Designing the Superplant: Increasing Lipid Signaling for Enhanced Phosphorus Uptake

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Abstract

Phosphate is fundamental for optimal growth and reproduction for plants. However, due to low solubility, not all phosphorus in the soil is available to the plant. It has recently been discovered that plants can take up complex phosphate-containing organic compounds, such as phospholipids. This depends on membrane transporters, more specifically lipid flippases. In the model plant *Arabidopsis*, the lipid flippases ALA10 is upregulated by phosphate deficiency. ALA10 form together with ALA9, ALA11 and ALA12 an evolutionary subfamily, suggesting that they might be involved in similar cellular functions.

The aim was to design a strategy to obtain proof of concept that hyperactivation of specific flippases can lead to plants with an increased phosphate utilization efficiency, using *Arabidopsis* as a model organism. This was done by creating yeast transformants containing C-terminal deletions of ALA10-ALA12 and a beta subunit. Lipid translocation was tested using flow cytometry. A small plant experiment was conducted to see if plants can grow under a phosphate deficiency.

It was found that the lipid uptake assay tested in this study was not enough to tell if ALA10-ALA12 with C-terminal deletions results in more lipid translocation and further investigation is needed. Preliminary results of ALA9 specificity suggest that it is a phosphatidylserine (PS) transporter. Results from a growth experiment suggest that *Arabidopsis* can grow under phosphate deficiency with a media containing lipids.

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List of abbreviations

ALA	Aminophospholipid ATPase
ALIS	ALA Interacting Subunit
GluCr	Glucosylceramide
LB	Luria Bertani medium
NC	Negative control
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol
PIC	Protease Inhibitor Cocktail
PMSF	Phenylmethylsulfonyl fluoride
PS	Phosphatidylserine
SCD	Synthetic complete medium with glucose
SCG	Synthetic complete medium with galactose
SDS	Sodium dodecyl sulfate
SM	Sphingomyelin
YPD	Yeast extract-peptone-dextrose medium

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1 Introduction

Millions of tons of fertilizers are used every year in agricultural production (FAO, 2017). Fertilizers contain several important plant nutrients and one of them is phosphorus (Better Crops, 1999). These fertilizers are expensive, and they are also responsible for a large proportion of the freshwater pollution (Cordell and White, 2014). One important factor contributing to this problem is the low phosphate uptake exhibited by many plants under normal conditions. However, when there is shortage of phosphate, high affinity phosphate transporters will be activated, which in turn will increase the uptake efficiency of plant cells (Poulsen et al., 2015).

1.1 Phosphate in agricultural production

Phosphate is a fundamental element for plant growth. No other nutrient can perform its functions and with an adequate supply of phosphate, optimal growth and reproduction can be acquired (Better Crops, 1999). Key molecules such as nucleic acids, phospholipids and ATP all contain phosphorus and plants can therefore not grow without a reliable supply of this nutrient. Phosphorus is the second most frequently limiting macronutrient for plant growth, after nitrogen (Schachtman et al., 1998). Soils with less-than-ideal amounts of phosphorus will lead to agricultural losses in the range of 10 to 15 % of the maximal yields. Phosphorus compounds tend to have low solubility which results in less available phosphorus to the plants in the soil. As a countermeasure to this, more than 45.9 million tons of phosphate fertilizers are used around the world every year. Ninety percent of the phosphate extracted globally from mines is used for fertilizers for food production (FAO, 2017). The mineral phosphate reserves are a finite resource, and as the reserves decrease, import prices increase, leading to high economical costs for the area, and increasing food prices. With the increased mining of phosphate, the global phosphorus cycle has been altered by mobilizing four times the natural level of phosphorus from mineral phosphate into the environment (Cordell and White, 2014). Phosphorus does not cycle between plants and the atmosphere. It does, however, cycle between plants and soil, and is lost through soil erosion and water runoff into creeks and rivers (figure 1). It contributes to the nutrient pollution of many of the world's lakes, rivers and oceans, with the Baltic sea being one of the most polluted seas in the world (Zimdahl, 2015).

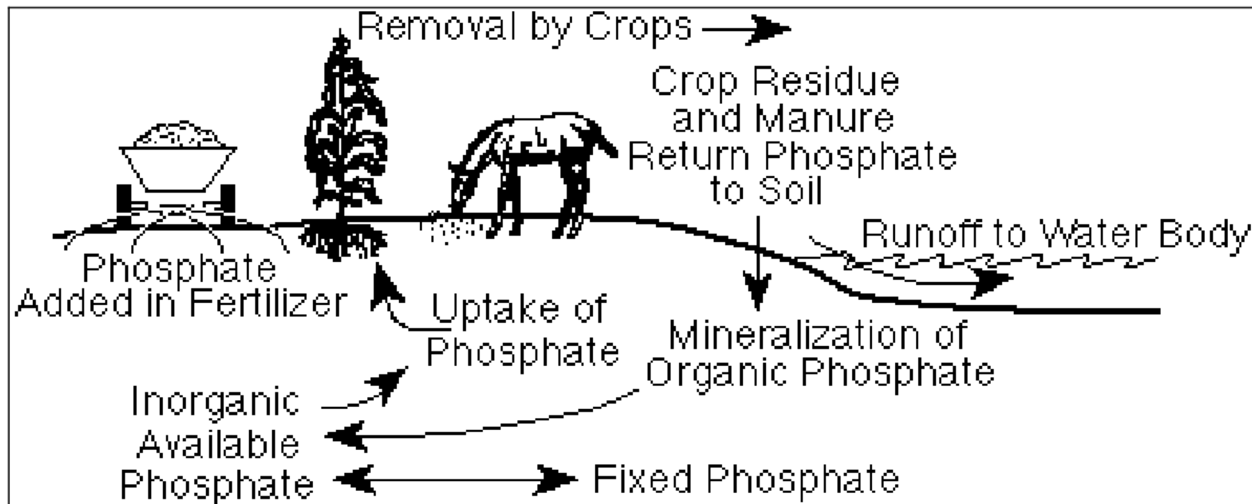

Figure 1. The phosphorus cycle in relation to agricultural practices, adapted from Busman et al., (2001). Phosphate is added to the soil in the form of fertilizers, but far from everything is available to the plant. When the plant takes up phosphorus, it is converted to phytic acid, which in turn cannot be taken up by the animals. Therefore, the manure is rich in phosphate-containing organic compounds. Plants can only use this phosphate if it has been released by microorganisms in the soil. This results in most of the phosphate being washed out and is responsible for the pollution of watercourses.

1.2 Lipid uptake by plants

It was long thought that plants could only take up inorganic compounds through the root (Schachtman et al., 1998), leading to a lot of research about the development of genetically enhanced plants capable of using soil phosphorus more efficiently. The focus has been on the mechanisms behind the phosphate uptake in plants, transportation from the roots to the rest of the plant, and internal utilization. Alternatives to genetically enhanced plants have also been tested, such as soils with microbes. The microbes solubilize mineral phosphate and increase available phosphate in the soil. Inconsistency in trials under field conditions have deemed this method not helpful (Shenoy and Kalagudi, 2005).

It has been demonstrated in recent years that plants are indeed capable of taking up complex phosphate-containing organic compounds, such as phospholipids, through the roots. The uptake of lipids through the roots depends on a group of proteins called flippases (Poulsen et al., 2015). They are membrane transporters that use ATP hydrolysis to energize lipid movements from the outside to the inside of the cell. Flippases control the asymmetric distribution of lipids between the two leaflets of biological membranes in eukaryotic cells (Yang et al., 2018; van Meer, 2011). It was first thought that their only function was connected to general plant fitness and tolerance to cold weather (Gomes et al., 2000). However, now it is known that their functions include developmental and reproductive functions and signaling events, as well as adaptation response to biotic and abiotic stress (Nintemann et al., 2019).

1.3 P-type ATPases and P4-ATPases

Lipid flippases can be further categorized as P-Type ATPase. The name comes from the protein's use of ATP during transportation and that it undergoes a cycle of phosphorylation and dephosphorylation that drives the conformational changes necessary for substrate transport (Axelsen and Palmgren, 1998). There are several subfamilies, all depending on their substrate specificity (figure 2). Type 1 P-Type ATPase transports potassium and heavy metals, type 2 calcium, type 3 protons and type 4 transports phospholipids. It is not known which specific substrate type 5 transports. P4 ATPases differ from the other groups since they are exclusive to eukaryotic organisms and that their substrates are considerably larger than the cations transported by the members of the family (Palmgren et al., 2019).

