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"TRANSPORT OF *ARABIDOPSIS* GPI-ANCHORED PROTEINS ALONG THE EARLY SECRETORY PATHWAY: ROLE OF GPI-ANCHOR REMODELLING AND P24 PROTEINS"

Tesis Doctoral con Mención Internacional presentada por

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València, Septiembre 2019

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MARIA JESÚS MARCOTE ZARAGOZA, Profesora titular del departamento de Bioquímica y Biología Molecular de la Universidad de Valencia,

CERTIFICAN:

Que la presente memoria titulada:

"TRANSPORT OF *ARABIDOPSIS* GPI-ANCHORED PROTEINS ALONG THE EARLY SECRETORY PATHWAY: ROLE OF GPI-ANCHOR REMODELLING AND P24 PROTEINS"

ha sido realizada por el Graduado en Farmacia César Bernat Silvestre bajo nuestra dirección, y que, habiendo revisado el trabajo, autorizamos su presentación para que sea calificada como tesis doctoral y obtener así el TITULO DE DOCTOR CON MENCIÓN INTERNACIONAL.

Y para que conste a los efectos oportunos, se expide la presente certificación en Burjassot, a 19 de septiembre de 2019.

Fdo: Fernando Aniento Company Fdo: María Jesús Marcote Zaragoza

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“Dadme un punto de apoyo y moveré el mundo”

Arquímedes de Siracusa

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ABBREVIATIONS

ABCR: *Arabidopsis* biological research center
AGD: ADP ribosylation factor GTPase activating protein domain
AGP: Arabinogalactan protein
ALP: Alkaline phosphatase
APS: Ammonium persulfate
Arabidopsis thaliana: Arabidopsis
Arf: ADP ribosylation factor
At: *Arabidopsis thaliana*
BAK: Brassinosteroid insensitive associated receptor kinase
Bmp: Blocked magnetic particles
BFA: Brefeldin A
BSA: Bovine serum albumin
CC: Coiled-coil domain
CCVs: Clathrin coated vesicles
cDBA: Complementary DNA
CESA: Cellulose synthase
CERK: Receptor kinase for chitin signalling
CFP: Cyan fluorescent protein
CGN: *cis*-Golgi network
CLSM: Confocal laser scanning microscopy
Col-0: Columbia-0
COPI: Coat protein I
COPII: Coat protein II
CPS: Classical or conventional protein secretion
CREST hydrolase superfamily: Alkaline ceramidase, PAQR receptor, Per1, SID-1 and TMEM8
CW: Cell wall
Dm: *Drosophila melanogaster*
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
DRMs: Detergent-resistant membranes
DTT: Dithiothreitol
ECL: Enhanced chemiluminescence method
EDTA: Ethylenediaminetetraacetic acid
EE: Early endosome
EFR: Elongation factor Tu receptor
EMP: Endomembrane protein
ER: Endoplasmic reticulum
ERAD: Endoplasmic reticulum degradation system
ERAS: Endoplasmic reticulum arrival sites
ERES: Endoplasmic reticulum export sites
ERD2: Endoplasmic reticulum-retention defective 2 receptor (K/HDEL receptor)
ERGIC: Endoplasmic reticulum-Golgi intermediate compartment
ERIS: Endoplasmic reticulum import sites
EtNP: Ethanolamine phosphate
FER: FERONIA protein
FLA: Fasciclin-like arabinogalactan protein
FLS: Flagelling sensing
GA: Golgi apparatus
Gal: Galactose
GalNAc: N-acetyl galactosamine
GAP: GTPase activating protein
GEF: GTP/GDP-exchange factor
GFP: Green fluorescent protein
GlcN: Glucosamine
GOLD: Golgi dynamics domain
GPI: Glycosylphosphatidylinositol
GPI-AP: Glycosylphosphatidylinositol anchored protein
GPI-TA: Glycosylphosphatidylinositol transamidase
H0: Null hypothesis
H1: Alternative hypothesis
HB: Homogenization buffer
HRP: Horseradish peroxidase
Hs: *Homo sapiens*

ABBREVIATIONS

IgG: Immunoglobuline G
IPs: Inhibitor proteases
IPC: Inositolphosphoceramide
LB: Luria-Bertani
LE: Late endosome
LEA: Late embryogenesis abundant
LTPL: Lipid transfer protein-like
LYM: Lysin-motif domain
Man: Mannose
MBOAT: Membrane bound O-acyl transferase
MeOH: Methanol
Mm: *Mus musculus*
MS: Murashige and Skoog
MVBs: Multivesicular bodies
NASC: Nottingham *Arabidopsis* Stock Centre
Nicotiana benthamiana: Nicotiana
Os: *Oryza sativa*
P: Phosphate
PAGs: Plastocyanin-like arabinogalactans
PAMPs: Pathogen associated molecular patterns
PAR: Protease-activated receptor
PCR: Polymerase chain reaction
PD: Plasmodesmata
PEG: Polyethylene glycol
PI: Phosphoinositol
PI-PLC: Phosphatidylinositol phospholipase C
PI-PLD: Phosphatidylinositol phospholipase D
PLA2: Phospholipase A2
PM: Plasma membrane
PMSF: Phenylmethylsulfonyl fluoride
PNS: Post nuclear supernatant
PSI: Plant-specific insert
PSV: Protein storage vacuole
PVC: Prevacuolar compartment
RabGDI: Rab GDP-displacement inhibitor
RALF: Rapid alkalization factor
RFP: Red fluorescent protein
RLKs: Receptor like kinases
RNA: Ribonucleic acid
RT: Room temperature
RT-PCR: Reverse transcription polymerase chain reaction
RT-sqPCR: Reverse transcription semi quantitative polymerase chain reaction
SAR: Salicylic acid-mediated response
SB: Sample buffer
Sc: *Saccharomyces cerevisiae*
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE: Statistical error
SF: Soluble fraction
SNARE: N-ethylmaleimide-sensitive factor attachment protein receptor
SP: Signal peptide
SS: Signal sequence
TAIR: Arabidopsis Information Resource
TEMED: N, N, N', N'-tetramethilethilendiamine
TGN: *trans*-Golgi network
TM: Total membranes
TMD: Transmembrane domain
TMED: Transmembrane emp24 domain
t-SNARE: Target membrane N-ethylmaleimide-sensitive factor attachment protein receptor
TRAPPI: Transport protein particle I
TX-100: Triton X-100
UPR: Unfolded protein response
VSS: Vacuolar sorting sequence/signal
VSR: Vacuolar sorting receptor
v-SNARE: Vesicle N-ethylmaleimide-sensitive factor attachment protein receptor
v/v: Volume/Volume
YFP: Yellow fluorescent protein
w/v: Weight/Volume

INTRODUCTION

1 INTRACELLULAR MEMBRANE TRAFFIC

Eukaryotic cells have a complex endomembrane system composed by several membrane-bound compartments, which have a specific molecular composition and, therefore, are functionally different. In plants, the major endomembrane compartments are the endoplasmic reticulum (ER), the Golgi apparatus (GA), the *trans*-Golgi network (TGN), the prevacuolar compartment/multivesicular bodies (PVC/MVB) and the vacuoles.

1.1 MEMBRANE TRAFFICKING PATHWAYS

The different compartments which are part of the endomembrane network are connected through small membrane-enclosed transport vesicles to exchange proteins, polysaccharides and lipids (Figure 1). The membrane trafficking allows the delivery of several thousands of proteins to their site of action. Moreover, membrane trafficking is involved in multiple cellular functions such as cellular homeostasis, development, cell-to-cell communication and physiological responses to changes in the environment (Bassham et al., 2008; Park and Jürgens, 2012; Pfeffer, 2013). This membrane trafficking system consist of highly organized directional routes through the cell of which two are the main pathways, the secretory or biosynthetic pathway and the endocytic pathway (Figure 1) (Bassham et al., 2008; Chung and Zeng, 2017):

- **Secretory or biosynthetic pathway:** The secretory pathway starts when newly synthesized molecules are transported from the ER to different compartments or are secreted. In the conventional secretory pathway, proteins synthesized in the ER are transported to the plasma membrane

(PM) or the vacuole via the Golgi apparatus and the TGN (Drakakaki and Dandekar, 2013; Wang et al., 2018).

- **Endocytic pathway:** Endocytosis is a process by which plant cells capture molecules from the apoplast or internalize plasma membrane proteins or receptor-ligand complexes via vesicles generated at the plasma membrane (Marsh and McMahon, 1999). Endocytic vesicles are released from the plasma membrane and are transported into the cell to the TGN/EE (Early Endosomes), where the endocytic cargos are sorted to follow different destinations: They can be recycled back to the plasma membrane or be delivered to the lytic vacuoles for degradation through the PVC/MVB (Viotti et al., 2010; Paez Valencia et al., 2016).

- **"Retrograde" pathways:** The retrograde pathways recover material back from later steps in either pathway for a wide variety of reasons (Pagny et al., 2000; Brandizzi et al., 2003; Kleine and Leister, 2016). These retrograde pathways can serve to get back molecules which should remain in one compartment and have entered into vesicles in a nonspecific manner or for recycling the molecules involved in the vesicular transport formation.

- **Others:** It has also been shown the existence of different pathways which allow protein transport from the endomembrane system to peroxisomes and plastids (Nanjo et al., 2006; Titorenko and Mullen, 2006). It has also been suggested that secretion may be redirected to the cell plate during cell division (Richter et al., 2009).

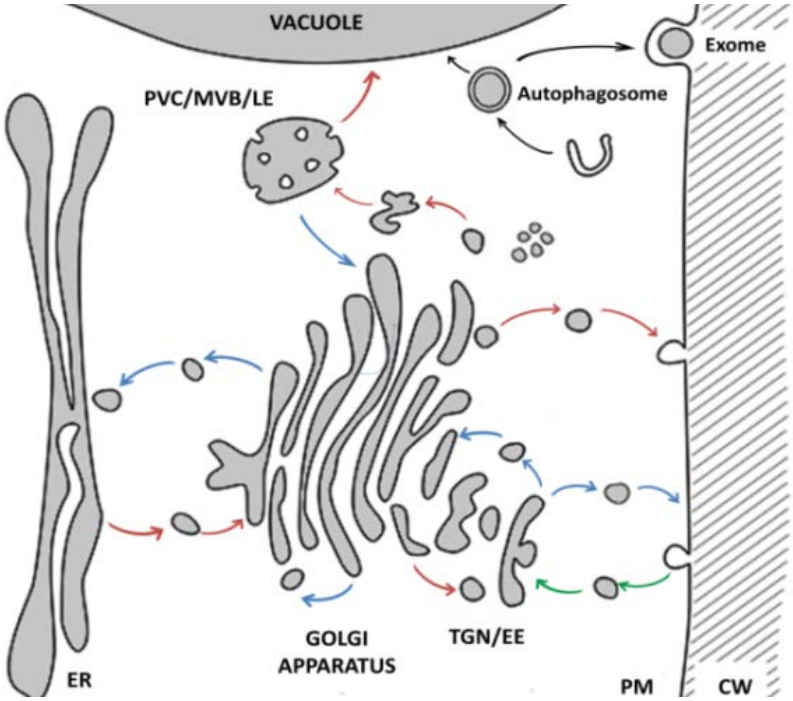

Figure 1. The endomembrane system of a plant cell (adapted from Vukašinović and Žárský, 2016). The compartments and organelles are all communicated with one another and the outside of the cell by vesicles. In the secretory pathway (red arrows), proteins are transported from the ER to the vacuole or to the PM. In the endocytic pathway (green arrows), molecules are endocytosed in endocytic vesicles formed at the PM and delivered to TGN/EE and then (via MVB/LE) to the vacuole. Some endocytosed molecules can be recycled from the TGN and returned to the cell surface, as some molecules are retrieved from MVB/LE and TGN to the Golgi apparatus or from the Golgi apparatus to the ER (blue arrows). CW, cell wall; ER, endoplasmic reticulum; PVC/MVB/LE, prevacuolar compartment/multivesicular body/late endosome; PM, plasma membrane; TGN/EE, *trans*-Golgi network/early endosome.

1.2 VESICLE TRAFFICKING

Nowadays it is widely accepted that molecular transport through the compartments of the membrane trafficking system occurs via small coated vesicles. These vesicles bud off from a donor compartment and fuse with a target compartment, carrying cargo molecules, including membrane and

soluble proteins, from one compartment to the next (Rothman and Wieland, 1996; Nickel et al., 1997; Paul and Frigerio, 2007; Klann et al., 2012).

1.2.1 Steps of the vesicular transport

The vesicular traffic between the different compartments of the plant endomembrane system occurs via similar mechanisms as in mammals and yeast. Each step of vesicle trafficking can be divided in three steps:

1.2.1.1 Budding

Transport vesicles bud from the donor compartment by the action of several ordered systems of coat proteins which are recruited and regulated by the activity of a specific GTPase (coat-GTPase) (Stenmark, 2009; Yorimitsu et al., 2014; Suda et al., 2018). This protein is recruited into the donor membrane through a GTP/GDP-exchange factor (GEF), which exchanges the GDP to GTP and activates the coat-GTPase. Once the coat-GTPase is activated, this is attached onto the donor membrane and then the other subunits needed to form the vesicle are also recruited. The subunits of the coat complex polymerize deforming the membrane surface to form the nascent vesicle. While the vesicle is forming, cargo molecules are captured into the vesicle together with those molecules that are necessary for transport and fusion with the target compartment, including soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), which carry out the membrane fusion between the vesicle and the target compartment (Chen and Scheller, 2001; Hong and Lev, 2014). Finally, a GTPase activating protein (GAP) triggers the hydrolysis of GTP to GDP of the coat-GTPase for uncoating the vesicle (Ross and Wilkie, 2000; Lamber et al., 2019).

Plant cells contain four major types of vesicles: COP ("Coat Protein") I and COPII coated vesicles, which are implicated in the transport in the early secretory pathway (Brandizzi and Barlowe, 2013); retromer coated vesicles, which are involved in post-Golgi trafficking, mostly recycling vacuolar sorting receptors from the PVC to the TGN (Niemes et al., 2010); and clathrin coated vesicles (CCV) which have been proposed to be involved in the late secretory pathway and in endocytosis (Ferreira and Boucrot, 2018).

1.2.1.2 Transport

Newly formed vesicles generated in the donor compartment have to be transported to the target compartment. This transport usually occurs along a cytoskeletal element via a motor-mediated process in which kinesin and dynein motors and other docking factors could be involved for transport of vesicles (Hafner and Rieger, 2016; Verdeny-Vilanova et al., 2017).

1.2.1.3 Fusion

Once the vesicles reach the target compartment, the membranes fuse and the cargo molecules are delivered into the target compartment. This requires specific mechanisms for recognition between the membrane of the vesicle and the target membrane. The identification is mediated by different family proteins, here summarized:

a) Rab family of small GTPases

Small GTPases of the Rab family are involved in vesicle formation, tethering, motility and docking, and also preceding vesicle fusion, recruiting proteins onto membranes for vesicle formation. Therefore, Rab GTPases contribute to membrane identity and the rightness of vesicle targeting

(Pfeffer, 2001; Woollard and Moore, 2008). Through genomic analysis, Rab GTPases are classified into eight types (RabA-RabH) which are conserved among the vast majority of eukaryotes (Pfeffer, 2017).

As introduced before, Rab GTPases are regulated by switching between GDP-bound and GTP-bound forms. The GDP-form is the inactive one, and is usually localized in the cytosol associated with a RabGDI (GDP-displacement inhibitor) (Wu et al., 1996), which masks the two prenyl-groups which are attached post-translationally to the C-terminus (Pereira-Leal et al., 2001). They are recruited onto the membrane by their interaction with RabGDI-displacement factors, which allow Rab attachment to the membrane using the prenyl-groups (Pylypenko et al., 2018). Then, specific GEFs convert the protein to the GTP-bound form which can recruit the subunits and effectors for protein assembly. Once the vesicle is formed, GAPs stimulate the intrinsic GTPase activity of the Rab GTPase, which finally produce the disassembly of the coated-vesicle (Woollard and Moore, 2008).

b) Tethering factors

Tethering factors are single long rod-like proteins or protein complexes which mediate the first specific contact between the vesicle and the target membrane. They connect newly assembled vesicles with the target membrane through their interaction with Rab GTPases, SNAREs and coat subunits, to ensure the appropriate docking and fusion. There are two classes of tethers that have been defined and characterized in eukaryotes: Elongated coiled-coil tethers and multisubunit tethering complexes (Sztul and Lupashin, 2009; Bröcker et al., 2010; Vukašinović and Žárský, 2016).

c) Soluble *N*-ethylmaleimide-sensitive fusion protein Attachment protein Receptors (SNAREs)

The SNARE family of proteins have a critical role in membrane fusion. SNAREs on a vesicle interact with the related SNAREs on the target membrane, forming a stable SNARE complex which provides energy for the membranes to fuse (Bombardier and Munson, 2015). They can be classified into two types depending on their localization: v-SNARE, as SNARE on the vesicle; and t-SNARE, as SNARE on the target membrane. On one hand, v-SNAREs on the vesicle interact with three t-SNAREs on the target membrane forming a parallel coiled-coil complex which produces membrane fusion (Kim and Brandizzi, 2012; Bombardier and Munson, 2015). On the other hand, t-SNAREs on the target membrane are bundled into three SNARE-helices which serves as binding site for the v-SNARE helix. Mutual twisting of the SNARE helices pulls the membrane into close proximity and drives fusion of the bilayers (Bassham et al., 2008). This post-fusion assembled SNARE complex is very stable and needs to be mechanically disassembled (Bombardier and Munson, 2015).

1.2.2 Principles of vesicular trafficking

The compartments which belong to the membrane trafficking system must maintain their unique composition of membrane and soluble proteins despite the constant exchange of vesicles. To this end, there are two key principles in the vesicular transport:

1.2.2.1 Molecular sorting

Vesicular transport requires that each transport vesicle includes the appropriate molecules, including cargo molecules and the components which are required for the traffic of the vesicle, and exclude the ones which should remain in the donor compartment. There are three different ways to achieve the accurate selection of the desired molecules into the transport vesicles:

- Sorting the cargo molecules into transport vesicles depends on the interaction between coat proteins involved in vesicle budding and the molecules that must be recruited into the vesicle. This interaction occurs through the recognition of particular motifs called sorting signals, localized in the cytoplasmic domain of the cargo molecules.
- Resident proteins can be retained through their interaction with other components of the donor compartment, avoiding their inclusion in the newly formed vesicle.
- Proteins that should remain at the donor compartment can be included into vesicles by mistake or randomly and be transported to the target compartment. From this compartment, these proteins can be recovered through “recovery or rescue pathways”.

1.2.2.2 Vesicle targeting

Transport vesicles must be transported towards the correct target compartment and recognize it to fuse correctly. To this end, vesicles contain Rab GTPases and SNARE proteins in their surface which identify them and target membranes display complementary receptors that recognize these proteins on their surface. The most important vesicle targeting markers for

vesicles are Rab GTPases and SNAREs (Pelham, 2001; Pfeffer, 2001; Sztul and Lupashin, 2009). Besides, these proteins have restricted and specific subcellular localization (Uemura and Ueda, 2014).

2 THE SECRETORY PATHWAY

The secretory pathway is a complex system of membrane-bound compartments which are specialized in the synthesis, transport, modification and secretion of a wide range of proteins, lipids and complex carbohydrates. It is the main exit route for secretory cargo proteins. Besides, the secretory pathway must response to specific cellular functional demands, which are continuously changing and, therefore, this implies a highly dynamic trafficking of molecules along the cell. Consequently, this system is of vital importance during the cell life (Kim and Brandizzi, 2016a; Wang et al., 2018).

The secretory pathway comprises first, the transport of newly synthesized proteins from the ER to the Golgi apparatus (early secretory pathway). Then, cargo molecules travel through the Golgi apparatus, from the *cis*- to *trans*- cisternae, to reach the TGN, where they are sorted into vesicles and delivered to the PM (late secretory pathway) or to the vacuole through the PVC/MVB (Figure 2).

There is an equilibrium between anterograde and retrograde transport. Retrograde transport is essential for the homeostasis of the cell because it is continually recycling proteins and lipids. Furthermore, disruption of retrograde transport inhibits anterograde transport (Hanton et al., 2005).

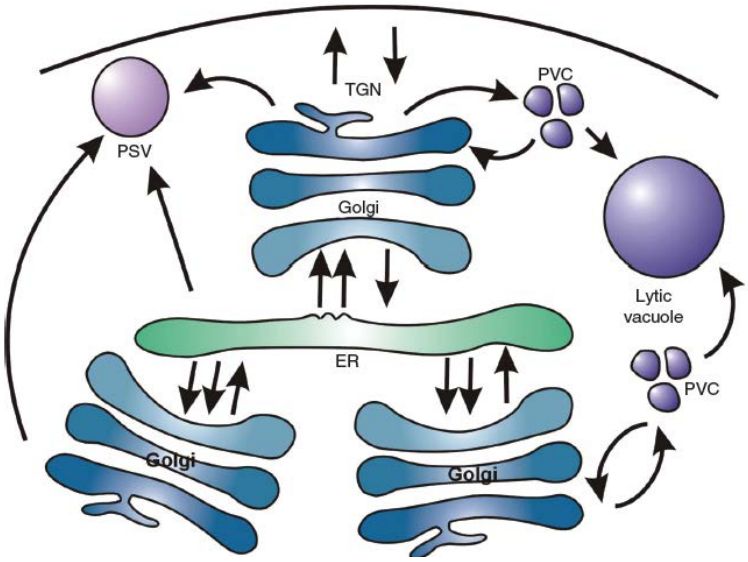

Figure 2. Overview of the secretory pathway in plants (Hanton et al., 2005). Schematic representation of organelles and their connecting protein transport routes in the plant-secretory pathway. ER, Endoplasmic reticulum; TGN, *trans*-Golgi network; PVC, prevacuolar compartment; PSV, protein storage vacuole.

In conventional protein secretion (CPS) pathway, secretory proteins are transported to the extracellular region via the ER-Golgi apparatus and the subsequent endomembrane system (Chung and Zeng, 2017). In this pathway, secreted proteins have in common three characteristics: They are post-translational modified through the secretory pathway (Walsh et al., 2005), they possess an N-terminal leader sequence (von Heijne, 1990; Petersen et al., 2011; Whitley and Mingarro, 2014) and their transport is blocked by the application of brefeldin A (BFA) (Fujiwara et al., 1988). For vacuolar proteins, they should contain an additional sequence-specific sorting determinant (Neuhaus and Rogers, 1998; Vitale and Hinz, 2005).

As it has been introduced before, protein transport through the secretory pathway is controlled by transport vesicles which carry cargo

molecules from one organelle to the next. This transport can occur in the forward direction (anterograde transport), from the ER to the PM, or in a reverse direction (retrograde transport) (Figure 2 and 3) (Hanton et al., 2005).

- **Anterograde transport:** Membrane traffic pathway in which cargo movement is from the ER towards the cell surface through the Golgi apparatus or to the lytic vacuole via PVC and protein storage vacuole (Brandizzi and Barlowe, 2013).

- **Retrograde transport:** Membrane traffic pathway in which a linear assembly of compartments facilitates cargo movement towards the ER (Brandizzi and Barlowe, 2013).

2.1 EARLY SECRETORY PATHWAY

The early secretory pathway includes the transport of newly synthesized proteins from the ER to the Golgi apparatus. Protein transport between ER and Golgi apparatus is bidirectional: Anterograde transport (from the ER to the Golgi) is mediated by COPII vesicles, while retrograde transport (from the Golgi to ER) is mediated by COPI vesicles (Figure 3) (Hawes et al., 2008; Marti et al., 2010; Gao et al., 2014; Brandizzi, 2018).

2.1.1 The ER network

The ER is the factory where proteins which have to be secreted are synthesized, and therefore, the early secretory pathway starts. These proteins contain an N-terminal signal peptide (SP) in their sequence and are translocated into the ER (von Heijne, 1990; Whitley and Mingarro, 2014; Bassham et al., 2008; Ding et al., 2012; Robinson et al., 2016). The ER network

morphology extends from the nuclear envelope to the cortical regions of the cell pushed by the large central vacuole. It is characterized by a network of interconnected membrane tubules and sheets, which are in continuous remodelling (Kriechbaumer et al., 2018; Pain et al., 2019). As the ER is extended along the cell, it is in close contact with every organelle in the cell, performing various roles with each contact (Sparkes et al., 2009, 2011; Stefano et al., 2014). Besides, ER network changes depending the necessities of the cell and environmental conditions (Brandizzi et al., 2014; Pain et al., 2019).

The ER is responsible for synthesis, folding and quality control, as well as the first steps of glycosylation of a large number of proteins (Sparkes et al., 2009, 2011; Stefano et al., 2014). In the ER, proteins acquire their proper conformation carried out by chaperones and undergo initial post-translational modifications (Boston et al., 1996; Miernyk, 1999; Webster and Thomas, 2012). *N*-glycosylation is initiated at the ER when a preformed oligosaccharide is transferred *en bloc* to the asparagine residue in an Asn-X-Ser/Thr motif (where X represents any amino acid residue except proline) of the nascent polypeptide chain during translation (Webster and Thomas, 2012). Then, chaperones recognize that are correctly *N*-glycosylated to continue for further modifications (Boston et al., 1996; Miernyk, 1999).

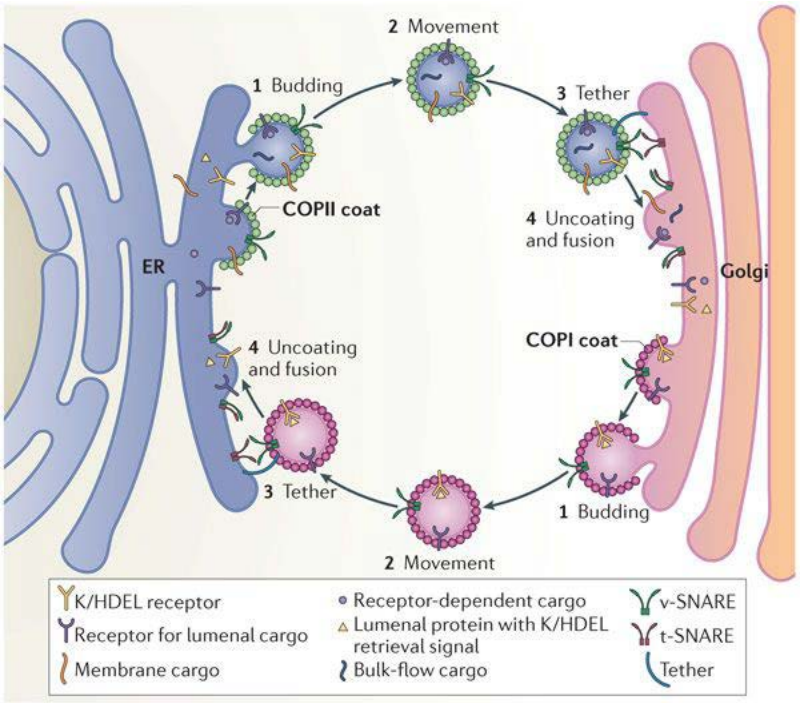

Figure 3. Bidirectional transport between the ER and the Golgi is mediated by COPI and COPII vesicles (Brandizzi and Barlowe, 2013). Bidirectional transport of secretory cargo between the ER and the Golgi requires budding, movement, tethering, as well as uncoating and fusion of COPII and COPI vesicles with their respective compartments. These include bulk-flow, membrane cargo and receptor-dependent luminal cargo. COPII vesicles facilitate selective and bulk-flow cargo export towards the Golgi apparatus. One important function of COPI vesicles is to facilitate retrieval of escaped luminal proteins containing K/HDEL retrieval signals that are recognized by the K/HDEL receptor ERD2 as well as other machinery required for optimal anterograde transport. Vesicle fusion is mediated by vesicular SNARE proteins (v-SNARE) a target-SNAREs (t-SNAREs) upon anchoring of the vesicles to their target compartment via tethers.

Proteins which are not properly folded have to repeat the folding cycle, but when folding fails, those proteins that are not correctly folded are targeted to degradation by the ER-degradation system (ERAD) (Meusser et al., 2005; Nishikawa et al., 2005). If the load of unfolded proteins increases, a signalling cascade, known as the unfolded protein response (UPR), is

activated in order to decrease the amount of unfolded proteins (Vitale and Boston, 2008; Wan and Jiang, 2016).

2.1.2 ER export sites and ER-to-Golgi transport

Once proteins and membrane cargos are correctly folded, they are transported from the ER to the Golgi through COPII-coated vesicles (Figure 3) which are known to be recruited and bud from specialized subdomains of the ER, called ER export sites (ERES) (Marti et al., 2010; Brandizzi and Barlowe, 2013; Brandizzi, 2018). Therefore, ERES are defined as the sites through which secretory proteins leave the ER and form vesicles, and they are also characterized by the absence of ribosomes and the local accumulation of COPII proteins, so that this is the reason by COPII components are used as ERES markers (Langhans et al., 2012; Brandizzi and Barlowe, 2013; Takagi et al., 2013; Luo et al., 2015; Zhao et al., 2016; Brandizzi, 2018). Moreover, ERES are rich in SEC16 proteins, which have been proposed to be required for ERES organization and function as scaffold and regulator of COPII coat assembly at ERES (Budnik and Stephens, 2009; Hughes et al., 2009; Miller and Barlowe, 2010; Brandizzi and Barlowe, 2013; Brandizzi, 2018).

In mammals the distance between the ER and the Golgi apparatus is relatively large, which may justify the existence of an intermediate compartment between both, known as the ER-Golgi Intermediate Compartment (ERGIC), which is involved in concentration of anterograde biosynthetic cargo, and COPI-dependent retrograde cargo (Figure 4) (Hauri and Schweizer, 1992; Appenzeller-Herzog and Hauri, 2006; Brandizzi and Barlowe, 2013). However, this compartment is not present in plants, and

COPII-coated carriers are supposed to fuse with other COPII-coated carriers to form the first *cis*-cisterna or to attach to the rims of the *cis*-cisterna (Yang et al., 2005; Kang and Staehelin, 2008). This fact involves that the organization of the ER-Golgi interface varies greatly among species. Furthermore, in plants the Golgi apparatus is present as multiple stacks which are distributed throughout the cytosol and it shows rapid motility (up to 4 µm/sec) and this requires the activity of actomyosin motors and close association with tubular ER strands. As the distance between both organelles is very small, ERGIC is no needed in plants (Figure 4) (Boevink et al., 1998; Hawes et al., 2008; Akkerman et al., 2011).

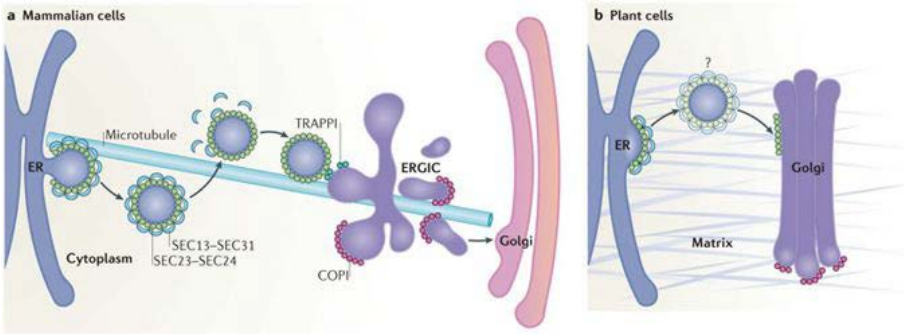

Figure 4. The ER-Golgi interface and ERES have a distinct organization in mammals and plants (Brandizzi and Barlowe, 2013). A) In mammalian cells, ER exit sites (ERES) are oriented towards a juxtaposed endoplasmic reticulum (ER)-Golgi intermediate compartment (ERGIC). COPII-coated vesicles originate within cup-shaped ER subdomains, which are associated with the plus end of microtubules. Upon fission of vesicles from the ERES, the SEC13-SEC31 cage is depolymerized, but the SEC23-SEC24 coat is partially retained. Vesicles reach the ERGIC in a microtubule-independent manner where they are tethered through the interaction between SEC23 and the TRAPPI (transport protein particle I) tethering complex. COPI mediates forward protein transport from the ERGIC towards the Golgi as well as recycling back to the ER membrane. B) In plant cells, ERES and Golgi are closely associated, possibly through a matrix (indicated in grey) that holds the ER and the Golgi together. The existence of COPII vesicles in plants is still debated. Unlike mammalian cells, plant cell ER-Golgi transport does not rely on the microtubule cytoskeleton.

Four possible mechanisms for protein transport from the ER to mobile Golgi stacks have been suggested (Figure 5) (Ito et al., 2014; Brandizzi, 2018):

- **"Kiss-and-run" or "stop-and-go" model:** ERES are relatively stable and the Golgi stacks are travelling from one ERES to another. It has been proposed in this model that an active ERES produces a "stop signal" which is recognized by the Golgi stack to stop it. Once this recognition is done, ER-to-Golgi transport takes place during this temporal association. Finally, after the transport is finished, the Golgi stack resumes its movement (Nebenführ et al., 1999; Kang and Staehelin, 2008).

- **Secretory unit model:** ERES are able to move over the ER and are continuously associated with Golgi stacks, so that both organelles move together. The transport between ER-to-Golgi stacks can occur while both are moving (daSilva et al., 2004; Stefano et al., 2006; Hanton et al., 2007; Takagi et al., 2013).

- **Hybrid model:** This is similar to the secretory unit model because some ERES are continuously associated with Golgi stacks. However, other ERES, which are not associated with the Golgi stacks, are smaller and move independently. They become active and stable when they associate with a Golgi stack (Ito et al., 2012).

- **Modified secretory unit model:** During the Golgi movement, COPII and COPI vesicles are continuously formed. The anterograde COPII transport is not restricted to temporary stationary Golgi stacks, nevertheless, fusion of COPI vesicles to the ER occur when the Golgi stacks temporally stop at the ER import sites (ERIS). However, both types of vesicles are

accumulated between the ER and the Golgi and move together with the Golgi stacks (Langhans et al., 2012; Lerich et al., 2012).

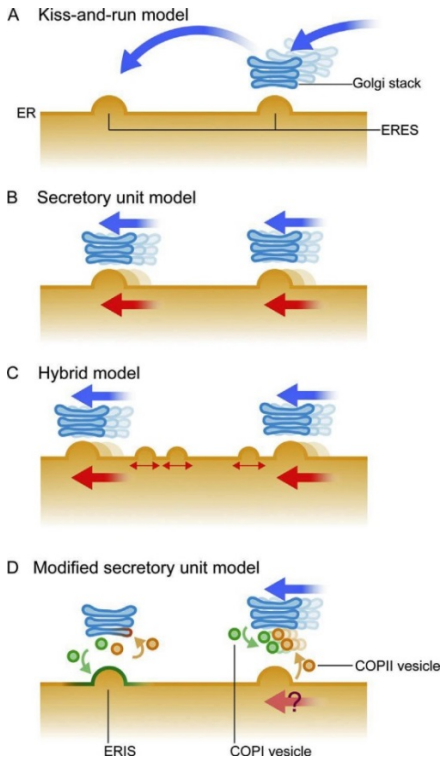

Figure 5. Models of ERES-Golgi organization in plant cells (Ito et al., 2014). Image of the four models of protein transport between ERES and the Golgi stacks (see text for details).

However, the major dispute is over the type of COPII-carriers involved in the ER-Golgi transport because vesicle-like structures have been rarely detected in electron microscopy analyzes, so the possibility of membrane connections between the ER and the Golgi through interconnecting tubules has been debated. Several different models have been proposed to explain the ER export in plants (Figure 6) (Robinson et al., 2015; Chung et al., 2016):

- **Vesicular-mediated ER export:** COPII-coated vesicles bud from the ERES to the *cis*-Golgi carrying cargoes.

- **Tubule-mediated ER export:** COPII carriers mediates the formation of tubules that connect the ER and the Golgi apparatus which allows the direct transport of cargo proteins.

- **Tubule and vesicle-mediated ER export:** Vesicles and tubules can mediate the ER export under specific conditions.

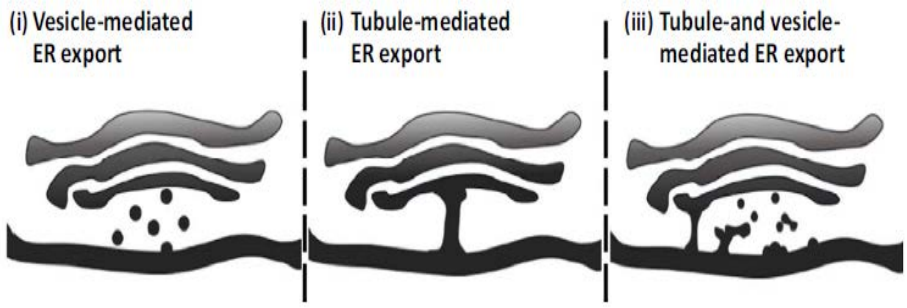

Figure 6. Models of ER export in plants (Chung et al., 2016). Picture of the three proposed models to explain the ER export in plants (see text for further details).

There are arguments based on experimental data which support each model, being difficult to arrive to any consensus about the modality of membrane traffic between both organelles in plants (Robinson et al., 2015).

It has been proposed that the close association between the ER and the Golgi stacks is due to the fact that both organelles are connected through a tethering matrix which might facilitate ER-Golgi COPII transport. Besides, cytoskeletal elements may not be needed to facilitate the bidirectional transport at the ER-Golgi interface so that in highly vacuolated cells, the Golgi

apparatus was found to be mainly associated with the ER due to the presence of a central vacuole that occupies most of the cell volume. So it is possible that different plant cell types could use diverse ERES-Golgi spatial organization to achieve ER export (Kang and Staehelin, 2008; Marti et al., 2010; Brandizzi and Barlowe, 2013; Robinson et al., 2015; Brandizzi, 2018).

2.1.3 COPII vesicles

2.1.3.1 Formation of COPII vesicles

The COPII coat is composed by five proteins: SAR1, SEC23, SEC24, SEC13 and SEC31, which are the minimal machinery required to form vesicles *in vitro* (De Craene et al., 2014; Chung et al., 2016). The COPII coat assembly is initiated by the activation of the small Rab GTPase SAR1 mediated by SEC12, which is a GEF localized at the ER which can recruit SAR1 changing GDP for GTP (Lee and Miller, 2007). When SAR1 is activated in its GTP-bound state, it exposes its N-terminal amphipathic helix to the ER membrane and induces membrane curvature (Lee et al., 2005). Next, two COPII coat heterocomplexes (SEC23/SEC24 and SEC13/31) are sequentially recruited to form two layers (Lee and Miller, 2007). To this end, activated SAR1 first interacts with SEC23 to recruit the SEC23/SEC24 complex for forming the "prebudding complex" SAR1/SEC23/SEC24 (Lee and Miller, 2007), where SEC23 is the GAP of SAR1 and SEC24 contains multiple independent domains to interact with specific cargo signals. At this point, the heterotetramic complex SEC13/SEC31 is recruited through the SEC23-SEC31 interaction, which also contributes for SEC23 GAP activity (Lee and Miller, 2007). Finally, GTP hydrolysis by SAR1 is maximal once the coat is completely assembled (Figure 7) (Bielli et al., 2005; Hanna et al., 2016).

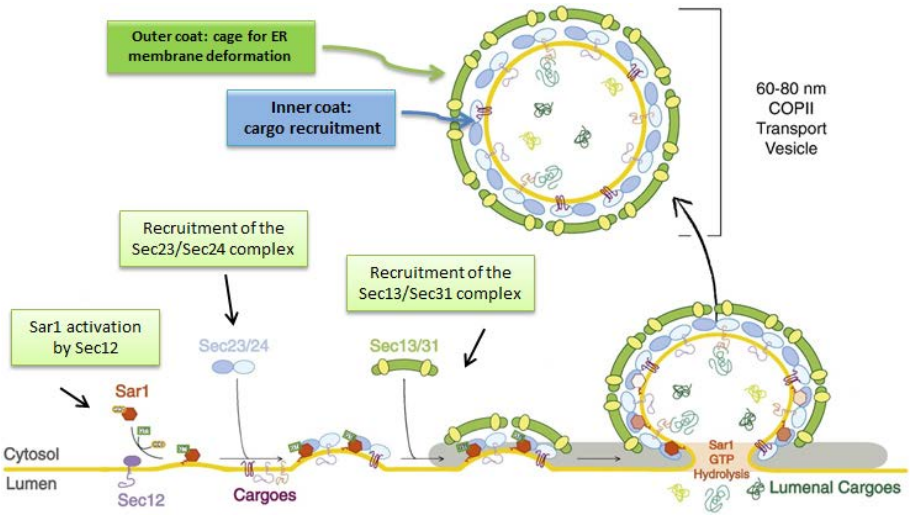

Figure 7. COPII assembly at the ER membrane (Adapted from D'Arcangelo et al., 2013). The formation of the COPII vesicle at the ER is initiated by the recruitment of SAR1 to the membrane in the GTP-state, enhanced by SEC12 GEF activity. SAR1-GTP recruits SEC23/24 heterodimer through interaction with SEC23. At the ER, SEC24 recruits cargo into prebudding complexes. Then, SEC13/SEC31 complex is recruited to the inner coat layer through interactions with SEC23. The assembly of SEC13/31 into the coat drives membrane curvature, facilitating membrane deformation. Nascent COPII vesicles typically measure within a 60 to 100 nm range. SEC16 facilitates COPII coat recruitment at the ER, likely through scaffolding for COPII components and contributing to ERES structure.

Thus, the fully assembled coat is composed by: The "inner" membrane layer of SAR1/SEC23/SEC24 (Bi et al., 2002), which contributes to cargo-binding function, and the "outer" membrane layer composed of SEC13/SEC31 (Stagg et al., 2006; Bhattacharya et al., 2012), which provides a scaffold that produces curvature to the nascent vesicle at the ER (Figure 7).

COPII components are highly conserved along eukaryotes (Chung et al., 2016). In *Arabidopsis thaliana* (*Arabidopsis*), nineteen paralogs have been identified, including five SAR1, seven SEC23, three SEC24, two SEC13 and two SEC31 isoforms in its genome (Table 1). Recent studies have suggested the

functional diversity of various COPII subunit paralogs in plants (De Craene et al., 2014), thus certain COPII subunit paralogs have shown organ specific or developmental stage-specific expression and several isoforms (SAR1 ad SEC31) seem to be regulated under stress (Chung et al., 2016; Bao and Howell, 2017; Gimeno-Ferrer et al., 2017).

SAR1	SEC23	SEC24	SEC13	SEC31
AT1G09180 (A)	AT4G01810 (A)	AT3G07100 (A)	AT2G30050 (A)	AT1G18830 (A)
AT1G56330 (B)	AT1G05520 (B)	AT3G44340 (B)	AT3G01340 (B)	AT3G63460 (B)
AT4G02080 (C)	AT2G21630 (C)	AT4G32640 (C)		
AT3G62560 (D)	AT2G27460 (D)			
AT1G02620 (E)	AT3G23660 (E)			
	AT4G14160 (F)			
	AT5G43670 (G)			

Table 1. Different isoforms for COPII coat subunits in *Arabidopsis thaliana*.

2.1.3.2 COPII interaction motifs

Cargo proteins can enter into vesicles in a nonspecific manner known as “bulk flow”; however, some cargos are dramatically enriched in some specific vesicles (Barlowe and Miller, 2013; Manzano-Lopez et al., 2015). Selective enrichment in COPII vesicles occurs via specific sorting signals. SEC24 acts as the cargo binding adaptor, with multiple binding sites for interaction with distinct sorting signals (Wendeler et al., 2007; Pagant et al., 2015).

Transmembrane cargo proteins also contain in their cytosolic tail different sorting signals for interacting with SEC24. However, soluble proteins cannot interact directly with SEC24, so they have to use cargo

receptors, such as the p24 proteins (for further details see section 3 of Introduction), which contain lumen-exposed domains that can bind cargo proteins to facilitate their incorporation into COPII vesicles (Barlowe, 2003).

Different sorting signals (ER export signals) have been identified which bind COPII subunits: A diacidic motif (DXD, DxE, EXE), a diaromatic motif (ϕϕ,FF) and a dibasic motif ([RX](X)[RX]) (Mossessova et al., 2003; Mancias and Goldberg, 2008). In plants, different studies have shown their differences between *Arabidopsis* SEC24 isoforms could reflect specific cargo recognition (Faso et al., 2011; Nishimura et al., 2013; Qu et al., 2014).

2.1.4 Golgi-to-ER transport

The transport from the Golgi apparatus back to the ER is mediated by COPI vesicles, which bud from the edges of *cis*-Golgi cisternae (Pimpl et al., 2000; Ahn et al., 2015). It has been proposed that ER export in plants is a continuous process, but COPI vesicles are tethered by the Dsl1 complex (Latijnhouwers et al., 2005; Ravikumar et al., 2017), while Golgi stacks are moving and can fuse with the ER during temporary pauses of the Golgi stack carried out by a domain of the ER containing COPI-tethering factors (Lerich et al., 2012; Ravikumar et al., 2017).

Fusion between COPI vesicles and the ER membrane seems to occur at specialized ER subdomains named ERAS (ER arrival sites) or ERIS, which are close and related to the ERES. The spatial separation of exit and entry sites may facilitate the simultaneous arrival and departure of proteins (Lerich et al., 2012; Schröter et al., 2016).

2.1.5 The Golgi apparatus and intra-Golgi trafficking

The plant Golgi apparatus is the main sorting station and the responsible for delivering cargo proteins to multiple destinations, so it plays a central role in the secretory pathway (Staehelin and Moore, 1995; Mollenhauer and Morre, 1966). It functions as a polysaccharide factory (*N*- and *O*-glycosylations) and its activity is essential for the formation of the cell wall during cytokinesis and growth (Driouich et al., 1993; Dupree and Sherrier, 1998; Ito et al., 2014). As described before, it consists of numerous individual stacks which are usually dispersed through the cytoplasm and can travel along actin filaments (Boevink et al., 1998). Each Golgi stack has a polarized structure, from the *cis* side which receives cargo proteins from the ER and the *trans* side which send the cargo proteins to their final destination (Zhang and Staehelin, 1992). Along the Golgi stacks there are many glycosylation enzymes which are arranged as a gradient, so that cargo proteins can be modified sequentially by these enzymes, while they traverse the Golgi stacks (Driouich et al., 1993; Staehelin and Moore, 1995). Two major models have been proposed to explain how cargo molecules are transported through the stack of cisternae (Figure 8) (Ito et al., 2014):

- **Vesicular transport model:** The Golgi cisternae are stable compartments and each Golgi cisterna is viewed as a different suborganelle with a characteristic set of resident proteins. Cargo proteins are transported from one cisterna to the other by anterograde or retrograde COPI vesicles, while resident proteins are excluded from them and remain in the cisterna. Finally, cargo proteins arrive to the TGN.

- **Cisternal progression model:** Each *cis*-cisterna is newly formed by the homotypic fusion of COPII vesicles and functions as a transient compartment which progresses from the *cis* to the *trans* side to become the TGN. Thus, the nature of the cisternae gradually changes while they progress along the secretory pathway. Retrograde COPI vesicles recycle Golgi proteins from later to earlier cisternae.

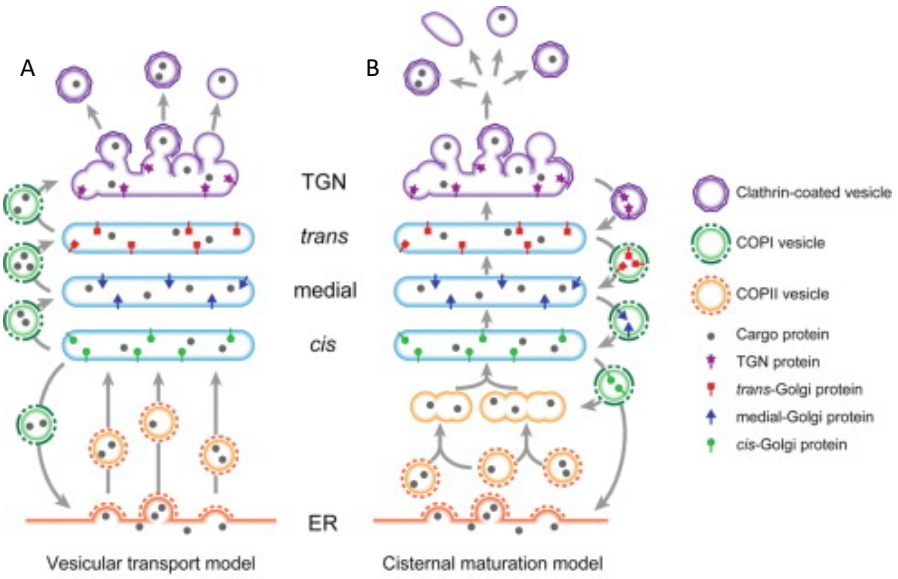

Figure 8. Two major models for intra-Golgi trafficking (Ito et al., 2014). A) The vesicular transport model. B) The cisterna maturation model.

2.1.6 COPI vesicles

2.1.6.1 Formation of COPI vesicles

The retrograde transport from the Golgi apparatus to the ER is mediated by COPI vesicles. The COPI coat complex consists of a heptameric complex (α, β, β', γ, δ, ε and ζ) named coatomer, which is composed of two

subcomplexes: The β/δ/γ/ζ F-complex, which is proposed to be the inner layer core that binds cargo; and the α/β'/ε B-complex, which has been proposed to function as the outer layer and confer curvature to the membrane (Figure 9) (Jackson, 2014). Nevertheless, recent structural studies suggest that the seven subunits are highly connected one to each other, which means that the COPI coat may not form a distinct two-layered structure like the COPII coat (Donovan and Bretscher, 2015).

COPI assembly to the Golgi membrane is initiated by the activation of the ARF1 GTPase (Takeuchi et al., 2002). This activation involves GDP/GTP exchange mediated by a family of GEFs proteins, and produces a conformational change to ARF1, which exposure a myristoylated amphipathic N-terminal helix that is inserted into the Golgi membrane (Jackson and Casanova, 2000; Lundmark et al., 2008). Then, ARF1 recruits *en bloc* the coatomer complex through its interaction with the F-complex (Figure 9) (Jackson, 2014).

In order to fuse with the target membrane, COPI vesicles need to be uncoated, which is mediated by the GAP activity of the ARF GAP1 protein, which is accelerated by binding with the coatomer, but this effect is inhibited by p24 proteins which are proposed to act as cargo receptors. *Arabidopsis* genome encodes fifteen ARF GAP domain (AGD) proteins (Ito et al., 2014). Once the hydrolysis of GTP is produced by a GTPase activating protein, ARF1-GDP is displaced from the membrane for future rounds of recruitment.

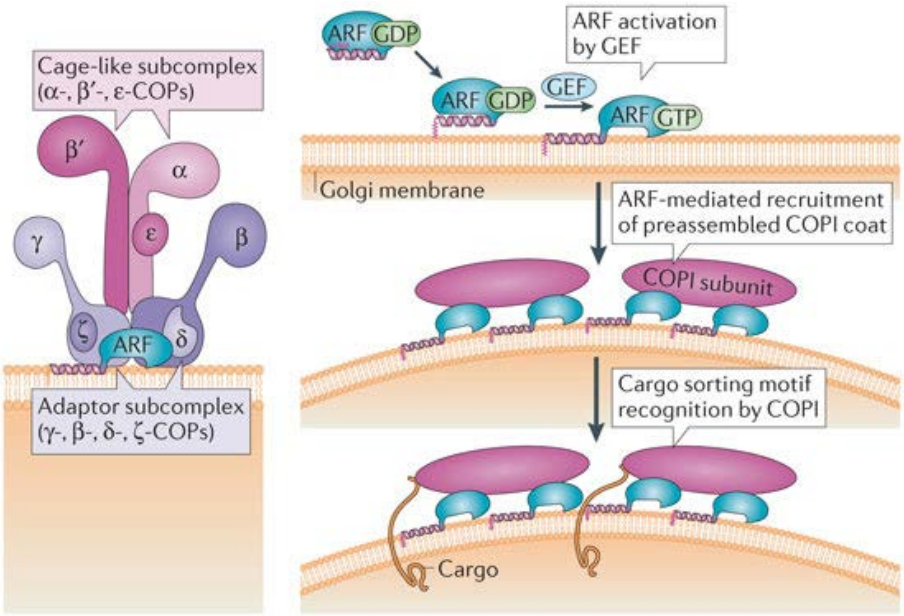

Figure 9. COPI assembly at the Golgi membranes (Brandizzi and Barlowe, 2013). Once ARF is activated by ADP-ribosylation factor (ARF) guanine nucleotide exchange factor (GEFs), myristoylated membrane-anchored ARF GTPases recruit COPI coatomer *en bloc* to Golgi membranes. The coatomer subunits α-COP, β'-COP, γ-COP and δ-COP can recognize sorting motifs on the cytosolic domain of membrane cargo and mediate cargo incorporation into nascent COPI vesicles.

