

The Development of Genetically Encoded Tools to Modulate Phosphatidic Acid Level at the Plasma Membrane *in planta*

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IV. List of Abbreviations

AH	amphipathic helix
AtPAH1	<i>Arabidopsis thaliana</i> PAH1
Avr	avirulence protein
CBL1	Calcineurin B-like protein 1
CBL1n12	12 first amino acids of CBL1
C-LIP	C-terminal LIPIN domain
cMT	cortical microtubule
DAG	diacylglycerol
DGK	DIACYLGLYCEROL KINASE
DGPP	diacylglycerol polyphosphate
DPP	diacylglycerol pyrophosphate phosphatase
ER	endoplasmic reticulum
HAD-like	haloalkanoic acid dehalogenase-like
h.p.i.	hours post infiltration
LPP	phospholipid phosphatase
MAP65-1	65-kDa microtubule-associated protein 1
MKK7	Mitogen-activated protein kinase kinase 7
MKK9	Mitogen-activated protein kinase kinase 9
M-LIP	middle LIPIN domain
MmLIPIN	<i>Mus musculus</i> LIPIN (mouse PAP)
NCR	non-conserved region

N-LIP	N-terminal LIPIN domain
NLR	Nucleotide-binding leucine-rich repeat receptor
PA	phosphatidic acid
PAH	Phosphatidic acid hydrolase
PAP	Phosphatidic acid phosphatase
PBR	poly basic region
PBS1	AvrPphB susceptible protein 1
PC	phosphatidyl choline
PE	phosphatidyl ethanolamine
PG	phosphatidyl glycerol
PI	phosphatidyl inositol
PI(4)P	phosphatidylinositol-4-phosphate
PI(4,5)P ₂	phosphatidylinositol-4,5-bisphosphate
PLA	PHOSPHOLIPASE A
PLC	PHOSPHOLIPASE C
PLD	PHOSPHOLIPASE D
RIN4	RPM1-interacting protein 4
RPM1	<i>Pseudomonas syringae</i> pv. <i>maculicola</i> 1
RPS5	Resistance to <i>Pseudomonas syringae</i> 5
ScPah1p	<i>Saccharomyces cerevisiae</i> Pah1p (yeast PAP)
SOS1	Sodium/hydrogen exchanger 1
TAG	triacylglycerol
UBQ10	UBIQUITINE10
W-domain	Tryptophan domain

1 Introduction

1.1 The plasma membrane as a signaling compartment – more than just a barrier

For a long time, the plasma membrane was considered to be nothing but a simple barrier separating the inner cell from the external environment. This point of view changed over the decades thanks to countless studies. Today we know that the plasma membrane hosts a huge variety of different proteins which fulfill important functions for the cell and the organism, such as for example transducing chemical signals into cellular responses or perceive and translate signals from outside the cell into an inner cellular action (1-3).

The plasma membrane mainly consists of different lipids, such as phospholipids and cholesterol, proteins that are embedded into the membrane bilayer and carbohydrate groups which are connected to different lipids and proteins (3). Today we know that the huge variety of different lipids at the plasma membrane, but also at all other compartments of the cell, fulfill more functions than just being structural components forming the bilayer. Especially low abundant lipids, like e.g. PA, PS and PI, also act as signaling lipids. These lipids are synthesized or degraded by phosphatases, lipid kinases and phospholipases, which manage a rapid turnover of those signaling lipids hereby regulating their function as secondary messenger but also alter the physiochemical properties of the membranes (e.g. membrane curvature or charge) (3-5).

This dynamic system underlines the importance of the plasma membrane as a signaling compartment and opens up the question how the plasma membrane modulates cell mechanics (3).

1.2 Glycerophospholipids – A heterogeneous lipid family

Plants are confronted with all kinds of environmental changes and stressful situations throughout their whole life and as sessile organisms, they have to adapt to these new challenges as quickly as possible. One key player to regulate stress responses is the signaling molecule phosphatidic acid (PA), a glycerophospholipid that can be found in membranes in all kingdoms of life (6).

Glycerophospholipids are essential biomolecules and are best known as structural components of membranes, but also form a diverse group of signaling molecules (Figure 1 A) (6). All glycerophospholipids are fatty acids and consist of a diacylglycerol (DAG) backbone. The simplest of all glycerophospholipids is PA, where the hydroxy group of the DAG headgroup is phosphorylated (6). PA can be further modified to other lipids such as diacylglycerol

polyphosphate (DGPP) or phosphatidyl inositol (PI) (which can be further modified by phosphorylation), making PA to an important precursor for phospholipid biosynthesis (6-8). Glycerophospholipids can be classified due to different chemical or structural properties but are mainly distributed into two classes: high and low abundant (8). PC and PE are considered as high abundant glycerophospholipids which mainly (but not only) fulfill a function as “*structural*” lipids forming the lipid layer of membranes. PS, PA and PI and its derivatives are considered as low abundant lipids. These lipids are thought to be “*signaling*” lipids which function as secondary messengers, but also affect the physiochemical properties of the membranes (8).

1.3 Phosphatidic acid distribution and properties

PA forms a group of different molecular species that vary in terms of length and saturation of the fatty acid tail that can be esterified on the sn-1 and sn-2 hydroxyls (6).

PA is found in membranes due to its hydrophobic nature (Figure 1 B). It makes up to 16% of all phospholipids and up to 1% of all lipids found in membranes of plant cells (9). The distribution and level is very much dependent on the life cycle and environmental conditions a plant has to face. In plants PA can be found in the membranes of the ER and plastids, where the main phospholipid turnover takes place, but it can also be found in the plasma membrane and in the nucleus, as nicely illustrated by the PA specific biosensor “*PA biosensor with superior sensitivity*”, also referred to as PASS (7, 10, 11).

Yao and colleagues (2023) demonstrated that members of the Arabidopsis Sec14 protein family are able to transport PA from the ER to the chloroplasts (12). The Sec14 protein family is generally known to transport lipids in eucaryotes from one compartment to another (13). So far, 32 members of the Sec14 protein family have been identified in *Arabidopsis thaliana* (14). Two of those members, Sec14-homologs 5 (SFH5) and SFH7, have been shown to bind and transport PA using *in vitro* assays (12). Yao and colleagues also demonstrated that both, SFH 5 and SFH7, localize to the ER and chloroplasts (12). Furthermore, quantitative lipid analysis of chloroplast lipids from SFH5 and SFH7 deficient Arabidopsis plants revealed significantly decreased level of PA in chloroplasts. Together, these results indicate a disrupted PA transport from the ER to the chloroplasts (12).

PA has the smallest headgroup among all glycerophospholipids, which is shaped in a cone like form and makes it unique compared to the others and affects the shape of membranes high in PA level (6, 15). Biophysical studies of PA in artificial membranes demonstrated that PE is able to form a hydrogen bond with an oxygen of the phosphate group of PA, resulting in the

deprotonation of PA, changing its charge from a mono-ionic (-1) to a di-ionic (-2) molecule (16, 17). This raises the question, if membrane domains of PA that are enriched in PE regulate the localization and/or function of certain proteins in a charge dependent manner and if so, how these membrane domains might be regulated (6, 16, 18).

1.4 Phosphatidic acid biosynthesis

PA level and distribution are very dynamic and can change within a very short amount of time. Here for, a very precise regulatory machinery is needed. The biosynthesis of PA is based on several pathways (Figure 1 C). PA can be synthesized by dephosphorylation of DGPP by the DPPs (also called LPP) and through the sequential acylation of glycerol- 3-phosphate and lysoPA, a PA species that has only one fatty acid tail (6).

The diacylglycerol kinase (DGK) dependent synthesis of PA is another important pathway. In *Arabidopsis thaliana*, this kinase family consists of seven members and is able to phosphorylate DAG to PA in an ATP dependent manner. The DGKs can be separated into 3 subgroups: The Type 1 that contain a transmembrane-domain (DGK1/2), Type 2 (DGK 3/4/6/7) and Type 3 that contains a putative calmodulin domain (DGK5) (19). The activity of the DGKs can directly be inhibited by the drug R59022, even though the effect of the drug on the different kinases seem to dependent very much on the concentration of the drug (6, 19, 20).

But more importantly is the hydrolysis of PC or PE catalyzed by the phospholipase D family (PLD). The *Arabidopsis* PLD protein family consists of 12 members, which can be differentiated into six subgroups: PLD α , PLD β , PLD γ , PLD δ , PLD ϵ and PLD ζ . These six subgroups can be further divided into C2-domain (PLD α 1-3, PLD β 1/2, PLD γ 1-3, PLD δ and PLD ϵ), or phox/pleckstrine homology domain (PLD ζ 1/2) containing proteins (21). The PLD dependent synthesis of PA can be blocked by treatment of primary alcohols such as ethanol, propanol or butanol, even though butanol is the usual choice, as it has three derivatives of which only n-butanol is able to interact with PLDs while the treatment with sec-butanol and tert-butanol can serve as negative controls. The primary alcohols substitute the water molecule during the hydrolysis, resulting in the formation of e.g. phosphatidyl butanol instead of PA (6, 21, 22).

PA levels were shown to be very dynamic and can increase in a short time. Especially stress can cause such a dramatic and fast increase, most likely by specifically increasing the activity of the PLD and DGK protein families. This wide range of (at least) 19 different enzymes gives *Arabidopsis* the opportunity to react very specifically to a specific stress as well as to tightly regulate PA level. Interestingly, PA seems to be a general stress signal, as even “*opposite*

stresses” as cold and heat stress lead to increasing PA level (Figure 2) (6, 23, 24). It was shown by a multitude of studies, that PA biosynthesis is upregulated as soon as plants face stress. This leads to a PA specific recruitment of certain proteins to the plasma membrane, hereby affecting their activity, which leads to the regulation of numerous signaling pathways in response to stress (Table 1) (for more details see chapter 3.2.8, 3.2.9 and 3.2.10) (22-24).

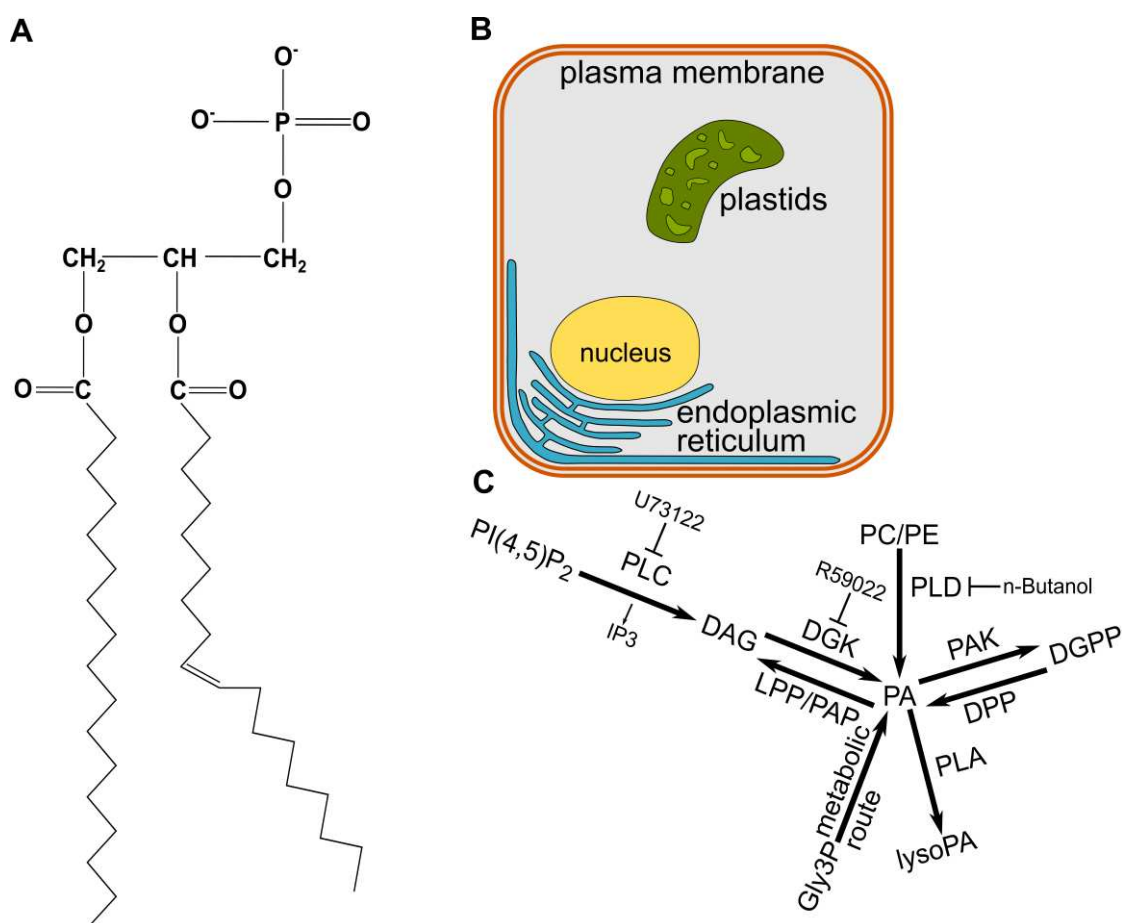


Figure 1 Schematic phosphatidic acid structure, phosphatidic acid distribution and biosynthesis

A) The fatty acid DAG forms the backbone for all glycerophospholipids. Phosphorylation of its hydroxy-group results in the formation of PA, which can be further modified.

B) PA is due to its hydrophobic nature found in membranes of the ER, plasma membrane, plastids and the nuclear laminar.

C) Formation and attenuation of phosphatidic acid. DAG = diacylglycerol; DGPP = diacylglycerol polyphosphate; DGK = diacylglycerol kinase; DPP = diacylglycerol pyrophosphate phosphatase; Gly3-P = glycerol-3-phosphate; IP3 = inositol-tri-phosphate; LPP = phospholipid phosphatase; n-Butanol = alternative PLD substrate that leads to the synthesis of phosphatidyl butanol instead of PA; PA = phosphatidic acid; PAK = phosphatidic acid kinase; PAP = phosphatidic acid phosphatase; PC = phosphatidyl choline; PE = phosphatidyl ethanolamine; PG = phosphatidyl glycerol; PI = phosphatidyl inositol; PI(4,5)P2 = phosphatidylinositol-4,5-bisphosphate; PLA = phospholipase A; PLC = phospholipase C; PLD = phospholipase D; R59022 = DGK inhibitory drug; U73122 = PLC inhibitory drug

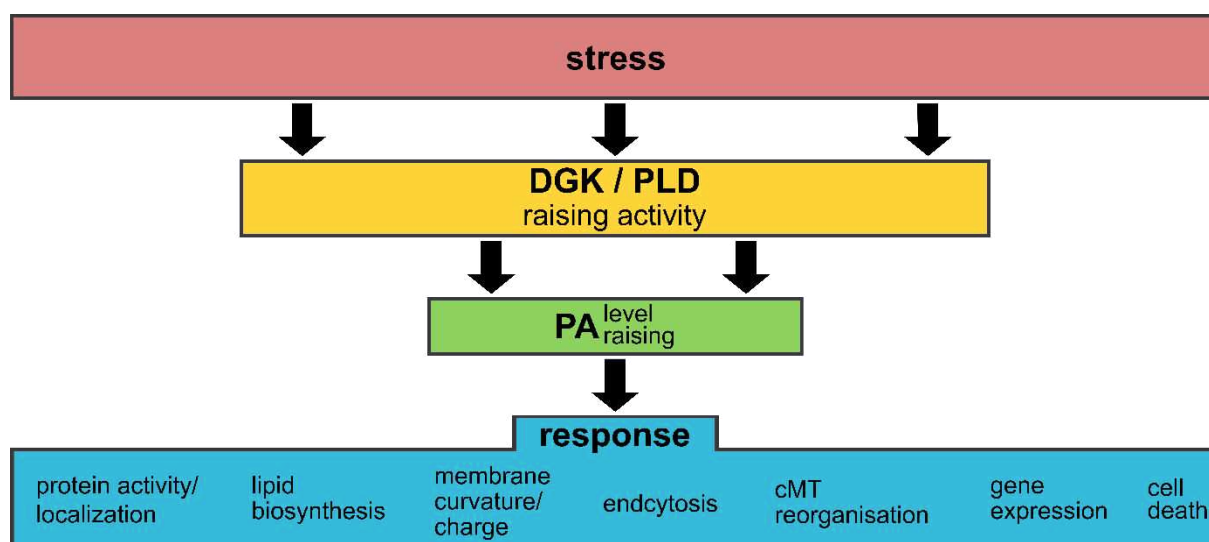


Figure 2 Simplified scheme illustrating that phosphatidic acid forms a bottleneck in plants for proper response to stress

The PA dependent stress response is a complex network. Stress increases the activity of specific DGKs and PLDs, leading to raising PA biosynthesis in specific membrane domains. These elevated PA level lead to distinct and stress specific cellular response. PLD = phospholipase D; DGK = diacylglycerol kinase; PA = phosphatidic acid; cMT = cortical microtubule; HR = hypersensitive response.

Table 1 Raising PA level in response to stress leads to specific protein recruitment to the plasma membrane

This list is adapted from Yao, Xue (2018), Pokotylo et al. (2018) and Li, Wang (2019) and was updated according to the latest literature (22-24).

stress	PA-binding Protein	function	reference
abiotic	ABA2 (abscisic acid deficient 2)	ABA biosynthesis	(25)
salt	ABI1 (abscisic acid insensitive 2 protein phosphatase C2)	dephosphorylation of SnRK2.4	(26)
cold	ACBP1 (acyl-CoA-binding protein)	ABA signaling	(27, 28)
not defined	CTR1 (constitutive triple response 1)	blocks activity of the ethylene receptor ETR1	(29)
salt	KAB1 (Probable voltage-gated potassium channel subunit beta)	not defined	(30)
salt	MAP65-1 (Microtubule associated protein 65)	cortical microtubule bundling	(31, 32)

salt	MKK7 (Mitogen-activated protein kinase 7)	SOS1 phosphorylation	(33)
salt	MKK9 (Mitogen-activated protein kinase 9)	SOS1 phosphorylation	(33)
salt, biotic, cold, chilling, draught, heat	MPK6 (Mitogen-activated protein kinase 6)	SOS1 activation, PIN6 transport, PAMP recognition	(34-38)
biotic stress	MtDef4 (<i>Medicago truncatula</i> Defensin)	antifungal activity	(39)
pathogen, basal resistance	PDK1 (3-phosphoinositide dependent protein kinase-1)	auxin regulation via PINOID	(40-42)
salt, drought	PI4K- γ (Phosphatidylinositol(4) Kinase gamma)	PI(4)P synthesis	(43)
salt	PID (PINOID)	PIN phosphorylation	(44)
dehydration	PLD ρ p1 (PLD-regulated protein 1)	not defined	(45)
salt, osmotic	PTEN2a (phosphatase and tensin homologue)	not defined	(46)
ROS	RbohD/F (respiratory burst oxidase homolog D/F)	stomatal closure	(47)
salt, cadmium	SnRK2.4 (sucrose non-fermenting-1-related protein kinase 2)	tethering to membranes upon stress	(48)
salt, cadmium	SnRK2.10 (sucrose non-fermenting-1-related protein kinase 2)	tethering to membranes upon stress	(48)
phosphate deficiency	SNX1 (Sorting Nexin 1)	endocytosis, PIN2 cycling	(49)

1.5 Aim of the thesis

Plants must face a lot of environmental changes and stressful situations throughout their whole life. These problems are increasing nowadays as global warming and spreading diseases are putting plants, and especially crops, under high selective pressure. These problems lead to a loss in yield and might lead to problems for our modern societies. To better understand how plants react to stress is therefore crucial for a successful agriculture economy, the bases of our modern living. Also, intense agriculture led to salinization and acidification of the soil, resulting

in more problems for farmers around the world. PA was shown to be an important second messenger for plants to regulate all kinds of different stress responses, but so far, we only scratched the surface. To understand how PA regulates stress response might also help to develop new tools to protect crops and/or increase the yield. It is therefore crucial to invent new and optimize already existing techniques to gain further insights in the distribution and dynamics of PA and how this affects transcription, protein activity and cellular response, but also how these changes affect plant physiology.

In this work we wanted to tackle the questions, how the phosphatidic acid phosphatase *Arabidopsis thaliana* PAH1 is structured, where it is localized and if we could use a modified version of this protein to downregulate PA level specifically at the plasma membrane *in planta*. Additionally, we wanted to adapt the *Streptomyces sp.* strain PMF phospholipase D, which was used by R. Tei and J.M. Baskin to upregulate PA level at different compartments in mammalian cells, to specifically upregulate PA level at the plasma membrane *in planta* (50).

The development of genetically encoded tools to manipulate phospholipid abundance at specific compartments is a technique that gained a lot of interest in the past. For example, Simon and colleagues introduced in 2016 a genetically encoded PI(4)P phosphatase to specifically downregulate PI(4)P level at the plasma membrane *in planta*. With the help of this tool they were able to show that the electronegativity of the plasma membrane is mainly driven by PI(4)P abundance (5). Using a similar approach as Simon and colleagues (2016) and R. Tei and J.M. Baskin (2020) we wanted to introduce two new genetically encoded tools to specifically up- and downregulate PA level at the plasma membrane *in planta*. To further verify the functionality of the tools we wanted to test the effect of both tools on the localization and activity of certain proteins that were shown in the past to specifically interact with PA.

We hope that our research sets the ground for new opportunities in the research field of PA in plants and gives scientists new insights that lead to more progression in stress regulation of plants.

2 Identification of a N-terminal helix and a M-LIP domain in *Arabidopsis thaliana* PAH1

2.1 Introduction – Phosphatidic Acid Phosphatases

Lipids are key players in membrane biogenesis and homeostasis, they act as signaling molecules or regulate the energy metabolism. One of key player in regulating these mechanisms is PA. PA is a fatty acid, consisting of a DAG backbone that is phosphorylated at its hydroxy group. PA on the other hand can act as a precursor for the synthesis of other lipids (6, 24). But PA is also an important signaling molecule in a variety of stressful situations in all organisms. This is why a tight regulation of PA level is crucial to ensure proper growth and development (24). Key players in regulating PA distribution are the phosphatidic acid phosphatases (PAPs). PAPs form a unique protein family that catalyze the dephosphorylation of PA to DAG to tightly regulate PA level and/or upregulate triacylglycerol (TAG) level, which is organized in lipid droplets and serves as the major form of fat. (51). There are two different PAP family described so far. The PAP1 family acts in a Mg^{2+} dependent manner, while the PAP2 family (encoded by the *Lipid phosphate phosphatase (LPP)* gene family) acts in a Mg^{2+} independent manner (52). In plants the *LPP* gene family encodes for two proteins that regulate DGPP and PA level. It was shown that LPP1 has a higher affinity for DGPP, while LPP2 binds to DGPP and PA equally well (53). We will focus in the following only on the PAP1 family (from here on just referred to as PAP) which specifically catalysis the dephosphorylation of PA to DAG.

So far there is no PAP described in bacteria or archaea, making them unique to the eukaryotic kingdom. Here on the other hand PAPs can be found in all organisms and in all organisms, they seem to work in a very similar way. The structure of all PAPs is quite comparable. They consist of a N-terminal N-LIP domain and C-terminal C-LIP domain, as well as N- and C-terminal non-conserved-regions (NCR) that have multiple phosphorylation sites. Besides the highly conserved domains one can also find other domains that are not found for all PAPs: the N-terminal amphipathic helix (AH), the polybasic region (PBR), which is by now only described in mammals, the C-terminal tryptophan domain (W-domain), and a semi-conserved M-LIP domain (Figure 3) (54, 55).

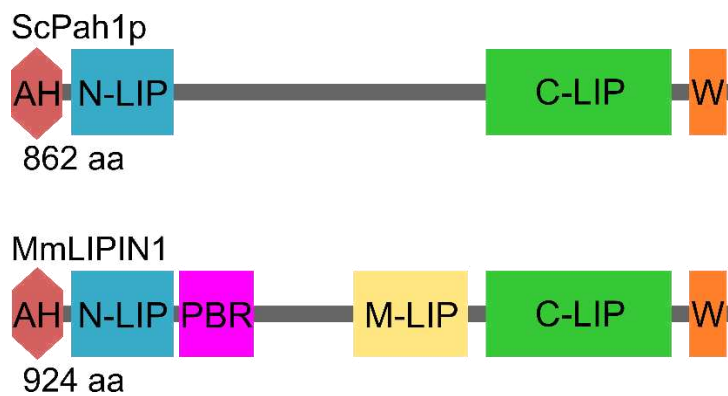


Figure 3 Scheme of yeast Pah1p and mouse LIPIN1 representing different type of PAPs

Schematic protein structure of *Saccharomyces cerevisiae* Pah1p (ScPah1p) and *Mus musculus* LIPIN1 (MmLIPIN1) based on the latest literature (54, 56-59). The grey area represents the non-conserved region. AH = amphipathic helix; PBR = polybasic region; W = tryptophan domain

2.1.1 Phosphatidic acid Phosphatase function and expression

Studies have revealed that PAPs act in a lot of different processes in multiple organisms. For example, in *Saccharomyces cerevisiae* Pah1p (ScPah1p) regulates PA level, TAG content and phospholipid level in general by regulating the transcription of many genes that encode for lipid biosynthetic enzymes (52, 55, 60). Mammals have three PAP encoding genes called *LIPIN1*, *LIPIN2* and *LIPIN3*. In mice, the knockout of *lipin1* causes lipodystrophy, a disease that is characterized by a significant reduction in fat mass and lack of adipocyte differentiation (55, 61). Also, LIPIN1 was shown to be regulated by internal stimuli, as for example insulin seems to regulate its phosphorylation and localization (62). Plants express the two paralogues *PAH1* and *PAH2*. In *Arabidopsis thaliana* PAH1 and PAH2 were shown to be important for phospholipid homeostasis in the ER and in the regulation of the thylakoid membrane in chloroplasts as well as their photosynthetic activity, especially when the plants are growing in the absence of nitrogen (63, 64).

Despite their function as a phosphatase PAPs can also act as transcription co-factors. Santos-Rosa *et al.* (2005) nicely showed that ScPah1p acts in lipid biosynthesis by regulating the expression of key enzymes (60). ScPah1p was shown to be phosphorylated by Cdc28/Cdk1 and dephosphorylated by the Nem1/Spo7 complex. Loss of either *Scpah1* or mutation in the phosphorylation sites cause the transcriptional upregulation of *INO1*, *INO2* and *OPI3*, key enzymes being involved in lipid biosynthesis. They showed that ScPah1p directly binds the promoters of phospholipid biosynthetic enzymes in a Nem1/Spo7-dependent, and hereby in a phosphorylation status dependent, manner. This illustrates, that the influence of PAPs on lipid biosynthesis seems to be more complex than just regulating PA level (60).

PAP expression can vary a lot, depending on the organism. In *Arabidopsis thaliana* PAH1 and PAH2 were shown to be expressed in all tissues, organs and stages of development, but seems to be the highest for seeds during maturation (64).

2.1.2 An N-terminal amphipathic helix regulates the localization ScPah1p

The site of action of ScPah1p was shown to be at the ER and the nucleus. Karanasios *et al* (2010) showed that an amphipathic helix at the very N-terminus of ScPah1p is essential for a proper localization (55). They have identified, with the help of the bioinformatic tool HeliQuest and mutations in the sequence of the putative amphipathic helix, that the first 18 amino acids of the N-terminus are necessary for proper localization (65, 66). Amphipathic helices are described to mediate the reversible binding of proteins with membranes. They were also able to show that point mutations in the HAD-like domain, which result in a catalytic dead phosphatase (D398A, D400A), do not affect ScPah1p localization (55). The HAD-like domain consist of two aspartic acids, which are separated by one random amino acid, can be found in the C-LIP domain of PAP's and is crucial for phosphatase function (for more details see chapter 2.1.5) (55, 67). Karanasios *et al.* designed two ScPah1p mutants to describe the function of the amphipathic helix. One is missing the first amino acids of the N-terminus (Pah1p- Δ [3-16]-GFP) and one with three mutations in the bulky residues (L8A, V11A, W15A, also called PAH1-AH3A) of the putative helix (55). They showed that in both cases the recruitment of the protein to the membrane was completely abolished, highlighting the importance of the N-terminal amphipathic helix. Furthermore, the access of the amphipathic helix to the membrane is dependent on the phosphorylation status of ScPah1p. The Nem1p-Spo7p complex, a protein phosphatase complex that localizes to the nuclear/ER membrane and dephosphorylates ScPah1p, affects the function of the amphipathic helix. The PA-dependent ScPah1p-GFP localization at the ER and nucleus was blocked in *nem1 Δ /spo7 Δ* yeasts, indicating that the Nem1p-Spo7p complex is necessary for this regulation. The authors were able to restore the membrane translocation by expressing a PAH1-7A construct, which is a phosphorylation deficient mutant where seven serine's have been replaced by alanine's, in *nem1 Δ /spo7 Δ* cells. They were also able to show that a ScPah1p-AH3A-7A-GFP fusion was not recruited to the ER or nucleus. This illustrates that dephosphorylated ScPah1p needs the amphipathic helix but not the Nem1p-Spo7p-complex for membrane association and suggests that dephosphorylation of ScPah1p allows the helix to bind to membranes (55).

2.1.3 The N-LIP domain – Crucial for phosphatase activity but of unknown function

The N-LIP domain is highly conserved among all PAPs and was shown to be crucial for PAP activity. For example, the deletion of the N-LIP domain in ScPah1p causes a loss of phosphatase function *in vivo* and is not able to rescue the *Δ pah1* deficient phenotype (56).

The N-LIP domain contains several highly conserved motifs of unknown function. The DI(V)I(V/L)A(V/C)V(I) and the V(I/L)RFGK(R) motifs as well as the conserved amino acids N, M and G can be highlighted. Exchanging the conserved glycine (G) for an arginine cause almost completely loss of protein function. This was shown for multiple PAPs from different organisms. For example, the mutation G80R of ScPah1p causes almost complete loss of phosphatase function *in vitro* and was shown to be not able to rescue the $\Delta pah1$ deficient phenotype in yeast (68). In 2001 Péterfy *et al.* first characterized the mammalian *LIPIN1* and described the homologs *LIPIN2* and *LIPIN3* from mouse and human as well as PAPs from other organisms (*Drosophila melanogaster*, *Caenorhabditis elegans*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Arabidopsis thaliana* and *Plasmodium falciparum*) (61). They described, using transgenic mice, a direct link between *LIPIN1* activity and normal adipose tissue development. These results were important to further understand human lipodystrophy, a sickness that is induced by *LIPIN1* deficiency and is characterized by loss of body fat, and to further understand genetic obesity, which can be induced by overexpression of *LIPIN1* (61, 69). Phosphorylation/dephosphorylation is major mechanisms regulating the activity of PAPs (57). A tight regulation of the phosphorylation/dephosphorylation of PAPs is therefore crucial for a correct function. It was shown that the serine/threonine protein phosphatase-1 catalytic subunit (PP-1c), an important protein dephosphorylation enzyme that is highly conserved in all kingdoms of life, is able to interact with mammalian *LIPIN1* and *LIPIN2* (70). Different regulatory proteins control PP1c activity and in most cases the protein-protein interaction relays on a conserved RVxF binding motif. It was demonstrated that *LIPIN1* and *LIPIN2* interact with PP-1c γ . The authors of this study hypothesize that a conserved HVRF motif in the N-LIP domain that is related to the conserved RVxF binding motif, is crucial for the protein-protein interaction, even though there is no proof for a direct interaction based on the HVRF motif so far. It is also puzzling, that PP-1c γ seems to have a higher affinity to interact with dephosphorylated *LIPIN1* then the phosphorylated protein (70).

Still, the exact mechanism that makes the N-LIP domain so very important for all PAP's need to be revealed.

2.1.4 The M-LIP domain regulates proper phosphatidic acid phosphatase subcellular localization and dimerization

In 2021 Gu and colleagues described a new domain for mouse *LIPIN1*, which they called middle *LIPIN* domain (M-LIP) (59). Gu *et al.* performed hydrogen deuterium exchange mass spectrometry (HDX-MS) to identify new secondary structure formations. HDX-MS determines secondary protein structure by measuring the exchange rate of amide hydrogen with deuterium

(59, 71). The HDX-MS assay highlighted several peptides where no H/D exchange took place when LIPIN1 bound to PC/PA enriched liposomes, suggesting that these peptides were associated with the liposomes. The peptides described were the amphipathic helix at the N-terminus, the Ig-like domain (that is predicted to lie at the membrane surface), the HAD-like domain, the C-terminal end of the C-LIP domain, as well as the C-terminal end of the M-LIP domain.

Mammalian LIPIN's were shown to homo and hetero-oligomerize (72). Gu *et al.* showed that the M-LIP is important for this dimerization, as all three crystal structures of the M-LIP domain formed symmetric or near-symmetric dimers. This result was further verified by the fact that a Δ M LIPIN1 protein shifted towards a lower molecular weight, which further underlines the importance of the M-LIP domain for LIPIN dimerization.

To further confirm the importance of the M-LIP domain for proper membrane binding of LIPIN1 they first performed a liposome sedimentation assay. They found, that deletion of the M-LIP domain resulted in a significant reduction of membrane binding of LIPIN1. They also showed that the M-LIP domain is important for proper subcellular localization. They transiently transfected Cos7 cells with full length and Δ M LIPIN1 (C-terminally fused with mEGFP) and illustrated their localization using confocal microscopy. They found that full length LIPIN1 localized to the ER, while Δ M LIPIN1 also localized to the ER and accumulated in the nucleus, highlighting the importance of the M-LIP domain for proper subcellular localization (59).

A phylogenetic analysis illustrated that this new domain is conserved among all mammalian PAPs but is also present in different other organisms (e.g. *Arabidopsis thaliana*) but cannot be found in ScPah1p (59).

2.1.5 The C-LIP and HAD-like domains provide the phosphatase activity

The C-terminal C-LIP domain is highly conserved among all PAPs and covers the very important haloalkanoic acid dehalogenase like (HAD-like) domain, that consists of two aspartic acids (DxD) which are separated by a random amino acid (in most cases valine or isoleucine) and was shown to be crucial for PAP phosphatase activity (67, 73). The HAD superfamily is a large, ubiquitous expressed enzyme superfamily that can be found in organisms of all three superkingdoms of life (bacteria, archaea and eukaryotes) (67). Approximately 79% of all HADs are phosphatases (also called phosphate monoester hydrolases), while the rest is represented by ATPases and phosphotransferases (67). The phosphatase activity is based on a two-step reaction, that is shared among all HAD family members (67, 74). The first step is the phosphoryl transfer from the substrate to the aspartic acid nucleophile, resulting in an aspartylphosphate,

and the second step is the phosphoryl transfer from the aspartylphosphate to a water molecule. The second aspartic acid residue, which is located two amino acids upstream of the first one, functions as a proton donor in the first step and deprotonates the water molecule in the second step. Mg^{2+} acts as a Co-factor in this two-step reaction. It stabilizes the binding of the nucleophile to its substrate and provides charge neutralization during the transition (67, 74). Besides the very well described phosphatase function, PAPs are also known to act as transcription co-factors. In 2006 Finck *et al.* presented the evidence that LIPIN1 is an important player within the PGC-1 regulatory pathway, which plays an important role in the synthesis of different fatty acids (75). They showed that LIPIN1, LIPIN2 and LIPIN3 interferes with the transcription factor PPAR α and its co-activator PGC-1 α via multiple mechanisms, but mainly by protein-protein interaction with PPAR α and PGC-1 α (as shown by Co-immunoprecipitation assays) and transcriptional activation of PPAR α (75). They also provided the evidence by further Co-immunoprecipitation assays that LIPIN1 also acts as a co-factor by interacting with other hepatic transcription factors including HNF-4 α , an important player for mammalian circadian rhythm and PPAR β and PPAR γ , both also very important for fatty acid synthesis. They have identified a conserved amino acid sequence LxxLL in the C-LIP domain of LIPIN1, which starts 9 amino acids upstream of the HAD-like domain, that is very important for interaction with PPAR α . This motif was also shown to be important for interaction with PGC-1 α . Site-directed mutations in the LXXIL motif of LIPIN1 (LXXIL motif to LXXFF) significantly reduced the interaction and coactivation of PPAR α . Site-directed mutation of the HAD-like domain of LIPIN1 (D712E), did not influence the interaction with, or coactivation of, PPAR α . This result could also be explained by changes in the protein structure and hereby in the accessibility of the substrate, even though the underlying mechanism is unknown. The LXXIL sequence in the C-LIP domain is not just found in mammals but in most animalia and some amoeba. If other organisms also have a similar interaction domain or display transcriptional activity (besides *Saccharomyces cerevisiae*, see introduction) still needs to be revealed (75).

2.1.6 The C-terminal Tryptophan-Domain – Crucial for the survival of yeast but not needed for phosphatase function

In 2017 Park *et al.* analyzed the function of the N- and C-terminal non-conserved regions (NCR) of *Saccharomyces cerevisiae* Pah1p (56). They have identified a WRDPLVDID domain in the C-NCR that is crucial for ScPah1p function *in vivo*. For analysis of the C-NCR of ScPah1p, the authors generated deletions from the C-terminal end and examined the effect *in vivo*. To do so, they performed yeast spotting assays using a temperature-sensitive *pah1 Δ* mutant (lethal at

37°C) and tried to rescue the phenotype with their constructs. They observed that ScPah1p C646 (lacking the residues 647-862) still complemented the temperature-sensitive phenotype of *pah1Δ*, while the C591 mutant (lacking residues 592–862) didn't. These results indicate that an amino acid sequence between the position 592 and 646 is crucial for ScPah1p function *in vivo*, while the rest of the C-NCR is not needed. To identify the crucial amino acids, they compared the ScPah1p sequence with different other PAPs and identified a conserved tryptophan residue at position 637. Mutations at this position (W637A, W637E, and W637R) were not able to rescue temperature-sensitive phenotype of *pah1Δ*, while the exchange of the tryptophan with a phenylalanine, another non-polar aromatic amino acid, was able to rescue the mutant. They also analyzed the effects of W637 mutations on TAG content and PAP catalytic activity using radioactive labeled cells. While the W637A, W637E, and W637R mutations were not able to rescue the defect in TAG content, the PAP activity was not compromised (56).

In 2022 Park *et al.* further examined the function of this C-terminal W-domain (57). They confirmed, using full length ScPah1p and the W637A mutant transformed to *Δpah1* cells analyzed with thin-layer chromatography, that DAG and TAG content was significantly decreased in W637A compared to the wild-type protein. Next, they wanted to address, if the membrane association of ScPah1p is Nem1-Spo7, a protein phosphatase complex crucial for ScPah1p dephosphorylation, dependent (76). They transformed cells deficient in *NEM1* and *SPO7* with wild type ScPah1p and W637A containing plasmids and compared them to cells containing the Nem1-Spo7 complex. The lack of Nem1-Spo7 leads to phosphorylated and therefor cytosolic ScPah1p. After subcellular fractionation and an Immunoblot assay they showed that both wild type ScPah1p and W637A need the Nem1-Spo7 complex to properly locate to membranes. Using a similar approach, they were able to show that in *pah1Δ* background more W637A protein located to the membrane fraction compared to the wild-type protein, while there was no difference in *pah1Δ nem1Δ* cells, indicating that W637 is important for ScPah1p dephosphorylation and therefor membrane association in a Nem1-Spo7 dependent manner. They also confirmed that ScPah1p W637A is still catalytically active. They examined ScPah1p and W637A phosphatase activity in *app1Δ dpp1Δ lpp1Δ pah1Δ* yeast cells. Using an *in vitro* assay with Triton X-100/PA-mixed micelles they confirmed that W637 ScPah1p is still capable of dephosphorylating PA (57).

Two earlier described PAPs from the ciliate *Tetrahymena thermophila*, TtPah1 and TtPah2, show, that the presence or absence of this W-domain is organism independent (77). While TtPah1 has the W-domain, TtPah2, a very small PAP lacking the N- and C non-conserved regions, does not have it, while still showing phosphatase function *in vitro* (58). Knock out

of *Ttpah2* in *Tetrahymena termophila* did not affect cell growth and, in contrast to most other known PAPs, TtPah2 did not regulate lipid droplet number and ER morphology. Also, TtPah2 is not able to rescue Δ *pah1* deficient phenotype in yeast, but it is able to rescue the respiratory defect in the Δ *pah1* yeast (78).

2.1.7 *Arabidopsis thaliana* PAH1 localization

PAP localization was intensively described for yeast Pah1p and mammalian LIPINs. They are described to be found in the cytoplasm, the ER, the nuclear laminar, the nucleus and mitochondria (54, 55, 79). There are, so far, two studies investigating the localization of *Arabidopsis thaliana* PAH1 and PAH2 *in planta*. In 2009 Nakamura and colleagues stated, based on subcellular fractionation and additional immunoblotting, that PAH1 and PAH2 are cytosolic (80). The localization has been further investigated by Eastmond and colleagues in 2010. They transiently expressed PAH1 and PAH2, both N-terminally tagged with a GFP, in *Nicotiana benthamiana* leaves and observed the localization using confocal microscopy. Based on the pictures they stated that both, PAH1 and PAH2, are only found in the cytoplasm (64). Both studies face several problems with their conclusion regarding the localization of PAH1 and PAH2. First, PAPs are conserved in terms of amino acid sequence, protein functions and localization and as described, yeast and mammalian PAPs can be found in a variety of different subcellular compartments. The protein structure of plant PAPs is similar to the ones from yeast and mammals, raising the question why *Arabidopsis* PAH1 and PAH2 should behave completely different to all other well described PAPs. Second, the substrate of PAH1 and PAH2 is PA, a phospholipid only found in membranes of different subcellular compartments and one might expect that a protein is found where its substrate is (24). Third, transient expression of *Arabidopsis* PAH1 was shown to be able to rescue the yeast Δ *pah1* phenotype, which is interesting as ScPah1p localized to different subcellular compartments (81). Also, the author found that AtPAH1 was found in the cytoplasm as well as the nucleus in yeast (81).

That PAPs are predominantly, but not only, found in the cytoplasm has been stated in other studies for other organisms before (81). Thus, we hypothesize that *Arabidopsis* PAPs are not localized only in the cytoplasm. However, in which other compartments and how the localization is mediated remain to be investigated. In the following we tackle the question, where else, besides the cytoplasm, AtPAH1 localizes and how this localization might be mediated.

2.2 Results

2.2.1 Localization of *Arabidopsis thaliana* PAH1 transiently expressed in *Nicotiana benthamiana* leaves

PA phosphatases are highly conserved among all eukaryotes. To gain further insights into the domains of AtPAH1 and thus predict its localization we aligned the amino acid sequence of Arabidopsis PAH1 and PAH2 (AtPAH1, AtPAH2) against mouse LIPINs and yeast Pah1p. Our alignment illustrates that the N-LIP as well as the C-LIP domain of AtPAH1 and AtPAH2 are similar to the ones of mouse and yeast (Figure 4, Figure 5). Our alignment also suggests the presence of a C-terminal W-domain, but whether it is functional in Arabidopsis still remains elusive (Figure 6, Figure 7 A). To further investigate the structure of AtPAH1, we analyzed the protein using AlphaFold2, an artificial intelligence predicting the protein structure based on different parameters (Figure 7 B) (82). This model suggests the presence of the N-LIP and C-LIP domain, comparable to the crystal structure of TtPAH2, as well as a N-terminal helix and a domain in the mid-section of the protein that were described to be important for proper PAP localization in yeast and mammals (Figure 7 A) (58).

To analyze the localization of the protein we transiently expressed *AtPAH1* under control of the ubiquitous promoter *UBIQUITIN10* (*pUBQ10*), with an N- or C-terminal *mTURQUOISE2*, in *Nicotiana benthamiana* leaves (mTurquoise2-AtPAH1; AtPAH1-mTurquoise2) (Figure 7 C). We co-infiltrated mTurquoise2-AtPAH1 and AtPAH1-mTurquoise2 with marker for endoplasmic reticulum (*p35S::VMA12-RFP*), plasma membrane (*pUBQ10::CBL1n12-3x(mCherry)*) and cytosolic (*p35S::RFP*) localization and analyzed the co-localization using ImageJ.

We co-infiltrated mTurquoise2-AtPAH1 and AtPAH1-mTurquoise2 with the ER marker VMA12, a yeast protein important for vacuolar ATPase assembly, and detected the localization using confocal microscopy (Figure 8 A) (83). We analyzed the pictures for co-localization of the proteins using two different tools of ImageJ. First, we used the line intensity plot profile tool of ImageJ (Figure 8 B&C). The line intensity plot profile indicates a colocalization of mTurquoise2-AtPAH1 and AtPAH1-mTurquoise2 with the ER marker VMA12. Second, we used the ImageJ plug-in JACoP (Just Another Colocalization Plugin) (Figure 8 D&E) (84). JACoP calculates the likelihood for colocalization of two proteins imaged using microscopy based on the Pearson's coefficient. Additionally, we also calculated Mander's coefficient where M1 and M2 are the sum of intensities of colocalizing pixels from a channel and dividing it by its integrated density (84, 85). Both coefficients vary from -1 to 1, with -1 stating exclusion, 0 the absence of correlation and 1 a full correlation. Our analysis suggests with a Pearson's

coefficient above 0.8 and a Mander's coefficient above 0.6 that both mTurquoise2-AtPAH1 and AtPAH1-mTurquoise2 co-localize with the ER marker VMA12.