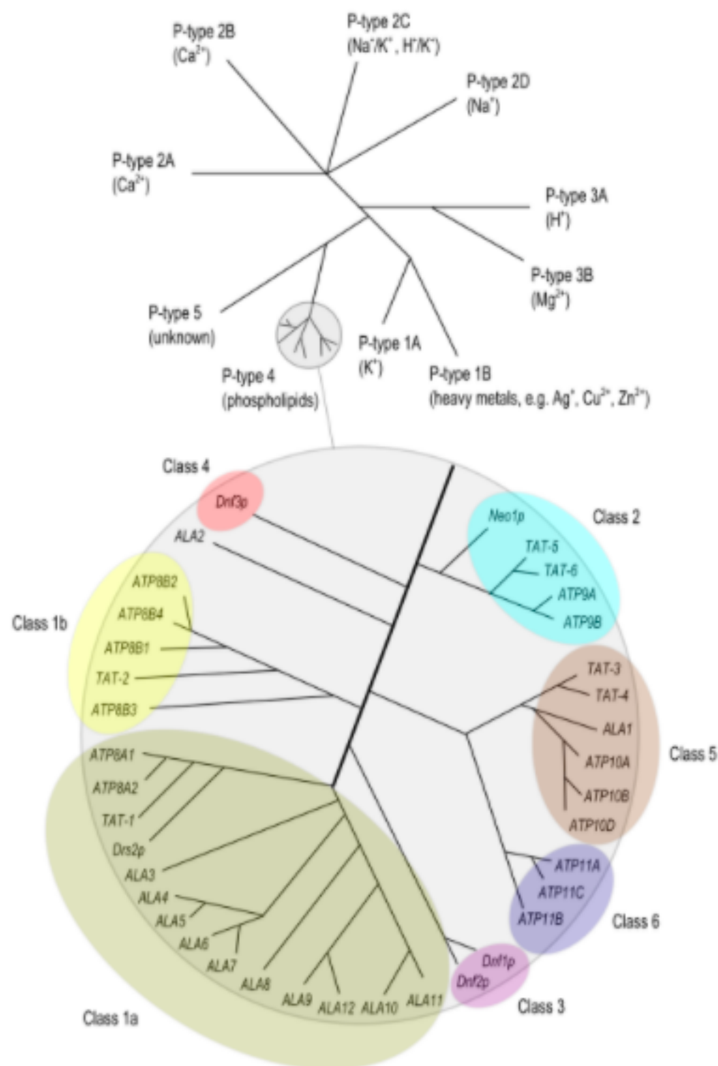

Figure 2. Phylogenetic tree of the P-type ATPase superfamily and the P4 subfamily proposed by Van der Mark et al. (2013). The substrate(s) transported by the different P-type ATPases are displayed in the brackets.

1.4 P4-ATPase in model organisms

In *Saccharomyces cerevisiae*, five members of the P4-ATPase family can be found, and they are all well characterized (Catty et al., 1997). They are Neo1p, Drs2p, Dnf1p, Dnf2p and Dnf3p and are involved in the transport of phospholipids and the formations of vesicles (Prezant et al., 1996; Ripmaster et al., 1993; Hua et al., 2002). Neo1p is the only essential lipid flippase with all the rest of the lipid flippases being able to be knocked out, except all at once, without leading to death of the yeast (Prezant et al., 1996; Hua et al., 2002). They are located in different parts of the cell, with Neo1p in the early endosome, Drs2p and Dnf3p in the trans-Golgi network and Dnf1p and Dnf2p in the plasma membrane (Wicky et al., 2004; Hua et al., 2002). The 14 P4-ATPases identified in humans have similar locations in the plasma membrane, the Golgi and early endosomes and have also been linked to phospholipid translocation (Axelsen and Palmgren 1998; López-Marqués et al., 2014). In *Arabidopsis thaliana*, there are 12 members of P4-ATPases, namely ALA1-12 (Aminophospholipid ATPase1-12). They constitute the largest family of P-Type ATPase pumps (Axelsen and Palmgren 2001).

1.5 The P4-ATPase beta subunit

It is not only their unusual substrate that sets P4-ATPases apart from most other P-type pumps, but also their association with a non-catalytic Cdc50 protein (beta subunit) (figure 3), with which they form heteromeric complexes (Lenoir et al., 2009). These subunits are necessary for flippase activity. In *Arabidopsis*, there are five members from this family of subunits, ALIS1-5 (ALA Interacting Subunits1-5) (Poulsen et al., 2008). Lipid substrate specificity is independent of the nature of the ALIS the ALA is interacting with. The beta subunit can interact and function with different ALAs, however does not display binding specificity to all ALAs. The beta subunit also helps in maturation of the catalytic α-subunit and assist the α-subunit in the exit from the ER. (López-Marqués et al., 2010).

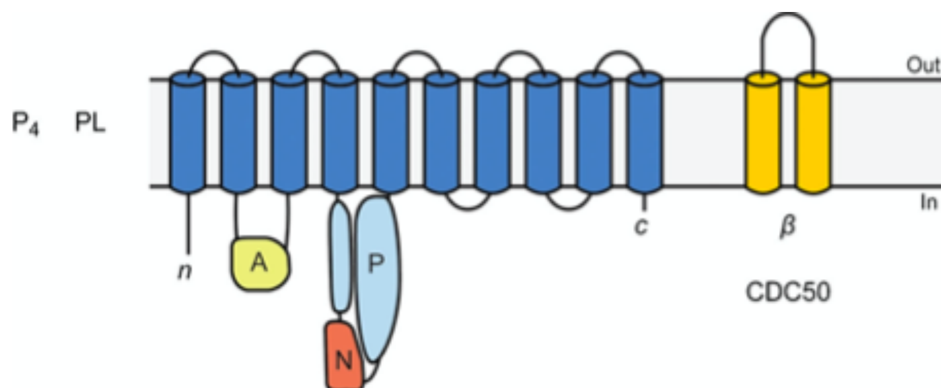

Figure 3. Diagrammatic representation of the P4-ATPase and the beta subunit CDC50 (yellow) proposed by López-Marqués et al. (2014). P4-ATPases are composed of 10 transmembrane domains (dark blue) and 3 cytoplasmic domains which are N nucleotide binding (red), P Phosphorylation (light blue) and A actuator (green). N-and C termini both face the cytosol.

The cytoplasmic region of the C-terminal of Drs2p acts as a regulatory domain. It autoinhibits the enzymatic activity by interacting with its N-domain (Natarjan et al., 2009; Azouaoui et al., 2017; Timcenko et al., 2019). The mammalian P4-ATPase ATP8A2 also seems to have an autoinhibitory domain in its C-terminal region, the regulation is however more complex. The enzymatic activities of P4-ATPases seems to be affected by their C-terminal domains.

1.6 Characteristics of *Arabidopsis* P4-ATPases

The first member of P4-ATPases in *Arabidopsis* to be identified is ALA1. It localizes in the plasma membrane, however the function and substrate specificity is still unknown. ALA2 transports phosphatidylserine (PS) in endosomes and ALA3 is located in the Golgi apparatus and has broad-specificity of phospholipids. ALA6 and ALA7 have many different functions, including pollen fitness (Poulsen et al., 2015).

ALA4-ALA12 have more than 75% sequence similarity (Axelsen and Palmgren, 2001). Together they form a monophyletic cluster. To have many closely related isoforms indicates that these isoforms have important redundant functions. It can also mean that they are differentially controlled in different cells under different conditions (Poulsen et al., 2015).

As mentioned before, plants are capable of taking up lipids through the root, and this is done using ALA10. ALA10 translocate several different phospholipids, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), PS and mammalian lipid sphingomyelin (SM). It seems like ALA10 transport SM better than PC. ALA10 is located at the plasma membrane of epidermal root cells, indicating the possibility of the flippase scavenging soil lipids, since SM can be found in the decomposition of organic material (Nintemann et al., 2019). Studies suggest that ALA10 is important for the uptake of lysoPC, a signaling lipid involved in plant development. It is also suggested that ALA10 transcription is upregulated under phosphate starvation and ALA affects root growth to phosphate and lysoPC (Poulsen et al., 2015). Three other *Arabidopsis* flippases (ALA9, ALA11 and ALA12) form an evolutionary subfamily with ALA10 (proteins 75% identical) (Axelsen and Palmgren, 2001), which suggests that they might be involved in similar cellular functions. The lipid specificity of ALA9 is unknown, whereas ALA11 is mainly a PC transporter and ALA12 is a PS transporter (unpublished).

1.7 Aim

This project will contribute to a better understanding of how plants can be engineered in order to improve phosphate utilization and remove the need for inorganic phosphate-based fertilizers, which are a big source of pollution of groundwater.

The aim of this degree project was to design a strategy to obtain proof of the concept that hyperactivation of specific flippases can lead to plants with an increased phosphate utilization efficiency, using *Arabidopsis* as a model organism. This was done by deletion of the C-terminal

of ALA9-12 using PCR and then transformation into yeast together with an ALIS. Consequently, the research questions (RQ) for this degree project were:

RQ1: Yeast and mammalian lipid flippases are activated by removal of their C-terminal end. Are ALA9-ALA12 activated in the same way?

RQ2: What amount of lipids in the soil will be enough for plants to grow under phosphate deficiency?

1.8 Experimental strategy

DNA constructs containing truncated versions of ALA9-ALA12 for yeast expression are prepared using first PCR, then cloning into a cloning vector and then ligation to a yeast expression vector. Yeast transformations are made, and the lipid transport is tested using a NBD-lipid uptake assay. The assay commonly used to measure lipid uptake is based on flow cytometry (Grant et al., 2001). Fluorescent 7-nitrobenz-2-oxa-1,3-diazole (NBD) acyl-labelled lipid reporters are used. Yeast cells are incubated with NBD-labelled lipids and following the removal of surface-exposed probe, the remaining fluorescence is measured. Fluorescence-activated cell sorter (FACS) is a flow cytometer equipped to separate the identified cells. Dead cells labeled with the dye will have a higher fluorescence signal than living cells. To exclude the dead cells from the analysis, a gate is set to excluded this highly labeled population. The remaining living cells are plotted on a histogram with the percentage fluorescence intensity relative to the negative control as a measure of the accumulated NBD-lipids (Grant et al., 2001).

A hydroponic experiment with be performed to test if *Arabidopsis* is able to grow in a media using lipids as a phosphate source. Three different media will be used: full nutrition, no phosphate and no phosphate but with lipids. Seeds from wild type *Arabidopsis* and a mutant *Arabidopsis* will be used to see if growth will be obtained in a media containing lipids.