COPI machinery is well conserved among eukaryotes, including plants. While yeast contains only one isoform for each COPI subunit, in mammals, γ and ζ subunits have two isoforms, which allows the formation of four alternative coatomer complexes. In clear contrast, all coatomer subunits in *Arabidopsis* have more than one isoform (except δ- and γ-COPI) (Table 2) (Gao et al., 2014). Interestingly, morphological studies in *Arabidopsis* have identified two structurally distinct types of COPI vesicles, named COPIa and COPIb, located between the ER and the Golgi apparatus or around Golgi cisternae, respectively (Donohoe et al., 2007; Hwang and Robinson, 2009;

Gao et al., 2014). These different subpopulations of COPI vesicles might be formed by different coatomer isoforms.

Organism	α-COP	β-COP	β'-COP	δ-COP	ε-COP	γ-COP	ζ-COP
Sc	Ret1	Sec26	Sec27	Ret2	Sec28	Sec21	Ret3
Hs	α	β	β'	δ	ε	γ1 γ2	ζ1 ζ2
At	At1g62020 At2g21390	At4g31480 At4g31490	At1g52360 At3g15980 At1g79990	At5g05010	At2g34840 At1g30630	At4g34450	At1g60970 At3g09800 At1g08520

Table 2. Plant COPI subunits in different organisms (Gao et al., 2014). Abbreviations: Sc, *Saccharomyces cerevisiae*; Hs, *Homo sapiens*; At, *Arabidopsis thaliana*

2.1.6.2 COPI interaction motifs

The α, β', γ and δ subunits of COPI coatomer have been proposed to recognize sorting motifs on the cytosolic domain of membrane cargo and mediate its incorporation into COPI vesicles (Brandizzi and Barlowe, 2013; Gao et al., 2014). In particular, δ-COPI has been suggested to be involved in efficient retrieval of HDEL proteins (Jackson, 2014; Arakel et al., 2016).

The best characterized ER retention signals are a canonical dilysine motif (KKXX or KXKXX) for membrane proteins (Letourneur et al., 1994; Benghezal et al., 2000; Contreras et al., 2004a) and the K/HDEL motif for soluble proteins. Soluble proteins containing the K/HDEL motif can bind to the retrieval receptor ERD2 at the Golgi apparatus for sorting into COPI vesicles for retrograde Golgi-ER transport (Xu et al., 2012; Gao et al., 2014). However, in *Arabidopsis* p24 proteins have been shown to directly interact with COPI proteins and ARF1, which depends on both dilysine and diphenylalanine motifs (Contreras et al., 2004b).

For Golgi retention signals the mechanisms usually are more diverse, thus KXD/E motif has been recently identified as a retention signal for integral membrane proteins for at least *Arabidopsis* endomembrane protein (EMP) family proteins (Gao et al., 2012, 2014). Due to the different population of COPI vesicles identified (Donohoe et al., 2007), it has been proposed that different sorting signals may be recognized by different coatomer isoforms for their sorting into COPIa vesicle for ER retrieval or COPIb vesicles to Golgi retention (Gao et al., 2014).

2.2 LATE SECRETORY PATHWAY

2.2.1 The *trans*-Golgi network (TGN)

Once the secretory proteins arrive to the *trans*-Golgi network through the Golgi stacks, this tubulo-vesicular compartment is transiently associated with an individual Golgi stack and can move away independently (Jurgens and Geldner, 2002; Viotti et al., 2010). This compartment is the major sorting station for exocytic cargo proteins except for some storage proteins which are sorted from the ER or *cis*-Golgi. Moreover, it also function as an early endosome (EE) in plants (Scheuring et al., 2011), being the first compartment which receives endocytosed proteins, so that the TGN is at the convergence of the secretory and endocytic pathways. However, there is no structural evidence for the existence of different TGN subdomains despite of distinct sorting functions performed by the TGN (Park and Jürgens, 2012). Finally, this compartment may be formed from the *trans*-Golgi cisterna, possibly by maturation (Matsuura-Tokita et al., 2006; Park and Jürgens, 2012).

2.2.2 Transport to the plasma membrane

The PM is the final destination for components of the extracellular matrix and secreted proteins which are delivered to the apoplast where they can become incorporated or diffuse away (Bassham et al., 2008; Kim and Brandizzi, 2016b). Traffic of soluble proteins from the ER to the PM through the Golgi apparatus requires a N-terminal signal peptide that allows their translocation across the ER membrane during protein synthesis (von Heijne, 1990; Park and Jürgens, 2012).

Membrane proteins at PM can function as membrane transporters, ion channels, signalling complexes or ligand receptors, or even as physical contact points for both the intracellular cytoskeleton network or for the extracellular matrix (Bassham et al., 2008; Kim and Brandizzi, 2016b). Membrane proteins with a single transmembrane domain seem to reach their destination along the secretory pathway according to the length of their hydrophobic region: Proteins with a shorter membrane span are held back in the Golgi stack whereas those with a longer membrane span are trafficked to the PM (Brandizzi et al., 2002).

Although secretory traffic to the PM appears to be the default pathway in interphase, in dividing cells, TGN-derived membrane vesicles that deliver the necessary material for building the PM and the cell wall are targeted to plane of cell division. Thus, the default pathway changes from the PM to the cell plate during cell division (Richter et al., 2009; Park and Jürgens, 2012).

2.2.3 Vacuolar cargo trafficking pathway

In the conventional secretory pathway, newly synthesized soluble cargo proteins destined to the vacuoles contain a N- or a C-terminal vacuolar sorting sequence/signal (VSS) or/and plant-specific insert (PSI) motif, which binds to vacuolar sorting receptors (VSRs) to be delivered to the PVC or MVBs and the vacuole (Neuhaus and Rogers, 1998; Vitale and Hinz, 2005; Pereira et al., 2013; Shimada et al., 2018).

Sorting of vacuolar cargo has been long thought to occur at the TGN, but other studies suggest that this could occur at the ER (Niemes et al., 2010). Once in the MVBs/PVC, they are released from the VSRs due to its acidic pH. Whereas soluble cargo proteins are delivered to the vacuole via membrane fusion of the MVBs/PVC with the vacuole, the VSRs are recycled back to the TGN through the retromer (daSilva et al., 2006; Oliviusson et al., 2006; Park and Jürgens, 2012).

Nevertheless, some published data claim the existence of multiple transport routes for tonoplasts proteins (Bottanelli et al., 2011; Viotti, 2014; Robinson and Pimpl, 2014; Sansebastiano et al., 2017). Some membrane proteins have been also suggested for an independent Golgi-to-vacuole transport, in which is involved a direct transport from the ER to the vacuole (Viotti, 2014; Uemura and Ueda, 2014). These different pathways show the existence of different types of vacuoles which are diverse in size, shape, content and function (Frigerio et al., 2008). Vacuoles have important functions in plant cells, such as space filling to increase the volume of the cell, defence responses, storage proteins and sugars and lytic function in lytic

vacuoles (Uemura and Ueda, 2014; Martinoia et al., 2018; Shimada et al., 2018).

3 P24 PROTEINS

The p24 family proteins have been known for a long time; nevertheless, they have recently emerged as essential regulators of protein trafficking along the secretory pathway, playing important specific functions in the composition, structure and function of different organelles in the pathway, especially the ER and the Golgi apparatus (Pastor-Cantizano et al., 2016). In addition, they seem to modulate the transport of specific cargos, including: Glycosylphosphatidylinositol-anchored proteins (GPI-APs), G-protein-coupled receptors and K/HDEL ligands bound to the K/HDEL receptor ERD2. As a result, they have been shown to play specific roles in signalling, development, insulin secretion and pathogenesis of Alzheimer's disease (Pastor-Cantizano et al., 2016).

p24 family proteins were first described by Wada et al. (1991), and subsequently they have been characterized in mammals and yeast and more recently, in plants. However, many functions of these proteins still remain elusive. It has been reported that p24 family proteins cycle between the ER and the Golgi apparatus (Aniento et al., 2006), the so-called early secretory pathway, as described before. p24 proteins have been shown to be efficiently packaged within COPI- and COPII-coated vesicles (Brandizzi and Barlowe, 2013), and based in these properties and in their topology, they have been proposed to function as cargo receptors and play a role in quality

control during transport along the secretory pathway (Pastor-Cantizano et al., 2016).

3.1 PHYLOGENY AND NOMENCLATURE

p24 proteins constitute a family of type-I transmembrane proteins of ~24 kDa. Based on sequence homology, p24 proteins can be classified in four subfamilies, named α, β, γ and δ (Figure 10) (Dominguez et al., 1998). The number of p24 proteins of each subfamily varies among species. The four p24 subfamilies are present in all animals and yeast but plants appear to have members of only p24δ and p24β subfamilies (Figure 10).

Phylogenetic analysis of vertebrate p24 proteins showed that the p24δ and p24α subfamilies have a common origin, as also seems to be the case for p24γ and p24β subfamilies (Figure 10) (Strating et al., 2009). In most vertebrates, the p24α and p24γ have several members, nevertheless the p24β and p24δ subfamilies contain only one single member. Within each of the four vertebrate p24 subfamilies, the degree of amino acid sequence identity is high, being the p24γ subfamily the one that shows largest variability (Theiler et al., 2014).

In plants, the p24δ subfamily seems to have greatly expanded independently from the animals/fungi. *Arabidopsis* has nine members of the delta subfamily which can be divided into two different subclasses, the δ-1 subclass, with four members (comprising p24 δ3-6) and the δ-2 subclass, with five (comprising p24 δ7-11), and two members of the beta subfamily (p24β2-β3) (Figure 11) (Chen et al., 2012; Montesinos et al., 2012).

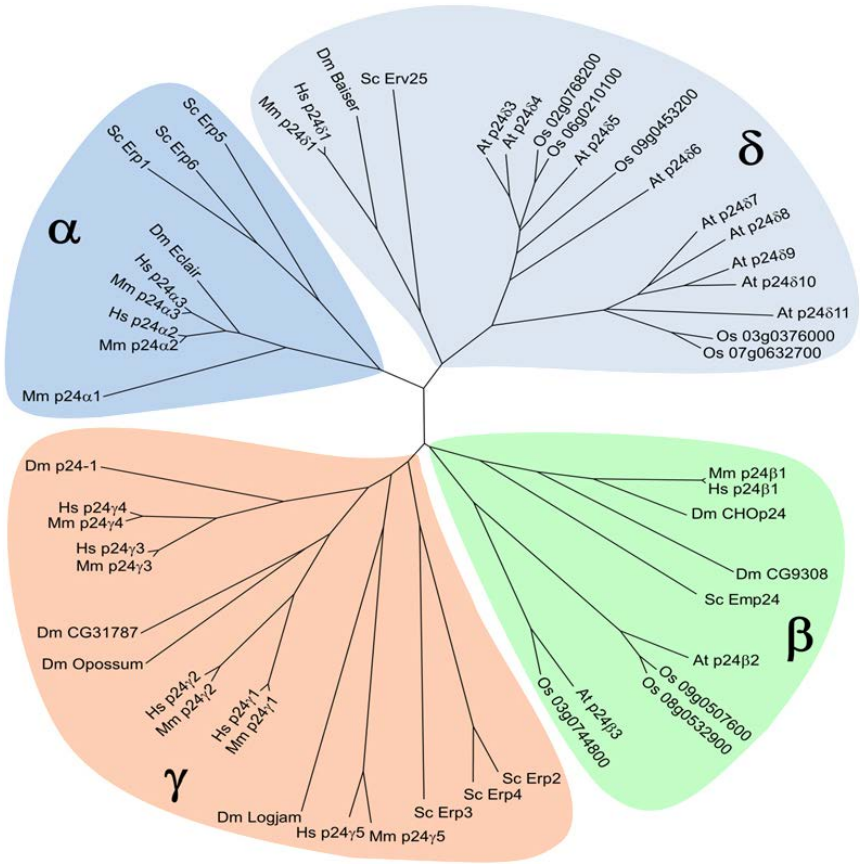

Figure 10. Phylogeny tree of the p24 protein family (Pastor-Cantizano et al., 2016). The four p24 subfamilies (α, β, δ, γ) are highlighted by different colours. Abbreviations: Hs *Homo sapiens*; Mm *Mus musculus*; Sc *Saccharomyces cerevisiae*; Dm *Drosophila melanogaster*, At *Arabidopsis thaliana*; Os *Oryza sativa*.

In the past, several nomenclatures for vertebrate p24 have been used. These proteins were originally named according to their apparent molecular weight in SDS-PAGE (22-24 kDa), for example: p23, p24, emp24, erv25… As new members have been discovered, they were named by analogy, p26, p27, p28, although their deduced molecular weight were also 22-24 kDa (Pastor-Cantizano et al., 2016). Most p24 proteins in the

vertebrate protein database are also named as TMED (transmembrane emp24 domain containing protein, emp24 was the first yeast p24 protein identified) (Schimmöller et al., 1995).

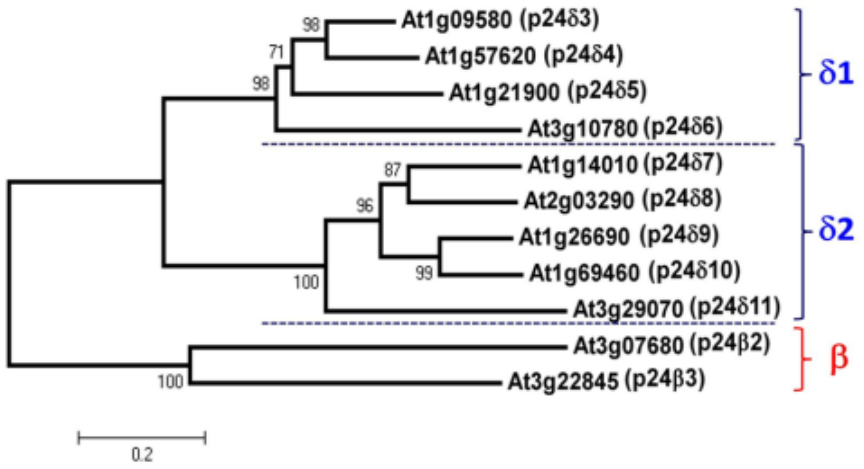

Figure 11. Phylogeny tree of the p24 protein family in *Arabidopsis thaliana* (Montesinos et al., 2012). *Arabidopsis* contains two different subfamilies (β and δ) of p24 proteins. The δ subfamily contains nine members and can be divided into two subclasses (δ-1 and δ-2), while the β subfamily only contains two members.

Dominguez et al. (1998) proposed a more systematic nomenclature involving the use of greek letters (δ, γ, β and α) to identify the subfamilies, followed by a number, starting with the first discovered member. In this thesis, we use this last nomenclature in order to facilitate their identification within the family and to avoid confusion (Figure 11).

3.2 TISSUE-SPECIFIC AND REGULATED EXPRESSION

Most p24 proteins are ubiquitously expressed (Rötter et al., 2002; Boltz et al., 2007; Strating et al., 2009; Pastor-Cantizano et al., 2018), although a few of them are expressed in a tissue-specific manner and show regulated expression (Denzel et al., 2000; Rötter et al., 2002; Hosaka et al., 2007; Vetrivel et al., 2008; Xie et al., 2014; Pastor-Cantizano et al., 2018). These specific expression patterns could reflect specialized functions, as the transport of a specific set of cargo proteins.

In *Drosophila*, the expression of some p24 genes is mediated by CRABA/CREB3-like family of bZIP transcription factors which have been suggested to be the direct and major regulators of the secretory capacity (Fox et al., 2010). Moreover, in mouse, several p24 proteins have been found to be highly expressed in secretory cell types for instance exocrine, endocrine and neural cells (Hosaka et al., 2007; Zhang and Volchuk, 2010; Wang et al., 2012).

In *Arabidopsis*, the expression pattern of the eleven p24 genes were studied by Pastor-Cantizano et al. (2017b). This study showed that p24δ4, p24δ5, p24δ7, p24δ9, p24δ10, p24β2 and p24β3 genes are widely expressed and have high or medium levels of expression in the different organs of the plant; however, p24δ3 expression was observed in all organs except flowers. In contrast, expression of p24δ6 and p24δ8 was mostly detected in flowers and siliques and p24δ11 expression was only observed in flowers, with no expression found in the other organs examined (Zimmermann et al., 2004; Pastor-Cantizano et al., 2018).

Therefore, most of p24 genes are widely expressed in *Arabidopsis*, indicating that these genes may play a housekeeping function. Nevertheless, the restricted expression patterns of three genes of the p24δ subfamily (p24δ6, p24δ8 and p24δ11), may reflect specialized functions for the p24 proteins coded by these genes in floral tissues (Zimmermann et al., 2004; Pastor-Cantizano et al., 2018).

Finally, some p24 proteins have been shown to be up-regulated in response to ER stress as p24β2 in *Arabidopsis* (Kamauchi et al., 2005) and p24γ4 in mammals (Hartley et al., 2010). This suggests that they may be induced as a part of the unfolded protein response to ER stress (Schuiki and Volchuk, 2012).

3.3 PROTEIN DOMAIN STRUCTURE

All p24 proteins have a similar structure: A large N-terminal region, which includes the Golgi dynamics (GOLD) domain, a linker region called the coiled-coil domain, a single transmembrane domain and a short (13-20 residues) cytosolic C-terminal tail (Figure 12).

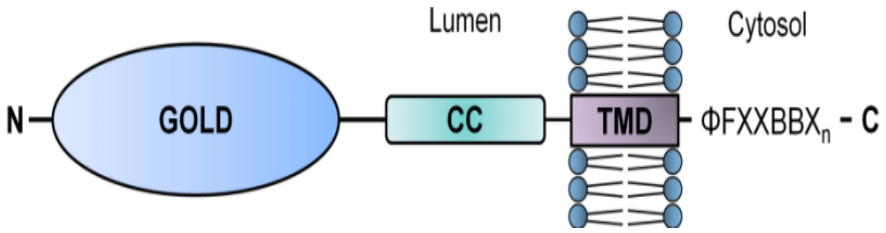

Figure 12. Domain organization of p24 proteins (Pastor-Cantizano et al., 2016). The structure includes two luminal domains, the Golgi dynamics (GOLD) domain and a coiled-coil (CC) domain, separated by a single transmembrane (TM) domain and a short cytosolic C-terminal tail.

- **The GOLD domain:** It is a β-strand-rich globular domain which is also present in several proteins related to Golgi dynamics and secretion hence its name (Golgi Dynamics). The GOLD domain is predicted to be involved in protein-protein interactions and cargo recognition (Gaskell et al., 1995; Anantharaman and Aravind, 2002; Carney and Bowen, 2004; Nagae et al., 2016). It also contains two cysteine residues, which may form a disulfide bridge and a site for *N*-glycosylation which is involved in recognition of the K/HDEL receptor ERD2 (Pastor-Cantizano et al., 2017).

- **The coiled-coil (CC) domain:** The CC domain has been shown to be involved in the interaction between p24 proteins and their oligomerization (Ciufo and Boyd, 2000; Emery et al., 2000; Montesinos et al., 2012; Liaunardy-Jopeace and Gay, 2014). Recently, it has been reported that the CC domain is important for recognition and transport of GPI-anchored proteins (Theiler et al., 2014).

- **The transmembrane (TM) domain:** The TM domain contains polar residues and a conserved glutamine at the membrane-cytosol interface. It seems to interact specifically with one single sphingomyelin species which is present in several G-protein-coupled receptors as potential cargos for p24 proteins (Contreras et al., 2012; Björkholm et al., 2014), and it has also been proposed to modulate the equilibrium between monomeric and oligomeric states of p24 proteins (Contreras et al., 2012).

- **The cytosolic tail:** The cytosolic tail of p24 proteins contains signals for binding COPI and COPII subunits, allowing p24 proteins to be efficiently packaged into COPI and COPII vesicles for their bidirectional transport between the ER and the Golgi apparatus (Aniento et al., 2006). All of

them have a conserved phenylalanine residue, which is often part of a diaromatic motif. Besides, many of them also contain a dibasic (dylisine) motif, in the form of a ϕFXXBB(X)n, where ϕ is a bulky hydrophobic residue, B is a basic residue, X can be any amino acid, and n≥2.

On one hand, the dilysine motif is directly involved in COPI binding (Béthune et al., 2006; Popoff et al., 2011; Jackson et al., 2012; Ma and Goldberg, 2013; Gao et al., 2014), and is only present in members of the α and δ subfamily. On the other hand, the diaromatic motif is present in all p24 proteins and has been shown to bind COPII subunits (Dominguez et al., 1998; Belden and Barlowe, 2001b; Barlowe, 2003; Contreras et al., 2004b; Aniento et al., 2006). In addition, this motif seems to be also involved in binding COPI subunits (Fiedler et al., 1996; Sohn, 1996; Dominguez et al., 1998; Goldberg, 2000; Belden and Barlowe, 2001b; Contreras et al., 2004b; Aniento et al., 2006).

Members of the same subfamily show significant differences among different organisms (Pastor-Cantizano et al., 2016). In *Arabidopsis*, all members of the p24δ subfamily have the dilysine motif in the -3,-4 position (relative to the C-terminus) and a diaromatic motif in the -7,-8 position, like members of the p24α subfamily in animals. The two members in *Arabidopsis* of the p24β subfamily only have the diaromatic motif in -7,-8 position but not the dilysine motif in -3, -4.

3.4 POST-TRANSLATIONAL MODIFICATIONS AND DEGRADATION

In animals, several reports have shown that some p24 proteins can be glycosylated, such as p24α2 (Dominguez et al., 1998; Füllekrug et al.,

1999; Lavoie et al., 1999), p24γ3 (Füllekrug et al., 1999) and p24δ1 (Osiecka-Iwan et al., 2014). This also happens in the yeast p24 protein Rtr6 (p24δ isoform), and glycosylation has been proposed to modulate cargo specificity (Hirata et al., 2013). Also in animals, Liu et al. (2015) has postulated that p24α2 could be phosphorylated.

Arabidopsis members of the p24δ-1 subclass (p24δ3-δ6) contain a putative *N*-glycosylation motif in its GOLD domain, which is not present in members of the p24δ-2 subclass (p24δ7-δ11) (Pastor-Cantizano et al., 2017). Indeed, p24δ5 (but not p24δ9) was found to be glycosylated in its GOLD domain. Furthermore, *N*-glycosylation of p24δ5 was shown to be important for its coupled transport with p24β2 at the ER-Golgi interface, for its interaction with ERD2 receptor and for its retrograde transport and therefore, the retrieval of K/HDEL ligands from the Golgi apparatus to the ER (Pastor-Cantizano et al., 2017).

Little is known about the degradation of p24 proteins. Liu et al. (2008) reported that p24δ1 in animals has a short half-life and is degraded by the ubiquitin-proteasome pathway. In *Arabidopsis*, co-expression of p24β2 with p24δ5 increases enormously its stability possibly because p24δ5 holds p24β2 in the early secretory pathway and prevents its transport to the vacuole (Montesinos et al., 2012). In addition, it has been shown that degradation of p24 proteins of the β subfamily in plants is mediated by cysteine proteases upon their transport to the prevacuolar compartment and the vacuole (Montesinos et al., 2013; Pastor-Cantizano et al., 2017).

3.5 OLIGOMERIZATION

p24 proteins can interact with each other through their CC domains (Ciufo and Boyd, 2000; Emery et al., 2000; Jenne et al., 2002; Langhans et al., 2008; Montesinos et al., 2012; Liaunardy-Jopeace et al., 2014) to form different types of oligomeric complexes (including members of the different subfamilies), which are important not only for their trafficking and localization but also for their stability.

In yeast, it was suggested that p24 proteins form a heterotetrameric complex containing members of the four p24 subfamilies, called the yeast p24 complex (Marzioch et al., 1999a). This complex has been proposed to contain Emp24 (p24β), Erv25 (p24δ) and different combinations of p24α and p24γ isoforms (Hirata et al., 2013). Nevertheless, in gel-filtration experiments it was also found that p24 proteins can also form p24 dimers and even p24 monomers (Marzioch et al., 1999a).

In mammals, the same experiments were carried out to identify the composition of p24 complexes. Immunoprecipitation or pull-down experiments also showed that p24 proteins form heterotetramers including members of the four subfamilies (Füllekrug et al., 1999; Fujita et al., 2011). However, it was also found that p24 proteins can also exist as monomers and dimers of different composition depending on their subcellular localization (Jenne et al., 2002). More recently, it was discovered that the composition of these complexes of p24 proteins depends on the cargo specificity (Theiler et al., 2014).

In both organisms, yeast and animals, it has been demonstrated that interactions between the p24 proteins are required for their stability, since deletion or knock down of a single member of the p24 family produces a decrease in the protein levels of other p24 proteins suggesting the formation of hetero-oligomeric complexes. In yeast, strains deleted of one member of each subfamily showed reduced levels of members of the other subfamilies (Marzioch et al., 1999a). The same happened in mammals, the stability of the complex was compromised when one member was silenced. This was the case of a knock down of p24δ1 (Denzel et al., 2000; Vetrivel et al., 2007; Takida et al., 2008; Zhang and Volchuk, 2010; Fujita et al., 2011; Theiler et al., 2014), p24γ2 (Koegler et al., 2010) and p24β1 (Jerome-Majewska et al., 2010).

All this data is consistent with a model where p24 proteins do not exist in a stable complex all the time, it is more likely that they exist in a dynamic equilibrium between complexes and individual proteins and maybe these heterotetramers could be a dimer of dimers, as proposed by Ciufo and Boyd (2000) (Figure 13).

In plants, as they only have members of the beta and delta subfamilies, any complex should be made with members of these two subfamilies. Previous studies have shown the interdependence between p24 protein levels in *Arabidopsis* (Montesinos et al., 2012, 2013). In these studies, single KO-mutants of p24δ4 or p24δ5 (p24δ-1 subclass) had similar protein levels of p24δ5 or p24δ4, respectively, but reduced protein levels of p24δ9 (p24δ-2 subclass), p24β2 and p24β3. Moreover, knock down lines of the two members of p24β subfamily showed also reduced protein levels of p24δ5

(p24δ-1 subclass), p24δ9 (p24δ-2 subclass) and p24β subfamily protein respectively.

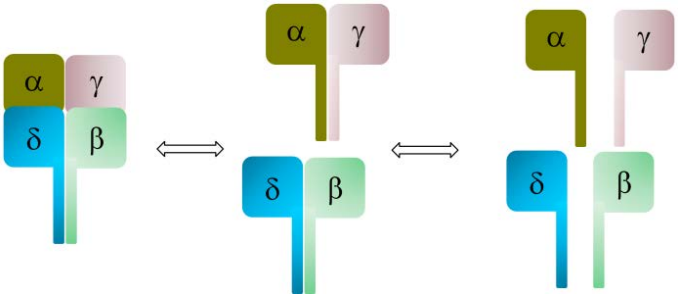

Figure 13. Oligomerization properties of p24 proteins (Pastor-Cantizano et al., 2016). Proposed model for a dynamic equilibrium between monomeric, dimeric and heterotetrameric forms of p24 family members.

Besides, a quadruple mutant of the four p24δ-1 subclass generated by Pastor-Cantizano et al. (2017b), showed undetectable protein levels of p24δ9 (p24δ-2 subclass) and a drastic reduction of the two members of p24β subfamily. However, the reduction in protein levels does not correlate with reduced mRNA levels of p24δ-2 subclass, p24β2 and p24β3, so this could be due to a decrease in protein stability. This is also consistent with *Arabidopsis* p24 proteins also form hetero-oligomeric complexes including p24 proteins from the p24δ-1 subclass, p24δ-2 subclass and the p24β subfamily (Pastor-Cantizano et al., 2018).

3.6 TRAFFICKING AND LOCALIZATION

p24 proteins have been shown to localize in the compartments of the early secretory pathway, which include the ER, the ERGIC (only mammals have this compartment), the *cis*-Golgi network (CGN) and the Golgi

apparatus (Stamnes et al., 1995; Belden and Barlowe, 1996; Blum et al., 1996; Sohn, 1996; Nickel et al., 1997; Rojo et al., 1997; Dominguez et al., 1998; Füllekrug et al., 1999; Gommel et al., 1999; Emery et al., 2000; Rojo et al., 2000).

They are also major constituents of both COPII-coated (Schimmöller et al., 1995; Belden and Barlowe, 1996) and COPI-coated vesicles (Stamnes et al., 1995; Sohn, 1996; Gommel et al., 1999), which facilitate their bidirectional transport between the ER and the Golgi apparatus. It has been also reported that p24 proteins can also be found in places different from the ER-Golgi interface, such as peroxisomes (Marelli et al., 2004), secretory granules (Hosaka et al., 2007) and even to the plasma membrane (Chen et al., 2006; Blum and Lepier, 2008; Langhans et al., 2008).

As described before, the CC domain was the domain of p24 proteins involved in the oligomerization of these proteins, therefore it has a strong influence in their trafficking. However, the minimal requirement for cycling p24 proteins between ER-Golgi interface has been described to be the transmembrane domain and the cytoplasmic tail with the dylisine motif (Emery et al., 2000; Blum and Lepier, 2008).

As mentioned before, in *Arabidopsis* the two members of the p24β subfamily are dependent on the protein levels of p24δ subfamily and their stability is depending on the presence of both subclasses of p24δ subfamily (Pastor-Cantizano et al., 2018), and protein levels of p24δ9 (p24δ-2 subclass) are also strongly dependent on p24δ-1 subclass proteins (Pastor-Cantizano et al., 2018). This interdependence between different members of the p24 family proteins indicates that these proteins may function together in

heteromeric complexes, as it was previously described (Figure 13) (Montesinos et al., 2013).

It has been proposed that these hetero-oligomeric complexes may contain members of the both subfamilies of p24 proteins, and also different members of the two subclasses of p24δ subfamily. However, the stoichiometry and composition of these complexes still remains elusive. It has been proposed that for "anterograde" transport (ER to Golgi) these complexes should include p24β2, which has been shown to facilitate transport of p24δ5 (p24δ-1 subclass) and p24δ9 (p24δ-2 subclass) from the ER to the Golgi apparatus. In contrast, for "retrograde" transport (Golgi to ER) these complexes should contain members of p24δ subfamily proteins, probably including members of both subclasses, which efficiently recruit the COPI coatomer to form COPI vesicles for their retrograde Golgi-ER transport (Montesinos et al., 2012, 2013). p24β3 may bind complexes containing p24β2/p24δ5 or p24β2/p24δ9 for its transport to the Golgi apparatus, and p24β3 may also be recycled to the ER in complexes containing both p24δ5 and p24δ9 proteins (Montesinos et al., 2012, 2013). This is consistent with the protein level of p24β3 been almost undetectable in the quadruple mutant of p24δ-1 subfamily, so it suggests that it needs p24δ proteins from both subclasses for its stability (Pastor-Cantizano et al., 2018).

3.7 FUNCTIONS OF P24 PROTEINS

p24 proteins can interact with COPII and COPI subunits through their cytosolic tail, and cycle between ER and the Golgi apparatus within COPII/COPI vesicles (Aniento et al., 2006). Due to these properties, numerous functions have been proposed for p24 proteins.

3.7.1 COPI and COPII vesicle formation

A large number of studies indicate that p24 proteins help in the formation of COPI vesicles from Golgi membranes (for reviews, see Popoff et al., 2011; Jackson, 2014; Pastor-Cantizano et al., 2016). Based on these reports, a model for the participation of p24 proteins in COPI vesicle formation has been proposed (Figure 14) (Pastor-Cantizano et al., 2016). Hetero-oligomers of p24 proteins (probably p24β/p24δ dimers) can directly interact with ARF1-GDP for the recruitment of ARF1 to the Golgi membranes via the cytosolic domain (Harter et al., 1996; Dominguez et al., 1998; Contreras et al., 2004a; Béthune et al., 2006). Once on the membrane, ARF1 is activated by GDP/GTP exchange which produces its dissociation from p24 proteins (Gommel et al., 2001). Then, ARF1-GTP carries out an interaction with the COPI coatomer which can also interact with p24 proteins (either through the γ-subunit of the F-complex or to the B-complex) (Hara-Kuge et al., 1994). At this stage, p24γ and p24α proteins can be also recruited forming a tetramer with p24β/p24δ dimers. Interaction with p24 proteins leads to coatomer polymerization and the formation of COPI vesicles. It has been reported that p24 proteins can also interact with the K/HDEL receptor ERD2, which can also interact with coatomer and has been proposed to be involved in a variety of interactions which contribute to COPI vesicle formation (Majoul et al., 2001; Montesinos et al., 2014; Pastor-Cantizano et al., 2017). Furthermore, p24 proteins can also control coat depolymerisation inhibiting GTP hydrolysis in ARF1 which is required for uncoating (Goldberg, 2000; Lanoix et al., 2001; Majoul et al., 2001). This may prevent immature uncoating and allow cargo selection.

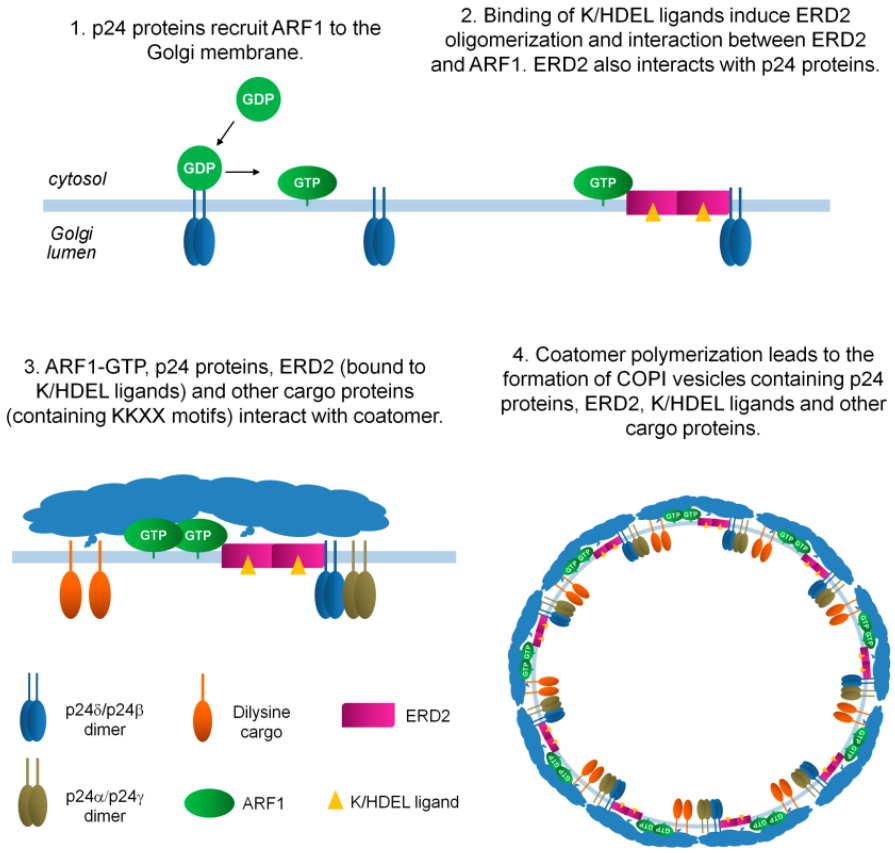

Figure 14. p24 proteins and COPI vesicle formation (Pastor-Cantizano et al., 2016). p24 proteins are involved in COPI vesicle formation, especially for recruitment of ARF1 to the Golgi membrane (1), the interaction with the K/HDEL receptor ERD2 (2) and the interaction with coatomer, which also binds to ARF1, ERD2 and other dilysine cargo (3). These interactions enhances coatomer polymerization and the formation of nascent COPI vesicles (4).

p24 proteins are also present in COPII vesicles and can interact with SEC23 and SEC24 subunits (Schimmöller et al., 1995; Belden and Barlowe, 1996; Dominguez et al., 1998; Miller et al., 2003; Contreras et al., 2004b). Although p24 are not essential for the formation of COPII vesicles, it has been proposed that p24 proteins influence this process through the alteration of

the physical properties of the ER membrane due to their abundance and asymmetric distribution (Čopič et al., 2012). It has been also suggested that p24 proteins could be involved in the formation of the ERES (Lavoie et al., 1999), helping the proteins folded at the ER to be packaged into COPII vesicles. Moreover, it has been proposed that asymmetrically localized proteins, like p24 proteins, are required for scaffolding function of the cargo adaptor Lst1p (a SEC24 homolog in yeast) to form larger COPII vesicles for special big cargoes (D'Arcangelo et al., 2015).

3.7.2 Maintenance of structure and organization of the early secretory pathway

Several reports have suggested that p24 proteins may play a role in the structure and organization of the compartments of the early secretory pathway. In particular, p24 proteins have been suggested to be involved in the formation of ERES (Lavoie et al., 1999), the structure of the ER and the ERGIC and the biogenesis and maintenance of the Golgi apparatus (Mitrovic et al., 2008; Koegler et al., 2010). Moreover, it has been proposed that p24 proteins may be important for recycling of components required for ER-Golgi transport or in ER function and maintenance.

In mammals, silencing of p24β1, p24α2 and p24γ2 led to Golgi fragmentation (Luo et al., 2007; Mitrovic et al., 2008; Koegler et al., 2010) and silencing of p24α2 also produced a reduction in the number of ERGIC clusters as well as the destabilization of the ERGIC (Mitrovic et al., 2008). Overexpression of p24β1 and p24δ1 led to Golgi fragmentation as well and the appearance of smaller Golgi fragments (Rojo et al., 2000; Blum et al., 1999; Gong et al., 2011). Nevertheless, inactivation of one allele of p24δ1

was shown to induce the dilation of the Golgi cisternae, which was more prominent at the rim (Denzel et al., 2000).

In *Arabidopsis*, single knock out mutants did not show any obvious ultrastructural alterations, perhaps due to a functional redundancy with other p24 family members (Montesinos et al., 2012). In contrast, the silencing of the four members of the p24δ-1 subfamily and the depletion of the other members of the p24 family caused a clear alteration in the Golgi, with dilated areas throughout the whole cisternae, which were also more prominent at the rim of the Golgi cisternae, and in some cases discontinuous cisternae (Pastor-Cantizano et al., 2018).

3.7.3 Cargo protein receptor

p24 proteins have been long proposed to function as cargo membrane receptors for protein transport in the early secretory pathway. Most putative p24 cargoes described until now are membrane proteins or lipid-linked proteins, in contrast to many classical cargo receptors (for a revision, see Pastor-Cantizano et al., 2016).

- **GPI-anchored proteins:** This is the cargo object of study in this thesis. Some studies in yeast have suggested that p24 proteins may be involved in the transport of GPI-APs (Schimmöller et al., 1995; Belden and Barlowe, 1996; Marzioch et al., 1999a; Muñiz et al., 2000). It has been proposed that the yeast p24 complex may function as an adaptor connecting remodelled GPI-APs with the COPII coat to facilitate their incorporation into COPII vesicles but do not participate in concentrating them into ERES (Castillon et al., 2009, 2011; Manzano-Lopez et al., 2015).

Besides, the p24 complex may be also involved in retrograde Golgi-to-ER transport of unremodelled GPI-APs (Castillon et al., 2011).

In mammals, p24 proteins have also been proposed to participate in efficient ER-to-Golgi transport of GPI-APs (Takida et al., 2008; Bonnon et al., 2010). Nevertheless, in comparison to yeast, p24 proteins appear to be required for concentration of remodelled GPI-APs at ERES and their packaging into COPII vesicles (Fujita et al., 2011). For the interaction between GPI-APs and p24 proteins, it was reported that it is needed the α-helical domain of p24 proteins (Theiler et al., 2014). This interaction has also been shown to be pH-dependent and takes place at the neutral pH of the ER but not at the mildly acidic pH of the Golgi apparatus (Fujita et al., 2011).

- **K/HDEL-receptor ERD2:** p24 proteins of the p24δ subfamily have been shown to be involved in retrograde transport of K/HDEL ligands through interaction between p24δ proteins and the K/HDEL receptor ERD2 in mammals and plants (Majoul et al., 2001; Montesinos et al., 2014; Pastor-Cantizano et al., 2017). In *Arabidopsis*, two p24δ members, p24δ5 and p24δ9, have been shown to interact with two different K/HDEL receptors, ERD2a and ERD2b, an interaction that requires the GOLD domain and its *N*-linked glycosylation of p24δ5 and it is also pH-dependent (Montesinos et al., 2014; Pastor-Cantizano et al., 2017). In particular, this interaction was optimal at acidic pH but very low at neutral pH, consistent with the interaction taking place at Golgi apparatus and the dissociation of p24 proteins from ERD2 at the ER lumen (Montesinos et al., 2014).

- **Other putative cargoes:** Some other putative cargo proteins have been proposed for p24 proteins, including Wnt glycoproteins, which are lipid-modified secreted signalling proteins involved in controlling animal development (Buechling et al., 2011; Port et al., 2011; Li et al., 2015b). Other putative cargoes proposed are: G-protein-coupled receptors including protease-activated receptors (PAR-1 and PAR-2), nucleotide P2Y and μ-opioid receptors (Luo et al., 2007, 2011); Toll-like receptors (Liaunardy-Jopeace and Gay, 2014); and GLL3, a putative myrosinase-associated protein in plants (Jancowski et al., 2014).

3.7.4 ER quality control

Several studies have proposed that p24 proteins prevent exiting from the ER of misfolded and aberrant proteins, suggesting a role of p24 proteins in the ER quality control of certain secretory proteins (Wen and Greenwald, 1999; Springer et al., 2000; Belden and Barlowe, 2001a; Vetrivel et al., 2007).

3.7.5 p24 proteins in physiology and pathology

Due to the implication of p24 proteins in a variety of specific functions in animals, they have been proposed to be involved in different physiological processes and diseases in mammals, including:

- Trafficking and metabolism of amyloid-β precursor and pathogenesis of Alzheimer's disease (Chen et al., 2006; Vetrivel et al., 2007, 2008; Hasegawa et al., 2010; Liu et al., 2015).
- Early embryotic mouse development and morphogenesis of the mouse embryo and placenta (Denzel et al., 2000; Jerome-Majewska et al., 2010).

- Normal insulin biosynthesis and subsequent secretion in pancreatic β-cells, with a putative role in diabetes (Zhang and Volchuk, 2010; Wang et al., 2012).

4 GPI ANCHORED PROTEINS

There are several ways to attach proteins to the plasma membrane. Transmembrane proteins contain domains with hydrophobic amino acid sequences which are embedded within the plasma membrane lipid bilayer, while other proteins use a post-translational attachment to lipids. On one hand, if the protein has to be on the intracellular face of the plasma membrane, it can be post-translationally modified by *S*-acylation, *N*-myristoylation, prenylation or palmitoylation (Figure 15) (Luschnig and Seifert, 2011; Hemsley, 2015). On the other hand, the protein can be attached to a GPI anchor during the secretion of the protein, targeting it to the outer surface of the plasma membrane.

Since the GPI-anchored proteins were discovered (Bordier et al., 1986; Conzelmann et al., 1988; Low, 1989), these proteins have raised a great interest due to their contribution to diverse crucial biological processes, including growth, morphogenesis, reproduction and disease pathogenesis (Cheung et al., 2014). They have been studied from yeast and trypanosomes to mammals and plants. In mammals, GPI anchoring is essential for mammalian embryogenesis, development, neurogenesis, fertilization and for the immune system. Mutations of genes involved in GPI anchor remodelling cause human diseases characterized by neurological

abnormalities (Takeda et al., 1993; Tarutani et al., 1997; Nozaki et al., 1999; Alfieri et al., 2003; Kondoh et al., 2005; McKean and Niswander, 2012; Park et al., 2013). In yeast, the GPI anchor is essential for the correct growth of *Saccharomyces cerevisiae* (Leidich et al., 1994).

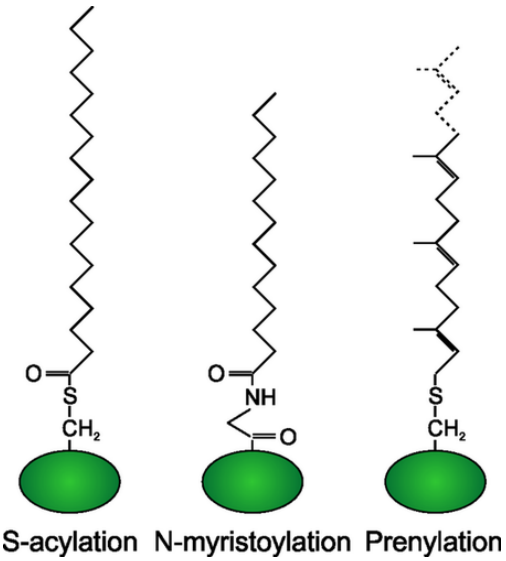

Figure 15. Structures of the three main lipid modifications of proteins found on the cytoplasmic face of cellular membranes (Hemsley, 2015). For *S*-acylation 18 carbon stearate is shown, for *N*-myristoylation the myristoyl group is shown attached to the α-amino group of Gly, and for prenylation a farnesyl moiety is depicted.

GPI-anchored proteins were identified based on their susceptibility to a treatment with bacterial phosphatidylinositol specific phospholipase C (PI-PLC) (Low, 1989), which hydrolyses the GPI anchor releasing a soluble protein. GPI-anchored proteins were first identified by Bordier et al., (1986) in the protozoan parasites *Leishmania spp*. and *Trypanosoma brucei* and in *Saccharomyces cerevisiae* (Conzelmann et al., 1988). Several years later, GPI-

anchored proteins were also discovered in plants (Morita et al., 1996; Kunze et al., 1997; Takos et al., 1997; Schultz et al., 1998).

The GPI anchor is newly synthesized in the ER and is then attached to the protein, which is also synthesized in the ER. The nascent peptide, which will be attached to the GPI anchor, has a N-terminal secretory signal peptide and a C-terminal GPI-specifying hydrophobic signal sequence (SS) (Yeats et al., 2018), which has to be cleaved before joining the GPI anchor.

Although the GPI anchor is largely conserved across eukaryotes, the repertoire of functional domains has diverged substantially. The GPI anchor is characterized for having two long fatty acids, a phosphatidylinositol ring, one N-acetyl glucosamine and three mannoses (Man) attached to ethanolamine phosphate (EtNP). The GPI anchor is attached to the polypeptide by an amide bond between EtNP and the C-terminal of the polypeptide (Kinoshita and Fujita, 2016).

Once attached, the GPI anchor of the GPI-anchored proteins is post-translationally remodelled by the addition of long saturated fatty acids replacing shorter insaturated fatty acids, and they are also modified by the addition or removal of EtNP at the glycan core of the GPI anchor. These modifications occur during their transport along the secretory pathway via Golgi apparatus to the outer surface of the plasma membrane (Kinoshita and Fujita, 2016).

Once the GPI anchor is correctly remodelled, some GPI-anchored proteins can also transiently homodimerize (Suzuki et al., 2012), acquire detergent resistance (Seong et al., 2013) and be sorted to the apical plasma

membrane in mammalian polarized cells (Paladino et al., 2006). Once at the plasma membrane, GPI-anchored proteins can associate with membrane microdomains enriched in sterols (cholesterol in mammals) and sphingolipids (Brown and Rose, 1992), also called lipid rafts (Simons and Gerl, 2010; Zurzolo and Simons, 2016).

The importance of GPI-anchored proteins for plants, mammals, yeasts and trypanosomes development has become obvious by the fact that GPI biosynthetic null mutants show embryo lethality, which was a problem for the understanding of the biosynthesis and remodelling routes of the GPI anchor in these organisms.

Most of the knowledge of the biosynthesis and modification on GPI anchors come from the studies in mammals and yeast, but these pathways are likely conserved in plants.

In plants, it has been predicted 248 GPI-APs in *Arabidopsis thaliana* (Borner et al., 2003), approximately 1 % of plant proteins and 10 % of secretory proteins, indicating the enormous importance of these proteins for plants. They play relevant functions in cell wall metabolism, cell wall polymer cross-linking, plasma membrane and cell wall signalling and plasmodesmatal transport (GPI-APs in plants will be further described below, section 4.8 of Introduction).

4.1 STRUCTURE OF THE GPI ANCHOR

The diverse eukaryotic lineages show a conserved glycan core structure with heterogeneity in the lipid composition of the anchor and the glycosyl and non-glycosyl substitutions of the glycan core structure.

The structure of the GPI anchor is composed by three α-linked Man residues as the glycan core structure. Yeast contains two additional mannoses (Fankhauser et al., 1993), of which Man4 is essential for the biosynthesis steps (Grimme et al., 2001), and Man5 is added at the Golgi apparatus (Sipos et al., 1995). This core structure is linked by N-acetyl glucosamine to the lipid moiety, which is composed by a phosphatidylinositol ring (myoinositol-P-lipid) anchored to long fatty acids (Figure 18). The lipid composition has been reported to be a phosphoceramide in yeast, consisting of phytosphingosine and tetracosanoic acid, with minor isoforms consisting of 4-hydroxy-8-sphingenine and/or docosanoic acid. In mammals a phosphatidylinositol of diacyl or 1-alkyl-2-acyl form with stearic acid (C18:0) or arachidonic acid (C20:4) have been found at *sn2* position (Käkelä et al., 2003; Houjou et al., 2005; Kinoshita and Fujita, 2016). On the other part, this core structure is linked to the C-terminal of the protein by an amide bond with a phosphoethanolamine residue (Figure 16).

The remodelling of the GPI anchor changes the glycan composition of the glycan core structure. EtNP residues or short mono-/oligo-saccharides like galactose or N-acetyl galactosamine, can be attached to several positions of the glycan core structure (Yeats et al., 2018).

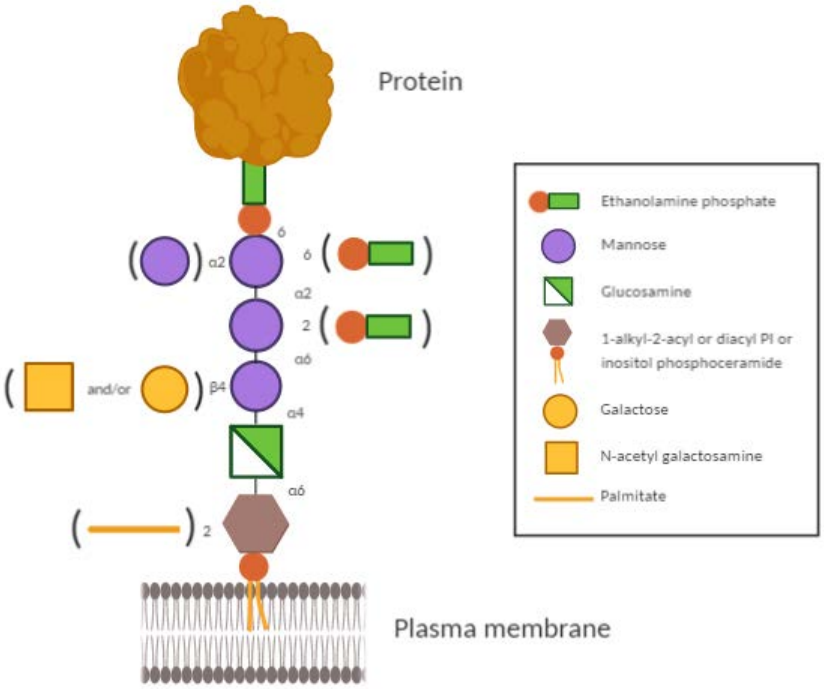

Figure 16. Structural features of GPI-anchored proteins (Created with BioRender). Common backbone of GPI anchors consisting of EtNP, three Mans, GlcN, and inositol phospholipid, is conserved in wide varieties of eukaryotes. Variable structural features that have been observed in a range of eukaryotic species are indicated with parentheses. The protein is anchored to the outer leaflet of the plasma membrane by fatty chains of inositol phospholipid.

