	For Protein
At-OEP7.2 NT	MVEKSGGEVNFPKLEKPTGKKQ	At-OEP7.2
At-OEP7.2 CT	LFKKLSSSKDKSDSDDATVPPPSGA	At-OEP7.2
Ps-FAX1-2	ERHEAETADTETKNTLSYAADESKLNVEEK	Ps-FAX1
Ps-FAX1-CT	GNPPPKKLKPSASVA	Ps-FAX1 /At-FAX1
At-FAX1-2b	TAEVSKPVVEKTSKPYSTVDETATNK	At-FAX1

7 Strain, vectors, clones and oligonucleotides

Cloning in *E. coli* was performed using the strains DH5-α (Invitrogen, Karlsruhe, Germany) or TOP10 (Invitrogen). The *Agrobacterium tumefaciens* GV3101::pMK90RK (Koncz and Schell, 1986) strain used for stable transformation of *Arabidopsis thaliana* was a kind gift of Dr. J. Meurer (Department Biologie I, Botany, LMU München). All plasmid vectors used in this work are shown in Table 2. Oligonucleotide primers in standard desalted quality used in this work were ordered from Qiagen /Operon (Köln, Germany) or Metabion (Martinsried, Germany) (Table 3). All plasmid constructs created in this work are listed in Table 4.

Table 2: Plasmid vectors used in this work

Name	Application	Origin
pCRblunt	Subcloning, sequencing	Invitrogen
pJET1.2	Subcloning, sequencing	Fermentas
pENTR/D/TOPO	Entry vector for GATEWAY recombination	Invitrogen
pH2GW7	Overexpression vector	Plant System biology (University of Ghent, Belgium)
p2GWF7	GFP fusion vector	Plant System biology (University of Ghent, Belgium)
pK7WGF2	GFP fusion vector	Plant System biology (University of Ghent, Belgium)

Table 3: Oligonucleotides used in this work

Oligoname	Sequence	Application
OEP7.1 LP	ATCGGTTGATACCACCGACACG	Genotyping *OEP7.1* gene, *oep7.1-2*, *oep7.2-3*
OEP7.1 RP	GCTCAAACTGTTATGATTAAGAACACATG	Genotyping *OEP7.1* gene, *oep7.1-2*, *oep7.2-3*
OEP7.1 LC fw	ATCGGTTGATACCACC	Real time RT-PCR
OEP7.1 LC rev	GCTCAAACTGTTATGATT	Real time RT-PCR
OEP6.7 fw garlic	TATTACCAACAAAACCCAAGACA	Genotyping *OEP7.2* gene, *oep7.2-1*
OEP6.7 rev garlic	AACGTGACCAGTCGAGATATTT	Genotyping *OEP7.2* gene
AtOEP7.1#1-35 fw	CACCATGGGAAAAACTTCGGGAGCGAAACAG	cloning of *AtOEP7.1#1-35* for GFP fusion
AtOEP7.1#1-35 rev	GAATTTATCGAGGAAAGGCTTGAAAGCG	cloning of *AtOEP7.1#1-35* for GFP fusion
AtOEP7.1#10-64 fw	CACCATGGCGACTGTGGTGGTCGCA	cloning of *AtOEP7.1#10-64* for GFP fusion
AtOEP7.1#10-64 rev	CAAACCCTCTTTGGATGTGGTTGCAG	cloning of *AtOEP7.1#10-64* for GFP fusion
AtOEP7.2#1-49 fw	CACCATGGTGGAGAAGTCAGGAGGAG	cloning of *AtOEP7.2#10-64* for GFP fusion
AtOEP7.2#1-49 rev	CAGCTTCTTGAACAATGGCTTAAACACG	cloning of *AtOEP7.2#10-64* for GFP fusion
AtOEP7.2#23-69 fw	CACCATGACAGCGACGGTTGTTGTGG	cloning of *AtOEP7.2#23-69* for GFP fusion
AtOEP7.2#23-69 rev	GGCGCCCGACGGGGGAGGGACG	cloning of *AtOEP7.2#23-69* for GFP fusion
AtOEP7.2#11-69 fw	CACCATGTTCCCAAAATTGGAGAAACC	cloning of *AtOEP7.2#11-69* for GFP fusion
AtOEP7.2#11-69 rev	GGCGCCCGACGGGGGAGGGACG	cloning of *AtOEP7.2#11-69* for GFP fusion
At-FAX1 LC fw	CCTATGATTCGTCCCCAG	Real time RT-PCR
At-FAX1 LC rev	CACTCACAACGAGACCA	Real time RT-PCR
At-fax1LP	TTTCTTCGCAACATTTTGACC	Genotyping *FAX1* gene
At-fax1RP	CCTCTACTGGCTCTGTGATGC	Genotyping *fax1-1*
At-fax1-2LP	AAGGAAACCTAAGCTTAAACCAGC	Genotyping *fax1-2*
GABI LB1	ATAATAACGCTGCGGACATCTACATTTT	Genotyping *fax1-2*
At-fax1RP2	AGTGGAGACACTATCAATCCC	Genotyping *FAX1* gene
LB1SAIL	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC	Genotyping SAIL mutant lines

At-FAX1 fw	CACCATGGCTTCACAAATCTCTCAGC	cloning of *At-FAX1*cDNA for overexpressing lines and complementation lines
At-FAX1 rev	GTATGAAGGACTAGTCGCAGATGG	cloning of *At-FAX1*cDNA for overexpressing lines and complementation lines
At-fax1 (+stop codon) rev	TCAGTATGAAGGACTAGTCGCAGATGG	cloning of At-FAX1cDNA
Ps-fax1 (cacc) fw	CACCATGGCGGCGACATCTCAGGCTCA	cloning of *Ps-FAX1*cDNA to pENTR/D/TOPO
Ps-fax1 (+stop codon) rev	TCAGGCTACACTGGCAGATGGCTTC	cloning of *Ps-FAX1*cDNA to pENTR/D/TOPO
attB1	ACAAGTTTGTACAAAAAAGCAGGCT	Gateway vectors related
attB2	ACCACTTTGTACAAGAAAGCTGGG	Gateway vectors related

Table 4: Constructs created in this work

Protein	Plasmid-vector	Application
FAX1(at3g57280)	pUNI151	Sequencing, subcloning
Ps-FAX1 contig	pCRblunt	Sequencing, subcloning
At-FAX1 (-stop codon)	pENTR/D/TOPO	Gateway recombination (GFP)
At-FAX1 (-stop codon)	p2GWF7	GFP fusion
At-OEP7.1(-stop codon)	pENTR/D/TOPO	Gateway recombination (GFP)
At-OEP7.1(-stop codon)	p2GWF7	GFP fusion (At-OEP7.1:GFP)
At-OEP7.2(-stop codon)	pENTR/D/TOPO	Gateway recombination (GFP)
At-OEP7.2(-stop codon)	p2GWF7	GFP fusion (At-OEP7.2:GFP)
At-OEP7.1	pENTR/D/TOPO	Gateway recombination (GFP)
At-OEP7.1	pK7WGF2	GFP fusion (GFP:At-OEP7.1)
At-OEP7.2	pENTR/D/TOPO	Gateway recombination (GFP)
At-OEP7.2	pK7WGF2	GFP fusion (GFP:At-OEP7.2)
At-OEP7.1#1-35	pENTR/D/TOPO	Gateway recombination (GFP)
At-OEP7.1#10-64	pENTR/D/TOPO	Gateway recombination (GFP)
At-OEP7.2#1-48	pENTR/D/TOPO	Gateway recombination (GFP)
At-OEP7.2#23-69	pENTR/D/TOPO	Gateway recombination (GFP)
At-OEP7.2#11-69	pENTR/D/TOPO	Gateway recombination (GFP)
At-OEP7.1#1-35	p2GWF7	GFP fusion (At-OEP7.1#1-35:GFP)
At-OEP7.1#10-64	p2GWF7	GFP fusion (At-OEP7.1#10-64:GFP)
At-OEP7.2#1-48	p2GWF7	GFP fusion (At-OEP7.2#1-48:GFP)
At-OEP7.2#23-69	p2GWF7	GFP fusion (At-OEP7.2#23-69:GFP)
At-OEP7.2#11-69	p2GWF7	GFP fusion (At-OEP7.2#11-69:GFP)

III. Methods

1 Plant methods

1.1 Plant material and growth conditions

All experiments were performed on *Arabidopsis thaliana* plants, ecotype columbia (Col-0, Lehle Seeds; Round Rock, USA). The TILLING lines *oep7.1-2* and *oep7.1-3* were ordered from the Seattle Arabidopsis TILLING Service (http://tilling.fhcrc.org; Till *et al*., 2003) and purchased from NASC (University of Nottingham, GB, Scholl *et al*., 2000). The T-DNA insertion lines (Alonso *et al*., 2003; Rosso *et al*., 2003) SAIL_813_F06 (*oep7.2-1*), SAIL_66_B09 (*fax1-1*) and GABI_599E01 (*fax1-2*) were purchased from NASC or GABI-Kat (MPI for Plant Breeding Research, Köln, Germany), respectively. Peas (*Pisum sativum*) var. "Arvica" were ordered from Bayerische Futtersaatbau (Ismaning, Germany).

Seeds were either sown directly on soil or on MS-plates (Murashige and Skoog, 1962) supplemented with 1% (w/v) sucrose. In some cases, seedlings were transferred to soil after 2-3 weeks. Before sowing on sterile plates, seeds of *Arabidopsis thaliana* were surface sterilized in 70% (v/v) ethanol for 1-2 min, 6% (v/v) NaClO with 0.05% (v/v) Tween-20 for 3-5 min, followed by washing in sterilized water for 3 x 1 min, and allowed to air-dry in a laminar flow hood. To synchronize germination, seeds were vernalized at 4°C in the dark for 1-3 days. For selection of transformed plants, seeds were grown on MS media (+1% sucrose) containing the adequate antibiotics (25 µg/ml hygromycin or 100 µg/ml kanamycin). Unless stated otherwise, plants were grown in a 16 h light (+21°C; 100 µmol photons $m^{-2} s^{-1}$) and 8 h dark (+16°C) cycle (long-day) and plant tissues were generally harvested during early light phase (2-5hrs of light).

1.2 Sub-cellular localization of GFP Fusion proteins in *Arabidopsis* protoplasts

Plant transformation and sub-cellular localization of GFP fusion protein in *Arabidopsis* protoplasts was performed as described in Duy *et al*., (2007).

1.3 Cross fertilization of *Arabidopsis thaliana*

Crossing lists of different *fax1* knockout mutants and wild type are depicted in Chapter IV.2.7. Cross fertilization was performed as described (Detlef and Glazebrook, 2002).

2 Molecular biological methods

2.1 General molecular biological methods

General molecular biological methods like growing of bacteria, DNA precipitation and determination of DNA concentration were performed as described (Sambrook *et al*., 1989). The preparation of transformation-competent cells was performed according to the protocol of Hanahan and co-worker (Hanahan, 1983). Preparation of plasmid DNA, restriction digests, ligations and agarose gel electrophoresis were performed as described (Sambrook *et al*., 1989). The reaction conditions were adjusted to the manufacturer's recommendations.

2.2 Isolation of genomic DNA from *Arabidopsis thaliana*

2-3 *Arabidopsis* leaves were cut and transferred to a 1.5 ml microtube, 450 µl extraction buffer (200 mM Tris-HCl with pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, 100 µg/ml RNase) and one small iron bead were added and the sample was lysed in a TissueLyser (Qiagen, Hilden, Germany) for 3 minutes at maximum speed. Then the sample was incubated at 37°C for 5-10mins. After centrifugation for 10 min at 16,000 x g and 4°C, the supernatant was transferred to a fresh tube. To precipitate the genomic DNA, 300 µl of isopropanol was added to the sample, carefully mixed and incubated for 5 min at room temperature. After centrifugation for another 5 min at 16,000 x g and +4°C, the pellet was washed once with 70% ethanol, subsequently air-dried and finally resuspended in 50 µl of sterilized H_2O or 10 mM Tris-HCl (pH 8.0) buffer.

2.3 RNA extraction from *Arabidopsis thaliana* and RT-PCR

Total RNA from leaves, stems or flowers of *Arabidopsis* plants was isolated using the Plant RNeasy Extraction kit (Qiagen, Hilden, Germany). The DNA was digested with RNase-free DNase I (Qiagen) and transcribed into cDNA using MMLV Reverse transcriptase (Promega, Mannheim). Detection and quantification of transcripts were performed as described previously (Philippar *et al*., 2004) using the LightCycler system (Roche, Penzberg).

2.4 Characterization of plant T-DNA insertion lines and TILLING lines

Genomic DNA of the T-DNA insertion lines was screened by PCR genotyping. To identify plants with the T-DNA insertion in both alleles (homozygous), a combination of gene-specific primers flanking the predicted T-DNA insertion sites and T-DNA-specific left border (LB) primers (Table 5) were used. Usage of a LB primer (in combination with a corresponding gene-specific primer) will only generate an amplification product in plants carrying at least one T-DNA allele (heterozygous or homozygous for the T-DNA insertion). On the other

hand, the combination of two gene-specific primers will generate a PCR product only in DNA of plants carrying alleles without a T-DNA (WT and heterozygous for the T-DNA insertion). In the TILLING lines, the site of the point mutation corresponds to the site provided by TILLING. The positions and orientations of the T-DNA inserts and the position of point mutation in TILLING line and oligonucleotide primers in *oep7.1-2, oep7.1-3, oep7.2-1*, *fax1-1* and *fax1-2* are shown in Figure 7 and Figure 18, respectively. To verify the T-DNA insertion sites, PCR genotyping products were cloned and subsequently sequenced.

Table 5: PCR primer combination for genotyping mutant lines of *OEP7* and *FAX1*
For primer sequence please refer to Table 1, and for position and orientation of primers please refer to Figure 7 and Figure 18.

Allele	Line	primers for wild-type	primers for T-DNA insertion or TILLING
oep7.1-2	TILL2	OEP7.1 fw, OEP7.1 rev	OEP7.1 fw, OEP7.1 rev
oep7.1-3	TILL3	OEP7.1 fw, OEP7.1 rev	OEP7.1 fw, OEP7.1 rev
oep7.2-1	SAIL_813_F06	OEP6.7 rev garlic, OEP6.7 fw garlic	LB1SAIL, OEP6.7 fw garlic
fax1-1	SAIL_66_B09	fax1LP, fax1RP2	LB1SAIL, fax1RP
fax1-2	GABI_599E01	fax1LP, fax1RP2	GABI LB1, fax1-2LP
fax1-2	Complementation	attB1, fax1rev	attB1, fax1rev
fax1-2	Overexpressing	attB1, fax1rev	attB1, fax1rev

2.5 Microarray analysis

150 mg tissue powder from flowers and from second to forth internode of inflorescent stems from more than 10 individual seven-week-old plants was used for preparation of RNA (identical sample pool that was used for fatty acid analysis). RNA (200 ng) of three samples (n = 3) from both wild type (Col-0) and *fax1-2* lines was processed and hybridized to Affymetrix "GeneChip *Arabidopsis* ATH1 Genome Arrays" using the Affymetrix "3' VIT express" and "Hybridisation wash and stain" kits (Affymetrix UK, High Wycombe, UK) according to the manufacturer`s instructions. The statistical significance of signal change was calculated as described in Duy *et al*., (2011). The hybridization of the microarrays and statistical analysis of data were performed by Karl Mayer and by Dr. Katrin Philippar, respectively (Department Biologie I, Plant Biochemistry and Physiology, LMU München).

3 Biochemical methods

3.1 General biochemical methods

SDS-PAGE was performed as described (Laemmli, 1970). For OEP7, Tricine-SDS-PAGE was used as described (Schägger, 2006). Gels were stained either by Coomassie Brilliant Blue R250 (Sambrook *et al*., 1989) or silver-staining (Blum *et al*., 1987, Ansorge et al., 1985). Determination of chlorophyll concentration was carried out as described by Arnon, (1949). Determination of protein concentration was performed by the Bio-Rad Protein Essay Kit (Bio-Rad, München, Germany) or the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, USA)

3.2 Total protein extraction from *Arabidopsis thaliana*

Rosette leaves or flowers (0.1 g) of four-week-old plants were harvested and flash-frozen in liquid N_2. The frozen material was thoroughly ground with mortar and pestle and extracted by one volume extraction buffer (50 mM Tris-HCl, (pH 8), 2% LDS (Lithium dodecyl sulphate), 0.1 mM PMSF). After incubation on ice for 30 min, the sample was centrifuged at 16,000 x g at +4°C for 15 m in to get rid of insolube components. The protein concentration was determined with the Pierce BCA protein Assay Kit. 50 mM EDTA and 0.15% DTT were added to the protein solution for storage and further experiment.

3.3 Immunoblotting

3.3.1 Electrotransfer and blocking of proteins

Proteins separated by SDS-PAGE were transferred onto either Nitrocellulose (PROTRAN BA83, 0.2 µm, Whatman/Schleicher & Schüll) or PVDF membranes (Zefa Transfermembran Immobilon-P, 0.45 µm, Zefa-Laborservice GmbH, Harthausen, Germany) by semi-dry-blotting (Amersham Biosciences) (Kyhse-Andersen, 1984) in blotting buffers (I: 300 mM Tris-HCl, 20% methanol, II: 25 mM Tris-HCl, 20% methanol, III: 25 mM Tris-HCl, 40 mM aminocapron acid, 20% methanol) for 1h at 0.8 mA per cm^2 membrane surface as described (Towbin *et al*., 1979). To be mentioned, the PVDF membranes have to be activated in 100% methanol before use. Proteins of the size markers were either stained with Ponceau S solution or amidoblack solution. Membranes with bound proteins were first incubated for 30 m in in blocking buffer containing skimmed milk powder (0-5% milk powder, 0-0.1M Tris, 0-0.15 M NaCl, 0-0.75% Tween 20). And then the membrane was incubated with the protein-specific primary antibody (diluted in blocking buffer 1:250-1:4000, depending on the antibody) for 2-3h at RT or overnight at 4°C. Non-bound antibody was removed from the membrane by

washing for 3x10 min in TTBS (0.1M Tris, 0.15 M NaCl with 0.1% Tween 20). The secondary antibody was selected according to the desired method of visualization.

3.3.2 Alkaline phosphatase (AP) detection

If the secondary antibody was alkaline phosphatase (AP)-conjugated secondary antibody (goat anti-rabbit IgG (whole molecule)-AP conjugated, Sigma-Aldrich Chemie GmbH, Taufkirchen), detection of AP signals was performed in developing buffer (100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$) with 6.6 µl/ml NBT (nitro blue tetrazolium chloride, 50 mg/ml in 70% N,N-dimethylformamide) and 13.2 µl/ml BCIP (5- bromo-4-chloro-3-indolyl phosphate, 12.5 mg/ml in 100% N,N-dimethylformamide). The membrane was incubated in 50 mM EDTA to stop the reaction.

3.3.3 Enhanced Chemiluminescence (ECL)

If the secondary antibody was a horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit (whole molecule)-peroxidase conjugated, Sigma), detection of signal was performed in 50% (v/v) solution 1 (100 mM Tis-HCl (pH 8.5), 1% (w/v) luminol, 0.44% (w/v) coomaric acid) and 50% (v/v) solution 2 (100 mM Tris-HCl (pH 8.5), 0.018% (v/v) H_2O_2). After incubation for 1 min at RT in the dark, the solution was removed and the luminescence detected with a film (Kodak Biomax MR; PerkinElmer, Rodgau, Germany).

3.4 Proteolysis experiments

To identify the orientation of chloroplast envelope proteins, proteolysis of *Arabidopsis* chloroplasts or of inner envelope vesicles from pea was performed using the protease thermolysin. The chloroplasts or envelope vesicles were incubated in wash buffer (330 mM sorbitol, 50 mM Hepes (pH 7.6) and 0.5 mM $CaCl_2$) with 10% (m/m) thermolysin and incubated for 0-30 min on ice. For envelope solubilisation, 1% triton was added. The proteolysis was stopped with 5 mM EDTA per 1 µg protease. The proteins were separated on SDS-PAGE or Tricine-SDS- PAGE and analysed by immunoblot.

4 Cell biology methods

4.1 Isolation of *Arabidopsis thaliana* chloroplasts

Chloroplast isolation was performed as described (Aronsson and Jarvis, 2002) with modification. Leaf material from 21-day-old plants grown on soil or on MS-plates supplemented with 1% (w/v) sucrose was homogenized in 25 ml isolation buffer (0.3 M sorbitol, 5 mM $MgCl_2$, 5 mM EGTA, 5 mM EDTA, 20 mM HEPES/KOH (pH 8.0), 10 mM $NaHCO_3$, 50 mM ascorbic acid). After homogenisation and filtration steps, the soluble

homogenate was pelleted at 1000 g for 4 min and resuspended in isolation buffer. The sample was separated on a two-step Percoll gradient (30/82% (w/v) Percoll) with centrifugation at 1500 g for 8 min. The lower band (intact chloroplasts) was transferred to a 10 ml tube and washed in wash buffer (50 mM HEPES/KOH (pH 8.0), 0.3 M Sorbitol, 2 mM DTT). The chloroplasts were pelleted at 1000 g for 4 min and resuspended in 0.5 ml wash buffer.

4.2 Preparation of inner and outer envelope from *Pisum sativum*

For isolation of IE and OE vesicles from chloroplasts, pea seedlings grown for 9-11 days on sand under a 12/12 hours dark/light regime were used. All procedures were carried out at 4°C. Pea leaves cut from ~ 20 trays were ground in a kitchen blender in 10-15 l isolation medium (330 mM sorbitol, 20 mM MOPS, 13 mM Tris, 0.1 mM MgCl2, 0.02% (w/v) BSA) and filtered through four layers of mull and one layer of gauze (30 µm pore size). The filtrate was centrifuged for 5 min at 1,500 x g, the pellet gently resuspended with a brush and intact chloroplasts reisolated via a discontinuous Percoll gradient (40% and 80%). Intact chloroplasts were washed twice with wash medium (330 mM sorbitol, Tris-base (~ pH 7.6)), homogenized and further treated according to the modification (Waegemann *et al*., 1992) of the previously described method (Keegstra and Youssif, 1986).

4.3 Preparation and transient transformation of protoplasts from *Arabidopsis*

Arabidopsis mesophyll protoplasts were isolated from 1 g leaves of 3 to 4-week-old *Arabidopsis* plants grown on soil under normal growth condition and transiently transformed according to the protocol from Jen Sheen (http://molbio.mgh.harvard.edu/sheenweb/main_page.html). GFP fluorescence was observed with a TCS-SP5 confocal laser scanning microscope (Leica, Wetzlar, Germany).

5 Light and transmission electron microscopy

Inflorescent stems, 1 cm above the first node and anthers of flower stage 11-12 (Smyth *et al*., 1990) from 2-3 individual *Arabidopsis thaliana* plants at the age of 34 to 40 days were harvested in the morning prior to illumination. Samples were prefixed in glutaraldehyde 2.5% (w/v) in 75 mM cacodylate buffer (pH 7.0), subsequently rinsed in cacodylate buffer and fixed in 1% (w/v) osmium tetroxide in the same buffer for at least 2.5 h at room temperature. The samples were stained with uranyl acetate 1% (w/v) in 20% acetone, dehydrated in a graded acetone series and embedded in Spurr's low viscosity epoxy resin (Spurr, 1969). Further steps and microscopic investigation were performed by Dr. Irene Gügel (Department Biologie I, Plant Biochemistry and Physiology, LMU München).

6 Wax analysis

The second to forth internode region of inflorescent stems from more than 10 individual, seven-week-old plants were used for wax analysis from *FAX1* mutants, wild-type and complementation lines. Wax analysis was conducted on pools of 3-4 stems as described (Prabhakar *et al*., 2010; Kurdyukov *et al*., 2006) and performed by Prof. Lukas Schreiber (Department of Ecophysiology, IZMB, University of Bonn)

7 Fatty acid analysis

Tissue of cauline leaves, second to forth internode of inflorescent stems and flowers from more than 10 individual, seven-week-old plants were grinded in liquid nitrogen and used for fatty acid analysis. Three samples of 50 mg tissue powder from homozygous *fax1* mutants and from wild-type plants were used. Fatty acid analysis was conducted as described (Hummel *et al*., 2011; Giavalisco *et al*., 2011) and performed by Dr. Patrick Giavalisco (Max Planck Institute of Molecular Plant Physiology, Potsdam-Golm, Germany).