2 Materials and methods

2.1 Plasmids, strains and media

The plasmids used in this degree project can be found in table 1. *Escherichia coli* transformants were plated on agar plates with LB medium (1 % (w/v) yeast extract, 2 % (w/v) tryptone, 2 % (w/v) NaCl, 2 % (w/v) agar) supplemented 100 mg/L ampicillin. *E. coli* cells were incubated overnight at 30°C. For transformation in *E. coli*, in-house made or commercially made Top10 cells (Thermo Fisher Scientific, Hvidovre, Denmark) were used. For yeast cultivation, cells were grown on YPD plates (2 % (w/v) glucose, 2 % (w/v) peptone, 1 % (w/v) yeast extract, 2 % (w/v) agar) at 30°C. Yeast transformations were plated on SCD–H–U–L–W (0.7 % Yeast Nitrogen base with ammonium sulfate without amino acids, 2 % glucose (SCD), 1x Drop out media without histidine, uracyl, leucin and tryptophan (Sigma Aldrich, Søborg, Denmark), 2 % (w/v) agar). The yeast strain (ZHY709) from *S. cerevisiae* used in transformation is a triple mutant, *Δdrs2Δdnf1Δdnf2* (Hua et al., 2002).

Table 1. Plasmid name, relevant genotype and reference. Ye stands for yeast enhanced.

Plasmid name	**Relevant genotype**	**Reference**
pMP5225	Gal1-10,leader,RGSH10 2-um,URA3	Unpublished
pMP5250	Gal1-10,leader, 2-um,URA3	Unpublished
pMP5331	ALA11	Unpublished
pMP5451	yeALA10	Unpublished
pMP5452	yeALA12	Unpublished
pMP3117	ALIS1	Unpublished
pRS423-Gal	empty plasmid	Burgers 1999
pRS426-Gal	empty plasmid	Burgers 1999
pMP5690	yeALA11	This study
pMP5691	yeALA10 lacking C-terminal	This study
pMP5692	yeALA11 lacking C-terminal	This study
pMP5693	yeALA12 lacking C-terminal	This study

2.2 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was used to create truncated gene fragments. These fragments were created using primers designed to not include the C-terminal in the amplification of ALA9-ALA12. Before the PCR, plasmids containing ALA10, ALA11 and ALA12 (pMP5451, pMP5690, pMP5452) were digested overnight with *EcoR1* to form linearized fragments. PCR reactions were performed with a mixture of 5 µL 5x HF Phusion Reaction Buffer, 10 nM dNTPs, 5 pmol/µL forward primer, 5 pmol/µL reverse primer, 1-5 ng/µL template DNA, 0.3 µL Phusion High-Fidelity DNA Polymerase (New England Biolabs, Herlev, Denmark) and water in a total volume of 50 µL. The program used, including temperatures, times and number of cycles can be found in table 2. The primers used in the PCR can be found in table 3. The same program was used for all of the primers.

Table 2. PCR settings including temperature, time and cycles.

Step	**Temperature °C**	**Time**	**Cycles**
1. Initial denaturation	98	3 min	1
2. Denaturation	98	30 sec	1
3. Annealing	55	30 sec	1
4. Extension	72	2 min	1
Repeat step 2-4 32 times			
5. Final extension	72	10 min	1
6. Hold	12		

Table 3. Primers used in this degree project. The restriction enzyme sequence is written in lowercase.

Primer name	**Forward or Reverse**	**Primer Sequence, 5´to 3´**	**Restriction enzyme**
oli 6113	F	gaattcATGGTTGGTGGTGGTACTAAGCGTAG	EcoR1
oli 6114	R	gtcgacTCAGGAGGCTGGTTCAGAAATTCTAACG	Sal1
oli 6176	F	gaattcATGGCTGGTCCATCTAGACG	EcoR1

oli 6177	F	gaattcATCACCAAGTGCAGAAGAAG	EcoR1
oli 6178	F	gaattcCATGGCTACTGTTTCTGGTAG	EcoR1
oli 6179	R	gtcgacTCAGATACATTGGGAAGAAAG	Sal1
oli 6180	R	gtcgacTCACTTTGGGAAGAATCTCATC	Sal1
oli 6181	R	GTCGACTCACCTGGGAAGAATCTCATC	Sal1
oli 6182	R	gtcgacTCACATTGGGAAGAATCTCATC	Sal1
oli6183	F	CTACGCTAAGTACCACCACCAACCATgaatt cGCCCTTACCACCTTGAAAATACAAATTT	EcoR1
oli6184	R	CGTTAGAATTTCTGAACCAGCCTCCTGAgt cgacGGTACCCAATTCGCCCTATAGTGAGT	Sal1

2.3 Gel electrophoresis

To verify that the fragments had been successfully amplified, gel electrophoresis was run. A gel with 1 % agarose in 1 x TBE buffer (0.089 M Tris base, 0.089 M Boric Acid, 0.002 M Ethylenediaminetetraacetic acid (EDTA)) was run as a control and when the samples needed to be purified from the gel, a gel with 0.7 % agarose in 1 x TBE buffer was run. Before the loading the samples in the wells, 10 µL sample + 2 µL 6x Gel Loading Dye Orange (NEB) containing gelred was used for the digestions. For the PCR reactions, 5 µl of the products were run on the gel. An O'Gene ruler 1kb ladder (Thermo Fisher Scientific, Hvidovre, Denmark) was loaded in one well as a reference. The gel electrophoresis was run at 110 V for 25-35 min.

2.4 Cloning of C-terminally deleted ALA10- ALA12

The PCR fragments containing C-terminally deleted ALA10-ALA12 were cloned into the cloning vector pCR4-blunt-TOPO (Thermo Fisher Scientific, Hvidovre, Denmark) and then transformed into *E. coli* and plated on agar plates containing LB medium with 50 mg/L kanamycin. The plates were incubated overnight in 30°C and then replated 4 colonies and incubated overnight again at 30°C. The next day, the plasmids were purified using a plasmid purification kit (GeneElute Plamid Miniprep Kit, Sigma Aldrich, Søborg, Denmark). A digestion with the restriction enzymes EcoR1 and Sal1 was done to verify the right construction. When the right construction had been verified, the DNA concentration was measured using NanoDrop 1000 (Thermo Fisher Scientific, Hvidovre, Denmark) and then the plasmids were sent to be sequenced to make sure that no mutations occurred in the newly constructed plasmid.

The genes in the pCR4-blunt-TOPO plasmid were then transferred to a yeast expression vector (pMP5225). Before the genes were transferred, an overnight digestion with EcoR1 and Sal1 was performed. The yeast expression vector was also digested using the same restriction enzymes. The digestion product was run on a 0.7 % agarose gel, then cut out and purified using a GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Hvidovre, Denmark) The DNA was eluted from the column with 10 µl water and then a ligation with the ALA-genes and the expression vector was started with ligase buffer and T4 DNA Ligase (New England Biolabs, Herlev, Denmark). The ligation product was left at room temperature overnight and was then transformed into *E. coli* and plated on plates containing LB medium and ampicillin. After plasmid purification and verification, the DNA concentration was measured again using NanoDrop 1000 and the plasmids were sent for sequencing.

2.5 Cloning of full length ALA9

Homologous recombination was used to amplify ALA9 in yeast. Firstly, primers were designed to overlap with full length ALA9 and a yeast expression vector (pMP5225). Then two PCRs were performed, one with ALA9 and one with the expression vector, and verified on a gel. The two PCR products were transformed in yeast by a method using lithium acetate, ssDNA and polyethylene glycerol (PEG) (Gietz and Schiestl, 2007). The yeast (ZHY709) was plated on a YPD plate and incubated at 30°C for 20-22 h. The following day, ¼ of a 10 µL inoculation loop of cells were resuspended in 1 mL of sterile MilliQ water in an Eppendorf tube and centrifuged at 6000 rpm for 1 min. One tube was prepared for each transformation. The supernatant was discarded and the cell pellet was resuspended in 1 ml of 100 mM lithium acetate. The cells were incubated for 20 min at 30°C. A master mix was prepared containing 240 µL PEG 3350 50 %, 36 µL 1 M lithium acetate, 24 µL MilliQ water, 10 µL ssDNA (Sigma Aldrich, Søborg, Denmark) (10mg/mL) per transformation. After the incubation, the cells were spun down at 6000 rpm for 1 min. The supernatant was discarded and 310 µL of the master mix, 25 µL of each PCR product (approx. 2-5 µg) was added. The cells were resuspended using a vortex then incubated at 42°C for 20 min. After the incubation period, the cells were spun down at 8000 rpm for 2 min. The supernatant was removed and the pellet was resuspended in 100 µL sterile MilliQ water and plated on SD-URA plates. The plates were incubated for 4 days at 30°C.

After growth of the transformants, the DNA was extracted from one colony using a lysis buffer (0.2 M Lithium acetate, 1 % SDS solution) and ethanol 96 % and 70 %. PCR with primers annealing to ALA9 was run to verify the right construct.

2.6 Yeast transformation

After the homologous recombination, ALA9 was transformed again using the same method containing lithium acetate, as described in section 2.5. 5-10 colonies from the SD-URA plates were resuspended in 1 mL of sterile MilliQ water. When the master mix was added, 8 µL of

plasmid containing ALIS1 was also added. The same procedures as described above were otherwise performed. The transformants were plated on plates containing SCD-H-L-U-W.

A high frequency protocol was used for the transformation of ALA10-ALA12. The yeast (ZHY709) used in the transformation was grown O/N in 10 mL of YPD and one extra incubation at 30°C for 20-30 min before the heat shock at 42 °C, otherwise same protocol as the lithium acetate method. For each transformation, two plasmids were co-transformed, one ALA-plasmid and one plasmid containing ALIS1. Transformants were plated on plates containing SCD-H-L-U-W and incubated for 4 days in 30°C. Negative control with two empty plasmids and positive controls with plasmids ALA10, ALA11 and ALA12 were made.