The GPI anchor remodelling pathway also changes the lipid composition of the anchor. The nascent GPI anchor has two fatty acid chains, one of them is unsaturated and the other one saturated. In mammals, the saturated one is usually stearic acid (C18:0), whereas the unsaturated one is mainly arachidonic acid (C20:4) (Käkelä et al., 2003; Houjou et al., 2005). After the remodelling of the lipid part, both fatty acid chains are saturated and long in a vast majority of the GPI-APs, which is important for their association with membrane microdomains (“rafts”) and their transient

homodimerization (Maeda et al., 2007; Seong et al., 2013). As we will see below, the palmitate attached to the *sn2* position of the phosphatidylinositol ring is normally removed during the processing and transport of GPI-APs, although in some mature GPI-APs, this fatty acid is not removed during remodelling.

Despite the GPI anchor structure has been studied in a range of different kingdoms, only one single GPI anchor has been characterized in plants. This GPI anchor was found in the arabinogalactan protein AGP1, which was isolated from *Pyrus communis* cell suspension cultures (Figure 17). This GPI-AP has a simple GPI anchor structure lacking EtNP residues or saccharides attached to the glycan core (Oxley and Bacic, 1999). The lipid moiety is based in a ceramide, which has been also detected in fungal GPI anchors. A ceramide was also observed as the lipid component of the GPI anchor of an arabinogalactan protein isolated from *Rosa sp.* cell suspension culture (Svetek et al., 1999).

The GPI anchor of the yeast *Saccharomyces cerevisiae* contains two additional mannoses in the glycan core which are important for the following steps of the biosynthesis (Fankhauser et al., 1993; Sipos et al., 1995; Grimme et al., 2001). The lipid moiety of mature yeast GPI-APs is composed by a very long chain fatty acid at the position *sn2* hexanoic acid (C26:0) or a ceramide containing phytosphingosine with a very long chain fatty acid (C26:0) (Fujita and Jigami, 2008).

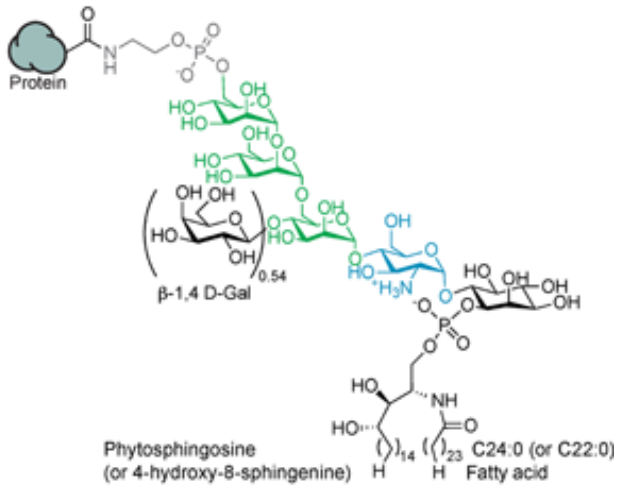

Figure 17. Structure of *Pyrus communis* AGP1 GPI anchor identified by Oxley and Bacic, 1999 (Adapted from Yeats et al., 2018). Aside from the core GPI glycan structure, only a β-1,4-linked Gal side-chain was observed. The lipid was found to be a ceramide consisting primarily of phytosphingosine and tetracosanoic acid, with minor isoforms consisting of 4-hydroxy-8-sphingenine and/or docosanoic acid.

4.2 BIOSYNTHESIS OF THE GPI ANCHOR

The pathway of the biosynthesis of the GPI anchors has been largely studied in different model systems as mammals, parasites and yeast. They all share a conserved pathway and it is possible that plants share similarities in this pathway. Even the archaea species have also a conserved biosynthetic pathway (Eichler and Adams, 2005).

First, the GPI anchor is synthesised *de novo* in the ER and then is attached *en bloc* to the C-terminus of the protein, which contains a GPI anchor signal, by GPI transamidase (GPI-TA). After this, the GPI anchor is remodelled by several enzymes also localized at the ER (Figure 18).

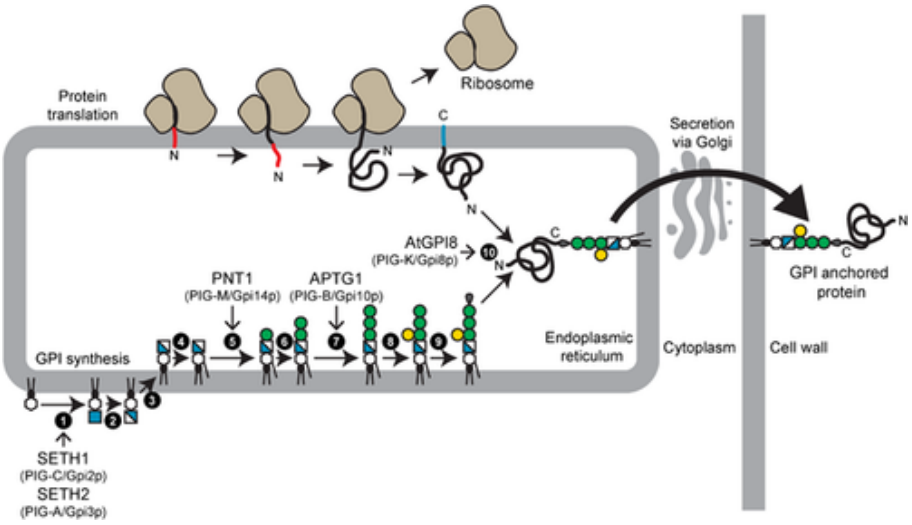

Figure 18. Biosynthesis of GPI-anchored in plants (Yeats et al., 2018). The scheme is based on the pathway described in mammalian, protozoan and yeast systems. GPI anchor biosynthesis is initiated by generation of N-glucosamine-phosphoinositide (steps 1 and 2) on the cytoplasmic surface before flipping by an unknown mechanism (is proposed to be a "flippase") to the ER lumen (step 3). This is followed by acylation of inositol (step 4) and synthesis of the trimannosyl core (steps 5 to 7); elaboration of the GPI-anchor in plants is proposed to include galactosylation (step 8) and the potential addition of EtNP side-chains (not shown). A GPI transamidase complex transfers the GPI anchor to the protein (step 10) before export from the ER. Most biosynthetic genes have single copy orthologs in the *Arabidopsis thaliana* genome. The orthologous human (PIG) and yeast (Gpi) proteins are indicated.

Around 30 genes have been identified to be involved in the synthesis, assembly and remodelling of the GPI anchor and there is homology between mammals and yeast genes. A study made in plants reported that *Arabidopsis* genome have single copy homologs of most of these genes (Luschnig and Seifert, 2011). Mutations in five of these genes have experimentally supported the importance of these enzymes during the biosynthesis of the GPI anchor.

Biosynthesis of GPI anchor is initiated on the cytoplasmic face of the ER, generating a N-glucosamine-phosphoinositide (GlcN-PI) by the enzymatic complex SETH1 and SETH2 (Figure 18, steps 1 and 2) (Watanabe et al., 1998, 1999; Lalanne et al., 2004; Murakami, 2005). Next GlcN-PI is translocated to the luminal face of the ER by an unknown mechanism (Figure 18, step 3). Nevertheless it has been proposed that this process is mediated by a "flippase", although it has not been identified and characterized yet (Vishwakarma and Menon, 2005). Then, the phosphoinositol (PI) ring is acylated at the position 2 (Figure 18, step 4) (Doerrler et al., 1996; Murakami, 2003).

Once the lipid part is completed, the next step is the synthesis of the trimannosyl core. The first Man is added by the enzyme PNT1 (Figure 18, step 5) (DeGasperi et al., 1990; Maeda et al., 2001; Sugimoto et al., 2005), while the second and third Mans are added sequentially by the APTG1 enzyme (Figure 18, steps 6 and 7) (Kang et al., 2005). In order to finish the glycan core, it has been proposed for plants to include a galactosylation (Figure 18, step 8) and the potential addition of lateral EtNPs (Hong et al., 1999) apart of the top one (Figure 18, step 9) to attach the C-terminus of the protein (Yeats et al., 2018).

Finally, the GPI transamidase complex (AtGPI8) catalyzes the attachment between the C terminus of the protein and the EtNP of the GPI anchor by an amide bond (Figure 18, step 10) (Maxwell et al., 1995; Sharma et al., 1999). After this, the nascent GPI-AP is remodelled during the secretory pathway before reaching the plasma membrane.

These enzymes of the biosynthetic pathway have each a single copy ortholog in the *Arabidopsis thaliana* genome (Table 3).

	Enzyme	Mammal	*Saccharomyces cerevisiae*	*Arabidopsis thaliana*
Step 1	GPI-GlcNAc transferase	PIG-C	Gpi2p	SETH1 (AT2G34980)
	GPI-GlcNAc transferase	PIG-A	Gpi3p	SETH2 (AT3G45100)
Step 2	GlcNAc-PI de-N-acetylase	PIG-L	Gpi12p	Not studied (AT2G27340)
Step 3	Flippase	Not identified	Not identified	Not identified
Step 4	Inositol acyltransferase	PIG-W	Gwt1p	Not studied (AT4G17910)
Step 5	α-(1-4)-Mannosyl-transferase	PIG-M	Gpi14p	PNT1 (AT5G22130)
Step 6	α-(1-4)-Mannosyl-transferase II	PIG-V	Gpi18p	Not studied (AT1G11880)
Step 7	α-(1-4)-Mannosyl-transferase III	PIG-B	Gpi10p	APTG1 (AT514850)
Step 8	EtNP transferase I	PIG-N	Mcd4p	Not studied (AT3G01380)
	EtNP transferase II	PIG-G	Gpi7p	Not studied (AT2G22530)
	Putative plant-specific GalT	---	---	Not identified
Step 9	EtNP transferase III	PIG-F	Gpi13p	Not studied (AT5G17250)
Step 10	GPI transamidase	PIG-K	Gpi8p	AtGPI8 (AT1G08750)

Table 3. Orthologs of the enzymes involved in the biosynthesis of the GPI anchor in mammalian, yeast and *Arabidopsis thaliana*.

These enzymes have been identified and characterized using mutants deficient in five of these enzymes (*seth1*, *seth2*, *pnt1*, *aptg1* and *atgpi8*) (Lalanne et al., 2004; Ellis et al., 2010).

4.2.1 Attachment of GPI anchor to proteins by GPI transamidase

Preproteins which are attached with a GPI anchor, have a N-terminal leader sequence for ER translocation (von Heijne, 1990; Petersen et al., 2011; Whitley and Mingarro, 2014) and a C-terminal sequence for GPI anchor attachment (Maxwell et al., 1995).

On the one hand, the signal peptide (SP) at the N-terminal sequence targets the protein to the ER translocon (Whitley and Mingarro, 2014), where the SP is cleaved off (Johnson and van Waes, 1999). On the other hand, the C-terminal sequence for GPI anchor attachment is composed by four consecutive parts from the N terminus of the *ω* site (where the GPI anchor is attached) to the C terminus. The first part consists of an unstructured linker of 10 amino acids from the site *ω-11* to *ω-1*; in the second part, *ω* and *ω+2* sites have short side chains; the third part has five to ten hydrophilic amino acids and the fourth part a 15 to 20 amino acid hydrophobic stretch (Figure 19) (Eisenhaber et al., 2001; Kinoshita and Fujita, 2016).

The *ω* site has been reported to include small residues such as Ala, Asn, Asp, Cys, Gly and Ser. If the *ω* site is changed for another amino acid such as proline, the GPI attachment is impaired (Eisenhaber et al., 2001). Currently, there is software which can predict the amino acid sequence of the *ω* sites and the GPI attachment (Eisenhaber et al., 2000).

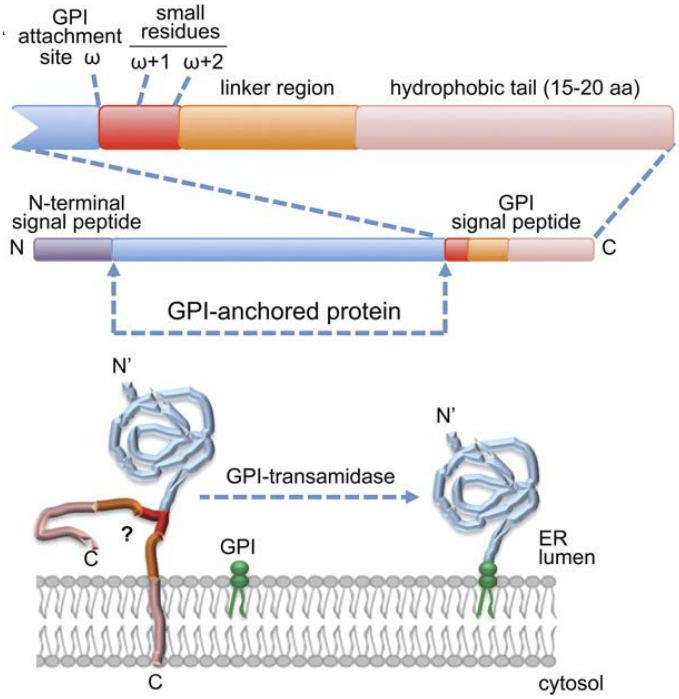

Figure 19. Schematic representation of the GPI signal architecture and the GPI-attachment reaction (Galian et al., 2012).

The preproteins with the SP in the N-terminal are translocated to the ER (Figure 20, step 1) (von Heijne, 1990; Johnson and van Waes, 1999; Whitley and Mingarro, 2014). Once the preprotein is translocated to the luminal part of the ER, the GPI attachment sequence at the C-terminal of the preprotein is recognized by the GPI transamidase AtGPI8 (Figure 20, step 2) (Hamburger et al., 1995; Yu et al., 1997; Luschnig and Seifert, 2011). The GPI transamidase recognizes the *ω* site and cleaves the amino acid sequence between the *ω* and *ω+1* sites, generating the substrate to the attachment (Figure 20, step 3) (Ohishi et al., 2000, 2001; Hong et al., 2003). This protein substrate is attached to the EtNP of the GPI anchor via an amide bond (Figure

20, step 4) (Benghezal et al., 1996; Eisenhaber et al., 2014), resulting in a GPI-AP which has yet to be maturated (Figure 20, step 5).

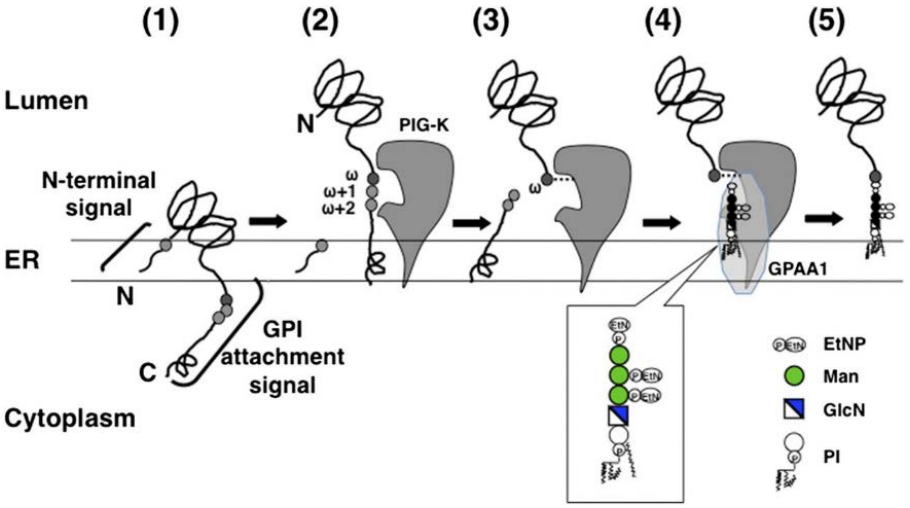

Figure 20. GPI attachment to proteins by GPI transamidase in mammalian (Kinoshita and Fujita, 2016). Preproprotein has an N-terminal signal for ER translocation and a C-terminal signal for GPI attachment (step 1). After translocation into the ER lumen, the N-terminal signal is removed and the C-terminal signal is recognized by the GPI transamidase (PIG-K) (step 2). GPI transamidase cleaves a peptide bond between *ω* and *ω*+1 amino acids, generating a substrate-enzyme complex linked via a thioester bond (step 3). The thioester-linked intermediate is attacked by GPI presented by GPAA1 (step 4) completing transamidation (step 5).

4.2.2 Remodelling of the GPI anchor

Before the delivery of the GPI-APs to the cell surface, these proteins are remodelled during their transport along the secretory pathway. The structure of the glycan core and the lipid composition of the GPI anchor change during this remodelling.

On the one hand, the change in lipid composition involves the removal of the acyl chain and the replacement of the unsaturated fatty acid

for a long saturated fatty acid. This allows the GPI-APs to follow a selective rather than a “bulk-flow” pathway (Muniz et al., 2000; Bonnon et al., 2010; Zurzolo and Simons, 2016). Due to this lipid composition, GPI-APs can associate with membrane domains rich in sphingolipids and sterols, also called membrane microdomains or lipid rafts (Yeats et al., 2018).

On the other hand, the composition of the glycan core is also changed. It has been proposed that p24 proteins are involved in transport of GPI-APs from the ER to the Golgi apparatus (for further details see section 3.7.3 of Introduction). To this end, p24 proteins can interact with the remodelled glycan core of GPI-APs and also recruit components of the COPI and COPII coat, to sort them within COPI or COPII vesicles (Muniz et al., 2000; Castillon et al., 2011; Fujita et al., 2011). In *Arabidopsis*, the role of the p24 proteins in sorting of GPI-APs has not yet been elucidated (Pastor-Cantizano et al., 2016).

In yeast, the remodelling of the GPI-APs is completed within the ER (Muniz and Zurzolo, 2014), while in mammals, this pathway starts at the ER but continues in the Golgi apparatus, where the remodelling is finally completed (Muniz and Zurzolo, 2014; Kinoshita and Fujita, 2016). For a summary of the orthologs of yeast and mammals involved in GPI anchor remodelling see Table 4. Little is known about the remodelling pathway in plants, which is one subject of study in this thesis.

GPI anchor remodelling enzymes	*Saccharomyces cerevisiae*		*Mammals*		**Function**
	Enzyme	Location	Enzyme	Location	
GPI inositol deacylase	Bst1p	ER	PGAP1	ER	Removes the acyl-chain linked to inositol
GPI phospholipase A2	Per1p	ER	PGAP3	Golgi	Removes the unsaturated fatty acyl chain in the *sn2* position
GPI O-acyltransferase	Gup1p	ER	PGAP2	Golgi	Inserts a long saturated fatty acid in the *sn2* position
GPI ethanolamine phosphate phosphoesterase	Cdc1p	ER	---	---	Removes a side-chain EtNP attached to Man1 from some GPI-AP
Ceramide remodelase	Cwh43p	ER	---	---	Exchanges lipid moieties from diacylglycerol to ceramide types
GPI ethanolamine phosphate phosphoresterase	Ted1p	ER	PGAP5	ER	Removes the side-chain EtNP attached to Man2

Table 4. Orthologs and function of the enzymes involved in the remodelling of GPI anchor in mammalian and yeast.

4.2.3 Remodelling pathway in mammals

The nascent GPI-APs formed by the action of the GPI transamidase are still immature and need some remodelling reactions to become mature GPI-APs (Kinoshita and Fujita, 2016). These remodelling reactions occur during their transport in the secretory pathway to the cell surface. As it is shown in Figure 21, these reactions are catalyzed by different enzymes located at the ER and the Golgi apparatus in mammals.

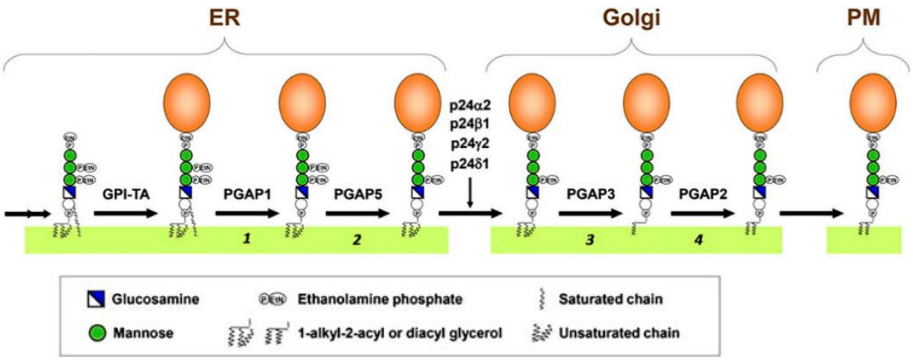

Figure 21. Maturation of mammalian GPI-AP during ER-to-plasma membrane (PM) transport (Kinoshita and Fujita, 2016). Nascent GPI-APs formed by GPI transamidase (GPI-TA) undergo two reactions, inositol-deacylation (step 1) and removal of the EtNP side branch from Man2 (step 2) in the ER. A cargo receptor consisting of four p24 proteins is involved in ER-to-Golgi transport. Once in the Golgi, GPI-APs undergo fatty acid remodelling (steps 3 and 4), generating mature GPI-APs.

The first reaction is in the ER carried out by the inositol deacylase, PGAP1 (Figure 21, step 1) (Chen et al., 1998; Tanaka et al., 2004), which removes the acyl chain from the inositol ring. This acyl chain is usually palmitate, but can also be myristate in some cases (Houjou et al., 2007). The enzyme has nine transmembrane domains with a typical lipase motif and is widely expressed in the ER (Tanaka et al., 2004).

The deacylation by PGAP1 makes GPI-APs sensitive to the bacterial PI-specific phospholipase C. There are some cells which exceptionally do not remove this acyl chain and therefore are resistant against PI-PLC, such as erythrocytes (Walter et al., 1990), CD52 on human spleen cells and sperm (Schröter et al., 1999) and ALP (alkaline phosphatase) proteins on certain cell lines (Yee Wah Wong and Low, 1992). In mammalian cells which are defective in PGAP1, GPI-APs are resistant to PI-PLC, and their transport from ER to Golgi is three times slower (Tanaka et al., 2004), probably because GPI-APs are not recognized by the p24 complex (Fujita et al., 2011) so the transport is impaired.

The second reaction is catalyzed by PGAP5, which removes the EtNP side branch linked to Man2 (Figure 21, step 2) (Fujita et al., 2009). PGAP5 is also in the ER but restricted to the ERES (Fujita et al., 2009). The lack of this enzyme cause that GPI-APs are not recognized by the p24 complex (Manzano-Lopez et al., 2015).

After these two remodelling reactions, the GPI-APs are recognized by the cargo receptors p24, which form a complex including different members, and concentrate them at the ERES and package them into COPII-coated vesicles in order to be transported from the ER to the Golgi apparatus (Kinoshita and Fujita, 2016). The GPI-APs need to be remodelled before their association with p24 transmembrane cargo receptors (Fujita et al., 2011).

Once at the Golgi, GPI-APs may dissociate from the p24 cargo receptor complex in the ERGIC/*cis*-Golgi lumen due to slightly acidic pH (Fujita et al., 2011). There, the lipid part of the GPI-APs is remodelled

replacing the unsaturated fatty acid at the *sn2* position by saturated fatty acid, which usually is a stearic acid (Kinoshita and Fujita, 2016).

The first enzyme which acts at the Golgi apparatus is PGAP3, a GPI-specific phospholipase A2 which has seven TMDs and belongs to the membrane bound hydrolase superfamily called CREST (alkaline ceramidase, PAQR receptor, Per1, SID-1 and TMEM8) (Pei et al., 2011). This enzyme removes the unsaturated fatty acid in the *sn2* position (Figure 21, step 3) (Maeda et al., 2007). The lack of this enzyme impairs lipid remodelling at the Golgi but the GPI-APs are still transported and expressed on the plasma membrane (Maeda et al., 2007), although the expression of GPI-APs at the plasma membrane is moderately affected. This unremodelled GPI-APs have unsaturated fatty acids and cannot associate with specific membrane domains (Maeda et al., 2007).

The last enzyme involved in the remodelling route of the GPI-APs is the Golgi-resident PGAP2, which has five TMDs. It is required for reacylation of the GPI-APs at the *sn2* position, adding a long saturated fatty acid, preferentially stearic acid (Figure 21, step 4) (Tashima et al., 2006). Cells defective in PGAP2 have severely affected the composition of GPI-APs at the cell surface, less than 10 % than in wild type cells, as a result of the secretion of GPI-APs with only one fatty chain (Tashima et al., 2006).

At the Golgi apparatus, GPI-APs are usually also modified by *N*/*O*-glycosylation, which mostly occurs in the Golgi apparatus. The addition of a GalNAc-containing side chain to some GPI-APs also occurs there (Kinoshita and Fujita, 2016). Three glycosyltransferases, β4GalNAc transferase, β3Gal

transferase and a sialyltransferase, which are not still identified, could be involved in the addition of the GalNAc (Brewis et al., 1995).

It has been reported that deficiencies of lipid/fatty acid remodelling and inositol acylation and deacylation can cause several diseases and cell abnormalities in peroxisomal disorders and the Zellweger syndrome (Braverman and Moser, 2012; Crane, 2014; Wanders, 2014; Kinoshita and Fujita, 2016). Loss-of-function of the enzymes PGAP2 (Hansen et al., 2013; Krawitz et al., 2013) and PGAP3 (Murakami et al., 2012; Howard et al., 2014) cause the Mabry syndrome (HPMRS) in patients with intellectual disability, seizures, and hyperphosphatasia. PGAP1 mutations cause neuronal abnormalities such as intellectual disability and encephalopathy in patients (Murakami et al., 2014; Williams et al., 2015) and male mice were infertile (Ueda et al., 2007).

4.2.4 Remodelling pathway in yeast

As it occurs in mammals, nascent GPI-APs at the ER need to be remodelled during the secretory pathway to achieve the mature form before being transported to the plasma membrane. The principal difference with mammals is that this remodelling route in yeast is completed at the ER (Fujita and Kinoshita, 2012).

The first step in the remodelling route is the elimination of the acyl-chain of inositol by the GPI inositol deacylase Bst1p, the ortholog of PGAP1 in mammals (Figure 22, step 1) (Tanaka et al., 2004). Next, the yeast PGAP3 homolog, Per1p, removes the unsaturated fatty acyl chain at the *sn2* position (Figure 22, step 2) (Fujita et al., 2006a). The third step is carried out by Gup1p, the yeast PGAP2 homolog, an enzyme from the MBOAT (membrane bound

O-acyl transferase) family (Bosson et al., 2006). This enzyme incorporates a very long chain fatty acid (C26:0) at the *sn2* position.

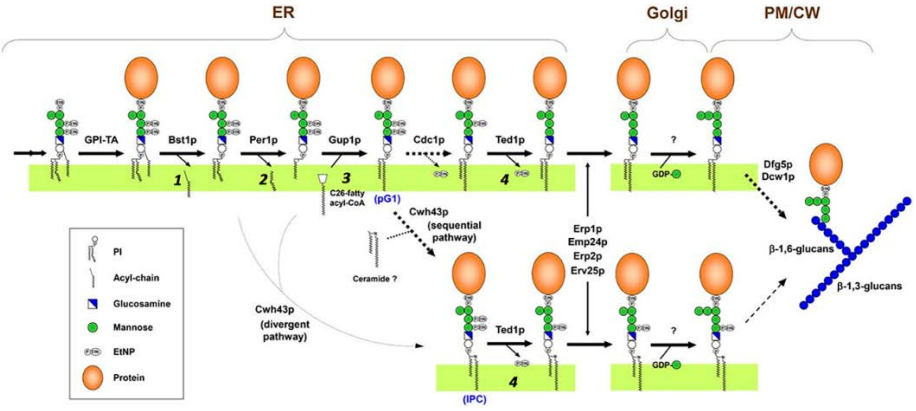

Figure 22. Remodelling of GPI anchors in yeast *Saccharomyces cerevisiae* (Kinoshita and Fujita, 2016). GPI anchor is synthesized in the ER and transferred to proteins by the GPI-transamidase complex. After GPI-attachment to proteins, an acyl-chain linked to inositol is eliminated by Bst1p. Then, an unsaturated fatty acyl chain in the *sn2* position is removed by Per1p, and a very long saturated (C26:0) fatty acid is reacylated to that position by Gup1p. C26-fatty acyl-CoA is used as the substrate. Many fractions of lipid moieties in GPI anchors are further exchanged from diacylglycerol types to ceramide types by Cwh43p. The substrate for the ceramide remodelling is still unclear. A side-chain EtNP attached to Man1 is removed from some fractions of GPI anchors by Cdc1p, but it is not clear which GPI-APs are recognized as substrates, whereas the experimental data suggest that GPI anchors having diacylglycerol types might be the preferential substrates (Vazquez et al., 2014). A side-chain EtNP attached to Man2 is removed by Ted1p. This reaction is important for recognition by the p24 protein complex. The order of the reactions mediated by Cdc1p and Ted1p is not known. After GPI-APs are transported to the Golgi, additional Man is transferred to the Man4 with α-1,2 or α-1,3-linkage by unidentified enzymes. Once on the cell surface, many GPI-APs are cleaved and cross-linked to β-1,6-glucans on the cell wall. Dfg5p and Dcw1p are involved in the cell wall anchorage of GPI-APs.

At this point, two different models have been proposed to describe the lipid remodelling pathway of GPI anchors in yeast, the sequential pathway and the divergent pathway (Ghugtyal et al., 2007; Umemura et al., 2007). In the sequential pathway, the PI moieties in GPI anchors are

sequentially modified from conventional PI to lysoPI by Per1p (Figure 22, step 2), then the C26:0 fatty acid is added at the *sn2* position by Gup1p (pG1) (Figure 22, step 3) to finally generate the inositolphosphoceramide (IPC) (Kinoshita and Fujita, 2016). The divergent pathway model involves the PI generated by Bst1p and the lysoPI generated by Per1p as substrates for the Cwh43p, an enzyme which carry out the changing to ceramide as the lipid part of the GPI anchor (Kinoshita and Fujita, 2016). Last studies suggest that the main pathway for lipid remodelling is the sequential pathway, but the other pathway is also conserved and exists (Yoko-o et al., 2013).

As indicated before, Cwh43p is the enzyme which carry out the addition of ceramide as lipid moiety to the GPI anchor (Ghugtyal et al., 2007; Umemura et al., 2007; Yoko-o et al., 2018). The substrate for the ceramide remodelling is still not clear, but it has been described that most lipid moieties of GPI anchors are exchanged from diacylglycerol to ceramide types (Ghugtyal et al., 2007). The N-terminal region of this enzyme shares homology with the mammalian PGAP2 (Ghugtyal et al., 2007; Umemura et al., 2007), but Cwh43p has an additional C-terminal domain consisting of 700 amino acids, which shares characteristics with exonuclease, endonuclease and phosphatase proteins (Kinoshita and Fujita, 2016). In the Cwh43p mutant, GPI-APs with diacylglycerol at the *sn2* position in GPI anchor are accumulated while ceramide as part as lipid moiety is completely lost (Ghugtyal et al., 2007; Umemura et al., 2007; Yoko-o et al., 2018).

In some GPI-APs, the enzyme Cdc1p removes a side-chain EtNP attached to Man1 (Vazquez et al., 2014). It remains elusive which GPI-APs are recognized as substrates, but experimental data suggest that the GPI-APs

with diacylglycerol as a lipid moiety could be preferential substrates (Vazquez et al., 2014). In the mutant of this enzyme, the transport of GPI-APs is not impaired in contrast to other enzymes of the remodelling route. Therefore, the reaction carried out by Cdc1p might be related to cell wall anchorage of some GPI-APs because the mutant shows defects in the cell wall (Vazquez et al., 2014).

In the step 4 (Figure 22), the enzyme Ted1p removes a side-chain EtNP attached to Man2, the homologue of PGAP5 in mammals (Fujita et al., 2009). This reaction is important for the recognition by p24 proteins complex (Emp24p, Erv25p, Erp1p and Erp2p) (Castillon et al., 2011). The order of reactions mediated by Cdc1p and Ted1p is not known yet (Kinoshita and Fujita, 2016). It has been reported that the mutant of Ted1p shows a delay in ER to Golgi transport of GPI-APs (Haass et al., 2007; Manzano-Lopez et al., 2015; Yoko-o et al., 2018).

After the GPI anchor is remodelled at the ER, the GPI-APs are transported to the Golgi apparatus via COPII vesicles. There, an additional Man is transferred to the Man4 by unidentified enzymes. Finally, many GPI-APs on the cell surface can be cleaved and cross-linked to β1,6-glucans on the cell wall (Fujii et al., 1999; Orlean, 2012). However, most GPI-APs are retained in the plasma membrane (Caro et al., 1997; Hamada et al., 1999; Frieman and Cormack, 2003). In this process, Df5p and Dcw1p enzymes are involved in the cell wall anchorage (Kitagaki et al., 2002, 2004; Pittet and Conzelmann, 2007).

4.3 EXPORT FROM THE ER

The remodelled GPI anchor acts as a transport signal that triggers the transport of GPI-APs from the ER to the Golgi apparatus (Muñiz and Riezman, 2016). The unique structure and composition of the GPI anchor confers special features to them and a special mode of interaction with membranes in the lumen of the organelles involved in the secretory pathway (Muñiz and Riezman, 2016). Therefore, transport of GPI-APs along the secretory pathway is different from other secretory membrane proteins (Lisanti et al., 1988; Brown et al., 1989; Muñiz et al., 2001; Castillon et al., 2009).

To initiate the transport to the plasma membrane, correctly remodelled and folded GPI-APs are selectively incorporated into COPII vesicles at ERES. For efficient ER export, proteins are concentrated at ERES by direct or indirect interaction with COPII proteins. In particular, the subunit SEC24 is the one specialized in cargo selection. The p24 protein complex acts as cargo receptor because it can interact with the COPII subunit SEC24 and also with cargos that have to be incorporated in COPII vesicles (Barlowe and Miller, 2013).

It has been observed in yeast that GPI-APs are accumulated in ERES distinct than those accumulating other secretory proteins. Therefore, GPI-APs and other secretory proteins are subsequently incorporated into distinct COPII vesicles (Muñiz et al., 2001; Castillon et al., 2009). Nevertheless, in mammalian cells it has been reported that GPI-APs are packaged into the same COPII vesicles as the other secretory proteins because GPI-APs were found in the same ERES and COPII vesicles as transmembrane proteins (Rivier et al., 2010). These facts suggest that GPI-APs use different mechanisms to

concentrate at the ERES and being packaged into COPII vesicles in these two organisms (Figure 23).

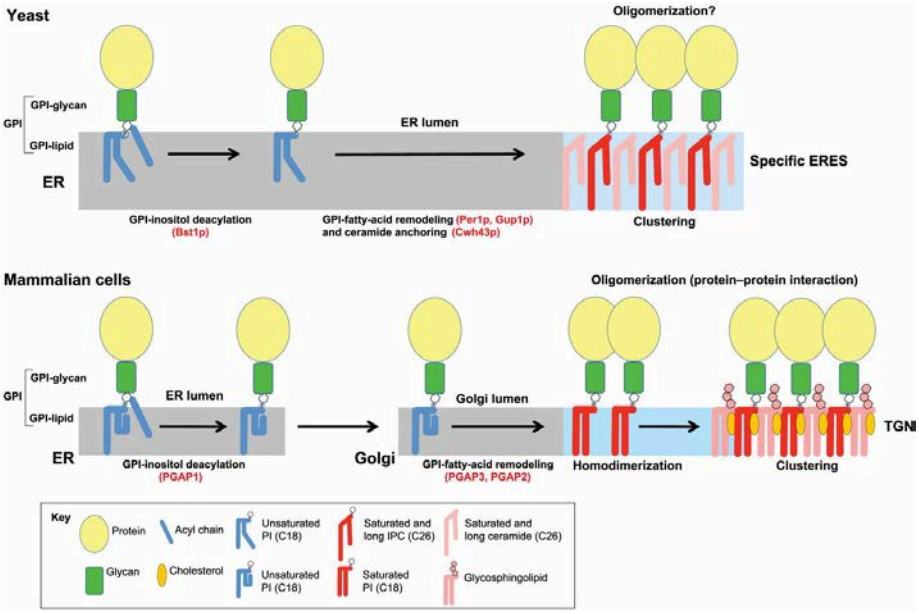

Figure 23. GPI anchor remodelling and export from the ER in yeast and mammalian cells (Muniz and Zurzolo, 2014). In yeast, the GPI anchor is completely remodelled in the ER and they can oligomerize forming clusters at specific ERES. In mammalian cells, the GPI anchor is not completely remodelled in the ER and this process continuous in the Golgi apparatus. Once GPI anchor is remodelled, they homodimerize forming clusters at the TGN.

4.3.1 Yeast

It seems that yeast do not need neither the COPII machinery nor p24 proteins in order to concentrate GPI-APs at ERES (Castillon et al., 2009), since mutants of several members of the p24 family, Emp24p and Erv25p, showed an impaired transport of GPI-APs (Schimmöller et al., 1995; Belden and Barlowe, 1996), but their concentration at ERES was not affected (Castillon et al., 2011). Therefore, an alternative mechanism for cargo concentration is

used for GPI-APs. It has been suggested that concentration of GPI-APs is based on a lipid-remodelling mechanism (Figure 24) (Castillon et al., 2009).

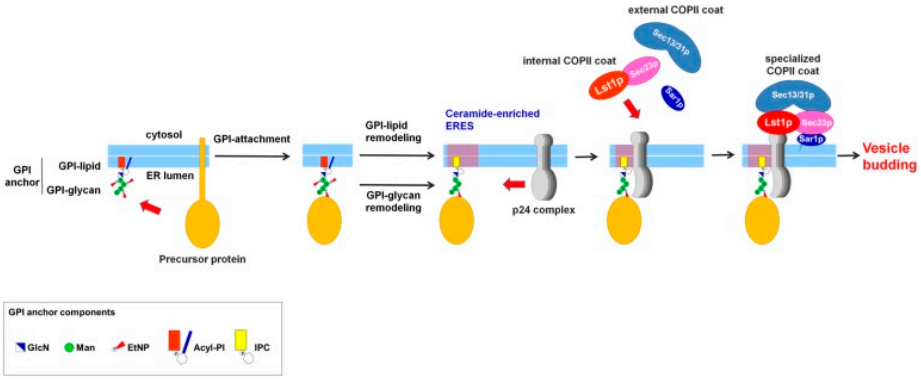

Figure 24. In yeast, GPI-APs use a specialized COPII vesicle budding system for ER export, which is actively regulated by structural remodelling of the GPI anchor (Muñiz and Riezman, 2016). After GPI-APs are remodelled, they are concentrated at specific ERES. The GPI-glycan remodelling allows the subsequent recruitment of p24 complex, which functions as a specific lectin by recognizing the remodelled GPI-glycan moiety of GPI-APs, to these ERES. This binding stimulates the p24 complex to selectively recruit and stabilize Lst1p-Sec23p pre-budding complexes to generate specialized COPII vesicles enriched in GPI-APs.

As it was explained before, newly synthesized proteins attached to the GPI anchor need to be remodelled in order to reach the plasma membrane. The GPI anchor remodelling starts just after the GPI transamidase catalyzes the formation of the amide bond between the GPI anchor and the C-terminal of the protein. As described before, the lipid remodelling of GPI anchor consist basically in changing unsaturated fatty acids to very long-chain saturated fatty acids, catalyzed by Bst1p, Per1p and Gup1p (Tanaka et al., 2004; Bosson et al., 2006; Fujita et al., 2006a). Mutants of these enzymes fail to concentrate GPI-APs at ERES (Castillon et al., 2009).

In yeast, remodelled GPI-APs have a lipid moiety composed by diacylglycerol (C26:0) or ceramide (C26:0). The remodelling of the GPI anchor is completed at the ER, and it is necessary for the isolation of GPI-APs in detergent-resistant membranes (DRM) (Fujita et al., 2006a; Maeda et al., 2007; Castillon et al., 2011). These long-chain saturated fatty acids change the physical properties of the GPI-APs and the association with the membrane forming ordered domains at the ER lipid membrane (Silva et al., 2006). Therefore, these domains would be selectively concentrated at specific ERES (Figures 23 and 24) (Muñiz and Riezman, 2016).

Once the GPI-APs have been concentrated at specific ERES, they have to be incorporated into COPII vesicles for transport to the Golgi. Because of their luminal topology, GPI-APs need a transmembrane cargo adaptor which connect these proteins with the cytosolic COPII proteins. This transmembrane cargo adaptor is the p24 complex (Muñiz et al., 2000; Castillon et al., 2011; Manzano-Lopez et al., 2015). These proteins act as a heteromeric complex that cycle between ER and Golgi (see section 3 of Introduction) (Marzioch et al., 1999b) and are required for the incorporation of remodelled GPI-APs to nascent COPII vesicles (Figure 24) (Muñiz et al., 2000; Castillon et al., 2011; Manzano-Lopez et al., 2015). Mutants that impaired the concentration of GPI-APs at ERES, showed a mislocalization of p24 proteins (Castillon et al., 2011).

It has been reported that the GPI-APs interact with the p24 complex when the glycan core is completely remodelled, acting as a lectin (Manzano-Lopez et al., 2015). This remodelling process is completed when the

phosphodiesterase Ted1p removes the side-chain EtNP on the Man2 (Haass et al., 2007; Fujita et al., 2009; Manzano-Lopez et al., 2015).

The biogenesis of specialized COPII vesicles containing GPI-APs in yeast involves the recruitment of a specialized COPII machinery, in particular the Lst1p subunit, which is one of the two paralogs of Sec24p (Manzano-Lopez et al., 2015; Lopez et al., 2019). It has been shown that p24 proteins can also interact with the two paralogs of Sec24p (Miller et al., 2003), but GPI-APs only are incorporated to COPII vesicles with the Lst1p subunit (Figure 24) (Manzano-Lopez et al., 2015). This could be explained by the fact that cargo binding could trigger a structural change of the p24 proteins and this increases the affinity to Lst1p and not to Sec24p (Peng et al., 2000; Miller et al., 2002; Iwasaki et al., 2015). The final scaffolding of COPII vesicles also requires the subunit Sec13p, a subunit of the outer layer of COPII coat (Čopič et al., 2012; D'Arcangelo et al., 2015). This specific requirement seems to be due to the luminal topology of both the p24 complex and GPI-APs; when both groups of proteins concentrate at specific ERES, they impose special biophysical requirements for vesicle budding. In particular, the size of the GPI anchors is short in comparison with the heavily glycosylated luminal ectodomains of GPI-APs, generating a negative curvature in ER membranes that has to be overcome by the COPII coat machinery. This appears to require both Lst1p, which creates buds with a larger diameter (Shimoni et al., 2000), and Sec13p, which confers rigidity to the coat. Therefore, both Lst1p and Sec13p could specifically cooperate to capture larger cargos, such as clusters of GPI-APs (Lopez et al., 2019).

Finally, some v-SNAREs and other specific tethering factors are required for the targeting of GPI-APs upon the ER exit. However, this mechanism is not still described (Morsomme and Riezman, 2002; Morsomme et al., 2003).

4.3.2 Mammals

In contrast to yeast, in mammalian cells GPI-APs are not segregated from other secretory proteins in different ERES and COPII vesicles for transport to the Golgi apparatus (Figure 23) (Rivier et al., 2010). This difference is caused by the fact that the glycan core of the GPI anchor is remodelled in the ER but the lipid remodelling is carried out in the Golgi apparatus (Tashima et al., 2006; Maeda et al., 2007). Therefore, GPI-APs cannot be concentrated at ERES by a lipid-based sorting mechanism. In this way, the p24 complex is responsible for the concentration at ERES (Figure 25) (Fujita et al., 2011). The mammalian p24 complex recognizes in the same way the GPI-APs as yeast. In mammals, the glycan core remodelling is also necessary for the interaction with the p24 complex, so PGAP5, Ted1p ortholog in yeast, is at the ER and is needed for GPI-APs recognition by p24 complex (Figure 25) (Fujita et al., 2009, 2011). The p24 complex domain involved in the interaction with GPI-APs have been reported to be the membrane adjacent α-helical region (CC domain) (Theiler et al., 2014).

Mammalian GPI-APS also exit the ER in specialized COPII vesicles. SEC24C and SEC24D are the specific isoforms of the COPII subunit SEC24 required for ER export of both GPI-APs and the p24 complex (Bonnon et al., 2010). This suggests that, similar to yeast, the mammalian p24 complex

interacts with the inner layer of the COPII coat through specific subunits for efficient packaging of GPI-APs into COPII vesicles (Lopez et al., 2019).

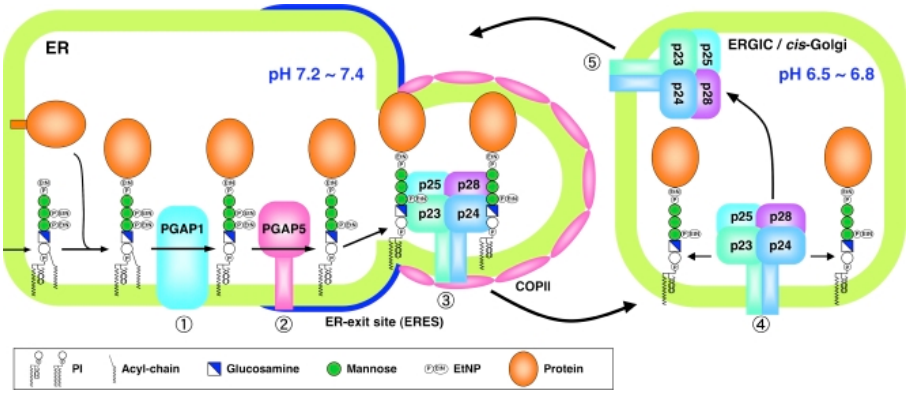

Figure 25. Mammalian model for selective sorting and transport of GPI-APs from the ER (Fujita et al., 2011). After GPI anchor is transferred to proteins by GPI transamidase, an acyl chain linked to inositol is removed by PGAP1, and then a side-chain on the Man2 is also removed by PGAP5. These two GPI remodelling reactions in the ER are critical for the sorting of GPI-APs to the ERES. Remodelled GPI-APs in the ER are recognized by the p24 complex that concentrates GPI-APs into the COPII-derived vesicles. Once to the ERGIC or *cis*-Golgi, GPI-APs dissociate from the p24 complex because of decreased luminal pH in these compartments. The p24 complexes are retrieved from the Golgi to the ER by the COPI vesicles.

4.4 GOLGI ARRIVAL AND POST-ER QUALITY CONTROL

Once at the Golgi apparatus, GPI-APs dissociate from the p24 complex. It seems that at slightly acidic pH of Golgi, the conformation of p24 complex changes and this causes the dissociation of GPI-APs from the p24 complex (Figure 25) (Fujita et al., 2011).

Then, p24 proteins are recycled to the ER within COPI vesicles, because some p24 proteins also have in their cytosolic part signals for binding COPI subunits (Bremser et al., 1999; Gommel et al., 2001; Aguilera-

Romero et al., 2008). In yeast, it has been suggested that p24 proteins play a post ER quality control because they can contribute to the retention of GPI-APs which are not correctly remodelled or function in the retrieval of escaped unremodelled GPI-APs from the Golgi to the ER in COPI vesicles (Castillon et al., 2011). The interaction of the p24 complex with unremodelled GPI-APs could only be detected by cross-linking pull down experiments (Castillon et al., 2011).

4.5 EXPORT FROM THE *TRANS*-GOLGI NETWORK

After being fully glycosylated and remodelled during transport along the different cisternae of the Golgi apparatus, GPI-APs have to exit from the TGN in secretory vesicles that transport them to the plasma membrane (Muñiz and Riezman, 2016). In yeast, GPI-APs exit from the ER in different vesicles than the other secretory proteins, but it is still unknown whether they continue travelling separately along the Golgi stacks to the plasma membrane or if they mix in these Golgi stacks.

However, this process have been further studied in mammalian cells, especially in mammalian polarized cells, because in these kind of cells GPI-APs can go to the apical or basolateral face (Figure 26) (Rodriguez-Boulan et al., 2005). In neurons and epithelial cells, which are polarized, GPI-APs go dominantly to the apical face (Ledesma et al., 1998; Keller et al., 2001; Hua et al., 2006; Paladino et al., 2006); nevertheless there are exceptions to this rule and some GPI-APs in different epithelial cells can also be transported and sorted to the basolateral face (Zurzolo et al., 1993; Sarnataro et al., 2002).

In mammalian cells, GPI-APs are segregated from other secretory proteins in different secretory vesicles at the TGN that can take on different routes to the plasma membrane (Figure 26) (Weisz et al., 2009; Cao et al., 2012). The GPI anchor seems to act as an apical sorting signal at the TGN (Lisanti et al., 1989). Interestingly, the sorting of GPI-APs correlates with the acquisition of the two saturated long-chain fatty acids by the GPI anchor after the lipid remodelling and leads to the formation of DRMs (Tashima et al., 2006; Maeda et al., 2007).

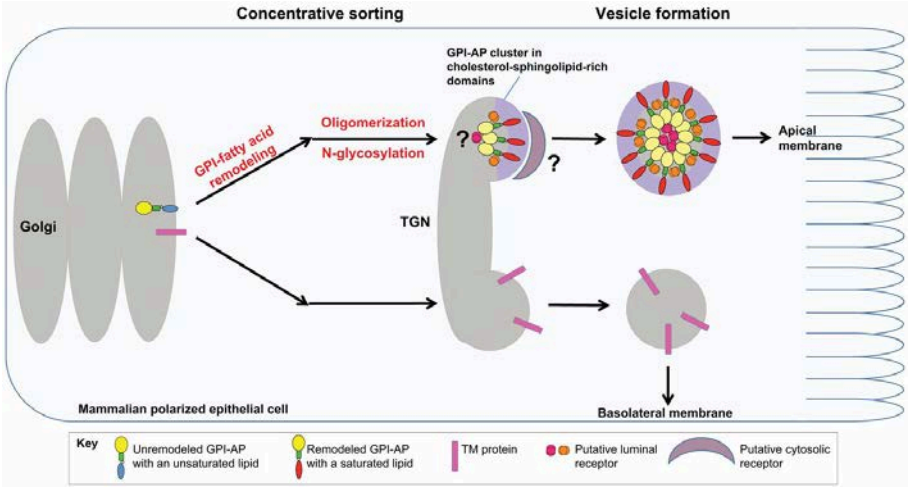

Figure 26. GPI-APs sorting upon TGN exit in polarized epithelial cells (Muniz and Zurzolo, 2014). Upon GPI-lipid remodelling with saturated fatty acid chains in the Golgi, GPI-APs can be segregated from other transmembrane proteins (TM) into sphingolipids and cholesterol-enriched domains. Further segregation would then occur as consequence of the oligomerization process. Vesicle formation and budding might derive from the coalescence of lipid domains that are driven by the protein oligomerization. Putative cytosolic receptors might also facilitate vesicle budding.

As we have introduced before, GPI-APs are principally sorted to the apical face. This seems to be dependent on their inclusion into sphingolipid and cholesterol-rich microdomains or rafts, which could act as apical sorting signal at the TGN (Simons and Ikonen, 1997). These rafts of GPI-APs have the feature to acquire resistance to detergent extraction (DRMs) (Brown and Rose, 1992; Zurzolo et al., 1994) after their complete lipid remodelling along the Golgi apparatus (Maeda et al., 2007; Fujita and Kinoshita, 2012). Besides, it has been studied that the removal of cholesterol or inhibitors of sphingolipid biosynthesis impairs their apical sorting (Mays et al., 1995; Lipardi et al., 2000; Paladino et al., 2004, 2014).