We further investigated the localization of mTurquoise2-AtPAH1 and AtPAH1-mTurquoise2 by co-infiltrating the constructs with CBL1n12-3x(mCherry), a marker for plasma membrane localization (see 3.2) (Figure 9 A). Again, we analyzed the localization of the marker and AtPAH1 by measuring the line intensity plot profile with ImageJ (Figure 9 B&C). Both, the images and the line intensity plot profile indicate a co-localization of mTurquoise2-AtPAH1 and AtPAH1-mTurquoise2 with CBL1n12-3x(mCherry).

We also checked mTurquoise2-AtPAH1 and AtPAH1-mTurquoise2 for cytosolic localization by co-infiltrating with *p35S::RFP* (Figure 10 A). We analyzed the pictures by measuring the line intensity plot profile with ImageJ. This time we draw 2 lines per cell, one line through the nucleus (Figure 10 B) and one through the cell including the plasma membrane (Figure 10 C). Together, both profiles as well as the confocal microscopy images indicate a co-localization of mTurquoise2-AtPAH1 and AtPAH1-mTurquoise2 with the free RFP within the cell, while, other than the free RFP, mTurquoise2-AtPAH1 and AtPAH1-mTurquoise2 were not present in the nucleus.

Together the results suggest that AtPAH1 localized to the ER, plasma membrane and the cytosol.

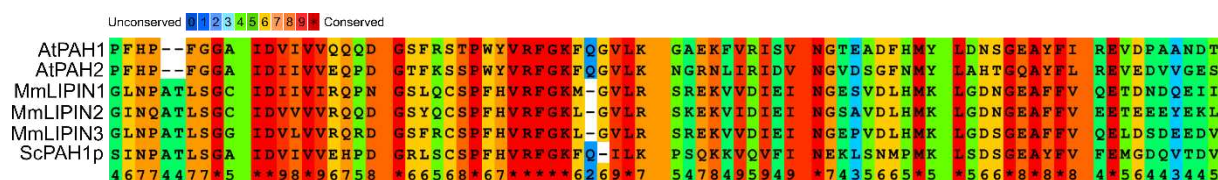


Figure 4 Protein alignment of *Arabidopsis thaliana* PAH1 and PAH2 against mouse and yeast PAPs illustrate the highly conserved N-LIP domain

The N-LIP domain is highly conserved among all PAP's and crucial for the phosphatase function. Nevertheless, the exact mechanism how and why this domain is so important still remains unknown. This protein alignment was done using the online platform PRALINE (6.11.1). At = *Arabidopsis thaliana*; Mm = *Mus musculus*; Sc = *Saccharomyces cerevisiae*

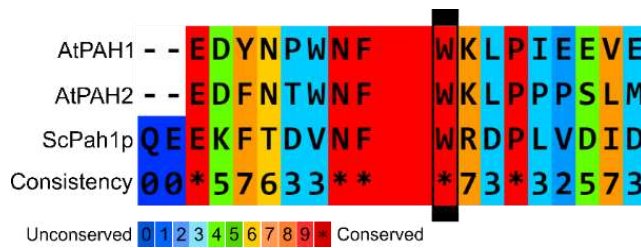


Figure 6 Identification of a potential W-domain in *Arabidopsis thaliana* PAH1 and PAH2

The tryptophan (W)-domain of ScPah1p was shown to be crucial to rescue the *pah1* deficient phenotype in yeast, but not for phosphatase function. If this W-domain also has a function in AtPAH1 and AtPAH2 still remain unknown. The protein alignment was done using the online platform PRALINE (6.11.1). At = *Arabidopsis thaliana*; Sc = *Saccharomyces cerevisiae*

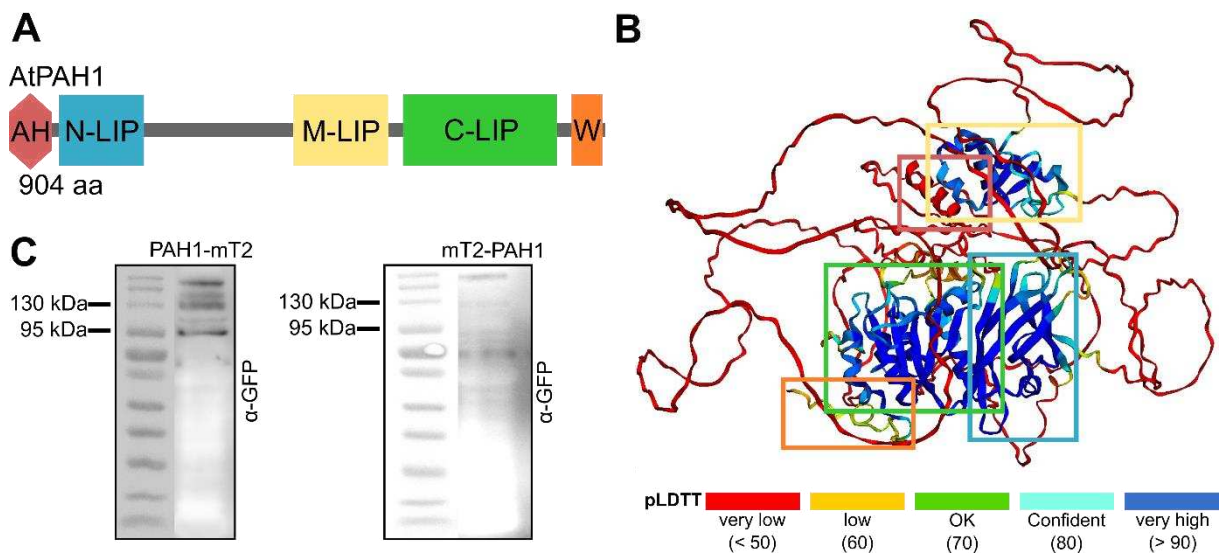


Figure 7 Putative Arabidopsis PAH1 structure

A&B) Putative protein structure based on amino acid sequence alignment against yeast and mouse phosphatidic acid phosphatases and 3D-protein model predicted by AlphaFold2 (6.11.1, 6.11.2). Both analysis predict a N-terminal helix (AH, red), eventhough AlphaFolds prediction is with a very low confidence, a N-LIP domain (blue), a M-LIP domain (yellow), a C-LIP domain (green) and the Trp-domain (W, orange).

C) Immunoblot analysis of mTurquoise2-PAH1 (101 kDa) and PAH1-mTurquoise2 (101 kDa) after transient expression under control of the *UBIQUITINE10* promoter in *Nicotiana benthamiana* leaves using α-GFP.

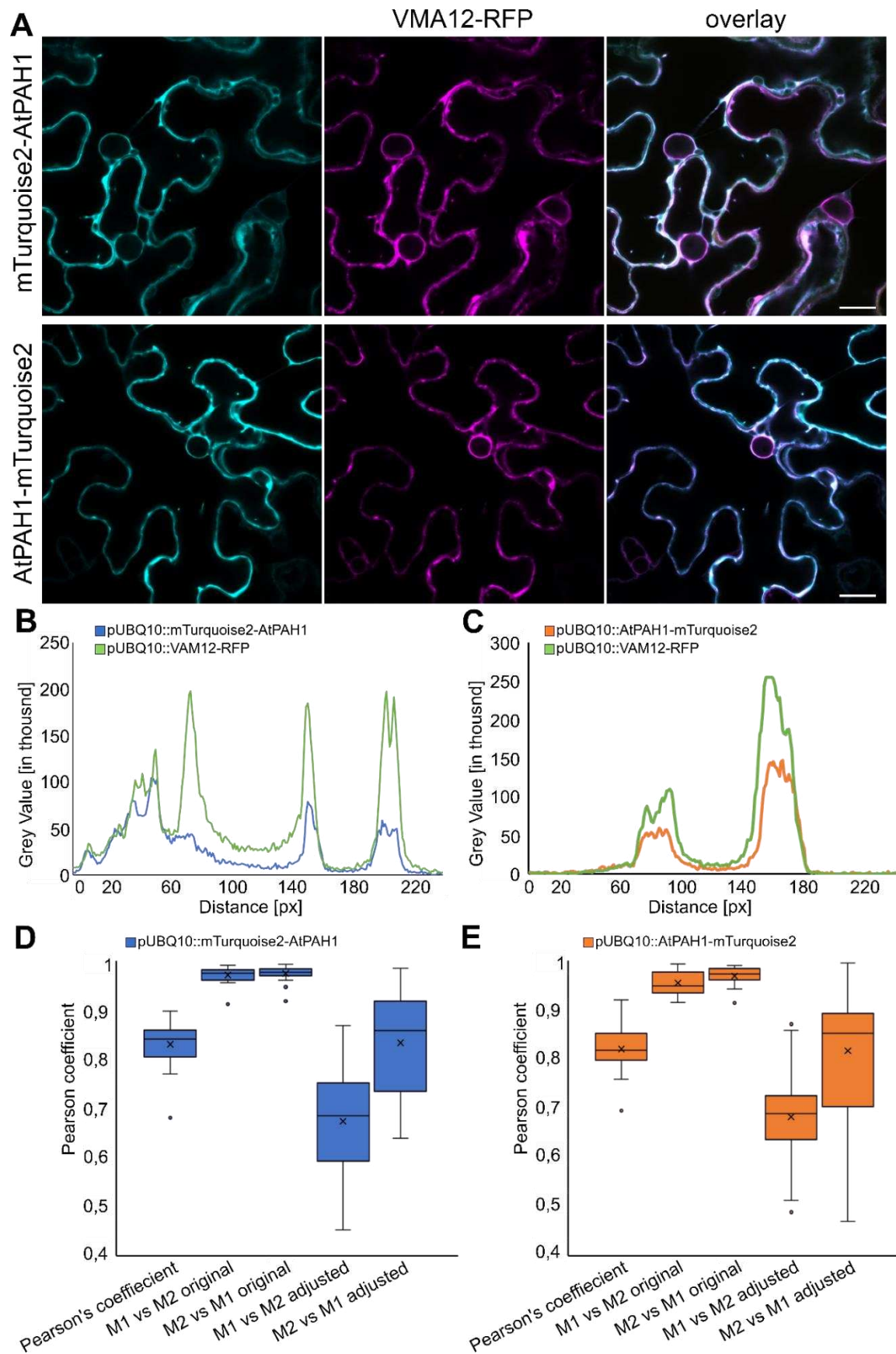


Figure 8 N- and C-terminal tagged AtPAH1 colocalizes with the endoplasmic reticulum-marker VMA12 after *Nicotiana benthamiana* leaf co-infiltration

A) Confocal microscopy images of mTurquoise2-AtPAH1 and AtPAH1-mTurquoise2 co-infiltrated with ER-marker VMA12 in *Nicotiana benthamiana* leaves. Images were taken approximately 40 h.p.i. White bar at the bottom right represents 20µm.

B&C) Line intensity plot profile of mTurquoise2-AtPAH1 and AtPAH1-mTurquoise2 against VMA12. ImageJ was used to analyze the pictures (6.9). For line intensity plot profile picture analysis, a line was drawn crossing the nucleus and / or the plasma membrane.

D&E) Calculations of the Pearson's coefficient and Manders coefficient between mTurquoise2-AtPAH1 and AtPAH1-mTurquoise2 and VMA12. The analysis was performed using the ImageJ PlugIn JACoP (6.9).

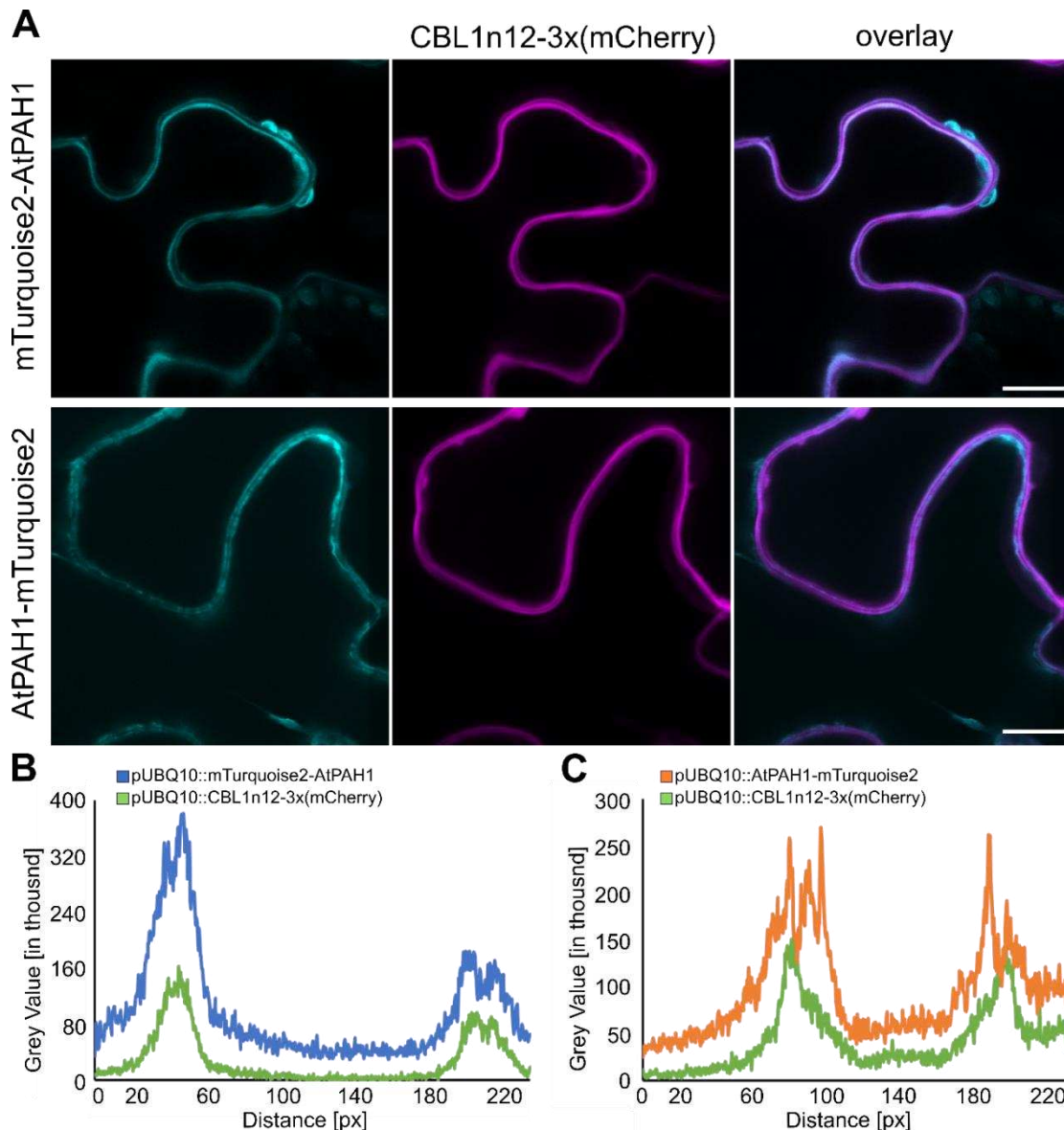


Figure 9 N- and C-terminal tagged AtPAH1 colocalizes with the plasma membrane-marker CBL1n12-3x(mCherry) after *Nicotiana benthamiana* leaf co-infiltration

A) Confocal microscopy images of mTurquoise2-AtPAH1 and AtPAH1-mTurquoise2 co-infiltrated with PM-marker CBL1n12-3x(mCherry) in *Nicotiana benthamiana* leaves. Images were taken approximately 40 h.p.i. White bar represents 10µm.

B&C) Line intensity plot profile of mTurquoise2-AtPAH1 and AtPAH1-mTurquoise2 against CBL1n12-3x(mCherry). ImageJ was used to analyze the pictures (6.9). For line intensity plot profile picture analysis, a line was drawn crossing two plasma membranes.

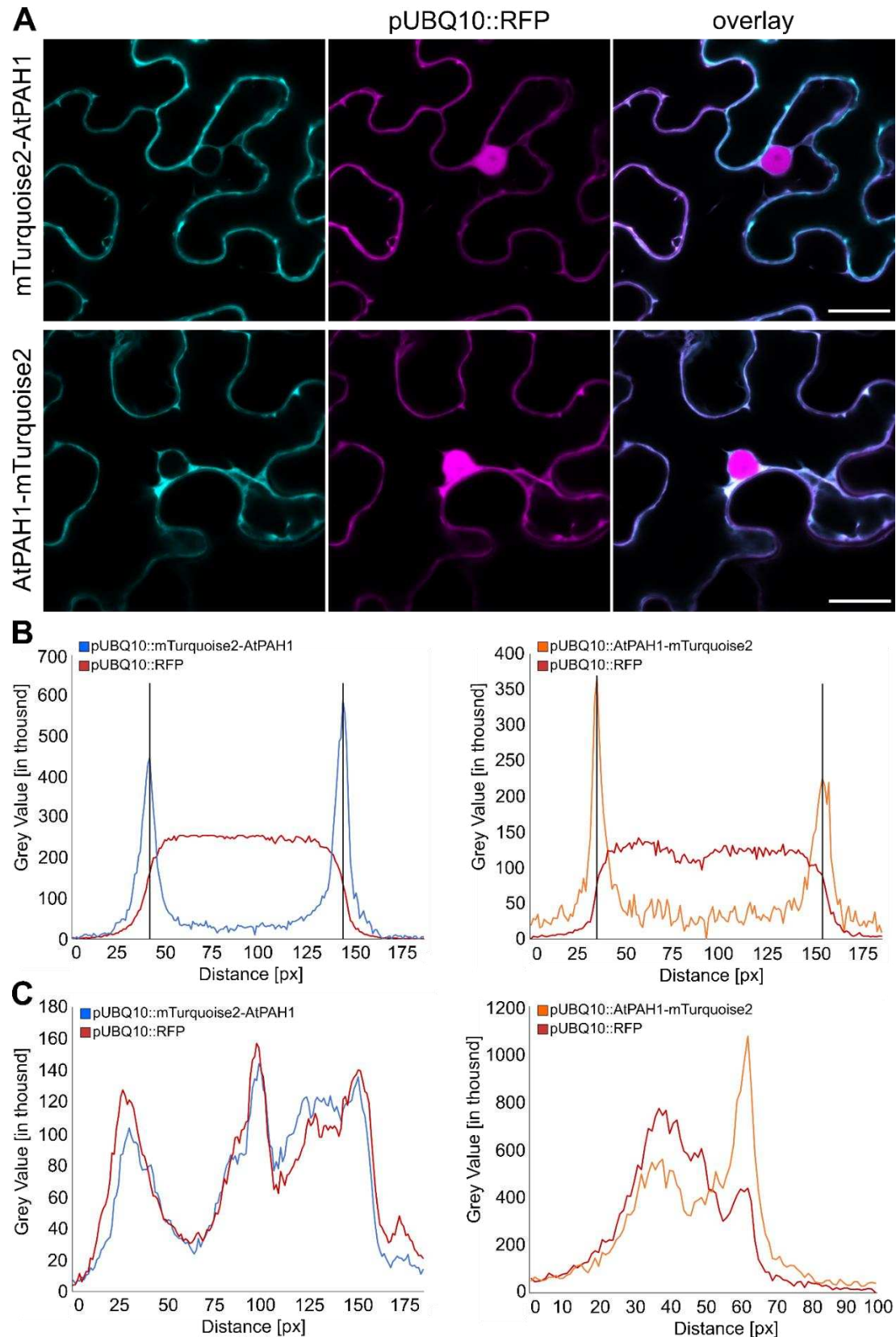


Figure 10 N- and C-terminal tagged AtPAH1 colocalizes in the cytoplasm with free RFP after *Nicotiana benthamiana* leaf co-infiltration

A) Confocal microscopy images of mTurquoise2-AtPAH1 and AtPAH1-mTurquoise2 co-infiltrated with *p35S::RFP* in *Nicotiana benthamiana* leaves. Images were taken approximately 40 h.p.i. White bar represents 35µm.

B&C) Line intensity plot profile of N- and C-terminal tagged AtPAH1 against free RFP. ImageJ was used to analyze the pictures (6.9). The plot in B represents the analysis of a nuclear signal while the plot in C represents the analysis of a plasma membrane and a cytosolic fraction.

2.2.2 Identification of a putative N-terminal helix in *Arabidopsis thaliana* PAH1

It was shown that a N-terminal amphipathic helix (AH) is important for proper localization of the yeast PAP ScPah1p (55). AH are defined by their hydrophobic and polar amino acid side chains which can be found on opposite sides, so that a lipophilic and a hydrophilic surface can form. Here we tried to find out, if AtPAH1 has such a N-terminal helix and if this was also important for proper localization. Therefore, we analyzed the protein using HELIQUEST, a bioinformatical online tool that is capable of predicting the presence of such helices (Figure 11 A) (65, 66). This tool was also used by Karanasios *et al.* to identify the presence of the AH of ScPah1p (55). HELIQUEST suggests the presence of a helix like structure at the very N-terminus of AtPAH1, which is comparable but slightly different to the helix found in ScPah1p. Also, we aligned the amino acid sequence of the N-terminus of AtPAH1 and AtPAH2 against each other and yeast Pah1p using the online tool PRALINE (Figure 11 B) (86-91). The alignment illustrates a certain degree of sequence conservation, were especially the amino acids VGR might to play an important role. These result fit to the prediction of a N-terminal helix in AtPAH1 by AlphaFold2 (see Figure 7 B).

To analyze the importance of the putative N-terminal helix we deleted the first 20 amino acids of AtPAH1 ((Δ AH)AtPAH1) (Figure 11 C). Protein structure analysis with AlphaFold2 suggests that the absence of the first 20 amino acids might not affect the overall protein structure (Figure 11 C&D).

Again, comparable to full length AtPAH1, we co-infiltrated (Δ AH)AtPAH1-mTurquoise2 with the ER marker VMA12 and detected the localization using confocal microscopy (Figure 12 A) (83). We analyzed the pictures for co-localization of the protein using the line intensity plot profile tool and the Plugin JACoP of ImageJ. First, we used the line intensity plot profile tool of ImageJ (Figure 12 B). We measured the line intensity plot profile of the line for each channel by drawing a line through the cell including the cell surface. The result indicates a co-localization of (Δ AH)AtPAH1-mTurquoise2 with the ER marker VMA12. Second, we used the ImageJ plug-in JACoP (Figure 12 C) (84). Also, with a Pearson's coefficient of >0.6 the analysis using JACoP suggests that (Δ AH)AtPAH1-mTurquoise2 co-localized with the ER marker VMA12.

We again investigated the localization of (Δ AH)AtPAH1-mTurquoise2 by co-infiltration with the plasma membrane marker CBL1n12-3x(mCherry) (Figure 13 A). Also, we analyzed the localization of (Δ AH)AtPAH1 by measuring the line intensity plot profile against the plasma membrane marker (Figure 13 B). The confocal microscopy images and the line intensity plot profile both suggest a co-localization of (Δ AH)AtPAH1-mTurquoise2 with CBL1n12-3x(mCherry).

We also co-infiltrated (Δ AH)AtPAH1-mTurquoise2 with *p35S::RFP* to investigate for cytosolic localization (Figure 14 A). Again, we measured the line intensity plot profile with ImageJ. Therefore, we draw 2 lines per cell, one line through the nucleus (Figure 14 B) and one through the cell including the plasma membrane (Figure 14 C). Together, both profiles as well as the confocal microscopy images suggest a co-localization of (Δ AH)AtPAH1-mTurquoise2 with the cytosolic RFP, while, other than the free RFP, (Δ AH)AtPAH1-mTurquoise2 was not present in the nucleus.

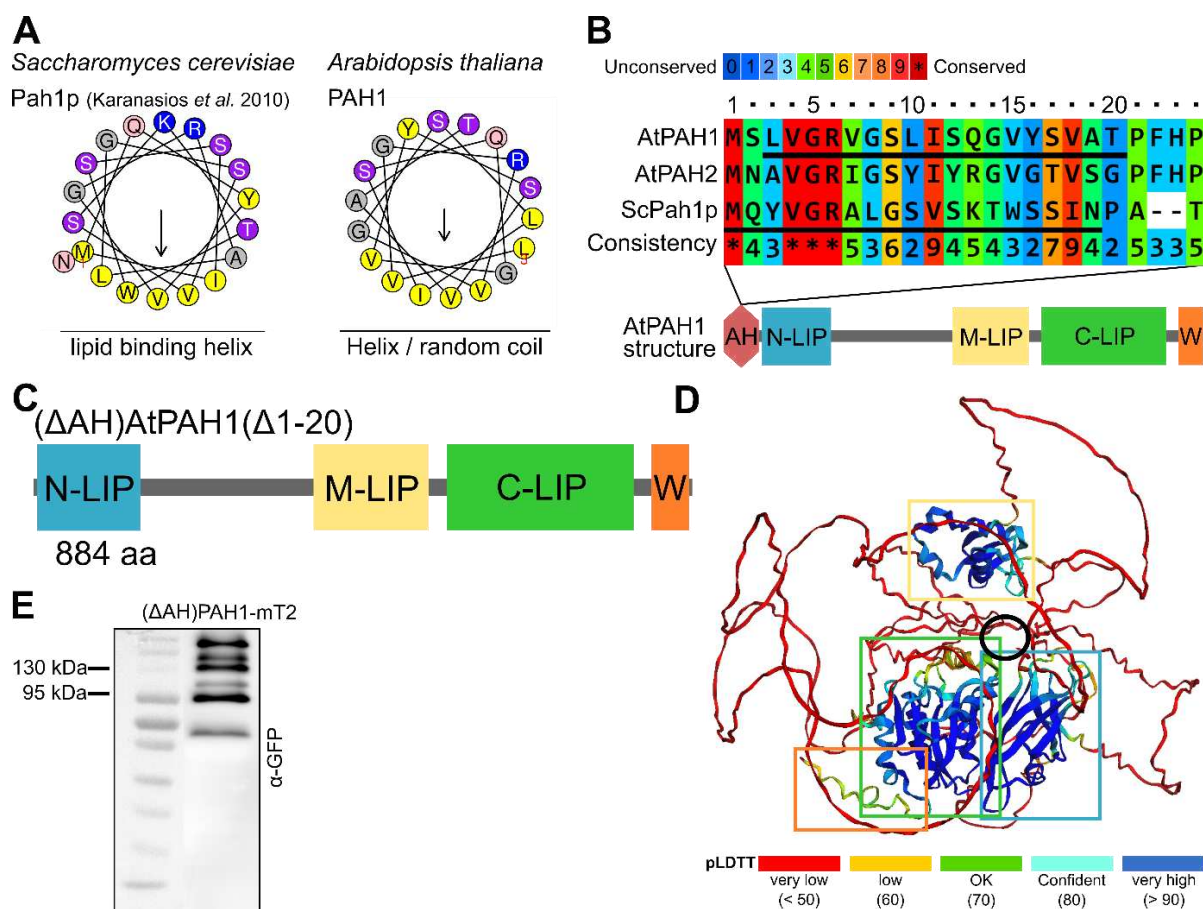


Figure 11 Prediction of a putative N-terminal helix of Arabidopsis PAH1

A) HeliQuest predicts a helix at the N-terminal end of AtPAH1 (right). On the left is the amphipathic helix of yeast Pah1p. The analysis was done using the settings published in Karanasios *et al.* (2010) (6.11.3) (55).

B) Amino acid sequence (1-24) alignment of AtPAH1 and AtPAH2 against ScPah1p.
C&D) Putative protein structure based on amino acid sequence alignment against yeast and mouse phosphatidic acid phosphatases and 3D-protein model made with AlphaFold2 (6.11.1, 6.11.2). The 3D-model was made without the N-terminal amino acid sequence (1-20) (black circle in the model marks the former position of the helix).
E) Immunoblot analysis of (Δ AH)PAH1-mTurquoise2 (99 kDa) after transient expression in *Nicotiana benthamiana* leaves using α -GFP.

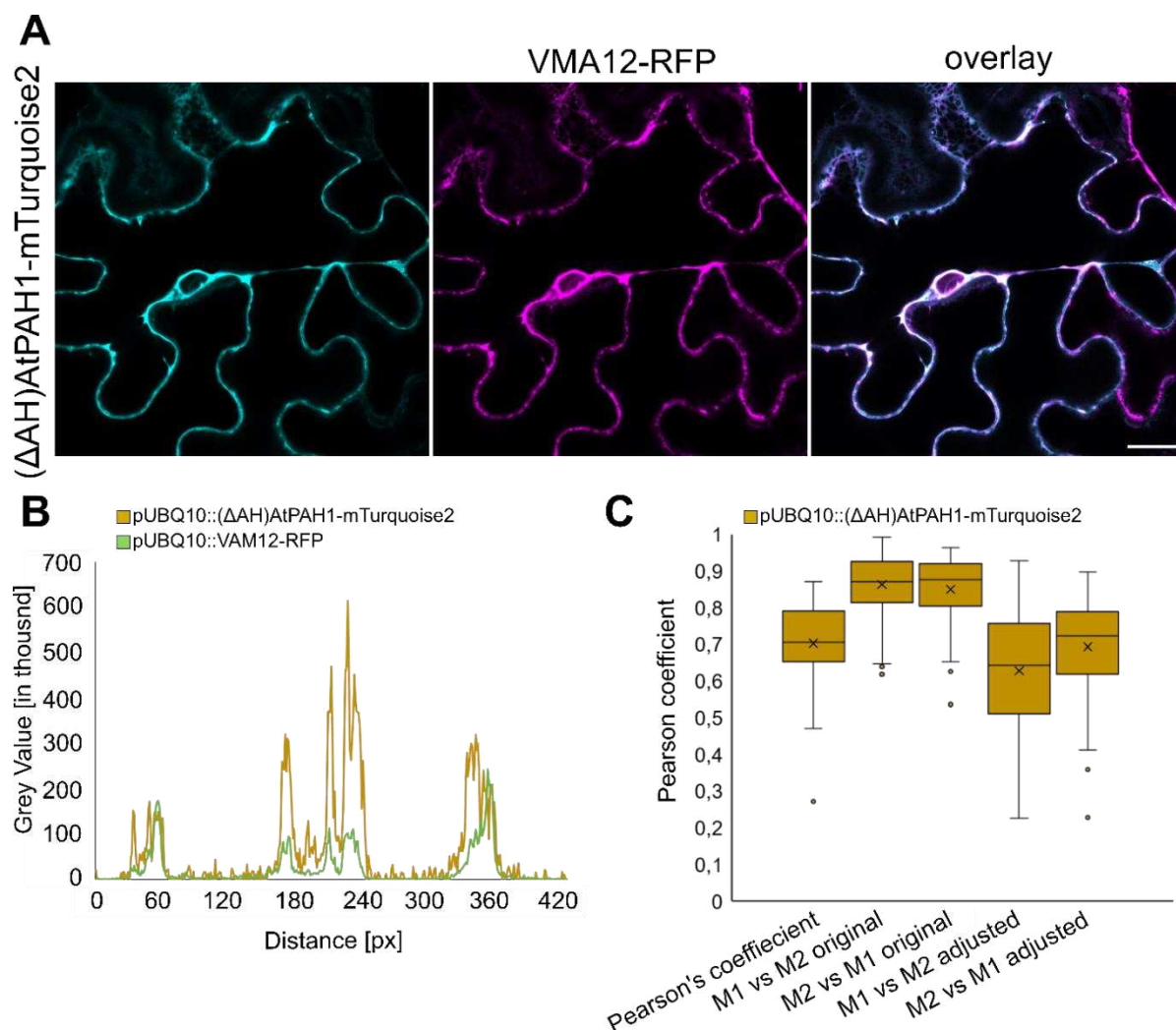


Figure 12 (Δ AH)AtPAH1 co-localizes with the endoplasmic reticulum-marker VMA12 after *Nicotiana benthamiana* leaf co-infiltration

A) Confocal microscopy images of (Δ AH)AtPAH1-mTurquoise2 co-infiltrated with ER-marker VMA12 in *Nicotiana benthamiana* leaves. Images were taken approximately 40 h.p.i. White bar at the bottom right represents 20 μ m.

B) Line intensity plot profile of (Δ AH)AtPAH1 against VMA12. ImageJ was used to analyze the pictures (6.9). For line intensity plot profile picture analysis, a line was drawn crossing the nucleus and / or the plasma membrane.

C) Calculations of the Pearson's coefficient and Manders coefficient between (Δ AH)AtPAH1 and VMA12. The analysis was performed using the ImageJ PlugIn JACoP (6.9).

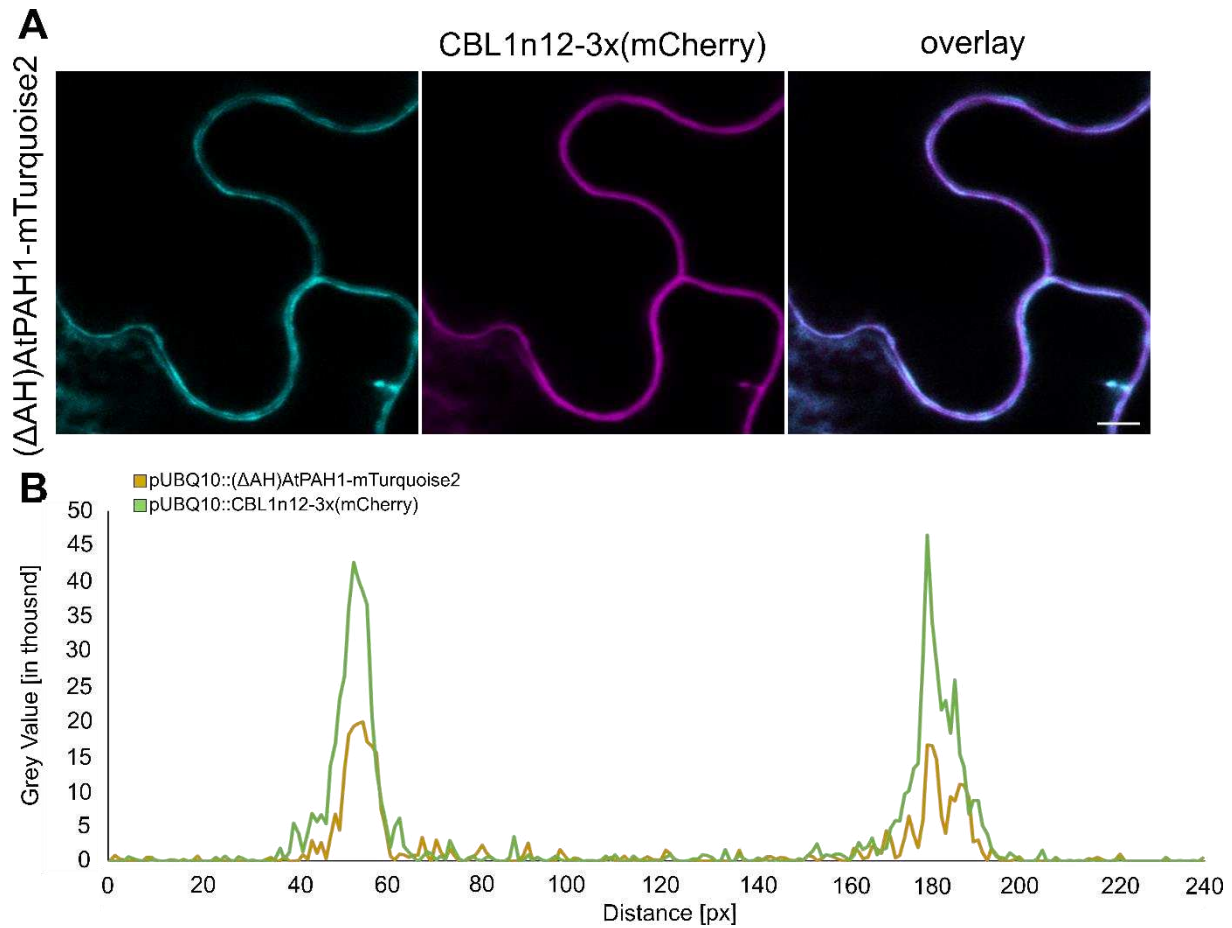


Figure 13 (Δ AH)AtPAH1 co-localizes with the plasma membrane-marker CBL1n12-3x(mCherry) after *Nicotiana benthamiana* leaf co-infiltration

A) Confocal microscopy images of (Δ AH)AtPAH1-mTurquoise2 co-infiltrated with PM-marker CBL1n12-3x(mCherry) in *Nicotiana benthamiana* leaves. Images were taken approximately 40 h.p.i. White bar represents 10 μ m.

B) Line intensity plot profile of (Δ AH)AtPAH1 against CBL1n12-3x(mCherry). ImageJ was used to analyze the pictures (6.9). For line intensity plot profile picture analysis, a line was drawn crossing two plasma membranes.

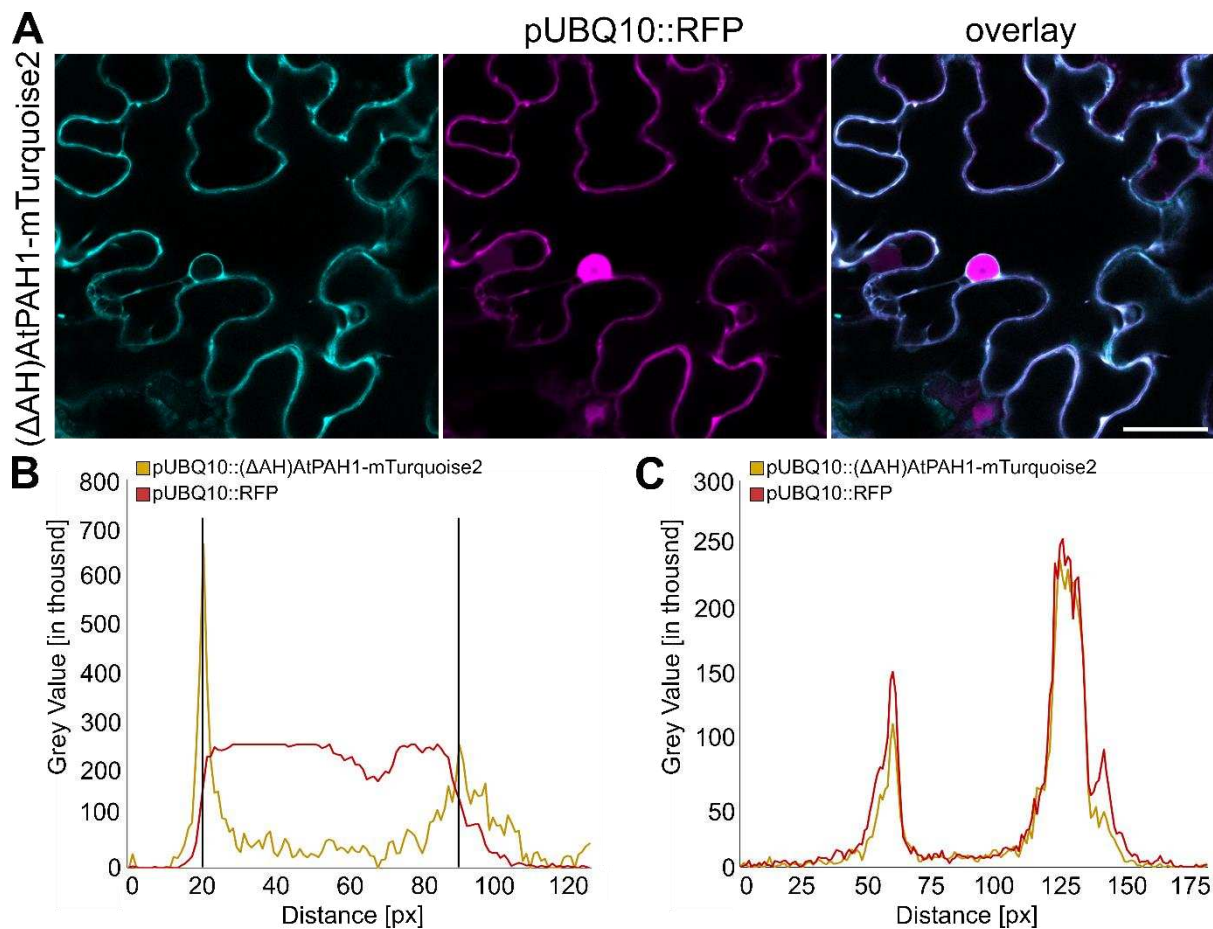


Figure 14 (ΔAH)AtPAH1 co-localizes in the cytoplasm with free RFP after *Nicotiana benthamiana* leaf co-infiltration

A) Confocal microscopy images of (ΔAH)AtPAH1-mTurquoise2 co-infiltrated with p35S::RFP in *Nicotiana benthamiana* leaves. Images were taken approximately 40 h.p.i. White bar represents 35 μm.

B&C) Line intensity plot profile of (ΔAH)AtPAH1 against free RFP. ImageJ was used to analyze the pictures (6.9). The plot in B represents the analysis of a nuclear signal while the plot in C represents the analysis of a plasma membrane and a cytosolic fraction.

2.2.3 Identification of a M-LIP domain in *Arabidopsis thaliana* PAH1

In 2021 Gu and colleagues identified the M-LIP domain in mammalian PAPs and showed the importance of this domain for proper localization (59). They also suggested the presence of this domain in *Arabidopsis* PAPs based on protein alignments. To identify the sequence of the putative M-LIP domain in AtPAH1 and AtPAH2 we also performed a protein alignment of both proteins against mouse PAPs (MmLIPIN1-3) using the online platform PRALINE (Figure 15 C) (86-91). We also found conserved regions in AtPAH1 and AtPAH2 that represent the M-LIP domain compared to the mammalian LIPINs.

To analyze the importance of the M-LIP domain we deleted the amino acids 553-675 of AtPAH1 ((ΔM)AtPAH1) (Figure 15 A). Protein structure analysis with AlphaFold2 suggests

that the absence of the amino acids 553-675 might not affect the overall protein structure (Figure 15 B).

Again, comparable to full length AtPAH1 we co-infiltrated (Δ M)AtPAH1-mTurquoise2 with the ER marker VMA12 and imaged the localization using confocal microscopy (Figure 16 A) (83). We also analyzed the pictures for co-localization of the proteins using the line intensity plot profile tool and the Plugin JACoP of ImageJ. First, we compared the localization of both proteins with the line intensity plot profile tool of ImageJ (Figure 16 B). We measured the line intensity plot profile of the line for each channel by drawing a line through the cell including the cell surface. The profile suggests a co-localization of (Δ M)AtPAH1-mTurquoise2 with the ER marker VMA12. Second, we used the ImageJ Plug-in JACoP (Figure 16 C) (84). Again, with a Pearson's coefficient of >0.5 the analysis with JACoP suggests that (Δ M)AtPAH1-mTurquoise2 co-localized with the ER marker VMA12 when infiltrated to tobacco leaves.

We also observed the localization of (Δ M)AtPAH1-mTurquoise2 when co-infiltrated with the plasma membrane marker CBL1n12-3x(mCherry) (Figure 17 A). We analyzed the localization of (Δ M)AtPAH1 against the plasma membrane marker using ImageJ's line intensity plot profile tool (Figure 17 B). Both, the confocal microscopy images and the line intensity plot profile indicate a co-localization of (Δ M)AtPAH1-mTurquoise2 with CBL1n12-3x(mCherry) when infiltrated into tobacco leaves.

To investigate the cytosolic localization of (Δ M)AtPAH1-mTurquoise2 we co-infiltrated the protein with *p35S::RFP* and imaged the cell using confocal microscopy (Figure 18 A). Again, we measured the line intensity plot profile with ImageJ. Therefore, we draw two lines per cell, one line through the nucleus (Figure 18 B) and one through the cell including the plasma membrane (Figure 18 C). Both line intensity plot profiles as well as the confocal microscopy images indicate a co-localization of (Δ M)AtPAH1-mTurquoise2 with the cytosolic RFP, while, other than the free RFP, (Δ M)AtPAH1-mTurquoise2 was not present in the nucleus.

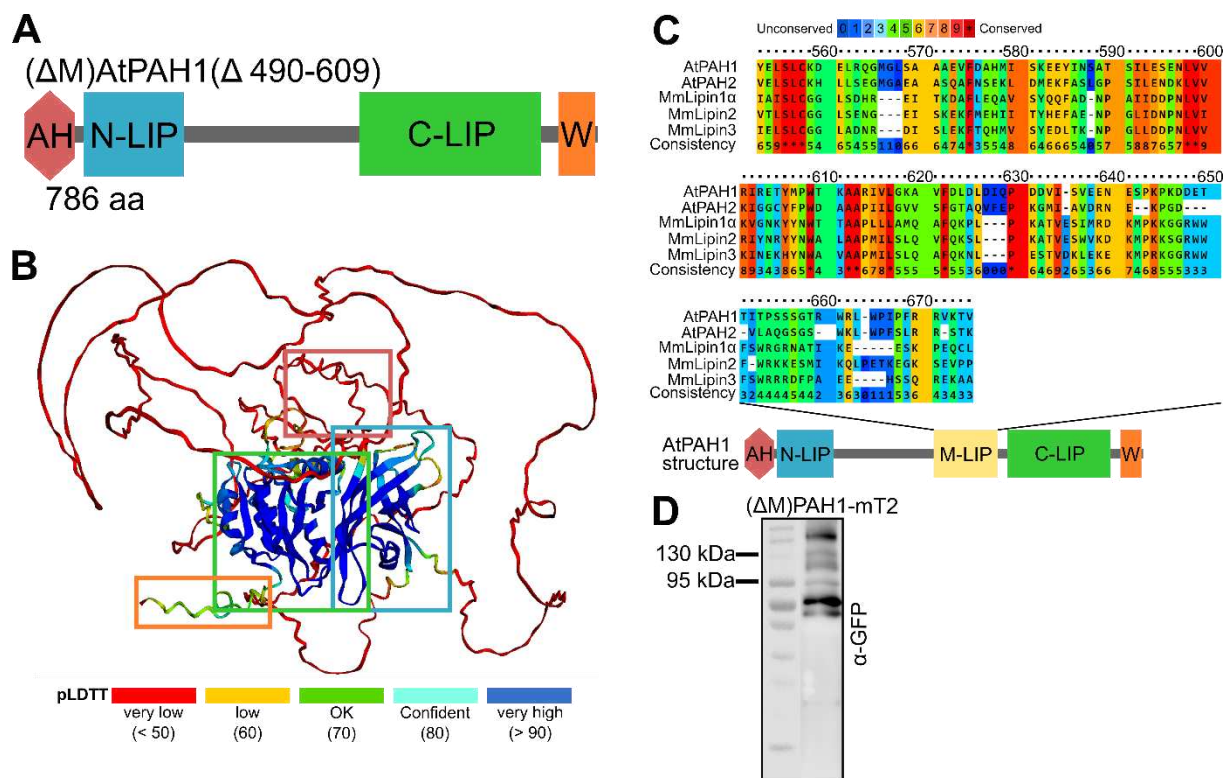


Figure 15 Prediction of a putative M-LIP domain of Arabidopsis PAH1

A&B) Protein structure and 3D-model predicted by Alphafold2 of AtPAH1 without the M-LIP domain (amino acids 490-609) (6.11.2).