2.7 FACS analysis on NBD lipid-labeled yeast

Overnight cultures in 2 mL SCD-H-U-L-W in 15 mL falcon tubes were made using about 5-10 colonies from the yeast transformation. The cultures were incubated overnight at 150 rpm and 30°C. After 22-24 h, the OD_{600} was measured using a 1/20 dilution. 50 mL cultures were prepared in SCG-H-U-L-W (same as SCD but with 2% galactose instead of glucose) in 100 mL shaking flasks at a starting OD_{600} = 0.06. They were grown at 24℃ and 100 rpm for 22-24 h. After this incubation time, the OD_{600} was measured, and the cultures were transferred to 50 mL falcon tubes and spun down at 900 g and resuspended in fresh selective SCG media at a concentration of 10 OD600/mL. The lipids tested were palmitoyl lipids: PC, PE, PS, SM and glucosylceramide (GlcCer). The lipids were dissolved in chloroform and transferred to a conical bottom glass tube. The lipids were dried by evaporating the chloroform under a N_2 current. Finally, the lipids are resuspended in DMSO with a final concentration of 4 mM. 125 µL of the yeast cells were transferred into glass tubes and then labelled with NBD lipid and incubated in a water bath at 25℃ for 30 min with periodical mixing. The labelling was stopped by adding 1 mL of ice-cold SSA+BSA media (0.7 % YNB, 1x Drop out media without histidine, uracyl, leucine and tryptophan, 2 % sorbitol, 3 % (w/v) bovine serum albumin and 20 mM NaN_3). Three washing steps followed at 700 g and 5 min at 4°C and then the cells were resuspended in 0.125 mL of SSA+BSA media and kept on ice. The cells that had not been used for labelling were transferred to Eppendorf tubes and spun down at 13,000 g for 30 seconds, the supernatant was discarded and the pellet was frozen with liquid nitrogen and kept at -80°C.

Measurements were carried out using a FACSCalibur which is a 4-Color flow cytometry Analyzer using Cell Quest software. 50 µL of the labelled yeast cells was mixed with 1 µL propidium iodide and 1 mL water. Twenty thousand cells were analyzed without gating during the acquisition. To identify the different cells, the live cells were selected based on gating using forward/side-scatter and propidium iodide exclusion. The mean fluorescence intensity of total cells were calculated using a histogram of the green fluorescence of living cells. Accumulation of NBD-lipids is given as the percentage fluorescence intensity relative to the negative control.

2.8 Isolation of total membranes

The unlabelled yeast cells stored at -80°C were immediately resuspended in 1 mL lysis buffer (1 mM PMSF, 10 µL PIC, 10 mM EDTA in 1x PBS (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4-$2H_2O$, 2 mM KH_2PO_4) after being taken out of the freezer. The pellets were then transferred to a conical glass tube with 1 g, 0.5 mm acid-washed glass beads. The cells were disrupted by vortexing 10 times for 1 min interspersed with 1 min cooling on ice. The cell lysates were then spun in 1.5 mL Eppendorf tubes at 500 g for 5 min at 4°C. Ultracentrifuge Eppendorf tubes were prepared and the supernatant from the previous spin were transferred and the weight were adjusted accurately (0.001 g) by adding Lysis buffer. The supernatant was spun at 100,000 g in a precooled Type TLA55 rotor for 45 min at 4 °C. After the centrifugation, the supernatant was removed and the membrane pellet was disrupted using a plastic micro pestle. The membranes were resuspended in 250 µL Lysis buffer. The membrane suspension was spun again at 500 g to get rid of the debris.

2.9 SDS PAGE and Western Blot

15 µL of each sample was mixed with SDS PAGE loading buffer and then placed on a water bath at 37°C for 5 min before loading into a 10% acrylamide gel. 7 µL prestained ladder (Color Protein Standard, Broad Range, NEB) was loaded into the first well. The gel was run for approx. 45 min at 160 V.

After SDS-PAGE, proteins were transferred to nitrocellulose membrane using a Pierce Fast Blotter. Precut Western Blot Whatman filter papers was soaked in a container with 1-Step™ Transfer Buffer (Thermo Scientific) for a couple of minutes. Then precut nitrocellulose membrane was also soaked. Two soaked Whatman filter paper were put in the bottom of a cassette. Then the membrane was put on top of the filter paper, the SDS gel was put on top of that and lastly two more soaked filter papers. Bubbles were removed and the cassette was closed. The cassette was transferred to a fast blotting machine and was fast blotting for 12 min at 25 V. The transfer stack was disassembled and the membrane was put in a small box with 20 mL 5 % non-fat milk in western wash. The membrane was shaken at room temperature for 1 h. After that, the membrane was incubated overnight at 4°C with 20 ml of the primary antibodies RGSHis™ Antibody BSA-free (Qiagen, Cat No. 36450) with 1:10000 dilution in 5 % non-fat milk powder in western wash. The blot was then washed in 5 x 5 min 25 mL western wash buffer, and incubated for 1 h at room temperature with the secondary antibody (Goat Anti-Mouse IgG(H+L)-HRP Conjugate, BIO-RAD, Cat No. 1706516) with the dilution 1:10000. Wash blot 3 x 5 min with western wash and 2 x 5 min with PBS buffer. Then the blot is left in PBS buffer before imaging with the Bio-Rad imager (ChemiDocTM MP System). 500 µL of each two substrates (SuperSignal® West Dura Extended Duration Substrate, Thermo Fisher Scientific, Hvidore, Denmark) were mixed in a 1.5 mL Eppendorf tube. The PBS was removed and the blot was covered with the substrate mix and incubated for 5 min. The residual substrate was collected and the blot was placed on the UV-tray of the machine and the image was taken.

2.10 Plant material and growth conditions

Arabidopsis wild type (accession Columbia, Col-0) and the ALA10 ALA11 (*ala10/11*) double mutant, which was a kind gift from Prof. Jeffrey Harper (University of Nevada-Reno), were used in all experiments. The seeds were surface sterilized by soaking for 5 min in 500 µL 95 % ethanol, followed by 5 min incubation in 500 µL of bleach solution (20 % (v/v) commercial bleach, 0.1 % soap). Seeds were rinsed three times with sterile water before they were transferred to the plant growth system. The system consists of a series of conical containers that are filled with media with agar, then the containers are loaded into a supporter and then those are placed on a tray. The set up can be seen in figure 4 (Araponics, Liège, Belgium). The first nine of the spots, marked A-I were from the wild type and J-R were the double mutant *ala10/11*. Three different media were prepared for the growth experiment: Full nutrition media containing phosphate, media containing no phosphate and media containing no phosphate but with lipids. The media contained 2 mM KNO_3, 1 mM NH_4NO_3, $CaCl_2$, $MgSO_4$, K_2SO_4, 1x oligoelements (150 mM H_3BO_3, 35 mM $MnSO_4$-H_2O, 2.5 mM $ZnSO_4$-$7H_2O$, 1.5 mM $CuSO_4$-$5H_2O$, 1 mM $NiSO_4$-$6H_2O$, 0.75 mM $(NH_4)_6Mo_7O_{24}$, 50 µM $CoCl_2$, 54 mM NaFeEDTA), 3 mM MES, 2.5 mM KCl, 4 mM L-glutamine. Phosphate sufficient media contained 3 mM K_2HPO_4/KH_2PO_4 with a pH of 5.7. The pH of the two remaining media were adjusted to 5.7 with a few drops of KOH. 50 mM L-α-Phosphatidylcholine (Sigma Aldrich, Søborg, Denmark) was added to the media with no phosphate but with lipids. 1 % plant agar (Goldbio) was added to 50 mL of no phosphate media to grow the seeds on. 2 L of each media was prepared. The growth systems were covered with plastic and put in 4 °C for approx. 48 hours for germination. Then the set ups were moved to a greenhouse and the containers were filled with the respective media. Containers were washed thoroughly with distilled water before use. The plastic that was wrapped around the containers was removed after one week in the greenhouse. Conditions in the greenhouse were 22°C at day and 20°C at night with 17 hours of day and 7 hours of night.

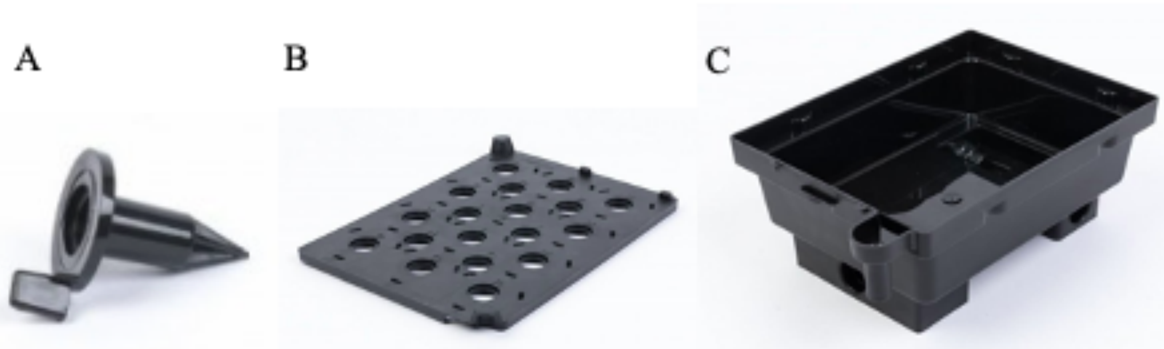

Figure 4. Different parts of the set up used for the growth experiment. (A) conical containers that were filled with agar. (B) supporter with holes for the conical containers. The holes are marked A-Q. (C) tray which can be filled with media and the supporter with the conical containers placed on top.