However, some GPI-APs are sorted to the basolateral face despite their association with DRMs (Zurzolo et al., 1993; Benting et al., 1999a; Sarnataro et al., 2002; Paladino et al., 2004), indicating that this is not the main mechanism for apical sorting of GPI-APs (Paladino et al., 2004). Nevertheless, it is postulated that the sorting of GPI-APs from the TGN is a lipid-based mechanism for the selective sorting in vesicles to the plasma membrane (Simons and Ikonen, 1997; Surma et al., 2012).

On one hand, GPI-APs have the intrinsic property to oligomerize, forming high molecular weight complexes at the Golgi apparatus (Paladino et al., 2004). This process has been identified as a requirement for apical sorting, because its impairment results in the missorting of GPI-APs to the basolateral face (Paladino et al., 2004, 2007). Oligomerization has been proposed to facilitate GPI-APs segregation from the other secretory proteins and favour their inclusion in specialized vesicles for the sorting to the apical plasma membrane (Paladino et al., 2004, 2014). This process is dependent

on lipid moiety remodelling of GPI-APs and their cholesterol and sphingolipids association forming the microdomains or rafts (Paladino et al., 2004; Seong et al., 2013). Interestingly, some specific GPI-APs which do not oligomerize, are transported to the basolateral face (Paladino et al., 2008).

On the other hand, the other process which seems to be involved in the apical sorting of GPI-APs is *N*-glycosylation (Benting et al., 1999b). Galectins have been proposed to recognize GPI-APs in a lectin receptor-based mechanism (Benting et al., 1999b) because they form oligomers which contain multivalent carbohydrate binding sites (Brewer et al., 2002). They are synthesized in the cytosol but are transported to the lumen of the TGN (Mishra et al., 2010), where due to galectin-carbohydrates interactions, it is supposed to induce clustering of glycoproteins and glycosphingolipids and facilitate vesicle formation at the TGN (Delacour et al., 2006; Mishra et al., 2010). It has been proposed that galectin 3 could interact with some GPI-APs in their apical sorting of the Golgi (Delacour et al., 2006). In favour of this hypothesis, some GPI-APs are modified with the addition of a N-acetylgalactosamine (GalNAc) residue to the glycan core (Ferguson et al., 2009), which could be recognized by these galectins.

Nevertheless, it is still unclear how these processes lead to sorting of GPI-APs for the apical or basolateral face and if at the TGN exists any specific transmembrane cargo-coat adaptor analogous to the p24 complex at the ER that facilitate the formation of vesicles for their transport from the ER to the Golgi apparatus.

4.6 GPI-APS AT THE PLASMA MEMBRANE

In yeast, which have cell wall as plants, glycoproteins are cross-linked to the glucans of the cell wall via *trans*-glycosylation, a process which is carried out by GPI-APs and is essential for correct development and growth. Most of these glycoproteins are GPI-APs, which are *N*- and/or *O*-glycosylated and are needed for yeast morphology and cell wall integrity (Bowman et al., 2006; Pittet and Conzelmann, 2007).

Sometimes, GPI-APs attached to the wall can be released after digestion of β-1,3-glucanase and β-1,6-glucanase suggesting that they are covalently linked to the polysaccharides of the cell wall (Fleet and Manners, 1977; Pettolino et al., 2012). This connection is made to β-1,6-glucan through EtNP and several mannose residues of the glycan core of GPI anchor (Kapteyn et al., 1997; Kollár et al., 1997; Fujii et al., 1999). Two enzymes have been identified in *Saccharomyces cerevisiae*, Dcw1p and Dfg5p, are involved in cleavage of the GPI anchor, allowing GPI-APs to be released to the cell wall (Kitagaki et al., 2002). Mutants of these two enzymes show important defects in cell wall integrity and the yeast viability is impaired (Kitagaki et al., 2002). In addition, it has been described that GPI anchors with C26:0 DAG determines cell wall destination. However, GPI-APs, whose fate is to remain to the plasma membrane, replace C26:0 DAG with ceramide. The molecular basis for this is still unknown (Yoko-o et al., 2018).

In mammals, several mechanisms have been proposed to release GPI-APs from the cell surface. The two classes of phosphatidylinositol-phospholipases (PI-PLs), PI-PLCs and PI-PLDs, can cleave the GPI anchor acting at different sites within the GPI anchor (Udenfriend and Kodukula,

1995). In mammals, PI-PLD has been reported to be involved in several cellular processes such as adhesion, differentiation, proliferation, survival and oncogenesis (Fujihara and Ikawa, 2016). Whereas PI-PLCs are known to act in unicellular organisms (Staudt et al., 2016), PI-PLCs can also cleave GPI-APs in mammals suggesting that the function of PI-PLCs may have been conserved (Song et al., 2006). The treatments with these enzymes support the idea that plasma membrane location of GPI-APs could be regulated by the action of PI-PLs (Borner et al., 2003; Lalanne et al., 2004).

In plants, *trans*-glycosylation of GPI-APs has not been reported yet; however, it is important for cell wall remodelling during growth that cell wall polysaccharides are cross-linked via *trans*-glycosylation (Franková and Fry, 2013). Although no clear Dcw1p and Dfg5p orthologues have been identified in plants, it cannot be excluded that the GPI anchor can link covalently to cell wall polysaccharides.

4.7 GPI-APS IN PLANTS

As introduced before, there are 248 predicted GPI-anchored proteins in *Arabidopsis thaliana* (Borner et al., 2003), a relatively large number compared with about 150 in mammals and 50 in the yeast *Saccharomyces cerevisiae* (Conzelmann et al., 1988). This means that approximately 1 % of plant proteins are predicted to be post-translationally modified with a GPI anchor, playing important roles in diverse plant biological processes focused at the interface of the plasma membrane and the cell wall including signalling, cell wall metabolism, cell wall polymer cross-linking and plasmodesmatal transport (Table 5) (Yeats et al., 2018).

Name	Gene	Family	Phenotype	References
Arabidopsis thaliana				
AGP4	AT5G10430	Classical AGP	Synergid degeneration	(Pereira et al., 2016)
AGP6 AGP11	AT5G14380 AT3G01700	Classical AGP	Collapse pollen development	(Levitin et al., 2008; Coimbra et al., 2009)
AGP17	AT2G23130	Lys-rich	Reduced biotic responses	(Nam et al., 1999; Gaspar, 2004)
AGP18	AT4G37450	Lys-rich	Defect in female game-togenesis and megaspore selection	(Acosta-Garcia, 2004; Demesa-Arevalo and Vielle-Calzada, 2013)
AGP19	AT1G68725	Lys-rich	Smaller growth	(Yang et al., 2007)
AGP24	AT5G40730	AG peptide	Deposition of Yariv-positive AGPs	(Stenvik, 2006)
FLA1	AT5G55730	Fasciclin-like AGP	Reduced shoot and regeneration in tissue culture	(Johnson et al., 2011)
FLA3	AT2G24450	Fasciclin-like AGP	Microspore development, cellulose deposition defect	(Li et al., 2010)
FLA4/SOS5	AT3G46550	Fasciclin-like AGP	Swollen root tips; salt sensitivity	(Shi et al., 2003; Xu et al., 2008b; Seifert et al., 2014; Xue et al., 2017)
FLA9	AT1G03870	Fasciclin-like AGP	Seed abor-tion under drought stress	(Cagnola et al., 2018)

FLA11 FLA12	AT5G03170 AT5G60490	Fasciclin-like AGP	Affected secondary cell wall mechanics and reduced cellulose	(MacMillan et al., 2010)
COBRA	AT5G60920	COBRA-like	Primary wall cellulose disorganized	(Schindelman et al., 2001)
COBRA-like 2	AT3G29810	COBRA-like	Seed coat mucilage reduced	(Ben-Tov et al., 2015)
COBRA-like 4/IRX6	AT5G15630	COBRA-like	Secondary wall cellulose affected	(Brown, 2005)
COBRA-like 9	AT5G49270	COBRA-like	Root hair development impaired	(Jones et al., 2006)
COBRA-like 10	AT3G20580	COBRA-like	Pollen tube impaired	(Li et al., 2013)
LORELEI	AT4G26466	Lorelei-like	Pollen tube reception	(Capron et al., 2008; Tsukamoto et al., 2010)
LLG1	AT5G56170	Lorelei-like	Rapid alka-lization factor (RALF) perception	(Li et al., 2015a)
ATBG_PPAP	AT5G42100	β-glucosidase	Control of symplastic connectivity	(Levy et al., 2007)
PdBG1 PdBG2	AT3G13560 AT2G01630	β-glucosidase	Control of symplastic connectivity, lateral root formation	(Maule et al., 2013)
A36 A39	AT5G36260 AT1G65240	Aspartic protease	Pollen and ovule development impaired	(Gao et al., 2017a, 2017b)

ENODL11 ENODL12 ENODL13 ENODL14 ENODL15	AT2G23990 AT4G30590 AT5G25090 AT2G25060 AT4G31840	Early-nodulin like/ plastocyanin	Pollen tube reception	(Huang et al., 2016)
LTPG	AT1G27950	Lipid-transfer protein	Cuticular wax export	(Jetter et al., 2009)
LTPG2	AT3G43720	Lipid-transfer protein	Cuticular wax export	(Kim et al., 2012)
LYM1 LYM3	AT1G21880 AT1G77630	Lysin-motif domain containing	Peptidogly-can sensing	(Lajunen et al., 2011)
NDR1	AT3G20600	Late embryo-genesis abun-dant (LEA) hydroxyprol-ine rich glycolprotein family	Mediates salicylic acid-mediated res-ponse (SAR), possibly double-anchored	(Coppinger et al., 2004)
PMR6	AT3G54920	Pectate-lyase-like	Powdery mildew resistance	(Vogel et al., 2002)
SHAVEN3	AT4G26690	SHAVEN3-like	Root hair defective	(Jones et al., 2006)
SHAVEN3-like 1	AT5G55480	SHAVEN3-like	Cellulose-deficient and sucrose-sensitive (in combination with SHV3)	(Wada et al., 2008; Yeats et al., 2016)
SKU5	AT4G12420	Skewed 5-like/cupre-doxin-like	Root skewing	(Sedbrook et al., 2002)
XYP1 XYP2	AT5G64080 AT2G13820	Lipid-transfer protein (xylogen)	Promotion of xylem diffe-rentiation, vascular development defects in mutant	(Motose et al., 2004)
ZERZAUST	AT1G64760	Beta-1,3-glucanase	Aberrant cell morphogene-sis	(Vaddepalli et al., 2017)

Oryza sativa				
SRL1/CLD1	Os07g01240	Unknown	Curled leaves, dwarf, reduced cellulose, enhanced water loss	(Xiang et al., 2012)
BRITTLE CULM 1	Os03g30250	COBRA-like	Secondary cell wall synthesis	(Li et al., 2003; Liu et al., 2013)
Zea mays				
BRITTLE STALK 2		COBRA-like	Secondary cell wall synthesis	(Ching et al., 2006; Johal et al., 2007)

Table 5. **Plant GPI-anchored proteins and their respective mutant phenotypes** (Adapted from Ellis et al., 2010 and Yeats et al., 2018).

Some important functions of GPI-APs have been found out studying some proteins, such as LORELEI family in the pollen tube-female gametophyte interaction (Capron et al., 2008; Tsukamoto et al., 2010; Liu et al., 2016b), the COBRA family in cell expansion and cell wall biosynthesis (Li et al., 2013) and the ARABINOGALACTAN proteins in megagametogenesis (Ellis et al., 2010; Demesa-Arevalo and Vielle-Calzada, 2013). This last family of proteins are predicted to be approximately the 40 % of plant GPI-APs encoding genes (Borner et al., 2002, 2003). Here are summarized some important families of GPI-APs in plants:

LORELEI family proteins

The success of plant reproduction depends on a series of cell to cell interactions between the male and female gametophytes (Kessler and Grossniklaus, 2011; Palanivelu and Tsukamoto, 2012; Beale and Johnson,

2013; Qu et al., 2015). The LORELEI proteins are encoded by three genes, which are highly expressed in synergid cells of the embryo sac (ovules) but not in the pollen or pollen tubes (Tsukamoto et al., 2010), and are involved in regulating pollen tube reception (Liu et al., 2016b).

A mutant of one of these proteins showed an impairment of fertilization in the embryo sac caused by the inability of the pollen tube to release the sperm cells upon arrival to the mutant embryo sac (Capron et al., 2008; Tsukamoto et al., 2010). As a consequence, the pollen tube experienced a continuous growth, resulting in an invasion of the embryo sac and consequently, the fertilization was prevented (Capron et al., 2008; Eckardt, 2008).

COBRA family proteins

These proteins are required for the oriented deposition of cellulose microfibrils in order to manage the cell expansion during plant morphogenesis (Roudier, 2005). The expression of COBRA family proteins focus in the root and specially in the more differentiated parts of the root (Schindelman et al., 2001). It is aligned in narrow bands perpendicular to the longitudinal axis in cells undergoing rapid elongation, a pattern which depends on cortical microtubule organization (Gendreau et al., 1997; Refregier, 2004). Mutants of these proteins showed severe growth defects (Roudier, 2005).

It has been reported that there are two mechanistically distinct growth phases during the expansion of leaf pavement cells (Fu, 2002). A mutant of COBRA protein used by Roudier et al. (2005) showed that this

protein is required for the second phase of growth, characterized by extensive cell growth and not for the initial morphogenesis of the different epidermal cell types. In this mutant, leaves of the epidermis were small, with roughly isodiametric shapes, which suggests that the ability of the cells to expand anisotropically is lost (Roudier, 2005).

COBRA proteins are modified by a GPI anchor and *N*-glycosylation and localize to compartments of the secretory pathway and at the plasma membrane (Borner et al., 2003; Foster et al., 2003; Lalanne et al., 2004). They are also detected at the cell wall, suggesting that during anisotropic expansion, the protein is released from the GPI anchor by a PI-specific phospholipase (Sharom and Lehto, 2002; Mayor, 2005) and can regulate the activity of this protein at the cell surface (Roudier, 2005).

ARABINOGALACTAN family proteins

As reported before, it has been postulated that up to 40 % of Arabidopsis GPI-APs are predicted to be ARABINOGALACTAN proteins (AGPs) (Borner et al., 2003). The roles that these proteins play are diverse, including plant growth and development as biological regulatory molecules. AGPs localize mostly to the plasma membrane, but they are also in the cell wall, apoplastic space and in secretions (stigma surface and wound exudates). The high degree of heterogeneity is a property of the complexity of both the carbohydrate structure and the protein backbone, due to this fact they have been proposed to act as ligands in signalling pathways (Ellis et al., 2010).

The complexity in the structure of the AGPs is due to the incredible diversity of glycans attached to the protein core, the peripheral sugars

decorating the large arabinogalactan chains, the degree of glycosylation of the structure and the diversity of the protein module (Figure 27). Several studies have suggested that the glycan chains are important for AGP function (Gaspar et al., 2001; Showalter, 2001; Seifert and Roberts, 2007; Ellis et al., 2010).

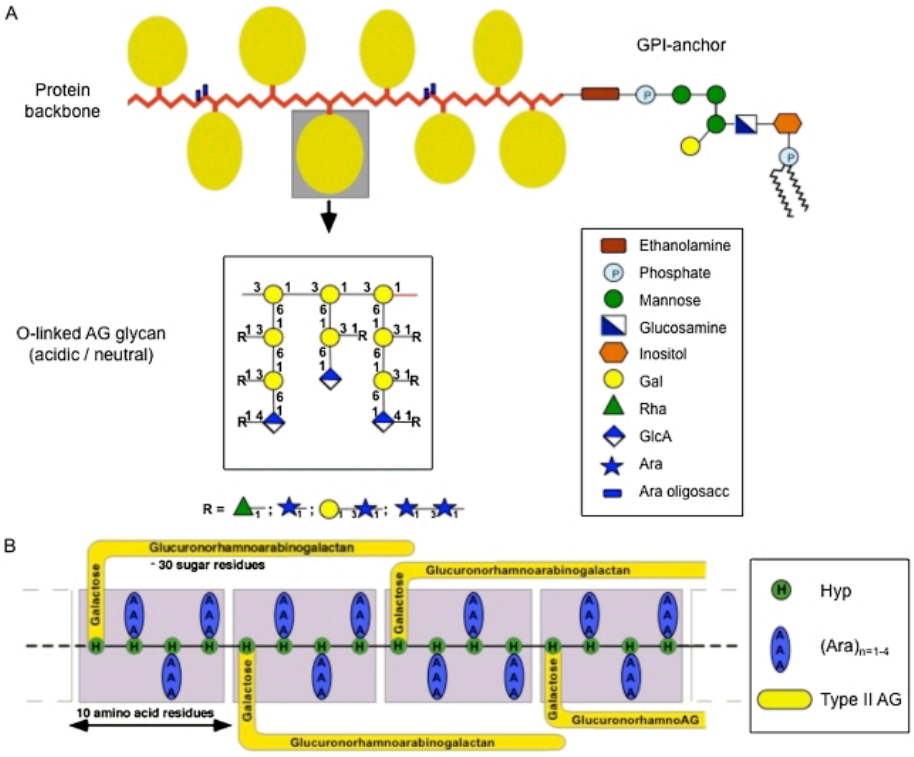

Figure 27. Model structure of AGPs with a GPI membrane anchor attached (Adapted from Ellis et al., 2010). A, In this model of AGP there are approximately 25 Hyp residues, most of these are non-contiguous and are predicted to bear an AG chain. Each AG chain may contain 15 or more repeats of a β-(1-3)-linked Gal oligosaccharide. The molecule as a whole is spheroidal. The structure of the GPI anchor is based from Oxley and Bacic, 1999. B, The twisted hairy rope model of the structure of the GAGP. A hypothetical block size of 7 kD contains 10 amino acid residues (1 kD), 30 sugar residues (4.4 kD), and three Hyp-triarabinosides (1.32 kD). This model is based from Qi et al., (1991).

There are some key features of the AGPs that confer their specificity and properties (Ellis et al., 2010): The carbohydrate part, *O*-linked to the Hydroxiproline (Hyp) residues of the protein core, constitutes 90 to 98 % of weight, while the protein backbone constitutes 2 to 10 % of the weight and this part is rich in Hyp/Pro, Ala, Ser and Thr; they are attached to a GPI anchor; and finally, most of AGPs have the ability to bind a class of synthetic chemical dyes (Yariv reagents) (Yariv et al., 1967).

Several roles have been suggested to this family of proteins due to their heterogenous nature. Some of these proteins are involved in somatic embryogenesis (Van Hengel et al., 2002), root growth and development (Van Hengel and Roberts, 2003), signalling (Schultz et al., 1998), resistance to *Agrobacterium tumefaciens* mediated infection (Gaspar, 2004), hormone responses (Park, 2003), cell wall plasticity (Lamport et al., 2006), salt tolerance (Shi et al., 2003; Lamport et al., 2006), xylem differentiation (Motose et al., 2004), initiation of female gametogenesis (Acosta-Garcia, 2004), promotion of pollen tube growth and guidance (Cheung et al., 1995; Wu et al., 1995, 2000; Mollet et al., 2002; Lee et al., 2008), cell expansion (Lee, 2005; Yang et al., 2007), secretion (Xu et al., 2008a), programmed cell death (Gao and Showalter, 1999), pollen grain development (Monteiro et al., 2005; Levitin et al., 2008; Coimbra et al., 2009) and self-incompatibility in pollen (Lind et al., 1996; Cruz-Garcia et al., 2005; McClure et al., 2008; Lee et al., 2008).

It has also been suggested that the GPI anchor of the AGPs could interact with plasma membrane bound receptors kinases while the soluble forms of the AGPs could also interact with receptors in neighbouring cells.

The Fasciclin-like AGPs (FLAs) have been proposed to be involved in such interactions because of having fasciclin-like domains (Johnson, 2003). One protein of this subfamily implicated in this kind of interactions could be FLA4/SOS5 (Xu et al., 2008b).

Sometimes, the function of the GPI-APs can be regulated by enzymatic cleaving of the GPI anchor, allowing the GPI-APs at the plasma membrane to diffuse into the extracellular space. Therefore, this kind of proteins can have additional functions like cross-linking of polysaccharides and glycoproteins at the cell wall (Kondoh et al., 2005; Watanabe et al., 2007; Fujihara et al., 2013; Park et al., 2013).

The importance of the GPI-APs in plants is deduced by the fact that losing of the GPI anchor in plants causes lethality, both embryogenic and gametophytic as indicated before. For instance, Gillmor et al., (2005) reported that a mutant of the *Arabidopsis* homolog of the mammalian PIG-M (PNT1), a mannosyltransferase enzyme localized at the ER which is required for the synthesis of the GPI anchor, showed defects in cell wall synthesis in the embryo and was lethal.

GPI-APs in plants can also play relevant roles associating with other structures of the cell for maintenance and signalling, such as:

4.7.1 Association of GPI-APs with plasmodesmata

Plasmodesmata (PD) are plasma membrane channels in plant cells that traverse the cell wall and connect adjacent cells to enable symplastic transport of RNA, soluble proteins and solutes. Therefore, PDs contain specific plasma membrane proteins which generate a curved membrane

organization (Mongrand et al., 2010; Bayer et al., 2014), and the cell wall lacks cellulose and is rich in pectins and callose (Knox and Benitez-Alfonso, 2014). Callose is deposited in the neck regions of PDs to structurally constrict the PD aperture, and therefore inhibit the molecular traffic.

The lipid composition of PDs is enriched in sterols and sphingolipids, with very long chain saturated fatty acids (Grison et al., 2015). Therefore, microdomains are formed in these regions to restrict lateral movement and segregate PD proteins from the rest of the plasma membrane (Raffaele et al., 2009; Simpson et al., 2009; Fernandez-Calvino et al., 2011).

Two GPI-APs were found in PDs, Callose Binding 1 (PDCB1) and β-1,3-glucanase (PdBG2) (Grison et al., 2015), which depend on the GPI anchor in order to localize to PDs (Grison et al., 2015; Zavaliev et al., 2016). Interestingly, the GPI anchor of two non-PDs GPI-APs, AGP4 and LTGP1, is able to target a reported protein to PD (Zavaliev et al., 2016). These proteins are predicted to provide a link between the PD and the cell wall, which is important for PD opening and for restricting lateral diffusion within the plasma membrane and PDs (Yeats et al., 2018).

There are also GPI-APs at the PDs that have important roles in defence responses against fungal pathogens. One of these proteins is the GPI-AP Lys motif domain 2 (LYM2), which is a chitin receptor-like protein responsible for changing the molecular flux through the PD upon chitin perception (Faulkner et al., 2013). This protein can bind chitin oligosaccharides and acts independently of the receptor kinase for chitin signalling CERK1 (Shinya et al., 2012; Faulkner et al., 2013). Therefore, this

component is essential for mediating cell to cell communication through PD during pathogen perception.

4.7.2 GPI-APs and cell wall biosynthesis, maintenance and signalling

GPI-APs that act to modify cell wall polymers in yeast are well characterized (Martínez-Núñez and Riquelme, 2015). In plants, GPI-APs also act modifying cell wall polymers like callose, xyloglucan and cellulose. Therefore, GPI-APs are really important modulating the synthesis and remodelling of the polysaccharides that form the cell wall.

The main component of the cell wall in plants is cellulose, which form strong microfibrils composed of 18-24 β-(1,4)-glucan chains. These microfibrils are synthesized by cellulose synthases (CESAs) at the plasma membrane, which are also associated with other protein complexes that regulate the activity of CESAs or the crystallization of the microfibrils (Richmond and Somerville, 2000; Taylor et al., 2000). Besides, the *S*-acylation of CESAs and their hydrophobicity provide a specialized membrane environment (Konrad and Ott, 2015; Kumar et al., 2016).

The complexes that regulate CESAs activity contain GPI-APs such as COBRA-like family (Hemsley et al., 2013), which regulate the deposition of cellulose into cell wall (Li et al., 2013; Ben-Tov et al., 2015) and microfibrils crystallinity (Liu et al., 2013). They also regulate the secondary cell walls providing rigidity (Zhong and Ye, 2015), and therefore are implicated in wood and fibre quality (Gritsch et al., 2015; Niu et al., 2015). The GPI-APs FLA11 and FLA12 can influence the microfibril angle of cellulose in secondary walls (MacMillan et al., 2010, 2015).

Two putative GPI-APs aspartic proteases, A36 and A39, also colocalize with COBRA proteins (Gao et al., 2017a, 2017b). In yeast, it has been reported that aspartic proteases have a role in cell wall integrity and remodelling (Krysan et al., 2005; Kaur et al., 2007).

Some GPI-APs are implicated in defence against fungal pathogens, by modulating the cell wall and limiting penetration. The protein PMR6 is a GPI-AP pectate lyase protein involved in pectin degradation and potentially release pectin oligosaccharides in order to defend against the fungal pathogen (Vogel et al., 2002; Engelsdorf et al., 2017).

As indicated before, GPI-APs may also play an important role in signalling as the LORELEI family (Liu et al., 2016b). These family proteins seem to interact with FERONIA (FER) protein, a receptor-like kinase implicated in cell wall integrity sensing, suggesting that they may act in the same pathway (Huck et al., 2003; Wolf and Höfte, 2014; Li et al., 2015a).

Recently, LORELEI protein has been shown to form complexes with FLAGELLIN SENSING 2 (FLS2), BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) and the elongation factor Tu receptor (EFR) in order to regulate the response to pathogen associated molecular patterns (PAMPs) (Shen et al., 2017).

The role of GPI-APs in pollen tube is also exemplified with ENOD-like 14 proteins (ENDOL/EN14), which interact also with FER. These proteins have a plastocyanin-like domain, arabinogalactan glycomotifs and GPI anchor, and are expressed in ovules and accumulate at the filiform apparatus (Huang et al., 2016).

Finally, in the seed coat, the GPI-AP FLA4/SOS5 is proposed to act in the same pathway as two leucine rich receptor like kinases, FER1 and FER2 (Basu et al., 2016; Showalter and Basu, 2016). It is suggested that they physically interact through the arabinogalactans glycans (Basu et al., 2016), which play a function as soluble extracellular factor as a ligand for receptor-like kinases (RLKs) (Xue et al., 2017).

OBJECTIVES

OBJECTIVES

GPI-anchored proteins (GPI-APs) are a family of proteins which are attached to the outer face of the plasma membrane by a GPI anchor. There are 250 predicted GPI-anchored proteins in *Arabidopsis*, approximately 10 % of secretory proteins and are involved in important functions such as signal transduction, cell-cell interactions, growth, host defence and cell wall biosynthesis. However, the molecular machinery involved in transport of GPI-APs to the plasma membrane is essentially unknown in plants.

GPI-APs are synthesized at the ER and, in mammals and yeast, ER export of GPI-APs requires p24 proteins. p24 proteins constitute a family of proteins which localize to the compartments of the early secretory pathway, including the ER and the Golgi apparatus, and to COPI- and COPII-coated vesicles. They play an important role in quality control during transport between ER and Golgi, possibly as cargo receptors. Several cargoes have been proposed for p24 proteins, including G-protein-coupled receptors, the K/HDEL receptor ERD2 and GPI-APs.

In mammals and yeast it has also been described that during their transport from the ER to the plasma membrane, GPI-APs undergo lipid remodelling of their GPI anchor, which is required for their efficient transport along the secretory pathway.

The main objective of this work is to characterize the transport to the plasma membrane of GPI-APs in *Arabidopsis*.

OBJECTIVES

Specific objectives:

1. To investigate whether *Arabidopsis* p24 proteins from the delta-1 subclass are implicated in ER export and plasma membrane localization of GPI-anchored proteins.

2. To study the role of the *Arabidopsis* enzymes PGAP1 and PER1L, which may be involved in the lipid remodelling of the GPI anchor, in the transport of GPI-APs from the ER to the plasma membrane.

MATERIAL AND METHODS

1 BIOLOGICAL MATERIAL

1.1 MICROORGANISMS

1.1.1 Growth of *Escherichia coli*

Escherichia coli DH5α strain (Invitrogen®) was incubated at 37°C and 220 rpm in liquid LB (Luria-Bertani) (Bertani, 1951) medium or solid LB medium (1 % (w/v) tryptone, 0.5 % (w/v) yeast extract and 1% (w/v) NaCl, pH 7; for solid medium 1.5 % (w/v) bacteriological agar (Pronadisa®) was added. Media for selection of transformants was supplemented with antibiotic (100 µg/mL ampicillin).

1.1.2 Growth of *Agrobacterium tumefaciens*

Agrobacterium tumefaciens C58 MP90 strain (Koncz and Schell, 1986) was incubated at 28°C and 220 rpm in liquid or solid LB medium plus 25 µg/mL gentamicin and 100 µg/mL kanamycin or 100 µg/mL spectinomycin, to ensure the presence of the Ti plasmid and T-DNA sequence of interest (when using T-DNA vectors with the kanamycin or spectinomycin resistance genes), respectively, to select and grow transformants.

1.2 PLANTS

1.2.1 *Arabidopsis thaliana*

1.2.1.1 Insertion mutants

The following table shows the *Arabidopsis* insertion mutants used in this work:

Mutant (Gene identifier)	Mutant (ID number*)	Ecotype	Origin
p24δ3 (AT1G09580)	GK_029E10	Col-0	GABI-KAT project (Kleinboelting et al., 2012)
p24δ4 (AT1G57620)	SAIL_664_A06	Col-0	SAIL collection (Sessions et al., 2002)
p24δ5 (AT1G21900)	SALK_016402C	Col-0	SALK collection (Alonso et al., 2003)
p24δ6 (AT3G10780)	GK_823G03	Col-0	GABI-KAT project (Kleinboelting et al., 2012)
p24δ3δ4δ5δ6		Col-0	Obtained by Pastor-Cantizano et al., 2017
pgapA-1 (AT2G44970)	SALK_067058	Col-0	SALK collection (Alonso et al., 2003)
pgap1A-2 (AT2G44970)	SALK_072702	Col-0	SALK collection (Alonso et al., 2003)
pgap1B-1 (AT3G27325)	SALK_078662	Col-0	SALK collection (Alonso et al., 2003)
pgap1B-2 (AT3G27325)	SAIL_1212_H07	Col-0	SAIL collection (Sessions et al., 2002)
pgap1C-1 (AT3G52570)	SAIL_302_A06	Col-0	SAIL collection (Sessions et al., 2002)
per1lA-1 (AT5G62130)	SALK_039375	Col-0	SALK collection (Alonso et al., 2003)
amiR-per1lB (AT1G16560)		Col-0 *per1lA-1*	Obtained in this work

Table 6. *Arabidopsis* insertion mutants

* All seeds were provided by NASC (Nottingham *Arabidopsis* Stock Centre)

* amiRNA technology was described by Ossowski et al. (2008).

1.2.1.2 Growth conditions in soil

Seeds were suspended in 1 mL distilled H_2O and kept in darkness for 2 days at 4°C to synchronize germination. Then, they were sown in plastic pots containing a mixture of compost:perlite:vermiculite (2:1:1). Two sizes of plastic pots were used, pots of 6 cm diameter (one plant per pot) to perform phenotypic analysis or to obtain protoplasts, or pots of 15 cm diameter (25 plants per pot) to transform the plants. After sowing, pots were covered with plastic film the first five days to maintain high humidity during germination and to prevent contamination of seeds from other plants nearby.

Plants were grown in the greenhouse or chambers under controlled conditions of temperature, 22°C, and 16h/8h photoperiod with 16 hours of white, cold and fluorescent light (150 µE m^{-2} s^{-2}, Sylvania Standard F58W/133-T8), watering them manually by immersion in distilled water twice a week.

1.2.1.3 Growth conditions in Petri dishes

Arabidopsis was cultured *in vitro* in culture chambers in Murashige and Skoog (MS) medium with agar, which contains 2.2 g/L MS salts (Duchefa®), 10 g/L sucrose, 0.1 g/L 2-(N-Morpholino) ethanesulfonic acid (MES), pH 5.9 and 0.6 % (horizontal oriented plates) or 1 % (vertical oriented plates) phytoagar.

Seeds were sterilized by immersion for 3 minutes in 70 % (v/v) ethanol and 0.05 % (v/v) Triton X-100 (TX-100), and for one minute in 96 % (v/v) ethanol. Then, seeds were left until they got dry over sterile Whatman™ paper at the laminar flow hood. After sowing them, plates were put for 2

days at 4°C and kept in darkness to synchronize germination and then they were moved into the growth chamber.

To select homozygous lines by segregation analysis, the seeds of the different insertion mutants were plated in Petri dishes containing selection medium (MS with antibiotic resistance).

1.2.1.4 Crosses of different transgenic lines

The technique to cross different *Arabidopsis* plants consist in rubbing gently the convex surface of the anthers from the male parent against the stigmatic surface of an exposed carpel on the female parent.

For the different crosses between single, double mutants, selection of F1 and F2 progeny was performed by PCR (section 4.2.3 of Materials and methods) using genomic DNA as a temple and specific primers of the different genes and their corresponding insertions (Table 9).

1.2.1.5 Isolation of *Arabidopsis* protoplasts

To obtain mesophyll protoplasts from *Arabidopsis* plants, the Tape-*Arabidopsis* Sandwich method was used as described previously by (Wu et al., 2009). In this protocol, two kinds of tape (Autoclave tape adhered to the upper epidermis and 3 M magic tape (Trademark Scotch®) to the lower epidermis) were used to make a "sandwich".

3-4 week rosette leaves were adhered to the autoclave tape with the abaxial surface down during manipulation. Then, the 3 M magic tape was adhered to the abaxial surface of the leaves. Tearing off the 3 M magic tape allowed easy removal of the lower epidermal layer and exposed mesophyll

cells to cell wall digesting enzymes. The autoclave tapes containing the leaves were incubated in an enzyme solution [1.5 % Cellulase R10 (Yakult Pharmaceutical®, Japan), 0.4 % Macerozyme R10 (Yakult Pharmaceutical®, Japan), 0.4 M mannitol, 20 mM KCl, 20 mM MES, 10 mM $CaCl_2$, 5 mM β-mercaptoethanol and 0.1 % Bovine serum albumin (BSA), brought to pH 5.7 with KOH] for 2 h at room temperature (RT) with gentle shaking.

After digestion, the suspension was filtered through a 100-µm nylon mesh and briefly washed of the cell debris to release further protoplasts from the tissue remnants with W5 medium which contains 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl and 2 mM MES and pH 5.7. The protoplast suspensions were then centrifuged in Falcon tubes (50 mL) for 5 min at 124 xg and 4°C. Protoplasts were washed twice with W5 medium and the protoplast pellets were used to PEG transformation.

1.2.2 *Nicotiana benthamiana*

Wild-type *Nicotiana benthamiana* plants were grown from surface-sterilized seeds on soil in the greenhouse at 24°C with 16 h daylength. Plants were everyday watered.

2 TRANSFORMATION PROCEDURES

2.1 TRANSFORMATION OF *Escherichia coli*

MAX Efficiency® DH5α™ competent cells from Invitrogen™ (Ref. #18258012) were used. Heat shock transformation was performed according to the manufacturer's protocol.

2.2 TRANSFORMATION OF *Agrobacterium tumefaciens*

Competent cells were prepared growing *A*. *tumefaciens* in liquid LB plus gentamicin (25 μg/mL) until an OD_{600} of 0.5-1.0 was reached. Then, the cells were collected and resuspended in 20 mM $CaCl_2$, as described previously (Weigel and Glazebrook, 2002). *A. tumefaciens* transformation was performed using the freeze-thaw method. Competent cells were incubated with 1 μg plasmid DNA for 5 minutes at 0°C. Then, they were transferred to liquid nitrogen for 5 minutes and after, they were incubated for another 5 minutes at 37°C. Finally, 1 mL of LB was added and the cells were incubated 3-4 hours at 28°C and 220 rpm. The cells were plated in solid LB medium with the antibiotic resistance of the plasmid used and incubated for 2 days at 28°C until colonies appeared.

Colony PCR was performed to identify *A. tumefaciens* colonies containing the plasmid of interest. A transformant colony was taken with a yellow tip and was spread vigorously inside a sterile PCR tube for 30 seconds to run a hot start PCR reaction (as described in section 4.2.3 of Materials and Methods). Then, the yellow tip was introduced in a culture tube containing 2

ml of LB medium with antibiotic and it was incubated 1-2 days at 28°C and 220 rpm to obtain a culture and store it at 4°C for further use.

2.3 TRANSIENT GENE EXPRESSION OF *Arabidopsis* PROTOPLASTS BY PEG TRANSFORMATION METHOD

For transient expression by PEG (polyethylene glycol) transformation method, the protocol described by Yoo et al., 2007 was followed. Protoplasts isolated from *Arabidopsis* rosette leaves (of wild-type, *p24δ3δ4δ5δ6*, *pgap1B-1*, *pgap1ABC*, *per1lA-1*, *per1lB-2* and *per1lAB-2*) (section 1.2.1.5 of Materials and methods) were washed with 40 mL of W5 pH 5.7 medium and collected by centrifugation at 124 xg and 4°C for 5 minutes.

For transformation, protoplasts were resuspended in 1 mL of W5 medium and incubated 30 minutes on ice. An aliquot of the protoplast suspension was taken and used to calculate the concentration of protoplasts (protoplasts/mL). Then, protoplasts were collected by centrifugation (at 124 xg and 4°C and for 5 min) and resuspended in MMG solution (0.4 M mannitol; 15 mM $MgCl_2$; 4 mM MES) to $5 \cdot 10^5$ cells/mL. 200 µl of protoplasts suspension was mixed with 50 µl of DNA(s) and 250 µL of PEG solution (0.1 M PEG 4000; 0.2 M mannitol; 80 mM $CaCl_2$) was added. After 5 minutes of incubation, 5 mL of W5 was added. Protoplasts were collected as described previously and washed twice with W5. Finally, transformed protoplasts were resuspended in 1 W5 and incubated for 16 hours at 25°C and darkness. Protoplasts were then analyzed by confocal laser scanning microscopy (CLSM), as described in section 6.1, used to perform pull-down assays, as described in section 5.5; or used to perform protein analyzes, as described in section 5.2.

The plasmids of the constructs used in this procedure were: pUC, pDH51 and pBP30.

2.4 AGROINFILTRATION OF *Nicotiana benthamiana* LEAVES

Two days before the agroinfiltration, fresh cultures of LB medium with correspondent antibiotics were prepared from the stock. Then, 10 mL of LB medium (containing the corresponding antibiotics) was inoculated with the fresh culture prepared from stock and incubated overnight under same conditions. Finally, the culture was collected by centrifugation (1525 xg, 15 min, room temperature) and the bacteria pellet was resuspended in 1 mL of water to measure the concentration of the *Agrobacterium* in the solution. A final OD of 0.6 for one construct was used and an OD of 0.3 of each marker was used when two different markers were used at the same time.

Once the solutions of *Agrobacterium* were prepared, *Nicotiana benthamiana* (*Nicotiana*) plants from the greenhouse were used (section 1.2.2 of Materials and Methods). *Nicotiana* plants were watered and humidified gently during 20 minutes for opening the stomatas of abaxial surface of the leaves. After this time, *Nicotiana* leaves were agroinfiltrated carefully with a syringe and kept them into darkness for three days. Finally, the leaf space infiltrated was analyzed to the confocal (section 6.1) or for pull-down (section 5.5) and Western Blot experiments (section 5.8).

2.5 TRANSIENT TRANSFORMATION OF *Arabidopsis thaliana* SEEDLINGS BY VACUUM INFILTRATION

This protocol was adapted from the protocol described by Marion et al., 2008. *Arabidopsis* seeds were sowed into small plates containing 4 mL of MS without sucrose. The plates were stored at 4°C in the dark for 2-4 days to stratify the seeds and synchronize germination. After this, plates were placed into the growth chamber for 4 to 5 days for getting a germinated and expanded cotyledons from the seedlings.

For preparation of *Agrobacterium* cultures used for agroinfiltration, the desired *Agrobacterium* was inoculated into 2.5 mL of LB containing the appropriate antibiotics. This culture was grown overnight at 28C in a shaking incubator. Next, the day before the experiment, it was inoculated 50 mL of LB containing the appropriate antibiotics with 0.5 mL of the 2.5 mL pre-culture. This culture was grown in the same conditions as indicated before.

Once the *Agrobacterium* culture reaches an OD around 2.2, it was centrifuged 30 mL of *Agrobacterium* overnight culture for 15 min at 6000 xg (RCF) at room temperature. The medium was removed and kept the bacterial pellet, which was resuspended with 2 mL of liquid MS medium. The bacterial suspension was measured in order to get the bacterial suspension OD. Then, the infiltration buffer was prepared with liquid MS at room temperature with 0.005 % Silwet L-77® and 200 µM acetosyringone (3',5'-Dimethoxy-4'-hydroxyacetophenone, 97 %, Fisher®, Ref. #115540010). The *Agrobacterium* suspension was diluted with the infiltration buffer to have an OD of 2. The *Agrobacterium* infiltration-buffer was kept for 30-45 min at room

temperature to let the acetosyringone be assimilated by the *Agrobacterium* and activate its virulence genes.

The *Agrobacterium* infiltration-buffer was placed onto the plates with the *Arabidopsis* seedlings, making sure that all seedlings were covered by the buffer. Plates were placed into the vacuum desiccator and vacuum was applied (300 mbar) with the help of a manometer for one minute. After, the pressure was increased slowly and applied the vacuum again for another minute. The *Agrobacterium* solution was removed from the plates and these were placed back to the growth chamber and covered with aluminium foil for 45 min-1 h. After 3 days, the healthy seedlings were selected and imaged the cotyledons by the abaxial side on the confocal microscope.

2.6 *Arabidopsis* STABLE TRANSFORMATION BY FLORAL DIP METHOD

For the generation of transgenic plants expressing the amiRNA of the gene PER1LB, wild-type (ecotype Columbia, Col-0) and *per1lA-1* plants were used. For the generation of transgenic plants expressing RFP-p24δ5 it was used the mutant *p24δ3δ4δ5δ6*. The transformations were performed following the protocol described by Clough and Bent, 1998. Approximately, 25 seeds of wild-type, *per1lA-1* or *p24δ3δ4δ5δ6* were sown and cultured for 5 to 6 weeks in pots, as described in section 1.2.1.2 of Materials and Methods. The first inflorescence shoots were removed as soon as they emerged, to promote secondary inflorescences development.

Three days before transformation, a culture of 10 mL LB medium (containing the corresponding antibiotics) inoculated with an *Agrobacterium*

strain carrying the construction of interest was incubated as described in section 1.1.2. Then, 600 mL of LB medium containing the corresponding antibiotic was inoculated with 6 mL of the preculture and incubated overnight under same conditions. Finally, the culture was collected by centrifugation (6000 xg, 20 minutes, room temperature) and the bacteria pellet was resuspended in 600 mL of infiltration medium (5 % (w/v) sucrose and 0.05 % (v/v) Silwet L-77®) with a final OD_{600} of 0.8.

The pots containing the plants were inverted and all the plants were immersed in the suspension of *Agrobacterium* in infiltration medium for 2 min with gentle shaking. Then, the pots were placed horizontally on trays which were covered with plastic film and a sheet of paper to avoid excess of light and were placed into the growth chamber. After 24 hours, the covers were removed and the pots with the transformed plants were placed as usual, letting plants to grow until the end of their reproductive cycle, when the seeds were harvested.

To select primary transformants, T1 seeds were sown on Petri dishes with MS medium supplemented with the corresponding antibiotic according to the antibiotic resistance gene of the construct (Table 8). After 7-10 days from sowing, transformants could be clearly distinguished by their green colour and developed roots. Selected plants were transferred into soil for growth under conditions described in section 1.2.1.2 of Materials and Methods. Secondary transformants from T2 seeds that showed a 3:1 (resistant:senstitive) ratio when grown in MS plus antibiotic plates were selected and at least 6 seedlings resistant to the corresponding antibiotic were transferred to soil. Finally, T2 transformants with seed that showed 100

% resistance to the corresponding antibiotic were selected as homozygous plants.

Selection of homozygous transformants lines was analyzed by RT-qPCR to test the silencing of the *PER1LB* gene of each line. The lines which were more silenced were chosen.

2.6.1 Segregation analysis of transgenic lines

To estimate the number of *loci* in which amiRNA and RFP-p24δ5 has been inserted in the different primary transformants of *A. thaliana*, 40 seeds from individual T1 plants were sown after sterilization and grown in MS solid medium supplemented with the corresponding antibiotic, as described in section 1.2.1.3 of Materials and methods. The counting of green and white seedlings, resistant or sensitive to the antibiotic, respectively, was performed 7-10 days after sowing. T2 homozygous and heterozygous plants were identified by analysing the T3 generation with the same technique.

To analyze segregation data of the corresponding antibiotic resistance in the progeny of the different T1 plants, the null hypothesis (H0) were that the data were compatible with a 3:1 segregation (resistant:sensitive), which corresponds with an unique insertion of the T-DNA in a locus or with a 15:1 segregation, which corresponds to the insertion of the T-DNA in two *loci*. The alternative hypothesis (H1) was that H0 was not true, setting as categories resistant and sensitive plants to the corresponding antibiotic.

To analyze segregation data of the corresponding antibiotic resistance in the progeny of the selected T2 transgenic plants with an unique

insertion, the null hypothesis (H0) were that the data were compatible with 40:0 segregation (resistant:sensitive), which corresponds with an homozygous line or with a 3:1 segregation, which corresponds with an heterozygous line. The alternative hypothesis (H1) was that H0 was not true, setting as categories resistant and sensitive plants to the corresponding antibiotic

The Chi-square (χ^2) statistical test was used to determine how well our sets of segregation data fit this particular hypothesis (H0). The formula is:

$$\boldsymbol{\chi^2 = \sum_{i=1}^{k} \frac{[(Oi - Ei) - 0.5]^2}{Ei}}$$

k = number of categories (2); *Oi* = number of plants observed in a category; *Ei* = number of plants expected in a category; the degree of freedom (*i* = *k*-1) is 1.

The calculated χ^2 value was then compared with computed critical values. In this case, for 2 different categories and one degree of freedom, a value of χ^2 equal or less than 3.841 should indicate that the null hypothesis (H0) was accepted.

2.7 PLASMIDS USED IN TRANSIENT GENE EXPRESSION IN *Arabidopsis* PROTOPLASTS

Table 7 shows all the plasmids encoding proteins of interest used in transient gene expression in protoplasts.

(X)FP protein	Origin
RFP-p24δ5	(Montesinos et al., 2012)
RFP-p24δ5ΔGOLD	(Montesinos et al., 2012)
RFP-p24δ5ΔCC	(Montesinos et al., 2012)
RFP-p24δ9	(Montesinos et al., 2013)
RFP-Calnexin	(Künzl et al., 2016)
GFP-PMA	(Martinière et al., 2012)
MAP-GFP	(Martinière et al., 2012)
GFP-PAP	(Martinière et al., 2012)
GFP-GPI	(Martinière et al., 2012)
GFP-AGP4	(Martinière et al., 2012)

Table 7. Plasmids used in transient gene expression in *Arabidopsis* protoplasts in this work.

2.8 PLASMIDS USED IN TRANSFORMATION MEDIATED BY *Agrobacterium*: TRANSIENT EXPRESSION IN *Arabidopsis* SEEDLINGS, *Arabidopsis* STABLE TRANSFORMATION AND AGROINFILTRATION OF *N. benthamiana* LEAVES

Table 8 shows all the plasmids encoding proteins of interest used in transformation mediated by *Agrobacterium* and the amiRNA construct for silencing PER1LB.

(X)FP protein	**Origin**
GFP-AGP4	(Martinière et al., 2012)
GFP-GPI	(Martinière et al., 2012)
PIP2A-RFP	ABRC stock
RFP-p24δ5	(Montesinos et al., 2012)
RFP-p24δ5ΔGOLD	Obtained in this work
RFP-p24δ5ΔCC	Obtained in this work
PER1LA-RFP	Obtained in this work
PER1LB-RFP	Obtained in this work
GFP-HDEL	(Pain et al., 2019)
YFP-ManI	DG Robinson lab, University of Heidelberg
amiRNA	**Origin**
amiR-PER1LB	The *Arabidopsis* Information Resource*

Table 8. Plasmids used in transformation mediated by Agrobacterium in this work.
* The Arabidopsis Information Resource. pAMIR vector.
http://www.arabidopsis.org/servlets/TairObject?type=vector&id=1001200233

3 TREATMENTS

3.1 GERMINATION UNDER NaCl TREATMENT

To study whether salt tolerance was affected in the mutants of the GPI anchor remodelling enzymes, seeds of wild-type (Col-0) and GPI anchor remodelling enzymes mutants were sown on Murashige and Skoog (MS) plates containing 120 and 150 mM NaCl. Plates were transferred to a controlled growth chamber after cold treatment in the dark for 3 days at 4°C. After 14 days, the rates of cotyledon greening were scored. Seeds harvested from Col-0 and GPI anchor remodelling enzymes mutants plants were grown under the same conditions and at the same time were used.

3.2 INFILTRATION OF BFA IN *N. benthamiana* LEAVES

In order to block the transport between the ER and Golgi apparatus it was decided to use the drug Brefeldin A (BFA), which blocks the activation of some ARF proteins involved in the regulation of the vesicular trafficking of COPI vesicles from Golgi apparatus to ER (Fujiwara et al., 1988; Ritzenthaler et al., 2002) and, therefore, this disrupts the secretion pathway avoiding GFP-AGP4 can go to the plasma membrane and would be retained into the ER, increasing the possibilities of the interaction between GFP-AGP4 and RFP-p24δ5 at the ER.

From a stock of 5 mg/mL BFA (from *Penicillium brefeldianum*, 99% (HPLC and TLC), Sigma-Aldrich®, Ref. #B7651) dissolved into ethanol, it was diluted into water to reach a concentration of 20 µg/mL. This was infiltrated 16 hours before taking the sample in the piece of leaves agroinfiltrated 2 days

before. The infiltration of the solution of BFA consists as it was explained in section 2.4 of Material and Methods.

3.3 STAINING OF PROTOPLAST PLASMA MEMBRANE USING FLUORESCENT PROBES

For staining the protoplast plasma membrane it was used the dye FM4-64 (*N*-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide) (Thermofisher®, Ref. #T3166). FM4-64 stains the plasma membrane and is endocyted by time. It is excited by laser at 520 nm and the CLSM detection window is 600-700 nm.

The dye stock was 2 mM in DMSO (dimethyl sulfoxide) and was diluted to 50 µM in W5. Protoplasts were centrifuged in an Eppendorf at 124 xg, 1 min and 4°C and the supernatant was removed. Next, the pellet (protoplasts) was resuspended with 100 µL of the 50 µM FM4-64 dilution in W5 and then incubated 15 min at 4°C. After this time, samples were washed by addition of 900 µL of W5 and then centrifuged at 124 xg, 5 min and 4°C. Finally, the supernatant was removed and the pellet (protoplasts) was resuspended with 80 µL for visualization to the CLSM.

4 NUCLEIC ACIDS

4.1 ISOLATION OF NUCLEIC ACIDS

4.1.1 Isolation of plasmid DNA

For small-scale preparations of plasmid DNA, an alkaline lysis method described by Green and Sambrook, 2016 was used beginning with 1 mL culture grown overnight in LB supplemented with the corresponding antibiotic.

Middle-scale preparations of plasmid DNA were performed beginning with 100 mL cultures grown overnight in LB with antibiotic, and following the manufacturer's instructions for extraction and purification of plasmid DNA indicated in Qiagen® Plasmid Midi Kit (Qiagen columns tip-100, Ref. #12143).

For large-scale preparations of plasmid DNA the Qiagen® Plasmid Maxi Kit (Ref. #12163) was used following the manufacturer's instructions. This method was used mainly to obtain plasmid DNA for PEG transformation.

