Complex roles of Rabs and SNAREs in the secretory pathway and plant development: a never-ending story

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COMPLEX ROLES OF RABS AND SNARES IN THE SECRETORY PATHWAY AND PLANT DEVELOPMENT: A NEVER-ENDING STORY …

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Summary

Membrane trafficking is critical for cell compartmentalisation, which allows for the maintenance of specialized environments required for specific cellular activities. To achieve this goal, cells need to tightly regulate vesicular transport between donor and acceptor compartments. This process involves several different protein families; including the SNAREs (65 genes) and small GTPases Rabs (57 genes), which show the highest number of isoforms and therefore are of most interest. We will focus on the roles of these proteins in the ER-Golgi-Plasma membrane pathway to illustrate how Rabs and SNAREs mediate a specific set of functions.

Keywords

Plant, Rab, SNARE, ER, Golgi, Plasma membrane, membrane trafficking.

INTRODUCTION

Professor Chris Hawes was, throughout his tremendous career, passionate about the study of cell compartmentalisation. Membrane trafficking through the secretory pathway from the endoplasmic reticulum (ER), to the Golgi and to the plasma membrane (PM) contributes to cell compartmentalisation. This is critical for many aspects of plant development (cell elongation, cell division, cell wall formation, organelle morphodynamics, and homeostasis, plant responses to environmental stresses, and so forth).

Contrary to mammalian cells where the Golgi apparatus is a singular organelle associated with the microtubule organizing center (MTOC), the Golgi apparatus in plant cells is made of several distinct individual stacks (with each collection of stacks comprising a single Golgi body, with potentially hundreds of Golgi bodies being present in each cell). Professor Hawes et al. determined that these Golgi bodies move on an ER-actin network (Boevink et al., 1998) and interact with ER export sites, constituting what was termed “single mobile secretory units” (daSilva et al., 2004). Therefore, the ER-Golgi interface in the secretory pathway of plant cells cannot be considered analogous to what is known and described in mammals (Brandizzi and Barlowe, 2013).

Vesicle trafficking through the secretory pathway allows for each subcellular compartment to be composed of unique combinations of proteins and lipids. This compartmentalisation of cells permits the maintenance of specialized environments required for specific cellular activities. To achieve this goal, cells need to tightly regulate a set of events. Vesicle transport generally requires formation and budding of the vesicle from the donor membrane (with appropriately sorted cargos),

correct/specific targeting of the vesicle to the acceptor membrane, and finally fusion of the vesicle with this target membrane (Söllner et al., 1993; Kim and Brandizzi, 2012; Brandizzi and Barlowe, 2013). At each step, several different protein families are involved: budding factors, small GTPases Sar/Arf (secretion-associated Ras-related protein/ADP-ribosylation factor) and Rab/Ypt (Ras-related in brain/ Yeast protein transport), GTPase effectors, coat proteins, tethering factors, SNAREs (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor), to name just a few (Rutherford and Moore, 2002; Vernoud et al., 2003; Sutter et al., 2006; Lipka et al., 2007; Moreau et al., 2007; Sanderfoot, 2007; Woollard and Moore, 2008; Saito and Ueda, 2009; Kim and Brandizzi, 2012; Brandizzi and Barlowe, 2013; Singh and Jürgens, 2018). The protein families comprising the trafficking machinery are well conserved and, at the ER-Golgi interface, anterograde trafficking is thought to be mediated by the coat proteins of the COPII machinery, and the retrograde Golgi to ER trafficking with the coat proteins of the COPI machinery, with these two pathways being interdependent (Stefano et al., 2006). We will see later that this scheme continues to evolve in its structure and that our view and understanding of the ER-Golgi interface in plant cells continue to progress.

Professor Hawes has worked on several members of these different protein families, especially on Rab proteins with Professor Ian Moore in Oxford, and to some extent on SNAREs with the team of Dr. Patrick Moreau in Bordeaux. This review will focus on these two families of proteins, and their essential function in the ER-Golgi-Plasma membrane pathway and various aspects of plant life and development.