8 Bioinformatical methods

Table 6: Software, databases and algorithms used in this work

Name	Reference	Link
NCBI BLAST	Altschul et al., 1997	http://www.ncbi.nlm.nih.gov/BLAST
Vector NTI	Invitrogen	
TopPred	Heijne 1992; Claros and Heijne 1994	http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::toppred
ARAMEMNON	Schwacke *et al*., 2003	http://aramemnon.botanik.uni-koeln.de
TAIR(The Arabidopsis Information Resource	Lamesch *et al*., 2011	http://www.arabidopsis.org
ChloroP 1..1		http://www.cbs.dtu.dk/services/ChloroP/
Arabidopsis eFP browser	Schmid *et al*., 2005	http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi
Genevestigator	Zimmermann *et al*., 2004	https://www.genevestigator.com/gv/plant.jsp
AtGenExpress Consortium		http://www.weigelworld.org/resources/microarray/AtGenExpress/
Genedoc	Nicholas and Nicholas,1997	http://www.psc.edu/biomed/genedoc
ExPASy PeptideCutter	Gasteiger *et al*., 2005	http://web.expasy.org/peptide_cutter/
EBI interpro protein sequence analysis & classification database		http://www.ebi.ac.uk/interpro/

MEGA 5.0	Tamura *et al*., 2011	http://www.megasoftware.net/
AT CHLORO database	Ferro *et al*., 2010	http://www.grenoble.prabi.fr/protehome/- grenoble-plant-proteomics/
Aida Image Analyzer v. 3.25	raytest	http://www.raytest.fr/index2.html

IV. Results

1 At-OEP7.2 structure and function analysis

1.1 OEP7 proteins in *Arabidopsis*

OEP7 was first identified and characterised as an outer chloroplast envelope protein in spinach (E 6.7, Salomon *et al*., 1990). Currently four isoforms of OEP7 have been identified in *Arabidopsis*: At-OEP7.1 (At3g52420), At-OEP7.2 (At3g63160), At-OEP7.3 (At3g19151) and At-OEP7.4 (At2g34585) (ARAMEMNON plant membrane protein database, Schwacke *et al*., 2003). The highest amino acid identity is 32% between At-OEP7.1 and At-OEP7.2. All four OEP7 isoforms have one α-helical transmembrane domain (Figure 3) (ARAMEMNON plant membrane protein database, Schwacke *et al*., 2003). It is predicted that At-OEP7.2 is localized at the outer envelope of chloroplasts, while At-OEP7.3 is in mitochondria and At-OEP7.4 of unknown subcellular localisation. Until the beginning of my thesis, no experimental data on the OEP7.2 topology and function was published.

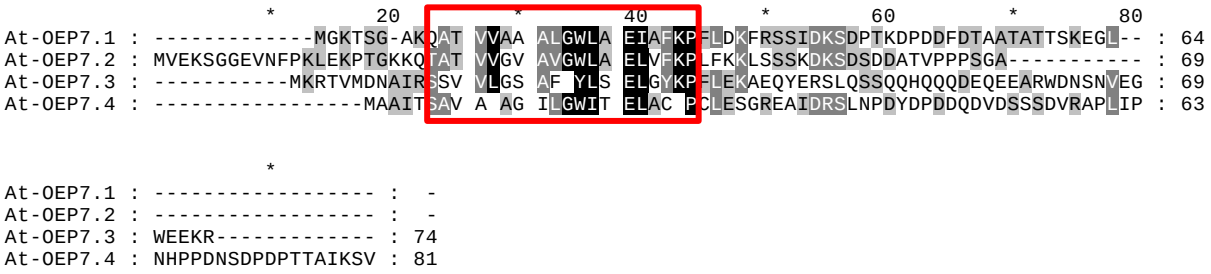

Figure 3: OEP7 proteins in *Arabidopsis*
Identical amino acids are shaded in black, similar amino acids in grey and the transmembrane domain is depicted as red box.

1.2 Membrane orientation of OEP7.2 in the outer envelope of chloroplasts

It is known that OEP7.1, a 14 kDa protein, has the N-terminus in the intermembrane space and the C-terminus in the cytosol orientation (Figure 4C left; Li *et al*., 1996). To analyse the orientation of At-OEP7.2, *Arabidopsis* chloroplasts were treated with proteases in the presence or absence of detergent and further subjected to immunoblot analysis (Figure 4).

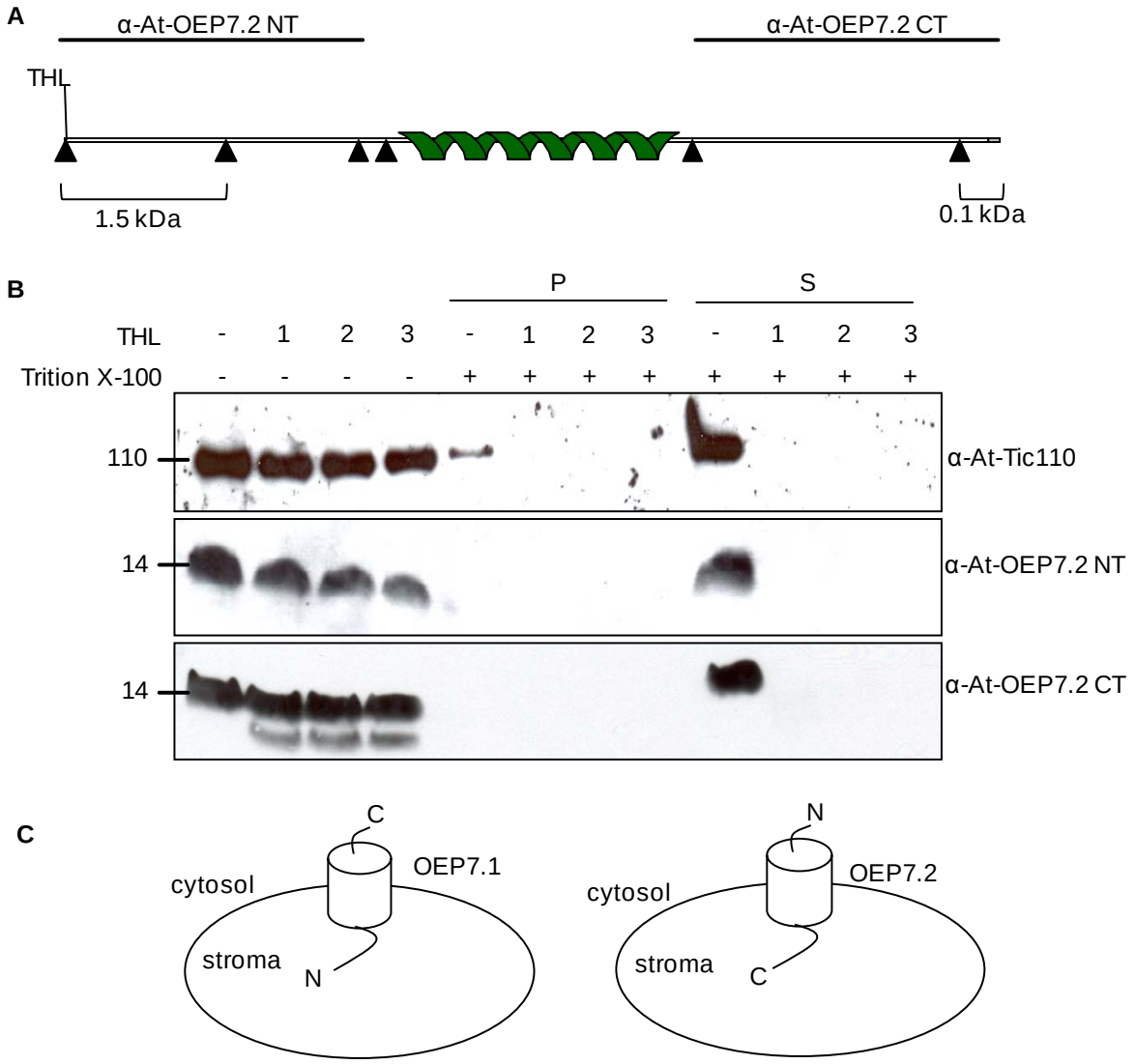

Figure 4: At-OEP7.2 protein topology analysis at the outer envelope of chloroplasts
A) Thermolysin digestion sites (THL) of At-OEP7.2. Six thermolysin digestion sites, the transmembrane α- helix and regions for two peptide antisera (α-At-OEP7.2 NT, CT) are shown, respectively. **B)** Immunoblot analysis of At-OEP7.2 with antiserums raised against At-OEP7.2 N-terminus (NT) or At-OEP7.2 C-terminus (CT) after protease treatment with or without 1% triton x-100. 1.5, 3, 4.5 µg thermolysin were added to chloroplasts (equal 15 µg chlorophyll) and incubated for 20 min on ice, respectively. P: pellet, S: supernatant. Tic110 is marker of inner envelope proteins, which should be prevented from thermolysin digestion. **C)** Topology model of At-OEP7.1 and At-OEP7.2.

In the presence of triton, which breaks the membrane structure, membrane proteins are fully accessible to proteolysis. However, in the absence of triton, only the proteolysis sites of membrane proteins at the cytosol site are accessible to proteolysis. It is firstly hypothesized that the N-terminus is in the cytosol and C-terminus is in the inter membrane space. A 1.5 kDa region would be digested and then a 12-13 kDa band would be recognized by the antiserum raised against At-OEP7.2 C-terminus (Figure 4A). In contrast, if the N-terminus is in the inter membrane space and C-terminus in the cytosol, only a 0.1 kDa region of OEP7.2 would be digested and therefore a 14 kDa band, which becomes weaker with the increase of

thermolysin amount, would be recognized by both antisera. The three thermolysin digestion sites near the transmembrane domain of OEP7.2 were not considered in the hypothesis because of possible membrane protection. It is clear that the immunoblot analysis results (Figure 4B) was matched with the first hypothesis, which revealed that At-OEP7.2 has its N-terminus in the cytosol (Figure 4C right).

1.3 Targeting of At-OEP7.2 to the outer envelope membrane of chloroplasts

To further confirm that At-OEP7.2 is localized at the envelope membrane of chloroplasts, I studied the At-OEP7.2: GFP and At-OEP fusion proteins in *Arabidopsis* protoplasts (*in vivo* GFP targeting; Figure 5).

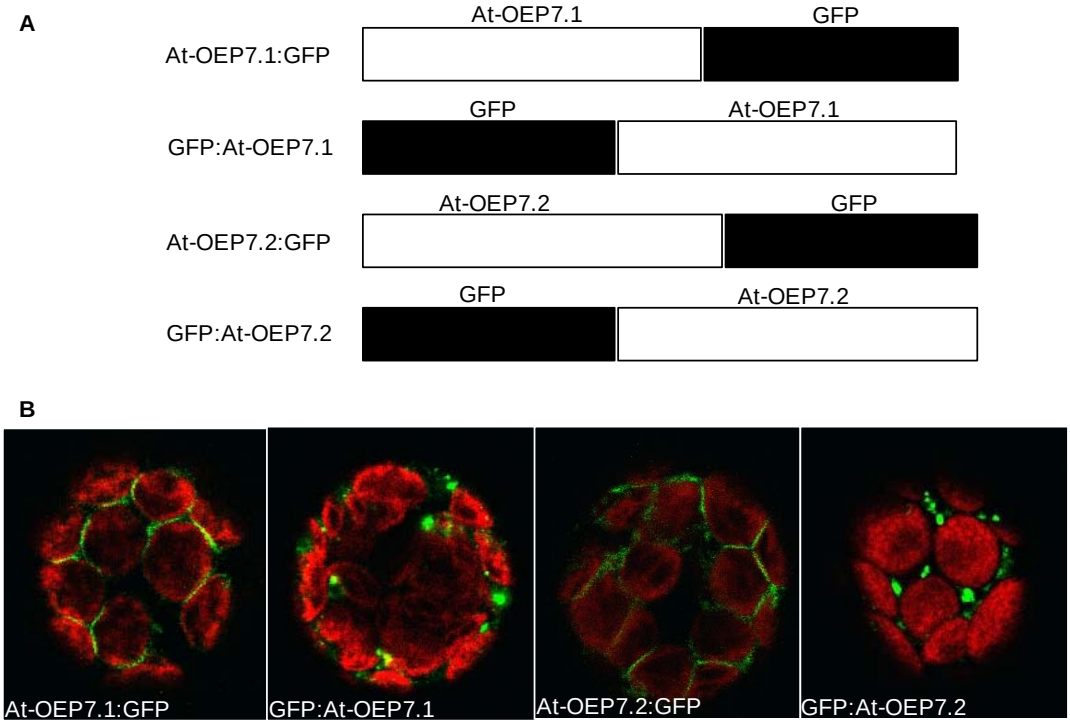

Figure 5: *In vivo* targeting of AtOEP7:GFP in protoplasts
A) The various fusion constructs used in the experiments. At-OEP7.1:GFP or At-OEP7.2:GFP: green fluorescent protein (GFP) was fused to the C-terminus of At-OEP7.1 or At-OEP7.2, respectively. GFP:At-OEP7.1 or GFP:At-OEP7.2: GFP was fused to the N-terminus of At-OEP7.1 or At-OEP7.2, respectively. **B)** *In vivo* targeting of fusion constructs in *Arabidopsis* protoplasts. Only overlaps of GFP (green) and chlorophyll (red) are shown.

As shown in Figure 5, At-OEP7.2, like At-OEP7.1, was targeted to the chloroplast envelope membrane when GFP-proteins were fused to their C-terminus. However, when GFP was attached to the N-terminus of both proteins, a punctate signals appeared, which revealed that GFP: OEP7 was mis-targeted. The results suggested that the N-terminus of At-OEP7.2 is critical for targeting to the outer envelope membrane. For OEP7.1, it was confirmed that the

seven-amino acid region at the C-terminus and the transmembrane domain are determinants for targeting to the chloroplast outer envelope membrane (Lee *et al*., 2001).

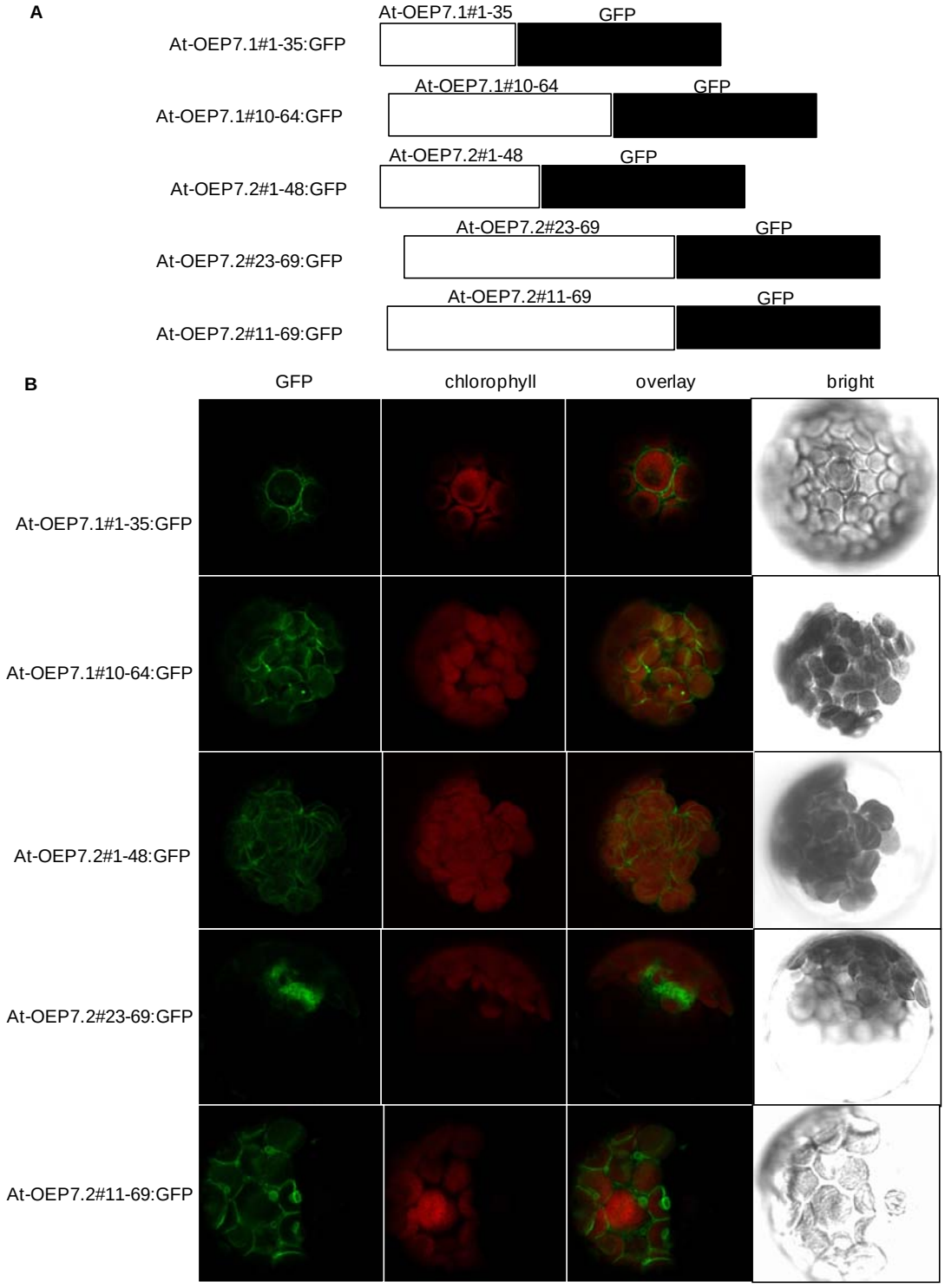

Figure 6: *In vivo* targeting of At-OEP7 deletion constructs fused with GFP in protoplasts
A) Scheme of the various deletion constructs fused with GFP. **B)** *In vivo* targeting of various At-OEP7.1 and At-OEP7.2 deletion constructs, which were fused to GFP N-terminus. Protoplasts were transformed with At-OEP7.1#1-35:GFP, At-OEP7.1#10-64:GFP, At-OEP7.2#1-48:GFP, At-OEP7.2#23-69:GFP and At-OEP7.2#11-69:GFP. GFP (green), chlorophyll (red), overlap of GFP and chlorophyll and bright-field images (bright) are shown, respectively.

To confirm which amino acid region is necessary and sufficient for the targeting of At-OEP7.2, I produced various deletion constructs of At-OEP7.2 and subsequently fused them to GFP (Figure 6A). Two deletion constructs of At-OEP7.1 with GFP were used as markers. All these constructs were expressed transiently in protoplasts (Figure 6B).

As shown in Figure 6, the fusion proteins At-OEP7.1#1-35:GFP, At-OEP7.1#10-64:GFP, At-OEP7.2#1-48:GFP and At-OEP7.2#11-69:GFP were targeted to the envelope membrane of the chloroplasts. In constrast, At-OEP7.2#23-69: GFP only gave a punctate staining pattern in the protoplasts, which revealed that it mis-targeted. Thus, these results suggest that the residues 11 to 22 of At-OEP7.2 are necessary and residues 1 to 48 of At-OEP7.2 are sufficient for targeting to the chloroplast envelope membrane.

1.4 OEP7 mutant analysis in *Arabidopsis*

Two TILLING lines *oep7.1-2* and *oep7.1-3* (Seattle Arabidopsis TILLING Project, Till *et al*., 2003) for OEP7.1, a T-DNA insertion mutant line (SAIL_813_F06), named *oep7.2-1*, for OEP7.2, and double mutant (*d.m.*) of *oep7.1* and *oep7.2-1* were available at the beginning of my thesis. In OEP7.1 TILLING lines, a point mutation at position +146 changed the nucleobase cytosine to thymine, which produced a stop codon at the beginning of first exon of the OEP7.1 open reading frame (Figure 7A). In *oep7.2-1*, the T-DNA insertion is localized at position +579 in the first exon of the OEP7.2 open reading frame (Figure 7B). For these single mutants and double mutants of *oep7*, immunoblot analysis showed that they were knockout mutant in *Arabidopsis* (Figure 7C).

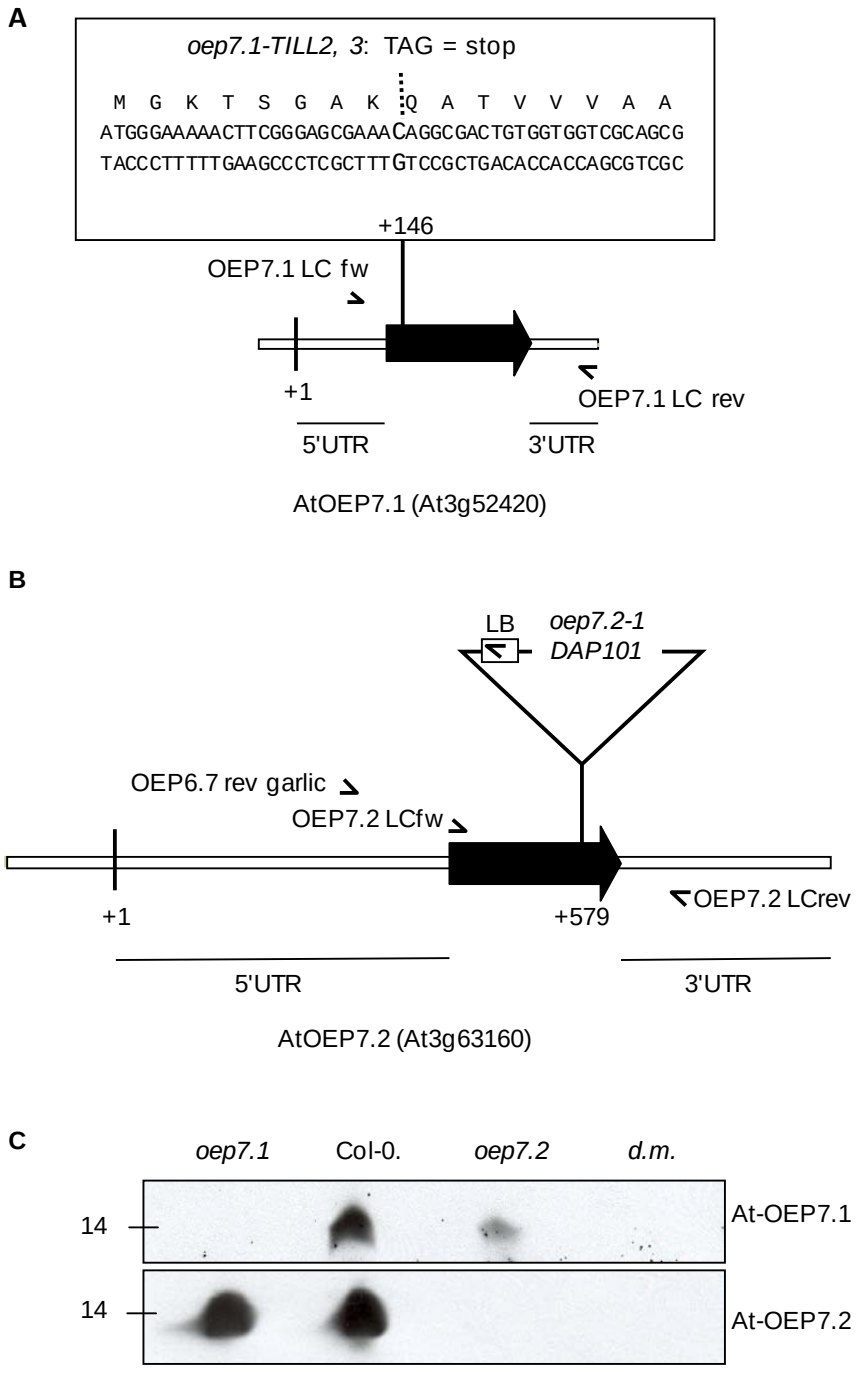

Figure 7: Characterisation of At-OEP7.1 and At-OEP7.2 mutant lines
A) *OEP7.1* from *Arabidopsis thaliana* (At3g52420). Black arrows denote exons and white line denote introns. The location of the point mutation in the TILLING line oep7.1 is detailed in the text box, which indicates a premature stop codon (C to T) at amino acid 146. Binding sites for OEP7.1 gene specific primers for real time RT-PCT are depicted. **B)** *OEP7.2* from *Arabidopsis thaliana* (At3g63160). Black arrows denote exons and white lines denote introns. The T-DNA insertion sites in line SAIL_813_F06 (*oep7.2-1*) are depicted by triangles. Binding sites for OEP7.2 gene specific primers and T-DNA specific left (LB) primer used for genotyping and for real time RT-PCT are depicted. **C)** Immunoblot analysis of At-OEP7.1 and At-OEP7.2 in *Arabidopsis* leaf protein extract (10 µg) of mutant lines and Col-0 using antisera against At-OEP7.1 and At-OEP7.2, respectively. *d.m.*: double mutant.

To further understand the function of OEP7, single and double mutant lines of oep7.1 and oep7.2 were grown under several growth conditions (1: 16 h light, +21°C, 100 µmol photons

m^{-2} s^{-1} and 8 h dark ,+16°C cycle; 2: 12 h light, +21°C, 100 µmol photons m^{-2} s^{-1} and 12 h dark ,+16°C cycle; 3: 8 h light, +21°C, 100 µmol photons m^{-2} s^{-1} and 16 h dark ,+16°C cycle; 4: 16 h light, +21°C, 180 µmol photons m^{-2} s^{-1} and 8 h dark ,+16°C cycle) but no obvious phenotype could be detected until now (data not shown). However, considering that *At-OEP7.2* was predicted as a light-sensitive gene according to Smith *et al*. (2004), real time RT-PCR and immunoblot analysis were performed on *oep7.2-1* and Col-0 throughout the diurnal cycle (Figure 8).