4.1.2 Isolation of genomic DNA from *Arabidopsis*

To isolate *Arabidopsis* genomic DNA, 100 mg of rosette leaves of 3-4-week-old plants, before the main shoot elongated, were collected and snap frozen in liquid nitrogen. The genomic DNA was obtained following a protocol described previously (Edwards et al., 1991).

4.1.3 Isolation of total RNA from *Arabidopsis*

To obtain *Arabidopsis* total RNA, the Rneasy® Plant Mini Kit (Qiagen®, Ref. #74904) system, which is specific for plants, was used. 75 mg of the indicated tissue was collected and snap frozen in liquid nitrogen. Samples were homogenized by grinding them in liquid nitrogen with a pestle. Total RNA extraction was performed following the instructions of the manufacturer. All samples were treated with DNase (Qiagen®, Ref. #79254).

RNA quantification was performed in a spectrophotometer (Ultrospec 2000, Pharmacia Biotech®) and total RNA was stored at -80°C for further use.

4.2 MANIPULATION AND ANALYSIS OF NUCLEIC ACIDS

4.2.1 Recombinant plasmid production

The coding sequence of fluorescent proteins PER1LA-RFP and PER1LB-RFP were commercially synthesized *de novo* (Geneart AG®) based on the sequence of RFP or GFP and the *Arabidopsis* genes of PER1LA (AT5G62130) and PER1LB (AT1G16560). The sequence of the fluorophore is at the end of the coding sequence of these genes. The coding sequence of these proteins were cloned into the pCHF3 vector (Ortiz-Masia et al., 2008) through the restriction enzymes: KpnI and SalI.

4.2.2 Agarose gel electrophoresis

DNA fragments were visualized in 0.8-2 % agarose gels (depending on the size of the fragments to be analyzed) in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0), stained with 10 µg/mL Real Safe®

(Durviz, S.L.), and separated by electrophoresis with a constant voltage between 100-150 V, immersed in TBE. Samples were diluted in 6X Loading buffer (50 % (v/v) glycerol, 0.05 % (w/v) bromophenol blue, 100 mM EDTA). DNA bands were visualized by lighting up the gel with ultraviolet light, using the UVITEC system® (Cambridge). This system allows also to photograph the gel.

4.2.3 Amplification by polymerase chain reaction (PCR)

Amplification reactions were performed in the cycler GeneAmp PCR system 2400® (Perkin Elmer®), following the instructions contained in the kit from WVR Red Taq DNA Polymerase Master Mix (Ref. #5200300-1250).

For genotyping by PCR, samples consisted of 2 µl from genomic DNA isolated as described in section 4.1.2., 2 µl of each primer at 10 µM (Table 9), 19 µl sterile Milli-Q water and 25 µl of WVR Red Taq DNA Polymerase Master Mix, which contains dNTPs (dATP, dCTP, dGTP and dTTP at 0.4 mM), 0.2 units/µl VWR Taq polymerase in Tris-HCl pH 8.5, $(NH_4)_2SO_4$, 3 mM $MgCl_2$ and 0.2% Tween ® 20. Total volume was 50 µl.

Genotypic analysis by PCR consisted in running a first denaturation step of 2 minutes at 95°C, followed by 36 cycles. Each cycle was divided in three sections: 30 seconds at 95°C (denaturation), 30 seconds at the respective annealing temperature of specific primers (usually 55°C) and 1 to 3 minutes according to the size of the fragment at 72°C (elongation). Finally, a final period of 7 min at 72°C was added to assure the elongation of all fragments.

The primers used in this work for PCR and RT-sqPCR are listed in the Table 9.

Name	Gene	Sequence (5'→3')	Tm (°C)
PGAP1 genes			
Bst1AR	*PGAP1A*	ACCGTATCTGACTTGAAGTAGC	60
Bst11AF	*PGAP1A*	TCGGCAGATGACATAGGATGGTTA	64
NRPBst1A	*PGAP1A*	GAAACTGTCTCCTTGTGTCTATG	61
RPBst1A	*PGAP1A*	TACGATCAACTTCCGGAGTTG	59
LPBst1A	*PGAP1A*	CTAAAGGATAAGGTCGCTGGG	61
Bst1BR	*PGAP1B*	ACGAGACCACTGTGAAGCTTGTGAG	67
Bst1BF	*PGAP1B*	GCTCTGCAAATTGCGTTGTTTCCC	65
RPBst1B	*PGAP1B*	ACCAGCTTAGGTCTATTGCCC	61
LPBst1B	*PGAP1B*	TTGGAAGGGAAATTTGGAAAC	55
Bst1CR	*PGAP1C*	CCATTTTCGTAGCGCATCGT	58
Bst1CF	*PGAP1C*	ACAGCTCTCATCCTTCATGG	58
PER1L genes			
RPPer1A	*PER1LA*	AATGTCAGAAAACTGGATGCG	60
LPPer1A	*PER1LA*	GAGCTTTCTTGATCTCGAGCA	62
Per1BR	*PER1LB*	GGATCCAAATCCTTGTAAAACTTAGC	63
Per1BF	*PER1LB*	GTCAAGGTGATTGCCGTTAT	56
Housekeeping genes			

A5	*ACT-7*	GGATCCAAATGGCCGATGGTGAGG	69
A3	*ACT-7*	GGAAAACTCACCACCACGAACCAG	67
Insertion			
LBb1	*T-DNA*	GGATCCGCGTGGACCGCTTGCTGCAACT	76
LB3	*T-DNA*	TTCATAACCAATCTCGATACAC	55
ami3	*amiRNA*	GGATCCGCAATTAACCCTCACTA	63
ami5	*amiRNA*	ATATAAGGAAGTTCATTTCATTTGGAG	61

Table 9. Primers used in this thesis.

4.2.4 Synthesis of cDNA by retrotranscription (RT-PCR)

This procedure allows obtaining complementary (cDNA) from RNA by the action of a reverse transcriptase which is a viral enzyme that synthesizes DNA using RNA as a template. To obtain cDNA, the Maxima® First Strand cDNA Synthesis Kit for RT-PCR (Fermentas®, Ref. #K1641) was used. The starting point was 3 µg of total RNA, to which 2 µl of Maxima enzyme mix, 4 µl of 5X Reaction mix and free ribonuclease water to a total volume of 20 µl were added. PCR tubes were incubated for 25 minutes at 25°C, then 30 minutes at 50°C and finally, the reverse transcriptase was inactivated incubating the PCR tubes for 5 minutes at 85°C. The cDNA obtained was stored at -20°C until its use.

4.2.5 Semiquantitative PCR (RT-sqPCR)

For semiquantitative expression PCR (RT-sqPCR) analysis, amplification reactions were performed in the cycler GeneAmp PCR system

2400 (Perkin Elmer®), following the instructions contained in the kit from Roche PCR Master®.

In this case, samples consist of 3 µl (initially) of cDNA from the retrotranscription reaction and 2 µl of each primer at 10 µM (Table 9) diluted in the H_2O provided by the kit, up to 25 µl. To avoid non-specific amplification, a "hot start" protocol was used. It consisted in running a first denaturation period of 2 minutes at 95°C, after which 25 µl of PCR Master (which contains dNTPs at 0.4 mM, 25 U of DNA polymerase thermophilic eubacterium *Thermus aquaticus* BM (Taq polymerase) in 20 mM Tris-HCl, 100 mM KCl, 3 mM $MgCl_2$ and 0.01 % (v/v) Brij 35 at pH 8.3) pre-heated at 50°C (total volume of 50 µL) was added.

The amplification cycles were divided into 15 initial cycles which consisted in three sections as described in section 4.2.3. To these initial cycles, a variable number of cycles were added depending on the level of mRNA expression of the analyzed gene in the tissue. 12 µL aliquots of the PCR products were taken at a consecutive number of cycles (to check the linear range) and incubated 7 min at 72°C, for further analysis. The temperature was kept at 4°C till the samples were removed from the thermal cycler.

The primers used in this work for PCR and RT-sqPCR are listed in the Table 9.

5 PROTEINS

5.1 TOTAL PROTEIN EXTRACTION OF *Arabidopsis* ROOTS

To obtain protein extracts from cytosolic and membrane fractions, roots of 7-day-old seedlings grown in MS plates (section 1.2.1.3 of Materials and methods) were cut with a scissors and frozen in liquid nitrogen until use. Next, they were homogenized in homogenization buffer (HB) (0.3 M sucrose; 1 mM EDTA; 1 mM dithiothreitol DTT; 20 mM HEPES pH 7.5; 20 mM KCl; pH 7.5) supplemented with 1 mM DTT and 10 % Protease Inhibitor Cocktail Sigma® (IPs) using a mortar and a pestle, maintaining always the mortar on ice. The homogenate was centrifuged for 10 minutes at 1215 xg and 4°C, and the supernatant was recovered (post nuclear supernatant (PNS)). TX-100 was then added to a final concentration of 0.5 % and the PNS was incubated in a rotating wheel during 30 min at 4°C. Then, the PNS was centrifuged again for 5 minutes at 15700 xg and 4°C. The final supernatant were adjusted to the same protein total concentration by adding HB.

5.2 TOTAL PROTEIN EXTRACTION OF *Arabidopsis* PROTOPLASTS FOR PULL-DOWN EXPERIMENTS

Protoplasts obtained as described in section 1.2.1.5 were collected in W5 medium by centrifugation for 5 minutes at 124 xg and 4°C. Pellet was homogenized in 1 mL of HB with 10 % IPs and protoplasts were disrupted by sonication (6 x 5 s). Protoplast extracts were separated from unbroken protoplasts by centrifugation for 10 min at 1215 xg and 4°C. To the supernatant (PNS) was added to a final concentration of 0.5 % TX-100 and the PNS was incubated in a rotating wheel during 30 min at 4°C. Then, the

PNS was centrifuged for 5 minutes at 15700 xg and 4°C to finally obtain the final supernatant which is incubated with the beads for pull-down experiments (section 5.5) or it is used as Input.

5.3 TOTAL PROTEIN EXTRACTION OF *N. benthamiana* LEAVES

Nicotiana leaves agroinfiltrated as described in section 2.4 of Materials and Methods were frozen in liquid N_2 and then grinded in a mortar adding HB with 10 % IPs until leaves are totally dispersed. Leaves extracts were separated from undispersed pieces by centrifugation for 10 min at 1215 xg and 4°C. Next, the supernatant (PNS) was added to a final concentration of 0.5 % TX-100 and the PNS was incubated in a rotating wheel during 30 min at 4°C, as in section 5.2. Finally, the PNS was centrifuged for 5 min at 15700 xg and 4°C to obtain the final supernatant which is used to do a pull-down as described in section 5.5, or it is used as Input.

5.4 PI-PLC TREATMENT

PI-PLC is an enzyme which can catalyze the hydrolysis of a phosphatidylinositol into an inositol triphosphate and a diacylglycerol. PI-PLC treatment was carried out in order to study if the precursors of GFP-AGP4 that interact with RFP-p24δ5 are PI-PLC sensitive.

A *N. benthamiana* leaf that was agroinfiltrated 3 days before with GFP-AGP4 was grinded in a mortar adding HB with 10 % IPs. *N. benthamiana* extracts were separated from unbroken cells by two centrifugations of 10 min at 1215 xg and 4°C to finally obtain the PNS.

Next, the PNS was centrifuged at 500000 xg, 10 min and 4°C in order to separate the cytosol from membranes. The sediments were resuspended by the addition of HB with 10 % IPs to obtain the membrane fraction. The membranes fractions were incubated with 2 U PI-PLC (from *Bacillus cereus*, 100 U/mL, Invitrogen®, Ref. #P6466) and without (as a control) for 1 h at 37°C. After this, samples were centrifuged for 1 h at 150000 xg and 4°C in order to separate membranes (pellet) from the GFP-AGP4 cleaved by the PI-PLC (supernatant). Membrane fractions were disrupted by the addition of 100 µL Lysis buffer (50 mM Tris-HCl pH 7.5 1 M, 0.5 % TX-100 20 %, 1 mM EDTA 0.5 M, 1 mM PMSF (phenylmethylsulfonyl fluoride) 0.1 M, 150 mM NaCl) with 10 % IPs and incubated 30 min in ice with occasional vortex. Next, broken membrane fractions were centrifuged for 5 min at 10000 xg and 4°C to get the supernatant. Finally, 3x Sample Buffer (SB) was added to the samples and heated for 5 min at 95°C and analyzed by Western blot as indicated in section 5.8.

5.5 PULL-DOWN EXPERIMENTS

Pull-downs experiments from leaves of *N. benthamiana* or *Arabidopsis* protoplasts co-expressing RFP-tagged proteins and GFP-tagged proteins were performed using RFP-Trap or GFP-Trap magnetic beads (Chromotek®), following the recommendations of the manufacturer, as described previously (Montesinos et al., 2013). The magnetic beads were incubated with 0.5 mL of PNS and 0.8 mL of CO-IP buffer (10 mM Tris-HCl pH 7.5 1 M, 150 mM NaCl, 0.5 mM EDTA; pH 7.5) for 2 h in a wheel at 4°C. After the incubation period, the beads were washed 3 times with CO-IP buffer and

then 2x SB was added to the beads and heated for 5 min at 100°C and analyzed by Western blot as indicated in section 5.8.

5.6 DETERMINATION OF PROTEIN CONCENTRATION

To quantify the protein concentration in a sample, the Bio-Rad® Protein Assay kit was used. It is based on the method described by Bradford (1976), which allows to correlate the variation of absorbance at 595 nm from an acidic solution of Coomasie Brilliant Blue G-250® with the quantity of proteins in a sample (optimum range of 0.2-2 mg/mL of protein), using different concentrations of BSA as a standard.

5.7 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Proteins from different samples were separated through electrophoresis in vertical gels of SDS-polyacrylamide, following the protocol described by Laemmli (1970), at constant voltage (100 V). Previously, samples were mixed 1:1 with 2X SB, which contains 125 mM Tris-HCl, 20 % glycerol, 4 % SDS, 25 µg/mL Bromophenol blue and 50 µl/mL of 14 M β-mercaptoethanol, pH 6.8. Then, samples were incubated for 5 minutes at 95°C.

The acrylamide/bisacrylamide gels consisted in two different parts:

- Running gel: 8-14 % polyacrylamide [30 % acrylamide/bis (29:1), Bio-Rad®], 0.39 mM Tris-HCl pH 8.8, 0.1 % SDS, 0.1% APS (ammonium persulfate, Bio-Rad®), 1/1000-1/2500 99 % TEMED (N, N, N', N'-tetramethilethilendiamine, Bio-Rad®).

- Stacking gel: 5 % polyacrylamide, 0.13 % Tris-HCl pH 6.8, 1 % APS, 1/1000 99 % TEMED.

The electrophoresis was performed with the Electrophoresis buffer, which contains 192 mM glycine, 25 mM Trizma® base, 0.1% SDS, pH 8.3. After SDS-PAGE, proteins were transferred to a nitrocellulose membrane to perform Western blot analysis.

5.8 PROTEIN DETECTION: WESTERN BLOT ANALYSIS

Proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane of 0.45 µm (Bio-Rad®) following the Burnette protocol (Burnette, 1981) through a humid transfer system (Bio-Rad®) with constant voltage (100 V) for 1 h, all immersed in Transfer buffer (25 mM Trizma® base; 192 mM glycine; 20 % MeOH; pH 8.5). The efficiency of the transference and the proper loading of the different samples were tested in the membranes staining them with Ponceau S 0.5 % solution (SIGMA®).

Western blot analysis is based on the indirect detection of proteins placed in a nitrocellulose membrane, using specific antibodies. To this end, membranes were blocked with blotto-Tween (powdered milk in 3-5 % PBS; 0.01 % Tween® 20) for 16 h at 4°C or 1 h at RT with gentle shaking (see-saw rocker SSL4, Stuart®), which was maintained during all the process. After blocking, membranes were incubated with the pertinent primary antibody (Table 10) diluted in PBS-BSA [PBS (8 mM Na_2HPO_4, 1.7 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl), 2 mg/mL BSA, 0.02 % sodium azide] for 1h at RT. Next, 6 incubations of 5 minutes with TBS-Tween [0.01 % Tween® 20 in TBS (24 mM

Tris-HCl, 150 mM NaCl, pH 7.5)) were performed to wash out the excess of antibody. The incubation with the secondary antibody (Table 11) which is conjugated to horseradish peroxidase (HRP) was performed for 1h at RT. After the incubation, the excess of secondary antibody was washed out as the primary antibody.

Developing was performed by the Enhanced chemiluminescence method (ECL). This method is based on the chemiluminiscence reaction of luminol. The enzyme HRP, which is linked to secondary antibodies, catalyz-expres the oxidation of luminol when there is hydrogen peroxide in alkaline conditions, generating a product that emits luminescence (Whitehead et al., 1979). Developing was performed following the instructions of the manufacturer (Western blotting detection reagents, Thermo Scientific®), using the automatic system Molecular Imager® ChemiDocTM XRS+ Imaging system (Bio-Rad®), with variable exposure times. The intensity of the bands obtained from Western-blots in the linear range of detection was quantified using the Quantity One software (Bio-Rad Laboratories®).

Nitrocellulose membranes can be reused for another Western blot analysis after stripping the antibodies. To this end, membranes were incubated with 0.5 M glycine pH 2.5 for 15 minutes at RT with constant shaking. Then, they were washed five times, twice with distilled water and three times with TBS-Tween. Finally, membranes were blocked again with blotto-Tween.

Target	Host	Dilution	Reference
p24δ5-Nt	Rabbit	1/500	(Montesinos et al., 2012)
p24δ9-Nt	Rabbit	1/500	(Montesinos et al., 2013)
p24β2-Ct	Rabbit	1/500	(Montesinos et al., 2012)
RFP	Rabbit	1/500	Rockland®
GFP	Rabbit	1/500	Rockland®

Table 10. Primary antibodies used in this work.

Target	Host	Dilution	Reference
IgG Rabbit	Donkey	1/7500	GE Healthcare®

Table 11. Secondary antibody used in this work.

6 VISUALIZATION OF PROTEINS

6.1 CONFOCAL MICROSCOPY (CLSM)

Confocal fluorescent images from protoplasts (section 2.3), *Arabidopsis* seedlings (section 2.5) or *N. benthamiana* leaves (section 2.4) were collected and analyzed using an Olympus® FV1000 confocal microscope with 60x water lens and a super-resolution microscopy Zeiss® LSM880 wit Fast Ayriscan® detector. Fluorescence signals for GFP (488 nm/496-518 nm), YFP (514 nm/529-550 nm) and RFP (543 nm/593-636 nm) were detected. Sequential scanning was used to avoid any interference between fluorescence channels. Post-acquisition image processing was performed using the FV10-ASW 4.2 Viewer® and ImageJ (v.1.45) for Olympus images and Zeiss ZEN 2 (Blue edition®) V.1.0 for Zeiss images.

RESULTS AND DISCUSSION

CHAPTER I. ROLE OF P24 PROTEINS IN ER EXPORT AND TRANSPORT TO THE PLASMA MEMBRANE OF GPI-ANCHORED PROTEINS

1 FUNCTIONAL REDUNDANCY AND PROTEIN STABILITY OF P24 FAMILY PROTEINS

Our group has shown previously that *Arabidopsis* p24 proteins form hetero-oligomeric complexes which are important for their intracellular trafficking and localization but also for their stability (Montesinos et al., 2012, 2013, 2014; Pastor-Cantizano et al., 2016, 2018). To test for the role of p24 proteins in localization of GPI-APs, we have used a quadruple KO mutant affecting the 4 members of the p24delta-1 subclass of the p24 delta subfamily (p24δ3, p24δ4, p24δ5 and p24δ6), which we named *p24δ3δ4δ5δ6* or *p24δ-1* mutant (Pastor-Cantizano et al., 2018).

We have shown previously that there is interdependence in the protein levels of p24δ proteins from the two subclasses and the two members of the p24 beta subfamily, which is consistent with *Arabidopsis* p24 proteins forming hetero-oligomeric complexes, as described in other systems, probably including p24 proteins from the p24δ-1 and p24δ-2 subclasses and the p24 beta subfamily (Montesinos et al., 2013). Indeed the *p24δ-1* mutant had reduced protein levels of p24δ9, a member of the p24delta-2 subclass of the p24 delta subfamily and also of the two members of the p24 beta subfamily (p24β2 and p24β3) without a change in their mRNA

levels (see Figure 28) (Pastor-Cantizano et al., 2018). Therefore, this mutant had reduced function of many different p24 proteins. Our previous experiments also suggested that there may be functional redundancy between members of the p24delta-1 subclass within the p24 delta subfamily (Pastor-Cantizano et al., 2018). To further address this question, we expressed a member of the p24delta-1 subclass, p24δ5 (RFP-p24δ5) in the *p24δ-1* mutant background (section 2.6 of Material and Methods), to see if p24δ5 could alone restore the protein levels of other p24 proteins. Indeed, expression of RFP-p24δ5 was enough to restore the protein levels of both p24δ9 and p24β2 (Figure 28).

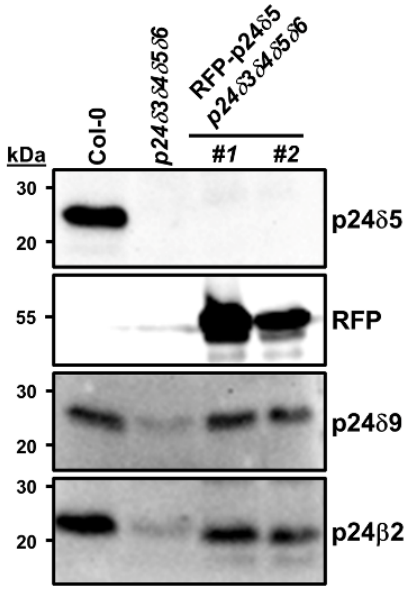

Figure 28. p24δ5 (p24delta-1 subclass) expression restores protein levels of p24 proteins in the *p24δ3δ4δ5δ6* mutant. Two independent transgenic lines were generated by transforming the *p24δ3δ4δ5δ6* mutant with RFP-p24δ5 (lines #1 and #2). Protein extracts were obtained from the roots of 7 days-old plants from these lines (section 5.1 of Materials and Methods), as well as from the *p24δ3δ4δ5δ6* mutant and wild-type (Col-0) plants and analyzed by Western blotting with antibodies against p24δ5, p24δ9, p24β2 and RFP (to detect RFP-p24δ5) (sections 5.7 and 5.8 of Material and Methods). A 20 µg aliquot of protein was loaded in each line (section 5.6 of Material and Methods).

These results suggest that p24δ5 is enough to restore the stability of other p24 protein family members, and thus can substitute for other members of the p24δ-1 subclass of the p24δ subfamily in putative p24 hetero-oligomeric complexes. These data also suggest the existence of functional redundancy within the p24delta-1 subclass.

2 P24 PROTEINS ARE NECESSARY FOR ER EXPORT AND PLASMA MEMBRANE LOCALIZATION OF GPI-ANCHORED PROTEINS

To test for the putative involvement of p24 proteins in transport to the plasma membrane of GPI-anchored proteins we used two different markers. The first one was a GFP fusion with arabinogalactan protein 4 (GFP-AGP4) (Martinière et al., 2012), a proteoglycan that seems to be implicated in diverse developmental processes such as differentiation, cell-cell recognition, embryogenesis and programmed cell death (Ellis et al., 2010). The second one was a glycosylphosphatidylinositol-anchored GFP (GFP-GPI) (Martinière et al., 2012). As a control, we used a transmembrane plasma membrane protein, the aquaporin PIP2A-RFP.

To study the localization of these proteins, we first used transient expression in *Arabidopsis* seedlings, as described in section 2.5 of Material and Methods. The localization of these markers was analyzed both in the *p24δ-1* mutant and in wild-type *Arabidopsis* seedlings. As shown in Figure 29 A, GFP-AGP4 was almost exclusively localized to the plasma membrane of cotyledon cells of wild-type *Arabidopsis* seedlings. In clear contrast, GFP-

AGP4 showed a predominant ER localization pattern in the *p24δ-1* mutant (Figure 29 D). The same happened with the second GPI-anchored marker protein, GFP-GPI, which localized to the plasma membrane in wild-type cells (Figure 29 B) but showed a predominant ER pattern in the *p24δ-1* mutant (Figure 29 E). In clear contrast, PIP2A-RFP, a transmembrane plasma membrane protein, mostly localized to the plasma membrane both in wild-type *Arabidopsis* cells and in the *p24δ-1* mutant (Figure 29 C and 29 F).

This suggests that p24 proteins are specifically required for transport to the plasma membrane of GPI-anchored proteins, and that loss of p24 proteins does not seem to affect transport to the plasma membrane of transmembrane proteins.

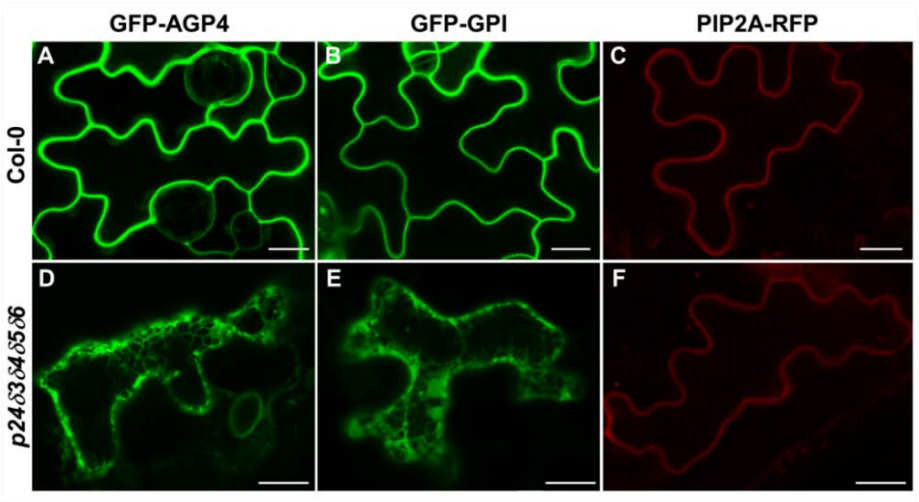

Figure 29. Localization of plasma membrane proteins in wild-type and *p24δ-1* mutant *Arabidopsis* seedlings. Transient gene expression in *Arabidopsis* seedlings. Two GPI-anchored proteins, GFP-AGP4 (A) and GFP-GPI (B), mainly localized to the plasma membrane in cotyledon cells from wild-type (Col-0) seedlings, as the transmembrane plasma membrane protein PIP2A-RFP (C). In the *p24δ-1* mutant, both GFP-AGP4 (D) and GFP-GPI (E) showed a predominant ER localization pattern, in contrast to PIP2A-RFP (F), which mainly localized to the plasma membrane. Scale bars, 10 µm.

We next analyzed the localization of GFP-AGP4 by transient expression in *Arabidopsis* protoplasts, as described in sections 1.2.1.5 and 2.3 of Material and Methods. As shown in Figure 30 A (and quantified in Figure 31), GFP-AGP4 mostly localized to the plasma membrane of protoplasts from wild-type *Arabidopsis* plants, where it colocalized with the FM dye FM4-64 (Figure 32 A-C). In contrast, GFP-AGP4 showed either a predominant or a partial ER localization pattern in protoplasts from the *p24δ-1* mutant (Figure 30 B-C), where it partially colocalized with the ER marker RFP-calnexin (Figure 32 G-I).

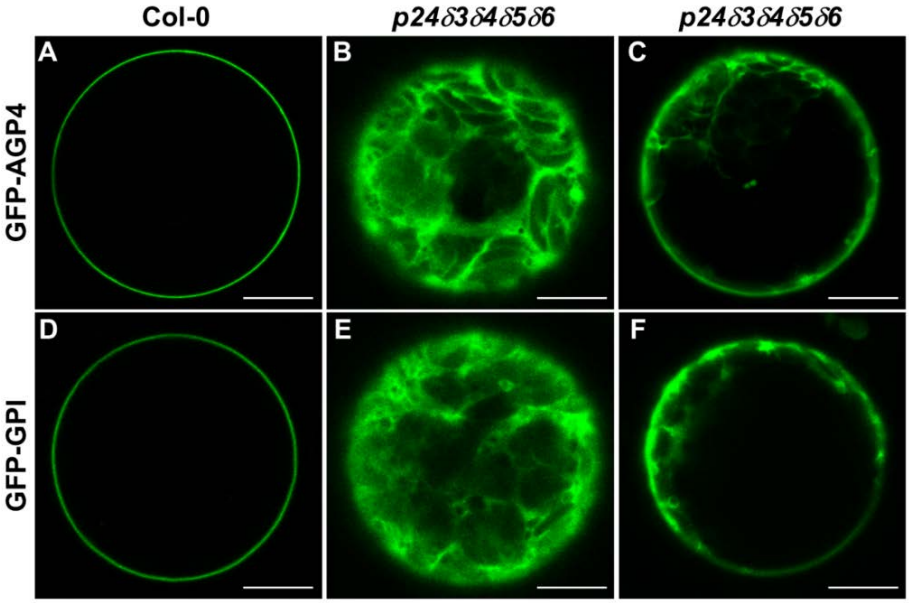

Figure 30. Localization of GPI-anchored proteins in wild-type (Col-0) and *p24δ-1* mutant *Arabidopsis* protoplasts. Transient gene expression in *Arabidopsis* protoplasts. GFP-AGP4 (A) and GFP-GPI (D) mainly localized to the plasma membrane in protoplasts from wild-type (Col-0) plants, but partially localized to the endoplasmic reticulum in the *p24δ3δ4δ5δ6* mutant (B-C, E-F) (see quantification in Figure 31). Scale bars, 10 µm.

Based on the localization patterns obtained under different experimental conditions, we analyzed a significant number of protoplasts and grouped them in three different categories depending on the main localization of GFP-AGP4: Mostly plasma membrane (PM), mostly ER (ER) and both ER and plasma membrane (ER + PM) (Figure 31).

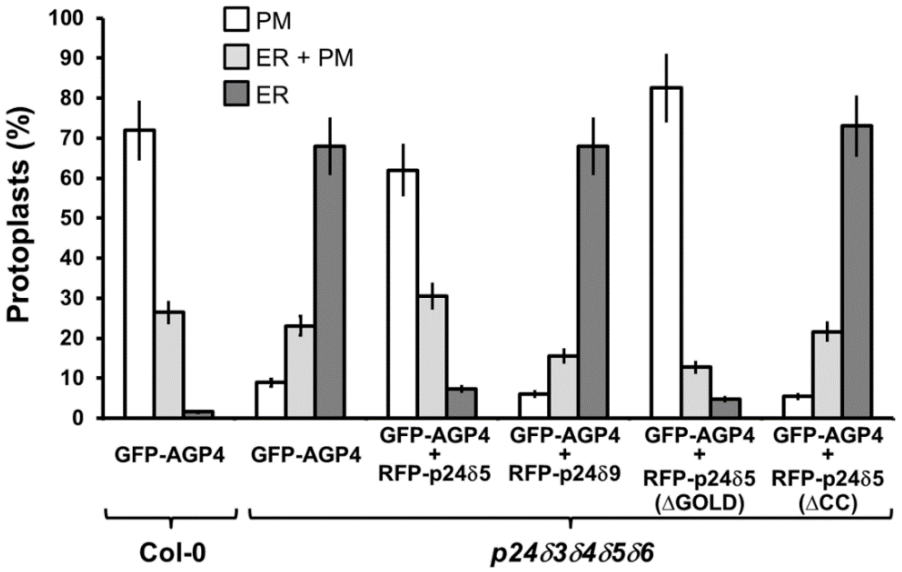

Figure 31. Quantification of the localization of GFP-AGP4 in transient expression experiments in protoplasts. A significant number of protoplasts (from at least four independent experiments), showing comparable expression levels of GFP-AGP4, in the absence or presence of RFP-p24δ5/9 (or mutant versions), were analyzed per condition, using identical laser output levels and imaging conditions. Number of protoplasts analyzed per condition: GFP-AGP4 (Col-0) (121); GFP-AGP4 (142); GFP-AGP4 + RFP-p24δ5 (206); GFP-AGP4 + RFP-p24δ9 (35); GFP-AGP4 + RFP-p24δ5(ΔGOLD) (63) GFP-AGP4 + RFP-p24δ5(ΔCC) (63). The localization of GFP-AGP4 was assigned as: Mostly plasma membrane (PM), mostly ER (ER) or ER and plasma membrane (ER + PM) and calculated as a percentage. Error bars represent SE of the mean.

Using these criteria, we found that in a percentage of protoplasts GFP-AGP4 also localized totally (9 % of protoplasts) or partially (23 % of protoplasts) to the plasma membrane in the *p24δ-1* mutant (Figure 30 C and 31), as shown by colocalization with FM4-64 (Figure 33).

This suggests that a proportion of GFP-AGP4 is still able to reach the plasma membrane in this mutant. The same happened with GFP-GPI, which mainly localized to the plasma membrane of wild-type *Arabidopsis* protoplasts (Figure 30 D and Figure 32 D-F) but showed a partial or predominant ER localization pattern in protoplasts from the *p24δ-1* mutant (Figure 30 E-F), where it partially colocalized with RFP-calnexin (Figure 32 J-L), but was also partially localized to the plasma membrane, where it colocalized with FM4-64 (Figure 33), as it is described in section 3.3 of Material and Methods.

To test if the lack of p24 proteins from the delta-1 subclass affects the localization of other plasma membrane proteins different from GPI-anchored proteins, we used different membrane-anchoring types of minimal constructs, including a myristoylated and palmitoylated GFP (MAP-GFP) and a prenylated GFP (GFP-PAP) (Martinière et al., 2012). We also used a transmembrane protein, a GFP fusion with the plasma membrane ATPase (GFP-PMA).

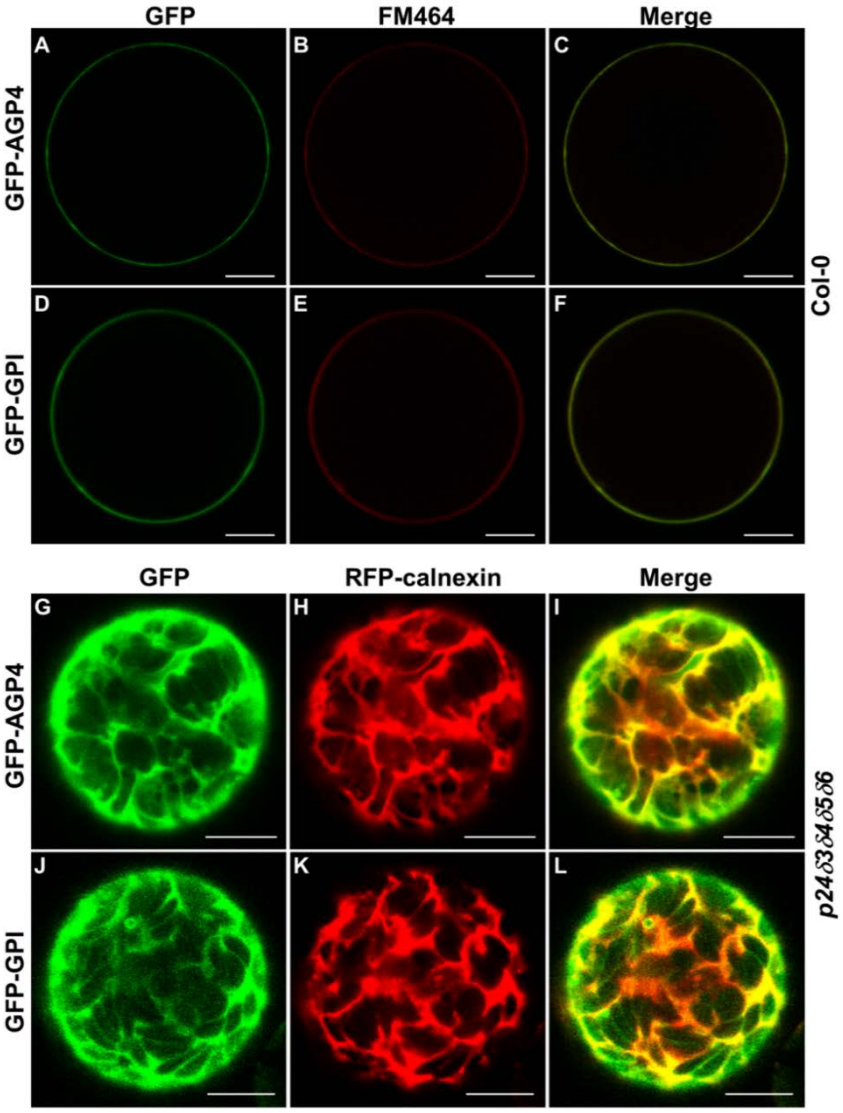

Figure 32. Localization of GFP-AGP4 and GFP-GPI in *Arabidopsis* protoplasts. Transient gene expression in *Arabidopsis* protoplasts. (A-F) In wild-type (Col-0) *Arabidopsis* protoplasts, GFP-AGP4 (A) and GFP-GPI (D) were mainly found at the plasma membrane (see quantification in Figure 31), where they colocalized with FM4-64 (B, E) (merged images in C and F) (see section 3.3 of Material and Methods). (G-L) In protoplasts from the *p24δ3δ4δ5δ6* mutant, GFP-AGP4 (G) and GFP-GPI (J) mainly localized to the endoplasmic reticulum (see quantification in Figure 31), where they partially colocalized with the ER marker RFP-calnexin (H, K) (see merged images in I and L). Scale bars, 10 µm.

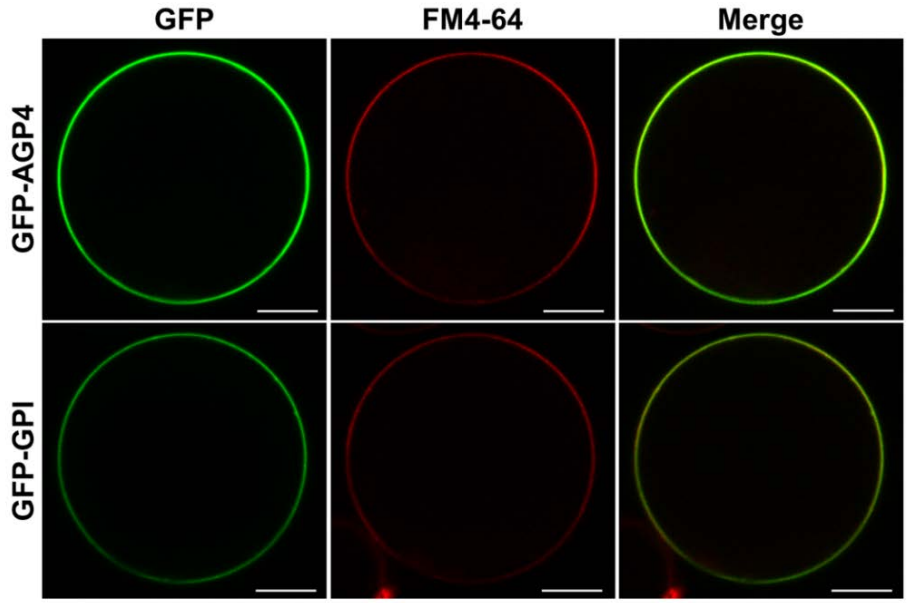

Figure 33. Localization of GFP-AGP4 and GFP-GPI in *p24δ-1 Arabidopsis* protoplasts. In protoplasts from the *p24δ3δ4δ5δ6* mutant, both GFP-AGP4 and GFP-GPI (left panels) localized to the plasma membrane in a small proportion of protoplasts (see quantification for GFP-AGP4 in Figure 31), where they colocalized with FM4-64 (medium panels) (merged images in right panels) (see section 3.3 of Material and Methods). Scale bars: 10 µm.

As shown in Figure 34, these 3 proteins mainly localized to the plasma membrane of *p24δ-1* mutant protoplasts (Figure 34 D-F), as in protoplasts from wild-type *Arabidopsis* plants (Figure 34 A-C). Therefore, p24 function seems to be specifically required for ER export and transport to the plasma membrane of GPI-anchored proteins.

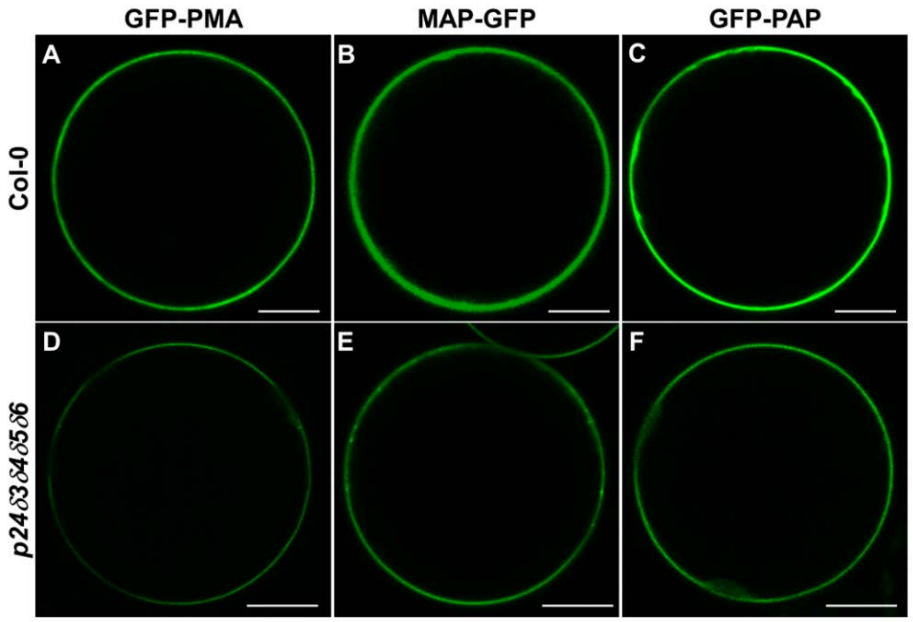

Figure 34. Localization of plasma membrane proteins without a GPI anchor in wild-type and *p24δ-1* mutant *Arabidopsis* protoplasts. Transient gene expression in *Arabidopsis* protoplasts from wild-type (Col-0) (A-C) or the *p24δ3δ4δ5δ6* mutant (D-F). The plasma membrane ATPase (GFP-PMA), a myristoylated and palmitoylated GFP (MAP-GFP) and a prenylated GFP (GFP-PAP) mostly localized to the plasma membrane both in wild-type and in mutant protoplasts. Scale bars, 10 µm.

3 P24δ5 (P24δ-1 SUBCLASS), BUT NOT P24δ9 (P24δ-2 SUBCLASS), PARTIALLY RESTORES PLASMA MEMBRANE LOCALIZATION OF GPI-ANCHORED PROTEINS IN THE *P24δ-1* MUTANT

As shown in Figure 28, p24δ5 was enough to restore the protein levels of different p24 proteins in the *p24δ-1* mutant, suggesting that there is functional redundancy between different p24 protein members (at least

within the p24δ-1 subclass). Indeed, we have previously shown that the function of p24δ5 was enough to restore normal trafficking of the K/HDEL receptor ERD2a in the *p24δ-1* mutant (Pastor-Cantizano et al., 2018). Therefore, we decided to test if p24δ5 function was sufficient to facilitate ER export and transport to the plasma membrane of GPI-anchored proteins in the absence of other p24 proteins from the delta-1 subclass. To this end, we co-expressed RFP-p24δ5 with GFP-AGP4 or GFP-GPI in protoplasts from the *p24δ-1* mutant.

As shown in Figure 35, RFP-p24δ5 expression was enough to partially restore plasma membrane localization of both GFP-AGP4 (Figure 35 A-C) and GFP-GPI (Figure 35 D-F). As quantified in Figure 31, GFP-AGP4 localized to the plasma membrane in more than 60 % of protoplasts, or had a dual ER/plasma membrane localization in around 30 % of protoplasts under these conditions. In clear contrast, expression of RFP-p24δ9 (which belongs to the p24δ-2 subclass) could not restore plasma membrane localization of GFP-AGP4 (Figure 35 G-I) and GFP-GPI (Figure 35 J-L) in the *p24δ-1* mutant. Instead, both proteins mainly showed an ER localization pattern (see also quantification in Figure 31), and partially colocalized with RFP-p24δ9 (Figure 35 G-L), which normally localizes at the ER (Montesinos et al., 2012). This suggests that members of the two p24delta subclasses are not functionally redundant.

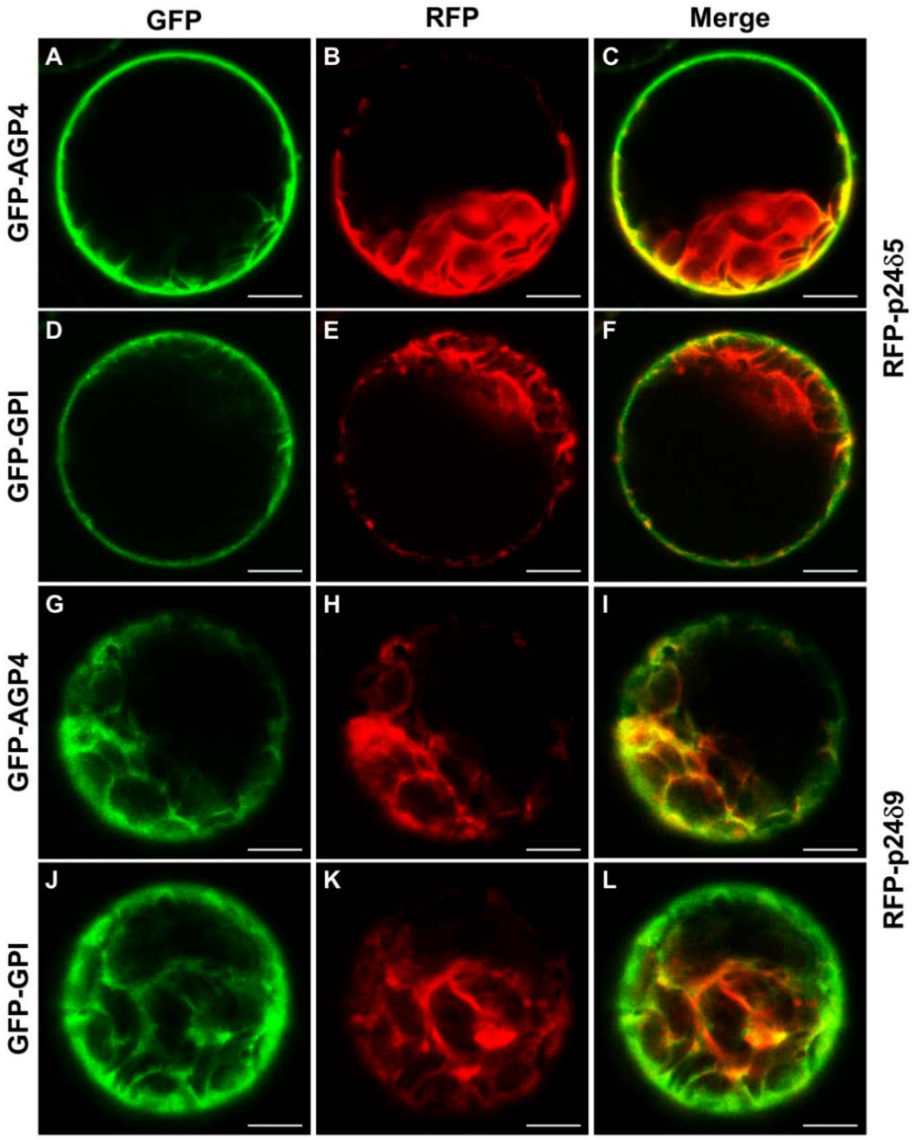

Figure 35. p24δ5 (but not p24δ9) partially restored the plasma membrane localization of GFP-AGP4 and GFP-GPI in the *p24δ-1* mutant. Transient gene expression in *Arabidopsis* protoplasts from the *p24δ3δ4δ5δ6* mutant. (A-F) Expression of RFP-p24δ5 (B, E) partially restored the plasma membrane localization of GFP-AGP4 (A) and GFP-GPI (B) (see merged images in C and F) (see quantification in Figure 4). (G-L) Expression of RFP-p24δ9 (H, K) could not restore the plasma membrane localization of GFP-AGP4 (G) and GFP-GPI (J) (see merged images in I and L) (see quantification in Figure 31). Scale bars, 10 µm.

4 TRANSPORT OF GPI-ANCHORED PROTEINS TO THE PLASMA MEMBRANE REQUIRES THE COILED-COIL DOMAIN, BUT NOT THE GOLD DOMAIN IN P24δ5

We next investigated which domain in p24 proteins was important for their role in transport of GPI-anchored proteins. Due to the luminal localization of the GPI anchor, we decided to test for the involvement of p24 luminal domains. The luminal part of p24 proteins includes two domains, a GOLD (Golgi Dynamics) domain and a coiled-coil (CC) domain.

To investigate which of these domains was necessary for transport of GPI-anchored proteins from the ER to the plasma membrane, we co-expressed GFP-AGP4 and GFP-GPI with RFP-p24δ5 deletion mutants lacking the GOLD or the coiled-coil domain (Montesinos et al., 2012).

As shown in Figure 36 (A-F), the RFP-p24δ5 deletion mutant lacking the GOLD domain was able to partially restore plasma membrane localization of both GFP-AGP4 and GFP-GPI, very similar to wild-type RFP-p24δ5 (see quantification in Figure 31). In contrast, the RFP-p24δ5 deletion mutant lacking the coiled-coil domain was unable to restore plasma membrane localization of GFP-AGP4 and GFP-GPI. Instead, both proteins mainly localized to the ER (see quantification in Figure 31), where they partially colocalized with the RFP-p24δ5 deletion mutant lacking the coiled-coil domain (Figure 36 G-L).

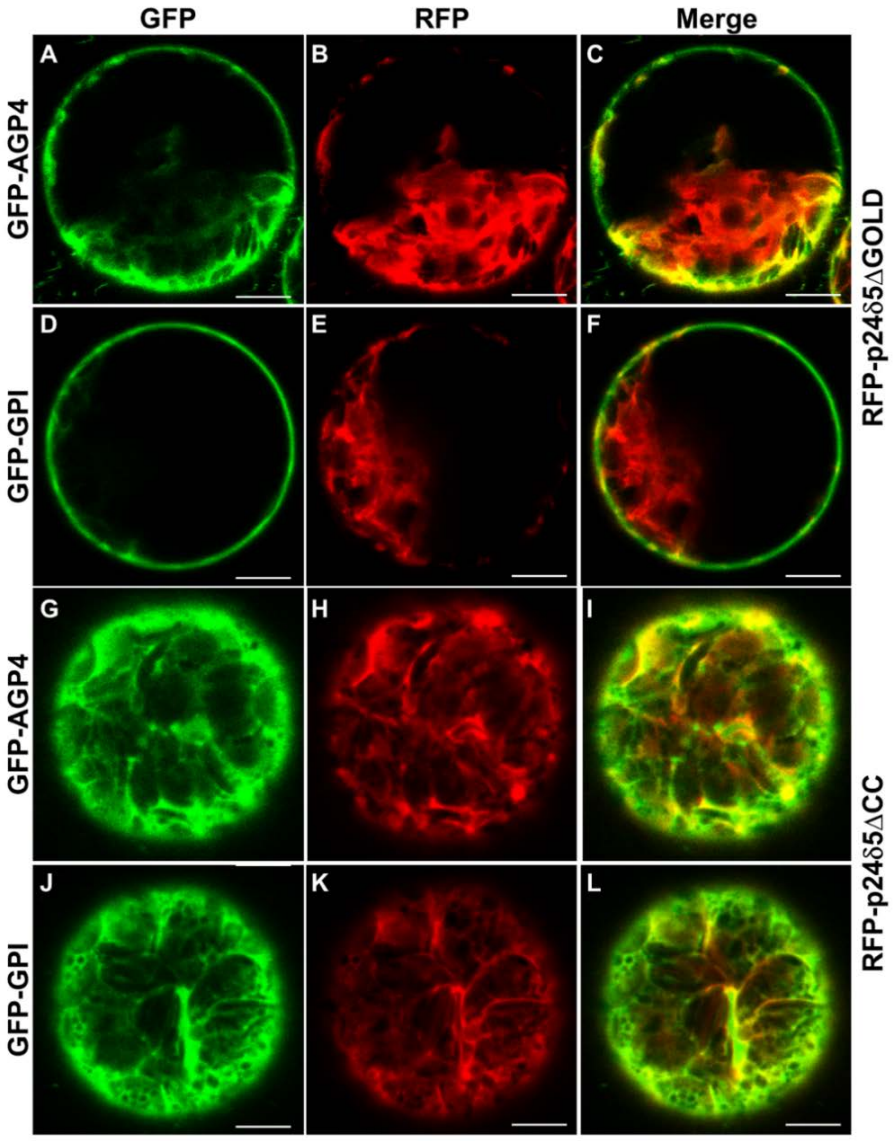

Figure 36. Transport of GPI-anchored proteins to the plasma membrane requires the coiled-coil domain, but not the GOLD domain in p24δ5. Transient gene expression in *Arabidopsis* protoplasts from the *p24δ3δ4δ5δ6* mutant. (A-F) Expression of RFP-p24δ5ΔGOLD (B, E) partially restored the plasma membrane localization of GFP-AGP4 (A) and GFP-GPI (B) (see merged images in C and F) (see quantification in Figure 4). (G-L) Expression of RFP-p24δ5ΔCC (H, K) could not restore the plasma membrane localization of GFP-AGP4 (G) and GFP-GPI (J) (see merged images in I and L) (see quantification in Figure 31). Scale bars, 10 µm.