3 Result and discussion

The first objective of this thesis was to find out whether the C-terminal end of the lipid flippases is autoinhibitory. As a model, the ALA9-ALA12 family of proteins were chosen. A construct containing ALA9 in a yeast vector was not available, so the first step was to prepare such a construct.

3.1 Cloning of full length ALA9 using different techniques

The plasmids in this degree project were constructed by using PCR, cloning and ligation. ALA9 had not previously been cloned into a plasmid and therefore also had to be amplified using PCR and then cloned using the same procedure as with ALA9-ALA12 genes with C-terminal deletions. When cloning the PCR fragments for ALA9 into the cloning vector and using heat shock transformation in *E. coli*, there were not enough colonies growing to be able to extract sufficient amounts of plasmid DNA. The PCR was repeated with the PCR products from the previous reactions to obtain a higher concentration of the fragments.

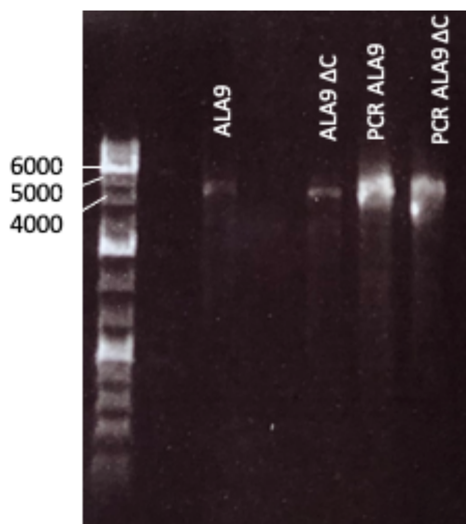

Figure 5. Agarose gel showing the result from the PCR with full length ALA9 and ALA9ΔC and the PCR of the PCR products of full length ALA9 and ALA9ΔC.

The PCR fragments from the second PCR, which showed stronger band as seen in figure 5, were cloned into the cloning vector and transformed in *E. coli*, however, this did not result in any colonies with the right construction. The cloning product was also transformed in *E. coli* using electroporation, but this did not result in any colonies. This indicates that ALA9 is possibly toxic for the bacteria. A different bacterial strain can be used in the experiments, but it will likely not change the outcome. Therefore, yeast homologous recombination was used to directly transform the PCR products of ALA9 to yeast. Primers creating overlaps with full length ALA9 and a yeast expression vector were used in the PCR and the products were then transformed into yeast. DNA from a few colonies of the transformants were extracted and a PCR was run to verify that ALA9 had successfully been cloned into yeast, which can be seen in figure 6.

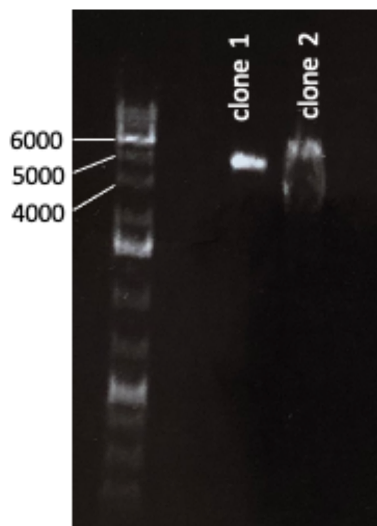

Figure 6. Agarose gel showing the result from the PCR with the extracted DNA from the yeast transformation of ALA9. Four clones were tested, however only clone 1 and clone 2 gave a result.

3.2 Preliminary result shows that ALA9 transports PC

Once ALA9 had been cloned into yeast and then transformed again together with a plasmid containing ALIS1, lipid uptake was measured to test the specificity of the lipid flippase. Fresh transformants containing ALA9 and ALIS1 were grown in selective media containing glucose and then in selective media containing galactose to express the flippase. The yeast cells were then labelled with NBD-lipids and the uptake was measured by measuring the fluorescence intensity by flow cytometry. Figure 7 shows preliminary results from a lipid uptake assay with three clones of ALA9. Both for clone nr 1 and 2, PC was the most transported lipid. ALA9 clone 3 shows different results, but as ALA9 was not successfully cloned in this clone, the difference in the result is to be expected.

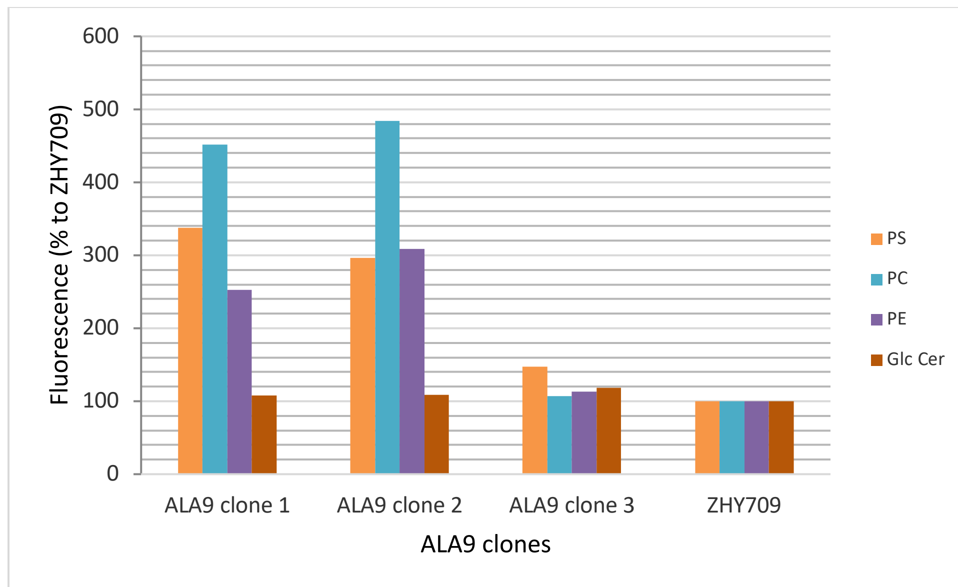

Figure 7. Preliminary result from lipid uptake assay with ALA9. A P4-ATPase deficient yeast strain (ZHY709) was transformed with three different clones of ALA9 and a plasmid containing ALIS1. The yeast cells were labelled with NBD-lipids and the fluorescence intensity was measured by flow cytometry. Yeast strain ZHY709 transformed with empty plasmids was used as a negative control. The lipids tested were PS, PC, PE and GlcCer. Accumulation of NBD-lipids is shown as a percentage of fluorescence intensity relative to ZHY709.

If this result can be repeated, it is interesting since ALA9 seems to be a PC transporter, but it is most similar to ALA12, which is a PS transporter. They have approximately 80 % similarity which means that only a few amino acids differ between the two flippases and thus control which transporter it is. This can be checked by doing an alignment, but this is outside the scope of the thesis. Unfortunately, due to reproducibility problems with the homologous recombination cloning, the lipid transport assay could not be repeated.

3.3 Cloning and ligation of ALA10ΔC-ALA12ΔC

When not being able to proceed with the experiments with ALA9, continued with the C-terminal deletion of ALA10-ALA12 to see if as previously shown, the C-terminal is an inhibitory region. Constructs containing ALA10-ALA12 as well as the truncated genes in a yeast vector was not available and had to be made. The first step was to delete the C-terminal using PCR. The truncated genes were cloned into a cloning vector and transformed into *E. coli.* The colonies of the transformants had to be replated twice before there were enough cells to extract plasmids from. The genes in the cloning vector were then transferred to a yeast expression vector. The cloning vector and the empty expression vector were digested with the same restriction enzymes, run on an agarose gel and cut and purified from the gel and a ligation was started. The ligation product was first only incubated for a few hours at room temperature, then transformed using in-house made heat competent *E. coli* but this did not result in an expression vector with the right inserts. The ligation was performed but with longer incubation time and was then transformed with in-house made heat competent *E. coli,* but the right construct was not obtained. A new ligase buffer was tested to see if the old one had lost its activity due to being thawed too many

times. Commercially made Top10 *E. coli* cells were used instead of in-house made *E. coli* cells since they have a higher efficiency. A different empty plasmid without a HIS-tag was also tested to see if the tag could have disturbed the insert of the truncated genes. Ligation with a new expression vector with a new ligase buffer, incubation overnight in room temperature and transformation using commercially made Top10 *E. coli* cells resulted in a successful construction of ALA10ΔC-ALA12ΔC.

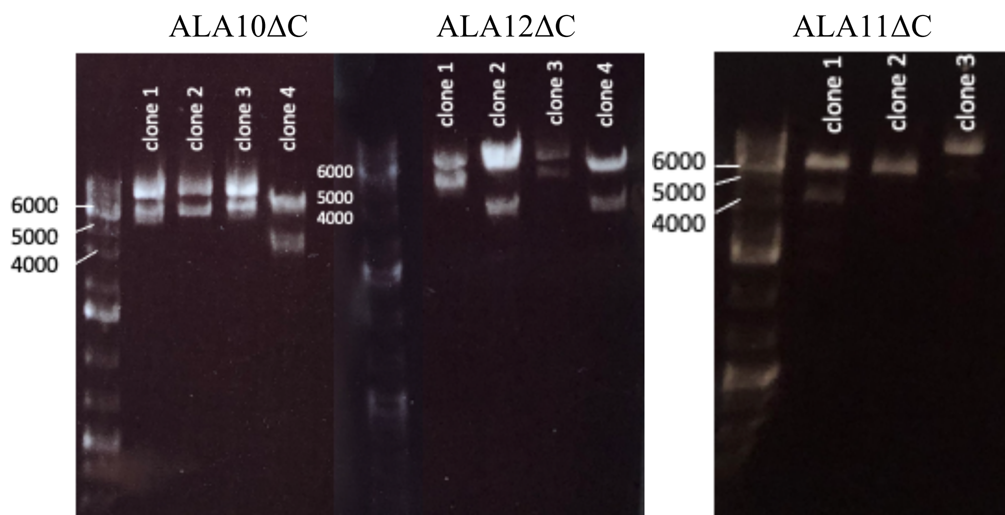

Figure 8. Agarose gel showing the digestion after the ligation for four clones of ALA10ΔC and ALA12ΔC respectively and three clones of ALA11ΔC. Clone 4 for ALA10ΔC, clone 2 and 4 for ALA12ΔC and clone 1 for ALA11ΔC showed the right construction.