Two classes of Ras-like small GTPases participate in the overall mechanism of membrane trafficking. First, Sar/Arf are required for vesicle formation at the donor membrane, and then Rab/Ypt are required for targeting and/or tethering of transport

vesicles to the acceptor compartment (Rutherford and Moore, 2002; Vernoud et al., 2003; Woollard and Moore, 2008; Saito and Ueda, 2009). Finally, vesicle fusion to the acceptor membrane is ensured by proteins known as SNAREs (Sutter et al., 2006; Lipka et al., 2007; Moreau et al., 2007; Sanderfoot, 2007; Saito and Ueda, 2009; Kim and Brandizzi, 2012; Brandizzi and Barlowe, 2013). The number of Rab/Ypt and SNARE genes are usually significantly higher than Sar/Arf. For example, in *Arabidopsis*, 57 Rab genes and 65 SNARE genes are present in the genome whereas only 16 Sar/Arf exist. From a phylogenetic point of view the diversity in Rab and SNARE genes was often associated with multicellularity and a high complexity of internal membranes and organelles (Sanderfoot, 2007; Woollard and Moore, 2008; Saito and Ueda, 2009; Kim and Brandizzi, 2012; Brandizzi and Barlowe, 2013). Compared to animals and fungi in terms of evolution, plants show both high conservation of some ancestral genes but also specialization of some, leading to novel functions of many proteins (Barlow and Dacks, 2018). This observation raised a fundamental question as to the functional role of such complexity, and especially how Rabs and SNAREs are able to achieve so many different specific functions (Lipka et al., 2007; Sanderfoot, 2007; Woollard and Moore, 2008; Saito and Ueda, 2009; Kim and Brandizzi, 2012; Brandizzi and Barlowe, 2013; Di Sansebastiano, 2013; Singh and Jürgens, 2018).

RAB PROTEINS

The Rab GTPase was one of the first elements involved in vesicle trafficking to be characterised (Gallwitz et al., 1983; Schmitt et al., 1986; Goud et al., 1990). First identified in yeast and called Ypt, their animal counterpart was found by

complementation approaches with cDNA isolated from rat brain (Touchot et al., 1987). During vesicular trafficking, Rab usually acts in targeting and/or tethering of transport vesicles to an acceptor membrane. A complex set of proteins functions to maintain the equilibrium between the active GTP-bound form and an inactive GDP-bound form of Rab. After a brief explanation of how Rabs are activated/inactivated and targeted to the membrane, we will further discuss their function in plant membrane trafficking.

Rab Prenylation

When associated with GTP, Rab typically binds to membranes through prenylation of its C-terminus. The Rab geranylgeranyl transferases (RGGT) are required for Rab prenylation in plants. They work as heterodimers of alpha and beta subunits. To be efficiently prenylated by RGGT, Rab needs to be in a complex with the Rab Escort Protein (REP), as it was demonstrated for RabA2a (Hála et al., 2005; Wojtas et al., 2007; Shi et al., 2016) (Figure1). In the absence of AtREP1, the *Arabidopsis* RGGT can prenylate other substrates, like Rho GTPases or G-proteins (Shi et al., 2016).

Going Apart with GDI

When Rabs are in their inactive, GDP-bound state, they dissociate from the membrane and are retained in the cytoplasm through the action of GDI proteins (GDP dissociation inhibitor) that mask their geranylgeranyl moiety (Figure1). Two isoforms of GDI are expressed in *Arabidopsis* vegetative tissues. They both interact with RabA5c and were shown to complement the defective yeast mutant *sec19/gdi1*, suggesting that they have a conserved function (Ueda et al., 1996; Andreeva et al., 1997; Ueda et al., 1998). A third isoform of GDI present in the *Arabidopsis* genome is

predominantly expressed in pollen, ovules, and embryos suggesting that it has an activity related to reproduction and the early stages of plant development.

Coming Back to the Membrane

To execute their function, Rabs have to dissociate from GDI and travel to the appropriate membrane. This is achieved by the GDI displacement factor (GDF), PRA1/YIP belongs to this family (Figure1).