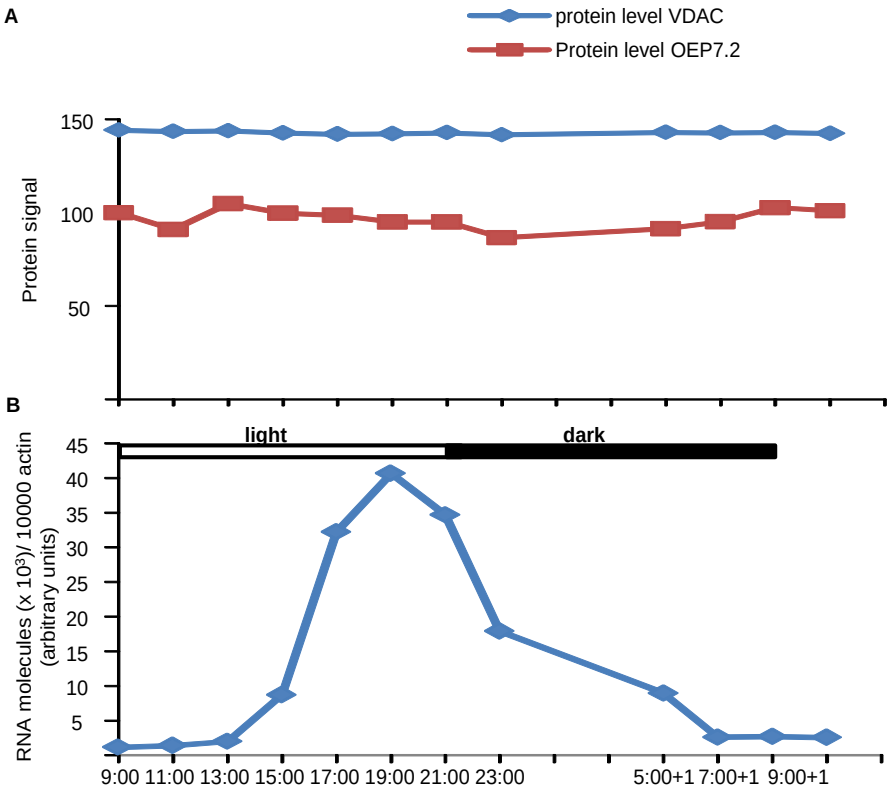

Figure 8: Expression of At-OEP7.2 throughout the diurnal cycle
Leaves were harvested from 4 weeks old Arabidopsis Col-0 plants under standard growth conditions (20 °C, in a 12-h-light/12-h-dark photoperiod with an irradiance of 180 µmol m^{-2} s^{-1}). **A)** Diurnal changes in amounts of At-OEP7.2 protein (shown as green triangle; n= 3±SD). Protein VDAC is one control which is stable expression. **B)** Expression patterns of OEP7.2 gene. The mRNA amount (arbitrary units) was normalised to 10000 actin transcripts.The curve represent mean values of n=3 independent experiments.

Under the conditions employed, transcripts of *OEP7.2* showed peak amount at 19:00, two hours before dark and changed relatively with light over 24 h , w hich indicated that *OEP7.2* transcription is regulated in a diurnal rythm. To further identify whether OEP7.2 proteins are regulated as well, the same samples used for real time RT-PCR were further used for immunoblot. The result revealed that the OEP7.2 protein amount changed relatively little throughout the diurnal cycle.

2 FAX1, a putative permease for fatty acid export from chloroplasts

2.1 Identification of a pre-senescence related chloroplast envelope protein

Until now, many chloroplast proteome databases were built up for plastid envelope protein analysis. In our research, the AT CHLORO database (http://www.grenoble.prabi.fr/protehome/-grenoble-plant-proteomics/; Ferro *et al.*, 2010) was used for selection of new envelope proteins. To provide a link to senescence processes, we used mutants of SAUL1 (Senescence-associated E3 ubiquitin ligase 1) that was identified as one E3 ubiquitin ligase preventing premature senescence in *Arabidopsis* plants (Raab *et al.*, 2009). To identify premature senescence associated chloroplast envelope proteins, we performed DNA microarray analysis of *SAUL1* knockout mutants under senescence inducing conditions (collaboration with Prof. S. Hoth, Plant Physiology, University of Hamburg; Vogelmann *et al.*, 2012).

Among three potential chloroplast envelope proteins from the AT CHLORO database (Ferro *et al.*, 2010) transcript of the protein At3g57280 was increased 5.8 and 3.2 fold after 24h and 48h of senescence induction in *saul1-1* mutants compared to wild type, respectively (Figure 9).

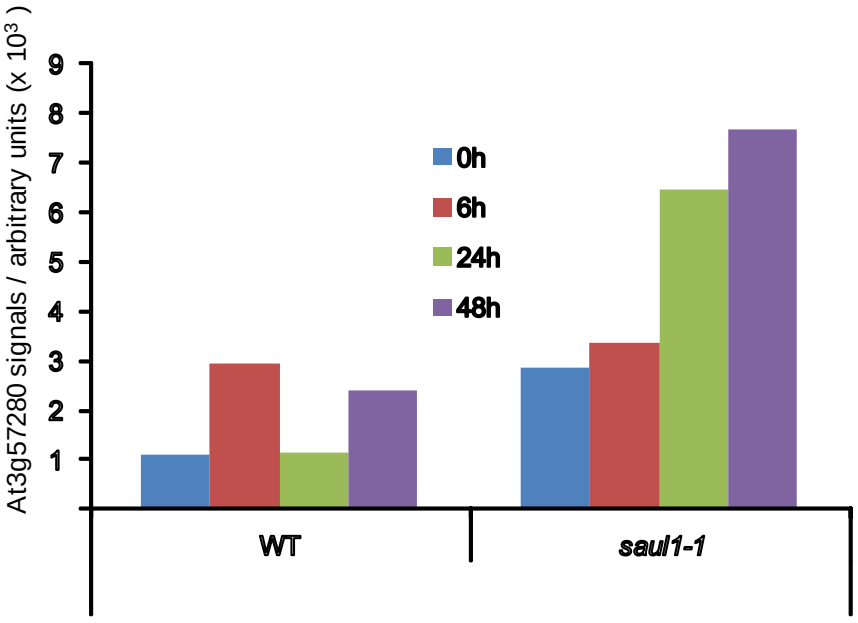

Figure 9: At3g57280 RNA content in *saul1-1* under senescence inducing conditions
Total RNA was isolated from wild-type and *saul1-1* mutant seedlings grown in permissive light (60 µmol m^{-2} s^{-1}) for 11 days and then challenged with low light (20 µmol m^{-2} s^{-1}) for 6h, 24h, or 48h. To provide biological replicates, mean values for three wild-type and three *saul1-1* mutant samples were harvested for each time point (Vogelmann *et al.*, 2012). Mean values for At3g57280 RNA (arbitray units, n= 3±SD) are shown.

Because of its potential function in chloroplast fatty acid transport (see Chapter IV. 2.9), the protein At3g57280 is named FAX1 (for fatty acid export 1) in the following. At-FAX1 was predicted to be an inner chloroplast envelope protein (Ferro *et al*., 2010) of 24.3 kDa, with an isoelectric point of 9.3 (TAIR, Lamesch *et al*., 2011) and four predicted α-helices (Table 9, ARAMEMNON plant membrane protein database, Schwacke *et al*., 2003). Further, a chloroplast signal peptide of 33 a mino acids was predicted (ChloroP, Emanuelsson *et al*., 1999). At-FAX1 cDNA was purchased as SSP pUNI151 clone U12755 (Yamada *et al*., 2003). At-FAX1 was also described as a putative solute transporter of chloroplasts in Tyra *et al*. (2007).

Table 9: Protein features of FAX1 from *Arabidopsis* and pea
The name, AGI code, the isoelectric point (IP), the molecular weight (Mw) in kDa, the predicted chloroplast signal peptide and the predicted number of transmembrane domains (TMs; ARAMEMNON) are shown. At-FAX1 by proteomic analysis was identified as chloroplast envelope protein in the listed references. The pea EST contig is published in Franssen et al. (2011).

Name	AGI codes/ contig	IP (TAIR)	Mw (kDa)	chloro signal peptide(AraM)	TMs (AraM)
At-FAX1	At3g57280	9.3	24.3	33	4
References: Fröhlich *et al*. (2003); Kleffmann *et al*. (2004); Zybailov *et al*. (2008); Ferro *et al*. (2003), (2010).					
Ps-FAX1	contig012718	9.16	25	39	4

For further experiments, the cDNA of *FAX1* in *Pisum sativum* was cloned by RT-PCR using pea seedling cDNA as template and primers designed according to an EST pea contig (Franssen *et al*., 2011). All general data of the Ps-FAX1 are shown in Table 9, the amino acid sequences of At-FAX1 and Ps-FAX1 are depicted in Figure 10. The amino acid identity between At-FAX1 and Ps-FAX1 is 49%, and for both proteins four α-helices transmembrane domains are predicted (Figure 10B).

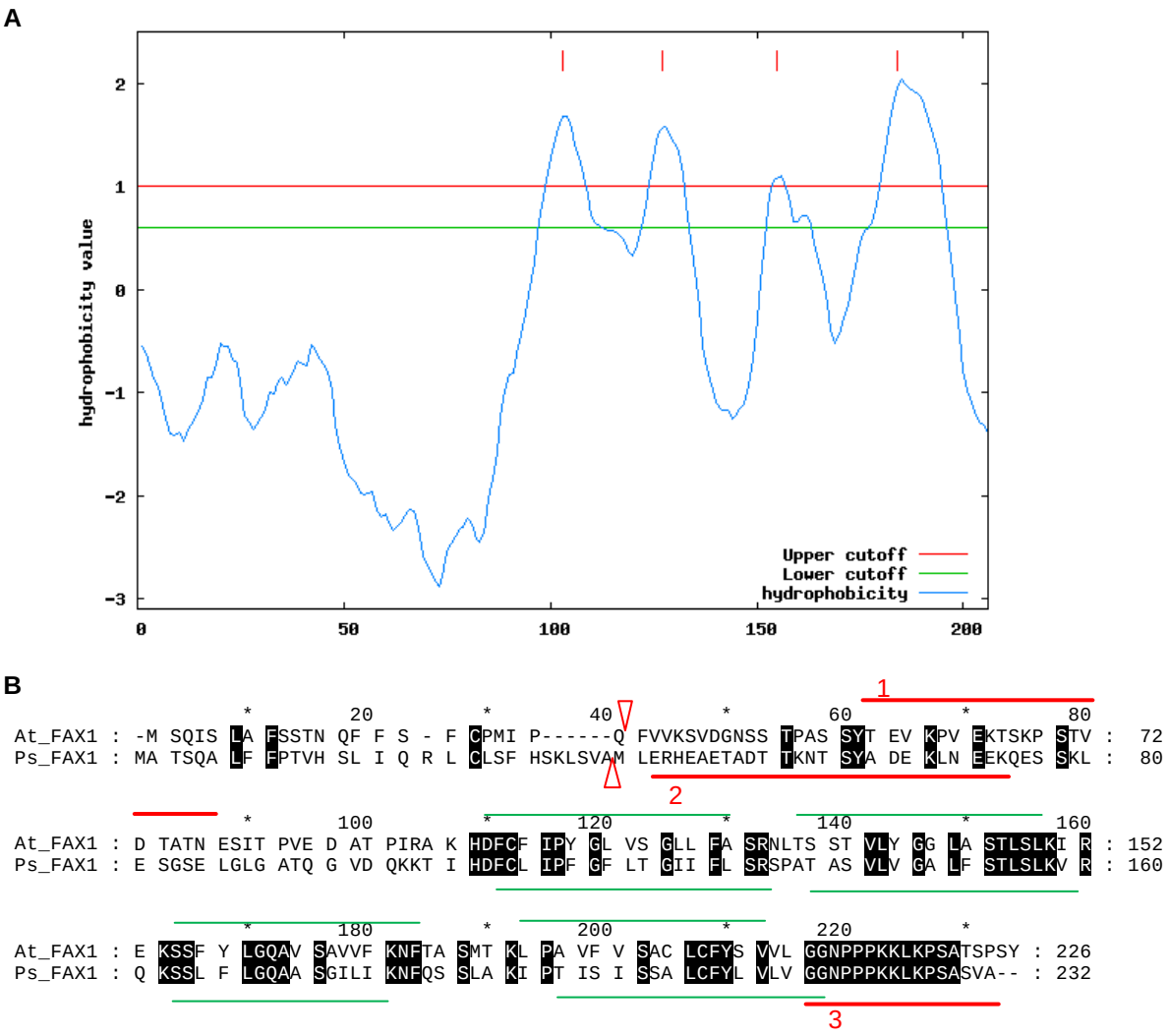

Figure 10: Sequence analysis of At-FAX1 and Ps-FAX1
A) Graphical output of At-FAX1 sequence showing four predicted hydrophobic transmembrane domains using the TopPred program (Heijne 1992; Claros and Heijne 1994)**. B)** Amino acid sequence of At-FAX1 and Ps-FAX1. Identical amino acids between At-FAX1 and Ps-FAX1 are shaded in black, the end of the predicted chloroplast transit peptides is marked as red triangle, the predicted transmembrane domains are lined in green, the regions used for peptide antisera are lined in red. 1: is for antiserum α-At-FAX1, 2: is for antiserum α-Ps-FAX1-NT, 3: is for antiserum α-Ps-FAX1-CT.

2.2 FAX1 is localized in the inner envelope of chloroplasts

Although At-FAX1 was predicted as an inner envelope protein according to proteomics data (Ferro *et al.*, 2003, 2010), no experimental data directly showed the sub-cellular localization. Therefore, the chloroplast localization of FAX1 was verified by *in vivo* GFP-targeting experiments. *At-FAX1* cDNA was fused C-terminally with the green fluorescent protein (GFP) and transiently transformed into *Arabidopsis* protoplasts (Figure 11).

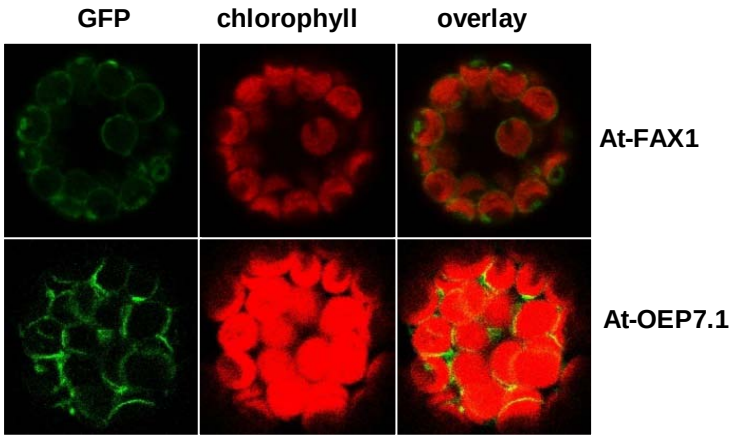

Figure 11: At-FAX1-GFP signals in *Arabidopsis* protoplast
At-FAX1-GFP and At-OEP7.1-GFP constructs were transiently transformed into *Arabidopsis* protoplasts. GFP, chlorophyll and overlay fluorescence of both constructs are shown. At-OEP7.1-GFP was used as marker for the chloroplast outer envelope.

GFP fluorescence signals only appeared surrounding the chloroplasts, which indicated that the FAX1-GFP fusion construct localized at the envelope membrane of chloroplasts. The At-OEP7.1-GFP fusion construct, as a marker for an envelope localized protein, also showed fluorescence signals only around the chloroplast.

To further confirm whether FAX1 is uniquely localized at the inner envelope of plastids, immuno-blotting analysis on fractionated pea chloroplasts was performed (Figure 12). Antiserum raised against peptides of Ps-FAX1-NT, named α-Ps-FAX1-NT was used in immunoblot (see Figure 10C).

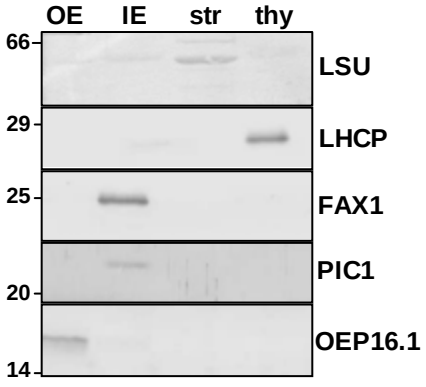

Figure 12: Immunoblot of Ps-FAX1 in pea chloroplast fractions
Same total protein amount (5 µg) of the pea chloroplast sub-fractions OE (outer envelope), IE (inner envelope), stroma (str) and thylakoid (thy) were separated by SDS-PAGE and immunoblots using antiserums raised against Ps-FAX1-NT were performed. LSU (large subunit of RuBisCO, 1 µg of protein) appears as a marker of the stroma, LHCP (light-harvesting chlorophyll a/b binding proteins, 0.2 µg of protein) as a marker of the thylakoids, PIC1 (permease in chloroplasts 1, 5 µg of protein) as a marker of IE, and OEP16.1 (outer envelope protein 16.1, 5 µg of protein) as a marker of OE.

The antiserum α-Ps-FAX1-NT showed a 24 kDa band only in the inner envelope fraction. Antisera raised against LSU, PIC1, LHCP and OEP16.1 were used to recognize marker proteins for stroma, inner envelopes (IE), thylakoids and outer envelopes (OE) of chloroplasts, respectively.

2.3 Membrane orientation of FAX1 in the inner envelope of chloroplasts

Considering that FAX1 is a four transmembrane domain protein, it means that both the N- and C-terminus should be localized either in the stroma or the inter membrane space. To verify the orientation of FAX1 at the inner envelope membrane, pea inner envelope vesicles were protease treated and further subjected to immunoblot analysis (Figure 13).

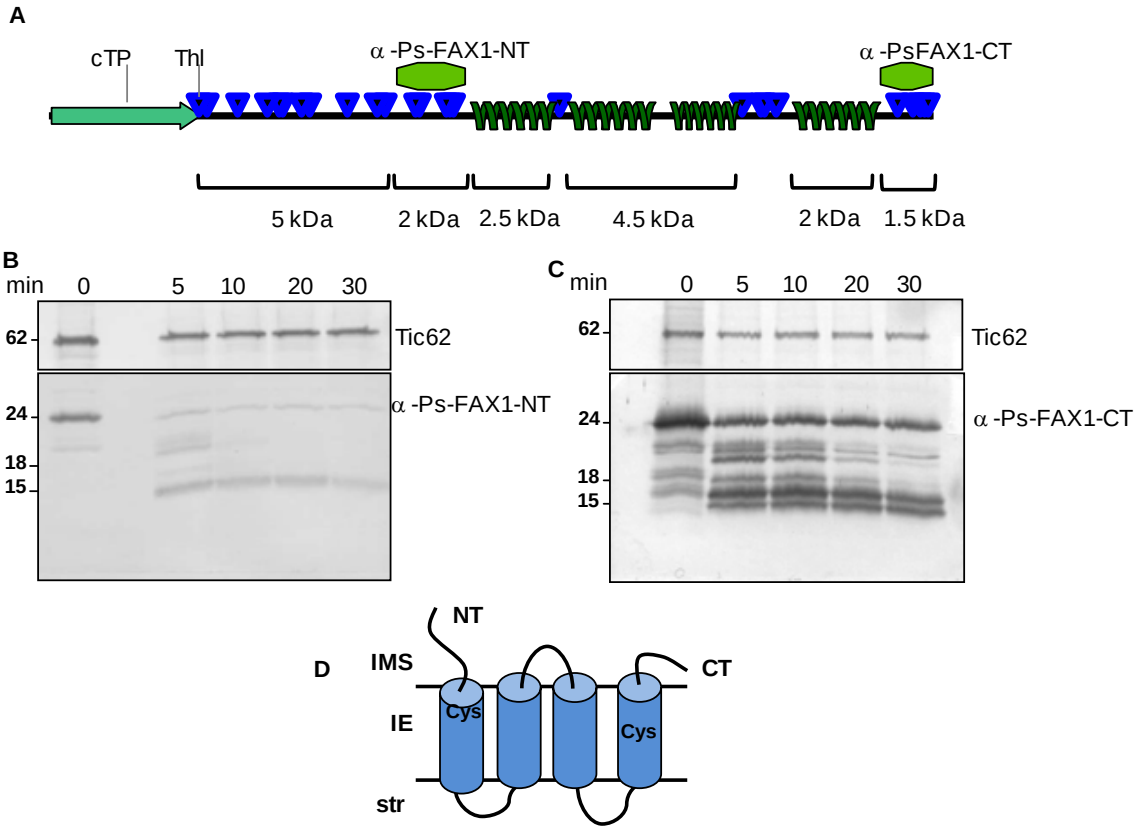

Figure 13: Ps-FAX1 protein topology analysis at the inner envelope of chloroplasts
A) Accessible proteolytic sites of Ps-FAX1 (without transit peptide and 4 tramsmembrane domain regions). THL: thermolysin digestion sites, cTP: the chloroplast transit peptide. The molecular weight in kDa is given for each possible THL fragment of the protein. Four transmembrane domains and two regions which were designed for antisera are also shown. **B)** Proteolysis using thermolysin and immunoblot with antiserum α-Ps-FAX1-NT. Inner envelope membrane vesicles (IE) of pea chloroplasts were used as sample. 5 µg thermolysin was added to 50ug IE and digested the proteins for 0, 5, 10, 20, 30 min. For each lane 10 µg of protein was separated by SDS-PAGE. Tic62 is a marker of the stromal side of inner envelope vesicles, which is not accessible for thermolysin digestion. **C)** Proteolysis using thermolysin and immunoblot with antiserum α-Ps-FAX1-CT. Procedure is same as in **B**. **D)** Topology model for FAX1 at the inner envelope of chloroplasts. IMS: inter membrane space, IE: inner envelope, Str: stroma, NT: N-terminus, CT: C-terminus of FAX1, Cys: Cysteine residues.

Because inner envelope membrane vesicles are isolated in a right-side out orientation, the size of proteolysis fragments of membrane proteins can help to determine their orientation (Keegstra and Youssif, 1986; Waegemann et al., 1992). It is firstly hypothesized that NT and CT are in the inter membrane space, which means that only the proteolysis sites at NT and CT are accessible to thermolysin and in immunoblot, many shifted bands between 15- 24 kDa are able to be recognized by antisera α-Ps-FAX1-NT or α-Ps-FAX1-CT antisera (see Figure 10B). However, if both NT and CT are in the stroma, which means only the proteolysis sites between the first and second and between third and forth transmembrane domains are accesible to proteolysis (Figure 13A), there would be only shift bands lower than 14 kD a recognized by antiserum α-Ps-FAX1-NT or lower than 10 kDa recognized by antiserum α-Ps-FAX1-CT. The immunoblot results (Figure 13B and C) matched with the first hypothesis. In addition a cysteine labelling assay (not shown) revealed that both cysteine residues of Ps-FAX1 are embedded in the membrane (Figure 13D). Conclusively, FAX1 is an inner envelope protein with four transmembrane domains and both NT and CT are in the inter membrane space.

2.4 FAX1 is a plant specific protein and a member of the transmembrane 14C family

Blast analysis of the At-FAX1 protein sequence (National Center for Biotechnology Information , NCBI, http://www.ncbi.nlm.nih.gov/BLAST) showed that FAX1 is a plant specific protein (Figure 14). FAX1 was also annotated as a plastid targeted solute transporter of putative "plantae-specific" origin (Tyra *et al.*, 2007). FAX1 exists not only in di-, and monocotyledonous plants like *Arabidopsis thaliana*, *Populus trichocarpa*, *Ricinus communis*, *Vitis vinifera*, *Plantago major*, *Glycine max*, *Pisum sativum*, *Picea sitchensis*, *Oryza sativa*, *Sorghum bicolor*, *Zea mays* but also in the green algae *Chlamydomonas reinhardtii* (Figure 14). Tyra *et al*. (2007) in addition identified more distantly related algae orthologs in *Ostreococcus tauri, Ostreococcus lucimarinus* and *Micromonas pusilla*.