After ALA10ΔC-ALA12ΔC was successfully ligated to a yeast expression vector, as seen in figure 8, the plasmids were transformed together with a plasmid containing ALIS1. First, transformation was done using a high efficiency protocol using lithium acetate, PEG and ssDNA, however, no transformants were made. Then a different protocol with higher frequency was tested, which included using yeast grown in a liquid media and an additional incubation step, resulting in successful transformants.

3.4 C-terminally deleted ALA10-ALA12 did not translocate more lipids

After ALA10-ALA12ΔC had been transformed into yeast together with a plasmid containing ALIS1, a lipid uptake assay could be performed. Figure 9 shows the mean of three independent biological samples of ALA10, ALA10ΔC, ALA11, ALA11ΔC, ALA12 and ALA12ΔC.

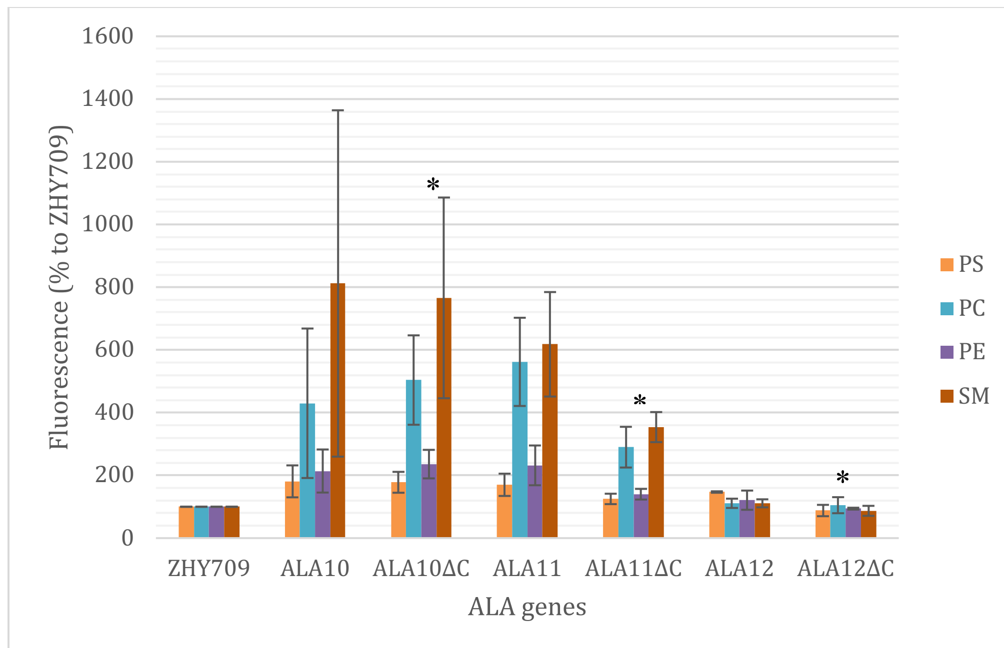

*Figure 9. The mean of three independent biological samples of ZHY709 (negative control), ALA10, ALA10ΔC, ALA11, ALA11ΔC, ALA12, ALA12ΔC. A P4-ATPase deficient yeast strain (ZHY709) was transformed with the different ALA-genes and a plasmid containing ALIS1. The yeast cells were labelled with NBD-lipids and the fluorescence intensity was measured by flow cytometry. Yeast strain ZHY709 transformed with empty plasmids was used as a negative control. The lipids tested were PS, PC, PE and SM. Accumulation of NBD-lipids is shown as a percentage of fluorescence intensity relative to ZHY709. * P≤0.05, significantly different with respect to the full length ALA gene. ** P≥0.05, not significantly different with respect to the full length ALA gene, according to two-way ANOVA.*

There was high variance was obtained for all of the flippases, especially for when transporting PC and SM. The amount of transported lipids varied in the different experiments resulting in large error bars. ALA12 and ALA12ΔC transport close to or even less lipids than the empty vectors. ALA12 is already expressed less than the other ALAs tested (López-Marqués, 2020), therefore less lipid translocation is expected, however, there should still be more transportation than the empty vectors.

The results show that deletion of the C-terminal does not result in more lipid transportation than the full length proteins. This can be due to three reasons: the expression level changes when the C-terminal is deleted, the location changes or that the flippase gets inactivated when the C-terminal is removed. To test whether the expression levels were changing upon deletion of the C-terminal, the amount of protein was tested in western blot.

3.5 Western blot can show if the expression level of the flippase changes when removing the C-terminal end

To test if the expression level changes when the C-terminal is deleted from ALA10-ALA12, a western blot was made. With a western blot, specific proteins can be tested and the amount of proteins can be compared. Samples from the third trial of lipid uptake assay was taken to test if

there is a difference in the amount of protein for the full length ALAs compared to the C-terminally deleted ALAs. The proteins were isolated using a lysis buffer and several steps of centrifugation. The proteins were then run on a SDS PAGE and then transferred onto a nitrocellulose membrane. To detect the desired protein, a primary antibody from mouse compatible with a HIS-tag was used and then a secondary antibody from goat binding to the primary antibody. The substrates added reacted with the enzyme bound to the secondary antibody. The change in color could be detected using a Bio-Rad Imager, the image can be seen in figure 10.

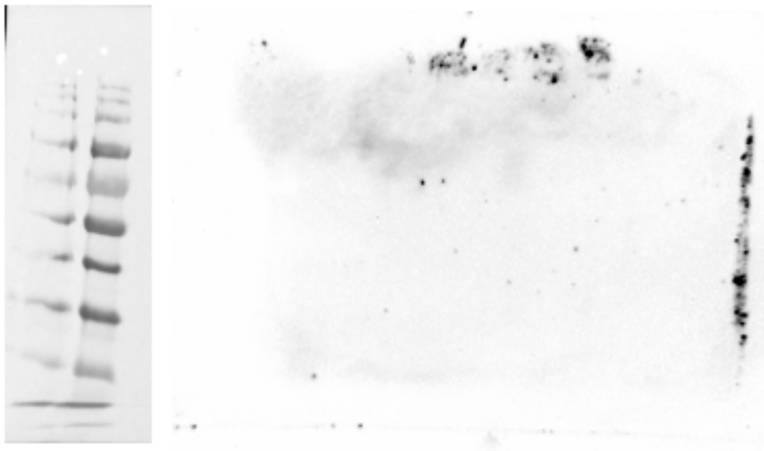

Figure 10. Western blot image. The proteins were transferred to the nitrocellulose membrane, which was incubated with the RGS-His antibody. Afterwards, the secondary antibody goat-anti mouse was incubated. The reaction with the SuperSignal ® West Dura Extended Duration Substrate allows the detection of the proteins coupled to the RGS-His tag. No proteins could be detected.

Unfortunately, the western blot did not work and due to time constraints, the experiments could not be repeated. It was found out after the experiment that the samples did not have the tag to match the primary antibody, and therefore any detection was impossible. It is important to have a positive control to see that the transfer has been made and that the antibodies work. There are many things that can go wrong when doing a western blot. There has to be a tag on the protein to match with the primary antibody. Then the secondary antibody has to be made in a different host than the primary antibody to be able to match. The gel in SDS-PAGE can be too old (dry) which results in the samples not running as they should. In this western blot however, the SDS-PAGE seem to have worked and the samples ran as they should and also the transfer to the membrane should have worked since the ladder was transferred. Another reason for no detection in western blot is the samples being too diluted and that there are not enough protein to be detected. The total protein membrane concentration should be tested and it can also be that there were some losses along the way. Based on the lipid transport assay and the western blot results, it cannot be concluded whether the C-terminal end of the proteins is autoinhibitory.

3.6 Growing plants under phosphate deficient conditions

After doing different experiments with flippases and lipids in yeast, it would be interesting to see how actual plants grow using lipids as a source of phosphorus. An experiment with wild type and a *ala10/11* mutant using three different kinds of media; full nutrition, no phosphate and lipids media was conducted. The seeds were sterilized and put onto the growing system. The growing system consists of conical containers filled with media containing agar, the containers are in turn placed onto a supporter which in turn is placed on a tray. The tray is filled with the media. In one grow system, there are 18 spots. Nine of them are wild type and nine are the double mutant. After germination at 4°C for 48 hours, the trays with the seeds are move to the greenhouse. Pictures were taken after 2 and 4 weeks in the greenhouse.