C) Amino acid sequence alignment of AtPAH1 and AtPAH2 against mouse PAPs (MmLIPIN1-3) using the online tool PRALINE (59). Numbers correspond to the amino acid sequence of AtPAH1.

D) Immunoblot analysis of (ΔM)PAH1-mTurquoise2 (85 kDa) after transient expression in *Nicotiana benthamiana* leaves using α-GFP.

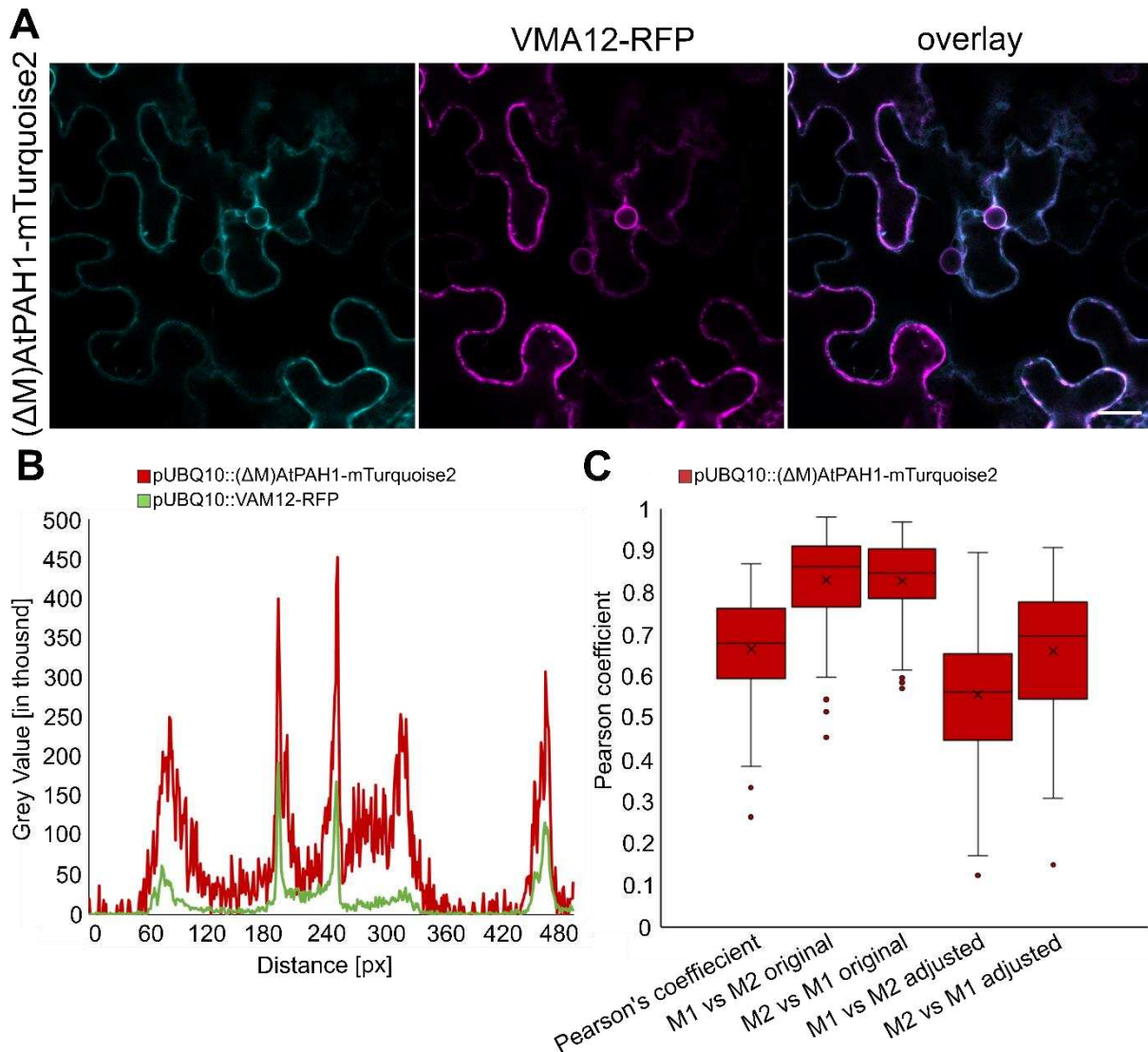


Figure 16 (Δ M)AtPAH1 co-localizes with the endoplasmic reticulum-marker VMA12 after *Nicotiana benthamiana* leaf co-infiltration

A) Confocal microscopy images of (Δ M)AtPAH1-mTurquoise2 co-infiltrated with ER-marker VMA12 in *Nicotiana benthamiana* leaves. Images were taken approximately 40 h.p.i. White bar at the bottom right represents 20 μ m.

B) Line intensity plot profile of (Δ M)AtPAH1 against VMA12. ImageJ was used to analyze the pictures (6.9). For line intensity plot profile picture analysis, a line was drawn crossing the nucleus and / or the plasma membrane.

C) Calculations of the Pearson's coefficient and Manders coefficient between (Δ M)AtPAH1 and VMA12 localization. The analysis was performed using the ImageJ PlugIn JACoP (6.9).

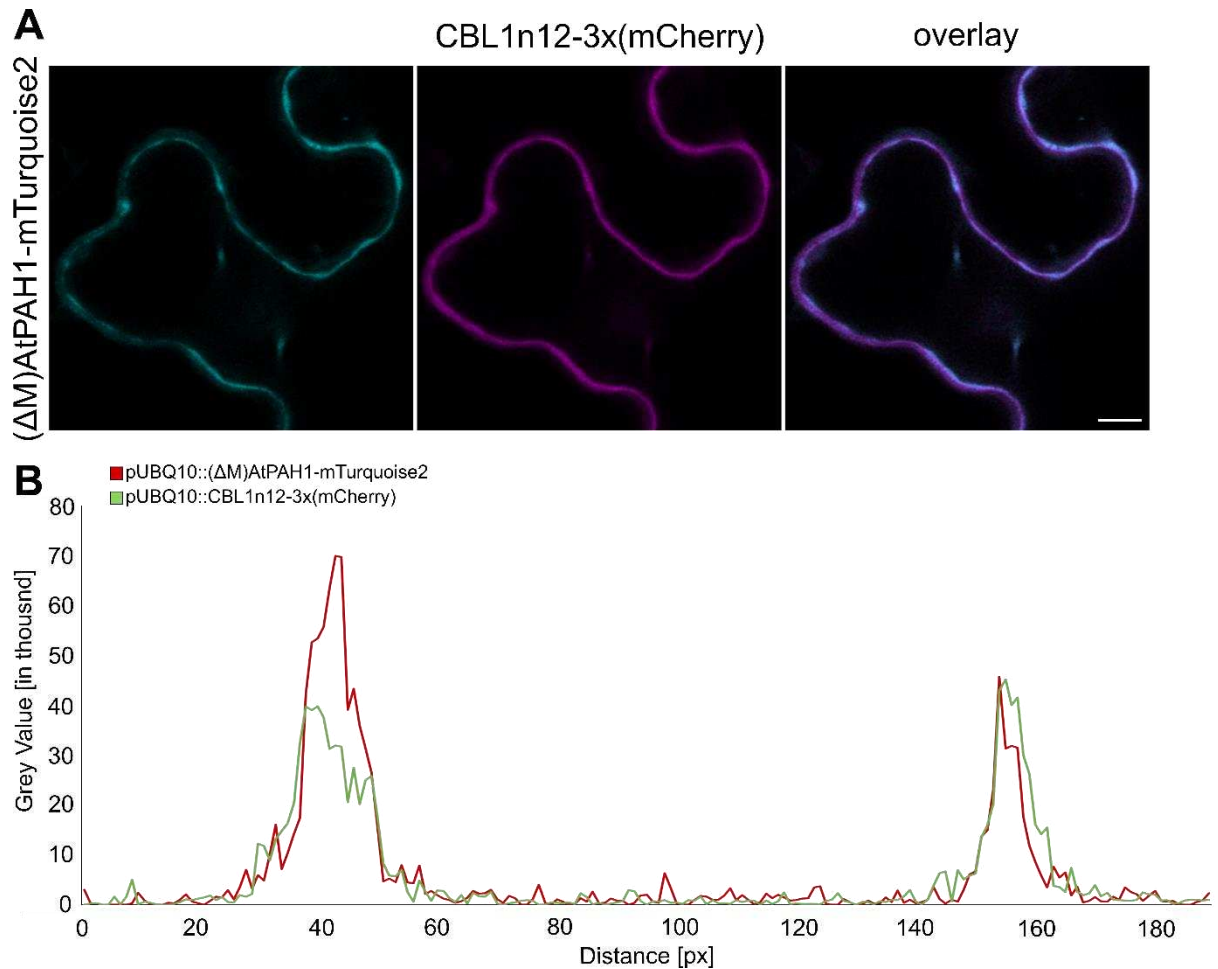


Figure 17 (Δ M)AtPAH1 co-localizes with the plasma membrane-marker CBL1n12-3x(mCherry) after *Nicotiana benthamiana* leaf co-infiltration

A) Confocal microscopy images of (Δ M)AtPAH1-mTurquoise2 co-infiltrated with PM-marker CBL1n12-3x(mCherry) in *Nicotiana benthamiana* leaves. Images were taken approximately 40 h.p.i. White bar represents 10 μ m.

B) Line intensity plot profile of (Δ M)AtPAH1 against CBL1n12-3x(mCherry). ImageJ was used to analyze the pictures (6.9). For line intensity plot profile picture analysis, a line was drawn crossing two plasma membranes.

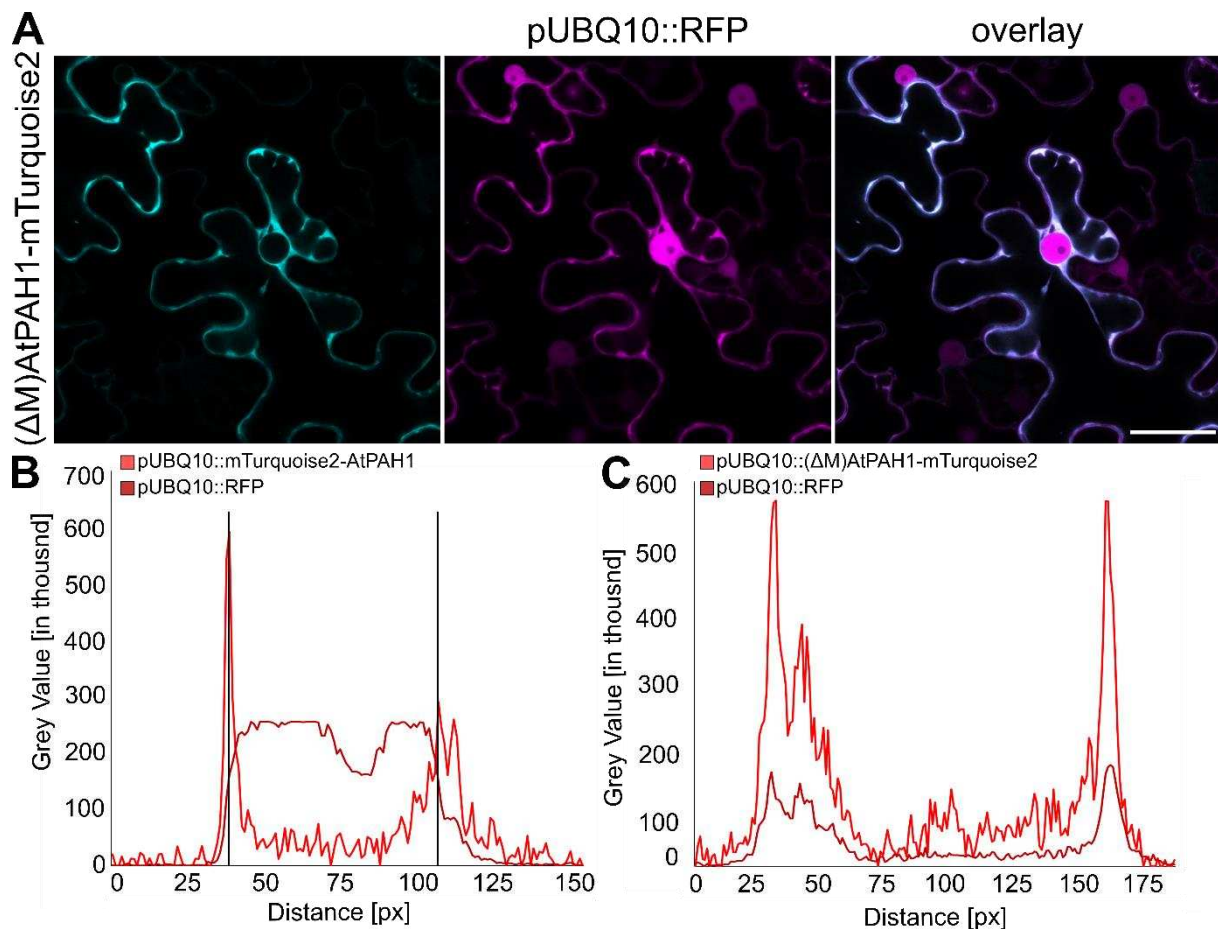


Figure 18 (M)AtPAH1 co-localizes in the cytoplasm with free RFP after *Nicotiana benthamiana* leaf co-infiltration

A) Confocal microscopy images of (ΔM)AtPAH1-mTurquoise2 co-infiltrated with *p35S::RFP* in *Nicotiana benthamiana* leaves. Images were taken approximately 40 h.p.i. White bar represents 35 μm.

B&C) Line intensity plot profile of (ΔM)AtPAH1 against free RFP. ImageJ was used to analyze the pictures (6.9). The plot in B represents the analysis of a nuclear signal while the plot in C represents the analysis of a plasma membrane and a cytosolic fraction.

2.2.4 Deletion of the N-terminal helix and the M-LIP domain of Arabidopsis PAH1 lead to developmental and stress related phenotypes when expressed in the *pah1/2* double mutant

To further investigate the importance of the N-terminal helix and the M-LIP domain we transformed Arabidopsis *pah1/2* double mutants with full length AtPAH1 N- and C-terminal tagged with mTurquoise2, as well as with (ΔAH)AtPAH1 and (ΔM)AtPAH1 C-terminally tagged with mTurquoise2. All constructs were expressed under control of the endogenous *PAH1* promoter. The *pah1/2* double mutant has a mild phenotype with slight developmental deficiencies such as smaller roots in the seedlings stage and a dwarfish growth in the rosette

stage as well as smaller and more roundish leaves. The *pah1* and *pah2* single mutants do not display any detectable phenotype in comparison to wildtype (64).

First, we wanted to investigate if truncating the N-terminal helix or the M-LIP domain lead to functional deficiencies. Therefore, we compared 5-days-old Arabidopsis seedlings that were germinated and grown in solid $\frac{1}{2}$ MS without sucrose. We compared the size of wild-type seedlings of the ecotype Columbia 0 (Col-0), the *pah1/2* double mutant and two independent lines for each of AtPAH1 N- and C-terminal tagged with mTurquoise2, as well as with (Δ AH)AtPAH1 and (Δ M)AtPAH1 C-terminally tagged with mTurquoise2 (Figure 19 A). Interestingly, non of the lines showed a major difference in growth. This is also true for the *pah1/2* double mutant, which did not show the published phenotype.

To further investigate the effect of truncating the N-terminal helix or the M-LIP domain of AtPAH1 we also observed the effect on growth in 5-week-old Arabidopsis plants that entered the rosette stage (Figure 19 B&C). Interestingly, the *pah1/2* double mutant showed the growth deficiencies as published when grown on soil, even though the seedlings were germinated on solid $\frac{1}{2}$ MS. Both lines expressing the N-terminal tagged full length AtPAH1 completely rescued the *pah1/2* growth deficient phenotype, while only one independent line of the C-terminally tagged full length protein showed a full rescue. The independent line PAH1-mT2(4) showed a mild phenotype with normal growth comparable to Col-0 but more roundish leaves comparable to the *pah1/2* double mutant. The same effect was visible for both lines expressing (Δ AH)AtPAH1, while the plants expressing (Δ M)AtPAH1 fully rescued the *pah1/2* growth deficient phenotype.

PA was shown to be very important for proper stress response in plants. Especially the PA related response to salt stress was investigated carefully in the past. We wanted to know if PAH1 expression and the deletion of the N-terminal helix or the M-LIP domain has any effect on the growth of Arabidopsis seedlings when treated with NaCl. We therefore germinated 5-days-old *Arabidopsis thaliana* seedlings on $\frac{1}{2}$ MS w/o sucrose with or without (Mock) 100mM NaCl and grew under them under long-day conditions (16h light/ 8h dark) (Figure 20). We compared the growth of wild-type (Col-0) seedlings, *pah1/2* double mutant and seedlings expressing AtPAH1 N- and C-terminal tagged with mTurquoise2, with the growth of seedlings expressing (Δ AH)AtPAH1 or (Δ M)AtPAH1. All tested seedlings showed a dramatic growth defect when grown on 100mM NaCl, except of both independent lines expressing (Δ M)AtPAH1, which responded less sensitive showing only mild growth deficiencies when grown on salt (Figure 20 B).

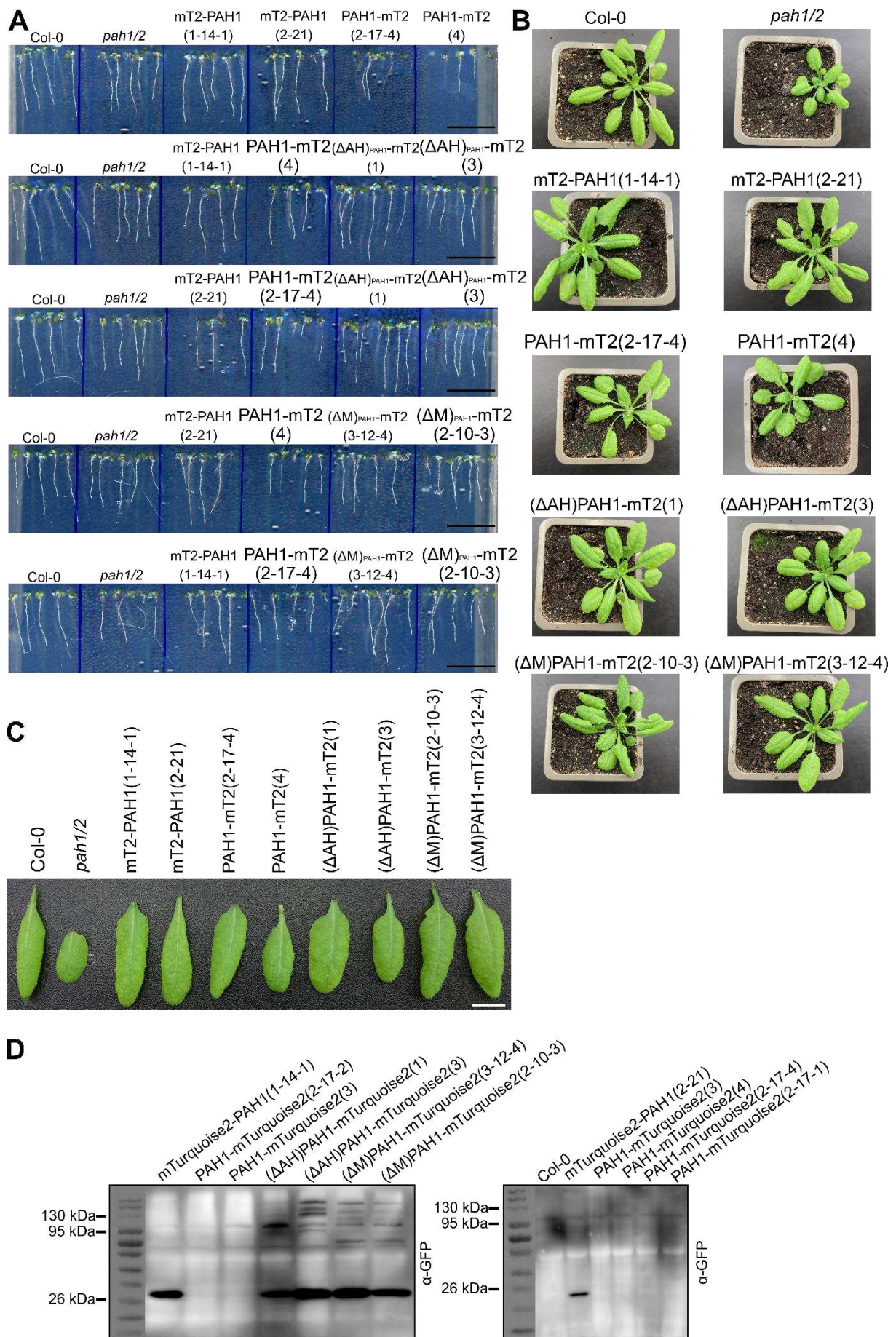


Figure 19 Full length PAH1, (Δ AH)PAH1 and (Δ M)PAH1 (partially) rescue the *pah1/2* deficient phenotype in *Arabidopsis thaliana*

A Representative pictures of 5-days-old *Arabidopsis thaliana* seedlings. The seedlings were grown on solid ½ MS w/o sucrose and grew under long-day conditions (16h light/ 8h dark). Col-0 represents the corresponding wild-type. All proteins were tagged with the fluorophore mTurquoise2 (mT2). All independent lines of PAH1-mT2, mT2-PAH1, (Δ AH)PAH1 and (Δ M)PAH1 are expressed under control of the *PAH1* promoter and in the *pah1/2* mutant background. Black bar represents 1cm. The experiment was repeated twice with the same results. n = 50 for PAH1-mT2 and mT2-PAH1; n = 10 for (Δ AH)PAH1 and (Δ M)PAH1

B Representative pictures of 5-week-old *Arabidopsis thaliana* plants. The plants shown correspond to the seedlings shown in A. The experiment was repeated one.

C Representative pictures of leaves of 5-week-old *Arabidopsis thaliana* plants. The corresponding plants are shown in B. White bar represents 1cm.

D Immunoblot analysis of PAH1-mT2 (101 kDa), mT2-PAH1 (101 kDa), (Δ AH)PAH1-mT2 (99 kDa) and (Δ M)PAH1-mT2 (85 kDa) after expression under control of *pPAH1* in *pah1/2* mutant background using α -GFP.

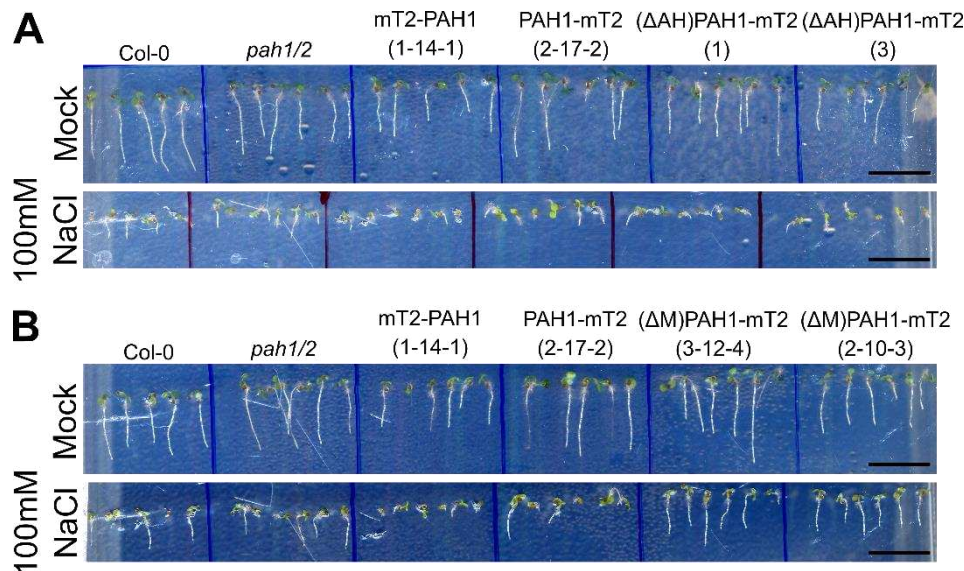


Figure 20 Arabidopsis seedlings expressing (Δ M)PAH1 are less sensitive to NaCl

A&B Representative pictures of 5-days-old *Arabidopsis thaliana* seedlings. The seedlings were germinated and grown on solid ½ MS w/o sucrose with or without (Mock) 100mM NaCl and grew under long-day conditions (16h light/ 8h dark). Col-0 represents the corresponding wild-type. All independent lines of PAH1-mT2, mT2-PAH1, (Δ AH)PAH1-mT2 and (Δ M)PAH1-mT2 are expressed under control of the *PAH1* promoter and in the *pah1/2* mutant background. Black bar represents 1cm. This experiment was repeated once. n = 15

2.2.5 Localization of PAH1 and the AH and M-LIP domain deletion mutants in *Arabidopsis thaliana*

Our results suggested that AtPAH1 as well as (Δ AH)AtPAH1 and (Δ M)AtPAH1 localized to the ER, plasma membrane and cytosol when transiently expressed in tobacco leaves (see 2.2.1-2.2.3). As heterologous and overexpression system may alter protein localization, we wanted to

further confirm the localization using AtPAH1, (Δ AH)AtPAH1 and (Δ M)AtPAH1 fluorescent lines that complement the *pah1/2* double mutant phenotype. Therefore, we imaged root cells of 4-days-old *Arabidopsis* seedlings using confocal microscopy (Figure 21). We observed a cytosolic localization for all seedlings tested. Unfortunately, no signal was visible for all tested seedlings expressing the C-terminally tagged full length PAH1. This goes along with the immunoblot detections shown earlier (Figure 19 D), where also no protein was detectable.

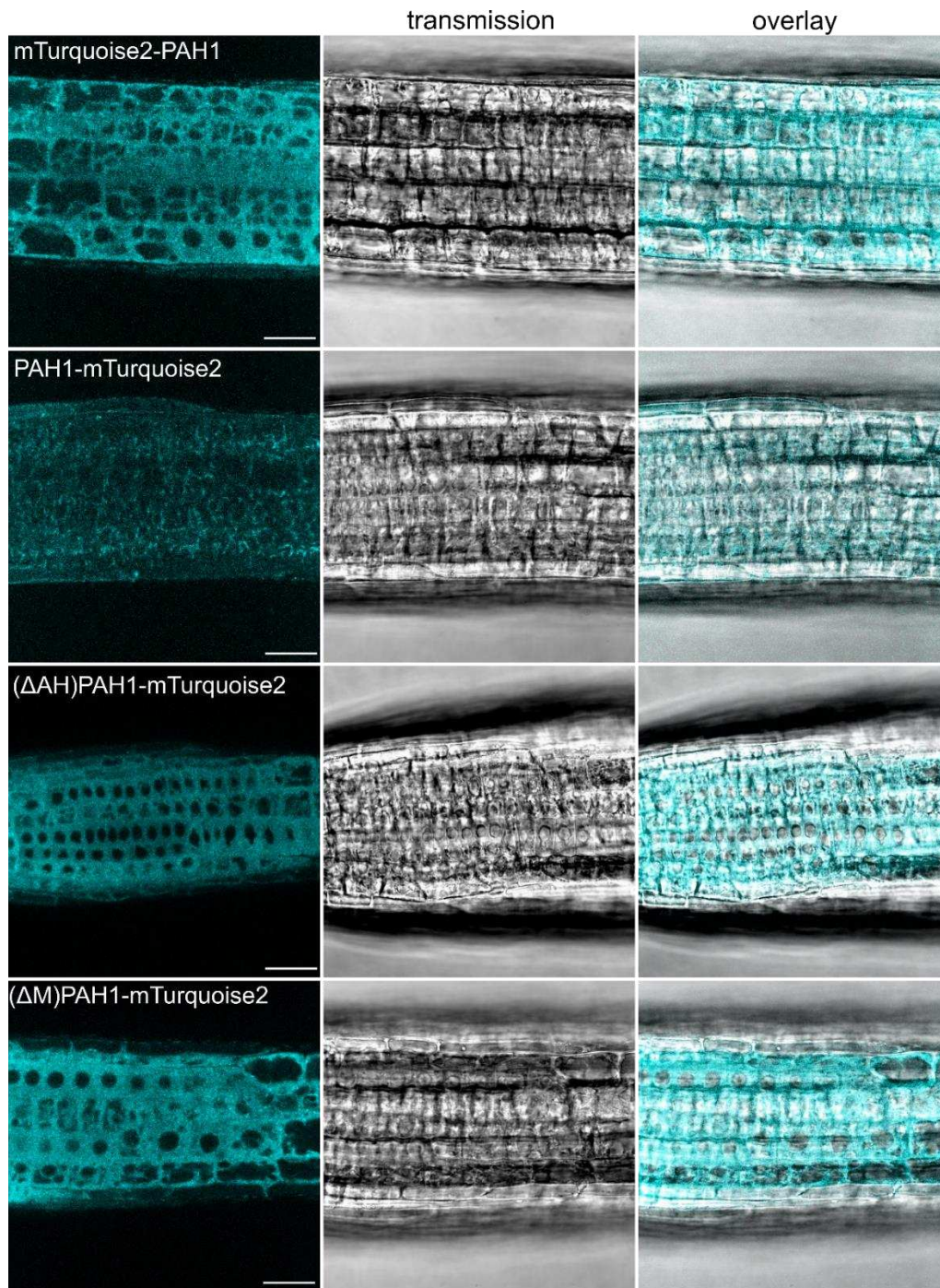


Figure 21 AtPAH1, (Δ AH)AtPAH1 and (Δ M)AtPAH1 localization in *Arabidopsis thaliana* roots

Confocal microscopy images representing the localization of AtPAH1 and the (Δ AH)AtPAH1 and (Δ M)AtPAH1 mutants tagged with mTurquoise2 in root cells of four days old Arabidopsis seedlings. *AtPAH1*, (*Δ AH*)*AtPAH1* and (*Δ M*)*AtPAH1* were expressed under control of the *pPAH1* promoter and in the *pah1/pah2* double mutant background. White bar represents 30 μ m.

2.3 Discussion

2.3.1 Localization of PAH1 after transient expression in *Nicotiana benthamiana* leaves and identification of a putative N-terminal helix and a M-LIP domain

We performed confocal microscopy to image the localization of N- and C-terminally tagged Arabidopsis *PAH1* when transiently expressed in tobacco leaves and analyzed the localization using different approaches. Our results suggest, other than published, a localization of full length AtPAH1 to the ER and plasma membrane as well as the cytosol. It is to mention that the technique we, but also Eastmond and colleagues (2010), were using has strong limitations (64). Using tobacco epidermal cells with their large central vacuole could lead to misinterpretations regarding cytoplasmic, plasma membrane or endomembrane localization. Tobacco leaf infiltrations are used due to its easy and fast protocol. Nevertheless, further approaches, like localization studies in smaller meristematic cells with much more cytoplasm per total volume, are crucial to confirm the results.

We further analyzed the protein-by-protein alignment against other PAPs, 3D-protein structure predictions and analyzed the protein for putative functional helices. All results together suggest the presence of a N-terminal helix and M-LIP domain, both of unknown function for Arabidopsis PAH1. Earlier studies in yeast and mammals showed that the N-terminal amphipathic helix and the M-LIP domain are both important for proper PAP localization. Studies in yeast cell showed that the N-terminal amphipathic helix, an α -helix whose hydrophobic and polar residues are on opposite sides, so that a lipophilic and a hydrophilic surface can form that anchors the protein to a membrane, is crucial for proper ER localization of ScPah1p, while studies in transfected Cos-7 cells revealed that basic-hydrophobic residues in the M-LIP domain mediate the ER localization. Therefore, we tested if truncating the first 20 N-terminal amino acids, which represent the putative helix, as well as the M-LIP domain, lead to any changes in localization when transiently expressed in tobacco leaves. Our results did not show any changes in localization compared to full length AtPAH1. In all cases the cytosolic localization of the protein was clear the dominant signal. As the immunoblot detection suggests, this signal was not due to free mTurquoise2, but actual protein localization. Both the cytosolic and the ER localization could of course also be an artifact, as the heterologous expression under control of *pUBQ10* might also lead to an oversaturation of

the cell leading to an AtPAH1 accumulation in the ER and cytosol. But as mammalian and yeast PAP were shown to localize to both, the cytosol and the ER, it is very likely that our results represent actual protein localization. The missing effect of (Δ AH)PAH1 and (Δ M)PAH1 on protein localization raises the question, if the N-terminal helix and the M-LIP domain regulate protein localization of AtPAH1 or other protein functions. If the N-terminal helix and the M-LIP domain are fine tuning mechanics regulating protein localization, the strong and heterologous expression could mask the effect of deleting the domains, as the system might be oversaturated. A more sensitive approach like subcellular fractionation and an additional immunoblot detection could lead to more insides, if the localization is actually affected or not.

2.3.2 PAH1 rescues the *pah1/2* deficient phenotype when expressed in *Arabidopsis thaliana* and the N-terminal helix and the M-LIP domain seem to be important for proper plant development and stress response

We wanted to further analyze the importance of the putative N-terminal helix and the M-LIP domain for proper AtPAH1 function. Therefore, we expressed, under control of the *PAH1* promoter, N- and C-terminally tagged *AtPAH1* as well as C-terminally tagged (Δ AH)*AtPAH1* and (Δ M)*AtPAH1* in *pah1/2* double mutant background. The *pah1/2* double mutant was shown to have a mild phenotype in seedling stage and has smaller and more roundish leaves in the rosette stage, while the *pah1* and *pah2* single mutants grow like wild-type plants (64). None of the tested full length AtPAH1, (Δ AH)AtPAH1 and (Δ M)AtPAH1 expressing Arabidopsis seedlings showed any difference compared to wild-type or the *pah1/2* double mutant, when we compared their size five days after germination. But it is to mention, that the *pah1/2* double mutant did not show the published phenotype at the seedlings stage, while we were able to see the growth deficit and the round leaves at the rosette stage of five-week-old plants. Both independent lines expressing the N-terminally tagged full length AtPAH1 and one tested line expressing the C-terminally tagged full length AtPAH1 were able to fully rescue the *pah1/2* deficient phenotype. The growth deficiency of PAH1-mT2(4) is most likely an artificial effect due to lower gene expression, as the growth deficit is rescued, while only the leave shape is not. Interestingly, the *pah1/2* double mutants expressing C-terminally tagged full length AtPAH1 show normal growth, which indicates the presence of AtPAH1, while we were not able to detect any protein, neither using immunoblot detection nor with confocal microscopy. Also, all tested independent lines expressing C-terminally tagged full length AtPAH1 carried the Phosphinothricin (PPT) resistance gene we used for plant selection (Supplementary Figure 1). One explanation might be that AtPAH1 is present, while the mTurquoise2 fluorophore carries an unknown point mutation leading false protein folding of the fluorophore. Another

explanation might be that the protein functions at very low concentrations and is therefore not easy to detect.

Both tested independent *pah1/2* double mutants expressing (Δ AH)AtPAH1 showed only a partial rescue of the *pah1/2* deficient growth in five-week-old plants. While the plant and leave size was comparable to wild-type plants, the leave shape was more roundish comparable to the *pah1/2* double mutant. This result indicates that the N-terminal first 20 amino acids are important for proper AtPAH1 function in plant development. The exact function of the putative N-terminal helix remains unknown.

Other than the putative N-terminal helix, the M-LIP domain seems not to be important for proper plant development. But interestingly, our results suggest that this domain seems to have a regulatory function in response to salt stress. Both tested independent lines showed less sensitivity in response to 100mM NaCl when compared to wild-type, the *pah1/2* double mutant other the seedlings expressing full length AtPAH1. This result indicates that the M-LIP domain somehow regulates AtPAH1 function in response to stress. To further verify this result, the experiment should be repeated. Also, it would be interesting to know if this is a specific response to salt stress or if other abiotic or biotic stresses lead to similar effects.

2.3.3 Confocal microscopy of AtPAH1 localization in transgenic *Arabidopsis thaliana* seedlings did not lead to further insides

Confocal microscopy images of transgenic *Arabidopsis* seedlings expressing *mTurquoise-AtPAH1*, (Δ AH)AtPAH1-*mTurquoise2* and (Δ M)AtPAH1-*mTurquoise2* under control of the PAH1 promoter and in the *pah1/2* double mutant background could unfortunately not give us further insides on the localization of AtPAH1 and whether or not the N-terminal helix and the M-LIP domain have an important function in regulating protein localization. The predominant signal in all cases was found in the cytosol. As the immunoblot detection suggests, this signal is predominantly free mTurquoise2, hereby masking the actual localization of AtPAH1. The strong accumulation of the fluorophore in the cytosol could be caused by a high turnover of AtPAH1, while the fluorophore gets cleaved and remains. For example, in yeast it was shown that ScPah1p is highly regulated by protein degradation. This high protein turnover could in our case affect that just AtPAH1 itself is degraded, while the fluorophore remains. Unfortunately, we were not able to detect any signal for C-terminally tagged full length AtPAH1. The missing protein detection fits to our negative results of the immunoblot detection.

2.3.4 Conclusion

Together, our results suggest that AtPAH1 localizes to the ER, plasma membrane but predominantly to the cytosol. Also, bioinformatical analysis and phenotyping experiments indicate the presence of an N-terminal helix and a M-LIP domain, both important for proper protein regulation but of unknown function. To verify the localization but also the protein structure further experiments are needed.

To analyze the localization of AtPAH1 in Arabidopsis, a more precise approach, as for example subcellular fractionation and immunoblot detection, could lead to further insights and give us an answer, whether or not the N-terminal helix and the M-LIP domain contribute to regulate the localization of AtPAH1. Also, how the helix and the M-LIP domain function remains elusive. Do they contribute to proper phospholipid binding? A PIP-strip and additional immuno detection could give a first answer.

As both, deleting the N-terminal helix and the M-LIP domain, did not lead to loss of protein function, as the phenotyping experiments suggest, it is very likely that these domain function very specifically and regulate protein function very precisely. Both domains are not crucial for proper protein function, but seem to regulate AtPAH1 in certain situations, as e.g. in distinct developmental processes or in response to stress.

In yeast and mammalian PAPs protein phosphorylation was shown to be a crucial mechanism to regulate protein function and localization. This is most likely also the case for Arabidopsis PAH1, but so far it remains elusive if, where and how AtPAH1 gets phosphorylated. Answering this question could also help to better understand how the N-terminal helix and the M-LIP domain function, as phosphorylation could also regulate their activity.

Besides PAH1, PAH2 was also shown to be an important PAP in *Arabidopsis thaliana*. It would also be interesting to test, where AtPAH2 localizes and whether or not the N-terminal first amino acids and the M-LIP domain are of importance for proper AtPAH2 function.

3 The development of genetically encoded tools to modulate phosphatidic acid level at the plasma membrane *in planta*

3.1 Introduction

Manipulating the abundance and amount of specific signaling molecules is a powerful way to verify the function and pathways these molecules are involved in. Classically this is done with either the help of certain drugs or by knocking out genes that encode for proteins crucial for the biosynthesis, uptake or transport of this molecule. Both methods have their up- and down-takes. Drugs are a very useful and a quick way to test e.g. protein responses. But most drug that are used to “*specifically*” manipulate signaling pathways often have unwanted side effects and when applied they rather effect the whole organism than specific tissues or cell layers. This makes it impossible to investigate the signaling molecule of interest in response to the drug in a locally restricted manner.

Knock outs of genes encoding for enzymes that are crucial for the synthesis or maintenance of the signaling molecule of interest is a direct and specific way to manipulate the level and abundance of this molecule. But this method also has its restrictions. Some enzymes have more than one function which might lead to unwanted cellular or physiological responses. Also do some enzymes localize to multiple subcellular compartments, making it impossible to investigate the function of the signaling molecule of interest in specific subcellular compartments when using knock out lines. Another problem might be a high redundancy among the enzyme families that are involved in the synthesis or maintenance of the molecule. This might make it necessary to knock out more than one gene, making the investigation more complex and time consuming.

These problems led to the development of a new kind of tool set to manipulate the amount of signaling molecules available: genetically encoded enzymes that are targeted to specific subcellular compartments to manipulate the level and abundance of specific signaling molecules. For example: in 2016 Simon et al. introduced SAC1, a PI(4)P phosphatase which is targeted to the plasma membrane to downregulate PI(4)P level (5). With the help of this tool it was possible to verify that the electronegativity of the plasma membrane is predominantly driven by PI(4)P. SAC1 was also used in the past to investigate the function of PI(4)P in different biological processes. For example, using SAC1 it was shown that the plasma membrane localization of the nucleotide-binding leucine-rich repeat receptor (NLR) RPM1, which is involved in pathogen-derived effector protein recognition and a quick immune response, is mediated by PI(4)P. This PM-localization of RPM1 is crucial for proper function (92).

PA is an important signaling molecule that was shown to act at the plasma membrane to regulate specific protein function in response to stress (see introduction) (6). To manipulate PA level, one can use certain drugs, such as DGK inhibitors or primary alcohols, which serve as an alternative substrate for the PLDs, to downregulate PA level or apply PA to upregulate PA level (6). Also, *dgk* and *pld* knock out mutants were generated to decrease the amount of PA available for the plant. Knock out mutants were shown to not be trivial to work with, as e.g. Arabidopsis has seven DGKs and 12 PLDs. Especially the PLDs were shown to act redundantly in plants (6). These problems led to the idea for an alternative tool set to manipulate PA level. In the following I will introduce two genetically encoded tools that are designed to theoretically up- or downregulate PA level specifically at the plasma membrane *in planta*.

3.1.1 PLD - A genetically encoded tool to upregulate phosphatidic acid level at the plasma membrane *in planta*

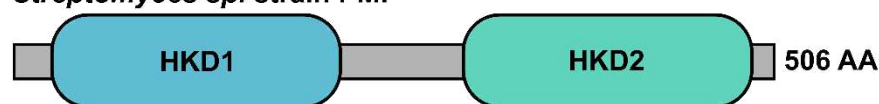
PLDs are enzymes found in all kingdoms of life, are involved in different cellular responses and are especially known for their key role in PA biosynthesis, where they catalyze the hydrolysis of PC or PE to PA (21, 24). Even though this protein family differs in terms of protein sequence and structure, the most important motif HxKxxxxD, also called HKD domain is highly conserved and defines the activity of these proteins (93). In eukaryotes, this domain is usually found at the C-terminus. Point mutations in the HKD domain lead to loss of catalytic activity and structural studies indicate that the catalytic site is comprised of a dimer of two HKD domains (93). Eukaryotic PLDs are usually classified by their N-terminal domain and are differed between phox/pleckstrine homology domain, which can interact with phosphatidyl inositol and its derivatives, and C2-domain, which can be activated by Ca^{2+} , containing proteins. Additionally, some PLDs also have a C-terminal protein binding domain for interaction with different regulatory proteins (Figure 22) (21, 93).

In 2020 Tei and Baskin introduced a new tool to upregulate PA level in mammalian cells. They have developed an optogenetic system using *Streptomyces sp.* strain PMF PLD (PLDpmf), tagged with 3x(mCherry) and fused to CRY2. To target the PLDpmf fusion protein to specific membranes they coinfect the cells with CIBN fused to a variety of different membrane associating peptides (50). CRY2 and CIBN dimerize upon blue light irradiation (488nm light) very rapidly forming a powerful tool to specifically target the protein of interest to different subcellular compartments. The authors chose PLDpmf for different reasons. The substrate of PLDpmf is PC, a very abundant phospholipid found in all membranes and PLDpmf displays an amino acid sequence homology of only 20% compared to mammalian PLDs, reducing the likelihood of unwanted protein-protein interactions that normally regulate mammalian PLD

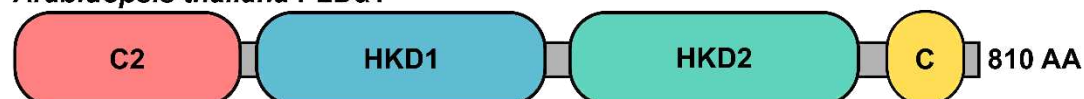
function. Also, this enzyme was already well described and the crystal structure is already known (94). They verified the activity of their tool using biosensors, click chemistry as well as different biochemical approaches such as LC-MS. The authors not just highlighted the effectiveness of their newly developed tool, but also showed that the activity of PLDpmf leads to biologically relevant effects in mammalian cells. They showed that the Hippo pathway, which controls size and proliferation of mammalian cells and was shown to be downregulated by PLD-derived PA, can be successfully manipulated by PLDpmf (50, 95).