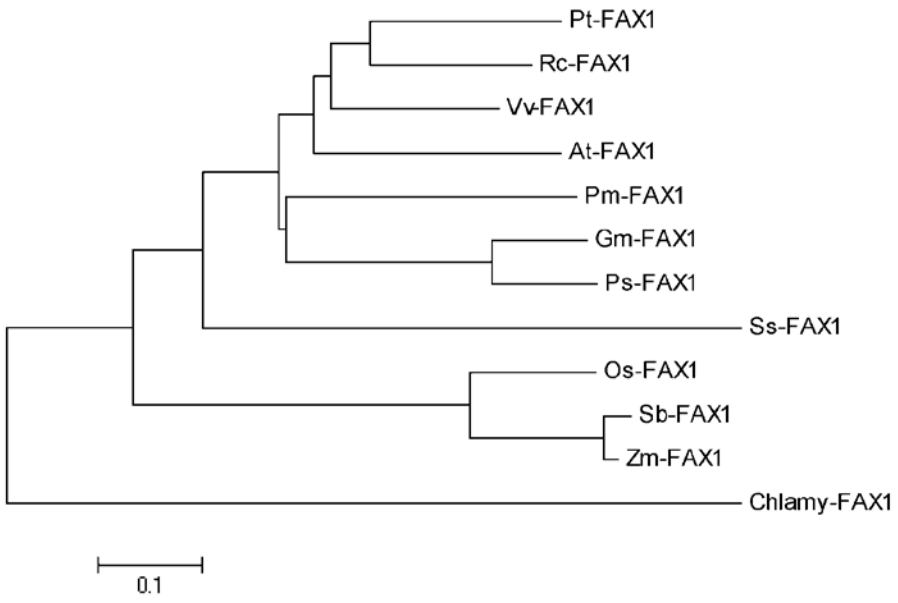

Figure 14: FAX1 is a plant specific protein
Phylogenetic tree of FAX1 was created by software MEGA 5.0 (Tamura *et al*., 2011). At: *Arabidopsis thaliana*, Pt. *Populus trichocarpa*, Rc: *Ricinus communis*, Vv: *Vitis vinifera*, Pm: *Plantago major*, Gm: *Glycine max*, Ps: *Pisum sativum*, Ss: *Picea sitchensis*, Os: *Oryza sativa*, Sb: *Sorghum bicolour*, Zm: *Zea mays*, Chlamy: *Chlamydomonas reinhardtii*.

Further, FAX1 is described as member of the transmembrane 14C family (Tmemb_14), which is an uncharacterised protein family UPF0136 with conserved membrane domains (EBI InterPro protein sequence analysis & classification database; http://www.ebi.ac.uk/interpro/). The Tmemb_14 family has members in all eukaryotes, including fungi, fruit fly, mammalians and plants but can be found in bacteria and cyanobacteria as well. In *Arabidopsis*, four Tmemb_14 proteins including FAX1, At2g38550, At3g43520 and At1g33265 are predicted to localize in chloroplasts with four similar transmembrane domains (Figure 15). Three other homologs At3g20510, At1g50740 and At2g26240 are predicted to localize at the plasma membrane. The highest amino acid identity to At-FAX1 is 23.8% with At3g20510 (putative plasma membrane protein).

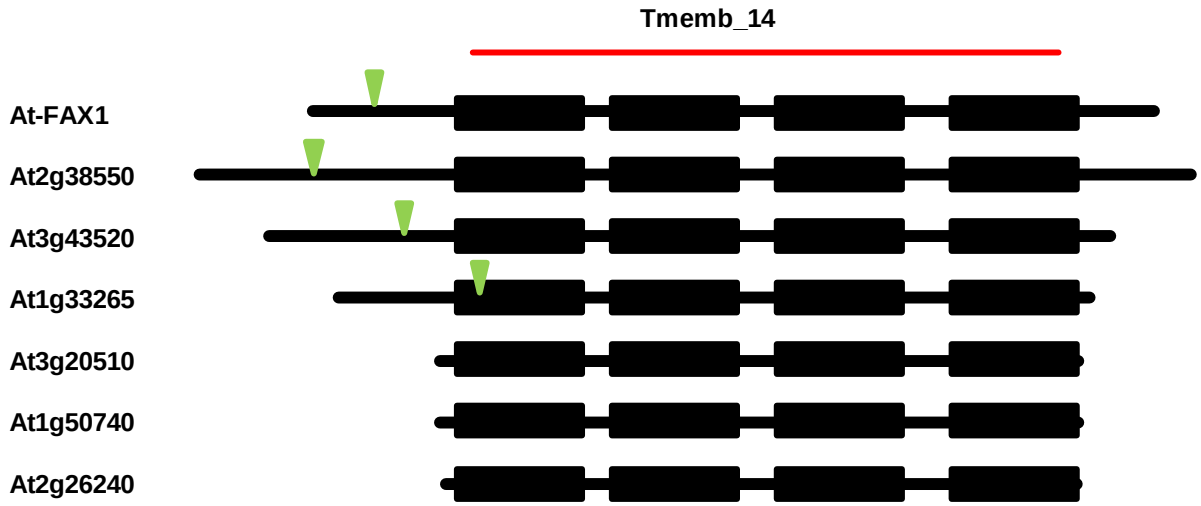

Figure 15: Graphical representation of all Tmemb_14 proteins in *Arabidopsis*
The Tmemb_14 conserved motif is lined in red. The four transmembrane domains are depicted in black boxes. The end of chloroplast transit peptides is marked by green triangles. The other three proteins (At3g20510, At1g50740 and At2g26240) are predicted to localize at the plasma membrane.

2.5 *FAX1* is expressed during all stages of *Arabidopsis* development

It is revealed that *FAX1* is expressed in all developmental stages and tissues of *Arabidopsis*, when screening the microarray data of the Arabidopsis eFP Browser (Winter *et al*., 2007), which depends on data from AtGenExpress (Schmid *et al*., 2005). According to data from Genevestigator (Zimmermann *et al*., 2004), At-*FAX1* is expressed throughout all *Arabidopsis* development stages as well with the highest level in young flower stages but the lowest level during seed germination and in mature siliques (Figure 16).

To further analyse the *FAX1* gene expression in flowers, microarray data of pollen development (Honys and Twell 2004) was used (Figure 17). Here, *FAX1* transcripts were high in young pollen stage but lower in mature pollen stages (Figure 17). Conclusively, *FAX1* may be an essential gene in all *Arabidopsis* developmental stages, especially in young flowers and early pollen development.

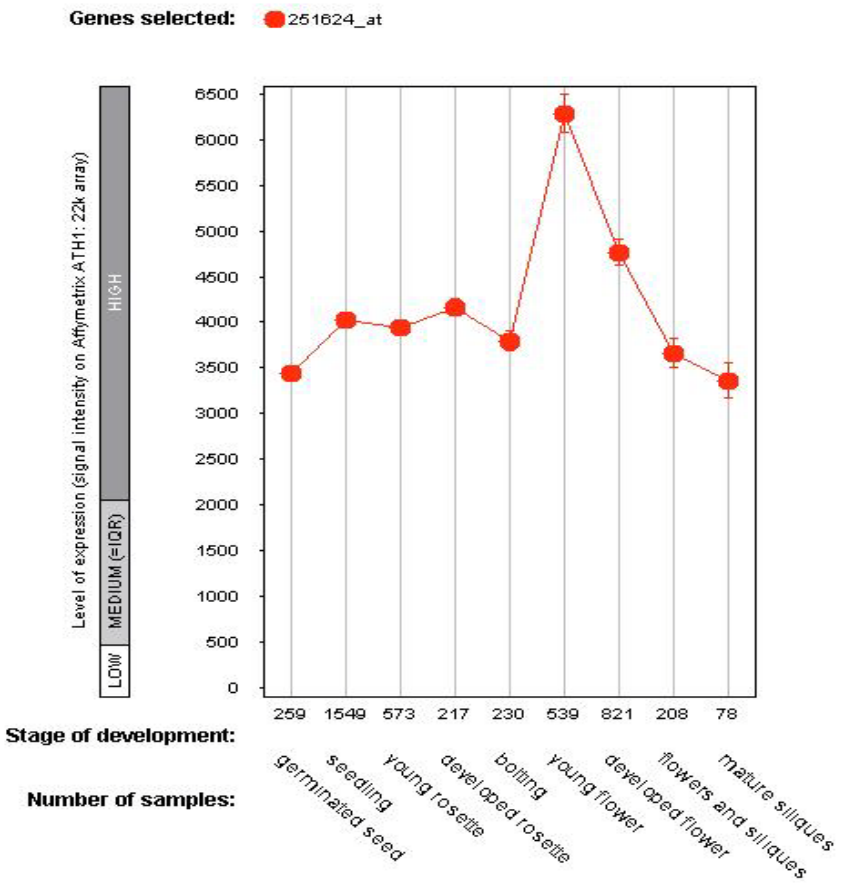

Figure 16: Gene expression of *FAX1* in different developmental stages of *Arabidopsis*
FAX1 gene code is 251624_at. Microarray data is from Genevestigator (https://www.genevestigator.com/gv/index.jsp, Zimmermann *et al*., 2004). Mean signal intensities (arbitrary units ± SD) were averaged from 2-3 replicates. *Arabidopsis* development was defined as 9 stages: germinated seed, seedling, young rosette, developed rosette, bolting, young flower, developed flower, flowers and siliques and mature siliques.

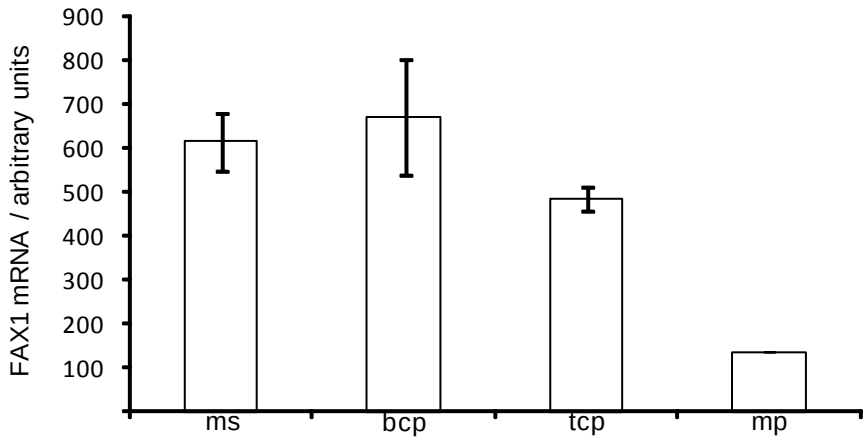

Figure 17: Transcript level of *FAX1* in *Arabidopsis* pollen development (Honys and Twell, 2004)
Mean signal intensity (arbitrary units±SD) of *FAX1* mRNA was averaged from 1-2 replicates. Arabidopsis pollen development was divided into four stages: ms (microspore), bcp (bicellular pollen), tcp (tricellular pollen), mp (mature pollen).

2.6 *FAX1* mutant analysis in *Arabidopsis*

The gene of *FAX1* in *Arabidopsis* consists of six exons and the mRNA is named *Arabidopsis thaliana* transmembrane 14C (At3g57280) in the NCBI database, with the reference sequence NM_15588. The mRNA sequence of *FAX1* is 1030bp long including 180bp 5' UTR and 169bp of 3' UTR (Figure 18).

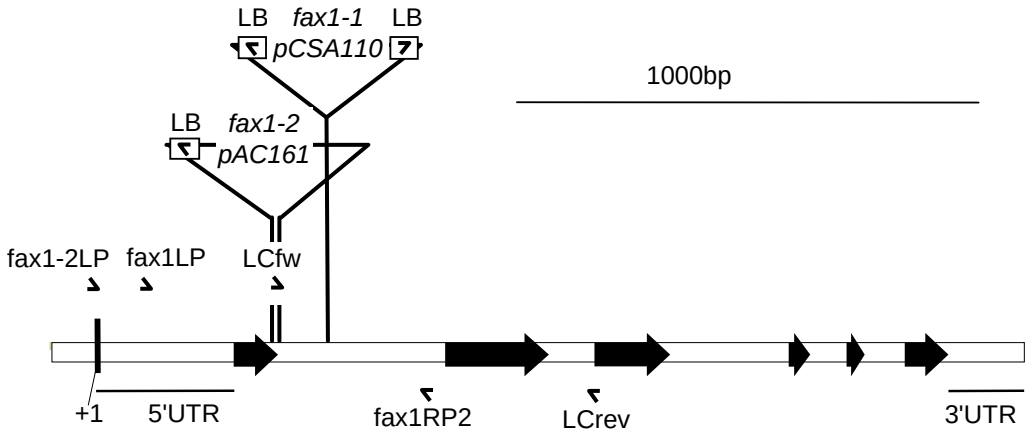

Figure 18: The *FAX1* gene in *Arabidopsis*
Scheme of the exon and intron localization of *FAX1* and T-DNA insertion sites of *Arabidopsis* mutants. Black arrows indicate the six exons, white lines introns. Two T-DNA insertion sites in the first intron (*fax1-1*, position +526) and in the first exon (*fax1-2*, position +388, +405, 17bp deletion of *FAX1*) are indicated by triangles. pCSA110 is the T-DNA sequence in the SAIL_66_B09 line (*fax1-1*). pAC161 is T-DNA sequence in the GABI-Kat line 599E01 (*fax1-2*). Binding sites for *FAX1* gene specific primers and T-DNA specific left (LB) border primers used for PCR genotyping and for real time RT PCR are depicted.

To understand the function of in the chloroplast inner envelope, two mutant T-DNA insertion lines in the *FAX1* gene were selected: SAIL_66_B09 (*fax1-1*) and GABI-Kat 599E01 (*fax1-2*; Figure 18). After PCR genotyping (Figure 19A and B) and sequencing of the PCR product, T-DNA insertion sites were identified in the first intron at position +526 (*fax1-1*) and in the first exon at position +388 to +405 with a 17bp deletion of the *FAX1* gene (*fax1-2*; Figure 18). Subsequent RT-PCR on RNA of homozygous *FAX1* mutants (Figure 19C) showed that both lines represent knockouts for *FAX1*.

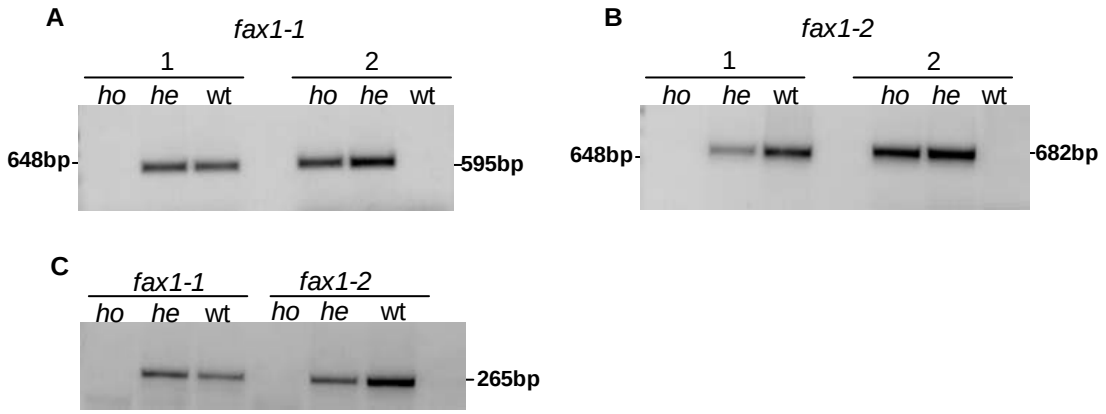

Figure 19: PCR genotyping of two *FAX1* T-DNA insertion lines in *Arabidopsis*
A) and **B)** PCR genotyping amplified a 648bp signal in heterozygous (*he*) and wild type (wt) lines by *FAX1* gene specific primers fax1LP and fax1RP2 (**A1** and **B1**). For the *fax1-1* T-DNA insertion line, a 595bp product was amplified in homozygous (*ho*) and *he* mutants by a T-DNA primer from SAIL (LB1 SAIL) and the gene specific primer fax1RP (**A2**). For the *fax1-2* T-DNA insertion line, a 682bp product in *ho* and *he* lines was detected by a T-DNA primer from GABI (GABI LB1) and a gene specific primer fax1-2LP (**B2**). **C)** RT-PCR on *FAX1* RNA extracted from young leaves of *fax1-1* and *fax1-2* mutants. LCfw and LCrev amplified a product of 265bp. *ho*: homozygous, *he*: heterozygous, wt: wild type. For position of primers, see Figure 16.

To complement the knockout of *FAX1*, the *FAX1* cDNA under control of the 35S promoter (plasmid vector pH2GW7; Karimi *et al*., 2002), was introduced into heterozygous *fax1-2* plants. After selection of the following T1 and T2 generations and PCR genotyping, two lines named Comp#7 and Comp#54 containing the complementation construct were identified as homozygous for the original T-DNA at the *FAX1* locus (Figure 20A).

To stable overexpress *FAX1* in the ecotype Columbia (Col-0), the same construct (35S::FAX1) used for the complementation lines was introduced into Col-0. T1 generation plants containing the construct 35S::FAX1 were also selected based on hygromycin resistance and PCR genotyping. In the T2 generation, two plants named FAX1ox2 and FAX1ox4 were identified as heterozygous overexpressing lines of FAX1 (Figure 20B).

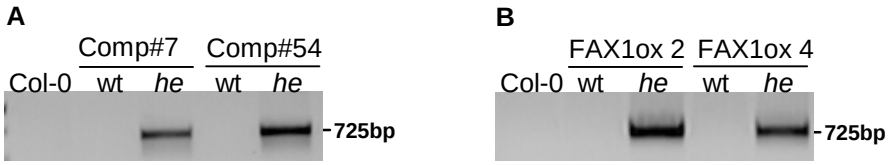

Figure 20: PCR genotyping of *FAX1* complementation and overexpressing lines
PCR genotyping amplified one 725bp signal by a *FAX1* cDNA specific primer (fax1rev) and a vector specific primer (attB1) in complemented *fax1-2* and overexpressing lines. *he*: heterozygous, wt: wild type.

In the following, the T-DNA insertion lines (*fax1-1*, *fax1-2*), complementation lines (Comp#7, Comp#54), overexpressing lines (FAX1ox2, FAX1ox4) and wild type (Col-0, wild type from *fax1-2* line) were analyzed for their FAX1 transcript and protein content by quantitative real time (qRT)-PCR and immunoblot analysis, respectively. The qRT-PCR result (n=1, data no shown) indicated that the *FAX1* mRNA amount in five weeks old cauline leaves of Comp#7

and Comp#54 is 0.8 and 12.1 fold higher, and in heterozygous FAX1ox2 and FAX1ox4 lines is 26 and 210 fold higher than in Col-0, respectively.

Immunoblot analysis was performed to analyze FAX1 protein expression level in five week old cauline leaves of all lines (*fax1-1*, *fax1-2*, Col-0, WT background of *fax1-2*, Comp#7, Comp#54, FAX1ox2 and FAX1ox4). The peptide antiserum α-At-FAX1 (see Figure 10) can recognize two bands between 23-25 kDa in the total membrane protein extract of cauline leaves (Figure 21A). Because signals in total protein extract of wild type were weak, I tested the FAX1 antibody on purified *Arabidopsis* wild type envelope, detecting two bands as well (Figure 21B). Alternative processing of signal peptide may cause the two bands signal. The results reveal that the amount of FAX1 protein in five weeks old cauline leaves of Comp#7 and Comp#54 is 1- and 1.8-fold more and of heterozygous FAX1ox2 and FAX1ox4 lines is 2.2- and 4-fold more than in Col-0, respectively (Signal intensities were determined by Aida Image Analyzer v. 3.25; Figure 21A).

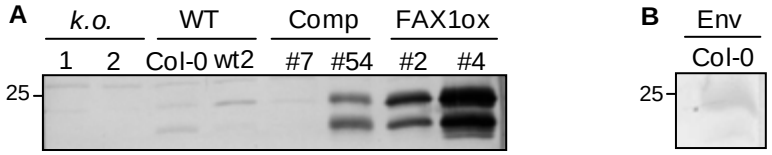

Figure 21: FAX1 protein expression in cauline leaves of wild-type and all mutant lines
A) Immunoblot analysis of FAX1 in protein extracts (50 µg per lane) from 5-week-old *Arabidopsis* cauline leaves. The antiserum against FAX1 protein (α-At-FAX1) can recognize two bands at 23-25 kDa at wild type (Col-0; wt2: WT background of *fax1-2*), complementation (Comp#7, #54) and FAX1 overexpressing (FAX1ox2, 4) lines. *k.o.*: knockout mutant, 1: *fax1-1*, 2: *fax1-2*, WT: wild type. **B)** Immunoblot analysis of FAX1 in isolated envelopes (Env; 10 µg protein per lane) of *fax1-2*, Col-0 and FAX1ox4 lines.

In summary, qRT-PCR and immunoblot analysis results indicate that the line Comp#7 contains wild-type levels of *FAX1* mRNA and protein, while the lines Comp#54, FAX1ox2 and FAX1ox4 represent overexpressors of FAX1.

2.7 Phenotype of FAX1 mutant lines

The heterozygous *fax1-1* and *fax1-2* mutants showed the same phenotype as Col-0 (data not shown). But the homozygous knockout mutant plants grew slower than Col-0 as visible after 30 days of growth (Figure 22A). However, the chlorophyll *a* and *b* amounts (per leaf fresh weight) were same as for Col-0 (data not shown). After 50 days of growth, homozygous *fax1* knockout mutant plants were smaller, with thin inflorescence stems and short siliques containing almost no s eeds (Figure 22B). Since the same phenotype was shown in both independent T-DNA insertion lines, *fax1-1* and *fax1-2*, it is deduced that the phenotype is caused by the loss of FAX1 function. To further confirm this correlation, complementation

and overexpressing lines were grown at the same conditions (Figure 22). Both complementation lines Comp#7, #54 showed the same growth phenotype as wild type at 30 and 50 days, except shorter siliques (see Figure 25). Remarkably, the overexpressing lines FAX1ox2 and 4 were slightly bigger and produced thicker inflorescence stems than wild type (Figure 22).

Figure 22: Growth phenotypes of *FAX1* mutants
A) 30-day-old plants of *FAX1* mutants and wild type. **B)** 50-day-old plants of *FAX1* mutants and wild type. *fax1-1*, *fax1-2*: homozygous knockout lines; Col-0, WT *fax1-2*: wild-type FAX1 alleles; Comp#7, Comp#54: FAX1 complementation lines; FAX1ox2, FAX1ox4: FAX1 overexpressing lines.

Conclusively, the phenotypes in *FAX1* knockout mutants and overexpressing lines in *Arabidopsis* are linked directly to the function of FAX1.

With the described growth phenotype of *FAX1* mutant lines, it is clear that FAX1 affects the biomass production, including leaves, stems, roots and flowers in *Arabidopsis* plants. Therefore, rosette weight, leaf weight, fresh stem weight, dry stem weight, root weight and stem diameter were measured in all *FAX1* mutant lines (Figure 23).

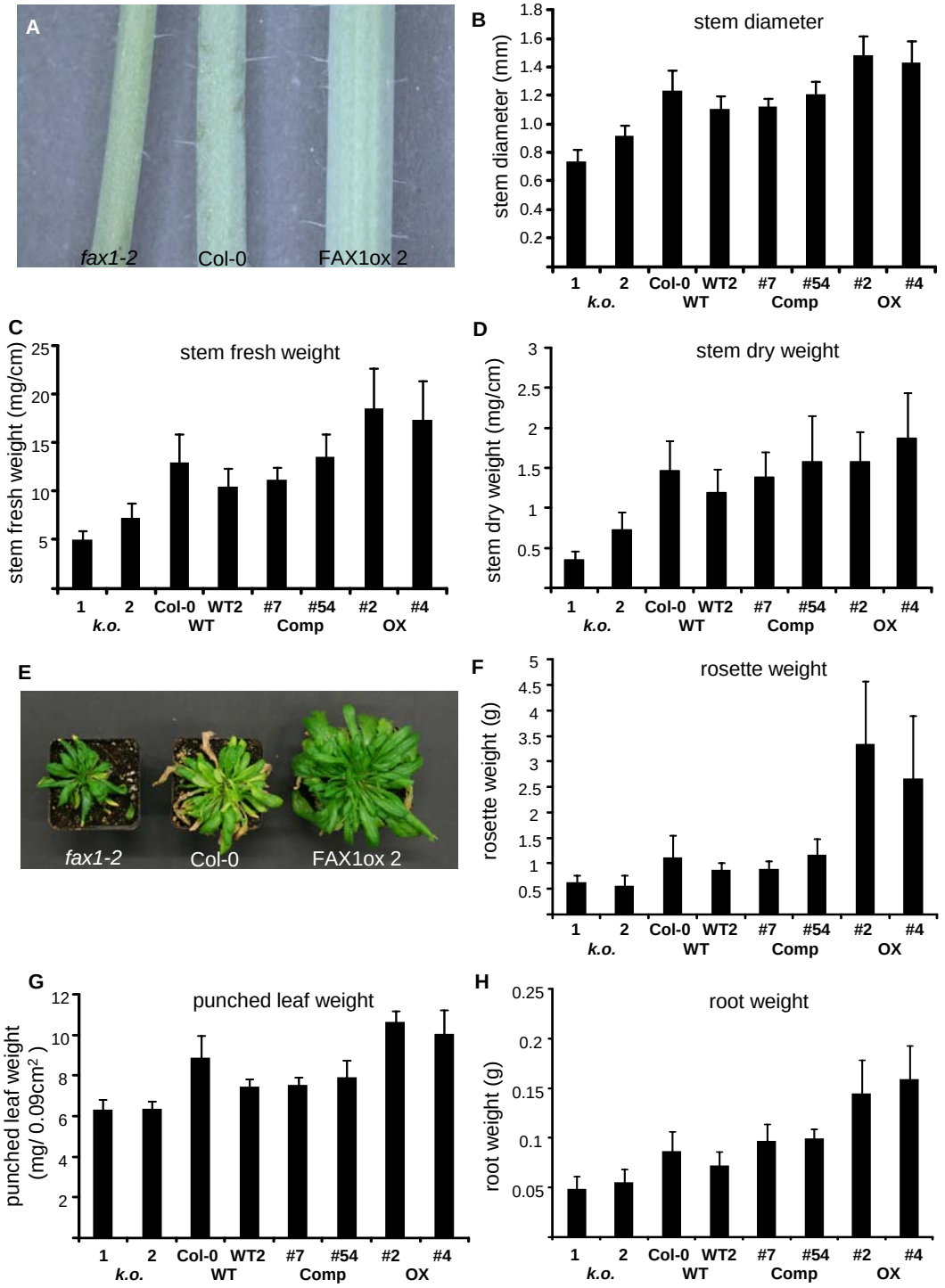

Figure 23: Plant biomass of *FAX1* mutants and wild type
Arabidopsis plants from *FAX1* mutants and wild type were harvested at 50 days.and dissected into different organs. **A)** Picture of stems. Bottom part of second internode of inflorescence stem from *fax1-*

2, Col-0 and FAX1ox2 was selected. **B)** Stem diameter in mm. Same internode part as in **A** (n=4-13±SD) was selected from *FAX1* mutants and wild type. **C)** Stem fresh weight (mg/cm, n=4-12±SD). 1cm stem at the bottom of the second internode of inflorescence stem, washed. **D)** Stem dry weight (same sample as in **C**, n=4-12±SD). **E)** Pictures of rosettes from *fax1-2*, Col-0 and FAX1ox2. **F)** Rosette weight (n=8-13±SD). **G)** Punched leaf weight (central of rosette leaf with similar size was punched as one 0.09 cm^{-2} circle and weighted, n=6-11±SD). **H)** Root weight (n=4-10±SD). *fax1-2, k.o.*: homozygous knockout lines; Col-0, WT2: wild-type FAX1 alleles; Comp#7, Comp#54: FAX1 complementation lines; FAX1ox2, FAX1ox4: FAX1 overexpressing lines.