This suggests that the coiled-coil domain in p24δ5, but not the GOLD domain, is essential for its role in ER export and transport to the plasma membrane of GPI-anchored proteins.

5 GFP-AGP4 INTERACTS WITH P24δ5, AN INTERACTION WHICH REQUIRES THE COILED-COIL DOMAIN IN P24δ5.

Once established that p24 proteins are important for ER export and transport to the plasma membrane of GPI-anchored proteins, and that p24δ5 is sufficient to facilitate this transport, we tested for a putative interaction between p24δ5 and GPI-anchored proteins. We first investigated the biochemical properties of GFP-AGP4 by transient expression using agroinfiltration in *N. benthamiana* leaves (section 2.4 of Material and Methods). A post-nuclear supernatant (PNS) from these leaves was analyzed by SDS-PAGE and Western blotting with antibodies against GFP (to detect GFP-AGP4) (sections 5.7 and 5.8 of Material and Methods). Western blotting showed a predominant band around 115 kDa, with a width compatible with its presumed high degree of glycosylation (Figure 37).

To test whether this was actually a GPI-anchored protein, we centrifuged the post-nuclear supernatant to obtain a total membrane fraction, which was treated in the absence or presence of Phosphatidyl Inositol-specific Phospholipase C (PI-PLC), as it is described in section 5.4 of Material and Methods. Membranes were then centrifuged again to separate

total membranes (TM), found in the pellet, from non-membrane proteins (soluble fraction, SF), found in the supernatant.

As shown in Figure 37, PI-PLC treatment for 1 h at 37°C produced a very significant decrease in the amount of the 115 kDa band found in the membrane fraction, and the appearance of a similar band in the soluble fraction, presumably released from membranes upon PI-PLC treatment. Another band of around 70 kDa, present in the membrane fraction (but not in the soluble fraction) was also sensitive to PI-PLC treatment, which probably represents the ER form of GFP-AGP4 (see below).

To test for interaction between GFP-AGP4 and RFP-p24δ5, we performed pull-down assays upon transient co-expression of these proteins in *N. benthamiana* leaves (section 5.5 of Material and Methods). Under these conditions we were unable to detect an interaction between these proteins (data not shown).

We reasoned that this could be due to the transient nature of these interactions and to different steady-state localization of both proteins. At steady-state, GFP-AGP4 mainly localizes to the plasma membrane, while RFP-p24δ5 mainly localizes to the endoplasmic reticulum (Figure 38 A-C). To increase the probability to detect the interaction between both proteins, we decided to infiltrate leaves with BFA 2 days after agroinfiltration and to allow expression for 1 extra day as it is described in section 3.2 of Material and Methods, to accumulate newly synthesized proteins at the ER. As shown in Figure 38 D-F, GFP-AGP4 and RFP-p24δ5 showed a high degree of colocalization upon BFA treatment, although a proportion of GFP-AGP4 was still found at the plasma membrane.

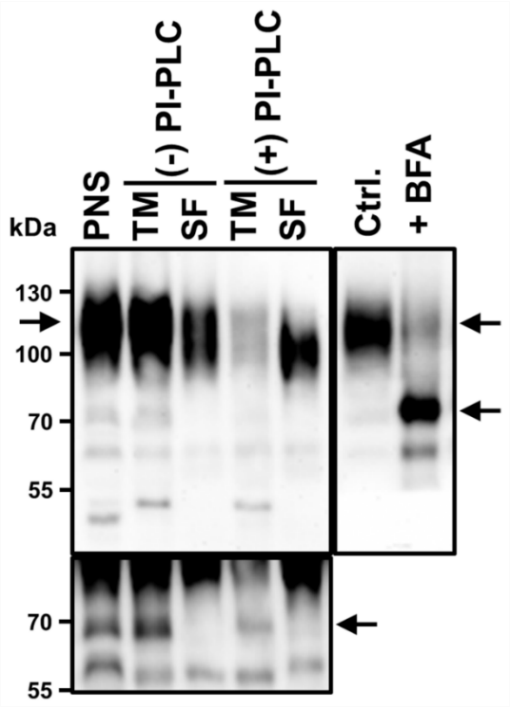

Figure 37. GFP-AGP4 is a GPI-anchored protein. A total membrane fraction was obtained using a post-nuclear supernatant (PNS) from *Nicotiana benthamiana* leaves transiently expressing GFP-AGP4 and treated in the absence (-) or presence (+) of Phosphatidyl-Inositol-Phospholipase C (PI-PLC). Total membranes were then centrifuged again to separate total membranes (TM) (pellet) from soluble proteins (soluble fraction, SF) (supernatant). PNS, TM and SF fractions were analyzed by SDS-PAGE and Western blotting with GFP antibodies (to detect GFP-AGP4). Lower panel shows a higher exposure of the 70 kDa region of the gel. Right panel shows a PNS from *N. benthamiana* leaves transiently expressing GFP-AGP4 and treated in the absence (-) or presence (+) of BFA for 24 h (see Figure 39). Arrows show the presence of a major band of 115 kDa in the absence of BFA and of a 70 kDa band which is much more prominent upon BFA treatment.

A PNS was obtained from leaves expressing both proteins in the absence or presence of BFA and analyzed by SDS-PAGE. As shown in Figure 37 (right panel), BFA treatment produced a drastic reduction of the 115 kDa protein and a concomitant increase in the band of 70 kDa, which was also present in the absence of BFA but at much lower levels. Therefore, we

hypothesize that the 70 kDa band correspond to the ER form of AGP4, while the 115 kDa form is the highly glycosylated form present at the plasma membrane.

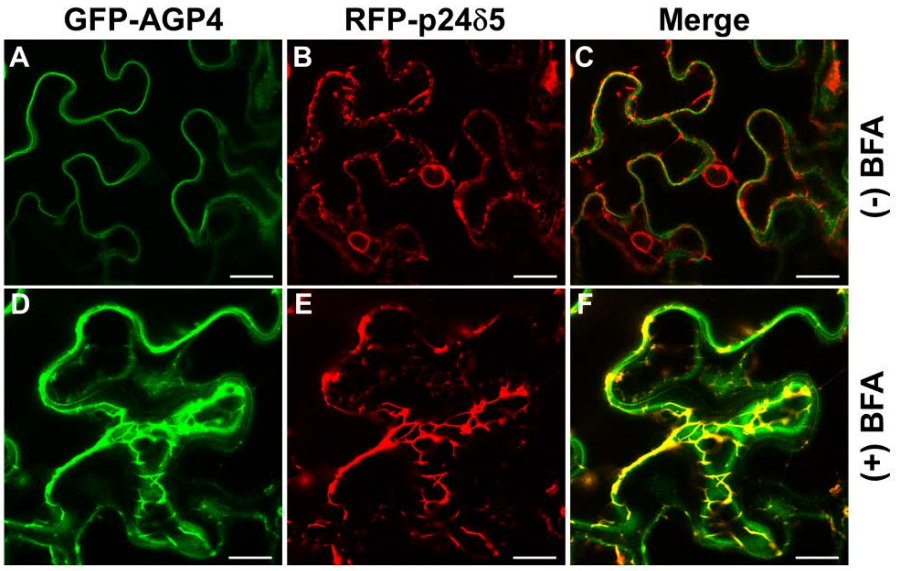

Figure 38. Effect of BFA treatment on localization of GFP-AGP4 and RFP-p24δ5. Transient expression of GFP-AGP4 and RFP-p24δ5 in *N. benthamiana* leaves. (+) BFA: Two days after agroinfiltration, leaves were infiltrated with BFA and left for an extra day before CLSM analysis. (-) BFA: Leaves were analyzed 3 days after agroinfiltration (see section 3.2 of Material and Methods). Scale bars, 10 µm.

We thus used PNS from leaves expressing GFP-AGP4 and RFP-p24δ5 and treated with BFA as input for GFP-trap, to pull-down GFP-AGP4, or RFP-trap, to pull-down RFP-p24δ5. Pull-downs were analyzed by SDS-PAGE and Western blotting with antibodies against RFP (to detect RFP-p24δ5) and GFP (to detect GFP-AGP4). As a control, PNSs were also incubated with blocked magnetic particles (Bmp), to detect unspecific binding. Additional negative controls included the incubation of GFP-trap or RFP-trap with extracts of leaves which did not express GFP-AGP4 and RFP-p24δ5 (Input Ctrl).

As shown in Figure 39 A, the GFP-trap pulled-down a major band of 70 kDa, presumably the ER form of GFP-AGP4 (top panel), but also RFP-p24δ5 (lower panel). The reverse was also true: RFP-trap pulled-down RFP-p24δ5 but also GFP-AGP4. No band was detected in the absence of expressed proteins or using blocked magnetic particles. Another band of around 60 kDa was also present in the specific input and the GFP-trap. Since this band was not present in the total membrane fraction (see Western blotting in Figure 37), we speculate this is not GPI-anchored. Similar experiments were performed upon expression of GFP-AGP4 and the RFP-p24δ5 versions lacking either the GOLD (Figure 39 B) or the CC domain (Figure 39 C).

These experiments showed the interaction of GFP-AGP4 with the p24δ5 deletion mutant lacking the GOLD domain, but not with the p24δ5 deletion mutant lacking the CC domain. These results suggest that the CC domain, but not the GOLD domain, is required for the interaction of p24δ5 with GFP-AGP4, in line with the experiments showing that the p24δ5 deletion mutant lacking the GOLD domain is able to partially restore the plasma membrane localization of GFP-AGP4, in contrast with the p24δ5 deletion mutant lacking the CC domain (Figure 36).

Finally, we also performed pull-down assays using post-nuclear supernatants from protoplasts of the *p24δ-1* mutant expressing GFP-AGP4 and RFP-p24δ5. Since both proteins partially accumulate at the ER in this mutant, they could also be used to detect the interaction, as after BFA treatment. Figure 39 D shows that the GFP-trap pulled-down the ER form of GFP-AGP4 but also RFP-p24δ5, while the RFP-trap pulled-down RFP-p24δ5 but also GFP-AGP4. No band was detected in the absence of expressed

proteins or using blocked magnetic particles. These experiments confirm the interaction between the ER form of AGP4 and p24δ5.

6 DISCUSSION

6.1 FUNCTIONAL REDUNDANCY BETWEEN P24 PROTEINS.

The p24 protein family includes 11 members in *Arabidopsis*, 9 of them belonging to the p24 delta subfamily (p24δ3 to p24δ11) and two belonging to the p24 beta subfamily (p24β2 and p24β3). p24 proteins of the delta subfamily can be divided into two different subclasses, the delta-1 subclass (including p24δ3 to p24δ6) and the delta-2 subclass (including p24δ7 to p24δ11) (Chen et al., 2012; Montesinos et al., 2012). We have shown previously that *Arabidopsis* p24 proteins form different heteromeric complexes (including members of the δ and β subfamilies) which are important for their stability and their coupled trafficking at the ER-Golgi interface (Montesinos et al., 2013). Consistent with this, the *p24δ-1* mutant, lacking the 4 members of the p24 delta subfamily, had reduced protein levels of other p24 proteins, including p24δ9 (p24 delta-2 subclass) and the two members of the p24 beta subfamily (p24β2 and p24β3) (Pastor-Cantizano et al., 2018). Strikingly, the expression of a single member of the p24 delta-1 subclass (p24δ5) in the *p24δ-1* mutant was enough to restore the protein levels of p24δ9 and p24β2. This suggests that the presence of p24δ5 is enough to compensate the absence of the other member of the p24 delta-1 subclass in putative oligomeric complexes, thus increasing protein stability of other p24 proteins. This increase in protein levels also correlates with increased p24 function.

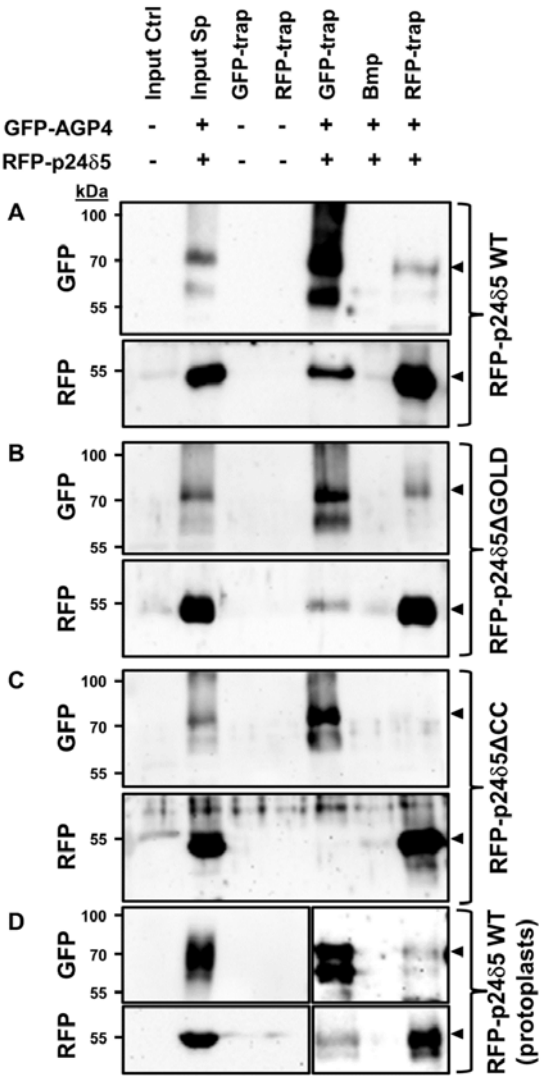

Figure 39. GFP-AGP4 interacts with RFP-p24δ5, an interaction which requires the coiled-coil domain in p24δ5. Pull-down experiments using GFP-trap (to pull-down GFP-AFP4) or RFP-trap (to pull-down RFP-p24δ5). As a negative control, we used blocked magnetic particles (Bmp). As inputs for the pull-downs we used PNS from *N. benthamiana* leaves expressing (+, Input Sp) or not (-, Input Ctrl) GFP-AGP4 and RFP-p24δ5 wild-type (WT) (A) or deletion mutants lacking either the GOLD domain (B) or the CC domain (C). Input in D was a PNS from *p24δ3δ4δ5δ6* mutant protoplasts expressing or not GFP-AGP4 and RFP-p24δ5WT (section 5.2 of Material and Methods). Inputs and pull-downs were analyzed by SDS-PAGE and Western blotting with antibodies against GFP (to detect GFP-AGP4) or RFP (to detect RFP-p24δ5). Arrowheads show the position of these proteins in the Western blotting.

We have shown previously that loss of p24δ-1 subclass proteins induced the accumulation of the K/HDEL receptor ERD2a (ER lumen protein-retaining receptor a) at Golgi membranes with an altered morphology, but normal ERD2 labelling was restored upon co-expression of p24δ5 (Pastor-Cantizano et al., 2018). Here we show that expression of p24δ5 (p24delta-1 subclass), but not p24δ9 (p24delta-2 subclass), partially restored plasma membrane localization of GPI-anchored proteins in the *p24δ-1* mutant. Altogether, these data suggest that the function of p24δ5 is enough to compensate the loss of function of p24 delta-1 subclass proteins, which indicates functional redundancy of *Arabidopsis* p24 family proteins, at least within the p24 delta-1 subclass.

6.2 P24 PROTEINS AND TRANSPORT OF GPI-ANCHORED PROTEINS.

In the absence of p24 proteins, GPI-anchored proteins partially accumulated at the endoplasmic reticulum. We could not detect a significant localization of these proteins at the Golgi apparatus, suggesting that p24 proteins are involved in the ER export of GPI-anchored proteins. In mammals and yeast, p24 proteins interact with GPI-anchored proteins at the endoplasmic reticulum and dissociate at the Golgi apparatus, presumably because of differences in pH between both compartments. In this manuscript we show that it is the ER form of GFP-AGP4 the one interacting with RFP-p24δ5, in line with the proposed role of p24 proteins in ER export of GPI-anchored proteins.

The coiled-coil (CC) domain seems to be the one involved in the function of p24 proteins to facilitate transport from the ER to the plasma

membrane of GPI-anchored proteins. This was confirmed by biochemical experiments showing the interaction of both p24δ5 and of a p24δ5 deletion mutant lacking the GOLD domain with GFP-AGP4, which was not the case for the p24δ5 deletion mutant lacking the coiled-coil domain. A previous report in mammals showed that the α-helical region of p24γ2 (but not the GOLD domain) was involved in the specific binding of GPI-anchored proteins, suggesting that this domain was responsible for the incorporation of these proteins within COPII vesicles for their ER export (Theiler et al., 2014). Plants do not contain p24 proteins from the gamma subfamily. Here, we show for the first time a direct interaction of a GPI-anchored protein with a p24 protein from the delta-1 subclass which involves its coiled-coil domain, and the role of this domain in the ER export of GPI-anchored proteins. Interestingly, p24δ5 (p24δ-1 subclass) and p24δ9 (p24δ-2 subclass) have different coiled-coil domains and this could explain that p24δ5, but not p24δ9, partially restores plasma membrane localization of GPI-APs in the *p24δ-1* mutant.

The function of p24 proteins seems to be restricted to GPI-anchored proteins, and is not required for plasma membrane proteins with different forms of membrane attachment, including transmembrane plasma membrane proteins (the aquaporin PIP2 or the plasma membrane ATPase), myristoylated and palmitoylated GFP and prenylated GFP. This is consistent with the fact that GPI-anchored proteins have special biophysical properties which may require a specialized trafficking machinery, different from that required for other secretory proteins, for their ER export, including specific COPII subunits (Lopez et al., 2019). In particular, the bulky nature of the GPI anchor in the luminal side of the ER opposes the membrane bending required

for COPII-dependent vesicle formation. In addition, the GPI-lipid appears to increase the rigidity of the ER membrane.

Sorting of GPI-anchored proteins at specific ERES seems to be different in yeast and mammals. In yeast, concentration of GPI-anchored proteins into specific ERES is lipid-based and does not require p24 proteins, which instead function as an adaptor to connect remodelled GPI-anchored proteins with the COPII coat subunits to facilitate their incorporation within COPII vesicles (Castillon et al., 2009, 2011). In contrast, concentration of GPI-anchored proteins at ERES and packaging within COPII vesicles in mammals is dependent upon p24 proteins (Fujita et al., 2011). In any case, since GPI-anchored proteins are entirely luminal cargo proteins, they need p24 proteins to recruit the cytosolic components of the COPII coat both in mammals and in yeast. Export of GPI-anchored proteins from the ER requires a specialized COPII system, both in mammals and in yeast. The biogenesis of specific COPII vesicles containing GPI-anchored proteins in yeast requires the specific COPII coat subunit isoform Lst1p, which together with Sec23p form the inner layer of the COPII coat. Mammalian GPI-anchored proteins also seem to use specific COPII coat isoforms, SEC24C and SEC24D (Bonnon et al., 2010; Lopez et al., 2019). Our data clearly show that p24 proteins are also required for ER export and transport to the plasma membrane of GPI-anchored proteins in plants. However, whether p24 proteins are required for the concentration of GPI-anchored proteins at specific ERES remains to be investigated. The same applies to the requirement of specific COPII subunits for ER export of GPI-anchored proteins in plants, although there is increasing evidence indicating that specific expression patterns in COPII subunit isoforms in *Arabidopsis* may reflect functional diversity (Chung et al., 2016).

Despite the accumulation of GPI-anchored proteins at the endoplasmic reticulum in the *p24δ-1* mutant, we could not find obvious phenotypic alterations in this mutant under standard growth conditions, although we found it was much more sensitive to saline stress (Pastor-Cantizano et al., 2018). There are several possible explanations for the lack of phenotypic alterations under standard growth conditions. Remaining levels of other p24 proteins (i.e. p24 proteins from the delta 2 subclass and the beta subfamily) in the *p24δ-1* mutant could still be sufficient to provide p24 function for normal plant performance. Indeed, despite the lack of p24 proteins from the delta-1 subclass, a proportion of GPI-anchored proteins is still able to reach the plasma membrane, which could be sufficient to provide their expected functions at this location. In addition, this mutant showed a constitutive activation of the unfolded protein response (UPR), which may help the plant to cope with the transport defects seen in the absence of p24 proteins (Pastor-Cantizano et al., 2018). The *p24δ-1* mutant also showed the transcriptional upregulation of the COPII subunit gene SEC31A, which encodes one of the two COPII SEC31 isoforms of *Arabidopsis*, but not SEC31B (Pastor-Cantizano et al., 2018). SEC31A shows 61% amino acid sequence identity with SEC31B and according to public microarray data (Zimmermann et al., 2004) SEC31B expression is about 10 times higher than that of SEC31A in *Arabidopsis* tissues. It would be interesting to study if this SEC31 isoform, together with specific SEC24 isoforms may play a role in ER export of GPI-anchored proteins in plants.

CHAPTER II. GPI ANCHOR REMODELLING AND TRANSPORT TO THE PLASMA MEMBRANE OF GPI-ANCHORED PROTEINS

Disrupting GPI-anchor synthesis in *Arabidopsis* is lethal. However, no studies have been reported of lipid GPI remodelling enzymes. In this study, we have used a loss-of-function approach to initiate the study of the role of *PGAP1/BST1* and *PGAP3/PER1* like *Arabidopsis* genes.

1 *PGAP1* GENES

Inositol deacylation of GPI-APs is mediated by mammalian PGAP1 and yeast Bst1p. As it is described in the Introduction (Table 4, Figure 21 and Figure 22), Bst1p and PGAP1 are ER membrane proteins with a catalytic serine containing motif that is conserved in a number of lipases. They function as a GPI inositol-deacylase and this deacylation is important for the efficient transport of GPI-anchored proteins from the ER to the Golgi apparatus. By searching for *Arabidopsis* putative GPI inositol-deacylase PGAP1-like (IPR012908, pfam07819) genes using Pfam and InterPro databases (Hunter et al., 2009; Finn et al., 2010), 7 *Arabidopsis* genes have been found (Table 12).

In this work, it was decided to begin the study of the putative function in lipid remodelling of GPI-APs of AT3G27325, the only *Arabidopsis PGAP1-like* gene that encodes a presumed ER protein. As a control, the

function of AT2G44970 and AT3G52570, which are not expected to localize to compartments of the secretory pathway, were also analyzed. From now on, AT2G44970, AT3G27325 and AT3G52570, will be referred as *PGAP1A*, *PGAP1B* and *PGAP1C*, respectively. We chose the mammalian name of the gene (*PGAP1*) because it is the name that appears in the *Arabidopsis* Information Resource (TAIR) and in addition, the yeast name had already been assigned to the *Arabidopsis* gene AT5G65090 (*BST1*, Bristled).

	Length (aa)	**Membrane Topology**	**Expected subcellular localization**
AT2G44970	503	Transmembrane	Nucleus
AT3G29790	144	Probably not a transmembrane protein	Nucleus
AT3G27325	1121	Transmembrane	ER/Plasma membrane
AT3G52570	335	Probably not a transmembrane protein	Mitochondria
AT4G34310	1228	Transmembrane	Chloroplast/Mitochondria
AT5G17670	309	Probably not a transmembrane protein	Chloroplast

Table 11. **Putative *Arabidopsis* GPI inositol-deacylase *PGAP1-like* genes.** For membrane topology it was used the TMpred program, (Hofmann and Stoffel, 1993) and the data for the expected subcellular localization was obtained from Tair (Arabidopsis.org).

To investigate the relative expression of the *PGAP1A-C* genes, we used the public available RNAseq expression database GENEVESTIGATOR (www.genevestigator.com) (Zimmermann et al., 2004; Hruz et al., 2008). As

it is shown in Figure 40, expression pattern of *PGAP1B* (AT3G27325) and *PGAP1C* (AT3G52570) genes are similar. Nevertheless, mRNA levels of *PGAP1A* (AT2G44970) are approximately double than *PGAP1B* and *PGAP1C* and a high expression in siliques is detected.

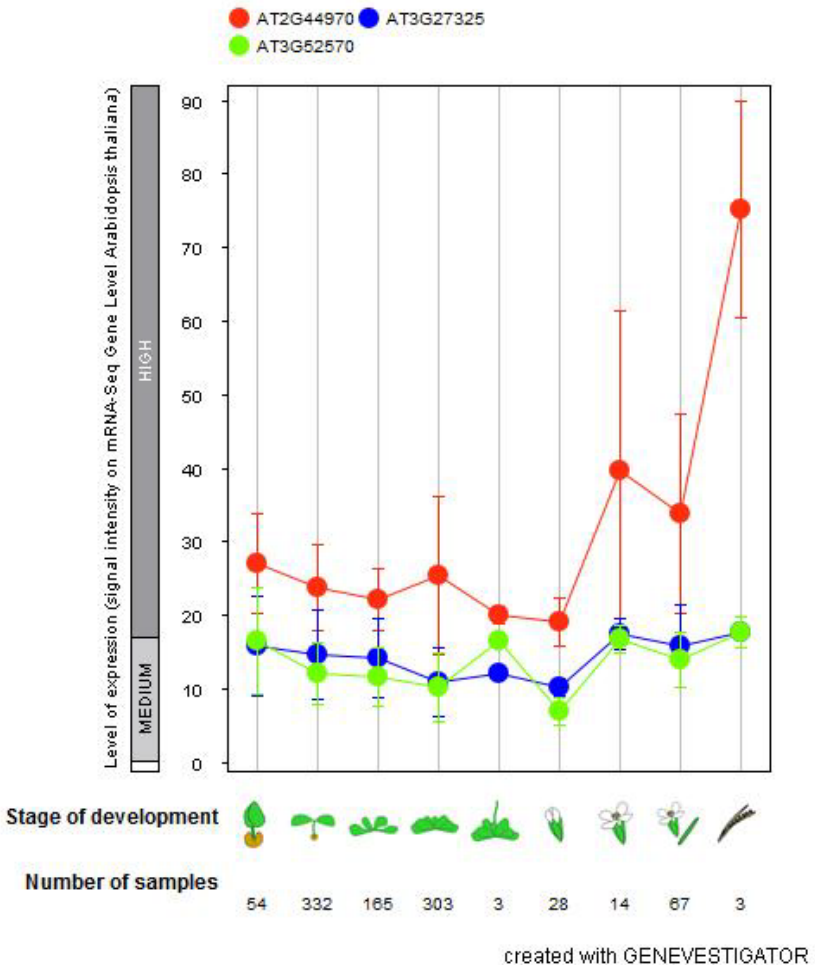

Figure 40. mRNA levels of *PGAP1A-C* in 9 developmental stages obtained from data of Genevestigator (Zimmermann et al., 2004; Hruz et al., 2008)**.**

1.1 IDENTIFICATION OF SINGLE T-DNA MUTANTS OF *PGAP1* GENES

To study the role of the *PGAP1B* gene, a reverse genetic approach was chosen. Several T-DNA insertion mutants of *PGAP1A-C g*enes were found in the *Arabidopsis* SALK collection (http://signal.salk.edu/cgi-bin/tdnaexpress).

1.1.1 *PGAP1A* mutants

Two mutants of *PGAP1A*, *pgap1A-1* (SALK_067058) and *pgap1A-2* (SALK_072702) were characterized (Figure 41 A). Homozygous plants were selected by PCR analysis. RT-PCR analysis showed that mRNA levels of *PGAP1A* from the *pgap1A-1* homozygous line were less than 10 % wild-type levels (Figure 41 B). No transcript of *PGAP1A* from the *pgap1A-2* homozygous line was detectable (Figure 41 B). Then, here we focused on the characterization of the *pgap1A-2* mutant for further analysis of *PGAP1A* loss of function.

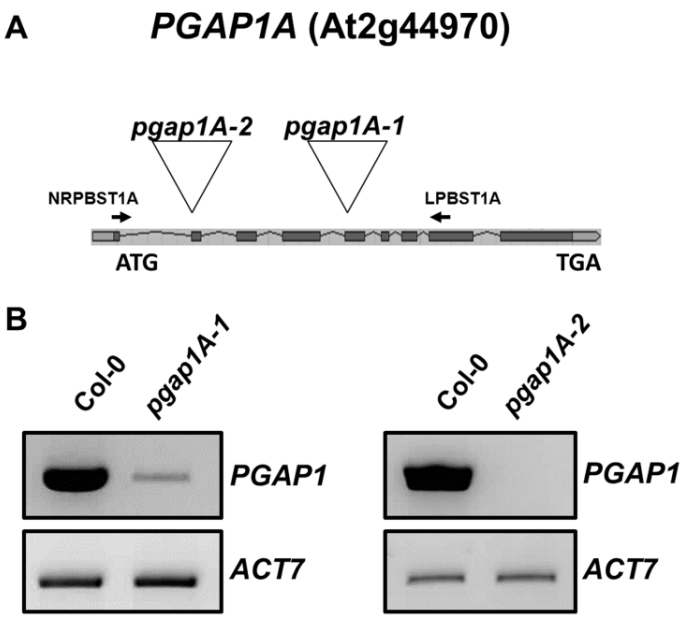

Figure 41. RT-sqPCR analysis of *PGAP1A* mRNA levels in the *pgap1A-1* and *pgap1A-2* mutants. **A.** Diagram of the *PGAP1A* gene and localization of the T-DNA insertion (triangle) in the *pgap1A-1* and *pgap1A-2* mutants. Black boxes represent coding regions. The positions of *PGAP1A* specific primers, NRPBst1A and LPBst1A (Table 9), are shown. PCRs were performed as described in sections 4.1.2 and 4.2.3 of Material and Methods. **B.** Total RNA from *pgap1A-1, pgap1A-2* and wild-type (Col-0) 4 day-old seedlings were used for the RT-PCR. In the PCRs, *PGAP1A* specific primers, NRPBst1A and LPBst1A, were used (Table 9). *Actin-7* (*ACT7*) was used as a control. RT-PCRs were performed as described in sections 4.1.3, 4.2.4 and 4.2.5 of Materials and Methods. PCR samples were collected at cycle 22 for *ACT7* and at cycle 36 for *PGAP1A*. It was observed that in wild-type and *pgap1A-1* plants, a cDNA fragment of 0,9Kb was amplified. In contrast, no fragment was detected in *pgap1A-2* plants.

1.1.2 *PGAP1B* mutants

Two mutants of *PGAP1B*, *pgap1B-1* (SALK_078662) and *pgap1B-2* (SAIL_1212_H07) were characterized (Figure 42 A). Homozygous plants were selected by PCR analysis. RT-PCR analysis showed that no transcript of *PGAP1B* from the *pgap1B-1 and pgap1B-2* homozygous lines was detectable

(Figure 42 B). Here we focused on the characterization of the *pgap1B-1* mutant for further analysis of *PGAP1B* loss of function.

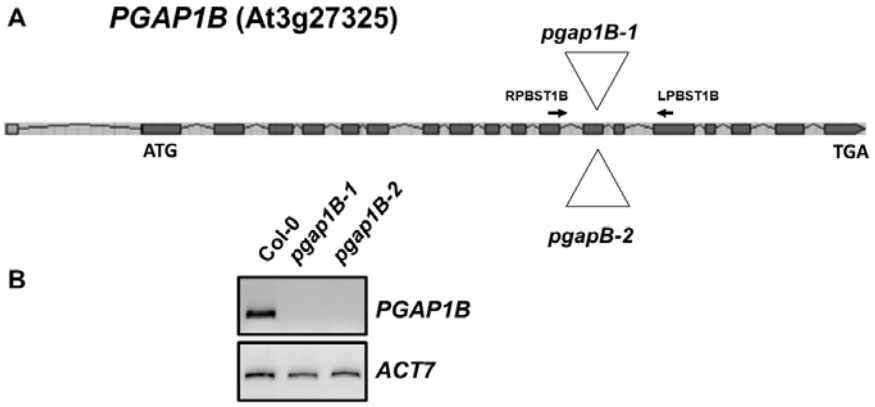

Figure 42. RT-sqPCR analysis of *PGAP1LB* mRNA levels in the *pgap1B-1* and *pgap1B-2* mutants. A. Diagram of the *PGAP1B* gene and localization of the T-DNA insertion (triangle) in the *pgap1B-1* and *pgap1B-2* mutants. Black boxes represent coding regions. The positions of *PGAP1B* specific primers, RPBst1B and LPBst1B, are shown**. B.** Total RNA from *pgap1B-1, pgap1B-2* and wild-type (Col-0) 4 day-old seedlings were used for the RT-PCR. In the PCRs, *PGAP1B* specific primers, RPBst1B and LPBst1B, were used (Table 9). *Actin-7* (*ACT7*) was used as a control. PCR samples were collected at cycle 22 for *ACT7* and at cycle 36 for*PGAP1B.* It was observed that in wild-type plants, a cDNA fragment of 650pb was amplified. In contrast, this fragment was not detected in *pgap1B-1 and pgap1B-2* plants.

1.1.3 *PGAP1C* mutant

One mutant of *PGAP1C*, *PGAP1C-1* (SAIL_302_A06) was characterized (Figure 43 A). Homozygous plants were selected by PCR analysis. RT-PCR analysis showed that no transcript of *PGAP1C* from the *pgap1C-1* homozygous lines was detectable (Figure 43 B).

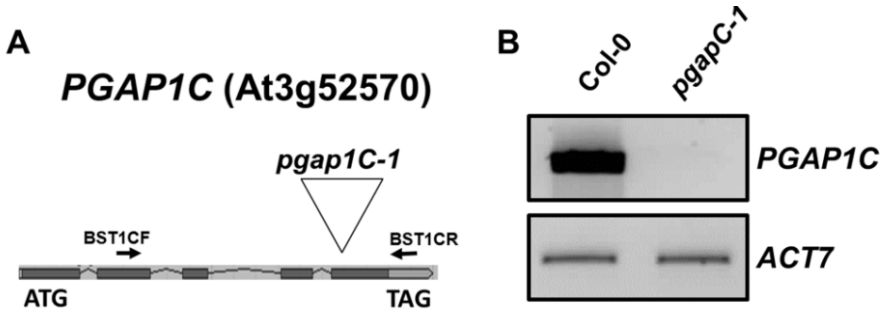

Figure 43. RT-sqPCR analysis of *PGAP1C* mRNA levels in the *pgap1C-1* mutant. A. Diagram of the *PGAP1C* gene and localization of the T-DNA insertion (triangle) in the *pgap1C-1* mutant. Black boxes represent coding regions. The positions of *PGAP1C* specific primers, Bst1CF and Bst1CR, are shown**. B.** Total RNA from *pgap1C-1* and wild-type (Col-0) 4 day-old seedlings were used for the RT-PCR. In the PCRs, *PGAP1C* specific primers, Bst1CF and Bst1CR, were used (Table 9). *Actin-7* (*ACT7*) was used as a control. PCR samples were collected at cycle 22 for *ACT7* and at cycle 36 for *PGAP1C.* It was observed that in wild-type plants, a cDNA fragment of 0,9Kb was amplified. In contrast, no fragment was detected in *pgap1C-1* plants.

None of the single mutants of *PGAP1A, PGAP1B* and *PGAP1C* showed any phenotypic alteration under standard growth conditions (Figure 44 B) or salt stress (data not shown).

1.1.4 Generation of the *pgap1ABC* triple mutant

The single mutants were crossed to obtain double mutants. Then, triple mutant was generated by crossing double mutants that share one allele. Genotype analysis of the progenies was performed by PCR in order to obtain the homozygous lines of the multiple mutants. The triple mutant homozygous line was named *pgap1ABC* and it did not show any phenotypic alteration under standard growth conditions (Figure 44 B) or salt stress (data not shown). To check the mRNA levels of *PGAP1A, PGAP1B and PGAP1C* in the *pgap1ABC* mutant, RT-sqPCR was performed with total RNA extracted from wild-type and *pgap1ABC* seedlings. Figure 44 A showed that the triple

mutant lacks the full transcript of *PGAP1A*, *PGAP1B* and *PGAP1C*.

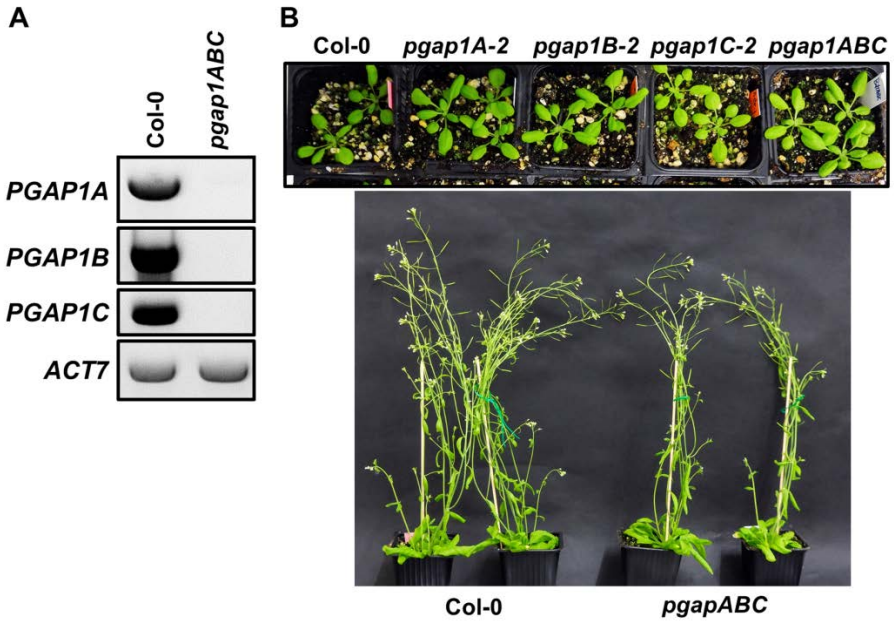

Figure 44. RT-sqPCR analysis of *pgap1ABC* mutant to show the absence of full-length *PGAP1A, PGAP1B and PGAP1C* mRNA. A. Total RNA from the triple T-DNA insertion mutant *pgap1ABC* and wild-type (Col-0) 4 day-old seedlings were used for the RT-PCR. In the PCRs, specific primers for *PGAP1A, PGAP1B* and *PGAP1C* genes were used (Table 9). *Actin-7* (*ACT7*) was used as a control. PCR samples were collected at cycle 22 for *ACT7* and at cycle 36 for *PGAP1A, PGAP1B* and *PGAP1C* genes. It was observed that in wild-type plants, the expected molecular weight cDNA fragments were amplified. In contrast no fragments were amplified in the mutant. **B**. *pgap1A-2*, *pgap1B-1*, *pgapC-1* and *pgap1ABC* mutants did not show a phenotype different from wild-type. Upper panel, 20 day-old plants and lower panel, 42 day-old plants of wild-type and the *pgap1ABC* mutant, respectively.

2 *PER1L* GENES

The lipid remodelling enzyme that removes an unsaturated acyl chain at the *sn-2* position of the PI moiety (see Table 4, Figure 21 and 22 of Introduction) is mediated by mammalian PGAP3 and yeast Per1p that belong

to the membrane bound hydrolase superfamily CREST (Pei et al., 2011). In this thesis, we have used a loss-of-function approach to study the role of *PER1* like *Arabidopsis* genes in GPI-APs metabolism. Two *Arabidopsis* genes, AT5G62130 and AT1G16560, have been assigned to belong to the PER1 family of fatty acid remodelling hydrolases for GPI-anchored proteins (Figure 45) (Pei et al., 2011). They share 60 % amino acid sequence identity. From now on, AT5G62130 and AT1G16560 will be referred as *PER1LA* and *PER1LB*, respectively. We chose this time the yeast name of the gene (*PER1*) because it is the name that appears in the *Arabidopsis* Information Resource (TAIR). We add the letter L at the end (*PER1L*, L stands for like) because the name *ATPER1* (1-cysteine peroxiredoxin 1) has already been assigned to the *Arabidopsis* gene AT1G48130.

Both *Arabidopsis* PER1L proteins are putative membrane proteins with expected subcellular localization at ER, Golgi apparatus or plasma membrane (Table 13).

Isoform	Mw (kDa)	Membrane Topology	Expected subcellular localization
*At*PER1LA	39,9	Transmembrane- C-terminus in the cytosol	ER/Golgi/PM
*At*PER1LB	39,4	Transmembrane- C-terminus in the cytosol	ER/Golgi/PM

Table 13. Putative *Arabidopsis* GPI inositol-deacylase *PER1-like* (*PER1L*) coding genes. For membrane topology it was used the TMpred program (Hofmann and Stoffel, 1993) and the data for the expected subcellular localization was obtained from Tair (Arabidopsis.org).

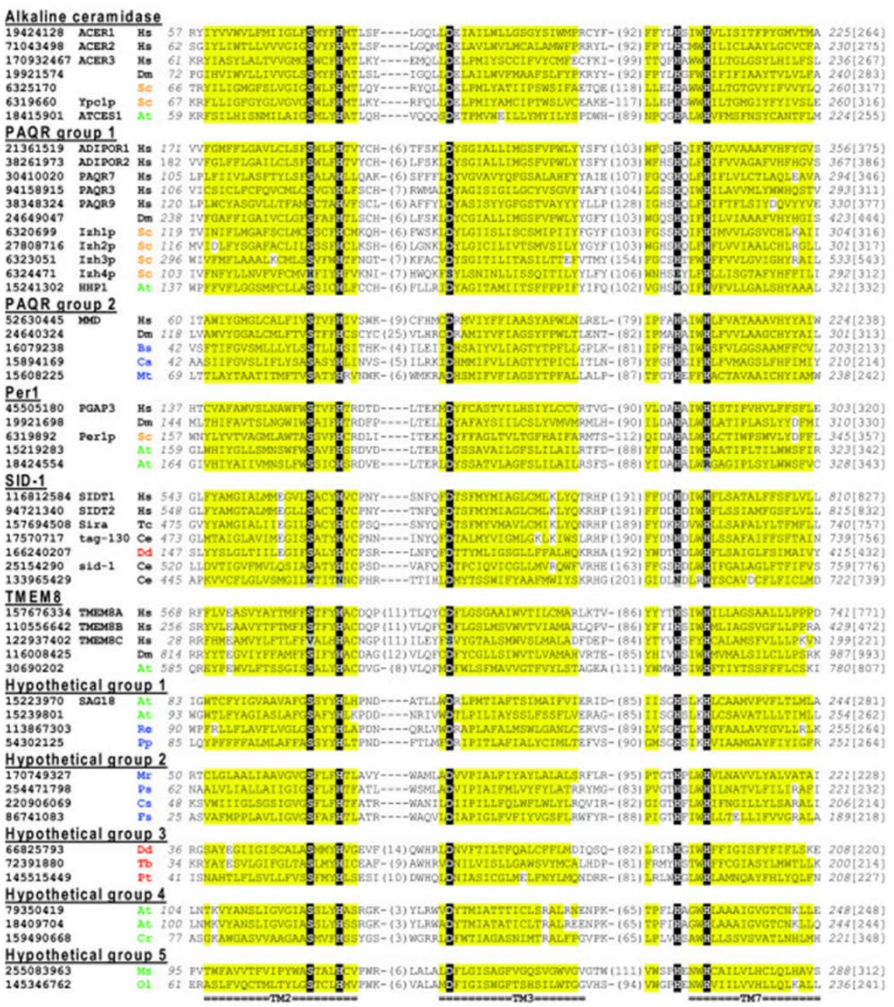

Figure 45. A multiple sequence alignment of CREST proteins (Pei et al., 2011). Three predicted core transmembrane segments (labeled TM2, TM3 and TM7 respectively below the sequences) with conserved motifs are shown for representative sequences of eleven CREST groups. Putative active site residues are shown on black background, whereas mutations in these positions are on grey background. Non-charged residues in mainly hydrophobic positions are on yellow background. NCBI gene identification numbers, along with common names for some proteins, are shown before the species abbreviations. The numbers of residues in between the three segments are shown in parentheses. Starting/ending residue numbers and sequence lengths are shown in italic font and in brackets, respectively. Species abbreviations are as follows: At, *Arabidopsis thaliana*; Bs, *Bacillus subtilis*; Ca, *Clostridium acetobutylicum*; Ce, *Caenorhabditis elegans*; Cr,

Chlamydomonas reinhardtii; Cs, *Cyanothece sp.*; Dd, *Dictyostelium discoideum*; Dm, *Drosophila melanogaster*; Fs, *Frankia sp.*; Hs, *Homo sapiens*; Mp, *Micromonas sp.*; Mr, *Methylobacterium radiotolerans*; Mt, *Mycobacterium tuberculosis*; Ol, *Ostreococcus lucimarinus*; Pp, *Photobacterium profundum*; Ps, *Pseudovibrio sp.*; Pt, *Paramecium tetraurelia*; Re, *Ralstonia eutropha*; Sc, *Saccharomyces cerevisiae*; Tb, *Trypanosoma brucei*; Tc, *Tribolium castaneum*. They are colored as follows: Metazoans, black; fungi, brown; plants, green; protists, red; and bacteria, blue.

To investigate the relative expression of the *PER1L* genes, we used the public available RNAseq expression database GENEVESTIGATOR (Zimmermann et al., 2004; Hruz et al., 2008) . As it is shown in Figure 46, the mRNA levels of *PER1LB* (AT1G16560) are higher than the mRNA levels of *PER1LA* (AT5G62130).

2.1 LOCALIZATION OF PER1L PROTEINS IN *N. BENTHAMIANA*

As described in the Introduction, Per1p (yeast) is an ER enzyme which removes an unsaturated acyl chain at the *sn-2* position of the PI moiety. However human PGAP3 is a functional homologue of Per1p but GPI anchor remodelling by PGAP3 occurs in the Golgi apparatus instead of at the ER. As a consequence, mammalian GPI-APs are segregated and sorted at the Golgi apparatus rather than at the ER, as it happens with yeast GPI-APs. Therefore, it would be of great interest to investigate the localization of the *Arabidopsis* PER1Ls.

In order to localize PER1LA-B *in vivo*, we prepared constructs of PER1LA and PER1LB with C-terminal RFP to be used for transient expression in *Nicotiana benthamiana* leaves, as it is described in section 2.4 of Material and Methods.

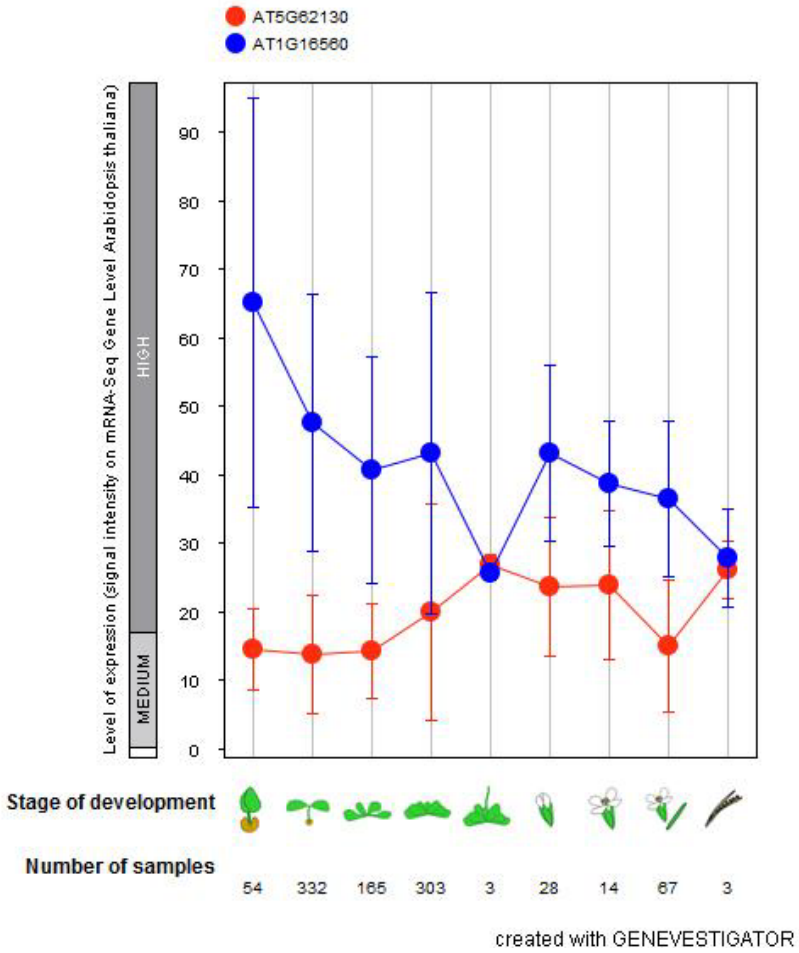

Figure 46. mRNA levels of *PER1LA-B* in 9 developmental stages obtained from data of Genevestigator (Zimmermann et al., 2004; Hruz et al., 2008)**.**

As shown in Figure 47 and 48, both PER1LA-RFP and PER1LB-RFP showed a punctate pattern and extensively colocalized with two Golgi markers, YFP-ManI and ST-YFP, but not with GFP-HDEL, an ER marker. These results clearly showed that both PER1LA and PER1LB proteins localize to the Golgi apparatus, as mammalian PGAP3, but in contrast to yeast Per1p, which is localized at the ER.

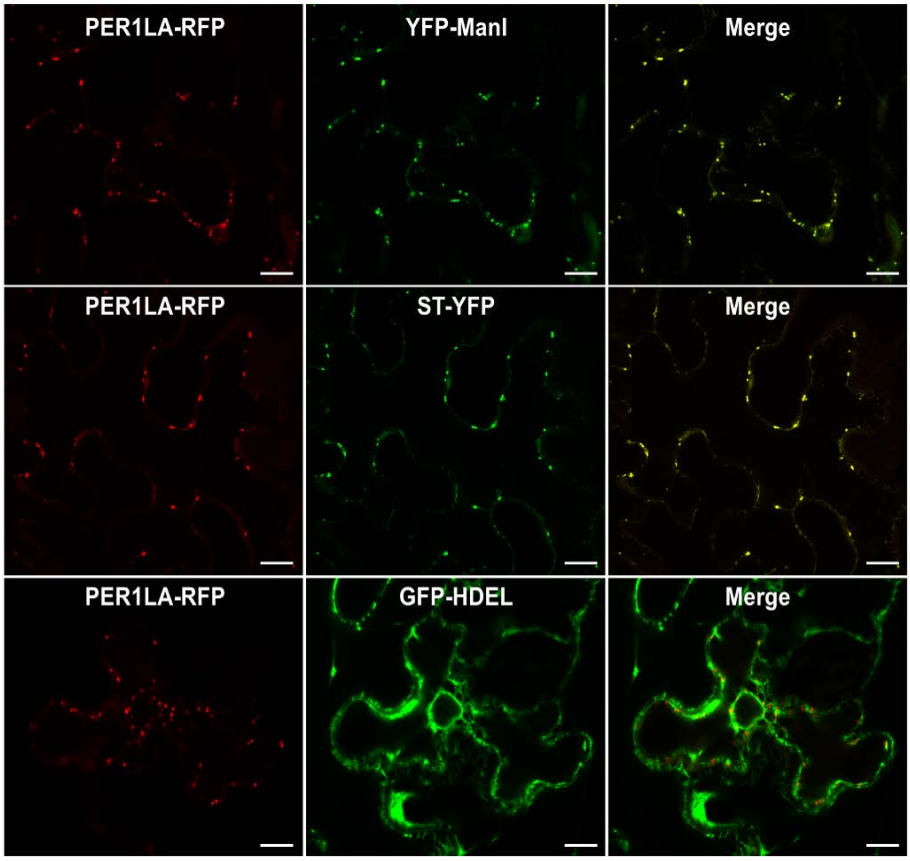

Figure 47. Localization of PER1LA-RFP in *N. Benthamiana* leaves. PER1LA-RFP (left panels) mainly showed a punctate pattern and extensively colocalized with the Golgi markers YFP-ManI and ST-YFP, but not with the ER marker GFP-HDEL (medium panels) (see merged images in right panels).