In this work we tried to adapt PLDpmf as a tool to upregulate PA level specifically at the plasma membrane *in planta*.

***Streptomyces sp.* strain PMF**



***Arabidopsis thaliana* PLD α 1**



***Homo sapiens* PLD1**

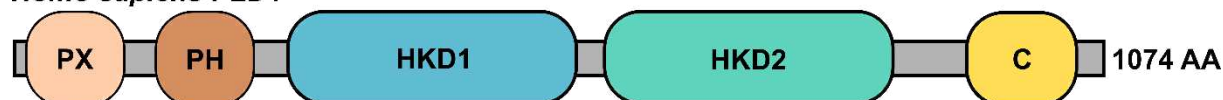


Figure 22 Scheme of PLDpmf, Arabidopsis PLD α 1 and human PLD1 structure illustrates the different types of Phospholipase D proteins

The three PLD shown above represent the different types of PLDs found in all kingdoms of life. The PLDs can be differed by the presence of either a phox/pleckstrine homology domain (PX / PH), which can interact with phosphatidyl inositol and its derivatives, and C2-domain, which can be activated by Ca^{2+} . Additionally, some PLDs also contain a C-terminal domain for protein-protein interaction (C). Also, some PLDs only consist of two HKD domains and non-conserved regions, as for example the PLD of *Streptomyces sp.* strain PMF represents. This scheme was adapted from Yao *et al.* (2021) (21).

3.1.2 PAP - A genetically encoded tool to downregulate phosphatidic acid at the plasma membrane level *in planta*

Phosphatidic Acid Phosphatases (PAPs) are thought to be highly conserved among all eukaryotic species at both level of their amino acid sequence and protein function (56). Their main function is to catalyze the dephosphorylation of PA to DAG (68, 73).

PAPs consist of different regulatory or catalytically active domains that were shown to be important for proper protein function. Especially the N-LIP and C-LIP domains are crucial for proper phosphatase function (58). While the N-LIP domain is of unknown function, we know

that the C-LIP domain contains a HAD-like domain. The HAD-like domain consists of two aspartic acids separated by a random amino acid and is crucial for the phosphatase function. More details about the different domains of the PAPs and their functions are precisely introduced in chapter 2.

In this work we aim to design a tool for the downregulation of PA using Phosphatidic Acid Phosphatases. As their structure is well known and a lot of studies showed that even minimalized PAPs still remain phosphatase function, we chose this protein family as a blueprint for the development of our tool. To guarantee protein function under physiological conditions (temperature, pH, etc.) we chose *Arabidopsis thaliana* PAH1.

3.2 Results

3.2.1 The N-terminal plasma membrane anchor and the fluorophore

PA was shown to be an important second messenger at the plasma membrane, which is why we want to address our tools to this compartment (6). Therefore, we designed in a first step the N-terminal part of the tool: the plasma membrane anchor and the fluorophore for protein detection (Figure 23 A).

To target the tools to the plasma membrane we chose the 12 first amino acids of the N-terminus of Calcineurin B-like protein 1 (CBL1), in the following referred to as CBL1n12 (Figure 23 B) (gifted by Prof. Dr. Alexis Maizel, University of Heidelberg). CBL1 acts as a calcium sensor in different developmental and regulatory processes in plants and was shown to be localized to the plasma membrane (96). In 2008 Batistic *et al.* showed that the 12 N-terminal amino acids of CBL1 are crucial for proper plasma membrane localization and that this is organized by a myristoylation and acylation. This modification was shown to be crucial for proper protein function and membrane association (96).

For protein detection we chose 3x(mCherry), where the three fluorophores are separated by small linker consisting of eight serine and seven glycine residues (8S7G), which are thought to give the tools the flexibility needed to target the substrate at the plasma membrane (Figure 23 C). This fluorophore was developed by Prof. Dr. Jan Lohmann (University of Heidelberg) and published in 2013 by Lampropoulos *et al.* (97).

The expression of *CBL1n12-3x(mCherry)* (in the following referred to as mCherry) was driven by the ubiquitous promoter of *UBIQUITIN10* (*pUBQ10*). Our results indicate that CBL1n12 successfully targeted 3x(mCherry) to the plasma membrane when infiltrated into *Nicotiana benthamiana* leaves (Figure 23 D). These results were further confirmed in chapter 3.2.6 by co-infiltrating mCherry with plasma membrane associated biosensors. Performing *Nicotiana*

benthamiana leaf infiltration and a following plasmolysis using 800mM mannitol further indicate the plasma membrane localization of mCherry (Figure 23 F). Besides the plasma membrane localization there was also a slight off target localization visible. This is most likely an artifact of the strong transient expression leading to an oversaturation of the system.

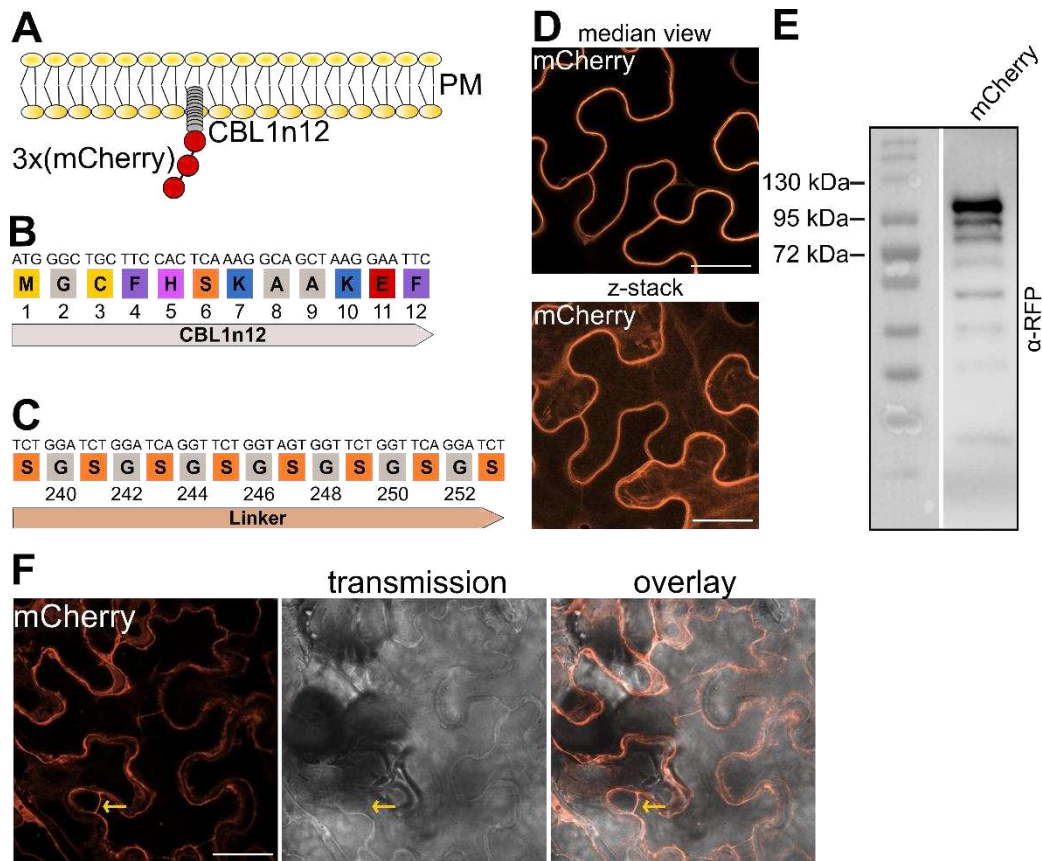


Figure 23 The N-terminal backbone of the tools mediated plasma membrane localization and protein detection

A-C) Schematic illustration of how the tools are targeted to the plasma membrane. For plasma membrane localization we used the N-terminal 12 amino acid peptide of calcineurin B-like proteins 1 (CBL1; grey). For detection of the proteins, we used a 3x(mCherry) fluorophore (red circles), where the fluorophores are separated by small linker (8S7G) to ensure protein flexibility and movement.

D) Transient *pUBQ10::CBL1n12-3xmCherry* expression in *Nicotiana benthamiana* leaves. The image on the top represent single plain pictures of the mid-section of a cell. Images on the bottom represent stacked pictures of the cell that had been made from the top to the mid-section. The images suggest plasma membrane localization of the CBL1n12-mCherry fusion protein. White bar represents 25µm. Confocal images were taken 40 h.p.i.

E) Immunoblot detection of mCherry (85 kDa) using α-RFP. The sample were harvested 40 h.p.i tobacco leaf infiltration.

F) Plasmolysis after transient expression of *pUBQ10::CBL1n12-3x(mCherry)* in *Nicotiana benthamiana* leaves confirm plasma membrane localization of the CBL1n12-3x(mCherry) fusion protein.. The pictures were taken 40 h.p.i. and 30 minutes after 800mM mannitol infiltration. Yellow arrows mark the detached plasma membrane. White bar represents 40µm.

3.2.2 Protein sequence of PLDpmf and *Arabidopsis thaliana* PLD's are very different but the catalytic active HKD-domains are conserved

Pairwise comparison of the amino acid sequence of PLDpmf (in the following referred to as PLD) against the amino acid sequence of the 12 *Arabidopsis thaliana* PLDs (PLD α 1-3, PLD β 1/2, PLD γ 1-3, PLD δ and PLD ϵ , PLD ζ 1/2) illustrated an amino acid sequence homology < 1% between PLD and the Arabidopsis PLDs (Table 2). This suggests that unwanted protein-protein interaction normally regulating the Arabidopsis PLDs might be avoided for our tool. An alignment of the HKD domains of PLD and the 12 Arabidopsis PLDs illustrated that the HKD domains are highly conserved among all tested PLDs (Figure 24 A). This result made us confident that the Streptomyces PLD might also be catalytically active in plants. As a negative control for PLD activity we also introduced the catalytically inactive mutant H167A (in the following referred to as dPLD), which was also used in the work of Tei, Baskin (2020) (Figure 24 B) (50).

Table 2 Protein sequence homology of PLD and Arabidopsis PLDs

Amino acid sequence homology of PLD against each Arabidopsis PLDs individually illustrates almost no similarities between the bacterial and the Arabidopsis PLDs (numbers shown are in %). The analysis was done as a Pairwise Comparison using the CLC Main Workbench 8.1 (www.clcbio.com, QIAGEN, Aarhus, Denmark).

	PLD α 1	PLD α 2	PLD α 3	PLD β 1	PLD β 2	PLD γ 1	PLD γ 2	PLD γ 3	PLD δ	PLD ϵ	PLD ζ 1	PLD ζ 2
PLD	0.23	0.23	0.15	0.13	0.14	0.22	0.07	0.07	0.15	0.4	0.13	0.46

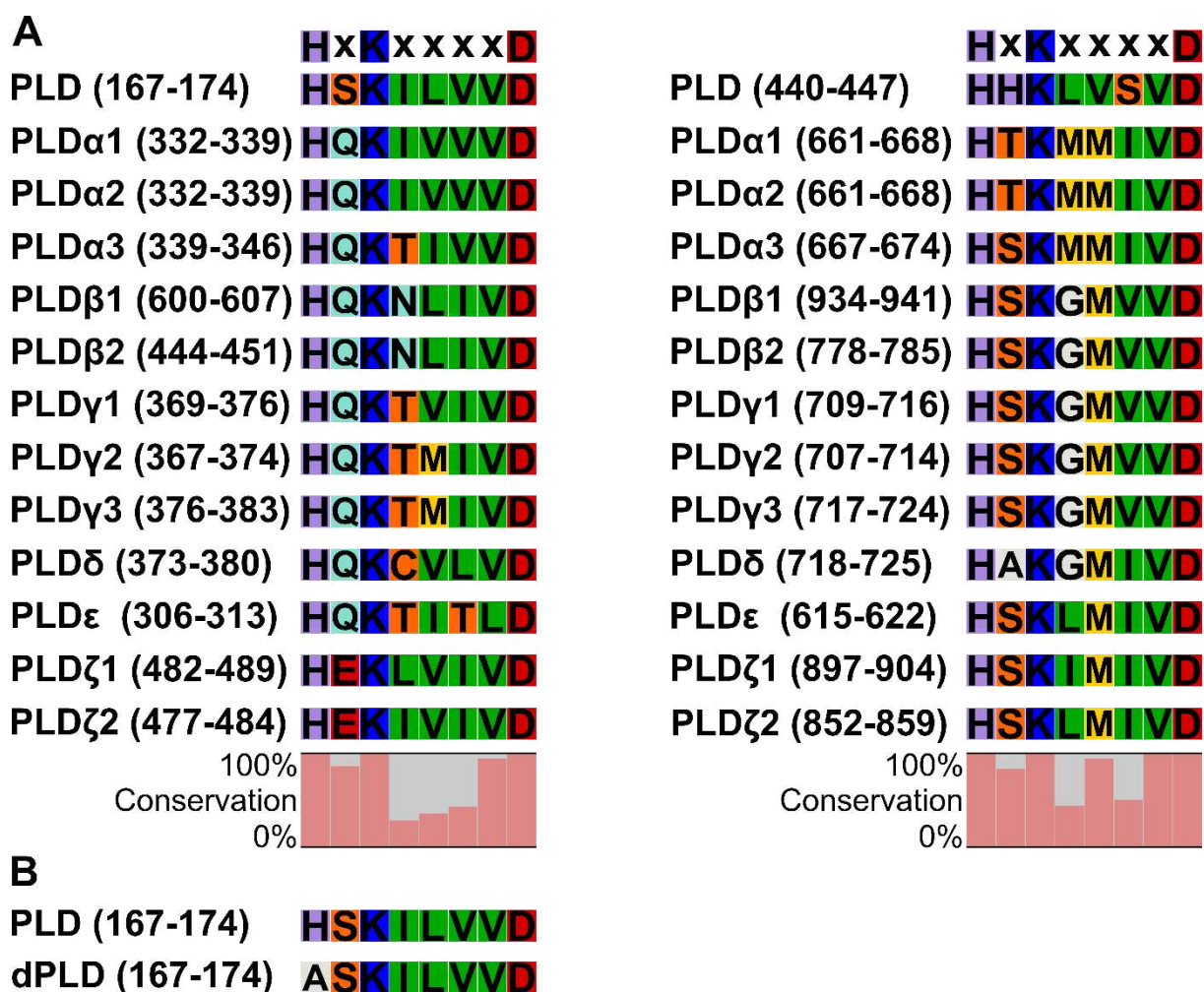


Figure 24 Protein alignment of the HKD-domains of the PLD and the 12 *Arabidopsis thaliana* PLDs

A) Both HKD domains are crucial for phospholipase function of the PLDs. Protein alignments of PLD and the 12 *Arabidopsis* PLDs shows that despite the little overall amino acid sequence homology of the PLD against the *Arabidopsis* PLDs, both HKD domains are highly conserved. B) Protein alignment of the HKD domain of the PLD and its catalytic inactive mutant, containing a point mutation (H167A) (50, 94). CLC Main Workbench 8.1 (www.clcbio.com, QIAGEN, Aarhus, Denmark).

3.2.3 Transient expression and localization of PLD in *Nicotiana benthamiana* leaves

We used the N-terminal backbone CBL1n12-3x(mCherry) (see 3.2) to target the PLD to the plasma membrane where it is supposed to hydrolyze PC hereby upregulating the PA level (Figure 25). The *PLD*, as well as the *dPLD*, in the following experiments were expressed under control of the *pUBQ10* promoter. Our results using *Nicotiana benthamiana* leaf infiltration indicate proper plasma membrane localization of PLD, as wells the catalytic inactive dPLD (Figure 26 A). Performing *Nicotiana benthamiana* leaf infiltration and a following plasmolysis using 800mM mannitol further indicate the plasma membrane localization of both PLD and

dPLD (Figure 26 D). These results were further confirmed in chapter 3.2.6 by co-infiltrating PLD and dPLD with plasma membrane associated biosensors. Again, comparable to the results of CBL1n12-3x(mCherry) we also observed a slight off target localization, most likely as an artifact of the strong transient expression.

We also observed a strong cell death activity in response of transient PLD expression 62 h.p.i., while the dPLD never showed any cell death response at all (Figure 26 C). This cell death response was not visible 40 h.p.i., which is why all experiments that included *Nicotiana benthamiana* leaf infiltration were performed until approximately 40 h.p.i.

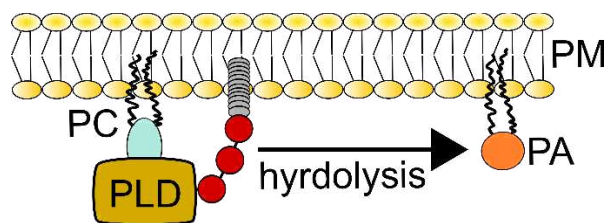


Figure 25 Schematic illustration of the PLD function at the plasma membrane

The PLD has a N-terminal CBL1n12 plasma membrane anchor (grey) and is tagged with a 3x(mCherry) fluorophore (red circles). The amino acid linker in-between the fluorophores give the tool the needed flexibility, to give the protein access to the plasma membrane associated substrate (PC) to upregulate PA level. PC = phosphatidyl choline; PA = phosphatidic acid.

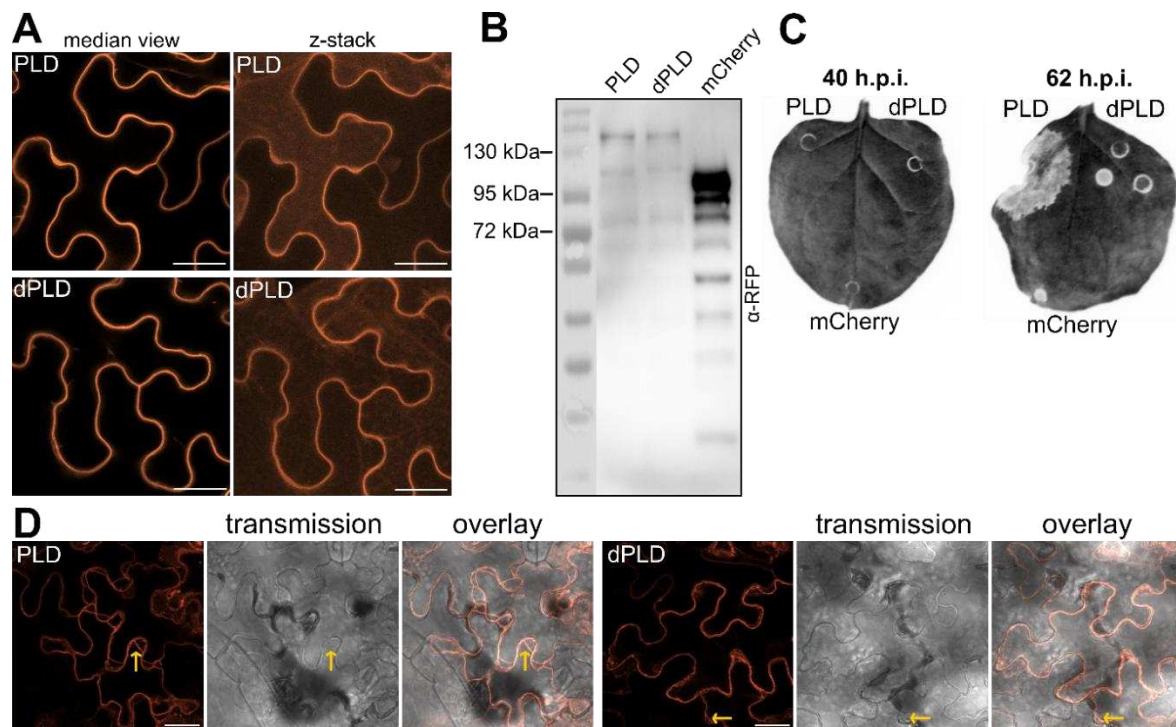


Figure 26 PLD localized to the plasma membrane when transiently expressed in *Nicotiana benthamiana* leaves

A) Transient *pUBQ10::CBL1n12-3x(mCherry)-PLD* (top) and the catalytic inactive *pUBQ10::CBL1n12-3xmCherry-dPLD* (bottom) expression in *Nicotiana benthamiana* leaves. Images on the left represent single plain pictures of the mid-section of a cell. Images on the right represent stacked pictures of the cell that had been made from the top to the mid-section. The images suggest plasma membrane localization of PLD and dPLD. White bars represent 25µm. Pictures were taken 40 h.p.i.

B) Immunoblot detection of PLD (138 kDa), dPLD (138 kDa) and mCherry (85 kDa) using α-RFP. The sample were harvested 40 h.p.i tobacco leave infiltration.

C) *Nicotiana benthamiana* leaves 40 h.p.i and 62 h.p.i. after infiltration with PLD and dPLD. Images were taken under UV light (6.7).

D) Plasmolysis after transient expression of *pUBQ10::CBL1n12-3x(mCherry)-PLD* (left) and *pUBQ10::CBL1n12-3xmCherry-dPLD* (right) in *Nicotiana benthamiana* leaves confirm plasma membrane localization of PLD and dPLD.. The pictures were taken 40 h.p.i. and 30 minutes after 800mM mannitol infiltration. Yellow arrows mark detached plasma membrane. White bar represents 40µm.

3.2.4 Design of the Phosphatidic Acid Phosphatase

We minimized, based on recent studies, the protein size of *Arabidopsis thaliana* PAH1 to avoid any unwanted regulatory process manipulating the localization, activity and stability of our tool. This minimization is based on protein alignments of *Arabidopsis thaliana* PAH1 against other well described PAPs from other organisms (see chapter 2). Earlier studies revealed that most domains of the PAPs are not needed to remain phosphatase function. For example, in 2010 Karanasios *et al.* showed that the very N-terminal end of yeast PAH1p contains an amphipathic helix, important for proper protein localization but not needed for phosphatase function (55). In 2017 Park *et al.* introduced the C-terminal tryptophan domain in yeast Pah1p (56). They showed that deletion of this tryptophan domain does not lead to loss of the phosphatase activity but is important for proper protein function. This result was further confirmed in 2022 by Park *et al.* (56, 57). Also, in 2020 Khayyo *et al.* showed that truncating the amino acid sequence between the N-LIP and C-LIP domain (the non-conserved regions and the M-LIP domain) are not required to remain proper phosphatase function (58). Moreover, they solved the crystal structure of a new kind of PAP from the fresh water living ciliate *Tetrahymena thermophila* (TtPAH2), which naturally only consist of the N-LIP and C-LIP domain. TtPAH2 confirmed that only the N-LIP and C-LIP domain are sufficient for proper phosphatase function (58).

Based on these studies as well as amino acid sequence alignments against well described PAPs, we truncated the 904 amino acids full length *Arabidopsis thaliana* PAH1 to 301 amino acids, including the N-LIP and C-LIP domain (Figure 27 A). This minimized *Arabidopsis thaliana* PAH1 is further referred to as PAP. We used the N-terminal backbone CBL1n12-3x(mCherry) (see 3.2) to target the PAP to the plasma membrane where it is supposed to catalyze the dephosphorylation of PA to DAG (Figure 27 B). As a negative control for PAP activity we also

introduced the catalytically inactive mutant D129E, further referred to as dPAP (Figure 27 C). This point mutation is in the catalytically crucial HAD-like domain and was shown to lead to complete loss phosphatase function (68, 81).

We used AlphaFold2 to analyze the theoretical 3D-protein structure of our minimized PAP compared to other truncated earlier described PAPs that were shown to still remain phosphatase function (Figure 28). The *in silico* protein analysis of AlphaFold2 confirmed that the structure of our tool theoretically remains stable and should guarantee phosphatase function.

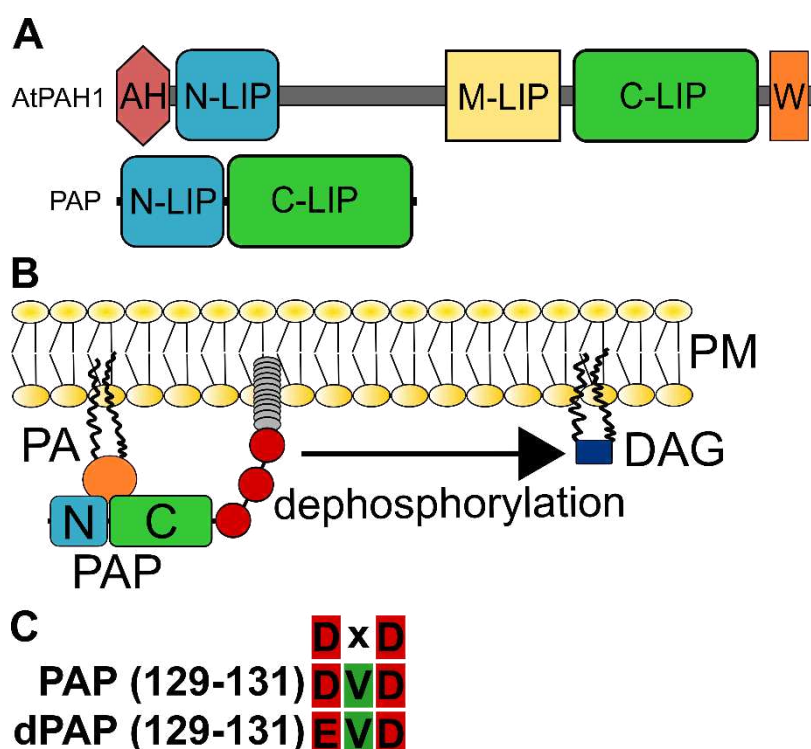


Figure 27 Schematic illustration of the PAP structure and function

A) Schematic protein organization of AtPAH1, and PAP, a minimized protein based on AtPAH1.

B) PAP has a N-terminal CBL1n12 plasma membrane anchor (grey) and is tagged with a 3x(mCherry) fluorophore (red circles). The amino acid linker in-between the fluorophores give the tool the needed flexibility, to give the protein access to the plasma membrane associated substrate (PA) and fulfill its phosphatase function.

C) Point mutation (D129E) in the HAD-like domain leads to loss of phosphatase function. The HAD-like domain is part of the C-LIP domain

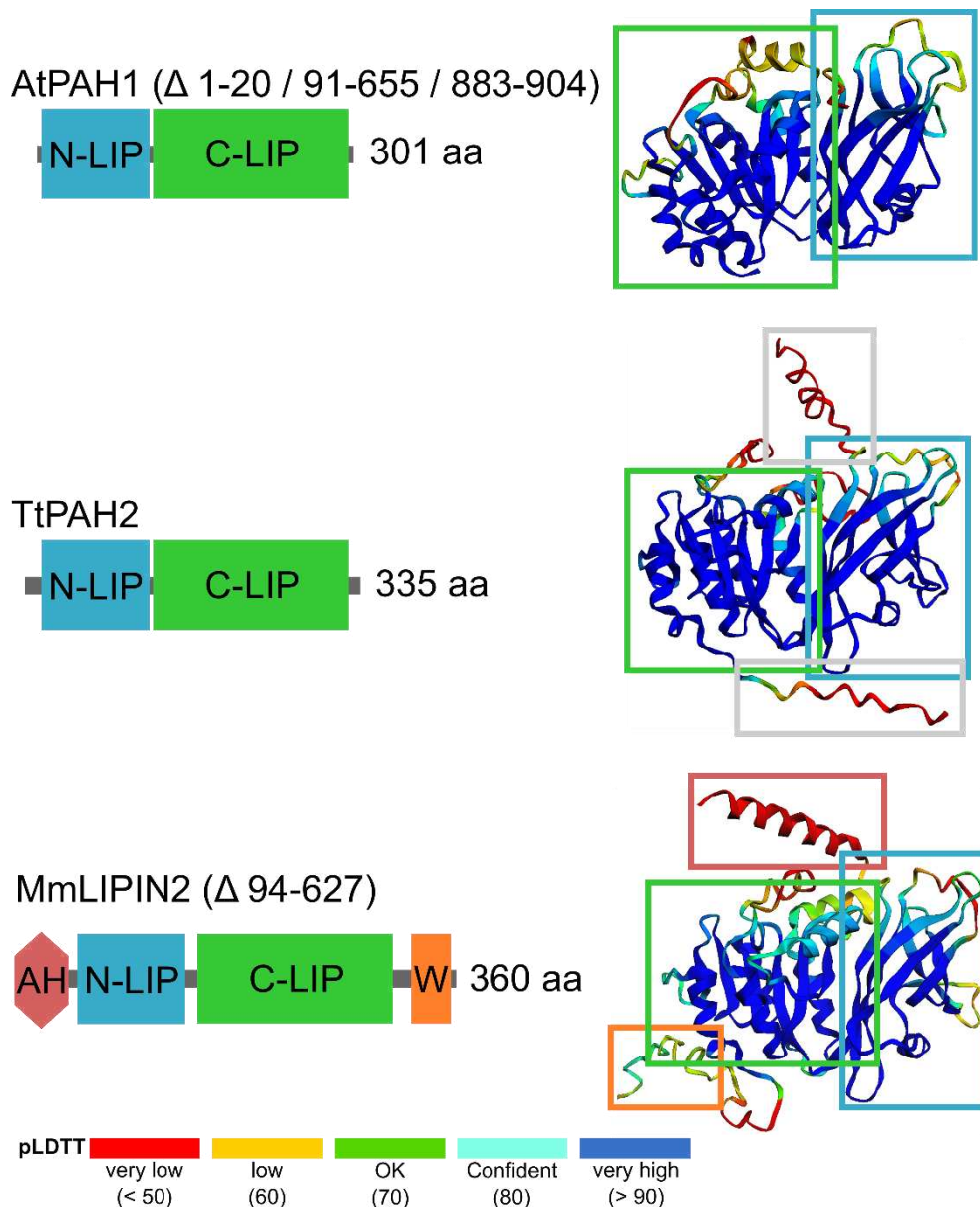


Figure 28 Protein domain organization and putative structure of the minimal PAPs of *Arabidopsis*, *Tetrahymena thermophila* and mouse

The theoretical protein 3D-structure of PAP (AtPAH1 (Δ 1-20 / 91-655 / 883-0904), *Tetrahymena thermophila* PAH2 (TtPAH2) and MmLIPIN2 (Δ 94-627) were made using AlphaFold2 AI (6.11.2). Alphafolds model for TtPAH2 fits with the published crystal structure in Khayyo *et al.* (2020) (58). The model predicts that the deletion of the different, for the phosphatase function dispensable domains, do still lead to proper N-LIP and C-LIP domain folding.

3.2.5 Transient expression and localization of PAP in *Nicotiana benthamiana* leaves

Our results using *Nicotiana benthamiana* leaf infiltration indicate plasma membrane localization of PAP and the catalytic inactive dPAP (Figure 29 A). These results were further confirmed in chapter 3.2.6 by co-infiltrating PAP and dPAP with plasma membrane associated

biosensors. *PAP* and *dPAP* expression in the following experiments were under control of the *UBIQUITINE10* promoter. Performing *Nicotiana benthamiana* leaf infiltration and a following plasmolysis using 800mM mannitol further indicate the plasma membrane localization of both *PAP* and *dPAP* (Figure 29 D). Comparable to the results of *CBL1n12-3x(mCherry)* we again observed a slight off target localization, most likely as an artifact of the strong transient expression.

Other than for the transient *PLD* expression, we never observed any cell death activity in response of transient *PAP* or *dPAP* expression at any tested time points (Figure 29 C).

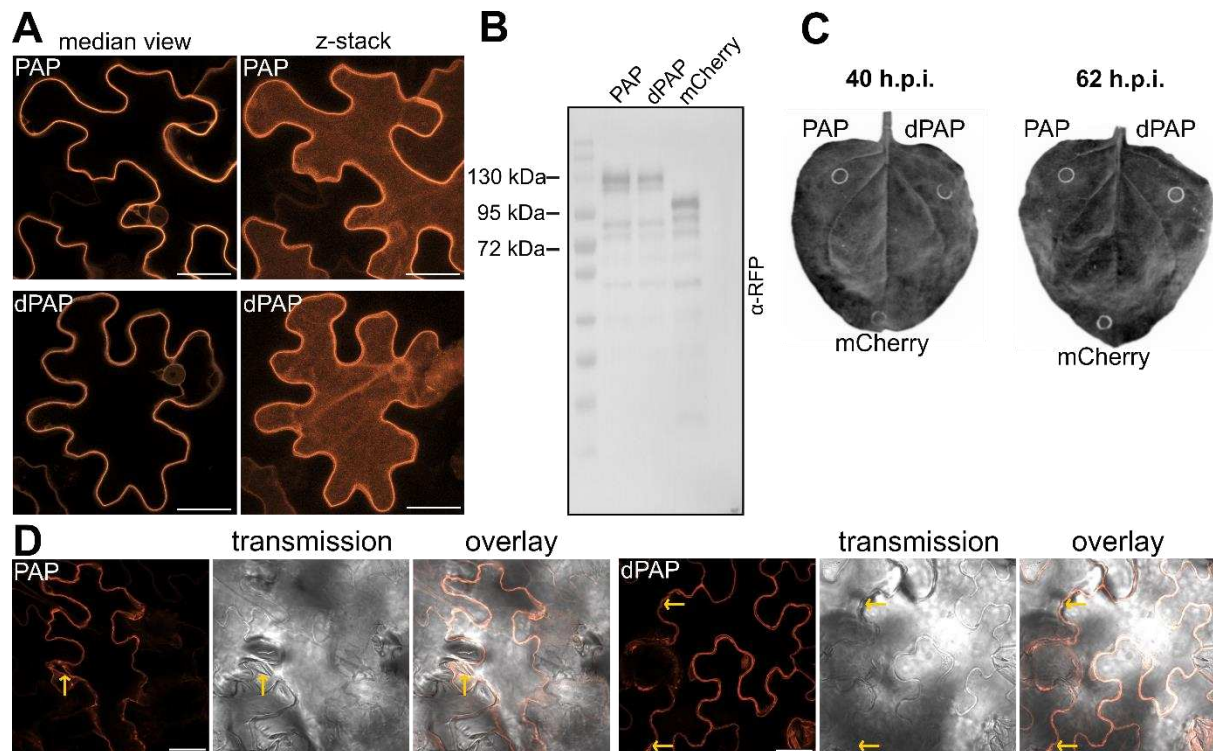


Figure 29 PAP localized to the plasma membrane when transiently expressed in *Nicotiana benthamiana* leaves

A) Transient expression of *pUBQ10::CBL1n12-3xmCherry-PAP* (top) and the catalytic inactive *pUBQ10::CBL1n12-3xmCherry-dPAP* (bottom) in *Nicotiana benthamiana* leaves. Images on the left represent single plain pictures of the mid-section of a cell. Images on the right represent stacked pictures of the cell that had been made from the top to the mid-section. The images suggest plasma membrane localization of *PAP* and *dPAP*. White bars represent 25 μm. Pictures were taken 40 h.p.i.

B) Immunoblot detection of *PAP* (118 kDa), *dPAP* (118 kDa) and *mCherry* (85 kDa) using α-RFP. The sample were harvested 40 h.p.i tobacco leave infiltration.

C) *Nicotiana benthamiana* leaves after infiltration with *PAP* and *dPAP* showed no cell death response 40 or 62 h.p.i.. Images were taken under UV light (6.7).

D) Plasmolysis after transient expression of *pUBQ10::CBL1n12-3xmCherry-PAP* (left) and *pUBQ10::CBL1n12-3xmCherry-dPAP* (right) in *Nicotiana benthamiana* leaves confirm plasma membrane localization of *PAP* and *dPAP*. The pictures were taken 40 h.p.i. and 30 minutes after 800mM mannitol infiltration. Yellow arrows mark detached plasma membrane. White bar represents 40 μm.

3.2.6 PA-associated biosensors don't show any changes in localization when co-infiltrated with PLD or PAP in *Nicotiana benthamiana* leaves

In order to analyze the functionality of the PA-level modifying tools, we tested the localization of biosensors in *Nicotiana benthamiana* leaves using confocal microscopy. As biosensors we used the two PA specific biosensors 1xPASS and 2xPASS as well as KA1, a biosensor for anionic lipids, since they should change their localization upon increasing or decreasing PA-levels. Past studies showed that 1xPASS and 2xPASS localize to the plasma membrane and detach of the plasma membrane when treated with DGK-inhibitors. KA1 also localizes to the plasma membrane and also alters its localization when treated with different drugs as well as when co-infiltrated into tobacco leaves with the PI(4)P phosphatase SAC1 (5, 10).

To analyze whether our tools affect PA distribution we co-infiltrated PLD, dPLD, PAP, dPAP and mCherry with the two PA specific biosensors “PA biosensor with superior sensitivity” *pUBQ10::mCitrine-1xPASS* and *pUBQ10::mCitrine-2xPASS* (Figure 30 A, Figure 31 A) (10, 98). PASS PA-specific biosensor consists of the PA-binding site of yeast Spo20p protein, tagged with a nuclear export signal (NES) to exclude nuclear localization to increase the cytoplasmic accessibility of the biosensor (10, 98). 1xPASS and 2xPASS both localized predominantly to the plasma membrane (and weakly to the nuclear laminar or ER) when infiltrated to *Nicotiana benthamiana* leaves. This localization pattern was not affected by PLD nor PAP co-infiltration as our images and the line intensity plot profile suggest. As control we also infiltrate the catalytically dead dPLD and dPAP as well as mCherry. Also, none of them affected 1xPASS nor 2xPASS localization according to the confocal microscopy images and the line intensity plot profiles (Figure 30 & Figure 31).

Besides the two PA specific biosensors we also tested the effect of our tools on *pUBQ10::cYFP-KA1* when infiltrated into tobacco leaves. Kinase Associated-1 (KA1) of the human microtubule affinity-regulated kinase (MARK)1 is a biosensor that was shown to bind unspecifically to anionic lipids (Figure 32) (5, 99). PA but also PI and PIP's, as well as PS are anionic phospholipids and are less abundant in membranes where they fulfill important regulatory functions (10). KA1 localized predominantly to the plasma membrane when infiltrated to *Nicotiana benthamiana* leaves. This localization pattern was not affected by PLD nor PAP co-infiltration as our images and the line intensity plot profiles suggest. As control we also infiltrate the catalytically dead dPLD and dPAP as well as mCherry. Also, none of them affected KA1 localization according to the confocal microscopy images and the line intensity plot profiles. Combined these results further confirm the plasma membrane association of all tested tools.

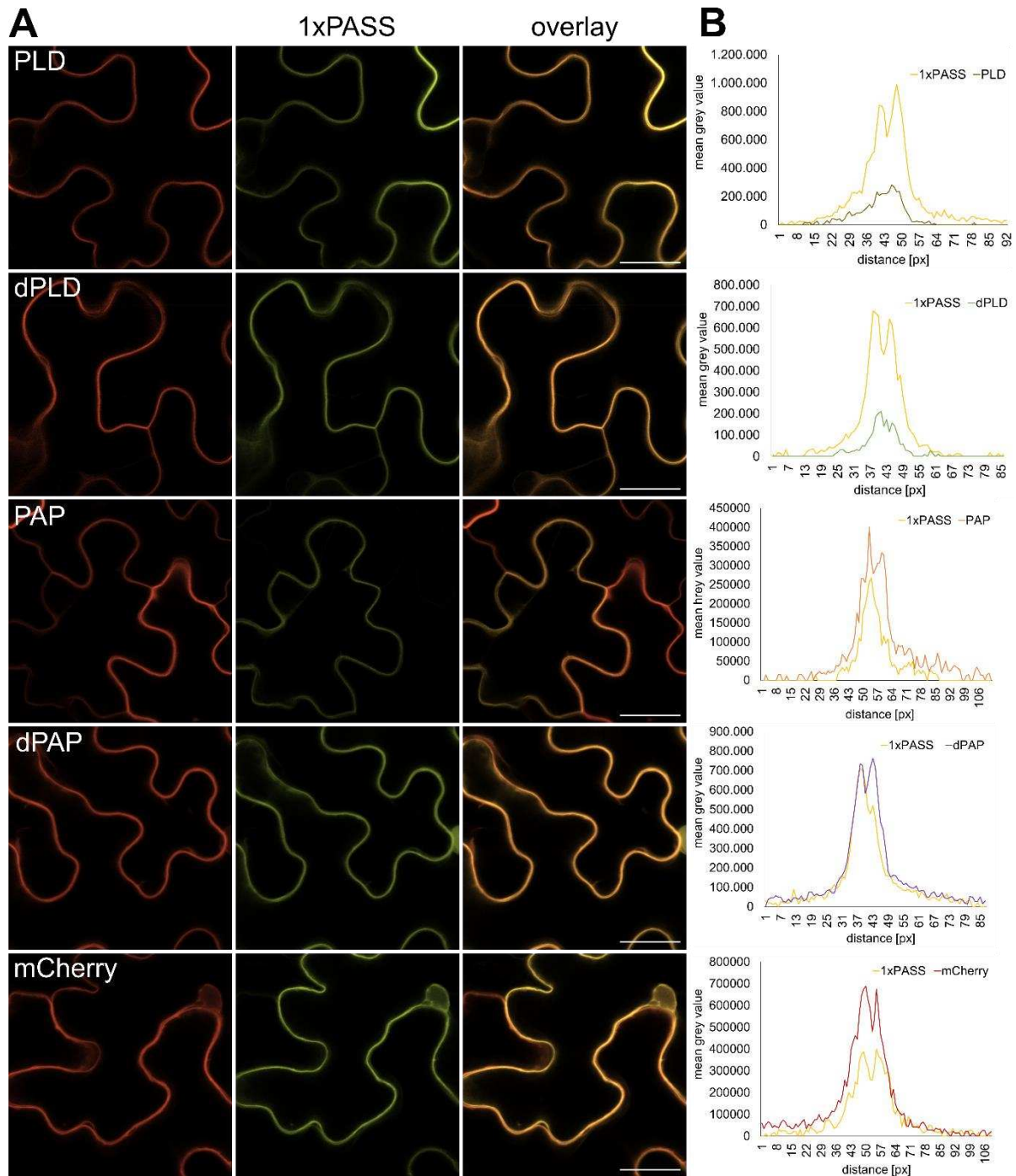


Figure 30 Co-infiltration of PLD and PAP with PA-associated biosensor 1xPASS in *Nicotiana benthamiana* leaves

A) Confocal microscopy images of PLD, dPLD, PAP, dPAP and mCherry (orange) co-infiltrated with PA-associated biosensor *pUBQ10::mCitrine-1xPASS* (yellow) into *Nicotiana benthamiana* leaves (10, 98). The Pictures were taken 40h.p.i. White bars at the bottom right indicate a length of 25µm. The experiment was repeated twice. n = 3

B) Line intensity plot profile of PLD, dPLD, PAP, dPAP and mCherry against 1xPASS localization. Measurements were done with ImageJ (6.9).

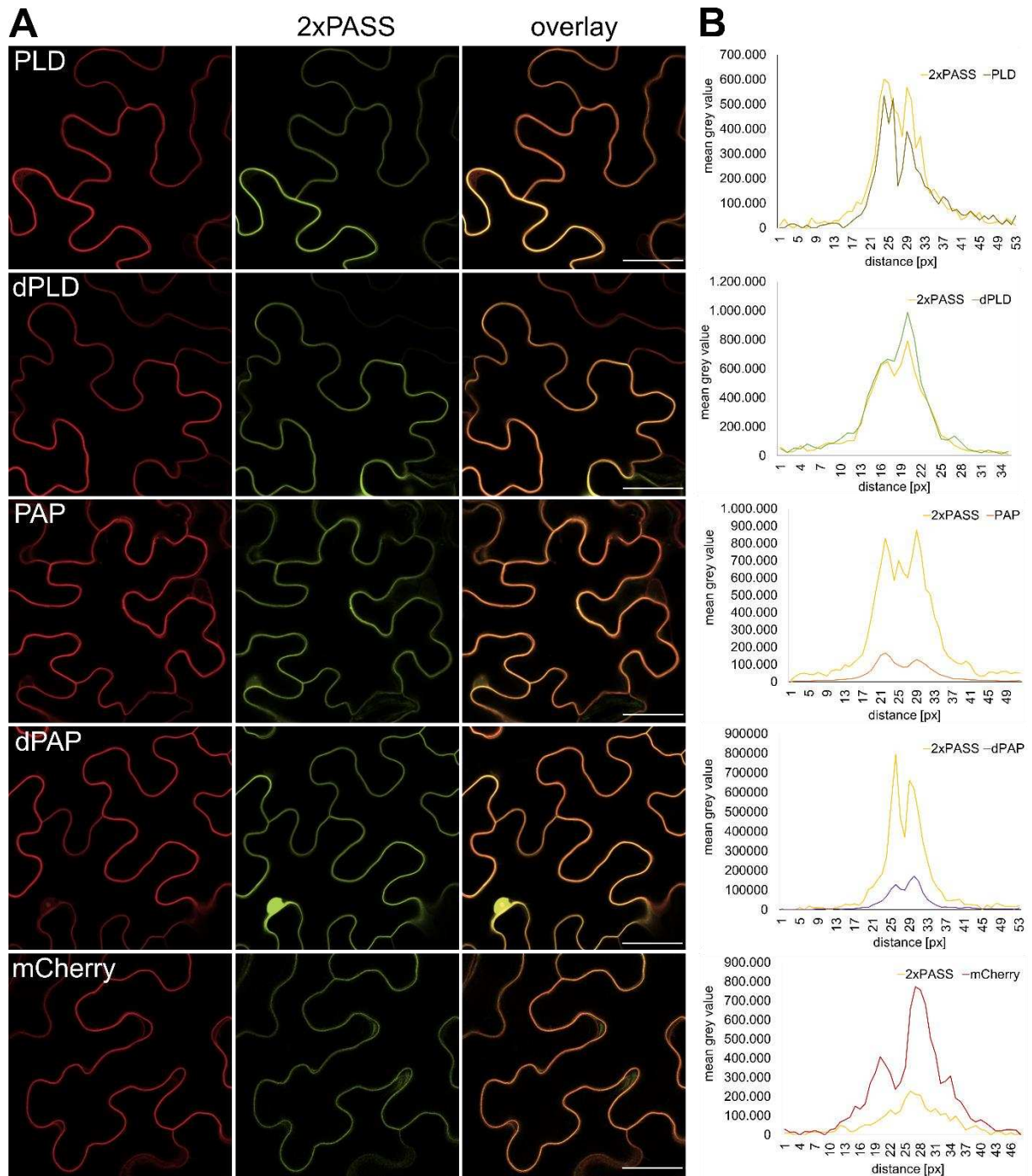


Figure 31 Co-infiltration of PLD and PAP with PA-associated biosensor 2xPASS in *Nicotiana benthamiana* leaves

A) Confocal microscopy images of PLD, dPLD, PAP, dPAP and mCherry (orange) co-infiltrated with PA-associated biosensor *pUBQ10::mCitrine-2xPASS* (yellow) into *Nicotiana benthamiana* leaves (10, 98). The Pictures were taken 40h.p.i. White bars at the bottom right indicate a length of 25µm. The experiment was repeated twice. n = 4

B) Line intensity plot profile of PLD, dPLD, PAP, dPAP and mCherry against 2xPASS localization. Measurements were done with ImageJ (6.9).