Generally, adult homozygous *fax1* knockout plants were smaller, and adult overexpressing lines plants were larger in size than wild type and complementation lines. When measured in 50-day-old plants, the stem diameter, stem fresh weight and stem dry weight of *FAX1* mutants were different from the corresponding wild type alleles and complementation lines: knockout lines were smaller and less, overexpressing lines were bigger and more (Figure 23A-D). Leaves and roots also showed a biomass reduction in *fax1* knockouts but an increase in FAX1ox lines when compared to wild type (Figure 23E-H).

Conclusively, all measurements in *FAX1* mutant tissues provided evidence that FAX1 is involved in plant biomass production.

fax1 knockout plants show reduced male fertility

To understand the loss of function phenotype of homozygous *fax1* mutants during flower development, especially during the pollination and fertilization procedure, genetic analysis of male and female crosses between T-DNA insertion lines and wild type was performed (Table 10).

Table 10: The backcrossing of *fax1* lines

♂ x ♀ : pollen and female gamtophyte pollination. No.: Number of plants analysed in the filial generation. "he *fax1-1* x he *fax1-1*" and "he *fax1-2* x he *fax1-2*" are self-pollination of the heterozygous *fax1-1*, *fax1-2* mutant, respectively. "wt *fax1-2* x ho *fax1-2*" is the backcrossing of wild type *fax1-2* pollen with homozygous *fax1-2* mutant female gametophyte. "he *fax1-2* x ho *fax1-2*" is the backcrossing of heterozygous *fax1-2* mutant pollen with homozygous *fax1-2* mutant female gametophyte. ho%: percentage of homozygous, he%: percentage of heterozygous, wt%: percentage of FAX1 corresponding wild-type alleles.

Crosses (♂ x ♀)	No.	ho %	he %	wt %
he *fax1-1* x he *fax1-1*	280	7	65	28
he *fax1-2* x he *fax1-2*	204	4	63	33
wt *fax1-2* x ho *fax1-2*	20*	/	100	/
he *fax1-2* x ho *fax1-2*	171	12	88	/

* mix from 10 crossing events

Self-fertilization of heterozygous *fax1-1* and *fax1-2* mutant revealed that the segregation ratio of homozygous progeny was 7% and 4% respectively, demonstrating an impaired pollination

or fertilization procedure. When the female gametophyte from homozygous *fax1-2* mutant was fertilized with wild-type *fax1-2* pollen, normal siliques with 100% heterozygous seeds were harvested at the filial generation, which indicates that the female gametophyte of homozygous *fax1-2* was fertile. However, when the wild-type *fax1-2* female gametophyte was fertilized with homozygous *fax1-2* mutant pollen, the same shorter siliques and reduced amount of homozygous progeny as during selfing of homozygous *fax1-2* mutant plants were harvested and seed yield was < 0.1% of wild-type (data not shown), which indicates that the male gametophyte of the knockout mutant was almost sterile. Furthermore, during this manual crossing, it became evident that pollen grains of homozygous *fax1-2* were improperly released by anthers. To minimize anther defects from the paternal tissue, I thus pollinated homozygous *fax1-2* gametophytes with pollen from heterozygous *fax1-2* anthers thereby achieving 12% of homozygous progeny (Table 10).

In summary, the strongly impaired male fertility phenotype of homozygous *fax1* mutants is mainly caused by impaired pollen anther development.

Anther and pollen development are impaired in homozygous fax1knockout mutants

To further analyse flower development, especially the fertilization phenotype, morphological studies of flower and silique development of all *FAX1* mutant lines were performed.

In wild-type alleles, complementation lines and overexpressing lines, bolting of flowers occurred normally and silique and seed development were also similar to each other. However, the stigma of homozygous *fax1* knockout mutants grew slower showing non pollinized papillae, which caused very short siliques with few seeds inside (Figure 25).

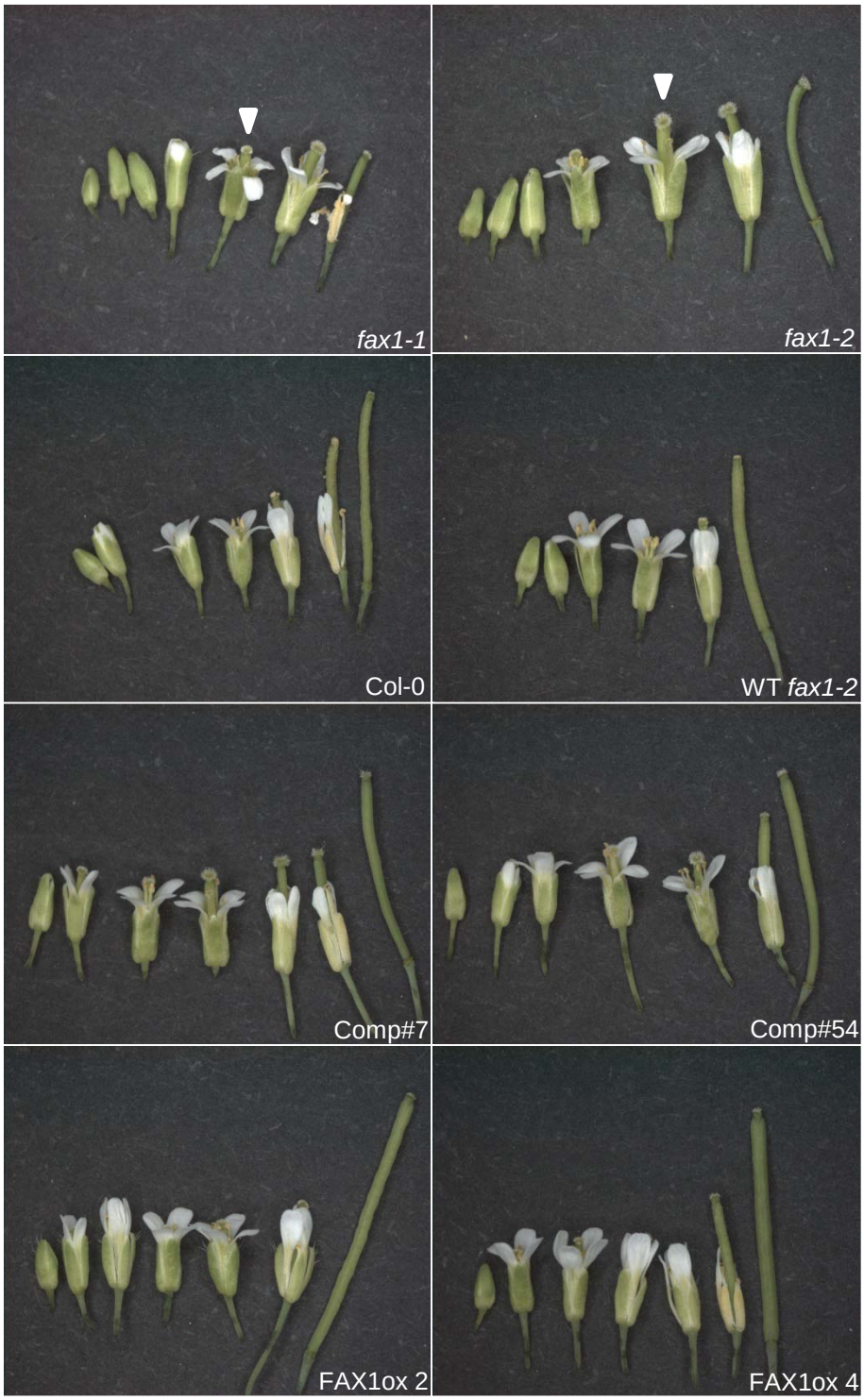

Figure 24: FAX1 function related phenotypes in flower development
6-7 flower buds and youngest siliques from one bouquet of mature *FAX1* mutant lines and wild-type were selected. *fax1-1*, *fax1-2*: homozygous knockout lines; Col-0, WT *fax1-2*: wild-type FAX1 alleles; Comp#7, Comp#54: FAX1 complementation lines; FAX1ox2, FAX1ox4: FAX1 overexpressing lines. Arrow heads indicate the non-pollinated stigma papillae of *fax1-1*, *fax1-2*.

After 55 days of growth, siliques of wild type alleles and complementation lines started to senescent, which firstly appeared at siliques on inflorescence stems (Figure 25). However,

homozygous *fax1* knockout mutants and overexpressing lines plants still only had green siliques, which imply that FAX1 may have function related to senescence.

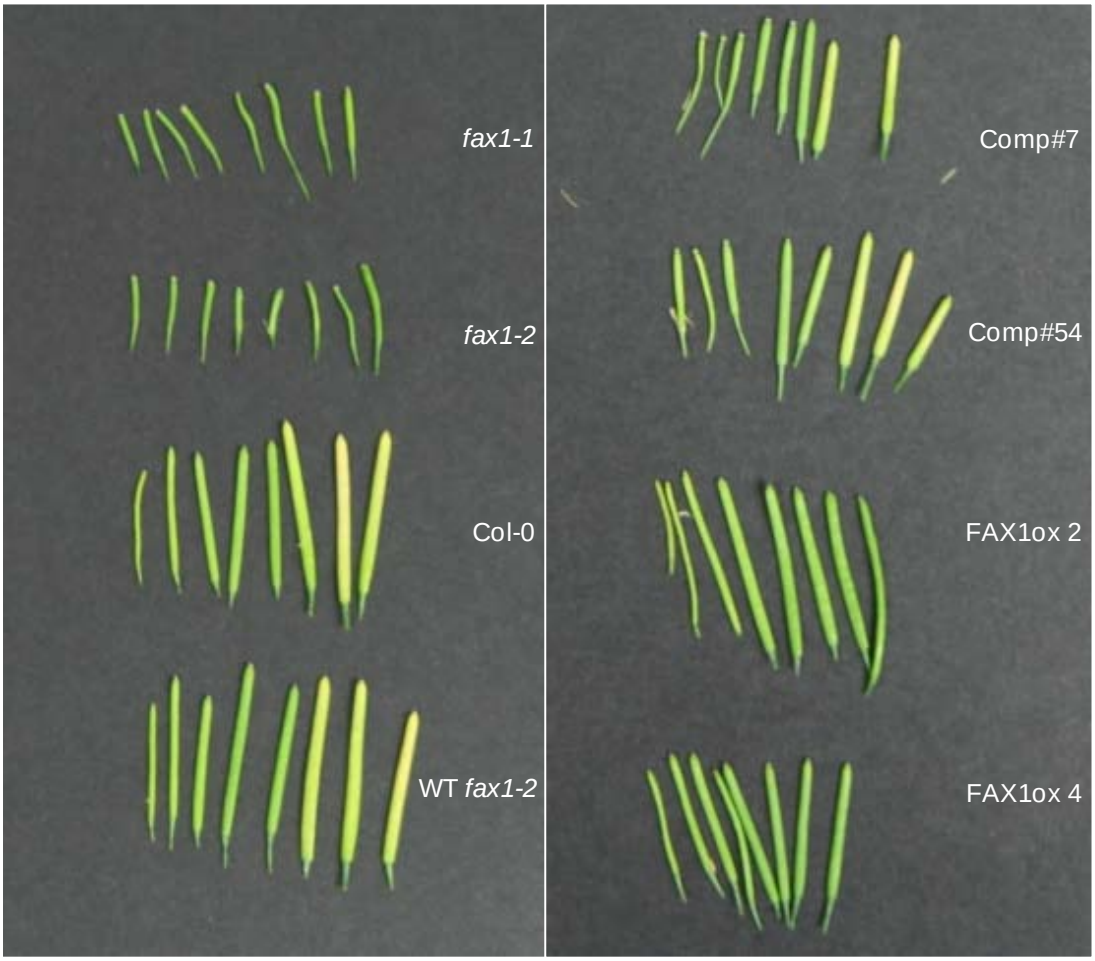

Figure 25: FAX1 function related phenotypes in silique development
8 siliques inflorescence stems of all lines (55 days old) were selected and arranged from left to right depending on the developmental stages (left side is the youngest, right side is the oldest). *fax1-1*, *fax1-2*: homozygous knockout lines; Col-0, WT *fax1-2*: wild-type FAX1 alleles; Comp#7, Comp#54: FAX1 complementation lines; FAX1ox2, FAX1ox4: FAX1 overexpressing lines.

To visualize the strongly impaired male fertility phenotype of homozygous *fax1* knockout mutants, open flowers at development stages 12-14 (Smyth *et al*., 1990) from all *FAX1* mutant lines and wild type were compared (Figure 26).

Figure 26: FAX1 function related phenotype in pollination
Flower buds at flower stage 12-14 (Smyth *et al.*, 1990) were selected. *fax1-1*, *fax1-2*: homozygous knockout lines; Col-0, WT *fax1-2*: wild-type FAX1 alleles; Comp#7, Comp#54: FAX1 complementation lines; FAX1ox2, FAX1ox4: FAX1 overexpressing lines

Both two *FAX1* wild-type alleles and overexpressing lines produced flowers with mature pollen grains being released by anthers and sticking to the papillae of stigmas. However, flowers of the two homozygous *fax1* knockouts had almost no free pollen grains and stigma papilla produced long hairs, which indicates that very few pollination happened. In

comparison to the knockout lines, flowers of complementation lines had more pollen outside of anthers but fewer than wild type, indicating that complementation of pollen release by anthers was not 100%. Furthermore, normal wild type pollen was yellow in colour because of the inclusion of flavonoid and carotenoid deposits (Dobritas *et al*., 2011). However, impaired pollen of homozygous *fax1* knockouts as well as of complementation lines was pale and colourless (Figure 27).

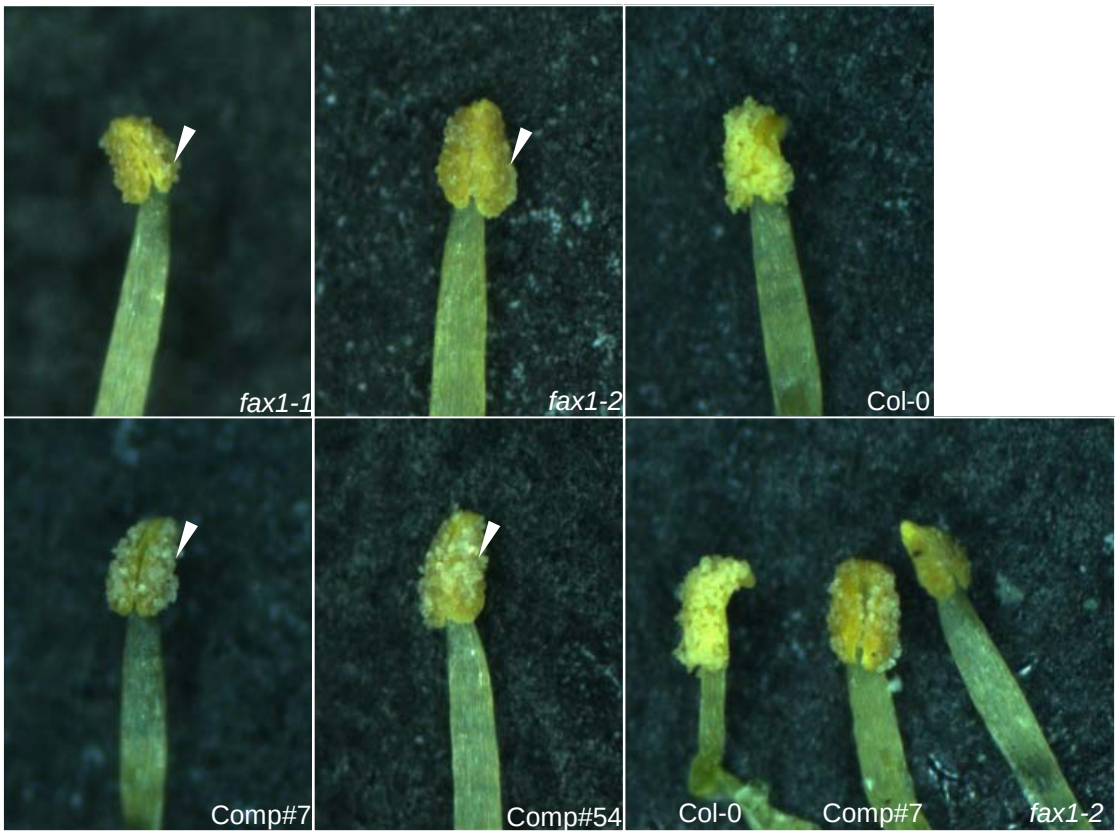

Figure 27: FAX1 function related phenotype of *Arabidopsis* mature pollen on the surface of anthers
Anthers after flower stage 13 were selected. *fax1-1*, *fax1-2*: homozygous knockout lines; Comp#7, Comp#54: FAX1 complementation lines. Arrow heads indicate the colourless and pale pollens of *fax1-1*, *fax1-2*, Comp#7 and Comp#54.

Ultrastructural analysis of FAX1 mutant lines

In the following, stem, anther and pollen morphology were analyzed in the *FAX1* mutants and Col-0 by light microscopy and transmission electron microscopy (TEM; Figure 28-30). Microscopic investigation were performed by Dr. Irene Gügle (Department Biologie I, Plant Biochemistry and Physiology, LMU München). Firstly, stems were pictured 1 cm above the first node of 5-week-old inflorescence stems (Figure 28). Here the amount of hypodermal cell layers appeared reduced (~3) in *fax1-2* but increased in FAX1ox (~5), when compared with Col-0 (~4; Figure 28A-C). Compared with Col-0 wild type, vascular bundles were smaller and xylem and sclerenchyma contained secondary cell walls reduced in homozygous *fax1-2*

mutants (Figure 28A, B, D and E). In the overexpressing line FAX1ox2 however, extended vascular strands (2-3 originating from cambium) and a multi-layered procambium appeared (Figure 28C and F). TEM of xylem cells revealed that secondary cell walls of tracheids were strongly reduced in *fax1-2* knockout stem when comparing with Col-0 wild-type (Figure 28G and H).

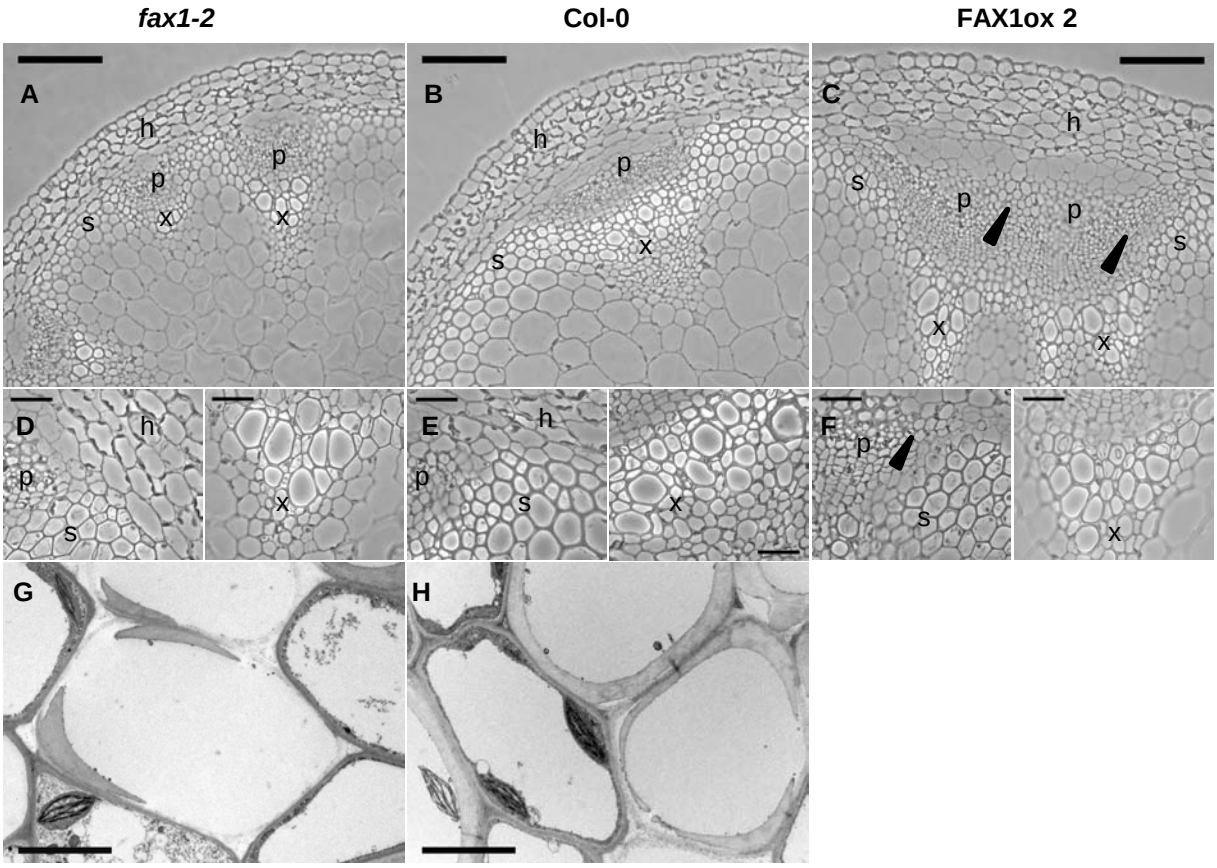

Figure 28 Stem tissue of *FAX1* mutants
Overview and vascular tissue of stem from homozygous *fax1-2* knockout (A, D, G), Col-0 (B, E, H) and FAX1ox2 (C, F). A, B, C: Overview of stem cross sections (light microscopy, bar=100 µm). D, E, F: Closeup of sclerenchyma and phloem cells (left) and xylem (right) (light microscopy, bar= 25 µm). G, H: Transmission electron microscopy (TEM) of cell walls of tracheids in xylem cell layer (bar=5 µm). h: hypodermis, s: sclerenchyma, p: phloem, x: xylem. Extended and multilayered procambium is depicted by arrowheads (C, F).

To further understand the stem phenotype, the section of epidermal cell wall at the stem surface in the *FAX1* mutants and Col-0 was analysed and compared (Figure 29). The ultrastructure revealed that the thickness of the outer epidermal cell wall was reduced in the homozygous *fax1-1* knockout, compared with Col-0 wild-type (Figure 29A-D). Furthermore, the thickness of the cuticular layer was reduced in *fax1-1*, but increased in the overexpressing line FAX1ox2 when compared with Col-0 wild-type (Figure 29G-I).