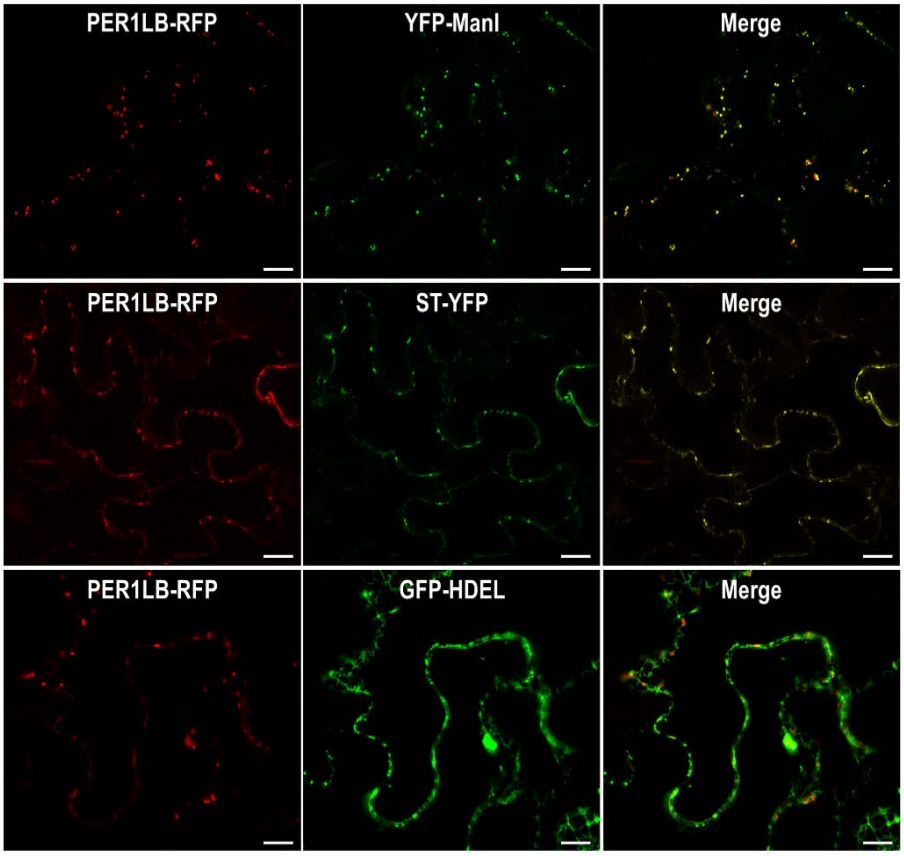

Figure 48. Localization of PER1LB-RFP in *N. benthamiana* leaves. PER1LB-RFP (left panels) mainly showed a punctate pattern and extensively colocalized with the Golgi markers YFP-ManI and ST-YFP, but not with the ER marker GFP-HDEL (medium panels) (see merged images in right panels).

2.2 CHARACTERIZATION OF LOSS OF FUNCTION MUTANTS OF *PER1L* GENES

2.2.1 *per1lA* mutant

per1lA-1 (SALK_039375) is the only one exon T-DNA insertion mutant of *PER1LA* found in the *Arabidopsis* SALK collection (http://signal.salk.edu/cgi-bin/tdnaexpress). Homozygous plants were selected by PCR analysis. RT-PCR analysis showed that the mRNA levels of *PER1LA* from the *per1lA-1* homozygous line were less than 10 % wild-type levels (Figure 49).

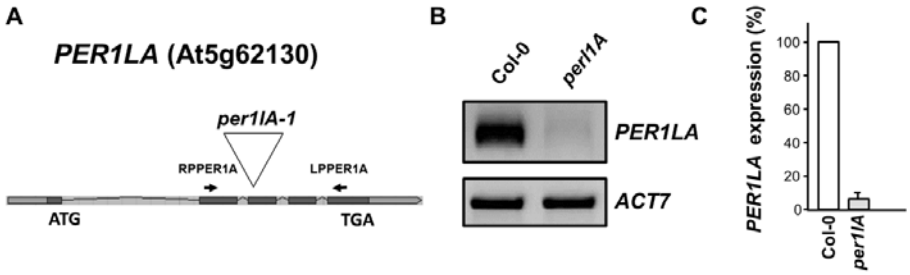

Figure 49. RT-sqPCR analysis of *PER1LA* mRNA in the *per1lA-1* mutant. **A.** Diagram of the *PER1LA* gene and localization of the T-DNA insertion (triangle) in the *per1lA-1* mutant. Black boxes represent coding regions. The positions of *PER1LA* specific primers, RPPER1A and LPPER1A (Table 9), are shown. **B.** Total RNA from *per1lA-1* and wild-type (Col-0) 4 day-old seedlings were used for the RT-PCR. In the PCRs, *PER1LA* specific primers, RPPER1A and LPPER1A, were used (Table 9). *Actin-7* (*ACT7*) was used as a control. PCR samples were collected at cycle 22 for *ACT7* and at cycle 36 for *PER1LA*. It was observed that in wild-type and *per1lA-1* plants, a cDNA fragment of 0,8Kb was amplified. **C.** Quantification of the experiments shown in B from three biological samples. Values were normalized against the *PER1LA* fragment band intensity in wild-type that was considered to be 100 %. Error bars represent SEM. The signal intensities of bands were measured using ImageJ software.

2.2.2 *per1lB* mutants

Due to the lack of *PER1LB* T-DNA insertion mutants in mutant collections, artificial microRNAs (amiRNAs) were used to knock down the expression of this gene. The *PERL1B* amiRNA construct CSHL_013451 was purchased from *Arabidopsis* Biological Resource Center (ABRC, https://abrc.osu.edu/). This amiRNA, that we called amiR-PER1LB, is targeted to a sequence of the last exon of *PER1LB*. After transformation with this construct, transgenic plants were selected by antibiotics and segregation of these lines were analyzed as described in section 2.6.1 of Material and Methods. T3 homozygous generation was used to characterize silencing by RT–PCR as above. Two independent homozygous lines, *amiR-per1lB-1* and *amiR-per1lB-2,* that showed the best silencing for *PER1LB* were selected (Figure 50).

2.2.3 Generation of the *amiper1lBper1lA* double mutant

per1A-1 plants were transformed with the amiR-PER1LB construct. Transgenic plants were selected by antibiotics and segregation of these lines were analyzed. T3 homozygous generation was used to characterize silencing by RT–PCR as above. Two independent homozygous lines, *amiR-per1lBper1lA-1* and *amiR-per1lBper1lA-2,* that showed the best silencing for *PER1LB* were selected (Figure 50 B) and from now on, they will be referred as *per1lAB-1* and *per1lAB-2,* respectively.

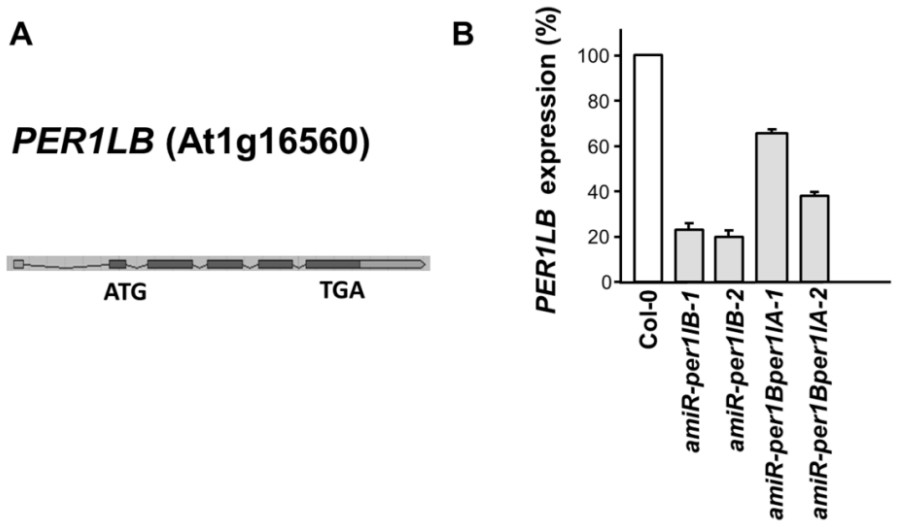

Figure 50. RT-sqPCR analysis of *PER1LB* mRNA in the *amiR-per1lB* mutants. **A.** Diagram of *PER1LB* gene. Black boxes represent coding regions. **B.** Total RNA from *amiR-per1lB-1*, *amiR-per1lB-2, amiR-per1lBper1lA-1 and amiR-per1lBper1lA-2* and wild-type (Col-0) 4 day-old seedlings were used for the RT-PCR. In the PCRs, *PER1LB* specific primers, Per1BR and Per1B were used (Table 9). *Actin-7* (*ACT7*) was used as a control. PCR samples were collected at cycle 22 for *ACT7* and at cycle 36 for *PER1LB*. Quantification of the experiments shown in B from three biological samples. Values were normalized against the *PER1LB* fragment band intensity in wild-type that was considered to be 100 %. Error bars represent SEM. The signal intensities of bands were measured using ImageJ software.

None of the single mutants of *PER1LA*, *PER1LB* and double mutants of *PER1LA-B* showed any obvious phenotypic alteration under standard growth conditions when comparing to wild-type plants (Figure 51). However, we found that *per1Al-1* and *per1lAB-2* showed enhanced sensitivity to salt stress (Figure 52).

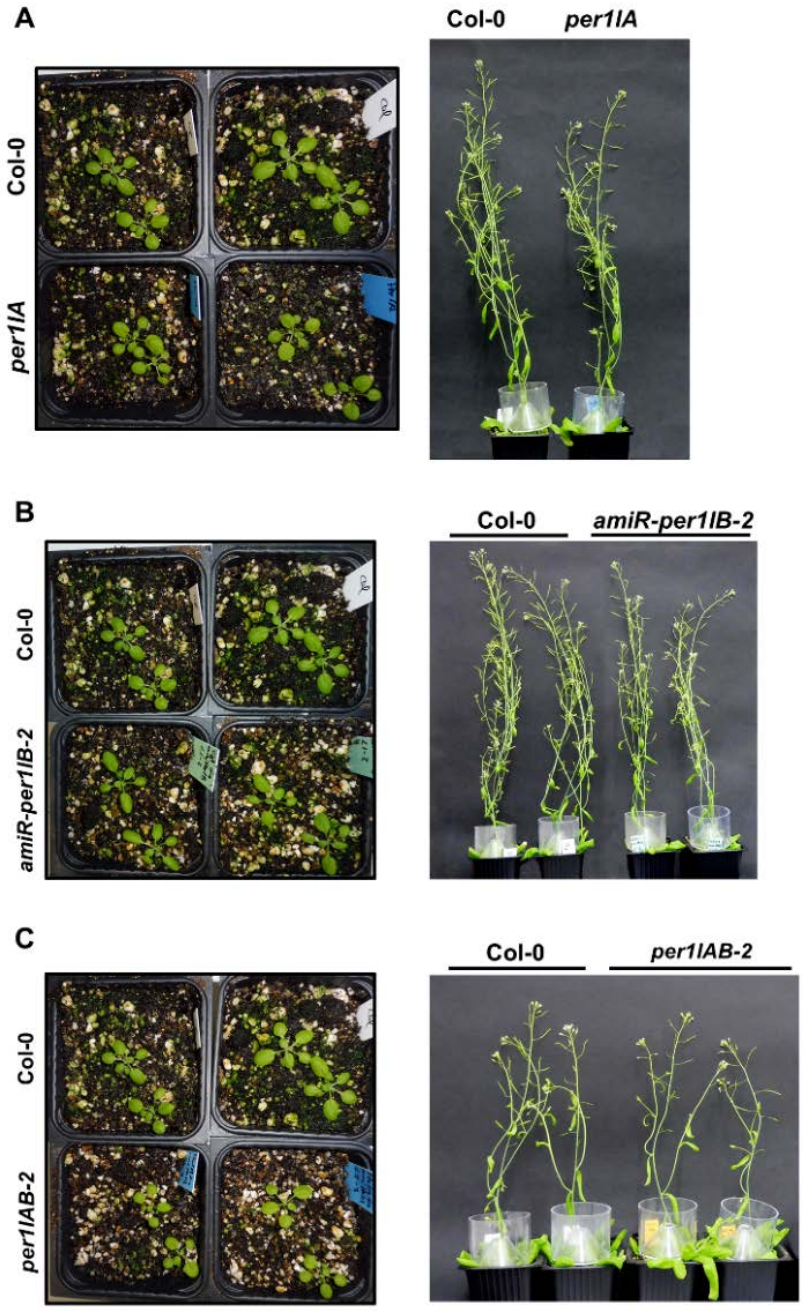

Figure 51. Phenotypic analysis of *per1IA-1, per1IB-2 and per1IAB-2* mutants under standard growth conditions.

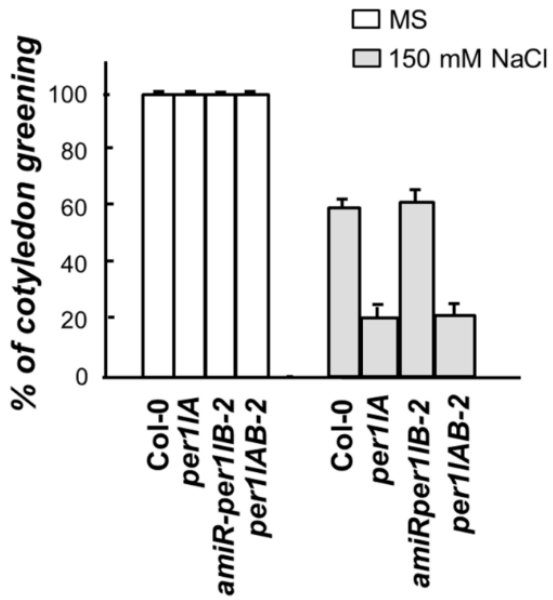

Figure 52. Phenotypic analysis of *per1lA-1, amiper1lB-2 and per1lAB-2* mutants exposed to salt (NaCl) stress. Wild-type (Col-0) and mutant seeds were sown on 0.5× MS for control conditions and 0.5× MS supplemented with 150 mM NaCl in Petri plates as described in section 3.1 of Material and Methods. The percentage of seedlings with green cotyledons was calculated after 12 days. Data are mean±s.e.m. (n=100) of three independent experiments.

3 LOCALIZATION OF GPI-ANCHORED PROTEINS IN *pgap1* AND *per1l* MUTANTS

3.1 LOCALIZATION IN *pgap1* MUTANTS

To test for the putative involvement of GPI anchor remodelling in the transport to the plasma membrane of GPI-anchored proteins, we used the same two markers as in Chapter I, a GFP fusion with the arabinogalactan protein 4 (GFP-AGP4) and a glycosylphosphatidylinositol anchor fused to GFP

(GFP-GPI) (Martinière et al., 2012). As a control, we used a transmembrane plasma membrane protein, the aquaporin PIP2A-RFP. To test for the localization of these proteins, we first used transient expression in *Arabidopsis* seedlings (see section 2.5 of Material and Methods), as in Chapter I.

The localization of these markers was analyzed in *PGAP1A*, *PGAP1B* and *PGAP1C* mutants and in wild-type (Col-0) *Arabidopsis* seedlings. As shown in Figure 53 A-B, both GFP-AGP4 and GFP-GPI were exclusively localized to the plasma membrane of cotyledon cells of wild-type *Arabidopsis* seedlings, as it was the case for PIP2A-RFP, a transmembrane plasma membrane protein (Figure 53 C), as shown in Chapter I. In both *pgap1A-2* (Figure 53 D-E) and *pgap1C-1* (Figure 53 J-K) mutants, GFP-AGP4 and GFP-GPI were also localized to the plasma membrane, as PIP2-RFP (Figure 53 F and 53 L), suggesting that PGAP1A and PGAP1C enzymes are not required for transport to the plasma membrane of GPI-anchored proteins. In clear contrast, GFP-AGP4 showed a predominant ER localization pattern in the *pgap1B-1* mutant; and in some cases, punctate structures were also observed, suggesting a partial Golgi localization (Figure 53 G). The same happened with the second GPI-anchored marker protein, GFP-GPI (Figure 53 H), which showed a predominant ER pattern and also a partial punctate pattern, which was more obvious than that of GFP-AGP4. In clear contrast, PIP2A-RFP mostly localized to the plasma membrane in this mutant (Figure 53 F), suggesting that PGAP1B enzyme is specifically required for transport to the plasma membrane of GPI-anchored proteins, and that loss of *PGAP1B* function does not affect transport of transmembrane proteins from the ER to the plasma membrane.

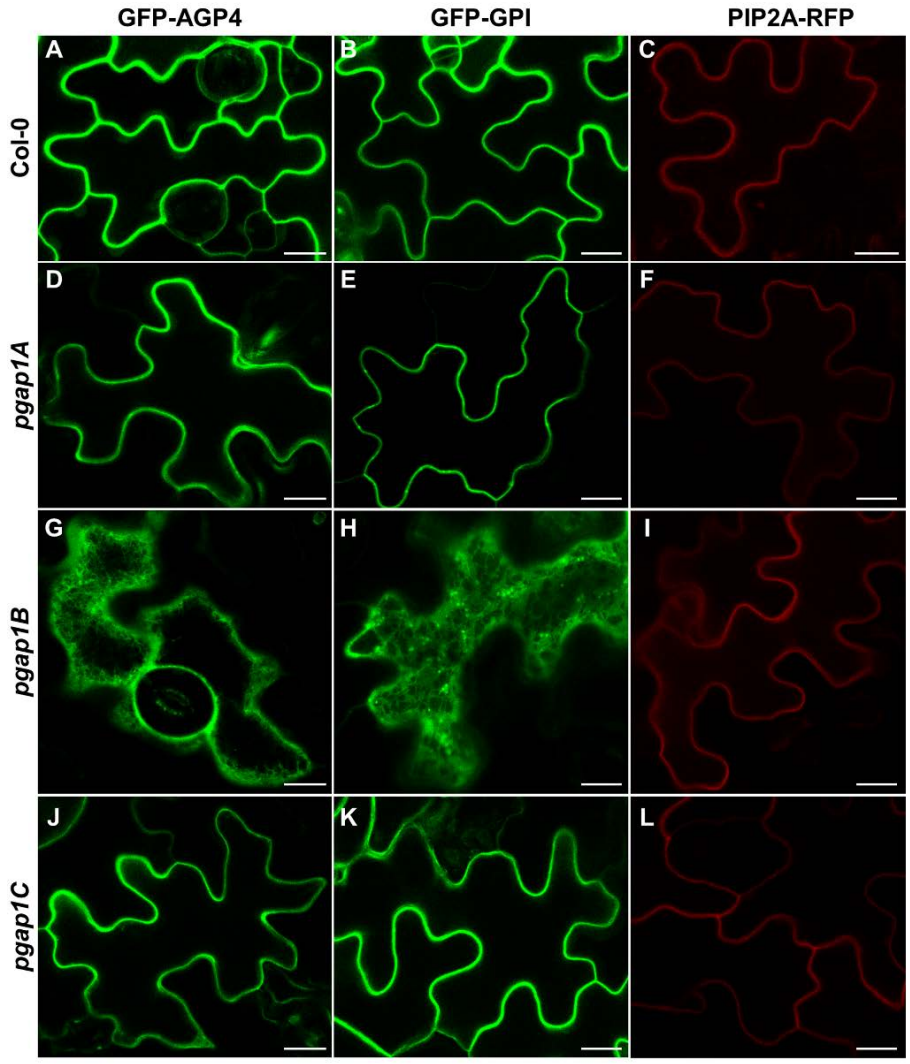

Figure 53. Localization of plasma membrane proteins in wild-type and *PGAP1* mutants *Arabidopsis* seedlings. Transient gene expression in *Arabidopsis* seedlings. Two GPI-anchored proteins, GFP-AGP4 (A) and GFP-GPI (B), mainly localized to the plasma membrane in cotyledon cells from wild-type (Col-0) seedlings, as the transmembrane plasma membrane protein PIP2A-RFP (C). In the *pgap1A-2* and *pgap1C-1* mutants, both GFP-AGP4 (D, J) and GFP-GPI (E, K) showed a predominant plasma membrane localization, as PIP2-RFP (F, L). In the *pgap1B-1* mutant, GFP-AGP4 (G) and GFP-GPI (H) showed a predominant ER localization pattern and also a partial punctate pattern, in contrast to PIP2A-RFP (I), which mainly localized to the plasma membrane. Scale bars, 10 µm.

To confirm these results, we next analyzed the localization of GFP-AGP4 and GFP-GPI by transient expression in *Arabidopsis* protoplasts as described in section 2.3 of Material and Methods. We have shown previously (Chapter I) that both GFP-AGP4 and GFP-GPI were mostly localized to the plasma membrane of protoplasts from wild-type *Arabidopsis* plants. In contrast, GFP-AGP4 showed a predominant ER localization pattern in protoplasts from the *pgap1B-1* mutant (Figure 54 A-B); occasionally, some punctate structures were also observed (Figure 54 A). The same happened with the second GPI-anchored marker protein, GFP-GPI (Figure 54 C-D), which showed a predominant ER pattern and also a partial punctate pattern, which was more obvious than that of GFP-AGP4, as observed in transient expression *Arabidopsis* seedlings experiments. Very similar results were observed in the triple *pgap1ABC* mutant (Figure 54 E-H).

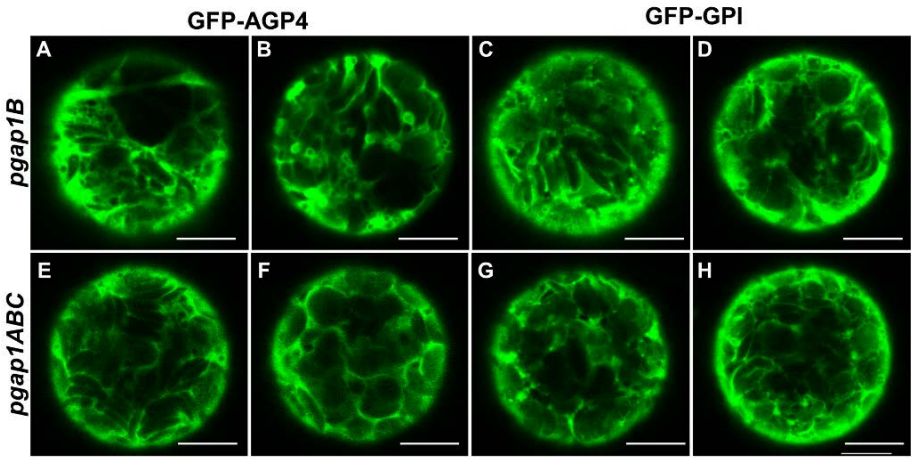

Figure 54. Localization of GPI-anchored proteins in *pgap1B-1* and *pgap1ABC* mutant *Arabidopsis* protoplasts. Transient gene expression in *Arabidopsis* protoplasts. GFP-AGP4 (A, B, E, F) and GFP-GPI (C, D, G, H) mainly showed an ER localization pattern and also a partial punctate pattern both in *pgap1B-1* (A-D) and *pgap1ABC* mutant (E-H) protoplasts. Scale bars, 10 μm.

To confirm the partial ER localization of both GFP-AGP4 and GFP-GPI in the *pgap1ABC* mutant, these markers were co-expressed with the ER marker RFP-calnexin. As shown in Figure 55, GFP-AGP4 and GFP-GPI partially colocalized with RFP-calnexin in the *pgap1ABC* mutant. In any case, both GFP-AGP4 and GFP-GPI could also partially reach the plasma membrane both in the *pgap1B-1* and *pgap1ABC* mutants.

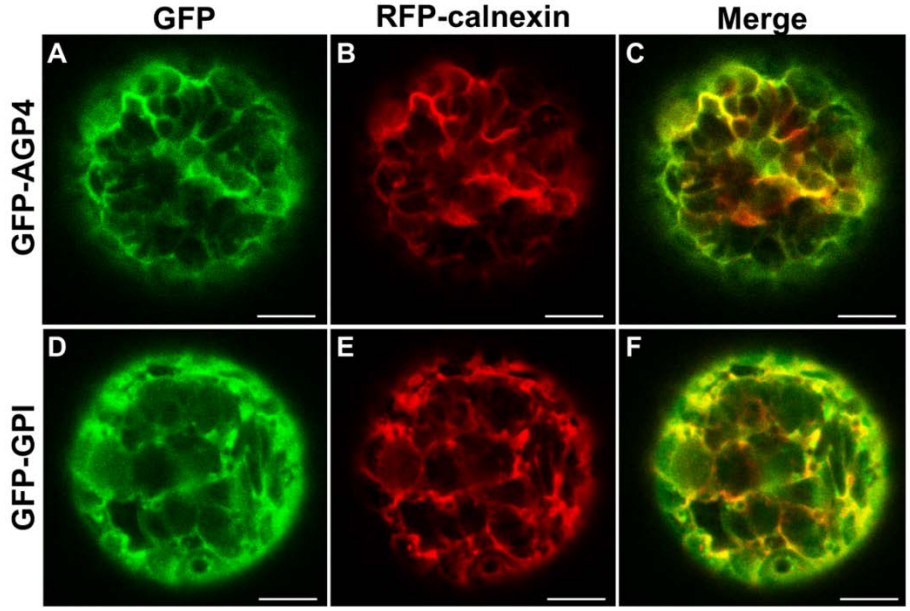

Figure 55. Localization of GFP-AGP4 and GFP-GPI in *pgap1ABC Arabidopsis* protoplasts. Transient gene expression in *Arabidopsis* protoplasts. In protoplasts from the *pgap1ABC* mutant, GFP-AGP4 (A) and GFP-GPI (D) mainly localized to the endoplamic reticulum, where they partially colocalized with the ER marker RFP-calnexin (B, E) (see merged images in C and F). Scale bars, 10 µm.

To test if the lack of PGAP1 enzymes affects the localization of other plasma membrane proteins different from GPI-APs, we used different membrane-anchoring types of minimal constructs, including a myristoylated and palmitoylated GFP (MAP-GFP) and a prenylated GFP (GFP-PAP)

(Martinière et al., 2012), as in Chapter I. We also used a transmembrane protein, a GFP fusion with the plasma membrane ATPase (GFP-PMA). As shown in Figure 56, these 3 proteins mainly localized to the plasma membrane of *pgap1B-1* (Figure 56 D-F) and *pgap1ABC* (Figure 56 G-I) mutant protoplasts, as in protoplasts from wild-type *Arabidopsis* plants (Figure 56 A-C). Therefore, PGAP1B function seems to be specifically required for ER export and transport to the plasma membrane of GPI-anchored proteins.

3.2 LOCALIZATION IN *per1l* MUTANTS

We next analyzed the localization of GFP-AGP4, GFP-GPI and PIP2A-RFP in *per1lA-1*, *per1lB-2* and *per1lAB-2* mutants by transient expression in *Arabidopsis* seedlings. As shown in Figure 57, GFP-AGP4 showed a predominant ER localization pattern in these three mutants, together with some punctate pattern which could reflect a partial Golgi localization. In clear contrast, GFP-GPI mostly localized to the plasma membrane in the three mutants, as it was also the case of PIP2A-RFP. This suggests that PER1L function may be required for transport to the plasma membrane of the GPI-anchored protein GFP-AGP4, but not to that of GFP-GPI or of a transmembrane plasma membrane protein (PIP2A-RFP).

We also analyzed the localization of GFP-AGP4 and GFP-GPI in protoplasts from these three mutants. As shown in Figure 58, GFP-AGP4 showed a predominant ER localization pattern in the three mutants, as well as a partial punctate pattern and also plasma membrane localization. GFP-GPI also showed a partial ER localization pattern, as well as a partial punctate pattern and also some plasma membrane localization in the *per1lA* and *per1lAB-2* mutants. In clear contrast, GFP-GPI mostly localized to the plasma

membrane in the *per1lB-2* mutant as it happened in transient expression in *Arabidopsis* seedlings.

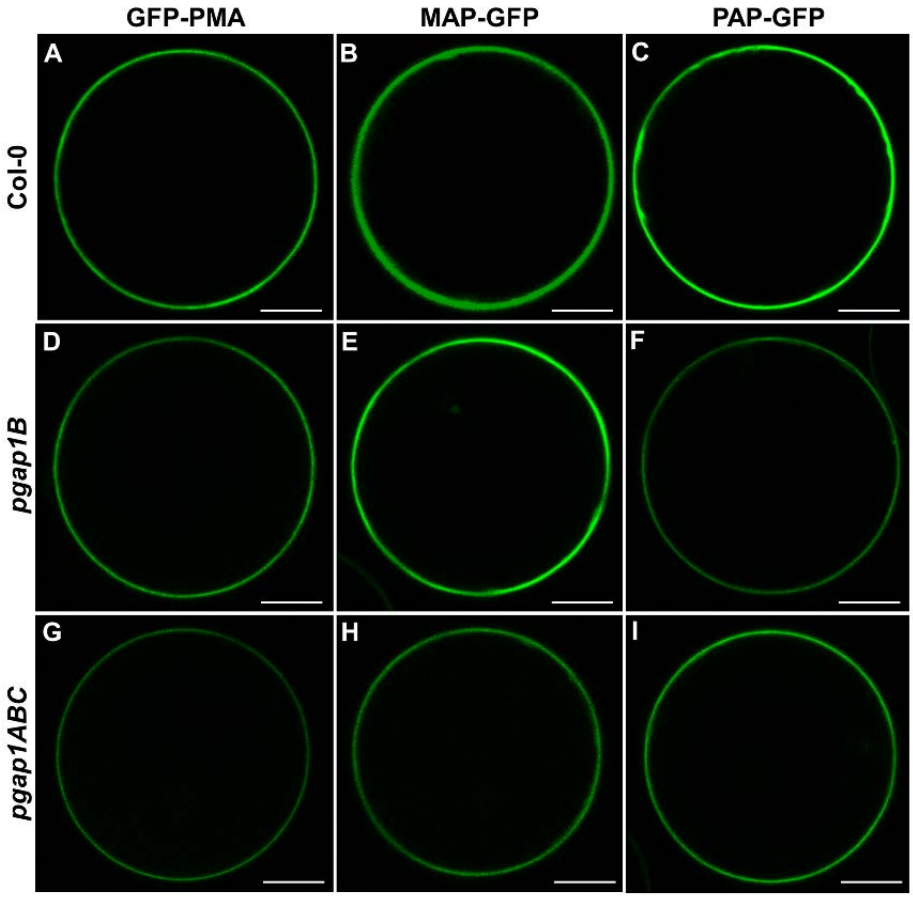

Figure 56. Localization of plasma membrane proteins without a GPI anchor in wild-type and *pgap1B-1* and *pgap1ABC* mutants *Arabidopsis* protoplasts. Transient gene expression in *Arabidopsis* protoplasts from wild-type (Col-0) (A-C), *pgapB-1* (D-F) and *pgap1ABC* (G-I) mutants. The plasma membrane ATPase (GFP-PMA), a myristoylated and palmitoylated GFP (MAP-GFP) and a prenylated GFP (GFP-PAP) mostly localized to the plasma membrane both in wild-type and in mutants protoplasts. Scale bars, 10 µm.

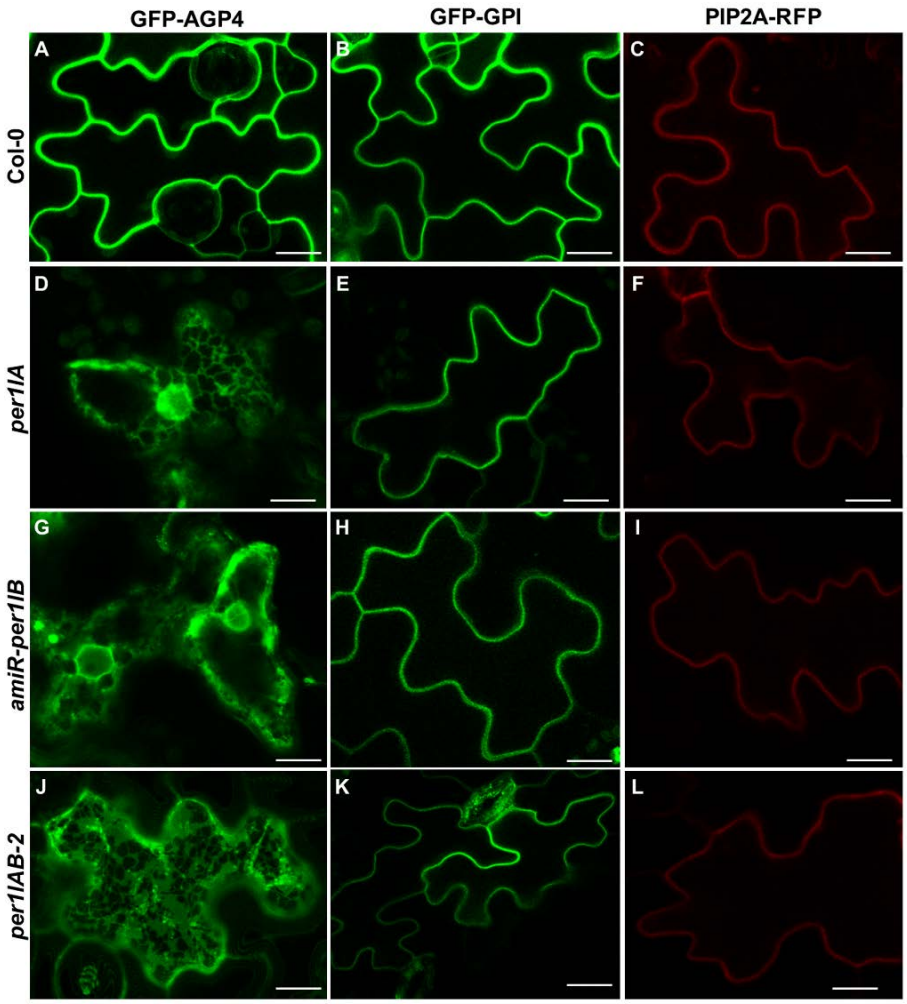

Figure 57. Localization of GFP-AGP4, GFP-GPI and PIP2A-RFP in wild-type and *per1lA-1*, *per1lB-2* and *per1lAB-2* mutants *Arabidopsis* seedlings. Transient gene expression in *Arabidopsis* seedlings. Two GPI-anchored proteins, GFP-AGP4 (A) and GFP-GPI (B), mainly localized to the plasma membrane in cotyledon cells from wild-type (Col-0) seedlings, as the transmembrane plasma membrane protein PIP2A-RFP (C). In the *per1lA-1*, *per1lB-2* and *per1lAB-2* mutants, GFP-AGP4 (D, G, J) mostly showed an ER localization and partial punctate pattern, in contrast to GFP-GPI (E, H, K), which showed a predominant plasma membrane localization, as PIP2-RFP (F, I, L). Scale bars, 10 µm.

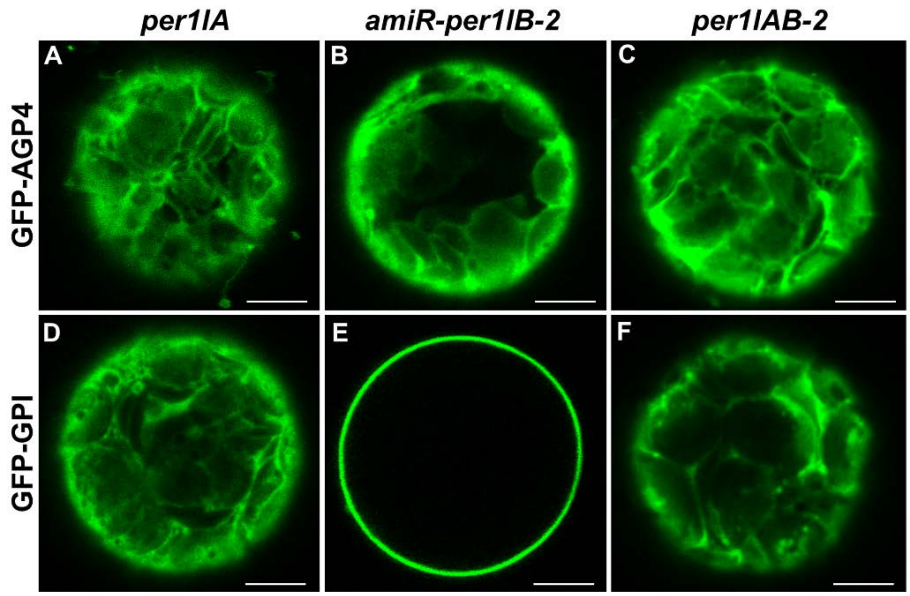

Figure 58. Localization of GPI-anchored proteins in *per1LA-1*, *per1lB-2* and *per1AB-2* mutants in *Arabidopsis* protoplasts. Transient gene expression in *Arabidopsis* protoplasts. GFP-AGP4 (A-C) mainly showed an ER localization and partial punctate pattern in *per1lA-1* (A), *per1lB-2* (B) and *per1lAB-2* mutants (C) protoplasts. The same was true for GFP-GPI in *per1lA-1* (D) and *per1lAB-2* mutants (F) protoplasts, but not in the *per1lB-2* mutant (E), where it mainly localized to the plasma membrane. Scale bars, 10 µm.

As a control, we used plasma membrane markers without a GPI anchor, including GFP-PMA, MAP-GFP and GFP-PAP. They mostly localized to the plasma membrane in these three mutants (data not shown), suggesting that transport of other plasma membrane proteins is not affected in these mutants.

4 DISCUSSION

Up to now, only one plant GPI anchor structure has been resolved, the structure of *Pc*AGP1, isolated from *Pyrus communis* (pear) cell suspension culture (Oxley and Bacic, 1999). From this structure, it can be said that the core structure of GPI anchors seem to be conserved in plant and non-plant eukaryotes. In addition, a survey of the *Arabidopsis* genome indicates that most of the genes involved in particular steps of GPI anchor assembly and their remodelling have orthologs in *Arabidopsis* (Luschnig and Seifert, 2011). However, it has to be established if all yeast and human orthologs of these enzymes are functional and if their function is conserved.

Five *Arabidopsis* orthologs of enzymes involved in the biosynthesis and attachment of the GPI anchor have been studied: SETH1, SETH2, PEANUT1 (PNT1), APTG1 and AtGPI8 (Lalanne et al., 2004; Gillmor et al., 2005; Dai et al., 2014; Bundy et al., 2016). SETH1 (PIG-C, mammals/Gpi2p, yeast) and SETH2 (PIG-A, mammals /Gpi3pA, yeast) are the orthologs of the GPI-GlcNAc transferase that initiates the anchor biosynthesis and is involved in the generation of the N-glucosamine-phosphoinositide (GlcN-PI). PEANUT1 and APTG1 are orthologs of the mammalian mannosyl-transferases PIG-M (yeast GPi14p) and PIG-B (yeast Gpi10p), respectively and *At*GPI8 (PIG-K/Gpi8p) is the GPI transamidase that catalyzes the transfer of an assembled GPI anchor to proteins. Studies with *Arabidopsis* null mutants of these enzymes results in lethality, either gametophytic or embryogenic. This indicates that GPI-anchored proteins are essential for plant growth and development.

Once the anchor is assembled in the ER and transferred *en bloc* to the processed C-terminus of a protein, subsequent remodelling of the anchor, particularly with regard to its lipid moiety, occurs during secretion. The lipid found in the only plant GPI anchor structure described was a ceramide consisting primarily of phytosphingosine and tetracosanoic acid. No studies have been reported of lipid GPI remodelling enzymes in *Arabidopsis*. In the second part of this thesis, we have undertaken the characterization of mutants of the *Arabidopsis* orthologs of two enzymes involved in lipid remodelling: Yeast Bst1p/mammal PGAP1 and yeast Per1p/mammal PGAP3.

7 *Arabidopsis* genes have been found that encode *Arabidopsis* putative PGAP1 genes. In this study we have characterized single mutants of three of them, *PGAP1A-C*, and we have also obtained and characterized the triple mutant. The three putative PGAP1 proteins have the typical lipase motif (GxSxG) containing a catalytic Ser. Only PGAP1B is expected to localize at the ER and therefore function as the GPI inositol-deacylase which cleaves the acyl chain from the inositol of the GPI anchor.

Interestingly, the trafficking of GPI-APs was altered in the *pgapB-1* and the *pgap1ABC* mutants but not in *pgap1A-2* or *pgap1C-1* mutants. Both, GFP-AGP4 and GFP-GPI, showed mainly ER localization both in protoplasts and seedling transient expression. This agrees with previous results in yeast and mammals that showed that in cultured cells null mutations of PGAP1 causes accumulation of GPI-APs in the ER due to inefficient exit from the ER (Tanaka et al., 2004; Fujita et al., 2011). However, steady state levels of cell surface GPI-APs were only mildly affected and therefore, GPI-APs with

unusual GPI structures were expressed at the cell surface. The studies with individuals with PGAP1 deficiency indicate that null mutations are compatible with life in humans but mainly affects functions of neuronal cells (Ueda et al., 2007; Murakami et al., 2014; Williams et al., 2015). *ScBst1p* is also non-essential in yeast (Komath et al., 2018) and its deletion causes delay in transport of GPI-APs from the ER to the Golgi (Fujita et al., 2006b). All these results are consistent with the fact that none of the *Arabidopsis* mutants have a phenotype different of wild-type under standard growth conditions. None of the mutant have altered salt response either. In the future, it would be interesting to determine the sensitivity of the mutants to other kind of stress and if the cell wall is affected, as it happens in *Candida albicans* (Liu et al., 2016a).

We have also characterized mutants of Per1p/PGAP3, other enzyme involved in lipid remodelling of the GPI anchor. In *Arabidopsis* there are two isoforms that we called PER1LA and PER1LB. They both belong, as Per1p/PGAP3, to the membrane-bound hydrolase superfamily name CREST (Pei et al., 2011). PER1LA and PER1LB share 60 % amino acid sequence identity and the expression of PER1LB is higher than PER1LA. We only found in T-DNA mutant public collections one exon T-DNA insertion mutant of *PER1LA* that has reduced expression of PER1A (less than 10 % wild-type expression). However, no *PER1LB* mutant was found and therefore an amiRNA was used to knock down the expression of *PER1LB*. Two independent amiRNA lines with reduced levels of *PER1B* were obtained (*amiR-per1lB-1* and *amiR-per1lB*-2). Both lines have reduced *PER1LB* expression (around 20 % *PER1LB* wild-type expression). In addition we obtained two independent lines of the double mutant, *per1lAB-1* and

per1lAB-2 (with around 65 % and 40 % *PER1LB* wild-type expression, respectively).

Interestingly, the trafficking of GPI-APs was altered in *per1lA-1*, *perlB-2* and *per1lAB-2* mutants. In all three mutants, GFP-AGP4 and GFP-GPI mainly localized to the ER and punctate structures (possibly Golgi apparatus) in transient expression experiments in seedlings or protoplasts. These results agree with previous results in yeast and mammals. In yeast, mature GPI-APs carry either a very long C26:0 at the *sn2* position or have a ceramide with phytosphingosine containing a C26:0 fatty acid. To replace the initial short fatty acid a series of events should take place, the first step of which involves the phospholipase A2 activity of the ER enzyme Per1p (Kinoshita and Fujita, 2016). This enzyme removes the fatty acid at *sn2* position to generate lyso-PI. The null mutant of Per1p was viable. However, in *per1p* cells, GPI-APs trafficking was altered and accumulate at the ER due to inefficient exit from the ER, consistently levels of cell surface GPI-APs were affected (Fujita et al., 2006a). Mammalian mature GPI-APs usually contained a stearic acid at the *sn2* position and PGAP3 is the enzyme that is involved in the removal of the initial unsaturated fatty acids at *sn2* position and generation of lyso-PI. PGAP3, as yeast Per1p, belongs to the CREST membrane-bound hydrolase superfamily although direct demonstration of this enzyme activity has not yet been shown (Pei et al., 2011). In contrast to yeast Per1p, which localized at the ER, mammalian PGAP3 is a Golgi enzyme (Maeda et al., 2007). The defect of PGAP3 also causes the surface expression of unremodelled GPI-APs, accompanying a decrease in the surface levels to different extents from almost normal to one-third of the normal level (Maeda et al., 2007). This variation may depend on proteins, cell types and animal species (Maeda et

al., 2017). It has also been observed that defects in PGAP3 causes that GPI-APs are not well associated with lipid rafts and their oligomerization is also altered (Murakami et al., 2012; Kinoshita and Fujita, 2016). There are several reports regarding *pgap3-ko* mice (Murakami et al., 2012). That mutant showed growth retardation and minor abnormalities as kinked tail and short heads. Mutations that produced low levels of PGAP3 in humans is associated with altered neuronal function (Howard et al., 2014).

We could not observe distinct phenotype in *per1lA-1, per1lB-2 and per1lAB-2* mutants when compared with wild-type plants under standard growth conditions. Nevertheless, *per1lA-1* and *per1lAB-2* mutants showed enhanced sensitivity to salt stress. Since many GPI-APs are signal receptors that help the cell response to the extracellular environment, GPI anchor remodelling defects are also expected to have altered cellular response to salt stress. *amiR-per1lB*, in contrast to other mutants, did not show enhanced sensitivity to salt stress. This suggests that the response to salt stress is PER1LA specific. Alternatively, this mutant may express enough PER1L to respond to salt stress.

The localization at the Golgi apparatus of PER1LA-RFP and PER1LB-RFP, may indicate that the fatty acid remodelling occurs at the Golgi, as it happens in mammalian cells. This fact suggests that the GPI anchor remodelling pathway of GPI-APs in *Arabidopsis* is likely as in mammals and not as in yeast, as it was previously supposed. Strikingly, in the *pgap1B-1* and *pgap1ABC* mutants, GFP-AGP4 and GFP-GPI showed the same localization pattern than in *per1lA-1*, *per1lB-2* and *per1lAB-2* mutants, in spite of the different localization of PGAP1B (ER) and PER1LA-B (Golgi) enzymes,

respectively. The predominant pattern of GPI-anchored proteins in these mutants was ER accumulation and some punctate structures that may correspond to Golgi apparatus. Co-localization experiments with Golgi markers and PGAP1B-RFP assays will be done in the future to corroborate that.

In the case of *pgap1B-1* and *pgap1ABC* mutants, this could be explained because GPI-anchored proteins which are not correctly remodelled could escape to the quality control of p24 proteins and when they would arrive to the Golgi, they could be retained by other mechanism involved in quality control into the Golgi apparatus or may not be recognized by PER1L enzymes at the Golgi. In the case of *per1lA-1*, *per1lB-2* and *per1lAB-2* mutants, it could be due to a regulation of the remodelling pathway. If GPI-AP traffic is blocked at the Golgi apparatus, p24 proteins or another mechanism could retain them at the ER, avoiding their trafficking along the secretory pathway. Nevertheless, a proportion of not remodelled GPI-APs can scape to this control and still reach the plasma membrane in all mutants.

Interestingly, GFP-GPI trafficking seems not to be altered in the *per1lB-2* mutant as it does in *per1lA-1*. In addition, *per1lB-2* is not sensitive to salt stress as it is *per1lA-1*. This could be due to different protein levels of PER1L enzymes in these mutants but it cannot be discarded that PER1LA and PER1LB are not redundant and have different specificities.

Finally, if PER1L in plants localized at Golgi, this indicates that once the lipid moiety is remodelled, this could act as a lipid-based mechanism for selective sorting in the Golgi instead of in the ER (as in yeast) within vesicles destined to the plasma membrane (Simons and Ikonen, 1997; Surma et al.,

2012). As a consequence, as it has been previously shown in mammals, plant GPI-APs may then homodimerize and associate to microdomains or rafts formed by cholesterol and sphingolipids association (Paladino et al., 2004; Seong et al., 2013) or not and this may decide whether a GPI-AP would go to the apical or basolateral face. Indeed, it is very important for mammalian cells the distribution along the secretory pathway of the enzymes implicated in the remodelling pathway of the GPI anchor, because mammals have polarized cells. As plants also have polarized cells, it makes sense that GPI anchor remodelling pathway would be as in mammals.

CONCLUSIONS

1. The absence of p24 proteins from the delta-1 subclass causes a decrease in the protein levels of other members of p24 protein family in *Arabidopsis*, which is due to a reduction in protein stability. The expression of only one single member of the delta-1 subclass (p24δ5) is enough to restore the protein levels of other p24 protein family members, suggesting the existence of functional redundancy within p24 proteins of the delta-1 subclass.

2. Loss of p24 proteins from the delta-1 subclass causes the accumulation of GPI-anchored proteins at the ER, indicating that p24δ-1 proteins are involved in ER export and plasma membrane localization of GPI-anchored proteins in *Arabidopsis*. However, a proportion of GPI-anchored proteins is still able to reach the plasma membrane in the *p24δ-1* mutant, which may explain why the *p24δ-1* mutant does not show any phenotypic alteration under standard growth conditions, although is more sensitive to salt stress.

3. p24δ-1 proteins seem to be specifically involved in ER export and transport to plasma membrane of GPI-anchored, since loss of p24δ-1 proteins does not affect bulk transport of other plasma membrane proteins, including transmembrane proteins and proteins anchored to the plasma membrane with different types of lipid anchors.

4. p24δ5 (delta-1 subclass) but not p24δ9 (delta-2 subclass) partially restores plasma membrane localization of GPI-anchored proteins in the *p24δ-1* mutant which suggest that members of the two p24delta subclasses are not functionally redundant. The coiled-coil domain and

not the GOLD domain of p24δ5 seems to be involved in the transport of GPI-anchored proteins. Pull-down experiments showed that p24δ5 interacts with the ER form of the GPI-anchored protein AGP4, an interaction which requires the coiled-coil domain in p24δ5.

5. The study of the role of *Arabidopsis* PGAP1/Bst1p and PGAP3/Per1p lipid remodelling enzymes of the GPI anchor in the transport of GPI anchored proteins reveals that:

   5.1 Mutants of PGAP1B (the only putative *Arabidopsis* PGAP1/Bst1p ortholog located at the ER), PER1LA and PER1LB (the two Arabidopsis PGAP3/ Per1p orthologs) show an accumulation of GPI-anchored proteins at the ER, suggesting the requirement of the lipid remodelling for efficient transport of GPI-anchored proteins from the ER to the plasma membrane. None of the mutants shows any evident phenotypic alteration under standard growth conditions; nevertheless, *per1lA* and *per1lAB* mutants are more sensitive to salt stress than wild type plants.

   5.2 Both PER1LA-RFP and PER1LB-RFP localize to the Golgi apparatus, indicating that in plants part of the remodelling pathway of GPI-anchored proteins may occur at the Golgi apparatus, as in mammals. This is in contrast to yeast, where all the remodelling pathway occurs at the ER.