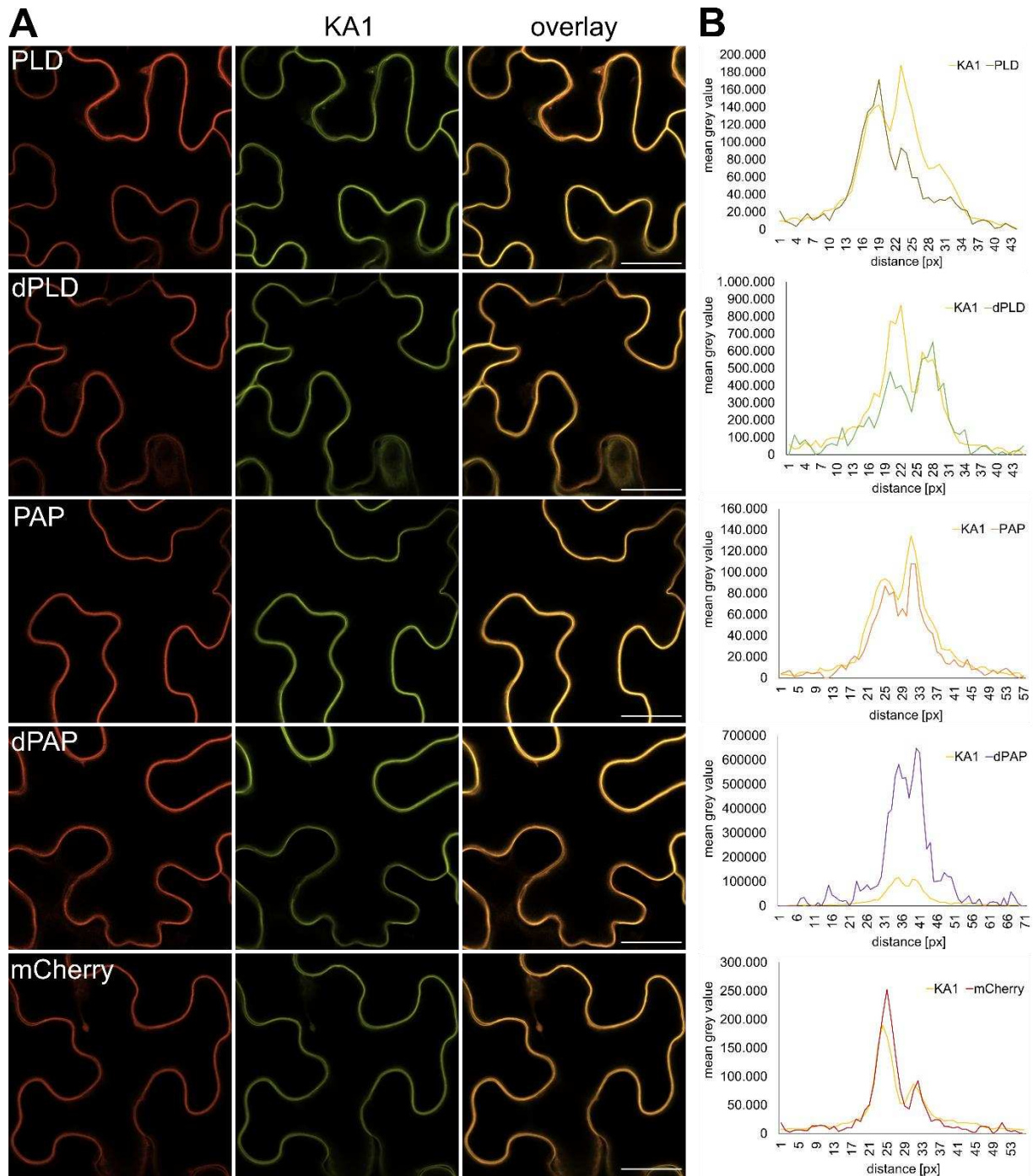


Figure 32 Co-infiltration of PLD and PAP with KA1 in *Nicotiana benthamiana* leaves

A) Confocal microscopy images of PLD, dPLD, PAP, dPAP and mCherry (orange) co-infiltrated with *pUBQ10::cYFP-KA1* (yellow) into *Nicotiana benthamiana* leaves. Kinase Associated-1 (KA1) binds unspecifically to every anionic lipid (5). The Pictures were taken 40h.p.i. White bars at the bottom right indicate a length of 25 μ m. The experiment was repeated twice. n = 5

B) Line intensity plot profile of PLD, dPLD, PAP, dPAP and mCherry against KA1 localization. Measurements were done with ImageJ (6.9).

3.2.7 Physical-chemical analysis of PLD and PAP function after *Nicotiana benthamiana* leaf infiltration using thin-layer chromatography and gas chromatography

To further analyze the effect of PLD and PAP when transiently expressed in *Nicotiana benthamiana* leaves we used two physical-chemical approaches: 2-dimensional thin layer chromatography (2D-TLC) and gas chromatography (GC). We started analyzing the samples using 2D-TLC. A TLC is a physical-chemical separation process using a silica gel as a stationary phase and a mobile phase, in our case optimized for phospholipid separation. Running the TLC for two dimensions allows us to precisely separate the different phospholipids, which gave us the opportunity to further analyze the samples. We transiently expressed *PLD*, *dPLD*, *PAP*, *dPAP* and *mCherry* in tobacco leaves and harvested the samples 40 h.p.i (Figure 33 C). After performing a lipid extraction, the samples were put on the silica gel and the first dimension was run. After drying, the plates were turned for 90° and the second dimension was run (Figure 33 A). The plates were then split for two different purposes: two plates per tool were burned on a hot plate at 180°C to analyze the phospholipid profile after the 2D-TLC (Figure 33 B), four plates were further prepared for harvesting the sample for GC analysis. We analyzed the phospholipid profile of the 2D-TLC by measuring the mean grey value of each lipid band using ImageJ (Figure 33 D). To quantify whether the PA level changed when expressing *PLD* or *PAP* we measured the mean grey value of PA divided through the mean grey value of either PC, PE, DGDG and MGDG, as these are the most abundant lipids that we measured. Unfortunately, there was no clear difference between the ratiometric PA level of PLD, dPLD, PAP, dPAP and mCherry detectable. It is to note that the lipid organization of the 2D-TLC for the dPAP samples varies strongly compared to the other constructs for unknown technical issues, making the results questionable.

To quantify the phospholipid content of each sample, the four other plates that have not been burned were further processed. Briefly, to visualize the bands in a non-destructive way for lipid extraction from the silica gel the plates were sprayed with anilino naphthalene sulfonic acid, a detergent that fluoresces under UV-light when bound to lipids. The bands were then scratched of the plate individually for GC analysis (Figure 34 B-E). To analyze the effect of PLD and PAP on the PA level of the tobacco leaves, we, comparable to the 2D-TLC, divided the PA content through the PC, PE, DGDG and MGDG content measured (Figure 34 F-H). We then compared the means for PLD with the once of dPLD and mCherry and also compared the means of PAP against mCherry. The results showed no obvious effects of PLD and PAP on PA content after transient expression in tobacco leaves.

A

First Dimension

Second Dimension

2D-TLC: 150 µg extract

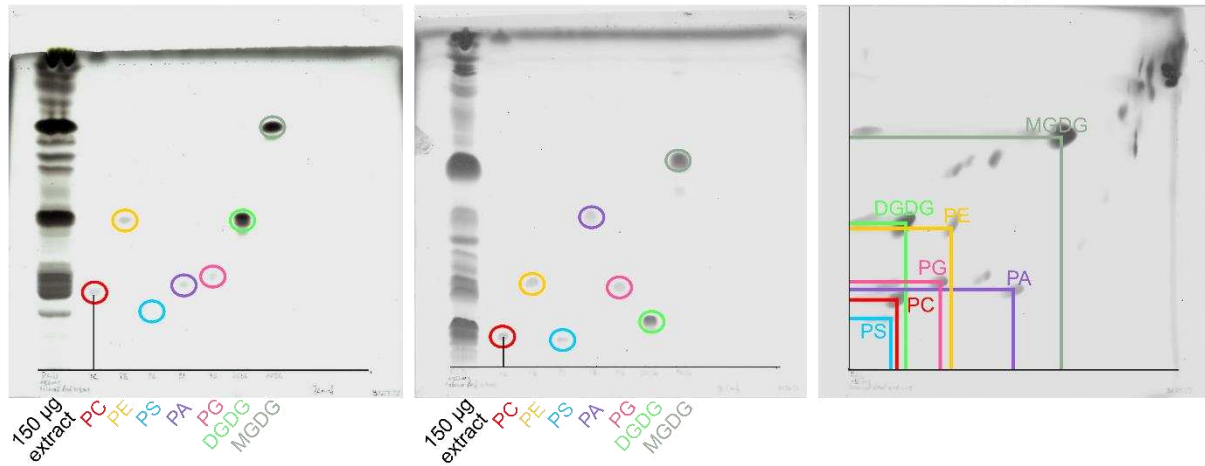
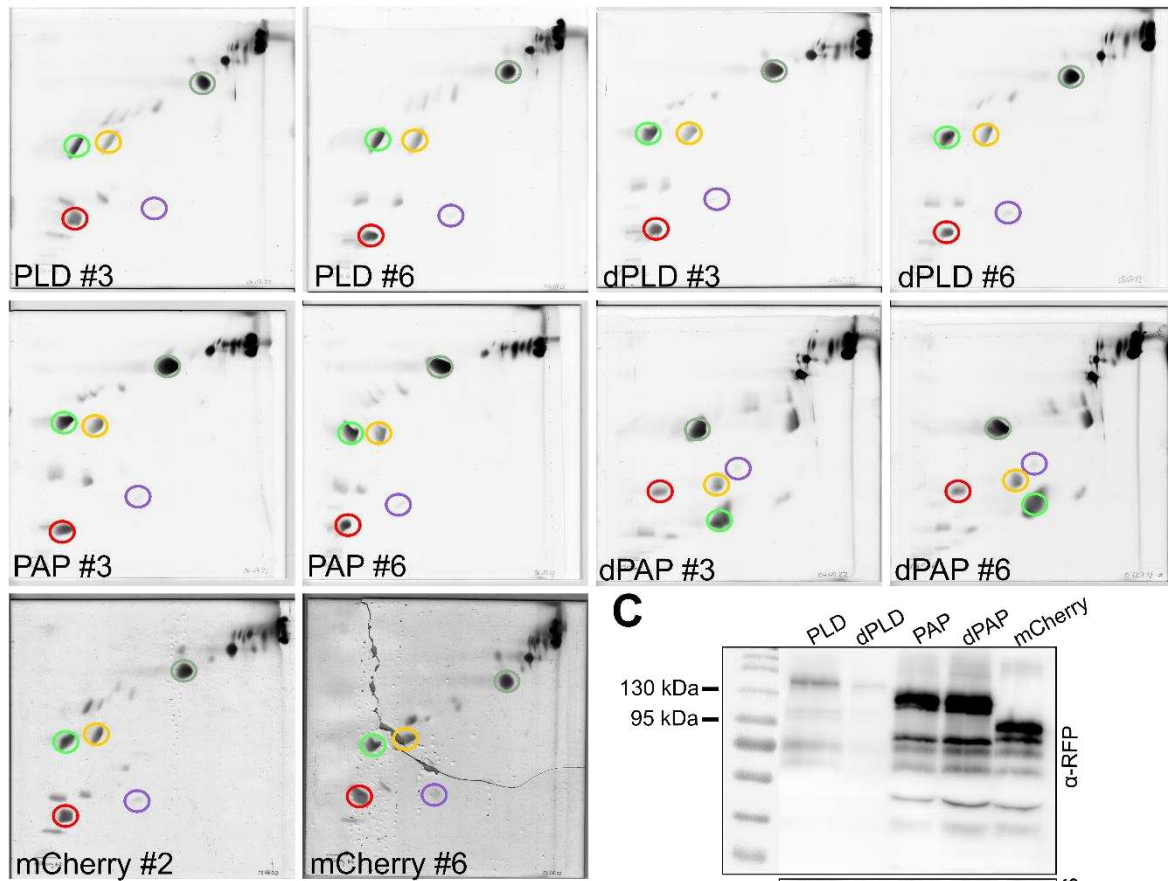
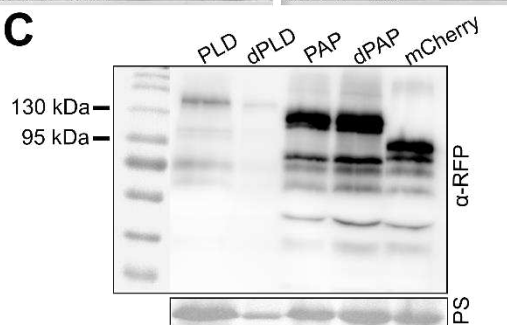
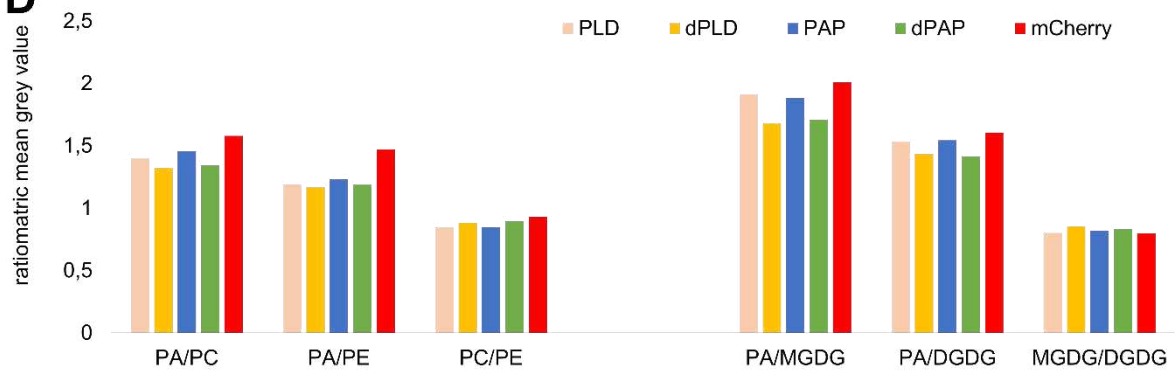
**B****C****D**

Figure 33 2D-Thin Layer Chromatography of lipid extracts from *Nicotiana benthamiana* leaves expressing *PLD* and *PAP* show no discernible differences in PA level

A) 1 dimensional (left, middle) and 2 dimensional (right) Thin Layer Chromatographies (TLC) to illustrate the identification of the different lipids. The left lane of the 1 dimensional TLCs are 150µg lipid extract of *Nicotiana benthamiana* leaves. The single bands highlighted in different colours are standards for the different lipids that were analyzed.

B) 2D-TLCs of lipid extracts from *Nicotiana benthamiana* leaves expressing *pUBQ10::CBL1n12-3x(mCherry)-PLD*, *pUBQ10::CBL1n12-3x(mCherry)-dPLD*, *pUBQ10::CBL1n12-3x(mCherry)-PAP*, *pUBQ10::CBL1n12-3x(mCherry)-dPAP* and *pUBQ10::CBL1n12-3x(mCherry)*. The phospholipid profile of the two dPAP 2D-TLC varies from the other 2D-TLC due to unknown technical issues. The samples were harvested 40 h.p.i. The coloured circles highlight the lipids according to A.

C) Immunoblot detection of PLD (138 kDa), dPLD (138 kDa), PAP (118 kDa), dPAP (118 kDa) and mCherry (85 kDa) using α -RFP. The samples were harvested 40 h.p.i.

D) 2D-TLC analysis was performed by measuring the mean grey value of each band using ImageJ. The mean grey value of PA was divided through the mean grey value of different lipids (PC, PE, MGDG, DGDG) to calculate differences in PA level. n = 2

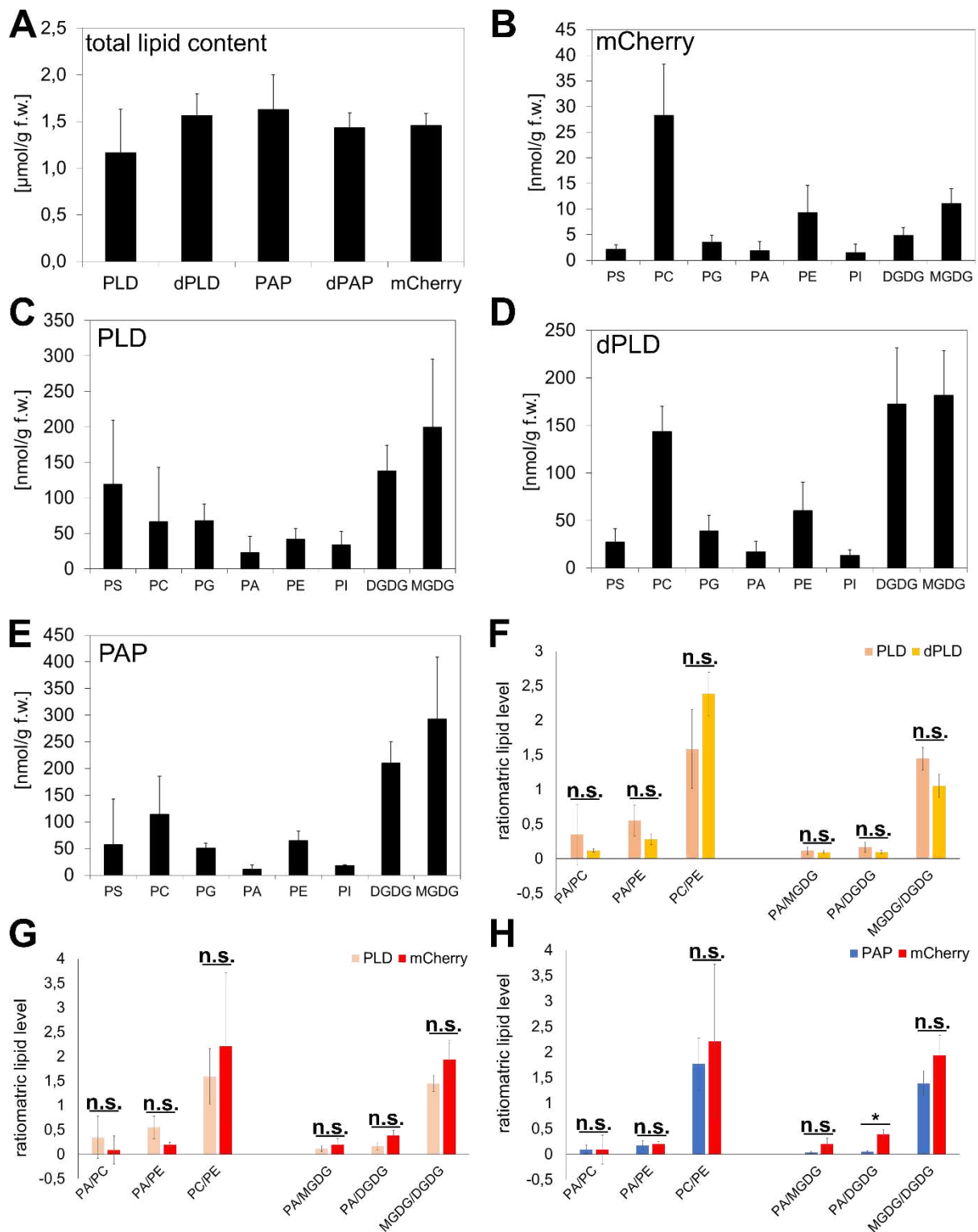


Figure 34 Phospholipid profile of *Nicotiana benthamiana* leaves transiently expressing PLD and PAP analyzed using gas chromatography show no significant differences in PA level

A) Total lipid content of *Nicotiana benthamiana* leaves transiently expressing *pUBQ10::CBL1n12-3x(mCherry)-PLD*, *pUBQ10::CBL1n12-3x(mCherry)-dPLD*, *pUBQ10::CBL1n12-3x(mCherry)-PAP*, *pUBQ10::CBL1n12-3x(mCherry)-dPAP* and

pUBQ10::CBL1n12-3x(mCherry) analyzed using gas chromatography.¹ The samples were harvested 40 h.p.i. n = 4

B-E) Phospholipid profile of PLD, dPLD, PAP, dPAP and mCherry after transiently expression in *Nicotiana benthamiana* leaves. The Lipid extract was first separated using 2D-TLC (see controls of the corresponding 2D-TLC's in Figure 33. To visualize the lipids under UV-light in a non-destructive way and subsequent GC analysis, the plates were then sprayed with anilino naphthalene sulfonic acid. The visualized lipid bands were marked and the lipids were then extracted by scratching them of the plate individually. n = 4

F-H) Ratiometric lipid level of PA against PC, PE, DGDG and MGDG for the lipid profile of PLD against dPLD (F), PLD against mCherry (G), and PAP against mCherry (H). Means were compared using Student T-Test. Asterisks indicate significant differences at *P < 0.05, **P < 0.01, and ***P < 0.001; n.s.= no significance.

3.2.8 MAP65-1 localization and activity are not affected when co-infiltrated in *Nicotiana benthamiana* leaves with PLD and PAP

3.2.8.1 Introduction

Salt stress is a tremendous problem in intense agriculture as NaCl leads to osmotic stress as well as NaCl toxicity. To face salt stress plants evolved a variety of different signaling pathways to respond and survive in this dangerous environment. It was shown in the past that PA plays an important role for plants in response to salt stress (33).

In 2012 Zhang and colleagues showed that PA regulates microtubule associated proteins 65-1 (MAP65-1) activity in response to salt stress (32). They described that PLD α 1 activity is important for proper cortical microtubule organization. They treated transgenic Arabidopsis wild-type seedlings and the *plda1*, both expressing the cortical microtubule biosensor *35S::GFP-TUA6*, with 50mM sodium chloride and counted the number of microtubules across a line of fixed distance in the hypocotyl and the cotyledons. After 1 day of salt treatment the number of cortical microtubules were significantly reduced in the *plda1* mutant seedlings compared to the wild type. They confirmed this result by treating wild type and *plda1* mutant seedlings with n-Butanol, an alternative substrate for PLDs hereby downregulating PA synthesis. While n-Butanol treatment alone had no effect on the number of microtubules, they illustrated that a combination of salt stress and n-Butanol treatment significantly reduced the number of microtubules in wild type and *plda1* mutant seedlings, while this effect can be partially rescued when the *plda1* mutant seedlings are additionally treated with PA. Using PIP-strip analysis and immunoblotting they also showed that MAP65-1 specifically binds to PA. Furthermore, they were able to show using a turbidimetric assay that PA promotes MAP65-1

¹ The gas chromatography data analysis was performed by Cornelia Herrfurth (Department for Plant Biochemistry, University of Goettingen). The statistical analysis was performed by Frank Ackermann.

mediated microtubule polymerization. They also identified three amino acid sequences in MAP65-1 at position 53-55 (KRK), 61-63 (KSR) and 428-429 (SK) which are important for proper PA binding of the protein (32).

In the following we wanted to test if PLD or PAP activity have any effect on MAP65-1 activity when co-infiltrated into tobacco leaves.

3.2.8.2 Results

To visualize the effect of the PA modifying tools on MAP65-1 we co-infiltrated PLD and PAP, as well as the catalytic inactive dPLD, dPAP and mCherry as negative controls, with *MAP65-1*, expressed under control of the *UBIQUITIN10* promoter and C-terminally tagged with *mCitrine*, into *Nicotiana benthamiana* leaves and imaged the samples using confocal microscopy (Figure 35 A). To quantify the effect of PLD and PAP on MAP65-1 we counted the number of microtubules within a cell along a distance of 40µm. To do so we chose a region within a cell where the microtubules were aligned most parallel. The images as well as the statistical analysis of the number of microtubules suggest that PLD nor PAP activity had any effect on MAP65-1 activity when transiently expressed in *Nicotiana benthamiana* leaves (Figure 35 B).

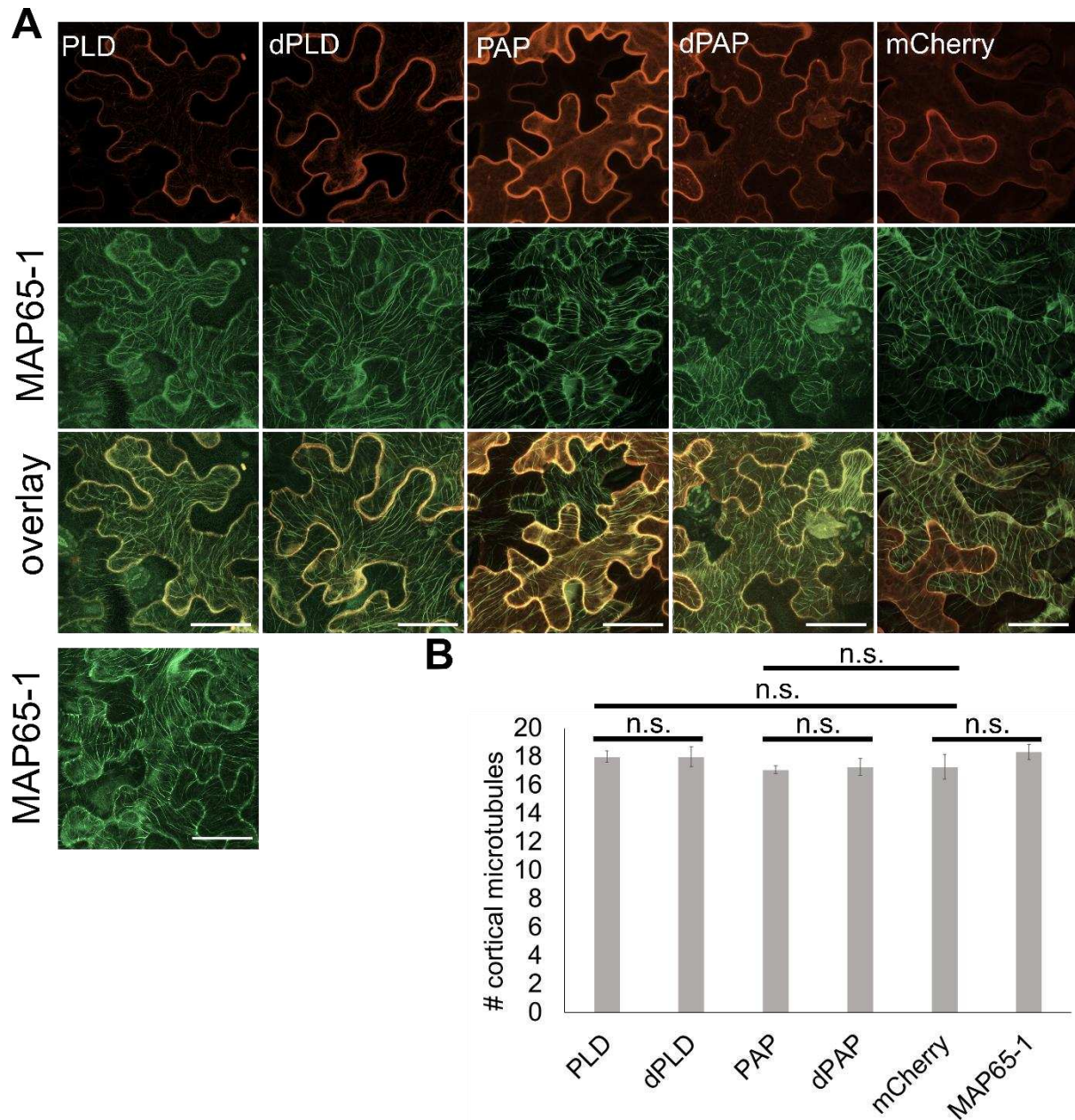


Figure 35 Number of cortical microtubules is not affected when MAP65-1 and PLD or PAP are co-infiltrated in *Nicotiana benthamiana* leaves

A) Maximum projection images of PLD, dPLD, PAP, dPAP and mCherry (orange) after co-infiltration with *pUBQ10::MAP65-1-mCitrine* (green) in *Nicotiana benthamiana* leaves. The pictures were taken 40 h.p.i. White bar illustrates a length 40μm.

B) Quantification of the number of cortical microtubules visualized with MAP65-1 when co-infiltrated with PLD and PAP. Number of microtubules were counted on a length of 40μm. Means were compared using students t-test. Asterisks indicate significant differences at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$; n.s.= no significance. The experiment was performed twice. For quantification, all samples were pooled. MAP65-1: n= 3; PLD: n=8; dPLD: n=10; PAP: n=12; dPAP: n=11; mCherry: n=7

3.2.9 MKK7 and MKK9 localization is not affected by PLD and PAP

3.2.9.1 Introduction

As described in chapter 3.2.8 PA is an important signaling molecule for salt stress response in plants. Mitogen-activated protein kinase 6 (MPK6) and salt overly sensitive 1 (SOS1) are two important players for salt response in plants. SOS1 is a plasma membrane localized sodium/hydrogen exchanger, which was shown to be phosphorylated and hereby activated by MPK6 (34, 100). Also, it was shown that MPK6 can bind to PLD α 1 derived PA, hereby increasing its activity (34). In 2019 Sheng and colleagues reported that PA recruits Mitogen-activated Protein Kinase Kinases (MKK) 7 and MKK9 in response to salt stress and how PA binding increases their activity (33). The authors also showed that PA-MKK7/9 binding increased their catalytic activity in this process. Using a PIP strip assay and additional immunoblotting they showed that MKK7 and MKK9 both specifically bind to PA (33). MKK7 and MKK9 are both able to phosphorylate and hereby activate MPK6. Furthermore, by transiently expressing MKK7 and MKK9 in mesophyll protoplasts they showed that both proteins are recruited to the plasma membrane in response to salt stress (which leads to increasing PA level), but also by treating the protoplasts with 20 μ M PA. They confirmed their findings by performing a cytosol/membrane fractionation and an additional immunoblotting (33).

In the following we wanted to use the specific PA-derived MKK7 and MKK9 recruitment to the plasma membrane to test the activity of the PLD and the PAP.

3.2.9.2 MKK7 and MKK9 localization are not affected when co-infiltrated in *Nicotiana benthamiana* leaves with PLD and PAP

To first test PLD and PAP activity and their effect on MKK7 and MKK9 we performed a *Nicotiana benthamiana* leaf infiltration. MKK7 and MKK9 were C-terminally tagged with 3x(GFP) and expressed under control of the *UBQUITIN10* promoter (Figure 36 D). Interestingly, transient expression in *Nicotiana benthamiana* leaves of MKK7 and MKK9 both led to a strong cell response approximately 65 h.p.i., but not 40 h.p.i (Figure 36 A&B). Confocal microscopy images suggest that MKK7 and MKK9 predominantly localized to the cytosol (Figure 36 D).

Co-infiltration of MKK7 and MKK9 with PLD and PAP, as well as the catalytic inactive dPLD, dPAP and mCherry, into *Nicotiana benthamiana* leaves and additional confocal microscopy imaging showed that PLD nor PAP activity had no impact on MKK7 and MKK9 localization (Figure 37 A, Figure 38 A). Also, the negative controls dPLD, dPAP and mCherry had no effect

on the localization of both proteins. We confirmed these finding by analyzing the pictures using ImageJs line intensity plot profiles (Figure 37 B, Figure 38 B).

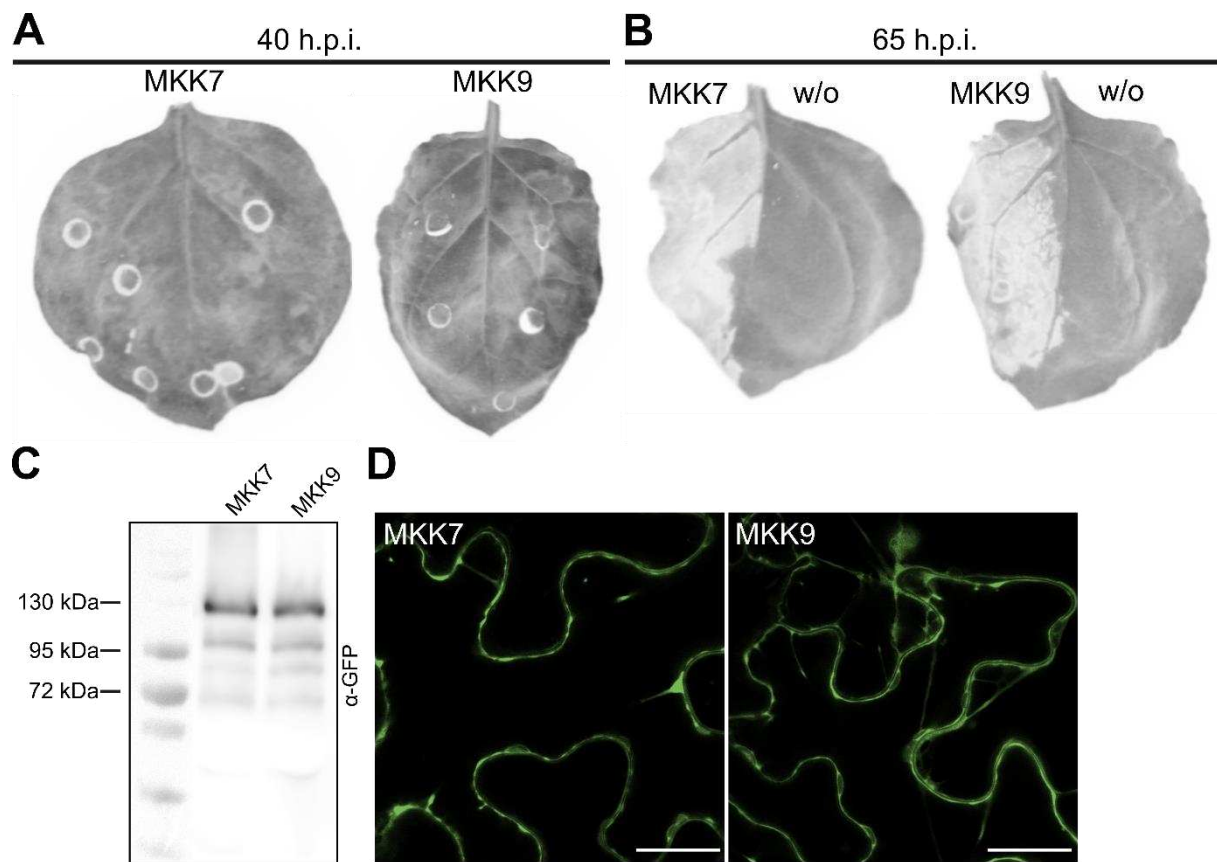


Figure 36 MKK7 and MKK9 localization after *Nicotiana benthamiana* leaf infiltration
A&B) *Nicotiana benthamiana* leaves 40 h.p.i and 65 h.p.i. after infiltration with *pUBQ10::MKK7-3x(GFP)* and *pUBQ10::MKK9-3x(GFP)*. Images were taken under UV light (6.7).
C) Immunoblot detection of MKK7-3x(GFP) (110 kDa) and MKK9-3x(GFP) (110 kDa) was done using α -GFP. Samples were collected 40 h.p.i of *Nicotiana benthamiana* leaf infiltration.
D) Localization of MKK7 and MKK9 40 h.p.i of *Nicotiana benthamiana* leaf infiltration. White bar at the bottom right indicates a length of 20 μ m.

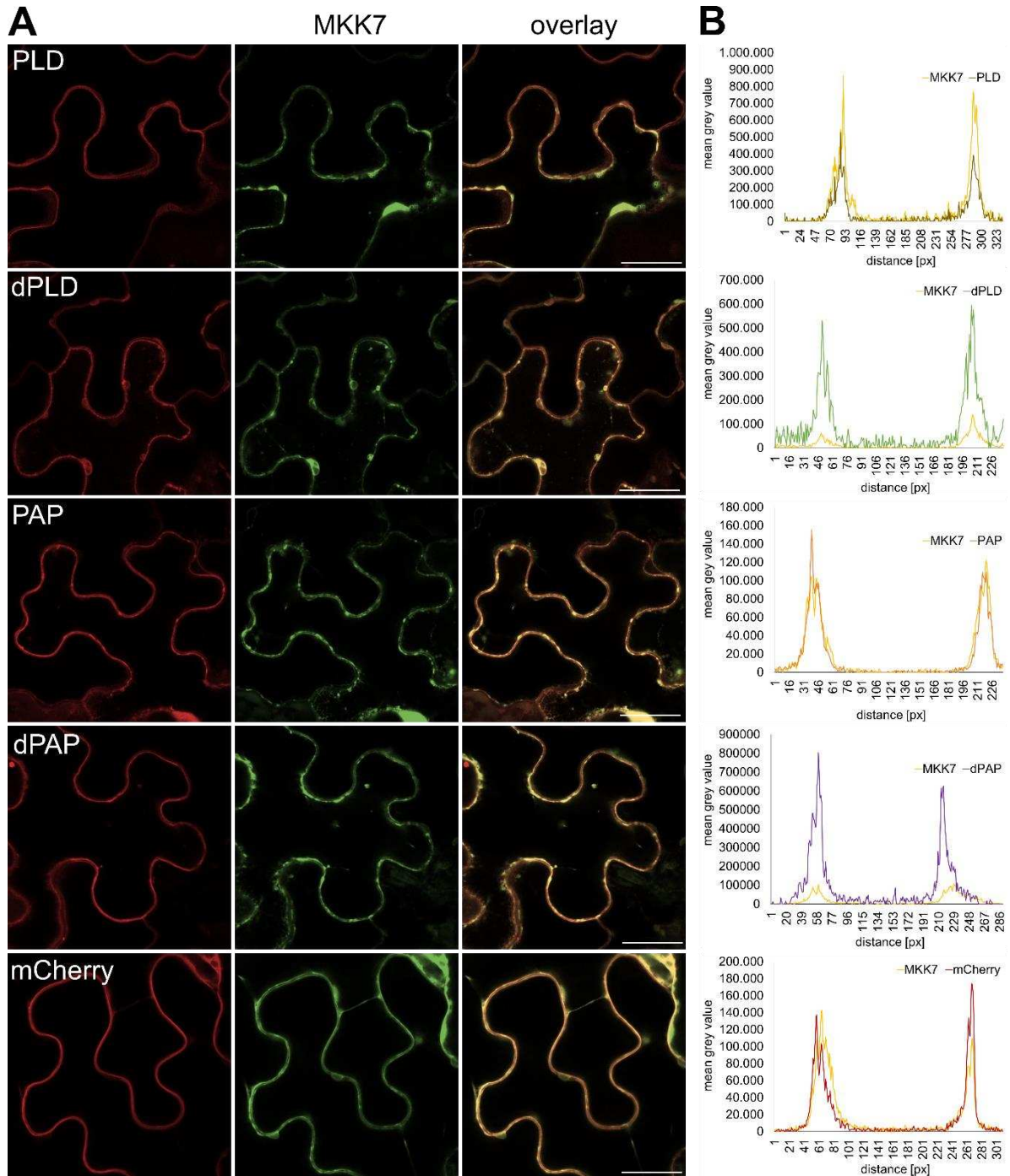


Figure 37 Co-infiltration of PLD and PA with MKK7 in *Nicotiana benthamiana* leaves

A) Confocal microscopy images of PLD, dPLD, PAP, dPAP and mCherry (orange) co-infiltrated with *pUBQ10::MKK7-3xGFP* (green) into *Nicotiana benthamiana* leaves. The Pictures were taken 40h.p.i. White bars at the bottom right indicate a length of 30 μ m. The experiment was repeated twice. n = 4

B) Line intensity plot profile of PLD, dPLD, PAP, dPAP and mCherry against MKK7 localization. Measurements were done with ImageJ (6.9).

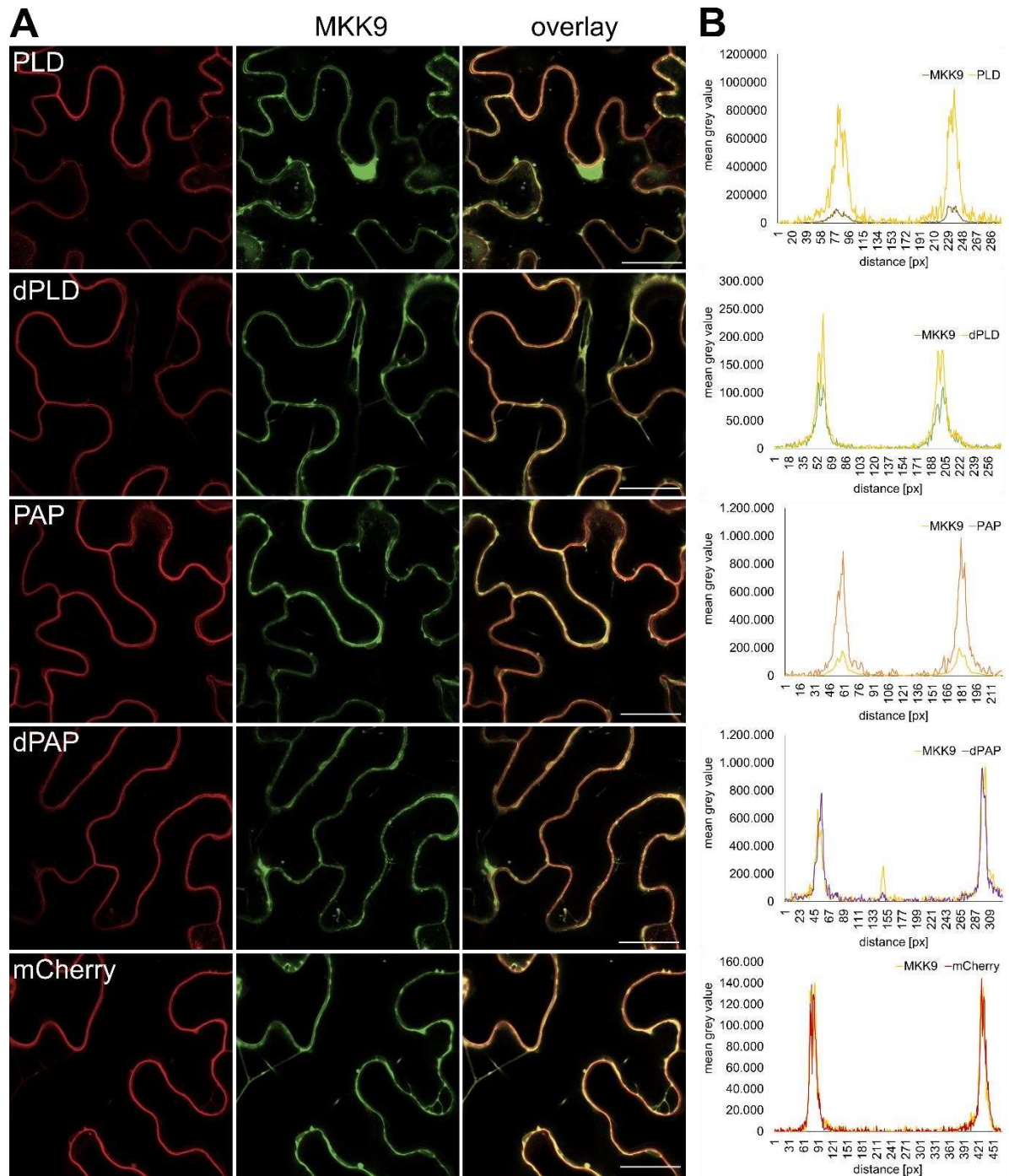


Figure 38 Co-infiltration of PLD and PA with MKK9 in *Nicotiana benthamiana* leaves

A) Confocal microscopy images of PLD, dPLD, PAP, dPAP and mCherry (orange) co-infiltrated with *pUBQ10::3xGFP-MKK9* (green) into *Nicotiana benthamiana* leaves. The Pictures were taken 40h.p.i. White bars at the bottom right indicate a length of 30 μ m. The experiment was repeated twice. n = 4

B) Line intensity plot profile of PLD, dPLD, PAP, dPAP and mCherry against MKK9 localization. Measurements were done with ImageJ (6.9).