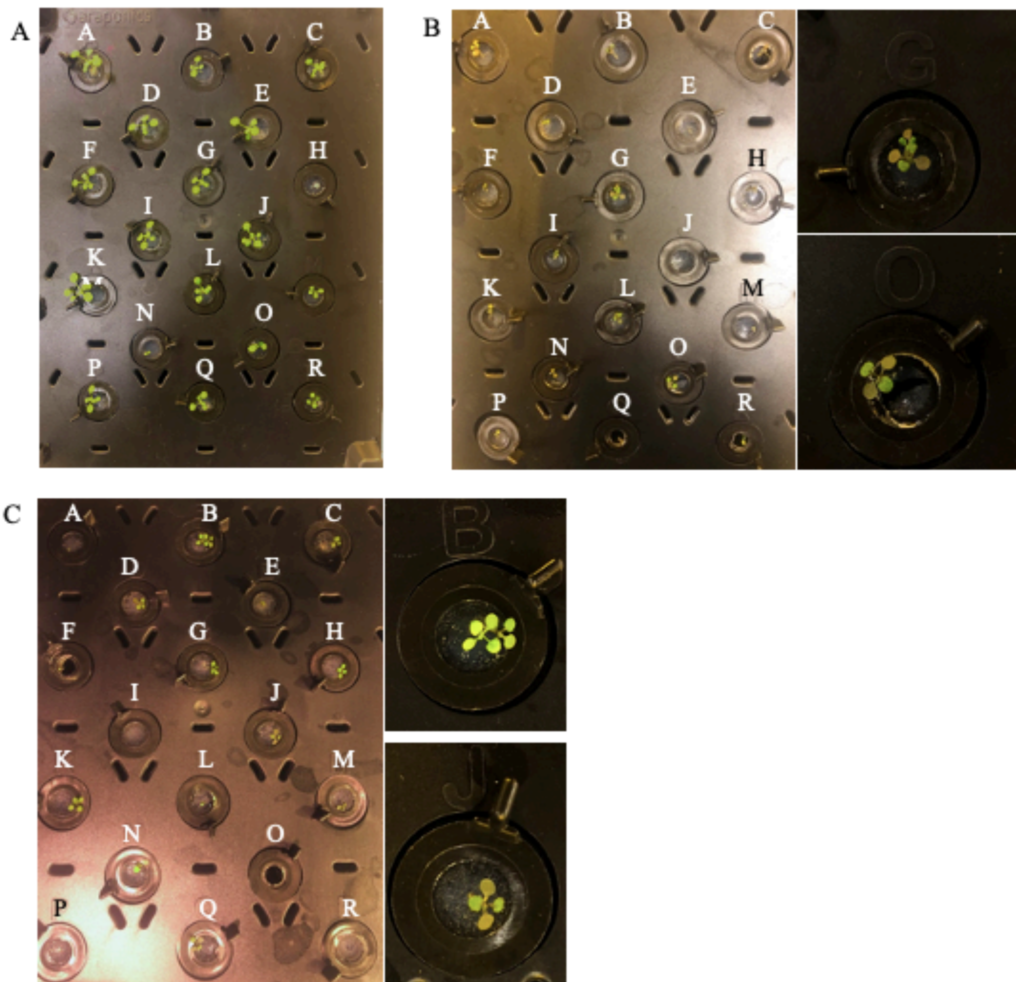

Figure 11. Plant growth after 2 weeks. A-I, wild type; J-R, ala10/11 mutant. A. Full nutrition media. B. Left: no phosphate media. Top right: wild type with purple leaves, no phosphate media. Bottom right: Mutant with purple leaves, no phosphate media. C. Left: Lipid media. Top right: Wild type with green leaves, lipid media. Bottom right: Mutant with purple leaves, lipid media.

For the plants with full nutrition media, all of them were growing, except for one wild type (H) and one mutant (N), as seen in figure 11A. The reason can be that the seed died during the sterilization or that there was not enough agar in the conical container for it to grow properly.

In figure 11B, for the plants with no phosphate media, all that have grown have grey/purple leaves. It can be seen that there is something wrong with the agar in the conical containers in C, R and Q, probably due to something happening when preparing the conical containers with agar. The mutant in J did not grow at all, probably due to the seed not being viable.

In figure 11C, for the plants with lipids media, some of the plants are green and some are grey/purple. Four of the wild type (A, E, F and I) and three of the mutant (O, P, and R) are not growing. The wild type plants that are still growing are still green and for the mutants, some are green and some have grey/purple leaves.

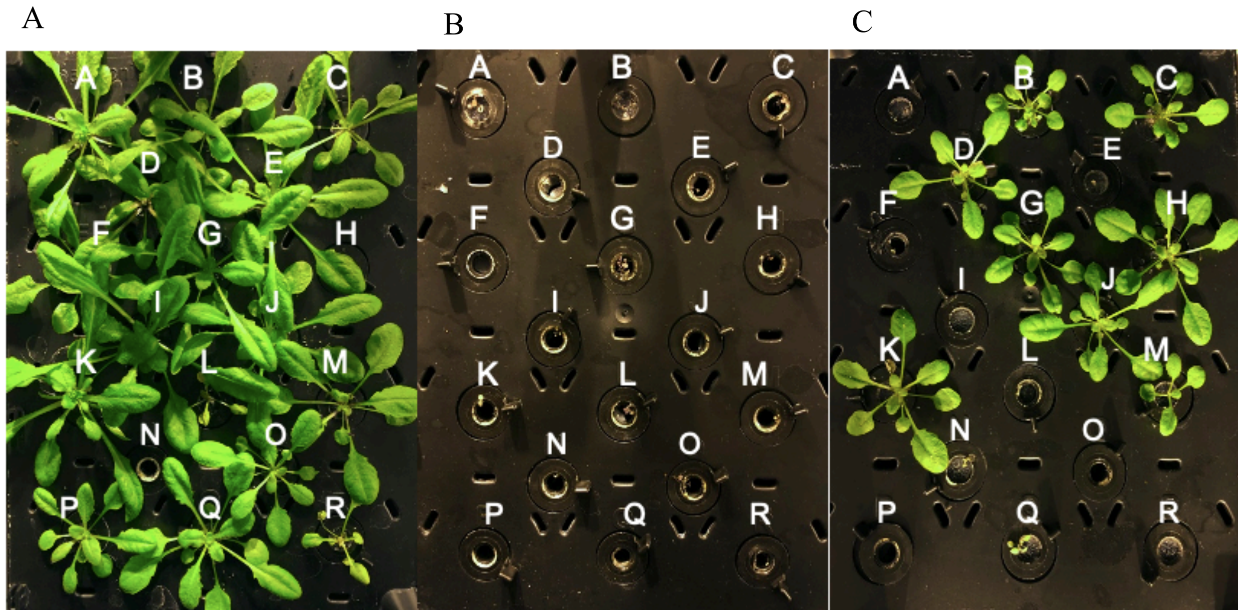

Figure 12. Plant growth after 4 weeks. A-I, wild type; J-R, ala10/11 *mutant. A. Plants grown with full nutrition media. B. Plants grown with no phosphate media. C. Plants grown with lipid media.*

After 4 weeks, for the full nutrition media, all of the plants are growing expect one wild type (H) and one mutant (N), same as for 2 weeks, which can be seen in figure 12A. For the plants growing in the phosphate free media (figure 12B), all of the plants are dead after 4 weeks. In figure 12C, five of the wild type plants are growing and three of the mutant plants. From the photo taken after 2 weeks, three of the mutants (L, N and Q) have died. All of the wild type plants still grew from after 2 weeks.

There are some aspects of this set up that result in uncertainty. Aeration was not implemented resulting in the lipids not being evenly distributed in the tray. They were also not easy to dissolve. The plants with full nutrition grew more than the plants with lipid media however, growth could still be seen for plants with lipid media. The experiment was conducted in as sterile conditions as possible, but it is still possible that the media could have been contaminated and that the lipids may have been degraded and therefore the plants were in fact taking up phosphate and not lipids. It did not look contaminated, which leads to the conclusion that the plants can grow with a media containing no phosphate but with lipids.

4 Perspective

Based on the results of this study, there are experiments that need to be done to be able to understand what the role of the C-terminal in *Arabidopsis* is. Since the deletion of the C-terminal did not result in more lipid translocation, the localization and the activity of the lipid flippases need to be tested. A recent paper on mammalian P4-ATPase suggests that the C-terminal sequence plays a key role in their intracellular trafficking (Okamoto et al., 2020). Mislocalized flippase will lead to the flippase not performing its functions properly. To test if the flippases change location after C-terminal deletion, staining and confocal laser scanning microscope can be used (Poulsen et al., 2015).

It can also be that the flippase gets inactivated when the C-terminal is deleted, contradicting studies done on flippases in yeast. The protein may not be processed correctly or it is not trafficked correctly as mentioned above, leading to the flippase not being able to transport any lipids. To test the activity, an arsenic-based Baginski assay can be used, which is a colorimetric assay for free inorganic phosphate (Timcenko et al., 2019).

5 Conclusion

In the present master thesis project, a set of plasmids containing C-terminal deletions of ALA10-ALA12 were constructed and the lipid transportation was tested for these flippases. The results could not show that the C-terminal deletion resulted in more active flippases. Preliminary results were also obtained from ALA9, showing that ALA9 is a PC transporter but unfortunately no conclusions could be drawn.

Regarding if removal of the C-terminal end results in higher activity, it is too early to say if C-terminal deletion lead to higher level of lipid translocation. Future research must be done to fully answer the research questions. The localization of the flippase with C-terminal deletion and enzyme activity needs to be tested. This study tested the plant flippases in yeast and the next step would be to continue with the experiments in *Arabidopsis*, to see if plants can be genetically modified to activate ALA9-ALA12.