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RESUMEN

1 INTRODUCCIÓN

TRÁFICO INTRACELULAR DE MEMBRANAS

Las células eucariotas tienen un complejo sistema de endomembranas compuesto por varios compartimentos rodeados a membranas, que tienen una composición molecular específica y por lo tanto, son funcionalmente diferentes. En plantas, los mayores compartimentos endomembranosos son: El retículo endoplásmico (RE), el aparato de Golgi (GA), el complejo *trans*-Golgi (TGN), el compartimento prevacuolar/cuerpos multivesiculares (PVC/MVB) y las vacuolas.

Estos compartimentos forman parte de un complejo endomembranoso que está conectado por pequeñas vesículas que transportan proteínas, lípidos y polisacáridos. Este tráfico de membranas permite el transporte de miles de proteínas hasta su sitio de acción. Además, este tráfico de membranas está implicado en múltiples funciones celulares, tales como la homeostasis celular, desarrollo, comunicación célula-célula y respuestas fisiológicas frente a cambios ambientales. Este sistema de tráfico membranoso puede clasificarse en las siguientes rutas:

- **Vía secretora o biosintética:** Esta vía la utilizan moléculas que son sintetizadas en el ER y son transportadas a otros compartimentos o son secretadas.
- **Vía endocítica:** La endocitosis es un proceso por el cual la célula captura moléculas del exterior o internaliza proteínas de la membrana plasmática mediante vesículas.

- **Vías retrógradas:** Vías encargadas de recuperar material y moléculas que por alguna razón se localizan en un compartimento pero están en otro.
- **Otros:** Se ha visto la existencia de diferentes vías que permiten transportar proteínas hasta peroxisomas y plastidios.

Tráfico vesicular

Hoy en día, está ampliamente aceptado que el transporte entre compartimentos celulares se produce mediante pequeñas vesículas. Éstas parten de un compartimento dador y son transportados hasta el compartimento diana en el que se fusionan, llevando así moléculas de un compartimento a otro.

El transporte entre estos compartimentos en plantas ocurre mediante mecanismos similares a animales y levaduras, y puede dividirse en tres etapas:

- **Gemación:** La formación de vesículas nacientes está regulada por la actividad específica de GTPasas. Las subunidades del complejo formador de vesículas polimerizan deformando la superficie membranosa para formar la vesícula. Mientras se produce esta deformación, las moléculas que deben ser transportadas van siendo capturadas por ésta.
- **Transporte:** Las vesículas son transportadas hasta el compartimento diana a través del citoesqueleto, mediante procesos mediados por proteínas motoras (quinesinas y dineinas).
- **Fusión:** Una vez las vesículas han llegado al compartimento diana, ambas membranas deben fusionarse. Esto requiere un proceso específico de reconocimiento entre las membranas de la vesícula y la diana, en el que

participan proteínas de diferentes familias como: Pequeñas GTPasas de la familia Rab, factores de anclaje y SNAREs.

Los compartimentos implicados en el sistema de transporte de endomembranas deben seguir unos principios básicos para mantener su composición única, a pesar del constante intercambio de vesículas:

- **Clasificación molecular:** El transporte vesicular debe incluir las moléculas apropiadas, tanto como cargo como aquellas apropiadas para el transporte de la vesícula.
- **Direccionalidad de la vesícula:** Las vesículas deben ir al compartimento adecuado para reconocerlo y fusionarse correctamente.

LA VÍA SECRETORA

Como hemos comentado anteriormente, la ruta secretora es la encargada de la síntesis, transporte, modificación y secreción de un amplio abanico de proteínas, lípidos y polisacáridos que además, debe responder a demandas celulares específicas, que están continuamente cambiando dependiendo de las necesidades de la célula por lo que requiere de un alto dinamismo en el transporte de moléculas a través de la célula. Para la homeostasis de las células, es importante que haya un equilibrio entre las rutas de transporte anterógrada (del ER al Golgi y la superficie celular) y retrógrada (en sentido contrario).

RESUMEN

Vía secretora temprana

Esta vía incluye el transporte entre el ER y el aparato de Golgi, en la que el transporte proteico es bidireccional. Está compuesta por:

- **Retículo endoplásmico:** Es la factoría donde se sintetizan las proteínas que van a ser secretadas y, por tanto, donde esta vía empieza. Las proteínas que van a seguir esta vía contienen un péptido señal (SP) en su extremo N-terminal, para ser translocadas al ER. El ER se extiende a través de la célula y está formado por una red de cisternas y túbulos interconectados que están en continuo remodelado.
- **Sitios de salida del ER y transporte ER-Golgi:** Una vez las proteínas y cargos membranosos son plegados correctamente, son transportados del ER al Golgi mediante vesículas COPII, las cuáles son reclutadas en subdominios especializados del ER llamados sitios de salida del ER (ER exit sites, ERES).
- **Vesículas COPII:** La cubierta COPII está compuesta por cinco proteínas: SAR1, SEC23, SEC24, SEC13 y SEC31. La formación de estas vesículas se inicia con la activación de SAR1, una pequeña Rab GTPasa, activada por SEC12. A continuación, se reclutan secuencialmente el complejo SEC23/SEC24 y SEC13/SEC31, formando dos capas. Se ha propuesto que la proteína SEC24 es la encargada de reconocer los diferentes cargos que entrarán en estas vesículas.
- **Transporte Golgi-ER:** El transporte desde el aparato de Golgi de vuelta al ER esta mediado por las vesículas COPI, que son formadas desde la cara *cis* del Golgi.
- **El aparato de Golgi y transporte intra-Golgi:** Es la estación de salida y el responsable del envío de proteínas a múltiples destinos, jugando un rol

central en la vía secretora. Está formado por múltiples cisternas que tienen una estructura polarizada, desde la cara *cis* donde recibe las vesículas procedentes del ER, a la cara *trans* desde donde se forman vesículas con diferentes destinos. Existen dos modelos para el transporte intra-Golgi, si éste se realiza mediante vesículas o más bien por progresión de las cisternas.

- **Vesículas COPI:** La cubierta COPI está formada por un complejo heptamérico (α, β, β', γ, δ, ε, ζ) llamada coatómero. Estas vesículas son las encargadas del transporte intra-Golgi y el transporte del aparato de Golgi al ER. Como en el caso de las vesículas COPII, el ensamblaje de las vesículas COPI está regulada por una proteína GTPasa, ARF1. Se ha descrito que las proteínas p24 y el receptor K/HDEL ERD2 son capaces de interaccionar directamente para la formación de estas vesículas.

Vía secretora tardía

Esta vía incluye el transporte de los cargos desde el aparato de Golgi hasta su destino final:

- **Red *trans*-Golgi (*trans*-Golgi Network, TGN):** Este compartimento es la estación de salida para las proteínas exocíticas excepto para las proteínas de almacenamiento. En plantas, también puede comportarse como un endosoma temprano.
- **Transporte a la membrana plasmática:** Este es el destino final para las proteínas secretadas o que forman parte de la matriz extracelular.
- **Vía de transporte vacuolar:** Destino de muchas proteínas y moléculas de almacenamiento en células vegetales. Las vacuolas juegan un papel importante en células vegetales como: Incrementando el volumen de la

célula, respuestas de defensa, almacenamiento de proteínas y azúcares y función lítica.

PROTEÍNAS P24

Filogenia y nomenclatura

Las proteínas p24 constituyen una familia de proteínas transmembrana de tipo I de alrededor de 24 kDa. Basándose en su homología de secuencia, éstas pueden clasificarse en cuatro subfamilias: α, β, δ, γ. El número de proteínas p24 en cada subfamilia varía dependiendo de la especie. Las plantas solamente poseen las subfamilias p24β (β2-β3) y p24δ (δ3-δ11), siendo esta última ampliamente expandida. *Arabidopsis* contiene 9 miembros de la subfamilia delta y 2 miembros de la beta.

Especificidad en el tejido y expresión regulada

Son ampliamente expresadas en todos los órganos por lo general, lo que indica que tienen una función de mantenimiento básica. En *Arabidopsis* se ha visto que algunas proteínas p24 se expresan exclusivamente en flores y silicuas, por lo que podrían tener una funcionalidad específica.

Estructura de las proteínas p24

Todas las proteínas p24 tienen una estructura similar, que se basa en: Una larga región N-terminal llamada dominio GOLD que está implicada en el reconocimiento de diferentes cargos; un dominio coiled-coil (CC) que

se encarga de la oligomerización de las proteínas p24; un dominio transmembrana y una pequeña (13-20 residuos) cola citoplasmática C-terminal que contiene diferentes motivos para interaccionar con las subunidades de las cubiertas COPI y COPII.

Modificaciones post-transduccionales y degradación

Se ha descrito que algunas proteínas p24 pueden ser *N*-glicosiladas o fosforiladas. En *Arabidopsis*, las proteínas p24δ de la subclase δ-1 (δ3-δ6) están *N*-glicosiladas, y se ha visto que esta modificación regula la especificidad de los cargos. También se ha visto que en *Arabidopsis*, las proteínas p24 son degradadas en la vacuola por cisteín-proteasas.

Oligomerización

Se ha visto que las proteínas p24 interaccionan entre ellas mediante el dominio CC formando diferentes complejos incluyendo miembros de las diferentes subfamilias y subclases. La formación de estos complejos es importante para su transporte y localización, pero también para su estabilidad.

Transporte y localización

Las proteínas p24 se localizan en los compartimentos de la vía secretora temprana incluyendo: El ER, el ERGIC (en animales), *cis*-Golgi y el aparato de Golgi, así como las vesículas COPI y COPII. Estas ciclan principalmente entre el ER y Golgi por medio de las vesículas COPII y COPI. Como se ha indicado anteriormente, el dominio citosólico de estas proteínas

es el encargado de interaccionar con las subunidades COPII y COPI para que su transporte bidireccional.

Funciones de las proteínas p24

- **Formación de las vesículas COPII y COPI:** Al contener diferentes motivos en la cola citosólica, pueden interaccionar directamente y, por tanto favorecer, la formación de vesículas COPI y COPII. Además, también se ha visto que son importantes para regular la función de las Rab GTPasas que son a su vez las encargadas de iniciar y regular, la formación de estas vesículas.
- **Mantenimiento de la estructura y organización de la vía secretora temprana:** Están implicadas en la formación de ERES, la estructura del ER y del ERGIC y la biogénesis de aparato de Golgi, pudiendo ser importantes para el reciclaje de componentes requeridos para el transporte ER-Golgi y función y estructura del ER.
- **Receptor de cargos proteicos:** Varias cargos se han propuesto para las proteínas p24 como: Las proteínas con anclaje GPI (objeto de estudio en esta tesis), el receptor K/HDEL ERD2 y receptores acoplados a proteínas G.
- **Control de calidad en el ER:** Varios estudios sugieren la posibilidad de que actúen en la prevención de la salida de proteínas mal plegadas y aberrantes.
- **Proteínas p24 en fisiología y patologías:** Parecen estar implicadas en diferentes procesos y enfermedades como la enfermedad de Alzheimer, desarrollo embrionario temprano en ratón y la síntesis y posterior secreción de la insulina.

PROTEÍNAS CON ANCLAJE GPI

Existen diferentes maneras de anclar una proteína a la membrana plasmática. Las proteínas transmembrana tienen un dominio hidrofóbico que le permite estar embebido en la bicapa lipídica mientras que otras proteínas son modificadas post-transduccionalmente mediante la unión a lípidos que les anclan a la membrana. Si las proteínas tienen que estar en la cara intracelular de la membrana plasmática, éstas son modificadas mediante *S*-acetilación, *N*-miristoilación, prenilación o palmitoilación. En el caso de que las proteínas deban anclarse a la cara externa de la membrana plasmática, deberán ser modificadas con ancla de glicosilfosfatidilinositol (GPI).

Las proteínas con anclaje GPI (GPI-APs), objeto de estudio en esta tesis, han sido ampliamente estudiadas en animales y levaduras debido a su elevada relevancia en procesos esenciales. En el caso de animales se ha estudiado que están involucradas en la embriogénesis, desarrollo, neurogénesis, fertilización y en el sistema inmunitario; mientras que en levaduras son imprescindibles para su correcto crecimiento.

En plantas también se presupone una elevada importancia a esta familia de proteínas, ya que se han predicho 248 en *Arabidopsis*, aproximadamente el 10 % de las proteínas de la vía secretora. Éstas juegan un papel relevante en el metabolismo, señalización y formación de polímeros en la pared celular; membrana plasmática; y transporte en los plasmodesmatas.

RESUMEN

Estructura del ancla GPI

La estructura principal del ancla GPI (ésta puede variar dependiendo de especies) se basa en una parte lipídica compuesta por una fosfoceramida, y una parte glicídica compuesta por una glucosamina, tres manosas y una etanolamina fosfato que une la proteína por el extremo C-terminal. La estructura del ancla GPI va cambiando mediante una ruta de remodelado desde su síntesis hasta su forma final.

Biosíntesis del ancla GPI

La síntesis del ancla GPI empieza en la cara citosólica del ER, siendo posteriormente translocada a la cara luminal mediante una flipasa para continuar su síntesis. Una vez sintetizada el ancla GPI, ésta es anclada a la proteína por el C-terminal mediante un complejo enzimático llamado GPI-transamidasa. Fruto de su importancia, no existen mutantes de ningún enzima en esta ruta para *Arabidopsis* ya que resultan ser letales.

Remodelado del ancla GPI

Una vez la proteína está ensamblada al ancla GPI, ésta se debe remodelar hasta alcanzar su forma madura. Ambas partes lipídica y glicano son remodeladas. El remodelado de la parte lipídica se basa en el cambio de los ácidos grasos cortos e insaturados por ácidos grasos de cadenas largas y saturadas para que de esta manera puedan asociarse a membranas ricas en esfingolípidos y esteroles, llamados microdominios o “rafts”. El remodelado del glicano se basa en la eliminación de etanolaminas fosfatos de las tres manosas, para que una vez finalizados ambos remodelados, puedan ser

reconocidas por las proteínas p24 y facilitar así su transporte al Golgi mediante las vesículas COPII.

Se ha descrito que en animales este remodelado sucede entre el ER y el aparato de Golgi, de manera que las proteínas con anclaje GPI acaban su remodelado en el Golgi, mientras que en el caso de las levaduras, el remodelado sucede exclusivamente en el ER, de manera que en estas proteínas el ancla GPI adquiere su forma final en el ER.

Salida de proteínas con anclaje GPI desde el ER

El ancla GPI una vez remodelada, actúa como señal de transporte desde el ER al aparato de Golgi. La estructura única del ancla GPI le confiere propiedades especiales y un modo especial de interacción con membranas en el lumen de los orgánulos. Una vez remodelas, son incorporadas a las vesículas COPII en los ERES. Como se encuentran en la cara luminal del ER y no pueden interaccionar directamente con la cubierta COPII, necesitan de un receptor de cargo para ser introducidas en estas vesículas COPII, como es el caso de las proteínas p24.

En el caso de las levaduras, al tener la parte lipídica del ancla ya remodelada, se concentran en ERES específicos por un mecanismo basado en lípidos, mientras que las proteínas p24 las ayudan a incorporarse a las vesículas COPII. En el caso de los animales, al no estar el ancla GPI completamente remodelada, las proteínas p24 son las encargadas de concentrarlas en los ERES y de facilitar su incorporación a las vesículas COPII.

También se ha observado que las proteínas con anclaje GPI se acumulan en ERES distintos al resto de proteínas que van a ser secretadas,

por lo que viajarán en distintas vesículas COPII. En la formación de estas vesículas COPII especiales parece estar implicada la subunidad SEC24 de la cubierta COPII, ya que en levaduras se ha visto que estas vesículas especiales contienen la isoforma Lst1p, mientras que en animales tienen las isoformas SEC24C y SEC24D.

Llegada al Golgi y control de calidad post-ER

Una vez en el aparato de Golgi, las proteínas con anclaje GPI se disocian de las proteínas p24, parece ser por el ligero pH acídico del Golgi. Aquellas proteínas cuyas anclas GPI no han sido correctamente remodeladas y han llegado al Golgi por error, son reconocidas por las proteínas p24 y las devuelven al ER mediante vesículas COPI.

Salida desde la red *trans*-Golgi (TGN)

Una vez las proteínas con anclaje GPI son completamente remodeladas y glicosiladas a través de las cisternas del aparato de Golgi, estas proteínas tienen que salir desde el TGN en vesículas secretoras para alcanzar la membrana plasmática. En levaduras, las proteínas con anclaje GPI son separadas del resto de proteínas secretoras desde su salida del ER, pero nada se sabe sobre su transporte por el aparato de Golgi. En cambio, este proceso ha sido más estudiado en animales, ya que al tener células polarizadas, las proteínas con anclaje GPI pueden dirigirse a la cara basolateral o a la apical. Se ha propuesto, que dependiendo de su asociación a microdominios o “rafts” ricos en colesterol y esfingolípidos, esto podría dirigir la secreción de estas proteínas hacia una cara u otra.

Proteínas con anclaje GPI en la membrana plasmática

En levaduras, se ha visto que las proteínas con anclaje GPI tienen una función fundamental en el correcto desarrollo y crecimiento de la pared celular. Muchas de estas proteínas controlan su morfología y su integridad. También se ha visto, que existen diversos enzimas capaces de degradar el ancla GPI de estas proteínas para liberarlas a la pared celular, donde realizan importantes funciones como la polimerización de polisacáridos en la pared celular.

En animales, también se ha visto que el ancla GPI puede ser digerida por enzimas fosfatilinositol-fosfolipasas (PI-PLs) para realizar diferentes funciones difundiendo hacia el espacio extracelular. Este proceso tiene implicaciones importantes en adhesión, diferenciación, proliferación, supervivencia y oncogénesis.

Proteínas con anclaje GPI en plantas

Se ha predicho que las plantas poseen alrededor de 248 proteínas con anclaje GPI, aproximadamente, un 10 % de las proteínas secretoras. Estas proteínas realizan funciones esenciales en los procesos biológicos de las plantas como señalización, metabolismo y formación de polímeros en la pared celular y transporte en el plasmodesmata. Existen algunas familias de estas proteínas con funciones importantes:

- **Familia LORELEI:** Tres proteínas forman parte de esta familia que se encarga de la interacción del gametofito femenino en el tubo polínico.
- **Familia COBRA:** Estas proteínas son requeridas para la deposición orientada de las microfibrillas de celulosa para dirigir la expansión celular

durante la morfogénesis de la planta. La expresión de las proteínas COBRA se concentra en la raíz.

- **Familia ARABINOGALACTANOS:** Son aproximadamente el 40 % de todas las proteínas con anclaje GPI. Se encuentran tanto en la membrana plasmática, como en la pared celular, apoplasto o secreciones. La complejidad en su estructura está formada por la gran diversidad de azúcares y el alto grado de glicosilaciones que están asociados a la estructura proteica de la proteína. Debido a su heterogeneidad en su estructura, pueden realizar múltiples funciones diferentes como: Embriogénesis somática, crecimiento y desarrollo de la raíz, señalización, resistencia frente a patógenos, plasticidad de la pared celular, tolerancia a la sal, diferenciación del xilema, iniciación de la gametogénesis femenina, expansión celular, secreción, muerte celular programada y desarrollo del grano de polen.

Fruto de la importancia de las proteínas con anclaje GPI en plantas es que no existen mutantes de los enzimas implicados en la síntesis del ancla, ya que resultan ser letales. También juegan papeles clave en su asociación con otras estructuras de la célula para su mantenimiento y señalización, como:

- **Asociación de proteínas con anclaje GPI a plasmodesmos:** Los plasmodesmos son canales en la membrana plasmática que conectan células adyacentes, permitiendo estar conectadas e intercambiar proteínas solubles, solutos y ARN. Se han descubierto varias proteínas con anclaje GPI que pueden regular la formación de plasmodesmos y su tráfico entre células.

- **Proteínas con anclaje GPI y señalización, mantenimiento y biosíntesis de la pared celular:** Las proteínas con anclaje GPI son capaces de modificar y regular la formación de polímeros de calosa, xiloglucanos y celulosa en la pared celular. Forman parte de complejos proteicos que regulan la actividad de las celulosa sintasas (CESAs), influyendo de esta manera en la deposición, cristalización y orientación de estas microfibrillas en la pared celular.

2 OBJETIVOS

Las proteínas con anclaje GPI (GPI-APs) son una familia de proteínas que están unidas a la cara externa de la membrana plasmática por un ancla GPI. En *Arabidopsis thaliana* se ha predicho la existencia de 250 proteínas con anclaje GPI, aproximadamente el 10 % de las proteínas secretoras, y además, participan en funciones muy importantes como la transducción de señales, las interacciones célula-célula, el crecimiento, defensa y la biosíntesis de la pared celular. Sin embargo, la maquinaria molecular involucrada en el transporte de las GPI-APs a la membrana plasmática es esencialmente desconocida en plantas.

Las GPI-APs se sintetizan en el ER y, en animales y levaduras, la salida del ER de las GPI-APs necesita de las proteínas p24. Las proteínas p24 constituyen una familia de proteínas que se localizan en los compartimentos de la vía secretora temprana, incluyendo el ER y el aparato de Golgi, y las vesículas recubiertas de COPI y COPII. Estas proteínas desempeñan un papel importante en el control de calidad durante el transporte entre el ER y el Golgi, posiblemente como receptores de cargo. Se han propuesto varios

cargos para las proteínas p24, incluyendo los receptores acoplados a proteínas G, el receptor K/HDEL ERD2 y las GPI-APs.

En mamíferos y levaduras también se ha descrito que durante su transporte desde el ER hasta la membrana plasmática, las GPI-APs son sometidas a un remodelado de la parte lipídica del ancla GPI, que es necesario para su transporte eficiente a lo largo de la vía secretora.

Por tanto, el objetivo principal de este trabajo es caracterizar el transporte a la membrana de las GPI-APs en *A. thaliana*.

Objetivos específicos:

1. Investigar si las proteínas p24 de *Arabidopsis* de la subclase delta-1 están implicadas en la salida del ER y en la localización en la membrana plasmática de las GPI-APs.
2. Estudiar el papel de los enzimas PGAP1 y PER1L de *Arabidopsis*, que pueden estar implicados en el remodelado de la parte lipídica del ancla GPI, en el transporte de las GPI-APs desde el ER a la membrana plasmática.

3 MATERIAL Y MÉTODOS

Material Biológico

Entre los diferentes materiales biológicos utilizados en esta tesis se encuentran los microorganismos *Escherichia coli* (cepa DH5α) y

Agrobacterium tumefaciens (cepa C58 MP90); y las plantas: *Arabidopsis thaliana* (ecotipo Columbia (Col-0)) y *Nicotiana benthamiana*.

Mecanismos de transformación

Los mecanismos de transformación que han sido utilizados en este trabajo se resumen a continuación:

- **Transformación de *Escherichia coli*:** Se utilizaron bacterias competentes MAX Efficiency DH5α para ser transformadas con los marcadores fluorescentes utilizados en esta tesis.
- **Transformación de *Agrobacterium tumefaciens*:** Se utilizaron bacterias competentes para ser transformadas con las construcciones que codifican los marcadores fluorescentes utilizados en esta tesis.
- **Expresión transitoria de proteínas en protoplastos de *Arabidopsis* mediante transformación con PEG:** Una vez obtenidos los protoplastos de plantas control o mutantes, esta técnica nos permite la rápida expresión de uno o varios marcadores fluorescentes en protoplastos.
- **Expresión transitoria de proteínas en hojas de *Nicotiana benthamiana*:** Se agroinfiltraron hojas de *N. benthamiana* con cultivos de *A. tumefaciens* conteniendo marcadores fluorescentes para analizar su localización subcelular o para la obtención de material para la realización de ensayos bioquímicos.
- **Expresión transitoria de proteínas en plántulas de *Arabidopsis* mediante infiltración por vacío:** Esta técnica nos permite la rápida expresión de un marcador fluorescente en plántulas de *Arabidopsis*, sin la necesidad de crear líneas transgénicas estables que necesitan de un largo proceso para su obtención.

- **Transformación estable de *Arabidopsis* mediante el método de sumersión floral:** Este método permite generar líneas transgénicas que expresen un gen de manera estable. Se utilizó en esta tesis para crear los mutantes del gen *PER1LB* con la construcción amiR-PER1LB y la expresión estable de RFP-p24δ5 en el mutante *p24δ-1*.
- **Análisis de segregación en líneas transgénicas:** Se realizaron para comprobar que el gen introducido en el genoma de *Arabidopsis* mediante el método anterior se había introducido solo una vez.
- **Plásmidos utilizados en expresión transitoria en protoplastos y plántulas de *Arabidopsis*:** Una gran variedad de construcciones fluorescentes fueron utilizadas a lo largo de la tesis para su visualización en microscopía de confocal o para la realización de ensayos "pull-down".

Tratamientos

Los tratamientos utilizados en este trabajo se resumen a continuación:

- **Germinación con NaCl:** Se realizó para comprobar la tolerancia a la sal en los mutantes del remodelado del ancla GPI.
- **Infiltración de brefeldina A (BFA) en hojas de *N. benthamiana*:** Se infiltró BFA en las hojas de *N. benthamiana* a los 2 días de ser agroinfiltradas para evitar la salida del ER de las proteínas sintetizadas por la agroinfiltración.
- **Tinción de la membrana plasmática de protoplastos:** Para la colocalización de marcadores fluorescentes verdes en la membrana plasmática, se utilizó el colorante FM4-64.

Ácidos nucleicos

Los siguientes procesos relacionados con el aislamiento, manipulación y análisis de ácidos nucleicos realizados en esta tesis se detallan a continuación:

- **Aislamiento de ácidos nucleicos:** Se aislaron plásmidos de ADN para su transformación en *Agrobacterium tumefaciens* y *Escherichia coli*; ADN genómico de *Arabidopsis* para el genotipado de plantas; y ARN total de *Arabidopsis* para medir la expresión de diferentes genes en plántulas.
- **Manipulación y análisis de ácidos nucleicos:** Se adquirieron comercialmente los marcadores PER1LA-RFP y PER1LB-RFP; se realizaron PCRs para el genotipado de mutantes y detectar inserciones de T-DNA, RT-sqPCRs para la obtención de cDNA a partir de RNA y medir la expresión de ciertos genes y se realizaron electroforesis en geles de agarosa para separar las bandas obtenidas en estos procesos que acabamos de detallar.

Proteínas

Los siguientes procesos relacionados con la manipulación de proteínas se explican a continuación:

- **Extracción de proteínas en raíces de *Arabidopsis*:** Se realizó la extracción de proteínas en raíces de 7 días para analizar la estabilidad proteica y la redundancia funcional de las proteínas p24.
- **Extracción de proteínas en protoplastos de *Arabidopsis:*** Se realizó para analizar si existía una interacción entre la proteína AGP4 y p24δ5 en protoplastos del mutante *p24δ-1* mediante ensayos “pull-down”.

- **Extracción de proteínas en hojas de *N. benthamiana*:** Se utilizó este procedimiento para la realización de ensayos "pull-down".
- **Tratamiento con PI-PLC:** Este tratamiento se llevó a cabo para comprobar si AGP4 es una proteína con anclaje GPI y su estudio bioquímico.
- **Experimentos "pull-down":** Estos ensayos nos permiten conocer si existe una interacción directa entre dos proteínas. En esta tesis se llevaron a cabo para conocer si existe una interacción directa entre AGP4 y p24δ5, así como qué dominio de p24δ5 está implicado en esta interacción.
- **Determinación de la concentración de proteínas:** Se utilizó el método de Bradford para conocer la concentración proteica de diferentes muestras.
- **Gel de electroforesis SDS-poliacrilamida y Western-Blot:** Esta técnica nos permite separar las proteínas por su tamaño y posteriormente ser reconocidas por un anticuerpo específico.

Estudio de localización de proteínas *in vivo*

Para la visualización de proteínas fluorescentes se utilizaron los microscopios confocales: Olympus® FV1000 y Zeiss® LSM880 con el detector Fast Ayriscan.

4 RESULTADOS Y DISCUSIÓN

CAPÍTULO I. PAPEL DE LAS PROTEÍNAS P24 EN LA SALIDA DEL ER Y TRANSPORTE A LA MEMBRANA PLASMÁTICA DE LAS PROTEÍNAS CON ANCLAJE GPI

Redundancia funcional y estabilidad proteica de las proteínas p24

Nuestro grupo demostró previamente que las proteínas p24 de *Arabidopsis* forman complejos hetero-oligoméricos, que son importantes para su transporte intracelular y para su estabilidad. Para estudiar la implicación de las proteínas p24 en el transporte de las proteínas con anclaje GPI se decidió utilizar un mutante KO para los cuatro miembros de la subclase δ-1 (mutante *p24δ-1*). Este mutante, resultó tener disminuidos los niveles proteicos de diferentes proteínas de la subclase p24δ-2 (p24δ9) y los dos miembros de la subfamilia p24β (β2 y β3), mientras que sus valores de RNA mensajero no variaban, lo que sugiere una pérdida de estabilidad del complejo p24 en ausencia de las proteínas de la subclase p24δ-1.

Otros resultados previos, sugirieron que existe redundancia entre los mismos miembros de la subclase. Para comprobar esto, se generaron líneas transgénicas estables del mutante *p24δ-1* expresando la proteína RFP-p24δ5 (un miembro de la subclase p24δ-1). Como se muestra en la Figura 28, se recuperaron los niveles proteicos de las proteínas p24δ-9 (subclase p24δ-2)

y p24β2, sugiriendo que existe una redundancia funcional entre los miembros de la subclase p24δ-1.

Las proteínas p24 son necesarias para la salida del ER y la localización en la membrana plasmática de las GPI-APs

A continuación, se analizó la localización de diferentes proteínas con anclaje GPI mediante técnicas de expresión transitoria en plántulas y protoplastos de *Arabidopsis*. Los marcadores utilizados fueron: GFP-AGP4, un arabinogalactano con anclaje GPI; y GFP-GPI, un ancla GPI fusionada a GFP. Además, se utilizaron 2 proteínas transmembrana, la acuaporina PI2A-RFP, y la ATPasa de membrana plasmática GFP-PMA; MAP-GFP (una GFP miristoilada y palmitoilada) y GFP-PAP (una GFP prenilada).

En experimentos de expresión transitoria en plántulas de *Arabidopsis* observamos que GFP-AGP4 y GFP-GPI se localizaban en la membrana plasmática en plantas silvestres pero se acumulaban en el ER en el mutante *p24δ-1*. En contraste, la acuaporina PIP2A-RFP se localizó en la membrana plasmática tanto en plantas silvestres como en el mutante *p24δ-1* (Figuras 29 y 30). Utilizando ensayos de expresión transitoria en protoplastos de *Arabidopsis* pudimos observar que los marcadores con anclas GPI se acumulaban en el ER, pero también podían llegar parcialmente a la membrana plasmática. Por ello decidimos realizar una cuantificación de estos experimentos en protoplastos (Figura 31), dividiendo éstos en tres categorías dependiendo de la localización principal de la proteína GFP-AGP4: Membrana plasmática (PM), retículo endoplásmico (ER) y ambos (ER + PM). Este análisis permitió comprobar que un porcentaje de estas proteínas

alcanzan la membrana plasmática, pero la mayor parte se acumula en estructuras con el patrón típico del ER.

Para corroborar estos patrones en el mutante *p24δ-1*, GFP-AGP4 y GFP-GPI se colocalizaron con el marcador de ER, y con una tinción de la membrana plasmática con FM4-64, como se puede observar en las Figuras 32 y 33, respectivamente.

También se expresaron proteínas de la membrana plasmática sin anclas GPI mediante expresión transitoria en protoplastos, tanto en el control como en el mutante *p24δ-1* (Figura 34). En particular, una proteína transmembrana, la ATPasa de la membrana plasmática (GFP-PMA), y proteínas unidas a lípidos diferentes del ancla GPI, incluyendo GFP miristoilada y palmitoilada (MAP-GFP) y GFP prenilada (GFP-PAP). Se observó que estos tres marcadores se encontraban en la membrana plasmática, tanto en el control como en el mutante cuádruple, de manera que la ausencia de las proteínas p24 no implica un retraso ni acumulación del resto de proteínas que no contienen un ancla GPI.

p24δ5 (subclase δ-1), pero no p24δ9 (subclase δ-2), recupera parcialmente la localización en la membrana plasmática de las proteínas con anclaje GPI en el mutante *p24δ-1*

Como se vio en la Figura 28, la expresión de p24δ5 fue suficiente para la recuperación del resto de proteínas p24 en el mutante *p24δ-1*. De manera que se decidió coexpresar ambos marcadores, GFP-AGP4 y GFP-GPI, con p24δ5 (subclase p24δ-1) y con p24δ9 (subclase p24δ-2) en expresión transitoria en protoplastos en el mutante cuádruple.

De esta manera, se observó que con la coexpresión de p24δ5 se recuperaba parcialmente la localización en la membrana plasmática de las proteínas con anclaje GPI, mientras que con la coexpresión con p24δ9 las proteínas con anclaje GPI seguían acumuladas en el ER (Figura 35 y cuantificación Figura 31).

El transporte de proteínas con anclaje GPI a la membrana plasmática requiere del dominio coiled-coil, pero no el dominio GOLD de p24δ5

A continuación se estudió qué dominio de las proteínas p24 es importante para el transporte de las proteínas con anclaje GPI a la membrana plasmática. Para esto, se utilizaron dos mutantes de p24δ5, uno sin el dominio GOLD y el otro sin el dominio CC, en expresión transitoria en protoplastos.

Como se puede observar en la Figura 36, cuando se coexpresaron GFP-AGP4 y GFP-GPI con el mutante sin el dominio GOLD, se restableció parcialmente el transporte de las proteínas con anclaje GPI a la membrana plasmática. En cambio, cuando se coexpresaron con el mutante sin el dominio CC, éstas se mantuvieron acumuladas en el ER (cuantificación en la Figura 31).

GFP-AGP4 interacciona con p24δ5, una interacción que requiere del dominio coiled-coil de p24δ5

Después de establecer que las proteínas p24 están involucradas en la salida del ER y el transporte a la membrana plasmática de las proteínas con

anclaje GPI, se decidió investigar si existe una interacción directa entre estos 2 tipos de proteínas. Para esto se agroinfiltraron los marcadores en *N. benthamiana* para luego realizar ensayos "pull-down".

Primero, se estudiaron las propiedades bioquímicas de GFP-AGP4 mediante un western-blot y se realizó un tratamiento con PI-PLC para ver si se trata de una proteína con anclaje GPI, ya que dicho enzima es capaz de degradar el ancla GPI soltando el resto de la proteína al medio. Como se observó en la Figura 37, GFP-AGP4 apareció en forma de tres bandas de tamaños 115, 70 y 60 kDa. La banda de 115 kDa es sensible al tratamiento con PI-PLC, y probablemente corresponde a la forma final de la proteína AGP4 en la membrana plasmática, al presentar un patrón consistente con su alto grado de glicosilación tras su paso por el aparato de Golgi; la banda de 70 kDa también resultó ser sensible al tratamiento con PI-PLC, y posiblemente corresponda con la forma de la proteína que se localiza en el ER; la banda de 60 kDa que no resultó ser sensible al tratamiento y además resultó ser soluble, por lo que no se trata de una proteína con ancla GPI.

A continuación se intentó co-inmunoprecipitar GFP-AGP4 con RFP-p24δ5, pero no se observó ninguna interacción, por lo que se decidió coexpresar ambas proteínas en presencia de brefeldina a (BFA) para evitar la salida de ambas proteínas del ER (Figura 38). Como se puede observar en la Figura 37, con estas condiciones se aumentó enormemente la proporción de la banda de 70 kDa, presumiblemente la forma de AGP4 en el ER. En estas condiciones, se puede observar en la Figura 39 como AGP4 es capaz de interaccionar con p24δ5, así como con el mutante sin el dominio GOLD pero no con el mutante sin el dominio CC. Esto indicaría que el dominio CC en

p24δ5 es el implicado en la unión a AGP4, lo que explicaría la incapacidad del mutante sin el dominio CC de facilitar el transporte de AGP4 a la membrana plasmática. También se realizó un ensayo "pull-down" de AGP4 con p24δ5 en protoplastos del mutante *p24δ-1*, observando la misma interacción.

Discusión

La familia de proteínas p24 en *Arabidopsis* incluye 11 miembros, 9 de ellos pertenecen a la subfamilia delta, que a su vez puede dividirse en subclase p24δ-1 con cuatro miembros (p24δ3-6) y subclase p24δ-2 con cinco miembros (p24δ7-11); y 2 miembros pertenecen a la subfamilia beta (p24β2 y β3). Previamente se ha demostrado que estas subfamilias y subclases son importantes para la estabilidad de los complejos de proteínas p24. En esta tesis se ha demostrado además, que la expresión de un miembro de la subclase p24δ-1 en el mutante *p24δ-1* es suficiente para restablecer la estabilidad del resto de proteínas p24.

En ausencia de las proteínas p24 se observó que las proteínas con anclaje GPI no pueden salir correctamente del ER y por lo tanto quedan retenidas en el retículo. La ausencia de las proteínas p24 no afecta al resto de proteínas secretoras, por lo que éstas pueden ser transportadas correctamente a la membrana plasmática o ser secretadas.

También se demostró que el dominio CC es el que está implicado en el reconocimiento del ancla GPI y por lo tanto, en el transporte de las proteínas con anclaje GPI a la membrana plasmática y que existe una interacción directa entre las proteínas con anclaje GPI y las proteínas p24. Interesantemente, p24δ5 (subclase p24δ-1) y p24δ9 (subclase p24δ-2)

tienen dominios CC diferentes, lo cual podría explicar por qué p24δ5 y no p24δ9, es capaz de restablecer parcialmente la localización de las proteínas con anclaje GPI en la membrana plasmática en el mutante *p24δ-1*.

Finalmente, queda aún por determinar en plantas si existen vesículas COPII especiales, como acurre en levaduras y animales, que transporten las proteínas con anclaje GPI del ER al aparato de Golgi. Interesantemente, se ha visto que el mutante *p24δ-1* tiene activada la respuesta a proteínas mal plegadas (UPR), en la que la isoforma SEC31A de COPII se encuentra sobreexpresada. Se requieren más experimentos para ver si esto pudiese tener alguna implicación en la salida de las proteínas con anclaje GPI del ER.

CAPÍTULO II. REMODELADO DEL ANCLA GPI Y TRANSPORTE A LA MEMBRANA PLASMÁTICA DE LAS PROTEÍNAS CON ANCLAJE GPI

Genes *PGAP1*

La desacilación del inositol de las proteínas con anclaje GPI es realizada por el enzima PGAP1 (mamíferos) o Bst1p (levaduras) y se trata de la primera reacción en la ruta del remodelado lipídico de las anclas GPI. En *Arabidopsis* se encontró en las bases de datos 7 genes que codifican ortólogos de este enzima. Se decidió utilizar la terminología PGAP1 para este enzima en *Arabidopsis*. En esta tesis se caracterizaron 3 de estas isoformas: AT2G44970 (*PGAP1A*), AT3G27325 (*PGAP1B*) y AT3G52570 (*PGAP1C*). Según la base de datos, PGAP1A debería localizarse en el núcleo, PGAP1B en el ER

y PGAP1C en la mitocondria, por lo que se decidió empezar el estudio de este enzima por la isoforma PGAP1B, utilizando PGAP1A y PGAP1C como controles (Tabla 11). La expresión de la isoforma PGAP1A fue aproximadamente casi el doble que la de las isoformas PGAP1B y PGAP1C, que tienen una expresión similar según las bases de datos (Figura 40).

Caracterización de mutantes de pérdida de función de *PGAP1A*, *PGAP1B* y *PGAP1C*

Para la isoforma PGAP1A se caracterizaron 2 mutantes: *pgap1A-1* y *pgap1A-2*; siendo el primero knock-down (menos del 10 % de expresión que el control), y el segundo knock-out, por lo que este útlimo fue el mutante seleccionado (Figura 41). Para la isoforma PGAP1B se caracterizaron 2 mutantes: *pgap1B-1* y *pgap1B-2*; los dos resultaron ser knock-out, por lo que se seleccionó *pgap1B-1* para el estudio de esta isoforma (Figura 42). Finalmente, para la isoforma PGAP1C se caracterizó el mutante *pgap1C-1*, que resultó ser knock-out (Figura 43).

A partir de estos 3 mutantes, mediante cruzamiento de los mutantes seleccionados se obtuvo el triple mutante *pgap1ABC*. Tanto este triple mutante como los mutante simples *pgap1A-2*, *pgap1B-1* y *pgap1C-1*; no mostraron fenotipo aparente bajo condiciones de crecimiento estándar, ni mayor sensibilidad al estrés salino (Figura 44).

Genes *PER1L*

Este enzima es el encargado de quitar el ácido graso insaturado de la posición *sn-2* de la parte lipídica del ancla GPI y está mediado por PGAP3 en

mamíferos o Per1p en levaduras. En *Arabidopsis* se ha visto que existen 2 isoformas para este enzima (Tabla 13): AT5G62130 (PER1LA) y AT1G16560 (PER1LB), que pertenecen a la superfamilia de proteínas CREST (Figura 45). En este caso se utilizó la terminología proveniente de levaduras PER1-like (PER1L). La expresión de la isoforma *PER1LB* resultó ser 3 veces mayor que la isoforma *PER1LA* según las bases de datos (Figura 46).

Localización de las isoformas PER1LA y PER1LB

Para saber la localización de este enzima en plantas, se expresaron las construcciones de ambas isoformas de PER1L, PER1LA-RFP y PER1LB-RFP, en hojas de *N. benthamiana*. Estos marcadores colocalizaron con dos marcadores del aparato de Golgi (YFP-ManI y ST-YFP) pero no con un marcador de ER (GFP-HDEL). Estos resultados sugieren que en plantas la ruta del remodelado del ancla GPI es como en animales, se produce en el ER y el aparato de Golgi, y no como en levaduras, que toda la vía de remodelado se completa en el ER.

Caracterización de mutantes de pérdida de *PER1LA* y *PER1LB*

Para la isoforma PER1LA se caracterizó el único mutante disponible, *per1lA-1*, que resultó ser knock down (menos del 10 % de expresión que el control) (Figura 49). Para la isoforma PER1LB no existe ningún mutante disponible, por lo que se utilizó la tecnología amiRNA para la obtención de mutantes con expresión disminuida (silenciamiento) de esta isoforma. De esta manera, se obtuvieron diferentes líneas, *per1lB-1* y *per1lB-2*, con una disminución de la expresión de menos del 20 % que las plantas silvestres en ambas líneas (Figura 50). Además, a partir de plantas *per1lA-1*, se transformó

con el amiR-per1lB para la obtención del doble mutante de ambas isoformas del enzima PER1L. Se obtuvieron dos líneas diferentes, *per1lAB-1* y *per1lAB-2*, con una disminución de la expresión del 65 % y 40 % respectivamente, por lo que se eligió la línea *per1lAB-2* (Figura 50).

Ninguno de estos tres mutantes (*per1lA-1*, *per1lB-2* y *per1lAB-2*) mostró un fenotipo diferencial bajo condiciones de crecimiento estándar (Figura 51), no obstante los mutantes *per1lA-1* y *per1lAB-2* resultaron ser más sensibles frente al estrés salino que las plantas silvestres (Figura 52).

Localización de GPI-APs en mutantes *pgap1*

Se utilizaron los mismos marcadores fluorescentes que en el capítulo I: GFP-AGP4 (una proteína arabinogalactana con anclaje GPI), GFP-GPI (un ancla GPI fusionada a GFP), PIP2A-RFP (una acuaporina transmembrana de la membrana plasmática), GFP-PMA (una ATPasa transmembrana de la membrana plasmática), MAP-GFP (una GFP con un ancla miristoilada y palmitoilada) y GFP-PAP (una GFP con un ancla prenilada).

En la técnica de expresión transitoria en plántulas de *Arabidopsis* se observó que en el mutante *pgap1B-1* GFP-AGP4 y GFP-GPI se acumulan en el ER y también aparecen en estructuras punteadas (posiblemente Golgi); además, un porcentaje de estos marcadores consigue llegar a la membrana plasmática. Sin embargo, estos marcadores se localizaron en la membrana plasmática en los mutantes *pgap1A-2* y *pgap1C-1*, por lo que estas dos isoformas no parecen estar implicadas en el remodelado del ancla GPI (Figura 53).

Estos resultados fueron refrendados utilizando la técnica de expresión transitoria en protoplastos de *Arabidopsis* con los mismos marcadores (Figura 44). También se colocalizaron ambos marcadores con RFP-calnexina para confirmar que este patrón coincide con el del ER (Figura 55). Finalmente, se comprobó que este efecto es específico para las GPI-APs y no para el resto de proteínas secretoras, ya que los marcadores antes indicados GFP-PMA, MAP-GFP y GFP-PAP se localizaron en la membrana plasmática, tanto en las plantas silvestres como en los mutantes *pgap1B-1* y *pgap1ABC*.

Localización de GPI-APs en mutantes *per1l*

Las mismas técnicas y marcadores del apartado anterior fueron utilizados esta vez para los mutantes *per1l*. En los 3 mutantes *per1lA-1*, *per1lB-2* y *per1lAB-2*, GFP-AGP4 quedó retenido en el ER y con un patrón punteado (posiblemente Golgi). Sin embargo no fue el mismo caso que para GFP-GPI, el cual se localizó en la membrana plasmática en estos 3 mutantes en expresión transitoria en plántulas de *Arabidopsis* (Figura 57).

Finalmente se utilizó la técnica de expresión transitoria en protoplastos para confirmar los resultados anteriores. Los patrones resultaron ser los mismos para estos 3 mutantes, excepto para GFP-GPI, que en los mutantes *per1lA-1* y *per1lAB-2* mostró un patrón reticular y punteado (característicos de ER y Golgi, respectivamente) en lugar de localizarse exclusivamente en la membrana plasmática (Figura 58). También se utilizaron los marcadores GFP-PMA, MAP-GFP y GFP-PAP para comprobar que esta acumulación es específica de las GPI-APs y no del resto de proteínas secretoras.

Discusión

Arabidopsis contiene 7 ortólogos putativos del enzima PGAP1 y 2 para el enzima PER1L. En esta tesis se han caracterizado 3 isoformas de PGAP1, centrándonos en la isoforma PGAP1B, la única que se ha predicho que se localiza en el ER, y las dos isoformas del enzima PER1L.

Por una parte, en los experimentos realizados en esta tesis, se ha visto que en el mutante *pgap1B-1*, las GPI-APs se acumulan en el ER, aunque aparece también un patrón punteado característico de Golgi (en el futuro se realizarán más experimentos para corroborarlo), y además un porcentaje de las GPI-APs consiguen llegar a la membrana plasmática. Este patrón coincide con lo descrito en animales y levaduras, donde las GPI-APs no remodeladas, sufren una salida ineficiente del ER y su transporte está retrasado. Además, este hecho explicaría que los mutantes de PGAP1, así como sucede con los mutantes de animales y levaduras, son viables. No obstante, se ha descrito en un mutante Bst1p de *Candida albicans*, que la pared celular está afectada, por lo que será de interés comprobar si en plantas ésta también está afectada.

Por otra parte, en los mutantes *per1lA-1*, *per1lB-2* y *per1lAB-2*, también se ha visto el mismo patrón que en el caso anterior, es decir, se observa una acumulación de GPI-APs en el ER, un patrón punteado, y que una proporción de GPI-APs, consiguen llegar a la membrana plasmática. Estos mutantes tampoco presentan ninguna alteración fenotípica en condiciones de crecimiento estándar, aunque a diferencia de los mutantes de PGAP1, *per1lA-1* y *per1lAB-2* son más sensibles al estrés salino, por lo que este enzima podría estar implicado en la resistencia a este estrés.

También se analizó la localización de las 2 isoformas de PER1L, las cuales se observaron en el Golgi, por lo que indica que la ruta del remodelado de anclas GPI podría ser parecida a la de animales, donde la ruta de remodelado se produce en el ER y Golgi, y no como en levaduras, como se pensaba, donde la ruta concluye en el ER. Esto adquiere especial relevancia ya que las células animales tienen polaridad, así como las vegetales, y el hecho de que esta vía concluya en el Golgi puede tener incidencia para dirigir a qué cara celular son enviadas las GPI-APs.

Por último, resulta interesante el hecho de que se observe el mismo patrón de localización de las GPI-APs en mutantes de enzimas que se sitúan en orgánulos diferentes. Esto podría ser debido, en el caso de PGAP1, a que en el Golgi existe un mecanismo de retención de las GPI-APs que no están correctamente remodeladas, o bien no son reconocidas por los enzimas de la ruta en el Golgi, quedando atrapadas. Y en el caso de PER1L, se podría deber a que esta ruta está regulada, y las proteínas p24 o algún otro mecanismo molecular podría retener las GPI-APs en el ER.

5 CONCLUSIONES

1. La ausencia de proteínas p24 de la subclase delta-1 provoca una disminución en los niveles de proteínas de otros miembros de la familia de proteínas p24 en *Arabidopsis*, lo que se debe a una reducción en la estabilidad de las proteínas. La expresión de un solo miembro de la subclase delta-1 (p24δ5) es suficiente para restaurar los niveles de proteína de otros miembros de la familia de proteínas p24, lo que sugiere

la existencia de redundancia funcional dentro de las proteínas p24 de la subclase delta-1.

2. La pérdida de las proteínas p24 de la subclase delta-1 provoca la acumulación de proteínas con anclaje GPI en el ER, lo que indica que las proteínas p24δ-1 están implicadas en la salida del ER y en la localización en la membrana plasmática de las GPI-APs en *Arabidopsis*. Sin embargo, una proporción de GPI-APs es todavía capaz de alcanzar la membrana plasmática en el mutante *p24δ-1*, lo que puede explicar por qué el mutante *p24δ-1* no muestra ninguna alteración fenotípica bajo condiciones de crecimiento estándar, aunque es más sensible al estrés salino.
3. Las proteínas p24δ-1 parecen estar específicamente involucradas en la salida del ER y el transporte a la membrana plasmática de las GPI-APs, ya que la pérdida de las proteínas p24δ-1 no afecta el transporte de otras proteínas de la membrana plasmática, incluyendo proteínas transmembrana y proteínas ancladas a la membrana plasmática con diferentes tipos de anclajes lipídicos.
4. p24δ5 (subclase p24δ-1) pero no p24δ9 (subclase p24δ-2), restaura parcialmente la localización en la membrana plasmática de GPI-APs en el mutante *p24δ-1*, lo que sugiere que los miembros de las dos subclases p24delta no son funcionalmente redundantes. El dominio coiled-coil y no el dominio GOLD de p24δ5 parece estar involucrado en el transporte de GPI-APs. Los experimentos realizados en la tesis mostraron que p24δ5 interacciona con la forma del ER de la proteína con anclaje GPI AGP4, una interacción que requiere el dominio coiled-coil de p24δ5.

5. El estudio de la función de los enzimas PGAP1/Bst1p y PGAP3/Per1p en *Arabidopsis,* en el remodelado lipídico del ancla GPI y en el transporte de las GPI-APs a la membrana plasmática, reveló que:

5.1 Los mutantes de PGAP1B (el único ortólogo de PGAP1/Bst1p en *Arabidopsis* que se encuentra en el ER), PER1LA y PER1LB (los dos ortólogos de PGAP3/Per1p en *Arabidopsis*) muestran una acumulación de las GPI-APs en el ER, lo que sugiere la necesidad del remodelado lipídico para un transporte eficiente de las GPI-APs desde el ER a la membrana plasmática. Ninguno de estos mutantes muestra una evidente alteración fenotípica bajo condiciones de crecimiento estándar; sin embargo, los mutantes *per1lA* y *per1lAB* son más sensibles al estrés salino que las plantas control.

5.2 Tanto PER1LA-RFP como PER1LB-RFP se localizan en el aparato de Golgi, lo que indica que en plantas la ruta de remodelado de las GPI-APs también ocurre en el aparato de Golgi, como en animales. Esto es contrario a lo sucedido en levaduras, donde toda la vía de remodelación ocurre en el ER.