3.2.9.3 FACS results show that PLD and PAP expression fail in protoplasts

As the effect of PA on MKK7 and MKK9 localization was visualized in protoplasts by Sheng and colleagues, we designed a similar experimental set up using our tools (33). First, we wanted to analyze the transfection efficiency of PLD, dPLD, PAP, dPAP, mCherry, MKK7 and MKK9 in protoplasts using Fluorescence activated cell sorting (FACS)², a method to analyze cells of a heterogeneous cell mixture based on their fluorescence profile, before starting to co-express the tools with MKK7 and MKK9 (Figure 39). Unfortunately, the FACS analysis showed that neither PLD (Figure 39 F), dPLD (Figure 39 G) nor PAP (Figure 39 H) expression can be detected, while dPAP (Figure 39 I), mCherry (Figure 39 J), MKK7 (Figure 39 K) and MKK9 (Figure 39 L) expression was successfully detected. Thus, we were not able to analyze the effect of our genetically encoded tools on MKK7 and MKK9 localization in protoplasts.

² The Fluorescence activated cell sorting as well as the analysis were performed by Kenneth Berendzen (ZMBP, University of Tuebingen)

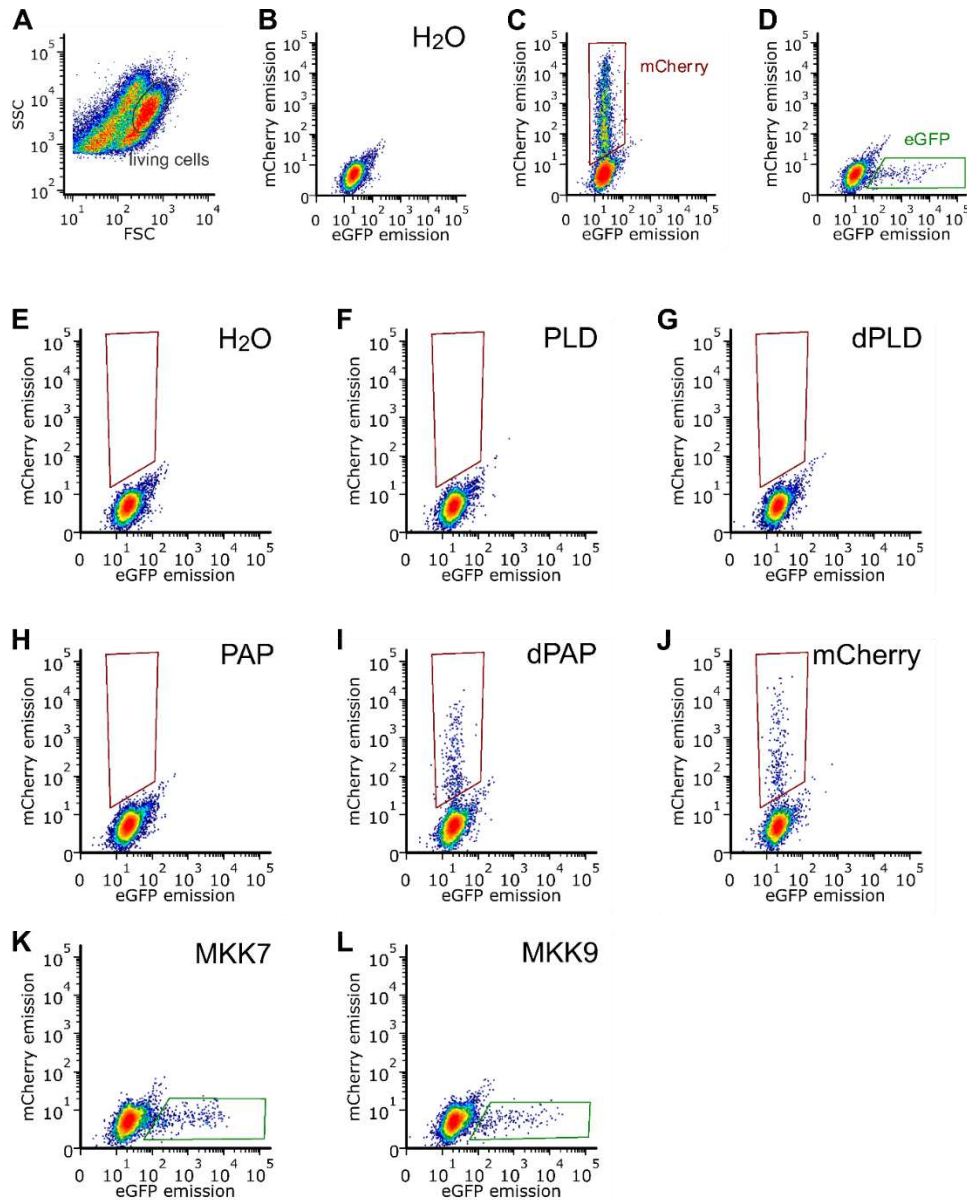


Figure 39 Fluorescent-activated cell sorting (FACS) plots to analyze PLD, PAP, MKK7 and MKK9 expression in protoplasts

A) Cytometric analysis was run on a Beckman Coulter MoFlo XDP (100 μ m CytoNozzle, 30.5/30.0 psi, PBS sheath), exciting eGFP at 488 nm (70 mW, elliptical focus) and peak emission in the FL1 (534/30); mCherry at 561 nm excitation (70 mW, spherical focus) and peak emission in the FL6 (620/29). Representative plot of a living cell population.

FACS analysis of the protoplasts was performed 6h after transfection.

B&E) Plots of non-transfected protoplasts using water as a control for mCherry and eGFP emission.

C&D) Emission range of 35S::mCherry and 35S::eGFP control constructs expressed in transfected protoplasts.

F-J) Plots for the mCherry emission range of protoplasts expressing *pUBQ10::CBL1n12-3x(mCherry)-PLD*, *pUBQ10::CBL1n12-3x(mCherry)-dPLD*, *pUBQ10::CBL1n12-3x(mCherry)-PAP*, *pUBQ10::CBL1n12-3x(mCherry)-dPAP* and *pUBQ10::CBL1n12-3x(mCherry)*.

K-L) Plots for the GFP emission range of protoplasts expressing *pUBQ10::3x(GFP)-MKK7* and *pUBQ10::3x(GFP)-MKK9*. The measurements were repeated twice with similar results.

3.2.10 RPM1 and RPS5 cell death response are not affected when co-infiltrated with PLD and PAP in *Nicotiana benthamiana* leaves

3.2.10.1 Introduction

Nucleotide-binding leucine-rich repeat receptor's (NLRs) are an important protein family in plants for the recognition of pathogen-derived effector proteins and the initiation of a strong immune response (101). Their activity often leads to a hypersensitive response (HR), a programmed cell death response of infected plant cells. Resistance to *Pseudomonas syringae* pv. *maculicola* 1 (RPM1) and Resistance to *Pseudomonas syringae* 5 (RPS5) are two well characterized plasma membrane localized NLRs (102, 103).

RPM1 recognizes the two *Pseudomonas syringae* effectors AvrRpm1 and AvrB (104-106). AvrRPM1, once entering the plant cell, localizes to the plasma membrane, where it interacts with RPM1-interacting protein 4 (RIN4). This interaction induces the phosphorylation of RIN4 at threonine residue 166, hereby activating the RIN4-RPM1 complex, finally leading to a HR (102, 107).

RPS5 recognizes the *Pseudomonas syringae* effector protein AvrPphB, a protein acting as a Cystein protease. To recognize AvrPphb, RPS5 needs the presence of the protein kinase AvrPphB susceptible protein 1 (PBS1) (108). PBS1 is cleaved by AvrPphb, which is crucial to activate RPS5. Activation of RPS5 leads to HR. Studies indicate that PBS1 interacts with RPS5 before it is cleaved (109, 110).

The abundance of phospholipids was shown to be important for proper RPM1 function and localization in the past. For example, it was shown that RPM1 localization, and hereby function, to the plasma membrane is mediated by direct interaction with PI(4)P. RPS5 activity and localization on the other hand is not affected by the presence or absence of PI(4)P, as the localization of this protein is mediated by the first 20 N-terminal amino acids which include a myristoylation (glycine-2) and palmitoylation (cysteine-4) membrane binding site (92) (110). PA, and especially PLD derived PA, was shown to generally be important in plant immunity. Past studies illustrated that changing PA level affect RPM1 immune response. So far, there is no general mechanism described for the function of PA in plant immunity. Some studies suggest a specific recruitment of certain proteins involved in plant immunity in response to rising or decreasing PA levels (22). One of the first links between plant immunity and PA was shown by Andersson and colleagues in 2006. They showed that the PA level is raising when *Arabidopsis* is infected with *Pseudomonas syringae* expressing the effectors *AvrRpm1* or *AvrRpt2* (111). They hypothesized that the PA biosynthesis is biphasic. First, the PLC-DGK pathway is activated, leading to decreasing PI(4,5)P₂ level and increasing PA level. Second, the PLD

mediated PA production starts, leading to highly increasing PA level. They were also able to show that extracellular Ca^{2+} influx is happening after the activation of the PLC-DGK pathway, but before the PLD mediated synthesis of PA. These findings are interesting, as the activation of some of the PLDs is Ca^{2+} dependent. In 2014 Johansson and colleagues wanted to gain further insights into this topic. They wanted to identify the PLDs being involved in response to infections with AvrRpm1 (112). They were able to show that n-Butanol treatment, an alternative substrate for PLDs, successfully blocked AvrRpm1 induced and RPM1-mediated HR, further highlighting the importance of this pathway for plant immunity, specifically NLR function. But they were not able to identify single PLDs being important for AvrRpm1 induced cell death. Even *pldβ1/β2/δ* triple mutants did not display any effect (112). These results underline the redundancy among the activity of the PLD enzyme family.

In the following we wanted to test if PLD or PAP activity have any effect on RPM1 or RPS5 induced HR when co-infiltrated into tobacco leaves.

3.2.10.2 Results

To examine the effect of PLD and PAP on RPM1 induced HR, we transiently expressed *DEX::AvrRpm1*, *35S::cYFP-RPM1* and *35S::RFP-RIN4* co-infiltrated with either *PLD* and *dPLD*, or *PAP* and *dPAP* in *Nicotiana benthamiana* leaves. *AvrRpm1* expression was induced 33 h.p.i. to make sure that PLD and PAP proteins are already expressed and the PA level already started to be modulated (Figure 40 B, Figure 41 B). Imaging of the tobacco leaves 48 h.p.i. and 14 hours after *AvrRpm1* expression was induced did not illustrate any effect of neither PLD nor PAP on RPM1 induced HR (Figure 40 A, Figure 41 A). The same experimental set up as for RPM1 was used to examine the effect of PLD or PAP on RPS5-mediated HR. We co-expressed Dex-inducible *AvrPphb*, *RPS5* and *PBS1* together with *PLD*, *dPLD*, *mCherry* or without any tool in tobacco leaves (Figure 42 B, Figure 43 B). Similar to the results for RPM1 we did not examine any effect of PLD or PAP activity on RPS5-mediated HR (Figure 42 A, Figure 43 A). To gain more detailed insights and a better resolution in time we performed a conductivity test (Figure 40 C, Figure 41 C, Figure 42 C, Figure 43 C). A conductivity meter measures the amount of ions leaked by cells when cell death starts to occur. Overall, the conductivity test illustrated that neither PLD nor PAP activity had a clear effect on RPM1- or RPS5-mediated HR. While some of the controls we used display significant changes in ion leakage when compared to other samples, there is no clear pattern, leading us to the assumption that these are technical issues due to e.g. sample size or transient expression.

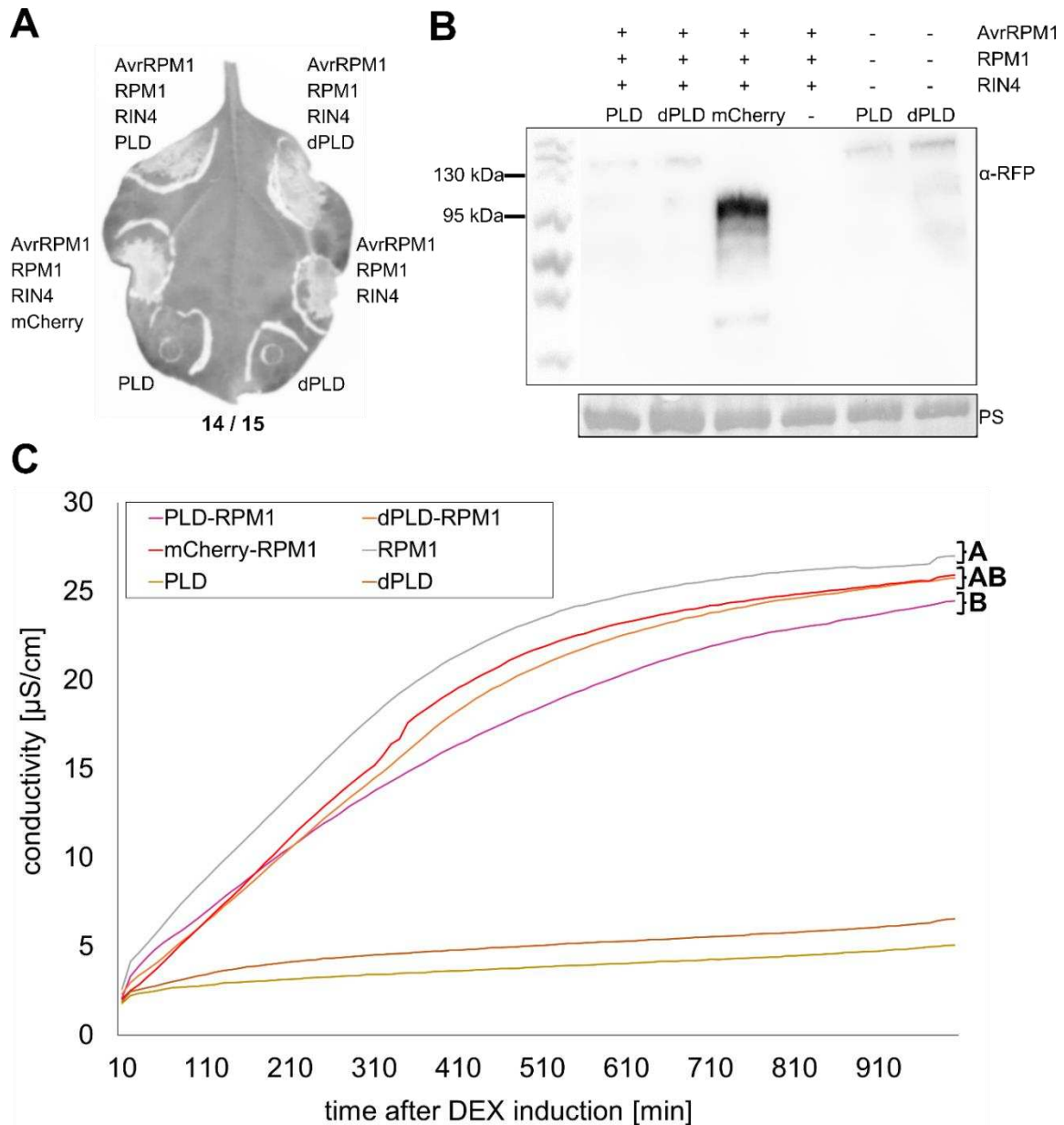


Figure 40 Cell death responses and conductivity tests of RPM1 co-expressed with PLD in *Nicotiana benthamiana* leaves

A) Pictures of *Nicotiana benthamiana* leaves expressing Dex-inducible *AvrRpm1*, *35S::cYFP-RPM1* and *35S::RFP-RIN4* together with *pUBQ10::CBL1n12-3x(mCherry)-PLD*, *pUBQ10::CBL1n12-3x(mCherry)-dPLD* or *pUBQ10::CBL1n12-3x(mCherry)*. The Pictures were taken 48 h.p.i. and 14 hours after dexamethason induction. Images were taken under UV light (6.7).

B) Immunoblot detection of PLD (138 kDa), dPLD (138 kDa) and mCherry (85 kDa) using α -RFP. The samples were harvested 34 h.p.i.

C) Conductivity measurements of *Nicotiana benthamiana* leaf discs expressing Dex-inducible *AvrRPM1*, *35S::cYFP-RPM1* and *35S::RFP-RIN4* together with *PLD*, *dPLD* or *mCherry*. The leaf discs were harvested 33 h.p.i. and washed for 1h in 20 μ M dexamethason in ddH₂O. In total, 100 conductivity measurements were performed every 10 minutes. The conductivity test was performed parallel to A. The experiment was performed twice with a sample size of 4 for each experiment. In the plot shown both results were pooled together. n = 8

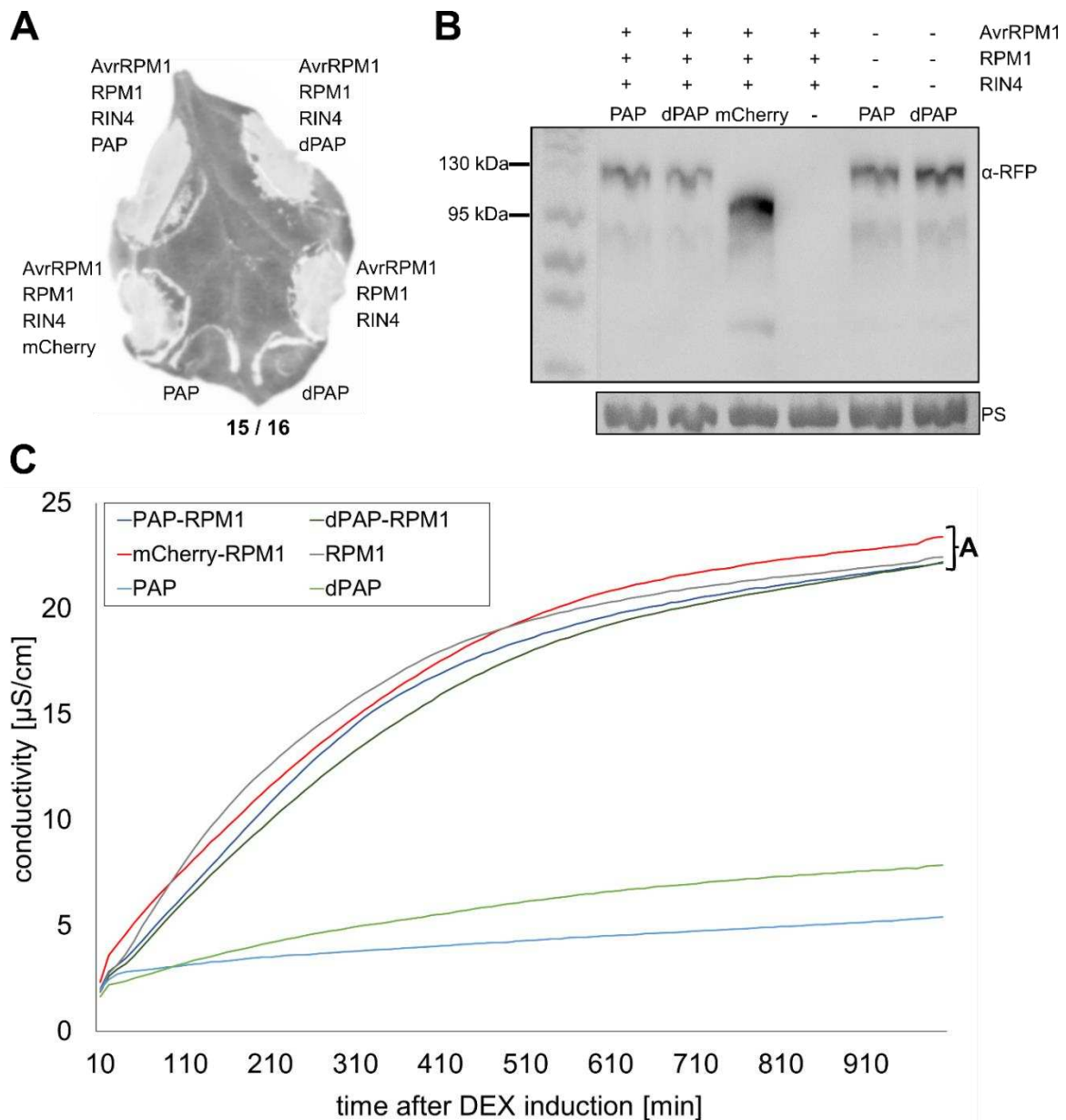


Figure 41 Cell death responses and conductivity tests of RPM1 co-expressed with PAP in *Nicotiana benthamiana* leaves

A) Pictures of *Nicotiana benthamiana* leaves expressing Dex-inducible *AvrRpm1*, *35S::cYFP-RPM1* and *35S::RFP-RIN4* together with *pUBQ10::CBL1n12-3x(mCherry)-PAP*, *pUBQ10::CBL1n12-3x(mCherry)-dPAP* or *pUBQ10::CBL1n12-3x(mCherry)*. The Pictures were taken 48 h.p.i. and 14 hours after dexamethason induction. Images were taken under UV light (6.7).

B) Immunoblot detection of PAP (118 kDa), dPAP (118 kDa) and mCherry (85 kDa) using α -RFP. The samples were harvested 34 h.p.i.

C) Conductivity measurements of *Nicotiana benthamiana* leaf discs expressing Dex-inducible *AvrRPM1*, *35S::cYFP-RPM1* and *35S::RFP-RIN4* together with *PAP*, *dPAP* or *mCherry*. The leaf discs were harvested 33 h.p.i. and washed for 1h in $20\mu\text{M}$ dexamethason in ddH_2O . In total, 100 conductivity measurements were performed every 10 minutes. The conductivity test was performed parallel to A. The experiment was performed twice with a sample size of 4 for each experiment. In the plot shown both results were pooled together. $n = 8$

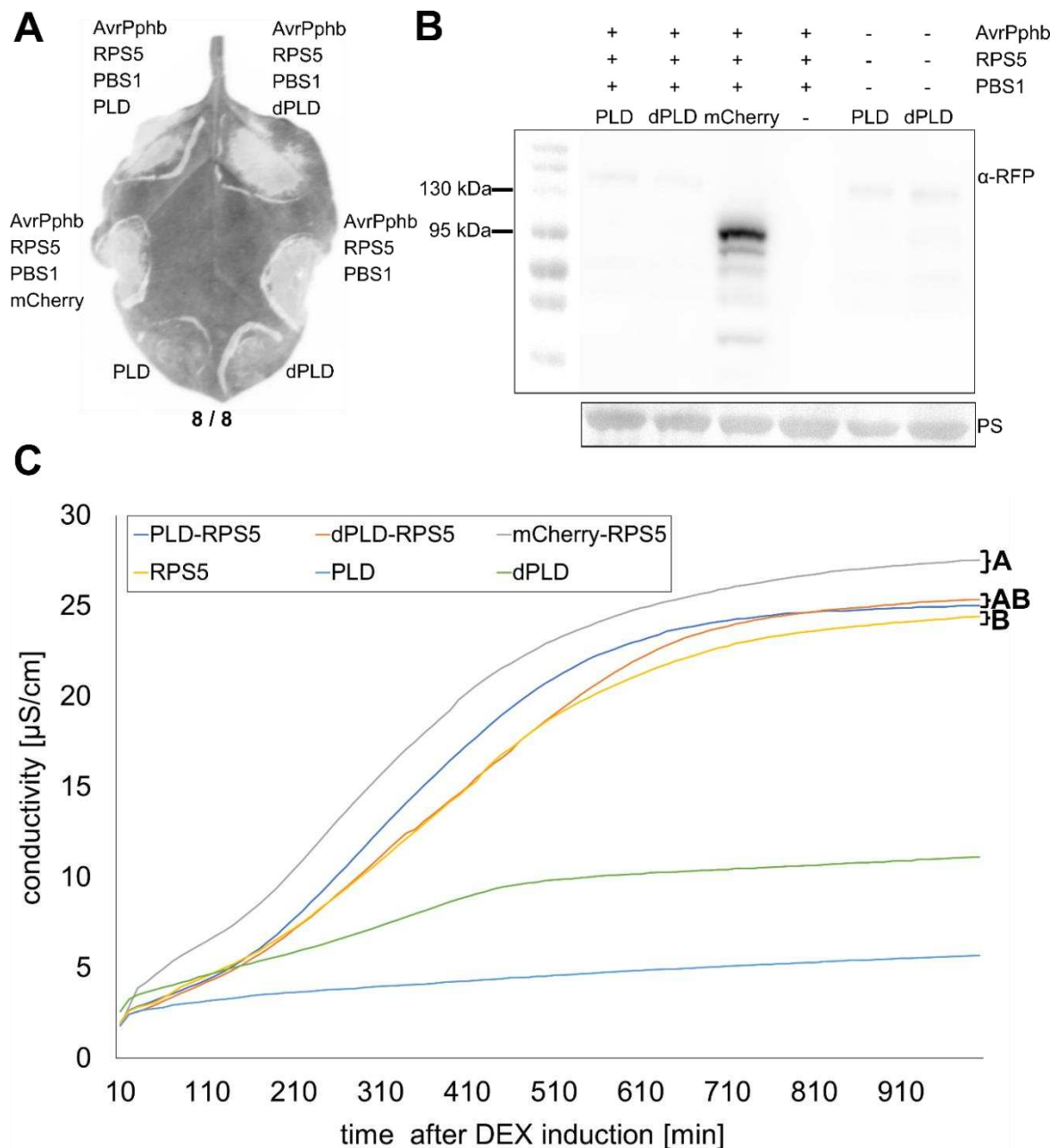


Figure 42 Cell death responses and conductivity tests of RPS5 co-expressed with PLD in *Nicotiana benthamiana* leaves

A) Pictures of *Nicotiana benthamiana* leaves expressing Dex-inducible *AvrPphb*, *RPS5* and *PBS1* together with *pUBQ10::CBL1n12-3x(mCherry)-PLD*, *pUBQ10::CBL1n12-3x(mCherry)-dPLD* or *pUBQ10::CBL1n12-3x(mCherry)*. The Pictures were taken 48 h.p.i. and 14 hours after dexamethason induction. Images were taken under UV light (6.7).

B) Immunoblot detection of PLD (138 kDa), dPLD (138 kDa) and mCherry (85 kDa) using α -RFP. The samples were harvested 34 h.p.i.

C) Conductivity measurements of *Nicotiana benthamiana* leaf discs expressing Dex-inducible dexinducible *AvrPphb*, *RPS5* and *PBS1* together with *PLD*, *dPLD* or *mCherry*. The leaf discs were harvested 33 h.p.i. and washed for 1h in 20 μM dexamethason in ddH₂O. In total, 100 conductivity measurements were performed every 10 minutes. Samples for Western Blots were taken 34 h.p.i (6.6, 6.7). The conductivity test was performed parallel to A. The experiment was performed twice with a sample size of 4 for each experiment. In the plot shown both results were pooled together. n = 8

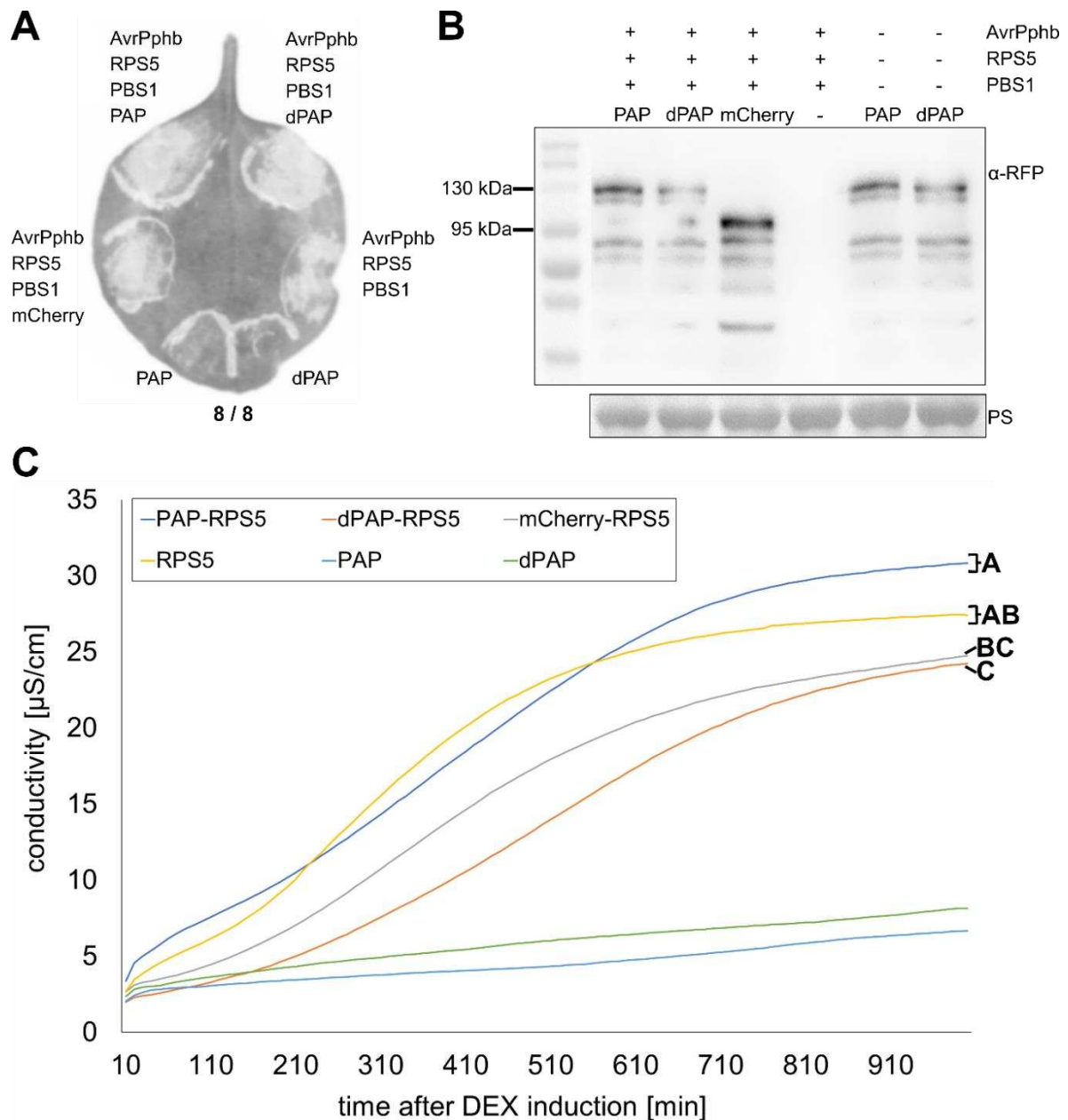


Figure 43 Cell death responses and conductivity tests of RPS5 co-expressed with PLD in *Nicotiana benthamiana* leaves

A) Pictures of *Nicotiana benthamiana* leaves expressing Dex-inducible *AvrPphb*, *RPS5* and *PBS1* together with *pUBQ10::CBL1n12-3x(mCherry)-PAP*, *pUBQ10::CBL1n12-3x(mCherry)-dPAP* or *pUBQ10::CBL1n12-3x(mCherry)*. The Pictures were taken 48 h.p.i. and 14 hours after dexamethason induction. Images were taken under UV light (6.7).

B) Immunoblot detection of PAP (118 kDa), dPAP (118 kDa) and mCherry (85 kDa) using α -RFP. The samples were harvested 34 h.p.i.

C) Conductivity measurements of *Nicotiana benthamiana* leaf discs expressing Dex-inducible dexinducible *AvrPphb*, *RPS5* and *PBS1* together with *PAP*, *dPAP* or *mCherry*. The leaf discs were harvested 33 h.p.i. and washed for 1h in 20 μM dexamethason in ddH₂O. In total, 100 conductivity measurements were performed every 10 minutes. Samples for Western Blots were taken 34 h.p.i. (6.6, 6.7). The conductivity test was performed parallel to A. The experiment was performed twice with a sample size of 4 for each experiment. In the plot shown both results were pooled together. n = 8

3.3 Discussion

3.3.1 PLD and PAP are not able to modulate PA level, but localize to the plasma membrane

In this work we tried to design two tools to up- and downregulate PA level specifically at the plasma membrane *in planta*. We were able to successfully target the PLD, dPLD, PAP and mCherry to the plasma membrane when transiently expressed in *Nicotiana benthamina* leaves. Unfortunately, all our efforts did not show any clear effect whether or not the enzyme we designed successfully manipulated the PA level at the plasma membrane. The problems we were facing are complex and there could be different reasons behind the missing effects.

We chose as time point for all our measurements 40 h.p.i. to tobacco leaves, while the first but weak protein expression was already detectable 33 h.p.i. We chose this timepoint, because of the strong expression and a good visibility at the microscope while no negative effects, especially the cell death response of the PLD, were detectable. It might be that 40 h.p.i. is too late, as the plant was already able to compensate the effect of the miss regulated PA level. In the opposite it could also be possible that the timepoint was too early and at a later timepoint we could have seen a stronger effect of the enzymes. However, a later time point was problematic, especially when working with PLD, as a first but weak physiological response in form of dying cells was visible 45 to 50 h.p.i. to tobacco leaves.

We chose for protein detection a 3x(mCherry) fluorophore where the single fluorophores were separated by small linker consisting of eight serine and seven glycine residues to give the protein the flexibility needed to target the substrate at the plasma membrane. But three fluorophores in a row are very bulgy and the linker might be too small to give the tools the flexibility needed. Being anchored to the plasma membrane and fused to three fluorophores might block the accessibility of the enzymes to the substrate (in case of the PLD PC and in case of the PAP PA) if the flexibility of the N-terminal part is not high enough. To answer the question an *in vitro* kinase assay could be used, where the enzymatic activity of each protein could be measured. On the other hand, the cell death response of the catalytically active PLD but the missing response for the inactive dPLD leads to the conclusion that an enzymatic activity was at least for the PLD possible.

3.3.2 Catalytically active PLD leading to cell death suggests that the enzyme is active

The cell death response activated by the catalytically active PLD, but not by the catalytically inactive dPLD, is a first hint that raising PA level might be the reason for this result. How this

cell death response is caused remains elusive. Several different reasons could lead to this effect. One might first think about a biological reason behind this extreme effect. The “*uncontrolled*” raising PA level at the plasma membrane might lead to unwanted recruitment of different proteins to the plasma membrane, hereby activating all kinds of different pathways that lead to a complete miss regulation of the plant cell. This overregulation could lead to a heavy oversensitivity of the plant cell finally resulting in the death of the cell. Another possibility is of physical-chemical nature. PA has a cone shaped head group and membrane sites enriched in PA have a negative curvature. Extremely increasing PA level at different membrane sites could lead to an increasing tension due to the negative curvature, finally resulting in a break of the membrane hereby killing the cell.

The missing effect of PLD on the tested biosensors 1xPASS, 2xPASS and KA1 is not surprising, as the biosensors all localize predominantly to the plasma membrane when infiltrated alone or with the negative controls into tobacco leaves.

3.3.3 Plants might be able to overcompensate PAP activity

We used a minimal version of *Arabidopsis thaliana* PAH1 as a tool to downregulate PA level. We therefore truncated the protein by deleting all regulatory domains, leaving only the domains crucial for phosphatase activity. We therefore adapted the protein based on the latest literature to PAPs and protein alignments. Even though structural simulations of our protein suggest that all domains are organized in a way that the enzyme should be active, it is still possible that the truncations led to a loss of phosphatase function. To test the phosphatase activity an *in vitro* kinase assay could be performed.

As shown in chapter 2 and by the latest literature describing PAPs, PA phosphatase abundance is very dynamic and PAPs are quickly degraded. Nevertheless, immunoblot analysis and confocal microscopy revealed that the protein is stable suggesting that most regulatory domains have been truncated successfully.

Co-expression of PAP and dPAP with the biosensors 1xPASS, 2xPASS and KA1 led to the first conclusion that the enzyme might not be active when tested in tobacco leaves. In all cases the probes did not show any effect in response to the enzyme. This could be explained on the one hand by the fact that the PAP we designed is inactive, on the other hand we also tested *Tetrahymena thermophila* PAH2, an endogenous minimal PAP, which also did not lead to any effect on the localization of the biosensors (Supplementary Figure 6). Agrobacteria infiltration into leaves is also a stress that the plant must adapt to. This stress might lead to increasing PA level, which could explain why the biosensors localize differently compared to when expressed

and analyzed in Arabidopsis root cells, where they also localize to the cytosol as well as to the ER. This stress induced upregulation of PA could neglect the effect of the PAP, as the plant overcompensates the enzyme activity.

Overcompensating the enzyme activity could also be a general problem when working with a genetically encoded tool to downregulate PA level. Externally manipulating the plasma membrane composition can also be seen as a stress that the plant needs to deal with. This stress might lead to increasing PA level hereby overcompensating the effect of the enzyme. With multiple enzymes and different pathways plants have a huge variety of possibilities to quickly increase PA level, hereby compensating the effect of the PAP.

3.3.4 Missing effect of PLD and PAP activity measured with TLC might reflect technical limitations

To physical-chemical measure the effect of PLD and PAP on PA level when infiltrated into *Nicotiana benthamiana* leaves we performed a thin layer chromatography and gas chromatography. Based on the result we concluded that there is no effect of the PLD nor the PAP on PA level when infiltrated into tobacco leaves. But this conclusion might be misleading, as there are several technical issues that could “*manipulate*” the result.

First, the tools are thought to manipulate the PA level at the plasma membrane while PA can be found in different other subcellular compartments as well. We measured with our set up the phospholipid profile of the whole cell. While the PA level might be up- or downregulated at the plasma membrane the effect on the other compartments could mask the effect at the plasma membrane.

Also, the transient expression in tobacco leaves leads to a very heterogenous expression pattern. Even though, we made sure that the different constructs were expressed when tested for TLC and GC, we have no idea how the expression pattern looked like for the samples that we took, especially in the cell layers beyond the epidermis, even though it was shown that they can be transfected too (113). This very heterogenous expression can be problematic as this conglomerate of infected and not infected cells could dilute the effect of the enzymes in single cells.

3.3.5 MAP65-1 activity is not affected when transiently co-expressed with PLD and PAP in *Nicotiana benthamiana*

MAP65-1 was shown to specifically bind to PLD-derived PA at the plasma membrane, hereby regulating its activity as cortical microtubule bundling protein (32). Here we tested whether the PLD and PAP can manipulate the activity of MAP65-1 in response to manipulated PA level

when co-infiltrated to tobacco leaves. We conclude that both, the PLD activity and the PAP activity had no effect on the activity of MAP65-1 as the number of cortical microtubules was not affected by the presence or absence of both enzymes when co-infiltrated with MAP65-1 in tobacco leaves. This could be a technical issue, as the *Agrobacterium* infiltration might lead to an upregulation of PA biosynthesis as response to the bacterial infection, hereby masking the effect of the enzymes. To verify the localization (and therefore activity) of MAP65-1 in response to PLD and PAP when co-infiltrated in *Nicotiana benthamiana* leaves, a more sensitive technique, as for example cellular fractionation and an immunoblot detection of MAP65-1, could give further insides. Also, analyzing MAP65-1 activity in response to PAP or PLD activity in a more regulated context, as for example in *map65-1* Arabidopsis mutants rescued with MAP65-1 expressed under control of the endogenous promoter, could lead to more detailed insides compared to the strong overexpression of MAP65-1 in tobacco leaves.

3.3.6 MKK7 and MKK9 localization is not affected by PLD or PAP when co-expressed in *Nicotiana benthamiana*

MKK7 and MKK9 are salt stress regulated kinases that have been shown to be specifically recruited to the plasma membrane in response to PLD-derived PA, but predominantly remain in the cytosol when no salt is treated (33). Here we tested whether the PLD and PAP can manipulate the plasma membrane localization of MKK7 and MKK9 in response to manipulated PA level when co-infiltrated to tobacco leaves. Unfortunately, our results suggest that both, PLD and PAP activity had no effect on the localization of MKK7 and MKK9. In combination with the previously described results we conclude that both enzymes, PLD and PAP, do not affect PA abundancy at the plasma membrane when infiltrated into *Nicotiana benthamiana* leaves. This could again be a technical issue, as the *Agrobacterium* infiltration might lead to stress regulated production of PA masking the effect of the enzymes. Also, the over expression of MKK7 and MKK9, both leading to cell death, could be addressed as stressful for tobacco leaves, resulting in raising PA level again masking the effect of PLD and PAP. To verify the localization of MKK7 and MKK9 in response to PLD and PAP activity when co-infiltrated to tobacco leaves one could also use a more sensitive technique, as for example cellular fractionation and an immunoblot detection of MKK7 and MKK9 to visualize the migration to or off the plasma membrane.

We also tried to express *PLD*, *dPLD*, *PAP*, *dPAP* and *mCherry* in protoplasts. Surprisingly, no signal for PLD, dPLD and PAP was detectable using FACS, while dPAP and mCherry both were detectable. We hypothesis that PLD, dPLD and PAP led to a strong cell death response when expressed in protoplasts. This conclusion of course raises further questions about the

cause. Assuming that PLD and PAP both were catalytically active in protoplasts, the manipulated PA level could cause cell death. For the catalytically inactive dPLD this explanation is invalid, raising the question if PLD has additional effects in plants that we don't know about. The missing cell death response of dPLD when expressed in tobacco leaves compared to the expression in protoplasts could be caused by the fact that protoplasts are a much more fragile system compared to tobacco leaves. Also, it might be that dPLD is able to target other proteins in tobacco compared to Arabidopsis, leading to a misregulation of signaling pathways that kill the protoplasts but not the tobacco leaves.

3.3.7 RPM1 and RPS5 induced hypersensitive response is not affected when PLD or PAP are co-expressed in *Nicotiana benthamiana* leaves

Past studies suggest that there is a direct link between plant immunity and PA maintenance, with PA acting as an important signaling molecule. Some studies focused on the effect of PA in response to *Pseudomonas* infection carrying the effector protein AvrRPM1 (111, 112). We wanted to use this link between PA distribution and plant immunity to test the effect of PLD and PAP. To do so we co-infiltrated *PLD*, *dPLD*, *PAP*, *dPAP* and *mCherry* with either *AvrRPM1*, *RPM1* and *RIN4* or *AvrPphb*, *RPS5* and *PBS1* into *Nicotiana benthamiana* leaves and analyzed the effect of the tools on the RPM1 or RPS5 induced hypersensitive response (HR), an important mechanism in plant immunity where cells die in a controlled way to locally restrict bacterial growth after infection (102). Our results show that neither PLD nor PAP were able to block or enhance RPM1 or RPS5 induced HR. Imaging the cell death response 48 h.p.i. illustrates that HR was not blocked when PLD or PAP were co-infiltrated with RPM1 or RPS5. In parallel to this experiment we also measured the ion leakage occurring during cell death every 10 minutes for up to approximately 16h, giving us the opportunity to visualize differences in the time point when HR starts to happen. Again, no clear difference for PLD or PAP co-infiltration with RPM1 and RPS5 was detectable. Our analysis showed significant differences for some of the measurements, but without any reliable pattern that would allow us to come to any other conclusion besides that PLD nor PAP had any effect on RPM1 or RPS5 function. This different outcomes could be due to several reasons. First, the heterologous expression of the proteins as well as the chemical induction of gene expression are factors that could affect the results. A higher sample size could minimize this variation. Second, the activity of RPM1 and RPS5 leads to intrinsic upregulation of PA biosynthesis. This increased PA level on the other hand could compensate the effect of PLD and PAP. While the strong upregulation of PA biosynthesis could mask the effect of PLD, the activity of PAP might not be high enough to compensate the intrinsic upregulation of PA biosynthesis.

To further analyze the effect of artificially up or downregulation PA production using genetically encoded tools one could also try to analyze the effect of the enzymes in Arabidopsis after infection with *Pseudomonas* expressing the effector *AvrRPM1* or *AvrPphb*, by measuring for example the effect of the tools on bacterial growth.

3.3.8 More controlled regulation of PLD and PAP expression could lead to the wanted effect

Our experiments suggest that both enzymes, PLD and PAP, have not the expected effect when infiltrated into tobacco leaves. The strong and uncontrolled expression of both, *PLD* and *PAP*, using a transient system could be one of the major problems when handling the proteins. A more controlled expression might be required to see an effect on PA level. Therefore, gene expression in transgenic Arabidopsis plants might be needed to gain further insights. But using transgenic plants also has its restrictions. A ubiquitous gene expression of PLD and PAP might negatively affect plant growth or germination or even result in a sudden death of the plant, as for example the cell death after PLD expression in tobacco leaves suggests. To compensate this effect, we tried to use a dexamethasone induced gene expression, using the system developed by Sebastian Wolf (University of Tuebingen), under control of the *UBQ10* promoter (114) (Supplementary Figure 7-9). But we were facing different problems using this system, as for example gene expression of our enzymes was only detectable in very few cells while the dexamethasone induced gene expression occurred in cells of the analyzed tissue, suggesting that gene silencing inhibits the expression of the enzymes. Another problem of chemical induced gene expression might also be the high stability of the compound within the plants. This stability leads to a strong and especially long and uncontrolled expression of the tools, again resulting in unwanted long-term effects, as for example cell death. To overcome this problem an optogenetic system might be helpful. In 2020 Ochoa-Fernandez and colleagues introduced such a system for plants called “*PULSE*” (Plant Usable Light-Switch Elements), where gene expression can be induced using red-light and can be deactivated by ambient white light, a process that can be repeated as often as needed (115). This system allows a short-term gene expression, also in a locally restricted manner, giving us the opportunity to analyze the effect of PLD and PAP for short term processes.