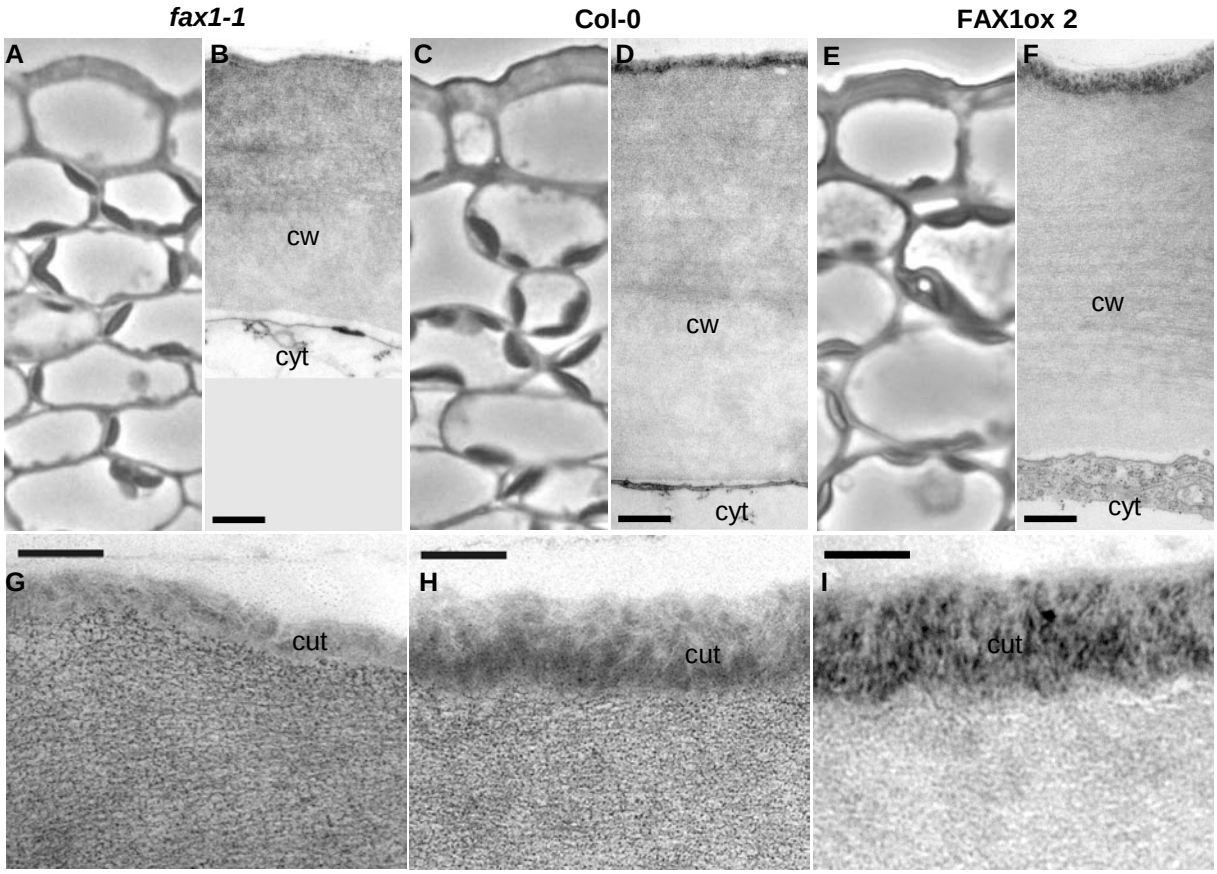

Figure 29 Stem epidermis of *FAX1* mutants
Epidermis and hypodermis of stem from homozygous *fax1-1* knockout (A, B, G), Col-0 (C, D, H) and FAX1ox2 (E, F, I). A, C, E: Overview of epidermal and hypodermal cell layers (light microscopy). B, D, F: Transmission electron microscopy closeups of epidermal cell wall (bar= 500 nm), and G, H, I: Cutin layer (bar= 200 nm). cut: cutin layer, cyt: cytosol, cw: cell wall.

Moreover, investigation of anther and pollen prior to dehiscence of homozygous *fax1-2* knockout, Col-0 wild-type and complementation line Comp#54 by light microscopy and TEM was performed (Figure 30). In line with the observations from stems, the vascular bundle of anthers appeared collapsed and cutin was reduced in *fax1-2* while comparing with Col-0 wild-type (data not shown). In the homozygous *fax1-2* knockout, the ultrastructure of anthers further showed that fragmented parts of tapetum still were present and bound together with pollen grains, while the tapetum had disappeared in Col-0 wild-type and only few parts of tapetum were bound with some pollen grains in the complementation line Comp#54 (Figure 30A-I). Surprisingly, the pollen grains in the homozygous *fax1-2* knockout were only surrounded by the intine layer, which revealed that the exine development was strongly impaired. In Comp#54, however bacula structures of the exine were restored, while both tectum and bacula of exine existed in Col-0 wild-type (Figure 30G-L). In addition accumulation of deposits unknown materials were detected in the complementation line Comp#54 (Figure 30I, L).

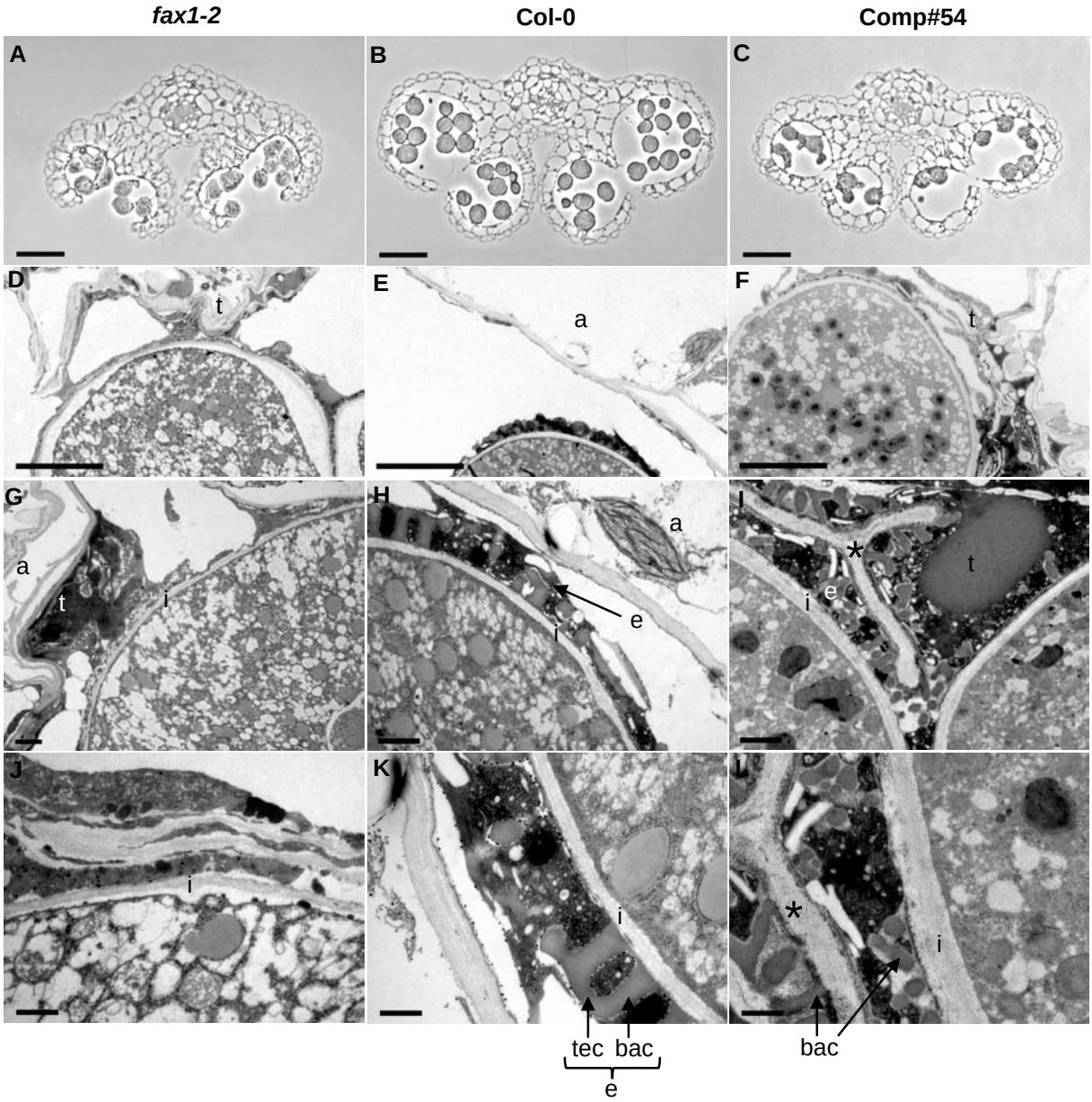

Figure 30 Anther and pollen of homozygous *fax1* knockout, Col-0 and complementation line
Anther and pollen of flowers from homozygous *fax1-2* knockout (A; D, G, J), Col-0 (B, E, H, K) and complementation line Comp#54 (C, F, I, L). A, B, C: Overview of anther cross section prior to dehiscence (light microscopy, bar= 50 µm). D, E, F: Overview of areas between pollen grain and anther cells (TEM, bar= 5 µm). G, H, I: Closeup of areas between pollen and anther cells (TEM, bar= 1 µm). J, K, L: Closeup of exine and intine of pollen grain (TEM, bar= 500 nm). t: tapetum, a: anther cell, e: exine, i: intine, tec: tectum of exine, bac: bacula of exine, * : material deposits in exine/tapetum region.

2.8 Wax content in stems of *FAX1* mutant lines

Considering the ultrastructural result on the stem cuticular layer (see Figure 29), the wax content on inflorescence stems of *FAX1* mutants and the corresponding wild-type alleles was analysed and compared (Figure 31-32). Wax analysis was performed by Prof. Lukas Schreiber (Department of Ecophysiology, IZMB, University of Bonn).

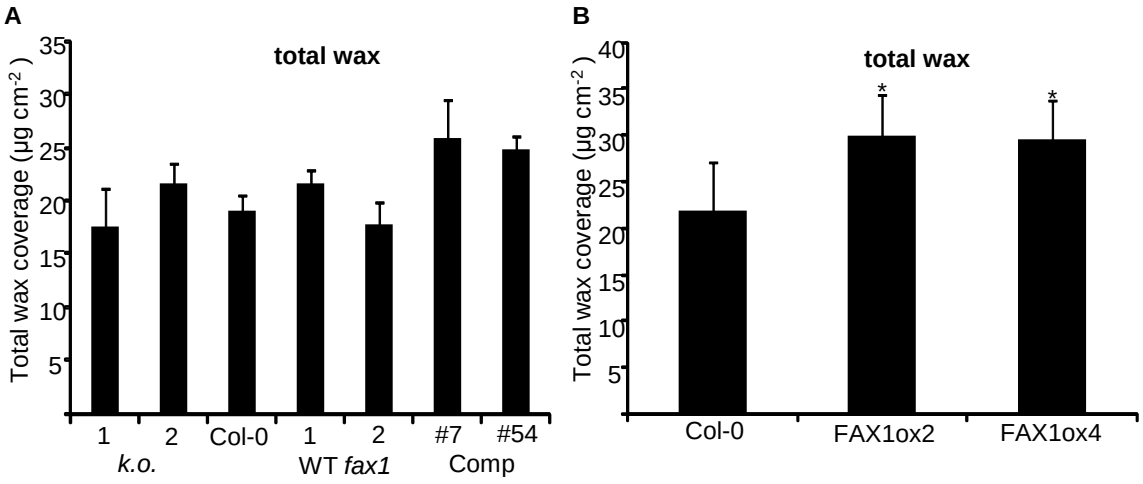

Figure 31: Wax analysis of *FAX1* mutant lines
A) Wax analysis of *FAX1* knockout and complementation lines. Total wax coverage in µg/cm^2 stem of surface (n= 3 ± SD, n=7 for *fax1-2*). For each replicate 3-4 stems (internode 2-4) were pooled. **B)** Wax analysis of FAX1 overexpressing lines and wild type. Total wax coverage in µg/cm^2 stem of surface (n= 6 ± SD). For each replicate 3-4 stems (internode 2-4) were pooled. The asterisks indicate significantly different wax levels (* P< 0.05) in FAX1ox2 and 4 when compared with Col-0 wild type. *k.o.* 1 and 2: homozygous knockout lines, WT *fax1* 1 and 2: wild-type FAX1 alleles, Comp#7, Comp#54: FAX1 complementation lines, FAX1ox2 and 4: FAX1 overexpressing lines.

The homozygous *fax1* knockout mutants had similar amounts of total stem wax coverage as the corresponding wild-type alleles, and interestingly, the complementation lines slightly increased the stem total wax amount when comparing with wild-type (Figure 31A). However, the total wax levels of FAX1 overexpressing lines FAX1ox2 and FAX1ox4 were increased to 136% and 134% of the Col-0 loads, respectively (Figure 31B).

Furthermore, the homozygous *fax1* knockout mutants differed significantly from wild type in the levels of ketones. The *fax1-1* and *fax1-2* ketone levels were reduced to 33% and 47% of the wild-type loads, respectively (Figure 32). But in FAX1 overexpressing lines, there is no difference in composition of wax, regarding aliphatic chain length or functional groups (eg. ketone, acid or aldehyde), when comparing with Col-0 wild type (data not shown).

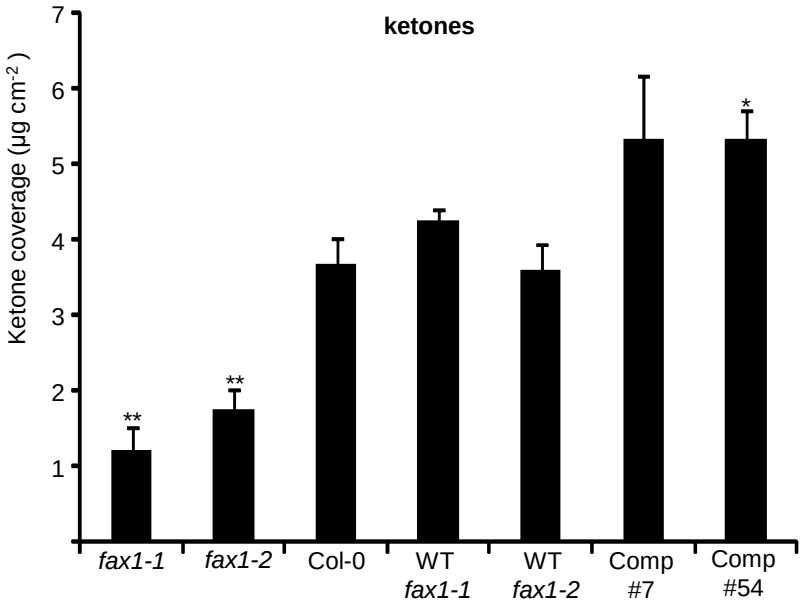

Figure 32: Ketone analysis of *FAX1* mutant lines
Total ketone coverage in µg/cm^2 stem of surface (n= 3 ± SD, n=7 for *fax1-2*). For each replicate 3-4 stems (internode 2-4) were pooled. The asterisks indicate significantly different signal levels (** *P* < 0.005, * *P*< 0.05) in homozygous *fax1* knockout mutants (*fax1-1* or *fax1-2*) and FAX1 complementation lines (Comp#7 or Comp#54) when compared with Col-0.

2.9 Fatty acid contents in *fax1* knockout mutants and wild type

Considering the phenotypes at the exine of pollen and stem outer layer of epidermis cells, the fatty acids composition in the flowers, stems and leaves of homozygous *fax1* knockout mutants and corresponding wild-type alleles were measured by Dr. Patrick Giavalisco (Max Planck Institute of Molecular Plant Physiology, Potsdam-Golm, Germany; Figure. 33-35).

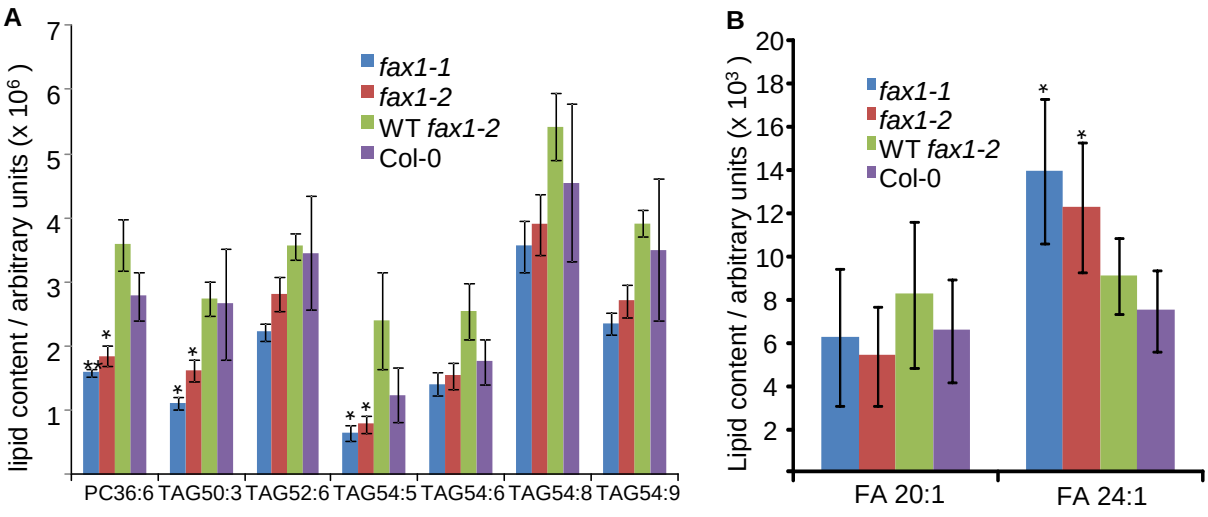

Figure 33: Polar lipid composition in flowers of wild type and the *fax1* knockout mutants
Flowers were harvested from 50 days old plants. Lipid content (n=6±SD) was normalised to fresh weight (FW) and the internal standard PC34:0. The asterisks indicate significantly different signal levels (** *P* < 0.005, * *P*< 0.05) in homozygous *fax1* knockout mutants (*fax1-1* or *fax1-2*) when compared with Col-0. **A)** Main differences of polar lipids except free fatty acids are shown. PC: phosphatidylcholine, TAG: triacylglycerol. **B)** Free fatty acids (FA) different in flowers of the homozygous *fax1* knockout mutants and corresponding wild-type alleles.

The measurement and analysis of flower lipid composition revealed that the amount of PC36:6, TAG50:3 and TAG54:5 were significantly reduced in the homozygous *fax1* knockout mutants comparing with Col-0 wild-type. Only amount of FA24:1 was increased in the *fax1* knockout mutants (Figure 33).

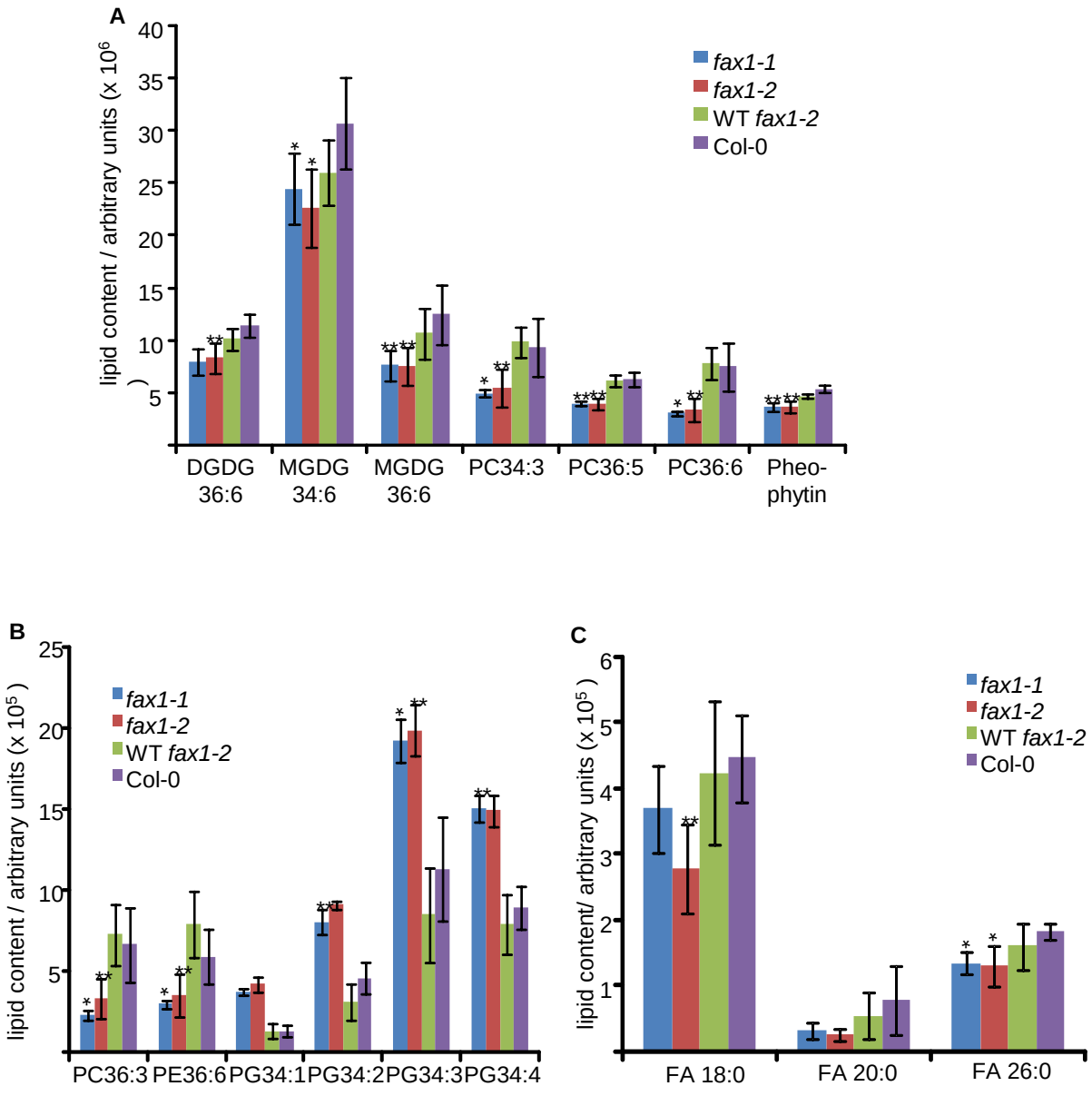

Figure 34: Polar lipid composition in cauline leaves of wild type and *fax1* knockout mutants
Cauline leaves were harvested from 50 days old plants. Lipid content (n=6±SD) was normalised to fresh weight (FW) and the internal standard PC34:0. The asterisks indicate significantly different signal levels (** $P < 0.005$, * $P < 0.05$) in homozygous *fax1* knockout mutants (*fax1-1* or *fax1-2*) when compared with Col-0. **A)** Main differences of polar lipids with high expression level are shown. PC: phosphatidylcholine, DGDG: digalactosyldiacylglycerol, MGDG: monogalactosyldiacylglycerol. **B)** Differences of polar lipids with lower expression level than in **A** are shown. PE: phosphatidylethanolamine, PG: Phosphatidylglycerol. **C)** Free fatty acids (FA) different in leaves of the homozygous *fax1* knockout mutants and corresponding wild-type alleles.

The measurement and analysis of leaf lipid composition of *fax1* knockout mutants and wild type showed that DGDG36:6, MGDG34:6, MGDG36:6, PC 34:3, PC 36:5, PC36:6, Pheophytin, PC36:3, PE36:6 were reduced in the homozygous *fax1* knockout mutants, but the PG34:2, PG34:3 and PG 34:4 were increased in the *fax1* knockout mutants. Furthermore, free fatty acids like FA18:0, and FA26:0 were also slightly reduced in the *fax1* knockout mutants (Figure 34).

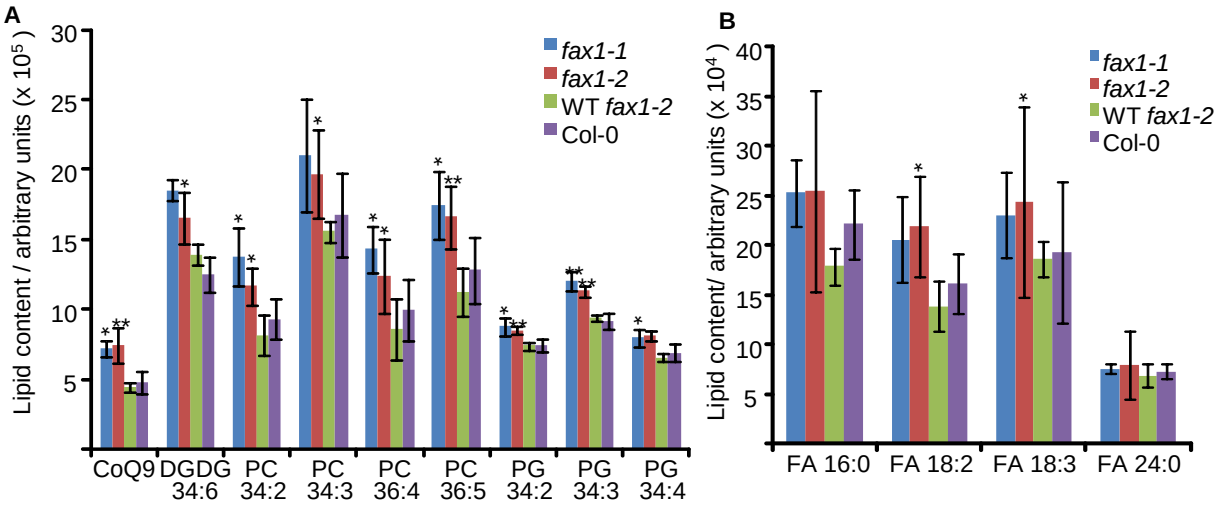

Figure 35: Polar lipid composition in stems of wild type and the *fax1* knockout mutants
Stem were harvested from 50 days old plants. Lipid content (n=6±SD) was normalised to fresh weight (FW) and the internal standard PC34:0. The asterisks indicate significantly different signal levels (** *P* < 0.005, * *P*< 0.05) in homozygous *fax1* knockout mutants (*fax1-1* or *fax1-2*) when compared with Col-0. **A)** Main differences of polar lipids except free fatty acids are shown. PC: phosphatidylcholine. **B)** Free fatty acids (FA) different in stems of the homozygous *fax1* knockout mutants and corresponding wild-type alleles.