Only preliminary results were obtained in attempts to answer what amount of lipids in the soil is enough for plants to grow under phosphate deficiency, but the result of plants being able to grow under phosphate deficiency is promising.

References

Axelsen, K. B. and Palmgren, M. G. (1998). Evolution of substrate specificiteis in the P-type ATPase Superfamily. *J Mol Evol*. 46**,** 84-101.

Axelsen, K. B. and Palmgren. M. G. (2001). Inventory of the Superfamily of P-Type Ion Pumps in Arabidopsis. *Plant Physiol.* 126(2), 696-706.

Azouaoui H, Montigny C, Dieudonne T, Champeil P, Jacquot A, Vazquez-Ibar JL, Le Marechal P, Ulstrup J, Ash MR, Lyons JA, et al. (2017). High phosphatidylinositol 4-phosphate (PI4P)-dependent ATPase activity for the Drs2p-Cdc50p flippase after removal of its N- and C-terminal extensions. *J Biol Chem*. 292, 7954–7970.

Better Crops (1999). Functions of Phosphorus in Plants. *Better Crops.* 83, 6-7.

Burgers, P. M. J. (1999). Overexpression of Multisubunit Replication Factors in Yeast. *Methods* 18(3), 349-355.

Busman, L., Lamb, J., Randall, G., Rehm, G. and Schmitt, M., (2002). *The Nature of Phosphorous in Soil*, Regents of the University of Minnesota.

Catty, P., de Kerchove d'Exaerde, A. and Goffeau, A. (1997). The complete inventory of the yeast Saccharomyces cerevisiae P-type transport ATPases. *FEBS Lett*. 409, 325–332.

Cordell, D. and White, S. (2014). Life's bottleneck: Sustaining the world's phosphorus for a food secure future. *Annual Review of Environment and Resources*. 39, 161–188.

FAO (Food and Agriculture Organization of the United Nations). (2017). *Current World Fertilizer Trends and Outlook to 2020.*

Gietz, R. D. and Schiestl, R. H. (2007). High-efficiency yeast transformation using the liAc/SS carrier/PEG method. *Nature Protocols*. 2, 31-34.

Gomès, E., Jakobsen, M. K., Axelsen, K. B., Geisler, M. and Palmgren, M. G. (2000). Chilling tolerance in Arabidopsis involves ALA1, a member of a new family of putative aminophospholipid translocases. *Plant Cell*. 12, 2441–2454.

Grant, A. M., Hanson, P. K., Malone, L. & Nichols, J. W. (2001). NBD-labeled phosphatidylcholine and phosphatidylethanolamine are internalized by transbilayer transport across the yeast plasma membrane. *Traffic.* 2, 37–50.

Hua, Z., Fatheddin, P. and Graham, T. R. (2002). An essential subfamily of Drs2p-related P-type ATPases is required for protein trafficking between Golgi complex and endosomal/vacuolar system. *Mol Biol Cell*. 13 (9), 3162-77.

Lenoir, G., Williamson, P., Puts, C. F., Holthuis, J. C. M. (2009). Cdc50p Plays a Vital Role in the ATPase Reaction Cycle of the Putative Aminiphospholipid Transporter Drs2p. *Enzyme Catalysis and regulation.* 284 (27).

López-Marqués, R. L., Poulsen, L. R., Hanisch, S., Meffert, K., Buch-Pedersen., M. J., Jakobsen, M. K., Pomorski, T. G., Palmgren, M. G. (2010). Intracellular Targeting Signals and Lipid Specificity Determinants of the ALA/ALIS P_4-ATPase Complex Reside in the Catalytic ALA α-Subunit. *Mol Biol Cell*. 21 (5), 791-801.

López-Marqués, R. L., Theorin, L., Plamgren, M. G. and Günther Pomorski, T. (2014). P4-ATPases: lipid flippases in cell membranes. *Pflugers Arch*. 466 (7) 1227-1240.

López-Marqués, R. L. October 29 2020.

Okamoto, S., Naitoa, T., Shigetomia, R., Kosugib, Y., Nakayamaa, K., Takatsua, H. & Shina, H-W. (2020). The N-or C-terminal cytoplasmic regions of P4-ATPases determine their cellular localization. *Molecular Biology of the Cell*. 31, 2115-2124.

Palmgren, M., Østerberg, J. T., Nintemann, S. J., Poulsen, L. R. and López-Marqués, R. L. (2019). Evolution and a revised nomenclature of P4 ATPases, a eukaryotic family of lipid flippases. *Biochimica et Biophysica Acta (BBA) - Biomembranes*. 1861, 1135–1151.

Poulsen, L. R., López-Marqués, R. L., McDowell, S. C., Okkeri, J., Licht, D., Schulz. A., Pomorski. T., Harper. J. F. and Palmgren. M. G. (2008). The Arabidopsis P4-ATPase ALA3 localizes to

the golgi and requires a b-subunit to function in lipid translocation and secretory vesicle formation. *Plant Cell*. 20, 658–676.
Poulsen, L. R., López-Marqués, R. L., Pedas, P. R., McDowell, S. C., Brown, E., Kunze, R., Harper, J. F., et al. (2015). A phospholipid uptake system in the model plant *Arabidopsis thaliana*. *Nature Communications*. 6, 7649.
Prezant, T. R., Chaltraw, W. E. Jr. and Fischel-Ghodsian, N. (1996). Identification of an overexpressed yeast gene which prevents aminoglycoside toxicity. *Microbiology*. 142, 3407–3414.
Ripmaster, T. L., Vaughn, G. P. and Woolford, J. L. Jr. (1993). DRS1 to DRS7, novel genes required for ribosome assembly and function in Saccharomyces cerevisiae. *Mol. Cell. Biol*. 13, 7901–7912.
Schachtman, D. P., Reid, R. J. and Ayling, S. M. (1998). Update on Phosphorus Uptake by Plants: From Soil to Cell. *Plant Physiology*. 116, 447–453.
Shenoy, V. V. and Kalagudi, G. M. (2005). Enhancing plant phosphorus use efficiency for sustainable cropping. *Biotechnology Advances*. 23, 501–13.
Timcenko, M., Lyons, J. A., Januliene, D., Ulstrup, J. J., Dieudonne, T., Montigny, C., Ash, M. R., Karlsen, J. L., Boesen, T., Kuhlbrandt, W., et al. (2019). Structure and autoregulation of a P4-ATPase lipid flippase. *Nature*. 571, 366–370.
Natarajan, P., Liu, K., Patil, D. V., Sciorra, V. A., Jackson, C. L., Graham T. R. (2009). Regulation of a Golgi flippase by phosphoinositides and an ArfGEF. *Nat Cell Biol*. 11, 1421–1426.
Nintemann, S. J., Palmgren, M. and Lopéz-Marqués, R. L. (2019). Catch You on the Flip Side: A Critical Review of Flippase Mutant Phenotypes. *Trends in Plant Science*. 24(3) 468-478.
van der Mark, V. A., Oude Elferink, R. P. J. and Paulusma, C. C. (2013). P4 ATPases: flippases in Health and Disease. *Int. J. Mol. Sci.* 14(4) 7897-7922.
van Meer, G. (2011). Dynamic transbilayer lipid asymmetry. *Cold Spring Harb. Perspect. Biol*. 3
Wicky, S., Schwarz, H. and Singer-Krüger, B. (2004). Molecular interactions of yeast Neo1p, an essential member of the Drs2 family of aminophospholipid translocases, and its role in membrane trafficking within the endomembrane system. *Mol. Cell. Biol*. 24, 7402–7418.
Yang, Y., Lee, M. and Fairn, G. D. (2018) Phospholipid subcellular localization and dynamics. *J. Biol. Chem*. 293, 6230–6240.
Zimdahl, R. L. (2015). Chapter 5- Phosphorus. In Zimdahl, R. L. (ed.) *Six Chemicals That Changed Agriculture*. 73-88.

Popular abstract

Phosphorus is a fundamental nutrient for plants without which plants cannot grow. However, phosphorus compounds tend to have low solubility which results in less available phosphorus for the plants in the soil. To compensate for this, millions of tons of phosphate-based fertilizers are used every year in the agricultural sector. These fertilizers are expensive, come from a limited resource and they are also responsible for a large proportion of freshwater pollution.

However, under conditions of phosphate limitation, the production of certain proteins, called flippases, is initiated, increasing the uptake efficiency of plant cells. These flippases transport complex phosphate-containing organic compounds, such as lipids, from the outside to the inside of the plant cells. In the model plant *Arabidopsis*, the flippase ALA10 is responsible for taking up lipids through the root. Three other flippases (ALA9, ALA11 and ALA12) are 75 % similar to ALA10, suggesting that they might be involved in similar functions.

The hypothesis is that hyperactivation of ALA10 (and/or its related ALA proteins) will increase lipid uptake, mimicking conditions of phosphate deficiency and enhancing phosphate uptake, even under normal nutritional conditions. The aim is to design a strategy to obtain proof of this concept. This was done by first deleting the inhibitory end part of the flippase and then perform a lipid uptake assay. The amount of lipids taken up was compared between the mutated version of the flippase and the full length flippase. An experiment involving growing *Arabidopsis* wild type and a mutant lacking ALA10 and ALA11 was also performed to see if the plants can growth with lipids as a phosphate source.

The result shows that removing the end part of the flippase is not enough for the flippase to increase the uptake efficiency. Further experiments are needed to fully understand what happens when the end part of the flippase is removed and if the activity can be increased. The growth experiment suggests that plants can grow with a media containing lipids. This project will contribute to a better understanding of how plants can be engineered in order to improve phosphate utilization and remove the need for inorganic phosphate-based fertilizers, which are a big source of pollution of groundwater.