3.3.9 Conclusion

All our experiments unfortunately suggest that neither PLD nor PAP affect PA level at the plasma membrane *in planta*. Both enzymes were shown to have no effect when infiltrated into tobacco leaves, as none of the phosphatidic acid specific biosensors or phosphatidic acid

binding proteins were affected by the presence or absence of the enzymes. Also, thin-layer- and gas chromatography analysis showed that there is no difference in the amount of PA when PLD or PAP were expressed compared to the corresponding controls. The cell death occurring in response to *PLD* expression, but not in response to *dPLD*, suggest that PLD is active and most likely upregulating PA level, making even more surprising that there is no other effect detectable. To sum up, both enzymes, PLD and PAP, need further improvements in term of gene expression and/or protein design to eventually be effective when expressed in plants.

4 Summary

Plants are confronted with a very dynamic climate throughout their whole life. These changes challenge the plants each and every day and put them, and especially crops, under high stress. To gain further insides on how plants react upon stress is there for an important task to for plant scientists, but also for agriculture economy, the bases of our modern living.

Phosphatidic acid (PA) was shown to be a very important second messenger in plants to response to stress. PA is a glycerophospholipid that acts as a signaling molecule, but is also the precursor for the synthesis of all other glycerophospholipids, underlining its importance for plants. Past studies showed that elevating PA level, especially at the plasma membrane, is a key mechanism to regulate protein function in response to stress. To better understand how PA regulates stress response is there for an important challenge. The development of new and optimized already existing techniques could help to gain further insides on how PA regulates stress response.

Phosphatidic Acid Phosphatases (PAPs) are important regulators of PA level in all eukaryotes, that dephosphorylate PA to diacylglycerol. The protein structure of yeast and mammalian PAPs was well described in the past. Today we know that PAPs generally consist of a N-LIP, C-LIP and Tryptophan domain, as well as of a N-terminal helix and a M-LIP domain, both important for proper protein localization. The presence or absence of the N-terminal helix and the M-LIP domain in the Arabidopsis PAP PAH1 remained elusive. Our bioinformatical analysis suggests the presence of both, a N-terminal helix as well as a M-LIP domain for Arabidopsis PAH1. Transient expression in tobacco leaves showed that full length PAH1 localized to the endoplasmic reticulum, plasma membrane and the cytosol, even though these results need to be verified with other approaches. If the N-terminal helix and the M-LIP domain contribute in regulating PAH1 localization still needs to be shown, while our phenotypic studies in transgenic Arabidopsis plants showed that both domains are important for proper PAH1 function. Deleting the N-terminal helix led to more roundish leaves, comparable to the *pah1/2* double knock out mutant. PAH1 without the M-LIP domain had no developmental defects, but seedlings treated with salt showed to be less sensitive compared to the controls.

Based on recent paper and experiments done in this work, we tried to develop two genetically encoded tools to modify PA level at the plasma membrane *in planta*. To target both PA modifying tools to the plasma membrane we used the twelve very N-terminal amino acids of CBL1. This amino acid sequence was shown to mediate plasma membrane localization in the

past. For protein detection we chose a triple mCherry fusion protein, where the single fluorophores are separated by small linker consisting of eight serine and seven glycine residues. Tei and Baskin introduced in 2020 a tool to upregulate PA level at different membranes in mammalian cells using the *Streptomyces sp.* strain PMF Phospholipase D (PLD). We tried to use this particular PLD to modify PA level at the plasma membrane *in planta*. Protein sequence alignments of the PLD against the twelve *Arabidopsis thaliana* PLDs revealed an amino acid sequence homology of less than 1%, making unwanted protein-protein interactions that regulate the *Arabidopsis* PLDs unlikely. Transient *PLD* expression in *Nicotiana benthamiana* leaves led to a strong cell death response 62 hours after the infiltration. The negative control, the catalytically inactive *dPLD*, did not lead to this response.

Additionally, we tried to develop a tool to downregulate PA level at the plasma membrane called PAP. We used *Arabidopsis thaliana* PAH1 and modified the protein, based on recent studies and experiments that were done in this work, to minimize the protein in size and avoid unwanted protein-protein interactions.

We used different approaches to verify the activity of both, the PLD and the PAP. We co-infiltrated the PLD and the PAP in tobacco leaves with PA specific biosensors and proteins that were shown to be recruited or activated by PA at the plasma membrane. Also, we used thin-layer and gas chromatography to analyze the activity. Unfortunately, all results lead to the conclusion, that both, PLD and PAP, did not modify PA level at the plasma membrane and need further modifications.

Taken together this thesis gave novel insights in the structure of *Arabidopsis thaliana* PAH1 and set the bases for the development of genetically encoded tools to modify PA level at the plasma membrane *in planta*.

5 Zusammenfassung

Pflanzen sind ihr ganzes Leben lang einem sehr dynamischen Klima ausgesetzt. Diese Veränderungen fordern die Pflanzen jeden Tag aufs Neue und setzen sie und insbesondere die Nutzpflanzen einem hohen Stress aus. Weitere Erkenntnisse darüber zu gewinnen, wie Pflanzen auf Stress reagieren ist eine wichtige Aufgabe für Wissenschaftler, aber auch für die Agrarwirtschaft, die Grundlage unseres modernen Lebens.

Es wurde gezeigt, dass Phosphatidsäure (PA) in Pflanzen ein sehr wichtiger sekundärer Botenstoff für die Reaktion auf Stress ist. PA ist ein Glycerophospholipid, das als Signalmolekül fungiert, aber auch die Vorstufe für die Synthese aller anderen Glycerophospholipid ist, was seine Bedeutung für Pflanzen unterstreicht. Frühere Studien haben gezeigt, dass die Erhöhung des PA-Spiegels, insbesondere an der Plasmamembran, ein Schlüsselmechanismus zur Regulierung der Proteinfunktion als Reaktion auf Stress ist. Es ist eine wichtige Herausforderung, besser zu verstehen, wie PA die Stressreaktion reguliert. Die Entwicklung neuer und die Optimierung bereits bestehender Techniken könnten dazu beitragen, weitere Erkenntnisse darüber zu gewinnen, wie PA die Stressreaktion reguliert.

Phosphatidsäurephosphatasen (PAPs) sind wichtige Regulatoren des PA-Spiegels in allen Eukaryoten, die PA zu Diacylglycerol dephosphorylieren. Die Proteinstruktur von Hefe- und Säugetier-PAPs wurde in der Vergangenheit gut beschrieben. Heute wissen wir, dass PAPs im Allgemeinen aus einer N-LIP, C-LIP und Tryptophan Domäne, sowie einer N-terminalen Helix und einer M-LIP-Domäne bestehen, die beide für die korrekte Proteinlokalisierung wichtig sind. Das Vorhandensein oder Fehlen der N-terminalen Helix und der M-LIP Domäne der *Arabidopsis* PAP PAH1, blieb unklar. Unsere bioinformatischen Analysen legen das Vorhandensein sowohl einer N-terminalen Helix als auch einer M-LIP-Domäne für *Arabidopsis* PAH1 nahe. Die heterologe Expression in Tabakblättern zeigte, dass PAH1 im endoplasmatischen Retikulum, in der Plasmamembran und im Zytosol lokalisiert war, auch wenn diese Ergebnisse mit anderen Methoden verifiziert werden müssen. Ob die N-terminale Helix und die M-LIP-Domäne zur Regulierung der PAH1-Lokalisierung beitragen, muss noch gezeigt werden, während unsere phänotypischen Studien an transgenen *Arabidopsis*-Pflanzen zeigten, dass beide Domänen für eine korrekte PAH1-Funktion wichtig sind. Das Entfernen der N-terminalen Helix führte zu rundlicheren Blättern, vergleichbar mit der *pah1/2* Doppel-Knockout-Mutante. PAH1 ohne die M-LIP-Domäne wies keine Entwicklungsstörungen auf, mit Salz behandelte Keimlinge erwiesen sich jedoch als weniger empfindlich im Vergleich zu den Kontrollen.

Basierend auf aktuellen Studien und in dieser Arbeit durchgeführten Experimenten haben wir versucht, zwei genetisch kodierte Werkzeuge zu entwickeln, um den PA-Spiegel an der Plasmamembran *in planta* zu verändern. Um beide PA-modifizierenden Werkzeuge an die Plasmamembran zu rekrutieren, verwendeten wir die zwölf N-terminalen Aminosäuren von CBL1. In der Vergangenheit wurde gezeigt, dass diese Aminosäuresequenz die Lokalisierung an die Plasmamembran vermittelt. Für den Proteinnachweis haben wir ein dreifaches mCherry-Fusionsprotein ausgewählt, bei dem die einzelnen Fluorophore durch einen kleinen Linker, bestehend aus acht Serin- und sieben Glycinresten, getrennt sind.

Tei und Baskin stellten im Jahr 2020 ein genetisch kodierte Werkzeug zur Hochregulierung des PA-Spiegels an verschiedenen Membranen in Säugetierzellen vor. Hierzu verwendeten sie die *Streptomyces sp.* Stamm PMF Phospholipase D (PLD). Wir haben versucht, dieses spezielle PLD zu verwenden, um den PA-Spiegel an der Plasmamembran *in planta* zu verändern. Proteinsequenzvergleiche der PLD mit den zwölf PLDs von *Arabidopsis thaliana* ergaben eine Aminosäuresequenzhomologie von weniger als 1 %, was unerwünschte Protein-Protein-Interaktionen, die die PLDs von *Arabidopsis* regulieren, unwahrscheinlich macht. Die heterologe PLD-Expression in Blättern von *Nicotiana benthamiana* führte 62 Stunden nach der Infiltration zu einer starken Zelltodreaktion. Die Negativkontrolle, die katalytisch inaktive dPLD, führte nicht zu dieser Reaktion.

Darüber hinaus haben wir versucht, ein Werkzeug namens PAP zu entwickeln, um den PA-Spiegel an der Plasmamembran herunterzuregulieren. Wir haben *Arabidopsis thaliana* PAH1 verwendet und das Protein auf der Grundlage aktueller Studien und Experimente, die in dieser Arbeit durchgeführt wurden, modifiziert, um die Proteingröße zu minimieren und unerwünschte Protein-Protein-Interaktionen zu vermeiden.

Wir haben verschiedene Ansätze verwendet, um die Aktivität sowohl der PLD als auch der PAP zu überprüfen. Wir infiltrierten PLD und PAP in Tabakblättern gemeinsam mit PA-spezifischen Biosensoren und Proteinen, von denen gezeigt wurde, dass sie von PA an der Plasmamembran rekrutiert oder aktiviert werden. Außerdem verwendeten wir Dünnschicht- und Gaschromatographie, um die Aktivität zu analysieren. Leider führen alle Ergebnisse zu dem Schluss, dass sowohl PLD als auch PAP den PA-Spiegel an der Plasmamembran nicht verändert haben und weitere Modifikationen erforderlich sind.

Insgesamt lieferte diese Arbeit neue Einblicke in die Struktur von *Arabidopsis thaliana* PAH1 und legte die Grundlagen für die Entwicklung genetisch kodierter Werkzeuge zur Modifizierung des PA-Spiegels an der Plasmamembran *in planta*.

6 Material and Methods

All of the following experiments were, if not stated differently, performed by *Frank Ackermann*.

6.1 Plant growth

For all experiments performed on solid plates *Arabidopsis thaliana* seeds were sterilized for 10 minutes in 70% (v/v) ethanol and briefly washed with 100% (v/v) ethanol. After drying the seeds, they were put on solid 0.5 MS (0.7% Phytagar, w/o sucrose, pH5.7) and stratified for two days at 10°C in the dark. Afterwards the seeds were germinating and grown for five to six days under long day conditions (16h light, 8h dark).

6.2 Genotyping

Plant material was collected from young leaves, placed in 1.5ml test tubes and immediately frozen in liquid nitrogen. The volume of the frozen plant material was reduced by punching repeatedly, then 440µl SENT (Table 4) was added. The samples were further centrifuged at speed 13000 rpm for 15 minutes. 350µl of the sample were then pipetted into a fresh test tube and 350µl isopropanol was added. The tube was inverted and incubated for 5 minutes. The sample were centrifuged for 10 minutes at speed of 13000 rpm. The DNA pellet was then washed in 200µl 70% ethanol. The ethanol was reduced and the DNA pellet was taken up in 50 µl ddH₂O.

The *pah1/2* double mutant was obtained from Peter J. Eastmond (116). For genotyping, the LB1.3 primer was used (3'ATTTTGCCGATTTTCGGAAC5') as well as 3'TAATTTTGGCTTGTTGTTGGG5' and 3'GTTTTGGTCAGCTCTGACTGC5' to verify the K.O of *pah1* and 3'AATGCAACAGGTAGACGCAAG5' and 3'GCAAATGCAAACAAACAGTTG5' to verify the K.O of *pah2*. Genotyping of *pah1/2* was performed by *Steffi Zimmermann* with the help of *Frank Ackermann*.

Table 3 *Arabidopsis thaliana* plants used in this work

line	background	resistance	description
WT	Col-0	-	Col-0 ecotype
<i>pah1/2</i>	Col-0	-	<i>pah1/2</i> K.O. mutant; received from Peter J. Eastmond (116)
pUBQ10::GR-LhG4	Col-0	Sulfadiazine	Driverline for dexamethasone induction; produced by Thomas Stanislas and Jutta Keicher (ZMBP, Uni Tuebingen)

Table 4 SENT DNA extraction buffer**500 ml SENT (DNA extraction buffer)**

Volume	compound
100ml	1M Tris HCL
25ml	5M NaCl
75ml	0.5M EDTA
25ml	10% SDS
rest	ddH ₂ O

6.3 Plasmid Design and Cloning

Plasmid construction was done using the GreenGate Cloning technique (97). *In silico* plasmid design and cloning was performed using Benchling (www.benchling.com).

The vectors in Table 5 and Table 15 were cloned by *Jutta Keicher, Steffi Zimmermann* and *Frank Ackermann*.

Table 5 Entry vectors cloned

The following plasmids were cloned for this work. Amp = Ampicillin (working concentration 100µg/ml)

name	backbone	resistance bacteria	forward primer	reverse primer
pPAH1	pGGA	Amp	AACAGGTCTCAACCTTGG GGTTTGTGCTCGATCTCTAA GACTAC	AACAGGTCTCATGTTTTTCATC AAATCTTTGAGAAAGTCTTCA ACTTTTACTGTCC
cMKK7	pGGB	Amp	AACAGGTCTCAaacaAATG GCTCTTGTTCTGTAACGCC	ACCAGGTCTCAagccTGCAGCT GCAGCAAGACTTTTACGGAG AAAAGGGTGAC
cMKK9	pGGB	Amp	AACAGGTCTCAaacaAATG GCTTTAGTACGTGAACGT CG	ACCAGGTCTCAagccTGCAGCT GCAGCAAGATCTTCCCGGAG AAAAGGATGAG
cMPK6	pGGB	Amp	AACAGGTCTCAaacaATGG ACGGTGGTTCAGGTCAAC C	ACCAGGTCTCAagccTTGCTGA TATTCTGGATTGAAAGCAAGC G
RPS5n20	pGGB	Amp	AACAGGTCTCAaacaATGG GAGGTTGTTTCTCTGTTTC ATTGCCATGTGATCAAGT AGTGAGTCAGTTCTCTCA G	ACCAGGTCTCAagccCTGAGAG AACTGACTCACTACTTGATCA CATGGCAATGAAACAGAGAA ACAACCTCCCAT
cAtPAH1	pGGC	Amp	AACAGGTCTCAGGCTCAA TGAGTTTGGTTGG	AACAGGTCTCACTGATTCAAC CTCTTCTATTG
c(ΔAH)AtPAH1	pGGC	Amp	AACAGGTCTCAGGCTATA TGCCATTTTCATCCCTTTGG TGG	AACAGGTCTCACTGATTCAAC CTCTTCTATTG
c(ΔM)AtPAH1	pGGC	Amp	AACAGGTCTCAggctTGATG AGTTTGGTTGGAAGAGTT GGG + AACAGGTCTCAactaTCACT GTTGAGCATACGGGAAG	AACAGGTCTCtagtTGATCT GTATCGGGTTCTCCTTCG + AACAGGTCTCActgaTTCAACC TCTTCTATTGGCAGTTTCC
cMAP65-1	pGGC	Amp	AACAGGTCTCAGGCTTGA TGGCAGTTACAGATACTG AA	AACAGGTCTCACTGATGGTG AAGCTGGAACCTTGATG
cPAP	pGGD	Amp	AACAGGTCTCATCAGCTA TGCCATTTTCATCCCTTTGG TGG +	AACAGGTCTCAATACAACCTTC CCTGATGAAATATGCTTC + AACAGGTCTCAGCAGTTAAG TTGGAGGAAACATGTCGTTG

			AACAGGTCTCAGTATATA GAACTAATGTCCCTACCA ATG	
MmLIPIN2Δ	pGGD	Amp	AACAGGTCTCATCAGCTatg aattatgtgggcagctctggct	AACAGGTCTCAGCAGTCAAG CCAGGTCATCCAGGTCCAGGT CAGGGATTGGaTCgCGCCAGT AGCA
PLD	pGGD	Amp	AACAGGTCTCATCAGGCA GATTCAGCTACACCACAT TTGGACG	AACAGGTCTCAGCAGAGCGT TGCAGATTCTCTTGCG
ScPAH1	pGGD	Amp	AACAGGTCTCATCAGCTca gtacgtaggcagagctcttggg	AACAGGTCTCAGCAGTtaattctcg aattcatcttcgtcgaa
ScPAH1Δ (1-600)	pGGD	Amp	AACAGGTCTCATCAGCTca gtacgtaggcagagctcttggg	AACAGGTCTCAGCAGTTAagga accatggaagtatt
TtPAH2	pGGD	Amp	AACAGGTCTCATCAGCTA TGATCAACGGTATCAAAA ACCTG	AACAGGTCTCATCAGCTATGA TCAACGGTATCAAAAACCTG

6.3.1 mRNA extraction and reverse transcription for cDNA synthesis

The mRNA extraction was performed using the Qiagen RNeasy Plant Mini Kit. The protocol was used as suggested by the manufacturer. The cDNA was synthesized using the First Strand cDNA Synthesis Kit (#K1612) of Fermentas Life Science. The protocol was used as suggested by the manufacturer.

6.3.2 Entry Vector cloning

The forward and reverse primers were designed using Benchling. Each primer was designed with suitable overhangs for GreenGate cloning, resulting in overhangs that were structured as the following: four random bases (AACA), BsaI recognition site (GGCTC), an extra base (A), BsaI cut site consisting of specific left and right border sequences (NNNN) (Table 6).

The polymerase chain reaction (PCR) was performed as described in Table 7 and Table 8.

The PCR product was isolated using gel electrophoresis. The product specific band was cut out and the DNA was extracted using the New England Biolabs Monarch Gel extraction Kit. The extracted DNA and the entry vector were digested together as described in Table 9. After the digestion the DNA was extracted using the New England Biolabs Monarch PCR & DNA Cleanup Kit and a ligation was performed as described in Table 10. For DNA amplification a *E. coli* transformation was performed as described in 6.4.1. The plasmid DNA was extracted

from the bacteria using the New England Biolabs Monarch MiniPrep Kit. The plasmid DNA concentration was measured using the ThermoFisher Scientific Nano Drop 2000.

Table 6 GreenGate cloning – specific left and right boarder sequences

Six almost identical pUC19 based entry vectors are used for the GreenGate cloning technique. Each vector is designed with specific left and right borders, giving each entry vector its identity.

entry vector	left border	right border
pGGA	ACCT	AACA
pGGB	AACA	GGCT
pGGC	GGCT	TCAG
pGGD	TCAG	CTGC
pGGE	CTCG	ACTA
pGGF	ACTA	GTAT

Table 7 Pipetting scheme for entry vector cloning

The template DNA was adjusted to a concentration of 100 ng. All compounds were added into a PCR tube and kept on ice before the PCR was started.

compound	volume
template DNA	100 ng
forward primer (10 μ M)	0.5 μ l
reverse primer (10 μ M)	0.5 μ l
dNTP's (5mM)	1.6 μ l
ThermoFisher Scientific High fidelity / GC 5x buffer	4 μ l
ThermoFisher Scientific Phusion Polymerase	0.2 μ l
ddH ₂ O	fill up to final volume
Σ	20 μ l

Table 8 PCR program that was used for entry vector cloning

The primer annealing temperature was based on melting temperature of the primer (based on the calculations of Benchling). The elongation time was based on the length of the replicated DNA. Thermo Scientific Phusion Polymerase replicates DNA with the speed of 1kb/30sec.

temperature	time in seconds	cycle
95°C	120	
95°C	30	x35
optimized based on melting temperature of the primer	30	
72°C	optimized based on DNA length	
72°C	420	
20°C	∞	

Table 9 Digestion of the extracted PCR product and entry vector

Extracted PCR product and entry vector are pooled and digested together.

compound	volume
PCR product	10 µl
entry vector	1 µl
New England Biolab BsaIV2	0.5 µl
New England Biolab CutSmart Buffer	1.5 µl
ddH ₂ O	2 µl
Σ	15 µl

Table 10 Digested PCR product and entry vector ligation after PCR & DNA clean up
Pipetting scheme for PCR product and entry vector digestion.

plasmid	volume
digested PCR product + entry vector	10 μ l
New England Biolab T4 Ligase	0.5 μ l
New England Biolab T4 Ligase 10x buffer	1.5 μ l
ATP (10mM)	1.5 μ l
ddH ₂ O	1.5 μ l
Σ	15 μ l

6.3.3 Site-directed mutagenesis

Site-directed mutagenesis was performed to integrate point mutations in the catalytic active domains of PLD and PAP (Table 11). The PCR was prepared as described in Table 7 and performed as described in Table 12. After the PCR a *E. coli* transformation was performed as described in 6.4.1.

Table 11 Site-directed mutagenesis – point mutations that have been integrated

name	backbone	resistance bacteria	forward primer	reverse primer
dPAP	pGGD	Amp	CAGATGTTGAGGGAAC	GTTCCcTCAACATCTG
dPLD	pGGD	Amp	CCTGGAACGCTAGCAAAATCTTG	CCAAGATTTTGCTAGCGTTCCAGG

Table 12 PCR scheme to perform a site-directed mutagenesis

Point mutations have been integrated using the following protocol. DpnI digestion was used to cut the template DNA.

temperature	time in seconds	cycle
95°C	120	
95°C	30	x20
optimized based on melting temperature of the primer	30	
72°C	optimized based on DNA length	
20°C → add DpnI (1µl)	digest for 1h30min	
95°C	300	
95°C	30	x15
optimized based on melting temperature of the primer	30	
72°C	optimized based on DNA length	
72°C	900	
20°C	∞	

6.3.4 Destination vector cloning

The destination vectors in Table 15 were cloned using the suggested protocol for GreenGate cloning (Table 13), with slide modification (Table 14). Also, instead of the destination vector pGGZ, which is suggested for the GreenGate cloning system, the destination vector PG800 designed by Gael Paszkiewicz was used (Figure 44) (117).

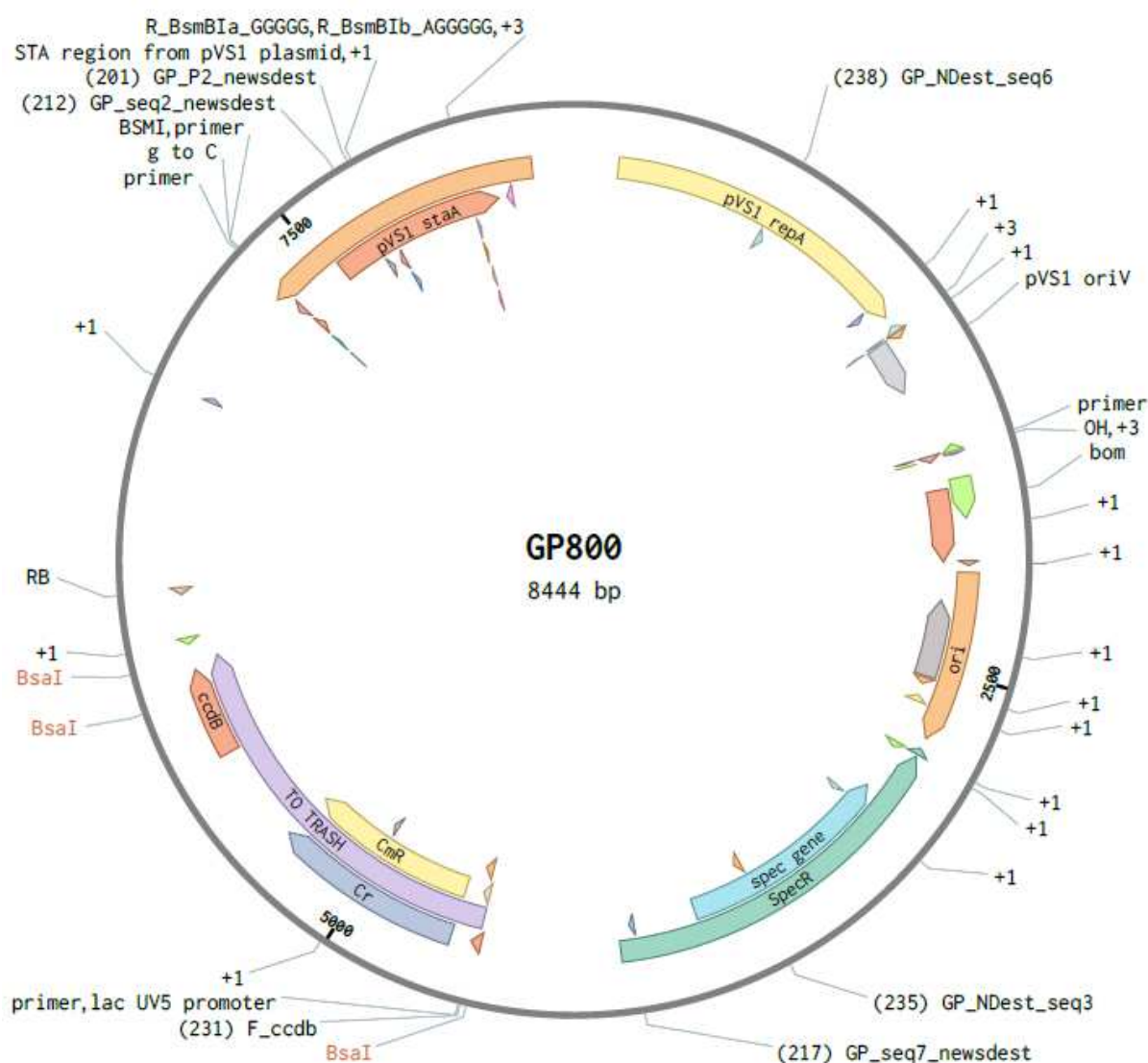


Figure 44 GreenGate cloning destination vector GP800

GP800 is a pCambia based plasmid and consists of a spectinomycin resistance gene (SpecR), an origin of replication (ori) and a VS1 replicon. Between the BsaI recognition sites and the left and right border sequences are a ccdB cassette and a chloramphenicol resistance gene (CmR), that are cut out after digestion. The plasmid was designed by Gael Paszkiewicz (117).

Table 13 final assembly pipetting scheme

plasmid	volume
entry vector (pGGA-F)	100 ng (each)
destination vector (GP800)	150 ng
New England Biolab BsaIV2	0.5 µl
New England Biolab CutSmart Buffer	1.5 µl
New England Biolab T4 Ligase	0.5 µl
ATP (10mM)	1.5 µl
ddH ₂ O	Fill up to final volume
Σ	15 µl

Table 14 Final assembly

temperature	time in seconds	cycle
16°C	300	
16°C	180	x30
37°C	180	
37°C	300	
50°C	300	
80°C	300	
20°C	∞	

Table 15 Destination vectors after final assembly

Spec = Spectinomycin (working concentration 100µg/ml); Kan = Kanamycin (working concentration 100µg/ml); PPT = Phosphinothricin (working concentration 50µg/ml)

name	backbone	resistance bacteria	resistance plants
pUBQ10::CBL1n12-3x(mCherry)-RBCS ^{Ter}	GP800	Spec	Kan
pUBQ10::CBL1n12-3x(mCherry)-PLD-RBCS ^{Ter}	GP800	Spec	Kan
pUBQ10::CBL1n12-3x(mCherry)-dPLD-RBCS ^{Ter}	GP800	Spec	Kan
pUBQ10::CBL1n12-3x(mCherry)-PAP-RBCS ^{Ter}	GP800	Spec	Kan
pUBQ10::CBL1n12-3x(mCherry)-dPAP-RBCS ^{Ter}	GP800	Spec	Kan
pUBQ10::CBL1n12-3x(mCherry)-TtPAH2-RBCS ^{Ter}	GP800	Spec	Kan
pUBQ10::MP-3xmCherry-ScPAH1-RBCS ^{Ter}	GP800	Spec	Kan
pUBQ10::MP-3xmCherry-ScPAH1Δ(1-600) -RBCS ^{Ter}	GP800	Spec	Kan
pUBQ10::MP-3xmCherry-dScPAH1-RBCS ^{Ter}	GP800	Spec	Kan
pUBQ10::CBL1n12-3x(mCherry)-MmLIPIN2Δ- RBCS ^{Ter}	GP800	Spec	Kan
pUBQ10::MP-3xmCherry-ScPAH1-RBCS ^{Ter}	GP800	Spec	Kan
pUBQ10::MP-3xmCherry-ScPAH1Δ(1-600) -RBCS ^{Ter}	GP800	Spec	Kan
pUBQ10::MP-3xmCherry-dScPAH1-RBCS ^{Ter}	GP800	Spec	Kan
pUBQ10::MP-3x(mCherry) -RBCS ^{Ter}	GP800	Spec	Kan
pUBQ10:: MP-3x(mCherry)-PLD-RBCS ^{Ter}	GP800	Spec	Kan
pUBQ10:: MP-3x(mCherry)-dPLD-RBCS ^{Ter}	GP800	Spec	Kan
pUBQ10:: MP-3x(mCherry)-PAP-RBCS ^{Ter}	GP800	Spec	Kan
pUBQ10::RPS5n20-3x(mCherry) -RBCS ^{Ter}	GP800	Spec	Kan
pUBQ10::RPS5n20-3x(mCherry)-PLD-RBCS ^{Ter}	GP800	Spec	Kan

pUBQ10::RPS5n20-3x(mCherry)-dPLD-RBCS ^{Ter}	GP800	Spec	Kan
pUBQ10::RPS5n20-3x(mCherry)-PAP-RBCS ^{Ter}	GP800	Spec	Kan
pUBQ10::RPS5n20-3x(mCherry)-dPAP-RBCS ^{Ter}	GP800	Spec	Kan
pop4::CBL1n12-3x(mCherry) -RBCS ^{Ter}	GP800	Spec	Kan
pop4::CBL1n12-3x(mCherry)-PLD-RBCS ^{Ter}	GP800	Spec	Kan
pop4::CBL1n12-3x(mCherry)-dPLD-RBCS ^{Ter}	GP800	Spec	Kan
pop4::CBL1n12-3x(mCherry)-PAP-RBCS ^{Ter}	GP800	Spec	Kan
pop4::CBL1n12-3x(mCherry)-dPAP-RBCS ^{Ter}	GP800	Spec	Kan
pUBQ10::MKK7-3x(GFP)-RBCS ^{Ter}	GP800	Spec	PPT
pUBQ10::MKK9-3x(GFP)-RBCS ^{Ter}	GP800	Spec	PPT
pUBQ10::MPK6-3x(GFP)-RBCS ^{Ter}	GP800	Spec	PPT
pUBQ10::mTurquoise-AtPAH1-RBCS ^{Ter}	GP800	Spec	PPT
pUBQ10::AtPAH1-mTurquoise-RBCS ^{Ter}	GP800	Spec	PPT
pUBQ10::(Δ AH)AtPAH1-mTurquoise-RBCS ^{Ter}	GP800	Spec	PPT
pUBQ10::(Δ M-LIP)AtPAH1-mTurquoise-RBCS ^{Ter}	GP800	Spec	PPT
pPAH1::mTurquoise-AtPAH1-RBCS ^{Ter}	GP800	Spec	PPT
pPAH1::AtPAH1-mTurquoise-RBCS ^{Ter}	GP800	Spec	PPT
pPAH1::(Δ AH)AtPAH1-mTurquoise-RBCS ^{Ter}	GP800	Spec	PPT
pPAH1::(Δ M-LIP)AtPAH1-mTurquoise-RBCS ^{Ter}	GP800	Spec	PPT

6.4 Bacterial transformation

6.4.1 *Escherichia coli* transformation

Escherichia coli strain DH5 α was used for all plasmid propagation. Before transformation, DH5 α cells were made chemical competent. The bacteria were put onto solid LB and grown over night at 37°C. Colonies were then collected and incubated overnight in 10ml liquid LB at 37°C. The 10ml bacterial suspension was further incubated in 500ml liquid LB. The culture

was grown until an OD600 of 0.35-0.4 was reached. When the final OD600 was reached, the suspension was incubated on ice for 20 minutes. The suspension was further centrifuged at 4°C with a speed of 3000g. The supernatant was decanted and the pellet was washed in 200ml cold 100mM MgCl₂. The suspension was again centrifuged for 15 minutes at 4°C with a speed of 2000g. The supernatant was decanted and the pellet was washed in 200ml cold 100mM CaCl₂. The suspension was again centrifuged for 15 minutes at 4°C with a speed of 2000g. The supernatant was decanted and the pellet was washed in 50ml cold 85mM CaCl₂ and 15% glycerol. The suspension was again centrifuged for 15 minutes at 4°C with a speed of 2000g. The pellet was collected and resuspended in 2ml 85mM CaCl₂ and 15% glycerol. 50 µl of the suspension was aliquoted into reaction tubes and frozen in liquid nitrogen for further storage.

Bacterial transformation was performed by using the heat shock method. The bacteria were defrozen on ice. 100-200ng of plasmid DNA was added to the bacterial suspension and incubated for 15 minutes on ice. The bacteria were then heat shocked at 42°C for 90 seconds. 1ml Liquid LB at room temperature was then added to the suspension. The bacteria were incubated for 60-90 minutes at 37°C and placed on solid selective LB media. The bacteria were then grown at 37°C overnight.

6.4.2 *Agrobacterium tumefaciens* transformation

Agrobacterium tumefaciens strain GV3101 was used for all plasmid propagation. Before transformation, GV3101 cells were made chemical competent. The bacteria were put onto solid LB (+ 50µg/ml rifampicin and 10µg/ml gentamicin) and grown over night at 28°C. Colonies were then collected and incubated overnight in 10ml liquid LB (+ 50µg/ml rifampicin and 10µg/ml gentamicin) at 28°C. The 10ml bacterial suspension was further incubated in 500ml liquid LB. The culture was grown until an OD600 of 0.5 was reached. When the final OD600 was reached, the suspension was incubated on ice for 20 minutes. The suspension was then centrifuged at for 15 minutes at 4°C with a speed of 3000 rpm. The supernatant was decanted and the pellet was washed in 100ml of 150mM NaCl. The suspension was again centrifuged for 15 minutes at 4°C with a speed of 3000 rpm. The supernatant was decanted and the pellet was resuspended with 10ml of 20mM CaCl₂. 100 µl of the suspension was further aliquoted in 1.5ml reaction tubes and frozen in liquid nitrogen.

Bacterial transformation was performed by using the heat shock method. The bacteria were defrozen on ice. 100-200ng of plasmid DNA was added to the bacterial suspension and incubated for 30 minutes on ice. The bacteria were then heat shocked at 37°C for five minutes. 1ml Liquid LB at room temperature was then added to the suspension. The bacteria were

incubated for 120 minutes at 28°C and placed on solid selective LB media. The bacteria were then grown at 28°C overnight.

6.5 Arabidopsis transformation – Floral dip

Arabidopsis plants were grown until they begin to bolt and produce floral inflorescences. Agrobacteria carrying the gene of interest were incubated over night at 28°C in 2ml liquid selective LB media. The Agrobacteria suspension was upscaled to 25 ml and further incubated over night at 28°C. The Agrobacteria suspension was centrifuged at a speed of 5000 rpm for 4 minutes. The pellet was collected in 50 ml infiltration medium (10 mM MgCl₂, 5% sucrose, 0.03% silwet). The 50 ml bacteria suspension was put into a box and further 100ml infiltration medium was added. the inflorescences were dipped into the Agrobacteria suspension for 10 seconds. The dipped plants were covered with a plastic bag over night. The plastic bag was then removed for seed propagation. The seeds were then selected on selective solid ½ MS plates. The plants in Table 16 were transformed and selected by *Steffi Zimmermann* and *Frank Ackermann*.

Table 16 *Arabidopsis thaliana* lines produced in this work

plasmid		background	resistance	description
pUBQ10::GR-LhG4 pop4::CBL1n12- 3x(mCherry)	/	Col-0	Kan	DEX-inducible
pUBQ10::GR-LhG4 pop4::CBL1n12- 3x(mCherry)-PLD	/	Col-0	Kan	DEX-inducible
pUBQ10::GR-LhG4 pop4::CBL1n12- 3x(mCherry)-dPLD	/	Col-0	Kan	DEX-inducible
pUBQ10::GR-LhG4 pop4::CBL1n12- 3x(mCherry)-PAP	/	Col-0	Kan	DEX-inducible
pUBQ10::GR-LhG4 pop4::CBL1n12- 3x(mCherry)-dPAP	/	Col-0	Kan	DEX-inducible

pPAH1::mTurquoise- AtPAH1	<i>pah1/2</i> (Col-0)	BASTA	<i>pah1/2</i> rescue line
pPAH1::AtPAH1- mTurquoise	<i>pah1/2</i> (Col-0)	BASTA	<i>pah1/2</i> rescue line
pPAH1::(Δ AH)AtPAH1- mTurquoise	<i>pah1/2</i> (Col-0)	BASTA	<i>pah1/2</i> rescue line
pPAH1::(Δ M-LIP)AtPAH1- mTurquoise	<i>pah1/2</i> (Col-0)	BASTA	<i>pah1/2</i> rescue line

6.6 Transient expression in *Nicotiana benthamiana*

Overnight cultures of *Agrobacterium tumefaciens* were centrifuged and resuspended in induction buffer (10 mM MgCl₂, 10 mM MES pH 5.6, 150 μ M acetosyringone). The optical density at 600 nm (OD₆₀₀) of all constructs was adjusted according to Table 17. Samples were mixed as indicated. Agrobacteria mixtures were infiltrated into leaves of 4 to 6week old *N. benthamiana* wild-type (WT) plants. For all infiltrations the silencing inhibitor P19 was also infiltrated.

Combined gene expression of more than one gene was induced by pooling different *Agrobacterium tumefaciens* suspensions and infiltrate them together at the same time.

For dexamethasone induced gene expression, 20 μ M dexamethasone (Sigma-Aldrich) and 0.001% (v/v) Silwet L-77 was sprayed on both sides of the *Nicotiana benthamiana* leaves 32 hours after *Agrobacterium tumefaciens* infiltration.

Table 17 Plasmids used for Agrobacteria transformation

Construct	OD ₆₀₀	Reference
35S::P19	0.05	Farid El Kasmi, ZMBP Uni Tuebingen
pUBQ10::CBL1n12-3x(mCherry)	0.3	done in this work
pUBQ10::CBL1n12-3x(mCherry)-PLD	0.3	done in this work
pUBQ10::CBL1n12-3x(mCherry)-dPLD	0.3	done in this work

pUBQ10::CBL1n12-3x(mCherry)-PAP	0.3	done in this work
pUBQ10::CBL1n12-3x(mCherry)-dPAP	0.3	done in this work
pUBQ10::CBL1n12-3x(mCherry)-TtPAH2	0.3	done in this work
pUBQ10::MP-3x(mCherry)-MmLIPIN2Δ	0.3	done in this work
pUBQ10::MP-mTurquoise2-ScPAH1	0.3	done in this work
pUBQ10::MP-mTurquoise2-ScPAH1(D398E)	0.3	done in this work
pUBQ10::MP-3x(mCherry)	0.3	done in this work
pUBQ10::RPS5n20-3x(mCherry)	0.3	done in this work
pUBQ10::RPS5n20-3x(mCherry)-PLD	0.3	done in this work
pUBQ10::RPS5n20-3x(mCherry)-dPLD	0.3	done in this work
pUBQ10::RPS5n20-3x(mCherry)-PAP	0.3	done in this work
pUBQ10::RPS5n20-3x(mCherry)-dPAP	0.3	done in this work
pUBQ10::mCitrine-KA1	0.2	Yvon Jaillais, École normale supérieure de Lyon
pPDFI::mCitrine-1xPASS	0.2	Yvon Jaillais, École normale supérieure de Lyon
pPDFI::mCitrine-2xPASS	0.2	Yvon Jaillais, École normale supérieure de Lyon
pUBQ10::MKK7-3x(GFP)	0.2	done in this work
pUBQ10::MKK9-3x(GFP)	0.2	done in this work
pUBQ10::GFP-MAP65-1	0.1	done in this work
pUBQ10::RFP	0.3	Farid El Kasmi, ZMBP Uni Tuebingen
35S::cYFP-RPM1	0.1	Farid El Kasmi, ZMBP Uni Tuebingen

35S::RIN4-RFP	0.1	Farid El Kasmi, ZMBP Uni Tuebingen
DEX::AvrRPM1	0.1	Farid El Kasmi, ZMBP Uni Tuebingen
DEX:AvrPphb	0.1	Farid El Kasmi, ZMBP Uni Tuebingen
DEX::RPS5	0.1	Farid El Kasmi, ZMBP Uni Tuebingen
DEX::PBS1	0.1	Farid El Kasmi, ZMBP Uni Tuebingen
pUBQ10::mTurquoise-AtPAH1	0.3	done in this work
pUBQ10::AtPAH1-mTurquoise	0.3	done in this work
pUBQ10::(Δ AH)AtPAH1-mTurquoise	0.3	done in this work
pUBQ10::(Δ M-LIP)AtPAH1-mTurquoise	0.3	done in this work
35S::VMA12-RFP	0.2	Farid El Kasmi, ZMBP Uni Tuebingen

6.7 Cell death assay and conductivity measurements

The samples for cell death and conductivity measurements were prepared as described in 6.6. Picture of the *Nicotiana benthamiana* leaves were taken at the indicated time points. Images were taken under UV light using the Amersham ImageQuant 800 Western blot imaging system and an integrated Cy5 filter (GE Healthcare; Chalfont St. Giles, UK). The images were processed using ImageJ (Wayne Rasband, National Institute of Health, Bethesda, Maryland, USA) for brightness and contrast.

For conductivity measurements single leaf discs were punched out of the infiltration site 33 hours after infiltration and one hour after dexamethasone induction. The leaf discs were washed for one hour in ddH₂O to remove the leaking ions after wounding the tissue. Conductivity measurements were performed using the CM100-2 Conductivity Meter (Reid & Associates, South Africa). For measurements, the leaves were then transferred to fresh 1ml ddH₂O. Measurements were taken every 10 minutes. In total, 100 measurements were done per sample.

6.8 Protoplast transformation and Fluorescent-activated cell sorting (FACS)

The following experiment was performed by *Dr. Kenneth Berendzen* (ZMBP, Univeristy of Tuebingen).

6.8.1 PEG mediated Protoplast transformation of *Arabidopsis thaliana* (Columbia) Cell Suspension Protoplasts

The protoplasts used in this work for transfection are derived from *Arabidopsis thaliana* (Columbia) Cell Suspension. The cell cultures are grown in the dark (at $\pm 23.5^{\circ}\text{C}$) in 250 mL Erlenmeyer flasks under constant shaking at a speed of 120 rpm. The cell culture is propagated every seven days by re-inoculating the culture to a new sterile 250 mL Erlenmeyer flask. 50 mL MSCol (4.3 g/L Murashige and Skoog salt; 1 mg/L Nicotin acid; 1 mg/L Pyridoxin-HCL; 10 mg/L Thiamin; 100 mg/L myo-inositol; 30 g/L sucrose; pH 5.8) medium was added.

Protoplasts transfection was performed as described in Mehlhorn *et al.* (2017) (118). For protoplast transfection 16 μg DNA was prepared in 20 μl ddH₂O, then 60 μl cell suspension was added and the suspension was gently mixed by shaking the reaction tube carefully. Furthermore, 60 μl 40% PEG-1500 (1.275 g Mannitol, 0.413 g Ca(NO₃)₂ (4xH₂O), 4mM MES, pH 6; 17.5 ml ddH₂O) was added. The suspension was again mixed carefully for 5 minutes. Then, 60 ml MM solution (0.4 M Mannitol, 5 mM MES, pH6) was added and mixed as described before. 500 μl K3 medium (100 ml: 10 ml macro stock, 0.1 ml micro stock, 0.1 ml vitamin stock, 0.5 ml EDTA stock, 1ml Ca-Phosphate stock, 10 mg myo-inositol, 25 mg D(+)-Xylose, 13.7 g sucrose, pH5.6) was added, and the medium was again gently mixed. The cells were then incubated for 6 hours in the dark.