Polar lipid composition measurement and analysis of stems showed other lipids to be different between the homozygous *fax1* knockout mutants and corresponding wild-type alleles than in flowers and leaves (Figure 35). Here the amounts of lipids like MGDG34:6 (data not shown), CoQ9, DGDG34:6, PC34:2, PC34:3, PC36:4, PC36:5, PG34:2, PG34:3, PG34:4 and free fatty acids FA16:0, FA18:2, FA18:3 were increased in mutant lines. However, none of the polar lipids were reduced in *fax1* knockout mutants.

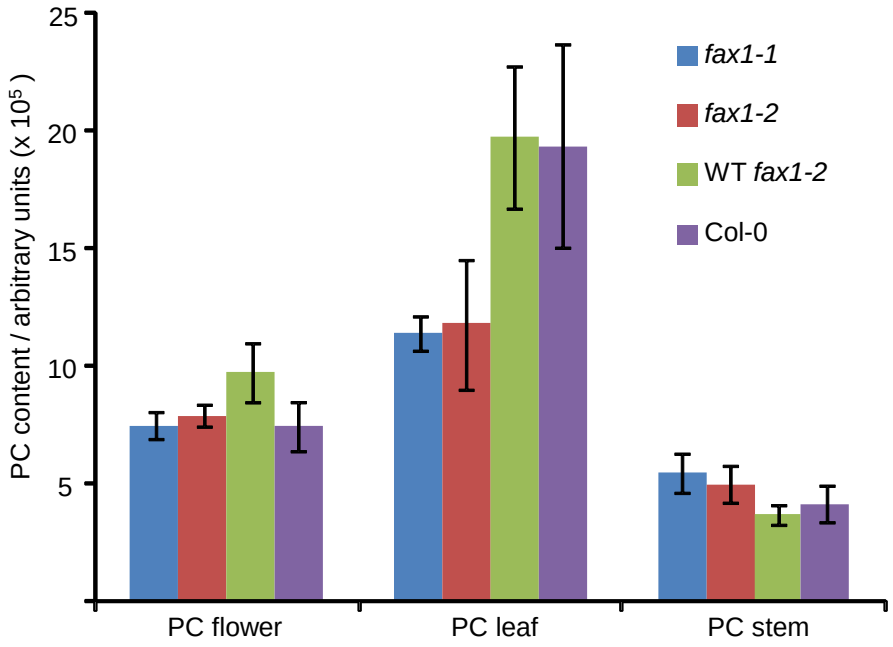

Figure 36: PC amount in flowers, leaves and stems of *fax1* knockout mutants and wild type
Flowers, leaves and stems were harvested from 50 days old plants. PC content (n=6±SD) was normalised to fresh weight (FW) and the internal standard PC34:0.

As a summary, total phosphatidylcholine (PC) amount in leaves and PC36:3 in flowers of the homozygous *fax1* knockout mutants were reduced significantly when compared with the corresponding wild-type, however in stems, total PC amount was increased (Figure 36). It is known that PC is a key chemical involved in fatty acid transport from plastids to ER (see Chapter I.2). Therefore, PC amount differences between the homozygous *fax1* knockout mutants and wild type provides evidence that FAX1 might be an inner envelope protein involved in fatty acid export from chloroplasts.

2.10 Transcriptomic analysis of *fax1* knockout mutant and wild type

Because of the impaired flower and stem development in homozygous *fax1* knockout mutants, gene expression in *fax1-2* versus the wild type was compared by microarray analysis (Affymetrix ATH1 GeneChip) in the same tissue pools that were used for lipid analysis and stem wax coverage analysis. The hybridization of the microarrays and statictical analysis of data were performed by Karl Mayer and by Dr. Katrin Philippar, respectively (Department Biologie I, Plant Biochemistry and Physiology, LMU München). I found that 1,058 genes could be identified as down-regulated and 515 genes were up-regulated in flowers of *fax1-2*, while 410 genes could be classified as down-regulated and 1136 genes were up-regulated in stems (Table 11). To be mentioned, the values for *FAX1* in homozygous *fax1-2* and wild-type

stems are 178.6 and 774.7 (p-value 0.073), respectively. The values for *FAX1* in homozygous *fax1-2* and wild-type flowers are 151.6 and 614.2 (p-value 0.022), respectively.

Table 11: Differential gene expression in *fax1-2* flowers and stems
Functional categories of differentially expressed genes in *fax1-2* versus wild-type flowers and stem are shown (Fold change >1.5, signal differeence >30). Classification of genes was performed by MAPMAN (Thimm *et al*., 2004).

	flowers		stems	
	Down, 1058 genes	Up, 515 genes	Down, 410 genes	Up, 1136 genes
cell wall modification	63	24	17	85
lipid metabolism	30	7	3	32
amino acid metabolism	7	9	3	18
secondary metabolism	22	32	15	32
hormone metabolism	34	17	10	33
Biotic stress	42	26	21	34
RNA	70	70	38	121
protein	108	40	42	135
signalling	85	18	37	70
development	46	16	24	28
transport	95	27	37	37
not assigned	265	133	91	347
miscellaneous	120	39	47	83
others	71	57	25	81

Interestingly, *fax1* knockout flowers mainly down-regulated gene expression (1058 of 1573) while loss of FAX1 in stems resulted in mostly increased transcript content (1136 of 1546). Further, the overlap of differentially expressed genes in flowers and stems was extraordinarily low (only 157), indicating different physiological reactions of flowers and stem tissues. Considering that FAX1 might function in fatty acid export across the chloroplast inner envelope membrane, we focused on e xpression of genes related to lipid biosynthesis or secondary cell wall development, which represented a hotspot of regulation with top fold changes. To be mentioned, none of the top regulated genes overlapped in flowers and stems. In stems, among 40 genes up-regulated with a fold change >10 (*fax1-2* versus wild-type) were 23 genes with a function related to lipid biosynthesis or secondary cell wall (Table 12). Interestingly, AtMAH1/AtCYP96A15 (At1g57750), which is highly up-regulated (FCH 12.7), is the midchain alkane hydroxylase responsible for formation of secondary alcohols and ketones in stem cuticular wax (Greer et al., 2007), indicating a correlation with the changed ketone content found in *fax1* knockouts (see Figure 32). Furthermore, both AtIRX15 (At3g50220) and AtIRX15L (At5g67210), which encode DUF579-containing proteins that are essential for normal xylan deposition in the secondary cell wall (Brown *et al*., 2011), are up-regulated in homozygous *fax1-2* versus wild-type *Arabidopsis* stem.

Of the 57 genes down regulated with a fold change >3 were 14 genes with a function related to lipid biosynthesis or secondary cell wall in *fax1-2* versus wild-type *Arabidopsis* stems.

Table 12: Top fold change genes in stems.
Highly regulated genes related to lipid biosynthesis or secondary cell wall biosynthesis in homozygous *fax1-2* versus wild-type *Arabidopsis* stems. u.k.: unknown.

Name	AGI Code	Description(ARAMEMNON)	Signal, *fax1-2*	Signal, wild type	Fold Change (FCH)
UP					
lipid biosynthesis					
u.k.	At1g74670	putative GASA/GAST/Snakin-type protein	310.63	18.75	16.57
AtMAH1	At1g57750	mid-chain alkane hydroxylase	2567.66	202.29	12.69
secondary cell wall					
AtXCP1	At4g35350	cysteine protease	2038.68	74.82	27.25
AtLAC2	At2g29130	putative laccase	2898.82	161.55	17.94
AtPGSIP1	At3g18660	putative xylan glucuronosyltransferase	3880.01	243.38	15.94
AtLAC10	At5g01190	putative laccase	559.23	37.16	15.05
AtXCP2	At1g20850	cysteine protease	5855.62	390.10	15.01
AtTED6	At1g43790	protein involved in secondary cell wall biogenesis	3852.57	269.13	14.31
AtLAC17	At5g60020	putative laccase	3469.41	245.58	14.13
AtMYB103	At1g63910	putative Myb-type transcription factor	1785.59	129.14	13.83
AtANAC073	At4g28500	NAC-type transcription factor	2988.57	233.74	12.79
u.k.	At5g01360	protein of unknown function	2045.68	170.28	12.01
AtXTH4	At2g06850	xyloglucan endotransglucosylase-hydrolase	4943.93	412.11	12.00
AtPer64	At5g42180	putative class-III peroxidase	8220.49	694.18	11.84
u.k.	At1g09610	protein of unknown function	1358.05	115.56	11.75
u.k.	At4g09990	protein of unknown function	1128.17	98.66	11.43
AtXTH6	At5g65730	xyloglucan endotransglucosylase-hydrolase	1889.98	169.11	11.18
AtLAC12	At5g05390	putative laccase	2584.48	231.61	11.16
AtIRX15L	At5g67210	protein probably involved in xylan biosynthesis	3214.46	288.30	11.15
AtCOBL4	At5g15630	putative glycosylphosphatidylinositol-anchored protein	4853.46	441.60	10.99
AtIRX15	At3g50220	protein probably involved in xylan biosynthesis	2541.10	237.34	10.71
AtXTH15	At4g14130	xyloglucan endotransglucosylase-hydrolase	387.87	37.11	10.45
AtXyn3	At4g08160	xylanase	931.23	90.12	10.33
DOWN					
secondary cell wall					
AtABCB16	At3g28360	putative subfamily B ABC-type transporter	39.31	328.67	8.36
AtVGD1	At2g47040	pectin methylesterase	66.25	458.57	6.92
u.k.	At1g19510	putative MYB_related-type transcription factor	17.43	80.19	4.60
u.k.	At5g07410	putative pectinesterase	15.88	68.17	4.29
u.k.	At1g10770	putative pectin methylesterase inhibitor	56.05	234.11	4.18
u.k.	At5g07430	putative pectinesterase	26.95	107.84	4.00
AtUGT73B5	At2g15480	UDP-dependent glycosyl transferase	36.20	138.66	3.83
u.k.	At4g02250	putative invertase/pectin methylesterase inhibitor	22.59	82.06	3.63
AtNAC2	At3g15510	NAC-type transcription factor	276.34	968.72	3.51
AtCslG1	At4g23990	putative cellulose synthase-like glycosyltransferase	103.34	321.57	3.11

In flowers, among 19 genes up regulated with a fold changes >3 and 33 genes down regulated with a fold change >5 (top fold changes of *fax1-2* versus wild-type) were 19 (7 up, 12 down) genes with a function related to lipid biosynthesis or secondary cell wall (Table 12).

To be mentioned, in the current model of the transcriptional network of secondary cell wall biosynthesis in *Arabidopsis* (Zhao and Dixon, 2010), a key myb-type transcription factor,

MYB103 and NAC-type transcription factors were also highly regulated in homozygous *fax1* mutant versus Col-0 wild-type (Table 12 and 13).

Table 13: Top fold change genes in flowers.
Highly regulated genes related to lipid biosynthesis or secondary cell wall biosynthesis in homozygous *fax1-2* versus wild-type *Arabidopsis* flowers. u.k.: unknown.

Gene	AGI Code	Description(ARAMEMNON)	Signal, fax1-2	Signal, wild type	Fold Change (FCH)
UP					
lipid biosynthesis					
AtAOx1a	At3g22370	alternative oxidase	2319.12	576.37	4.02
AtAOC1	At3g25760	allene oxide cyclase	261.04	78.81	3.31
secondary cell wall					
AtPer49	At4g36430	putative class-III peroxidase	625.76	82.84	7.55
AtPer58	At5g19880	putative class-III peroxidase	695.83	184.47	3.77
AtPer14	At2g18140	putative class-III peroxidase	190.58	55.92	3.41
AtPer39	At4g11290	putative class-III peroxidase	1163.18	366.35	3.18
AtNTL1	At1g32870	putatively NAC-type transcription factor	551.81	174.17	3.17
DOWN					
lipid biosynthesis					
u.k.	At5g47350	putative palmitoyl protein thioesterase	108.18	1671.07	15.45
AtANR	At1g61720	dihydroflavonol 4-reductase	70.44	1023.51	14.53
AtDFR	At5g42800	dihydroflavonol 4-reductase	76.63	494.19	6.45
PME49	At5g07420	putative pectinesterase	327.30	1778.40	5.43
AtPTEN1	At5g39400	phosphatidyl-inositol triphosphate phosphatase	203.49	1063.88	5.23
PME28	At5g27870	putative pectinesterase	98.03	511.79	5.22
secondary cell wall					
u.k.	At4g15750	putative invertase/pectin methylesterase inhibitor	94.44	3208.18	33.97
AtEXP23	At5g39280	putative expansin	73.00	561.75	7.70
AtGDPD6	At5g08030	putative glycerophosphoryl diester phosphodiesterase	27.28	195.03	7.15
AtXYLP4	At5g09370	putative xylogen-type arabinogalactan protein	70.32	422.77	6.01

V. Discussion

1 OEP7

Whereas the OEP7.1 isoform has been described in the literature (see Chapter I.1.1), OEP7.2 from *Arabidopsis* is firstly characterized in this work. In 1993, tail-anchored (TA) proteins were classified as integral membrane proteins with a cytosolic N-terminal functional domain, a single transmembrane domain (TMD) and a short C-terminal hydrophilic tail (Kutay *et al*., 1993). In the last few years, a considerable amount of TA proteins, especially the mechanisms of the targeting into specific membranes, was investigated in yeast and mammals. It is demonstrated that the targeting signal of almost all TA proteins is located within the C-terminal TMDs and flanking sequences (Borgese et al., 2007; Pedrazzini, 2009). The topology analysis result of At-OEP7.2 (see Figure 4) revealed that OEP7.2 is a TA protein in the outer envelope membrane of chloroplasts. With the *in vivo* targeting of At-OEP7.2 in protoplasts, I could show that the N-terminus of OEP7.2, not the C-terminus, is critical for targeting to the outer envelope. These findings are novel and complement those described for OEP7.1, where in contrast the C-terminal part of the protein is necessary for targeting (Lee *et al*., 2001). Further *in vivo* targeting of At-OEP7 deletion constructs fused with GFP in protoplasts revealed that the N-terminal amino acid residues 11 to 22 (neighboring TMD) of At-OEP7.2 are necessary and residues 1 to 48 (N-terminus, TMD and C-terminal flanking sequences) are sufficient for targeting to the chloroplast envelope membrane. Therefore, my findings on OEP7.2 targeting provide important evidence to understand the targeting of TA proteins into chloroplast membranes in plants. Furthermore, it could be demonstrated that the double knockout mutant of *oep7.1/oep7.2* in *Arabidopsis* showed no phenotype under various conditions. Considering that there are at least four isoforms of OEP7 in *Arabidopsis* and that the polypeptide of OEP7 is very short, which means that even more OEP7 isoforms might exist, it is likely that triple or quarter mutants could be necessary to investigate a plant phenotype of OEP7 loss of function. And as mentioned about SsOEP8, a homolog of OEP7 in *Suaeda salsa*, which was described to confer oxidative stress tolerance and to induce chloroplast aggregation in transgenic *Arabidopsis* plants (Wang *et al*., 2012), it could be also interesting to analyse the knockout mutant of OEP7.1 and OEP7.2 under oxidative stress conditions.

2 FAX1

In the second part of this work, I identified a gene encoding a novel membrane protein in the chloroplast inner envelope most likely involved in fatty acid or fatty acid derivative export.

2.1 Plastid fatty acid export and the pollen exine formation in *Arabidopsis*

The *Arabidopsis* mature pollen grain surface consists of three layers (from inside to outside): i) an inner intine, made primarily of cellulose; ii) an outer exine wall, multilayered and composed of the chemically resistant polymer sporopollenin and interrupted by openings called apertures; iii) a pollen coat, composed of lipids, proteins, pigment, and aromatic compounds, that fills the sculptured cavities of the pollen exine (Edlund *et al*., 2004). The sporopollenin in exine layers is a complex polymer of fatty acids and phenolic compounds (Guilford *et al*., 1988; Ahlers *et al*., 1999). The exine itself splits into outer sexine and the inner nexine layers. The sexine comprises the tectum and the bacula. The nexine comprises the nexine I and nexine II (Ariizumi and Toriyama, 2011; Figure 37). The ultrastructural investigation of the pollen in *FAX1* mutants showed that the pollen of *fax1* knockout mutants lacks the sexine and parts of the nexine (see Figure 30). In the recent years, several genes involved in exine formation were identified in *Arabidopsis*, such as male sterility 2 (MS2; Aarts *et al*., 1997; Chen *et al*., 2011), CYP703A2 (Morant *et al*., 2007), CYP704B1 (Dobritsa et al., 2009), Acyl-CoA sythetase 5 (ACOS5; Souza et al., 2009) and an ABCG transporter (ABCG26; Choi *et al*., 2011; Dou *et al*., 2011; Quilichini *et al*., 2010), which also showed a pollen phenotype similar to *fax1* knockout mutants. With the known function of proteins mentioned above, it was summarized that the pollen exine development precedure depends on fatty acids, which are synthesized in the plastids of tapetum cells and esterified with CoA by Acyl-CoA synthetase (e.g. ACOS5) and are sent to the ER, where the fatty acyl-CoAs are hydroxylated by CYP704B1 and CYP703A2, respectively. After the modification and other unknown steps, the modified fatty acids are exported from the tapetum cell for exine formation by the ABCG26 transporter and other unknown transporters (Choi *et al*., 2011). Recently, both MS2 and the probable ortholog of MS2, defective pollen wall (DPW) of rice (*Oryza sativa*) were identified as the plastid localized fatty acyl carrier protein reductase, which participates in a conserved step in primary fatty alcohol synthesis for anther cuticle and pollen sporopollenin biosynthesis (Chen *et al*., 2011; Shi *et al*., 2011).

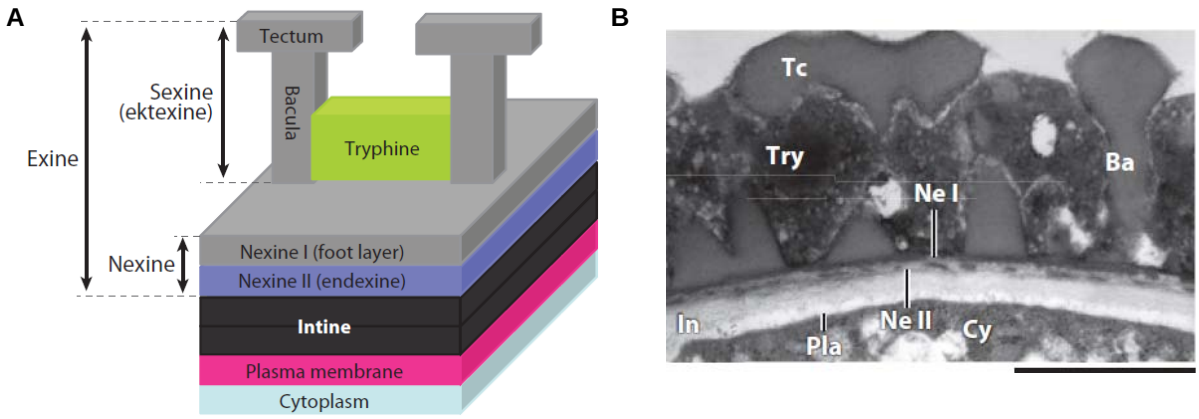

Figure 37 A typical angiosperm pollen grain structure (adapted from Ariizumi and Toriyama, 2011)
A) model of a pollen grain **B)** Transmission electron microscopy of a cross-section of exine architecture in Arabidopsis. Bar= 1 µm. Tc: tectum, Ba: bacula, Try: tryphine, In: intine, Cy: cytoplasm, Pla: plasma membrane, Ne I/II: nexine I/II.

According to the high similarity of phenotypes it is therefore proposed that FAX1 represents the missing transporter for export of fatty acid derivatives from plastids in tapetum cells (Figure 38). Transcriptomic analysis of flowers revealed that all the above mentioned genes for exine formation were not highly regulated in *fax1* knockout mutants while comparing with wild type. However, an effective genetic screen in *Arabidopsis* on sporopollen synthesis and exine assembly recently showed that many other genes are involved in the pollen morphology (Dobritsa *et al.*, 2011). Surprisingly, the genes of PTEN1 (a phosphatidyl-inositol triphosphate phosphatase), DFR (dihydroflavonol 4-reductase), ANR (dihydroflavonol 4-reductase) and the putative pectinesterases PME28 and PME49 were highly down regulated in *fax1* knockout mutant versus Col-0 (see Table 13). The *Arabidopsis* PTEN, a protein and lipid dual phosphatase, homologous to animal PTEN (a tumor suppressor), was identified to regulate autophagy in pollen tubes by disrupting the dynamics of phosphatidylinositol 3-phosphate (PI3P; Zhang *et al.*, 2011; Gupta *et al.*, 2002). Both DFR and ANR are key reductases during anthocyanins and flavonoid biosynthesis (Winkel-Shirley, 2001; Marinova et al., 2007). Considering the colorless pollen phenotype in *fax1* knockout mutant plants (see Figure 27), it is suggested that the absence of exine layers prevents inclusion of flavonoids and carotenoids thus leading to the transparent pollen and secondly affecting expression of genes (e.g. down-regulating) involved in flavonoid biosynthesis. It was reviewed that pectin methylesterases (PME) belong to large multigene families and are involved in vegetative and reproductive processes, including pollen formation (Pelloux *et al.*, 2007). Although the function of PME 28 and PME49 are unknown, another PME, named quartet, was indicated recently to affect the pollen tetrad separation during floral development, which may provide a

hint to explain the sticky pollen phenotype in *fax1* knockout mutant plants (Francis *et al*., 2006).

Furthermore, the complementation of *fax1* by 35S:: FAX1 (Comp #7, Comp#54) is able to rescue fertility and most of exine structures, because of 35S promoter is not active in pollen but in tapetum cells (Wilkinson *et al*., 1997). As mentioned above, fatty acids, which are necessary for pollen exine formation and fertility, are delivered by the anther. However, some exine material, which is necessary for interrelation of flavonoid and carotenoids, should be delivered by the pollen, such as pollen plastid fatty acids. Therefore, the complementation lines of FAX1 still show transparent pollen grains (see Figure 27)

Other interesting genes, which were highly regulated in *fax1* knockout mutant versus Col-0 and may have a function related to cutin, wax biosynthesis or structure of anther cells will be discussed together with the stem phenotype (see Chapter V. 2.2).

Furthermore, the genes of some other proteins which may also have function during fatty acid transport and lipid biosynthesis, were also regulated in flowers of *fax1* knockout mutants versus Col-0, such as PAP2 (purple acid phosphatase 2; down FCH= 4.28), MGD2 (Galactosyltransferase; down FCH=3.36), LACS5 (Long-chain Acyl-CoA synthetase; down FCH=2.70), ACS8 (Acyl-CoA synthetase; up FCH= 2.17), FAR7 (Fatty acid reductase 7; up FCH= 1.99).

Interestingly, the ultrastructure of anthers in *fax1* knockout mutants clearly showed that fragmented parts of tapetum still were present and bound together with pollen grains, while the tapetum was totally degraded in Col-0 (see Figure 30). The timing of the tapetal programmed cell death is critical for pollen development (Ku *et al*., 2003; Kawanabe *et al*., 2006; Phan *et al*., 2011). Because, FAX1 initially was identified as premature senescence upregulated (see Chapter IV. 2.1) and the siliques of *FAX1* knockout and overexpressing lines showed late a senescence phenotype compared to wild-type. Proper function of FAX1 seems to be necessary for controlled cell death of tapetum in anther and also silique development.