In *Arabidopsis*, 19 small membrane proteins grouped in 8 clades display sequence homology to animal or yeast PRA1/YIP (Kamei et al., 2008). Depending on the isoform, these membrane proteins can localise to all membranes in the secretory pathway, from the ER and Golgi to the TGN (trans Golgi network)/early endosomes and PVC (pre-vacuolar compartment) (Kamei et al., 2008). It was demonstrated that a unique PRA1/YIP isoform in rice, which interacts with OsRab7, is required for vesicular trafficking toward the vacuole (Heo et al., 2010). Also, the overexpression and RNAi approach demonstrated that AtPRA1.B6 regulates ER anterograde trafficking, perturbing post Golgi trafficking (Lee et al., 2011). Another isoform of PRA1/YIP, AtPRA1F4, is localized at the Golgi and participates in the sorting of cargo proteins to the TGN (Lee et al., 2011).

More recently, the YIP4a and b were shown to be associated with ECHIDNA at the TGN, suggesting that they may participate in PM sorting of specific cargo like AUX1 (Gendre et al., 2013; Gendre et al., 2019). The role of YIP4a and b on exocytotic routing could also be estimated since they interact with the Golgi localized RABH1b (Renna et al., 2018). Indeed, *rabh1b* shows a defect in CESA6 distribution and velocity at the PM. This default was associated with a slowdown of CESA6 trafficking

to the PM together with a thinner cell wall and a reduction in the growth of etiolated hypocotyls (He et al., 2018).

Rab Inactivation and Activation

As with many small GTPases, Rabs by themselves only have a weak GTPase activity. To be “turned off” by putting them back in their GDP-bound forms, they need the help of accessory proteins called GTPase-activating proteins (GAP) to hydrolyze GTP. In *Arabidopsis*, 20 genes show RabGAP catalytic core motifs and contain a conserved Arg residue critical for RabGAP activity, but the exact physiological role of the RabGAP proteins in plants remains mostly elusive (Albert et al., 1999; Vernoud et al., 2003).

After being associated with the appropriate membrane, Rabs can be converted to their activated form by RabGEF (Guanine nucleotide Exchange Factor) proteins. They mediate the exchange of GDP to GTP, allowing a conformational change of Rabs and ultimately affecting their interaction with effector proteins. AtVPS9a and b are the only genes containing a typical RabGEF domain in the Arabidopsis genome, suggesting a weak specificity of interaction between Rabs and RabGEFs (Saito and Ueda, 2009). This was illustrated by Goh et al., who demonstrated that VSP9a mediates the activation of RabF1, RabF2a, RabF2b but not RabG3f (Goh et al., 2007). The interaction of the VPS9a and RabF2b complex was resolved by crystallography and it was shown that the leaky allele *vps9a-2* could be rescued by the dominant active Q92L form of RabF2b (Goh et al., 2007; Uejima et al., 2010). This suggests that VPS9a acts upstream of RabF. In addition to the VPS9s, other

types of proteins that do not contain the typical RabGEF domain can participate directly or indirectly in Rab activation. For instance, PUF2 was shown to recruit VSP9a in the endosomal membrane and coordinates the activation of RabF2b and RabF1 (Ito et al., 2018a). Also, some proteins were suggested to have a GEF activity. Loss of TGN (LOT) displays a substantial homology with the yeast Rgp1 (Jia et al., 2018). RGP1 was suggested to act as a functional RabGEF for the yeast YPT6 and the animal Rab6 (Siniossoglou et al., 2000; Bonifacino and Rojas, 2006). The authors show that LOT cDNA was able to complement yeast *rgp1,* and that loss of function plants have massive defects in Golgi and TGN structure. These results suggest that LOT may act as a RabGEF protein in plants (Jia et al., 2018). GEF activity toward Rab proteins can also be mediated by tethering complexes. It is most prominently the case in yeast and animal cells through the transport protein particle (TRAPP) complex (Barrowman et al., 2010; Vukašinović and Žárský, 2016). In yeast, four forms of TRAPP complexes are described, where TRAPPI acts as a tethering factor for COPII derived vesicles, TRAPPII and TRAPPIII are involved in post Golgi trafficking and TRAPPIII and TRAPPIV in autophagy (Barrowman et al., 2010). More recently, this model has been challenged, and it was concluded that yeast may only possess TRAPPII and TRAPPIII complexes as in mammalian cells (Thomas et al., 2018). In plants, TRS130, one element of the TRAPPII complex, and RabA1c were found to co-localize at the TGN (Qi and Zheng, 2011). Overexpression of GTP-locked Q72L RabA1c was able to partially complement the *trs130* loss of function mutant, suggesting that TRAPPII is an upstream activator of Rabs in plants (Qi and Zheng, 2011). More recently, Rabs of several clades (E, D, B, A, and G) were identified in a TRS130 interactome and several elements of the TRAPPII complex, including TRS130, demonstrate a preferential binding with the dominant-negative S26N