Table 18 Protocol for 1 l Macro Stock used for protoplast transfection

Volume	Compound
1.5g	NaH ₂ PO ₄ x H ₂ O
9g	CaCl x 2xH ₂ O
25g	KNO ₃
2.5g	NH ₄ NO ₃
1.34	(NH ₄)SO ₄
2.5g	MgSO ₄ x 7xH ₂ O
1l	ddH ₂ O

Table 19 Protocol for 100ml Micro Stock used for protoplast transfection

Volume	Compound
75mg	KI
300mg	H ₃ BO ₃
1g	MnSO ₄ x 7xH ₂ O
200mg	ZnSO ₄ x 7xH ₂ O
25mg	Na ₂ MoO ₄ x 2xH ₂ O
2.5mg	CuSO ₄ x 5xH ₂ O
2.5mg	CoCl ₂ x 6xH ₂ O
100ml	ddH ₂ O

Table 20 Protocol for 100 ml Vitamine Stock used for protoplast transfection

Volume	Compound
100mg	Nicotinic acid
100mg	Pyridoxine-HCL
1g	Thiamine-HCL
100ml	ddH ₂ O

Table 21 Protocol for 1l EDTA Stock used for protoplast transfection

Volume	Compound
7.46	EDTA
Add 300 ml ddH ₂ O → dissolve	
5.56 g	Fe(II)SO ₄ x 7xH ₂ O
Add 300 ml ddH ₂ O → dissolve	
Combine both solutions	
Add 400 ml	ddH ₂ O

Table 22 Protocol for 200ml Ca-Phosphate Stock used for protoplast transfection

Volume	Compound
1.26g	CaHPO ₄ x 2H ₂ O
200ml	ddH ₂ O
Adjust to pH 3	

6.8.2 Fluorescent-activated cell sorting (FACS)

Cytometric analysis was ran on a Beckman Coulter MoFlo XDP (100 μ m CytoNozzle, 30.5/30.0 psi, PBS sheath), exciting eGFP at 488 nm (70 mW, elliptical focus) and peak emission in the FL1 (534/30); mCherry at 561 nm excitation (70 mW, spherical focus) and peak emission in the FL6 (620/29). Data were collected using Summit 5.5 (Beckman Coulter), plotted analyzed with FCS Express (DeNovo). The main light-scattering gate was determined by identifying the population expressing the greatest amount of reporter protein and termed “living cells”. The eGFP and mCherry populations are those cells whose expression in the respective channel exceeded that of the auto-fluorescent background observed in the mock transfected samples, marked in their respective gates.

6.9 Confocal Laser Microscopy and picture analysis

Protein localization was analyzed at the indicated time points with the upright confocal laser scanning microscope TCS SP8 from Leica (Wetzlar, Germany). Confocal imaging was done using a 40x or 63x water-immersion objective. mTurquoise2 was excited using a 448nm laser with a detected emission spectrum of 455-505 nm. GFP was excited using a 488nm laser with a detected emission spectrum of 495-520 nm. mCitrine was excited using a 512nm laser with a detected emission spectrum of 520-545 nm. mCherry was excited using a 552nm laser with a detected emission spectrum of 595-625 nm. Focal plane images were processed with the LAS X software (Leica) for adjustment of brightness and contrast. Maximum Z-projection images were processed with the LAS X software (Leica).

Line intensity plot profile image analysis were produced using ImageJ (Wayne Rasband, National Institute of Health, Bethesda, Maryland, USA). The line intensity plot profile was made by using the same line in both channels of the picture. The data achieved were further processed using Microsoft's Excel.

Co-localization of AtPAH1 and the ER-marker VMA12 were analyzed using the ImageJ plug-in JACoP (Just Another Colocalization Plugin) (84). JACoP calculates the likelihood for colocalization of two proteins imaged using microscopy based on the Pearson's coefficient. The Pearson's coefficient calculates the scattering of the dot cloud relative to the median line estimated by the correlation coefficient. One must keep in mind that Pearson's coefficient is no true quantification of co-localization of two proteins but rather an estimate. The Pearson's coefficient varies from -1 to 1, with -1 stating exclusion, 0 the absence of correlation and 1 a full correlation (84). Besides the Pearson's coefficient, also the Mander's coefficient was used

to estimate colocalization of two proteins using JACoP (84, 85). M1 and M2 are the sum of intensities of colocalizing pixels from a channel and dividing it by its integrated density. A pixel from channel A is considered as colocalized if it has a counterpart in channel B with a value above zero. This results in an estimate of colocalization for a signal of M1 and M2 from a channel over the other (84).

6.10 Protein detection using Western Blot analysis

For protein detection using Western Blot 500 mg of plant material was collected per sample, placed in 1.5ml test tubes and frozen immediately in liquid nitrogen. For protein extraction, the volume of the tissue was reduced by punching the tissue repeatedly and 150 μ l protein extraction buffer was added (100mM Tris HCl pH7.7, 2% SDS, 500mM DTT). Further 50 μ l 4X Laemmli buffer (900 μ l Laemmli + 100 μ l β -mercaptoethanol) was added and the sample were incubated for 10 minutes on ice. The samples were then boiled for 10 minutes at 95°C and then centrifuged at 4°C for 2 minutes with a speed of 13000 rpm. 150 μ l were then transferred into fresh 1.5ml reaction tubes.

The sodium dodecyl sulphate (SDS)-page was prepared as described in Table 23. 10-20 μ l of one protein extract was loaded per pocket. The gel was run at 80V for around 120 minutes using a 1x SDS running buffer (Table 24). 1.5l 1x Transferbuffer was prepared by adding 150 ml 1x Transferbuffer (Table 25) to 200 ml ethanol and 1150ml dd H₂O. The membrane was prepared to transfer the proteins by activating it using 100% methanol and then placing it between wet tissue and Whatman paper and a PVDF membrane was placed on top of the gel (Bio Rad Cat. #10026933). The proteins were then transferred over night at 30V using a wet blot system. The tank was filled up with the 1x Transferbuffer.

The membrane was then incubated for one hour in TBST (1x TBS + 0.1% Tween20) and 5% milk. The primary antibody α -GFP mouse (1:1000, Roche b3⁴d3) was used for GFP and mTurquoise2 detection, and the primary antibody α -RFP mouse (1:1000, ChromoTek 6G6) was used for mCherry detection. The primary antibodies were added in the right amount to TBST + 5% milk and incubated for one hour. The membrane was then washed three times for 5 minutes each with TBST. For protein detection, the secondary antibody α -mouse HRP (1:10000, goat, Sigma b3d1) was used. The secondary antibody was added in the right amount to TBST + 5% milk and the membrane was then incubated for one hour. Afterwards the membrane was washed twice for five minutes each with TBST and once for five minutes with 1xTBS. Before chemiluminescence detection, the membrane was prepared by adding the Amersham ECL Prime Luminol Enhancer Solution (Cytiva, RPN2232V1) and ECL Prime Peroxidase Solution

(Cytiva, RPN2232V2) (1:1). Ponceau staining was performed using the Ponceau S concentrate (Sigma-Aldrich, St Louis, Missouri, USA). Images were processed using GIMP (GNU Image Manipulation Program, The GIMP-Team) for brightness and contrast.

Table 23 Protocol to prepare a SDS-page

4 ml of the running gel solution and 1.5 ml of the stacking gel solution was used to prepare one gel.

volume	compound
running gel (10%)	
5.2 ml	ddH ₂ O
2.5 ml	Lower Tris (1.5 M Tris, pH8.8)
2.2 ml	Acrylamid (40%)
100 µl	10% SDS
100 µl	10% APS
15 µl	TEMED
stacking gel	
3.3 ml	ddH ₂ O
1.25 ml	Upper Tris (0.5 M Tris, pH6.8)
0.75 ml	Acrylamid (40%)
50 µl	10% SDS
50 µl	10% APS
15 µl	TEMED

Table 24 Protocol to prepare 1l of 10x SDS running buffer for Western Blot

10x SDS running buffer

volume	compound
30 g	Tris-Base
10 g	SDS
144.1 g	Glycin

Table 25 Protocol to prepare 1l of 10x Transferbuffer

10x Transferbuffer

volume	compound
39 g	Tris-Base
143 g	Glycin

Table 26 Protocol to prepare 1l of 10x TBS buffer

The pH needs to be adjusted to 7.5

10x TBS-buffer

volume	compound
87.6 g	NaCl
11.8 g	Tris-Base
63.5 g	Tris-HCl

6.11 *In silico* protein analysis

6.11.1 Protein alignment with PRALINE

PRALINE (<https://www.ibi.vu.nl/programs/pralinewww/>) is a protein alignment online tool that allows you to align multiple protein sequences at a time (86-91). The protein sequences were saved in one FASTA file and uploaded to the website. The alignment was performed with the settings in (Table 27).

Table 27 Settings used to analyze protein sequence alignment with PRALINE

Exchange weights matrix	BLOSUM62		
Progressive alignment strategy	PSI-BLAST pre-profile processing (Homology-extended alignment)	PSI-BLAST Iterations: 3	at an E-value cut-off of 0.01
DSSP-defined secondary structure search	YES		
Secondary structure prediction	PSIPRED		
Transmembrane structure prediction	NO		

6.11.2 Protein structure prediction with AlphaFold2

The protein structure modelling was made using AlphaFold2, an artificial intelligence capable of predicting protein structures based on amino acid sequences in a very reliable manner. As some of the proteins were modified during this work, all models were made using DeepMinds ColabFold/AlphaFold2 (DeepMind, London, England; <https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb>) (82, 119). The predicted local-distance difference test (pLDDT) is a pre-residue confidence metric, that is used to highlight the confidence of the predicted protein structure for accuracy.

6.11.3 α -Helix prediction with HeliQuest

HeliQuest (<https://heliquest.ipmc.cnrs.fr/>) analysis an amino acid sequence for its physicochemical properties and calculates based on defined parameters the likelihood for the presence of an α -helix (65, 66). To analysis AtPAH1 and AtPAH2, the whole amino acid sequences were saved as a FASTA file uploaded to the HeliQuest “Screening α -Helix” tool (<https://heliquest.ipmc.cnrs.fr/cgi-bin/SearchMotifs.py>) and analyzed as described in Karanasios *et al.* (2010) (55).

Table 28 Settings used to analyze AtPAH1 and AtPAH2 amino acid sequence for potential α -Helices with HeliQuest

Physico-chemical parameters			
	Reference value	Minimal value	Maximal value
Hydrophobicity	0.45	0.28	0.6
Hydrophobic moment	0.7	0.35	1.0
net charge	0	-1.0	2.0
Sequence statistics			
	Reference value	Minimal value	Comment
Minimal number of polar residues + glycine	8	8	
Minimal number of uncharged residues	4	4	Serine and Threonine
Minimal number of glycine	1	1	
Maximal number of charged residues	3	3	
Proline accepted	Yes		
Cysteine accepted	No		
Use geometric rules	yes		

6.12 Thin Layer Chromatography and Gas Chromatography

All of the following experiments were performed by *Frank Ackermann* in the lab of Prof. Dr. Ivo Feussner (Department of Plant Biochemistry, University of Goettingen). The GC data analysis was performed by *Dr. Cornelia Herrfurth* (Department for Plant Biochemistry, University of Goettingen).

6.12.1 Lipid extraction

For Lipid extraction, 3g of fresh *Nicotiana benthamiana* leaf material was weighed 40 hours after infiltrating *Agrobacteria* carrying the mentioned constructs. The samples were frozen in liquid nitrogen and stored at -80°C for further preparation. 10ml methanol / chloroform / formic acid (1:1:1; v/v/v) was added and the samples were then vortexed for 20 seconds. The samples were further sonicated for five minutes and shaken for another ten minutes. 5ml 1 M KCl + 0.2M H₃PO₄ were then added to the samples and they were again sonicated for five minutes and shaken for ten minutes. The suspension was then centrifuged for 2 minutes at 1500 rpm and the lower phase was pipetted using a disposable glass pipette into a 8ml glass tube. The suspension was then reduced under a nitrogen stream until no liquid phase was left. The lipid pellet was resuspended in 1 ml chloroform. To measure the total lipid content, 50µl were aliquoted for acidic methanolysis.

6.12.2 1D-TLC lipid separation

Before loading the samples, the silica gel (20cm x 20cm; glass, silica gel 60; Merck KGaA, Darmstadt, Germany) needed to be dried carefully for 90 minutes at 120°C. For the 1D-TLC in Figure 33 A, 150µl of lipid extract was reduced under a nitrogen stream until no liquid phase was left. The lipid pellet was then taken up in 50µl chloroform / methanol (2:1; v/v). The sample was put on the plate in a distance of 1.5cm to each border of the plate and along a straight line of 1.5cm. The sample was added on the plate by using a 10µl pipette and take up the samples using capillarity and gently applying on the plate along the 1.5cm line. The line was dried frequently using an argon stream. The lipid standards were applied similarly, with the only difference that the band size was reduced to 0.5 cm. In-between each sample applied, a distance of 1.5 cm was left to avoid crossovers.

Before starting the phase separation, the glass chamber was equilibrated for 1h by adding 205ml running buffer (for three plates; chloroform / methanol / ddH₂O; 150ml / 50ml / 5ml) and sealing it carefully. The plates were then put into the chamber in an upright position. The separation was stopped roughly 1.5cm before the phase reached the upper border of the plate (ca. 90 minutes after the separation was started). The plates were then dried carefully for around 120 minutes at room temperature. The lipids are then visualized by immersion of the plates in an acidic CuSO₄ solution (0.4 M CuSO₄ in 6.8 % (v/v) H₃PO₄). After drying of the plates using a warm air stream (ca. 30 minutes), the lipids were then visualized by heating the plates at 170 °C for 60 seconds.

6.12.3 2D-TLC lipid separation and sampling for GC

For the 2D-TLC, the first dimension was prepared similarly to the 1D-TLC described in 6.12.2, with the difference that 500µl lipid extract per sample was reduced. After the first dimension was finished the plates were dried carefully for 120 minutes. While that, the glass chambers were cleaned carefully and equilibrated for 60 minutes with 206ml of the running buffer for the second dimension (chloroform / methanol / acidic acid / ddH₂O; 160ml / 18ml / 24ml / 4ml). For the separation in the second dimension the plate was turned at 90°, so that the bands of the first dimension are at the bottom. The silica gel was put into the equilibrated chamber and the separation was stopped 1.5cm before the phase reached the border of the plate). The plates were then dried carefully for around 120 minutes at room temperature. Two of the plates per sample were then prepared as described in 6.12.2 for lipid visualization, the other four plates were first further prepared for lipid extraction. To visualize the lipids under UV-light in a non-destructive way and subsequent GC analysis, the plates were then sprayed with anilino naphthalene sulfonic acid (ANS). The visualized lipid bands were marked and the lipids were then extracted by scratching them of the plate individually (Figure 45). The lipid extract was stored in 8ml glass tubes for further preparations and 1ml FAME solution (Table 29) and 5µg Tri.15:0 were added. As a control, the lipids were then visualized as described in 6.12.2 (Figure 45).

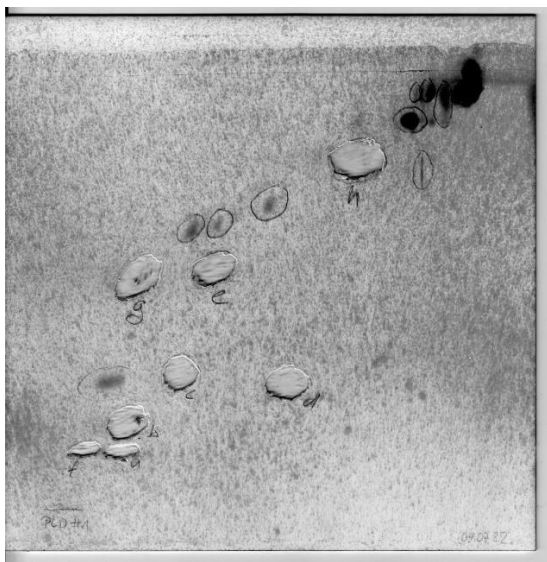


Figure 45 Extracted phospholipid bands on a silica gel after 2D-TLC

The plates were first sprayed with anilino naphthalene sulfonic acid (ANS) to visualize the lipids under UV light in a non-destructive way. The lipid bands were extracted separately by scratching them of the plate. As a control, the plates were then incubated in acidic CuSO₄ solution (0.4 M CuSO₄ in 6.8 % (v/v) H₃PO₄) and (after drying) burned at 170°C for 60 seconds to visualize the remaining lipids.

6.12.4 Acidic methanolysis

The lipid extracts to measure the total lipid content were first reduced under a nitrogen stream until no liquid phase was left. 1ml FAME solution (Table 29) and 5Ml Tri 15:0 were added. The samples, as well as the samples that were prepared as described in 6.12.3, were incubated in a 80°C water bath for 1h (don't let the sample run dry). Then 1.5ml saturated aqueous NaCl

solution and 1.2ml hexane were added and the samples were centrifuged for 2 minutes with a speed of 1500g. The upper phase was collected into a new 8ml glass tube and the sample were dried under a nitrogen stream until no liquid phase was left. The residue was dissolved in 100µl acetonitrile, carefully vortexed, transferred into a GC vial and analyzed using a gas chromatography-flame ionization detector (GC-FID) (Total extracts – FAME60, 1 µl).

Table 29 Protocol to prepare 1l FAME solution

1l FAME solution	
volume	compound
25ml	H ₂ SO ₄
20ml	dimethoxypropane
660ml	methanol
330ml	toluene

6.13 Statistical analysis

Statistical analysis for root length measurements was performed using Excel. Means were compared using Student's t test. Asterisks in the figures denote significant differences as follows: *P < 0.05, **P < 0.01, and ***P < 0.001.

The ion leakage assay was subjected to one-way ANOVA analysis with Scheffe's post hoc analysis with p < 0.05 considered significant. Significant differences are assigned as group letters A–D where there are significant differences between groups but not within groups.

7 Contributions

All experiments were, if not stated differently, performed by Frank Ackermann (ZMBP, University of Tuebingen). The experiments were designed by Thomas Stanislas and Frank Ackermann (ZMBP, University of Tuebingen). Plasmid cloning was designed by Frank Ackermann and performed by Frank Ackermann, Steffi Zimmermann and Jutta Keicher (ZMBP, University of Tuebingen). *Arabidopsis thaliana* transformation was performed by Steffi Zimmermann and Frank Ackermann (ZMBP, University of Tuebingen). Genotyping was performed by Frank Ackermann and Steffi Zimmermann (ZMBP, University of Tuebingen). Thin-Layer-Chromatography and Gas chromatography were performed in the lab of Ivo Feussner (Department for Plant Biochemistry, University of Goettingen) by Frank Ackermann (ZMBP, University of Tuebingen). The GC data analysis was performed by Dr. Cornelia Herrfurth (Department for Plant Biochemistry, University of Goettingen). Protoplast transformation and FACS was performed by Kenneth Berendzen (ZMBP, University of Tuebingen). All statistical analysis were performed by Frank Ackermann (ZMBP, University of Tuebingen).

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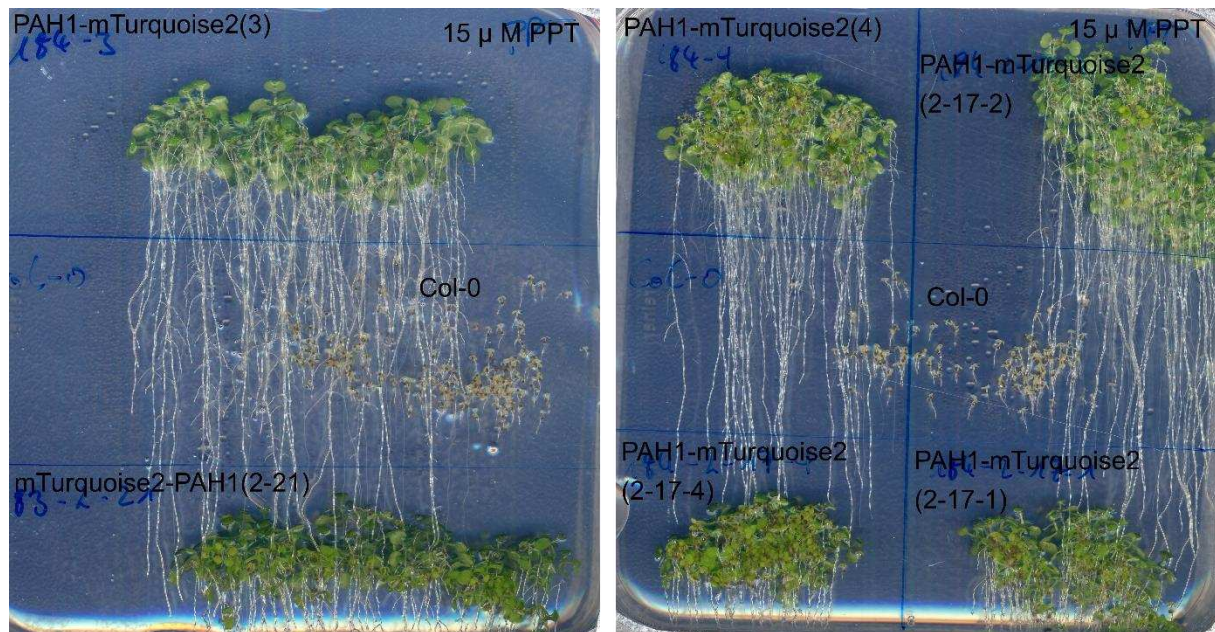
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9 Appendix

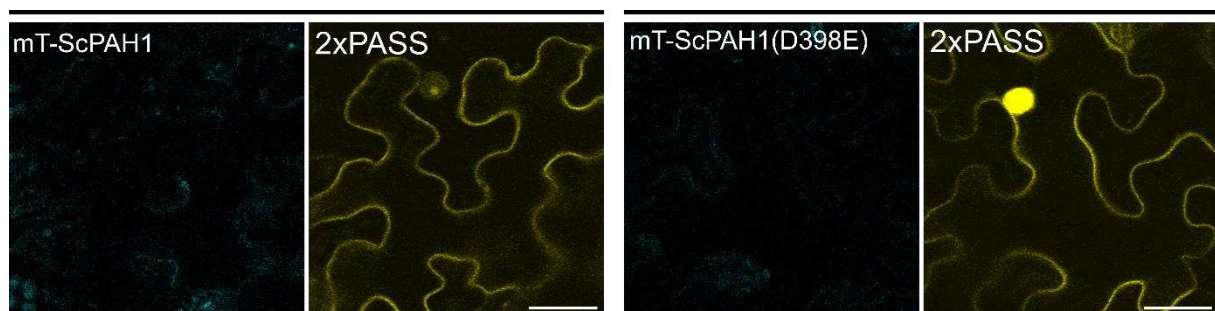
9.1 Identification of a putative N-terminal helix and a M-LIP domain in *Arabidopsis thaliana* PAH1



Supplementary Figure 1 Selection for transgenic *Arabidopsis* seedlings expressing full length PAH1 in the *pah1/2* deficient mutant background

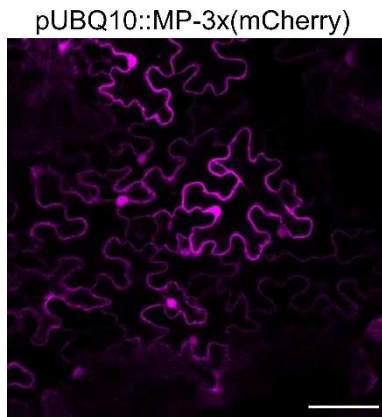
Representative pictures of 14 days old *Arabidopsis thaliana* seedlings. The seedlings were grown on solid ½ MS w/o sucrose containing 15μM Phosphinothricin (PPT) and grew under long-day conditions (16h light/ 8h dark). Col-0 represents the corresponding wild-type. All independent lines of PAH1-mT2 and mT2-PAH1 are expressed under control of the endogenous PAH1 promoter and in the *pah1/2* mutant background.

9.2 The development of genetically encoded tools to manipulate phosphatidic acid level at the plasma membrane *in planta*



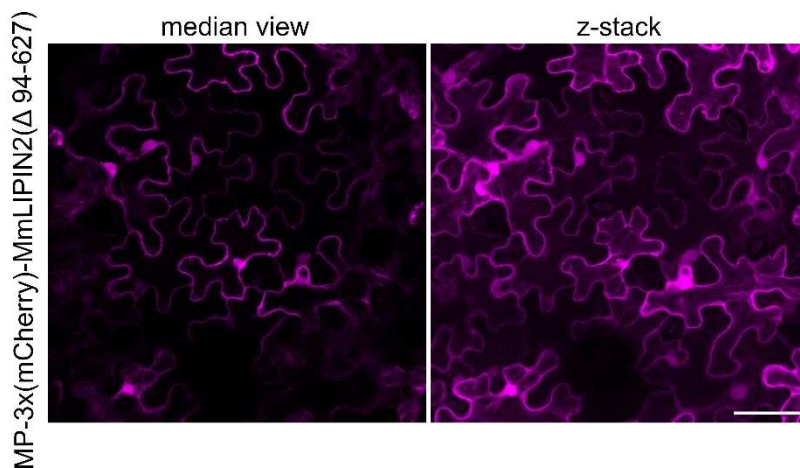
Supplementary Figure 2 *Saccharomyces cerevisiae* Pah1p was not detectable when transiently expressed in *Nicotiana benthamiana* leaves

Full length *Saccharomyces cerevisiae* Pah1p (ScPah1p) and the catalytical inactive ScPah1p(D398E) were transiently expressed, under control of the *UBIQUITINE10* promoter and N-terminally tagged with mTurquoise2, in tobacco leaves and imaged 40 h.p.i. using confocal microscopy. We co-infiltrated mT-ScPah1p with the PA probe pUBQ10::mCitrine-2xPASS. White bar represent 25 μm.



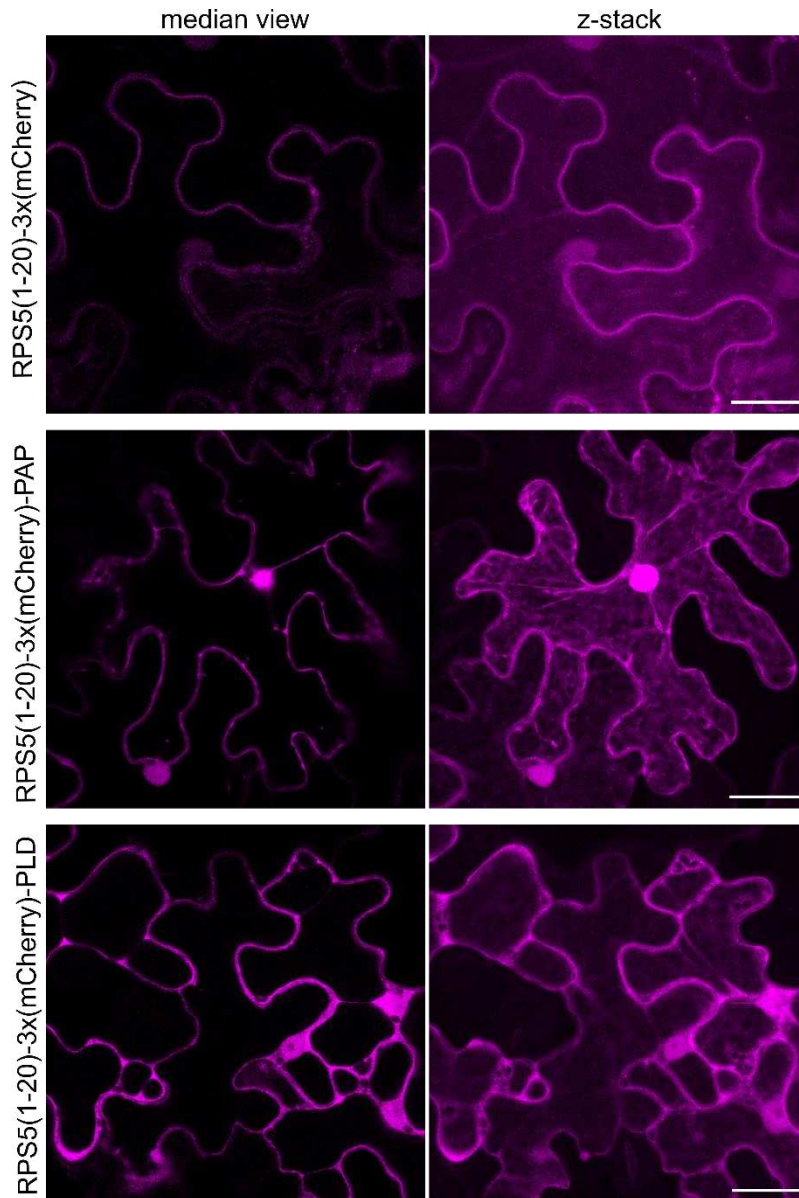
Supplementary Figure 3 The myristoylation and palmitoylation sequence used for plasma membrane localization of SAC1 localized to the cytosol when transiently expressed in *Nicotiana benthamiana* leaves

In 2016 Simon and colleagues established a genetically encoded to downregulate PI(4)P level at the plasma membrane (5). They used a myristoylation and palmitoylation (MP) sequence to anchor the protein to the plasma membrane. We tried to use the same sequence tagged with 3x(mCherry) and expressed under control of the *UBQUITINE10* promoter. We transiently expressed pUBQ10::MP-3x(mCherry) in *Nicotiana benthamiana* leaves and imaged the leaves 40 h.p.i. using confocal microscopy. White bar represents 50µm.



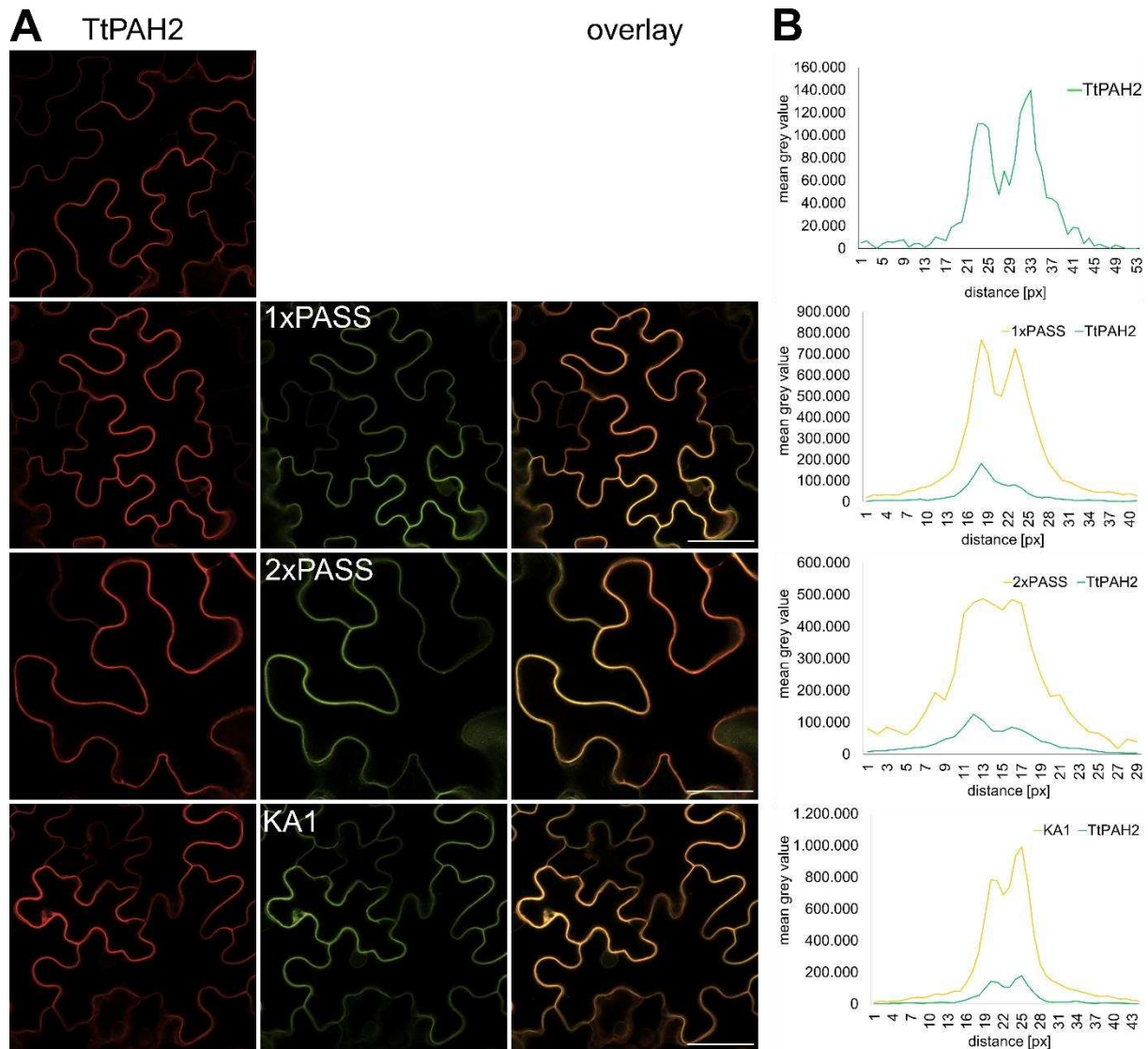
Supplementary Figure 4 Mouse LIPIN2(Δ 94-627) tagged with MP-3x(mCherry) localized to the cytosol when transiently expressed in *Nicotiana benthamiana* leaves

We transiently expressed pUBQ10::MP-3x(mCherry)-MmLIPIN2(Δ 94-627) in *Nicotiana benthamiana* leaves and imaged the leaves 40 h.p.i. using confocal microscopy. White bar represents 50 µm.



Supplementary Figure 5 **The myristoylation (glycine-2) and palmitoylation (cysteine-4) membrane binding site of RPS5 did not lead to plasma membrane localization of PAP and PLD**

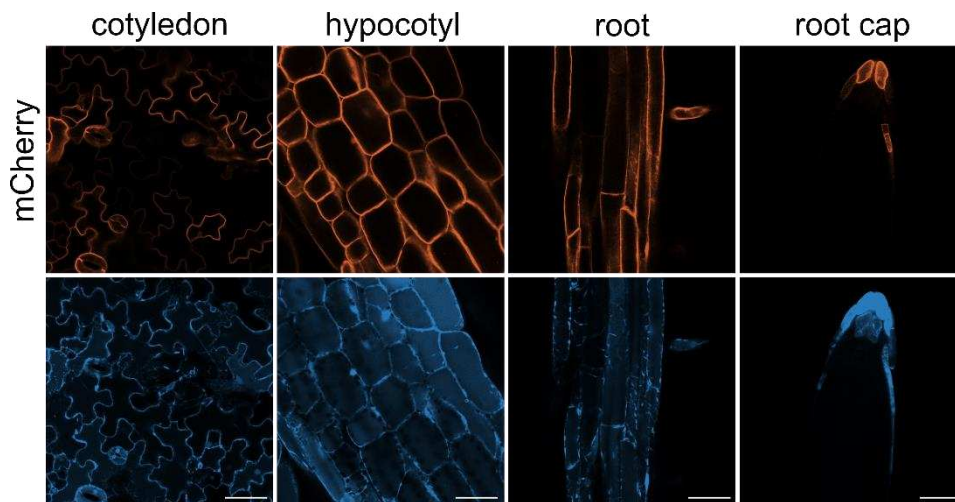
The localization of RPS5 was shown to be mediated by the first 20 N-terminal amino acids which include a myristoylation (glycine-2) and palmitoylation (cysteine-4) membrane binding site (103). We wanted to use these 20 amino acids as a plasma membrane anchor for to regulate PLD and PAP localization. We transiently expressed PLD and PAP under control of the *UBIQUITINE10* promoter N-terminally tagged with RPS5(1-20)-3x(mCherry) in *Nicotiana benthamiana* leaves and imaged the samples using confocal microscopy 40 h.p.i. White bar represents 40 μ m.



Supplementary Figure 6 Co-infiltration of minimal PAP *Tetrahymen thermophila* PAH2 with PA-associated biosensore 1xPASS and 2xPASS as well as KA1 in *Nicotiana benthamiana* leaves

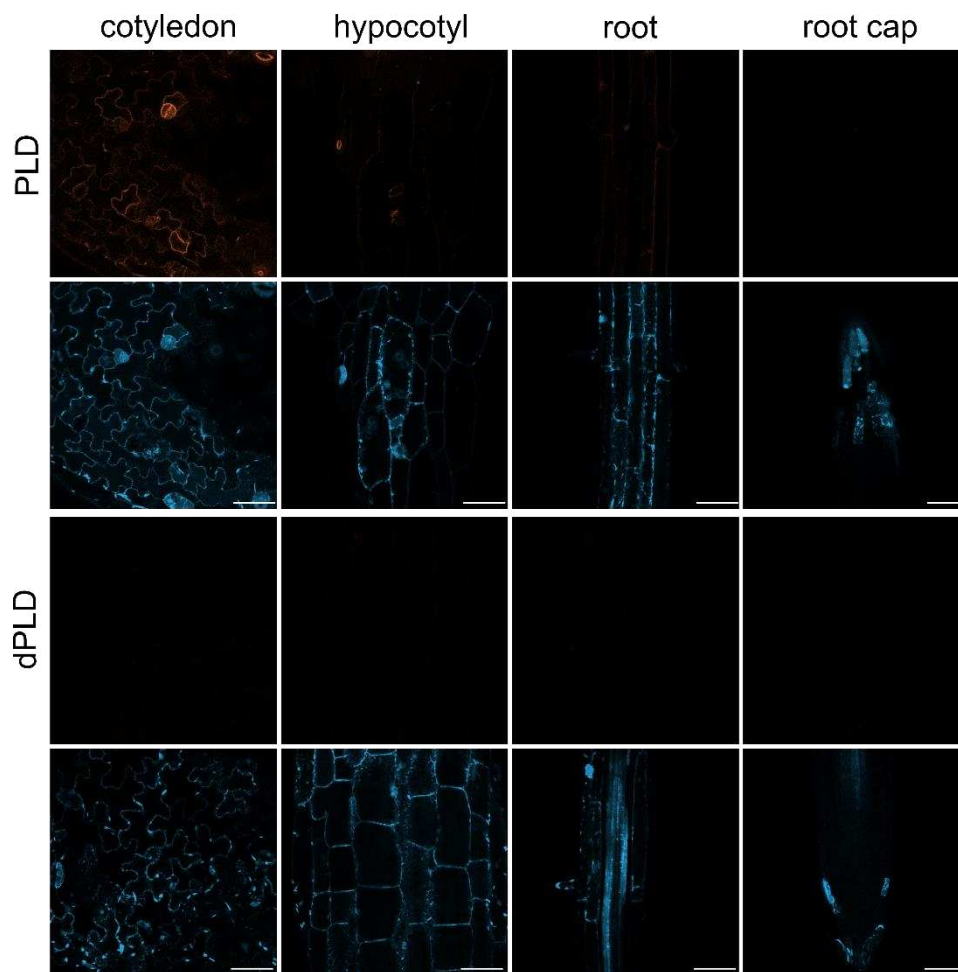
A) Confocal microscopy images of *Tetrahymen thermophila* PAH2 (TtPAH2) (orange) co-infiltrated with PA-associated biosensors pUBQ10::mCitrine-1xPASS and pUBQ10::mCitrine-2xPASS as well as pUBQ10::cYFP-KA1 into *Nicotiana benthamiana* leaves. The Pictures were taken 40 h.p.i. White bars at the bottom right indicate a length of 40 μm. The experiment was repeated twice.

B) Plot profile of TtPAH2 against 1xPASS, 2xPASS and KA1 localization. Measurements were done with ImageJ (6.9).



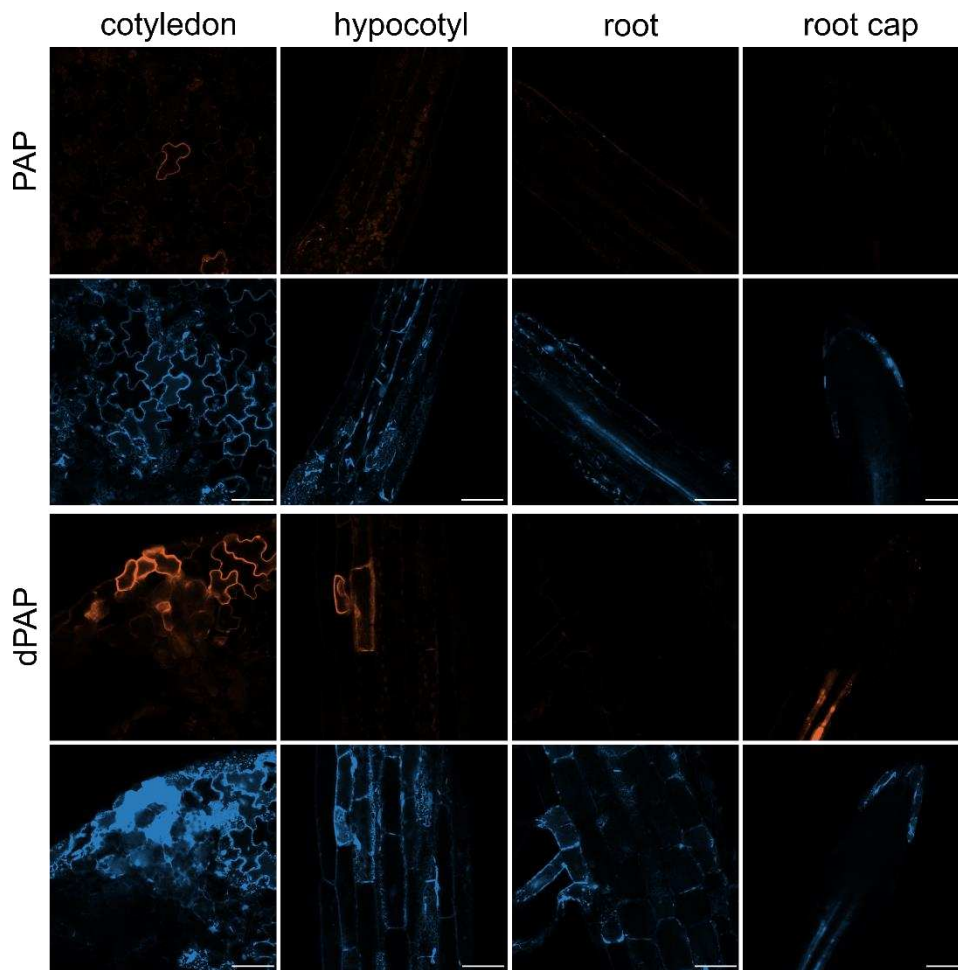
Supplementary Figure 7 Dexamethason inducible expression of CBL1n12-3x(mCherry) in *Arabidopsis thaliana* seedlings

To control gene expression of CBL1n12 more precisely we used the dexamethasone inducible cell type-specific transactivation system established by Schurholz and colleagues (114). The system is based on combination of the chimeric GR-LhG4 transcription factor and the synthetic *pOp* promoter. As a control for successful dexamethasone induced gene expression they established an intrinsic ER-localized mTurquoise2 reporter (blue). The GR-LhG4 transcription factor was expressed under control of the *UBIQUITINE10* promoter. We treated five-days-old *Arabidopsis* seedlings expressing CBL1n12-3xmCherry for 4 hours on solid $\frac{1}{2}$ MS without sugar with 2 μ M dexamethasone and transferred the seedlings to fresh solid $\frac{1}{2}$ MS plates. We imaged the seedlings approximately 26 hours after dexamethasone treatment using confocal microscopy. White bar represents 50 μ m.



Supplementary Figure 8 Dexamethasone inducible expression of CBL1n12-3x(mCherry)-PLD in *Arabidopsis thaliana* seedlings

To control gene expression of CBL1n12 more precisely we used the dexamethasone inducible cell type-specific transactivation system established by Schurholz and colleagues (114). The system is based on combination of the chimeric GR-LhG4 transcription factor and the synthetic *pOp* promoter. As a control for successful dexamethasone induced gene expression they established an intrinsic ER-localized mTurquoise2 reporter (blue). The GR-LhG4 transcription factor was expressed under control of the *UBIQUITINE10* promoter. We treated five-days-old *Arabidopsis* seedlings expressing either CBL1n12-3x(mCherry)-PLD or CBL1n12-3x(mCherry)-dPLD for 4 hours on solid $\frac{1}{2}$ MS without sugar with 2 μ M dexamethasone and transferred the seedlings to fresh solid $\frac{1}{2}$ MS plates. We imaged the seedlings approximately 26 hours after dexamethasone treatment using confocal microscopy. White bar represents 50 μ m.



Supplementary Figure 9 Dexamethasone inducible expression of CBL1n12-3x(mCherry)-PAP in *Arabidopsis thaliana* seedlings

To control gene expression of CBL1n12 more precisely we used the dexamethasone inducible cell type-specific transactivation system established by Schurholz and colleagues (114). The system is based on combination of the chimeric GR-LhG4 transcription factor and the synthetic *pOp* promoter. As a control for successful dexamethasone induced gene expression they established an intrinsic ER-localized mTurquoise2 reporter (blue). The GR-LhG4 transcription factor was expressed under control of the *UBIQUITINE10* promoter. We treated five-days-old *Arabidopsis* seedlings expressing either CBL1n12-3x(mCherry)-PAP or CBL1n12-3x(mCherry)-dPAP for 4 hours on solid $\frac{1}{2}$ MS without sugar with $2\mu\text{M}$ dexamethasone and transferred the seedlings to fresh solid $\frac{1}{2}$ MS plates. We imaged the seedlings approximately 26 hours after dexamethasone treatment using confocal microscopy. White bar represents $50\mu\text{m}$.