2.2 Plastid fatty acid export affects wax and cutin biosynthesis in stems

The hydrophobic plant cuticle, which coats the epidermis to protect tissues from environmental stresses, consists of two types of highly lipophilic materials: i) cutin, which contains w- and mid-chain hydroxy and epoxy C16 and C18 fatty acids and glycerol (Heredia, 2003; Nawrath, 2006); ii) cuticular wax, which contains straight chain C20 to C60 aliphatics and possible secondary metabolites. Furthermore, cuticular wax includes the intracuticular wax, which interspersed within the cutin layer, and the epicuticular wax on the outer surface

of cutin and intracuticular wax (Samuels *et al*., 2008). In *Arabidopsis*, it is estimated that more than half of the fatty acids made by epidermal cells of the rapidly expanding stem are transported into the cuticular cutin and wax (Suh *et al*., 2005). During the wax biosynthesis, the very-long-chain fatty acids, which are elongated in the endoplasmic reticulum (ER) are modified into wax compositions: alcohols, esters, aldehydes, alkanes and ketones (Samuels *et al*., 2008). In line with a proposed function of FAX1 in plastid fatty acid or fatty acid derivative export *fax1* knockout mutants showed decreased cutin and *FAX1* overexpressing lines had increased cutin and wax contents (see Figure 31). Until now, it is known that a cytochrome P450 enzyme (CYP96A15) is a mid-chain alkane hydroxylase (MAH1) responsible for the formation of secondary alcohols and ketones in the stem cuticular wax of Arabidopsis. The stem wax amount of T-DNA insertion mutants of MAH1 was measured to be devoid or reduced of secondary alcohols and ketones (Greer *et al*., 2007). In this work, the ketones amount of *fax1* knockout mutants were reduced while the MAH1 RNA level was highly up regulated in stem transcriptomic analysis comparing with Col-0 indicating a gene regulatory in response to the lack of ketone components. Considering no da ta on t he overexpressing of MAH1, it can be hypothesized that MAH1 not only catalyzes the hydroxylation reaction from alkanes to secondary alcohols and ketones but also is crucial for balancing ketones biosynthesis in stem cuticular wax of *Arabidopsis*.

In the recent years, many proteins involved in stem wax and cutin biosynthesis were identified, such as ABCG11 (ABC transporter for wax and cutin precursors transport; Luo *et al*., 2007; Panikashvili *et al*., 2007), ABCG12 (ABC transporter working together with ABCG11 for wax precursors; Pighin *et al*., 2004; Panikashvili *et al*., 2007; McFarlane *et al*., 2010), as well as enzymes like CER1 (Aarts *et al*., 1995), CER3 (Chen *et al*., 2003; Rowland *et al*., 2007), CER8/LACS1 (Lü *et al*., 2009; Jessen *et al*., 2011) and LACS2 (Lü *et al*., 2009). Interestingly, genes of CER1 (up FCH=5.77), CER8 (up FCH=3.87) and LACS2 (up FCH=2.48) are up regulated in stems of *fax1* knockout mutant versus Col-0. The analysis of stem wax of *cer1* mutants revealed that CER1 has the function as an aldehyde decarbonylase during the conversion of aldehydes to alkanes (Aarts et al., 1995). Both CER8/LACS1 and LACS2 are members of the nine Arabidopsis long-chain acyl-CoA synthetases. Analysis of the double mutant of *lacs1-1lacs2-3* revealed that LACS1 and LACS2 have overlapping functions in both wax and cutin synthesis, furthermore, LACS1 preferentially modifies both very long-chain fatty acids (VLCFA; > C24) for wax and long-chain (C16) fatty acids for cutin synthesis (Lü et al., 2009). Considering the ultrastructural phenotype of stem cuticle of *FAX1* mutants and the transcriptomic analysis result mentioned above, it is suggested that

FAX1, by exporting fatty acids across the inner envelope protein of plastids, affects wax and cutin biosynthesis.

Interestingly, some genes which also relate to lipid biosynthesis are also up regulated in stem of *fax1* knockout mutant versus Col-0: At1g06120 (fatty acid desaturase; up F CH=8.22), At1g65890 (putative thioesterase; up FCH=4.52) and LACS3 (At1g64400; up F CH=2.23). The detailed connection of fatty acid biosynthesis, transport and the LACS family will be discussed in V. 2.4.

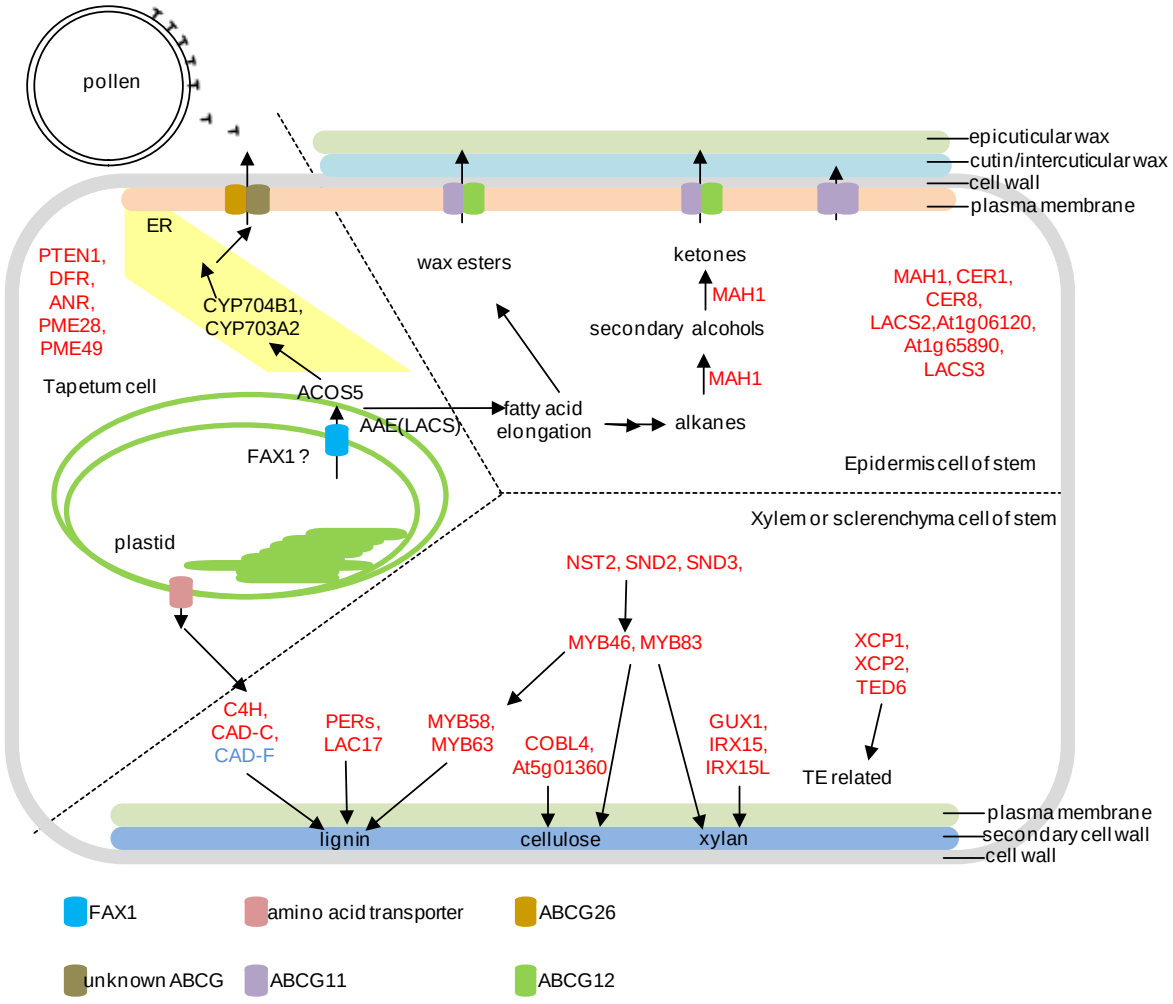

Figure 38: The function of FAX1 in plastid fatty acid or fatty acid derivative export affects pollen exine formation, wax and cutin synthesis in epidermis cells as well as secondary cell wall formation
Up-regulated genes related to lipid biosynthesis or secondary cell wall in *fax1* knockout mutant versus wild-type are colored red, down-regulated blue. All abbreviations and names are described in text.

2.3 Plastid fatty acid export and plant cell wall formation in stems

The plant cell wall functions as regulator of cell expansion, cell adhesion, a barrier to pests and pathogens and so on, and consists of three layers: i) the primary cell wall, which is created in the cell plate during cell division and rapidly increases in surface area during cell expansion; ii) the middle lamella, which forms the interface between the primary walls of

neighboring cells, and the cell corners, which are often filled with pectin-rich polysaccharides; iii) the secondary cell wall, which builds up complex structures exclusively suiting to different cells' functions (Buchanan *et al*., 2002; Brown *et al*., 2005; Caffall and Mohnen, 2009; Figure 39). In *Arabidopsis*, the xylem and interfascicular cells of the inflorescence stem form a thick secondary cell wall, which predominantly consists of cellulose, lignin and xylan (Turner and Somerville, 1997; Nieminen et al., 2004). As mentioned in Chapter IV 2.7, in the *fax1* knockout mutants, the vascular bundles were smaller and the secondary cell walls of xylem tracheids reduced while comparing with Col-0 wild type (see Figure 28). Therefore, I fcouse on the highly regulated genes which may function in xylem vascular bundles or the secondary cell walls of xylem in *fax1* knockouts.

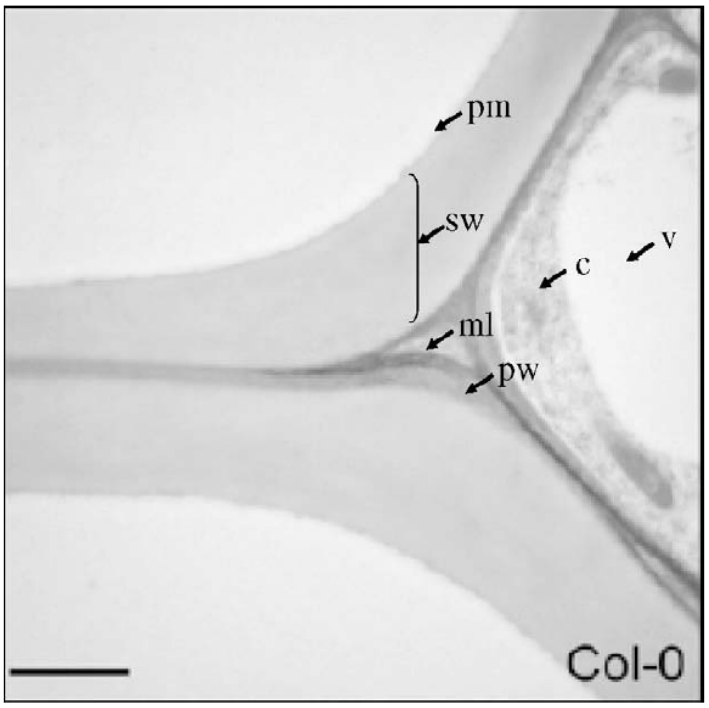

Figure 39 The cell wall of Arabidopsis thaliana (copied from Caffall and Mohnen, 2009)

The primary cell wall (pw), middle lamella (ml), and secondary cell wall (sw) of WT *Arabidopsis thaliana* Col-0 transverse root section are shown. pm: plasma membrane, c: cytosol, v: vacuole. Bar= 2 µm.

Here, the transcripts of xylem cysteine proteases XCP1 and XCP2, which were identified to aid micro-autolysis within the central vacuole of xylem tracheary elements during xylogenesis and secondary cell wall thickening (Avci *et al*., 2008), were highly up regulated in stem of *fax1* knockout mutant versus Col-0. Tracheary elements form xylem vessels strands, which are strengthened with secondary cell walls including cellulose, hemicellulose and lignin (Turner *et al*., 2007; Endo *et al*., 2009). Therefore, it is suggested that the phenotype of *fax1* knockout mutant stems may relate to the highly efficient micro-autolysis with increased

XCP1 and XCP2 proteases activity. Furthermore, it was described that inhibition of both TED6 (differentiation-related 6; transcripts up regulated in *fax1* knockout mutant stem versus Col-0) and TED7 enhanced the aberrant secondary cell wall formation of root vessel elements, but co-overexpression of TED6 and TED7 increased tracheary elements differentiation in cultured *Zinnia* cells (Endo *et al*., 2009). Another up regulated transcript in *fax1* is COBL4, which is known as a regulator that controls the culm mechanical strength and cellulose content in the secondary cell walls of sclerenchyma (Sato *et al*., 2010). Although cellulose synthase genes are not regulated in *fax1*, I found the gene TBL3 (Bischoff *et al*., 2010), TBR-like, to be highly up regulated in *fax1* knockout mutant versus Col-0. TBL3 is described to significantly contribute to deposition of secondary cell wall cellulose.

As a potential abundant source of carbohydrate, xylan limits the ability of cell wall degrading enzymes to access other cell wall polymers such as cellulose, and therefore contributes to the lignocellulosic biomass (Yang and Wyman, 2004). Transcripts of three key proteins involved in xylan deposition (GUX1, IRX15 and IRX15L) were highly up regulated in *fax1* knockout mutant versus Col-0. GUX1 is required for the addition of both glucuronic acid and 4-O-methylglucuronic acid branches to xylan in *Arabidopsis* stem cell walls. The T-DNA insertions of IRX15 (irregular xylem 15) and IRXL15 (irregular xylem like 15) contain less xylose because of decreased xylan content and exhibit mildly distorted xylem vessels (Brown *et al*., 2011).

Lignin is a product of the plant phenylpropanoid pathway. It is found mainly at the secondary cell wall of sclerenchyma and water-conducting cells of the xylem (high levels of G lignin) and in the structural fibers (higher level of S subunit; Ralph et al., 2004; Zhong and Ye, 2009). The lignified secondary cell wall formation occurs only after cells have attained their final shape and size, under the control of a complex network of tissue specific transcription factors, which mainly are NAC or MYB transcription factors (Umezawa, 2010; Zhong and Ye, 2007; Bonawitz and Chapple, 2010). It has been shown that class III peroxidases and laccase, two major classes of cell wall-related oxidoreduction enzymes, are involved in the polymeriations of lignin precursors (Berthet *et al*., 2011; Huis *et al*., 2012; Marjamaa *et al*., 2009). In the transcriptomic result of stems and flowers in *fax1* knockout mutant, transcripts of many class III peroxidases and laccases were regulated. Furthermore, recently, it was revealed that disrupting of LACCASE17 (highly up regulated in *fax1* knockout mutant) principally affected the deposition of G lignin units in the interfascicular fibers (Berthet *et al*., 2011). The transcripts of cinnamic acid 4-hydroxylase (C4H; up 2.72) and two members of cinnamyl alcohol dehydrogenase (CAD-C (down 2.71) and CAD-F (up 4.35)), the enzymes

involved in the monolignol biosynthesis (Schilmiller *et al*., 2009; Sibout *et al*., 2005), were also regulated in *fax1* knockout mutant versus Col-0.

As mentioned above, transcription factors, especially NAC or MYB transcription factors, regulate the secondary cell wall formation. Interestingly, many MYB and NAC transcription factors were regulated in stem and flower of *fax1* knockout mutant versus Col-0: MYB103 (up 13.83), SND2/ANAC073 (up 12.79), MYB63 (up 8.27), MYB58 (up 7.54), SND3 (up 6.89), MYB46 (up 6.72), NST2 (up 4.80), MYB4 (up 4.54), MYB83 (up2.86), NAC2 (down 3.51), NTL1 (flower; up 3.17) . In *Arabidopsis*, the NAC transcription factors NST2 and NST1 regulates secondary wall thickenings and are required for anther dehiscence, the latter providing a link to the *fax1* pollen phenotypes (see Chapter V. 2.1; Mitsuda *et al*., 2005). MYB58 and MYB63 are lignin-specific transcription factors which only affect lignin biosynthesis. MYB46 and MYB83 are direct downstream targets of NST transcription factors (Zhao and Dixon, 2011).

Another major component of plant cell walls, pectin, is methylesterificated ubiquitously by pectin methylesterases (PME). PMEs comprise a large multigene family, which has functions involved in vegetative and reproductive processes. Except the function of PME mentioned above on the exine development, PMEs play important roles in plant development, such as cell wall extension and stiffening (Moustacas *et al*., 1991; Al-Qsous *et al*., 2004), cellular separation (Sobry *et al*., 2004; Wen *et al*., 1999) and internode stem growth (Saher *et al*., 2005; Pelloux et al., 2007). In the *fax1* knockout mutant, transcripts of many putative PME and PME inhibitors were highly down regulated while comparing with Col-0.

Transcripts of proteins, which are involved in senescence (SAG21; down 2.68) and acyl activating enzyme (AAE12; up 4.80), are also regulated in *fax1* knockout mutant stem versus Col-0.

In summary, transcript regulation and stem secondary cell wall phenotypes of *fax1* knockout mutant do not directly relate to fatty acid or lipid transport and thus most likely represent secondary effects due to the lack of fatty acid compounds like phosphatidylcholine (see Chapter IV. 2.9).

2.4 Acyl-activating enzymes

The acyl-activating enzymes (AAE) superfamily includes the acyl-coenzyme A synthetases, 4-coumarate-coenzyme A ligases, luciferases, and non-ribosomal peptide synthetases. All members contain a 12-amino acid motif that activates their acid substrates by ATP via an enzyme-bound adenylate intermediate (Shockey et al., 2003). Until now, all nine members of

the long-chain acyl-coenzyme A synthetases (LACS) were cloned and isolated from *Arabidopsis* (Shockey et al., 2002). As mentioned in V. 2.2, LACS1 and LACS2 have overlapping functions in both wax and cutin synthesis and LACS1 preferentially modifies both very long chain fatty acids for wax synthesis and long-chain fatty acids for cutin synthesis (Lü *et al*., 2009). LACS1 and LACS4 together are required for proper pollen coat formation in Arabidopsis (Jessen *et al*., 2011). LACS6 and LACS7 might have overlapping functions to initiate β-oxidation in plant peroxisomes (Fulda *et al*., 2002). LACS1 and LACS9 have overlapping functions in triacylglycerol biosynthesis (Zhao *et al*., 2010). Furthermore, LACS9, which was localized at the outer envelope membrane of chloroplasts, is not identified to play a key role during fatty acid export, since the knockout mutants of LACS9 in *Arabidopsis* were indistinguishable from wild type in growth and appearance (Schnurr *et al*., 2002). Interestingly, LACS1 (stem, up 3.87), LACS2 (stem, up 2.48), LACS3 (stem 2.23) and LACS5 (flower, down 2.70) were regulated in *fax1* knockout mutant versus Col-0. Furthermore, LACS4 is a gene, expressed with *FAX1* (ATTED-II database; Obayashi *et al*., 2011; http://atted.jp/top_search.shtml#CoExSearch). Therefore, it is hypothesized that potential fatty acid or fatty acid derivative export by FAX1 plays an important role during the lipid biosynthesis procedure which has neighboring relation with the function of LACS.

Furthermore, long chain acyl-CoA synthetases exist in all fatty acid transport and trafficking systems in *E.coli*, yeast and mammalian, which were named FadD, faa1p/faa4p and ACSL, respectively (see Chapter I.2). Recently, the only homologous protein of LACS9 in the cyanobacteria *Synechoccystis* sp. PCC 6803, named SynAas, was identified to be essential and sufficient to mediate transfer of fatty acids across a biological membrane (Berlepsch *et al*., 2012). Therefore, the understanding of the function of the AAE superfamily, especially of LACSs, and the connection between FAX1 and LACS provide an important evidence to interpret the role of FAX1 during the fatty acid export procedure from plastids.

2.5 The role of phosphatidylcholine

As described in I.2, more knowledge on the proteins catalyzing the initial incorporation of nascent fatty acid into phosphatidylcholine (PC) will provide an important breakthrough for our current understanding of fatty acid export from plastids. The reduced PC content of *fax1* knockout mutant leaves clearly shows that FAX1 severely affects the procedure during fatty acid export to PC formation. Although it is unknown if the measured PC are mainly from ER

or outer envelope membrane, the result indicates that FAX1 is a key protein involved in plastid fatty acid export and subsequent PC formation.

2.6 Predicted role of FAX1

With the discussion above, I can conclude that FAX1 is involved in export of fatty acids or fatty acid derivatives from chloroplasts. Considering the other three homologs of FAX1 predicted in the envelope of plastids, it is implied that FAX1 is a key protein in lipid export from plastids but that the other three proteins have some redundant functions (Figure 40).

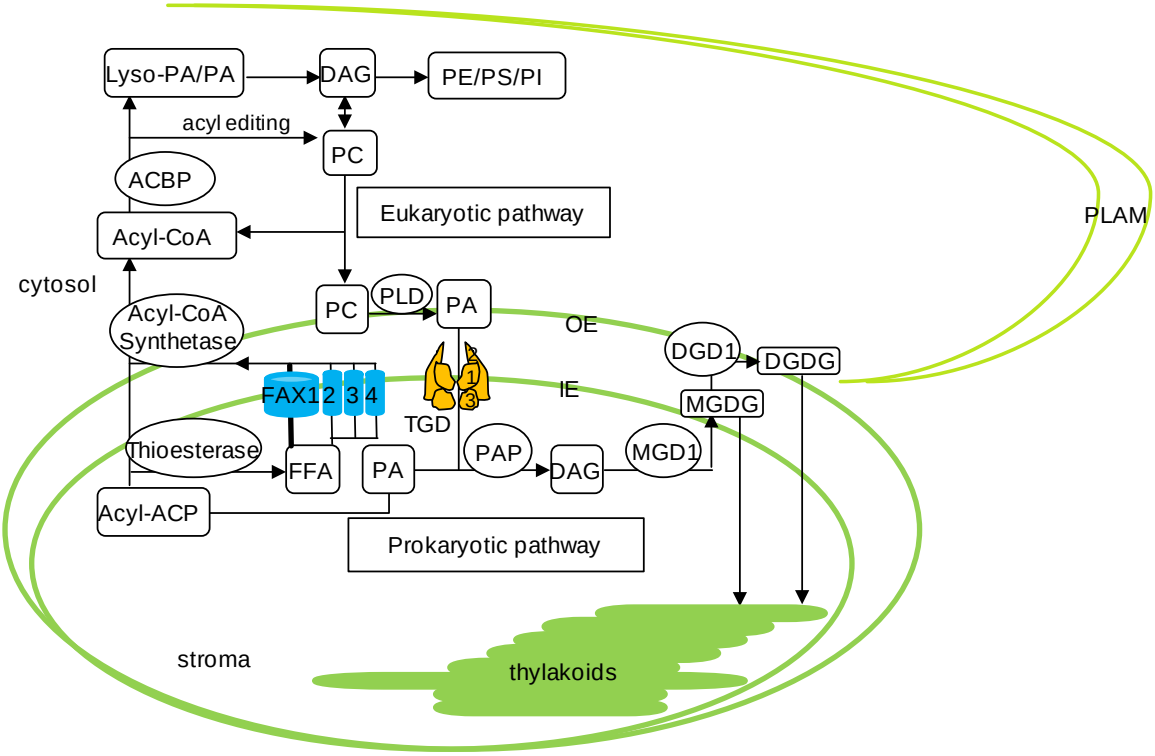

Figure 40 Model of plastid fatty acid export and the hypothesized function of FAX1 and its homologs
FAX1 is involved in export of free fatty acids (FFA) across the inner envelope of plastids. 2, 3, 4: three homologs of FAX1 in plastid inner envelope. For pathway description see Figure 2.

VI. Outlook

To investigate a potential role of FAX1 in fatty acid or fatty acid derivative export from plastids, it would be necessary to express the cDNA of *FAX1* and its *Arabidopsis* homologs in the plasma membrane (such as At3g20510) in the yeast *fat1* mutant, which is defective in fatty acid uptake through the plasma membrane of yeast cells (Zou et al., 2002). Similar assays recently showed that the function of the acyl-acyl carrier protein synthetase (SynAas) from the cyanobacteria *Synechocystis* sp. PCC 6803 is involved in mediating transfer of fatty acids across biological membrane (Berlepsch *et al*., 2012).

The ultrastructural investigation of *FAX1* mutants revealed that the thickness of the cuticular layer was affected. It is suggested that this layer most likely consists of cutin, since waxes were removed by fixation and embedding of tissue during microscopy sample preparation. Therefore, to be sure of the difference of cuticular layer phenotype in *FAX1* mutants, analysis of cutin content will be performed soon.

Transmission electron microscopy of xylem cells showed that secondary cell walls of tracheids were reduced in *fax1-2* knockout stem when comparing with Col-0 wild-type. However, as mentioned in V.2.3, secondary cell walls mainly consist of cellulose, hemicellulose and lignin. Lignin amount will be analyzed and compared between *FAX1* mutants and Col-0 to clarify the content difference of secondary wall of xylem cells.

It is clear that the ongoing lipid analysis and transcriptomic analysis of *FAX1* overexpressing lines will provide more evidence for the characterization and function of FAX1.

FAX1 is a member of Tmemb_14 family, which is an uncharacterized protein family. Therefore, the characterization of the other homologs in *Arabidopsis* and other origin, such as from green algae like *Chlamydomonas reinhardtii*, and the generation of double or triple mutants in *Arabidopsis* will be necessary.

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