RabA2a. In addition, a GTP locked Rab variant can complement a *trappii* mutant (Kalde et al., 2019). These results illustrate the ability of the plant TRAPPII complex to activate Rab proteins. The STOMATAL CYTOKINESIS DEFECTIVE1 (SCD1) contains a tripartite DENN domain that has been demonstrated in other systems to function as GEF for Rab GTPases (Marat et al., 2011). SCD1 acts on the exocyst complex and loss of function plants display perturbations of protein exocytosis and recycling of PM proteins like PIN1 (Mayers et al., 2017). SCD1 interacts preferentially with Rabs from the clade E and in a nucleotide state manner. Indeed, SCD1 interacts in vitro with a dominant negative form S29N, but not with a constitutively active form of RabE1c (Q74L). Mayer et al show that overexpression of wild type or Q74L RabE1c can rescue the phenotype of *scd1.1.* Consequently, it was postulated that SCD1 acts as a GEF to mediate RadE1c activation during the process of exocytosis.

RAB Complexes Talk with Effector Proteins

In their GTP-bound form, Rabs interact with effector proteins. Compared to the huge number of Rab isoforms in plants, only a few effectors have been characterized. A portion of them act as tethering factors and participate in the docking of vesicles to acceptor membranes. This is especially true in the case of Golgins, which are long coiled-coil proteins located at the surface of Golgi stacks. Six Golgin homologs were identified in the *Arabidopsis* genome and at least one, GC5, shares homology with the yeast Sgm1p and was shown to interact with RabH1b and RabH1c (Latijnhouwers et al., 2007).

Other well-described effectors of Rabs are phosphoinositide kinases and phosphatases. Indeed, the PI4KBeta1 and beta2 localise at the TGN and interact with the constitutively active form of RabA4b, Q68L, but not the dominant-negative

T22N form. This interaction contributes to the polarised secretion of cell wall components in tip-growing cells, but is also important in biotic interactions (Preuss et al., 2006; Kang et al., 2011; Antignani et al., 2015).

All five members of the Rab clade E were shown to interact with the phosphatidylinositol-4-phosphate 5-kinase 2 (PIP5K2). In addition, it was shown that PIP5K2 has a greater affinity for the GTP locked form of Rabs. Therefore, it seems that RabE may be able to regulate PIP5K2, most likely through protein retargeting (Camacho et al., 2009). It was also shown that the PI4P phosphatase Root Hair Defective 4 (RHD4) is required for the proper localisation of the RabA4b (Thole et al., 2008). Therefore, PI4P regulation at the tip of growing cells is a direct target of Rab GTPases and illustrates that the function of Rab effectors is not restricted to tethering factors involved in vesicle fusion.

FUNCTIONAL ROLE OF RAB PROTEINS IN PLANTS

In plants, Rabs are divided into 8 clades based on their sequence homology, which correlates to some extent to their subcellular localisation and function (Vernoud et al., 2003) (Figure 2).

Rabs in ER-Golgi / Golgi-ER Trafficking

ER-Golgi trafficking mostly involves Rabs from the clades B1 and D. Specifically in *Arabidopsis*, ER-Golgi trafficking is thought to involve at least three isoforms of RabB1. Indeed, some of the plant Rab1 isoforms were shown to complement the yeast Rab1 homolog, Ypt1 (Park et al., 1994). Furthermore, the expression of a dominant-negative version of RabB1b in tobacco leaves slows down the recovery of Golgi fluorescence, in BFA (brefeldin A) treated cells. This indicates a role for the

Rab clade B1 in ER-Golgi anterograde transport (Saint-Jore et al., 2002). RabDs are Golgi localized and *Arabidopsis* loss-of-function mutants of the three isoforms show a bushy phenotype and low fertility. Interestingly, by using a dominant-negative approach it was possible to show that RabD1 and D2 are both needed for protein targeting, but most likely function in two independent pathways (Pinheiro et al., 2009).

The Rab clade H is also involved in ER-Golgi trafficking. Members of the Rab clade H are homologous to Rab6 andYpt6, identified in animal and yeast cells respectively. RabHs are typically cis-Golgi resident and are described as being involved in Golgi to ER retrograde transport. There is some evidence of a conservation in protein function of RabHs across different phyla as RabH1b can functionally complement the yeast homolog Ypt6 (Bednarek et al., 1994). RabH1b and c were also reported to localise to the Golgi and to interact with Golgins, putative Golgi tether proteins (Latijnhouwers et al., 2007; Johansen et al., 2009). RabH1b was also shown to participate in an anterograde trafficking route from the Golgi, as its expression was necessary for CESA6 localisation at the PM (He et al., 2018). Identification of the exact role of Rab clade H in retrograde trafficking in plants warrants further investigation.

Rabs in post-Golgi Trafficking to the PM and Cell Plate

In plants, Rab proteins from clade E regulate trafficking at or after the Golgi. The expression of a dominant-negative inactive form of RabE1d (N128I) results in an accumulation of sec-GFP in the ER and Golgi; and induces the re-localisation of the sec-GFP signal to the vacuole (Zheng et al., 2005). In this study, the authors also demonstrate that RabE1d is acting downstream of RabD2. YFP-tagged RabE1d was shown to localise at the Golgi in a prenylation and nucleotide-dependent mechanism

(Zheng et al., 2005). Interestingly, the RabE1c was also shown to regulate the localisation and function of the peroxisome receptor PEX7, demonstrating that Rabs from the same clade can have extended functions in plants (Cui et al., 2013). In *Arabidopsis*, RabA constitutes the biggest clade and contains almost half of the known Rab isoforms. The remarkable expansion of this clade is one of the most striking features of plant Rab GTPases. RabAs are classically associated with post-Golgi secretion. The subgroup A1 contains 9 members and clear homologs have not been identified in either yeast or animals. RabA1a, A1b, and A1c are thought to be linked to auxin signaling. RabA1a loss of function mutants show a deficit in auxin response (Koh et al., 2009). When functionally linked to TRAPPII, RabA1c was shown to accumulate in a VHa1 positive TGN compartment (Qi and Zheng, 2011). Its correct localisation is sensitive to the specific inhibitor endosin1 and determines PM targeting of PIN2 and AUX1 (Qi and Zheng, 2013). BEX5/Rab1b, which was identified in a reverse genetic screen for enhanced susceptibility to BFA, participates in both exocytosis and transcytosis of PM proteins including PIN1 and PIN2 (Feraru et al., 2012).

Other Rabs identified in clade A were demonstrated to have a role in cell plate formation. Though RabA1d and 1e typically localize to the TGN, during cell plate formation both instead localise to the cell plate, albeit in a different zone to RabA2a (Berson et al., 2014). Interestingly, RabA1e was also identified as being upregulated in response to salt stress in *Arabidopsis* roots. This is suggestive of a potential role for this specific isoform during stress acclimation (Geng et al., 2013). In other plant species, the NtRab11b has been demonstrated to play a key role in pollen tube growth. It localizes to the apical clear zone of the elongating pollen tubes and is

required for secretion and endocytosis at the tip of the pollen tubes (de Graaf et al., 2005).

Subsequently, the role of the tomato Rab11a in secretion has been investigated in tobacco protoplasts. Rehman *et al.* have suggested that Rab11a can regulate the anterograde transport from the TGN to the PM in a SYP122-dependent manner, and did not involve SYP121, supporting the hypothesis that SYP121 and SYP122 may function in independent secretory events (Rehman et al., 2008). Secretome proteomic analysis of the culture medium surrounding tobacco protoplasts expressing dominant-negative transgenes thereafter revealed a potential specific relationship between Rab11 and SYP122 (Rehman et al., 2011).

The Rab subgroup A2 is somewhat similar to both the mammalian Rab11 and the yeast Ypt31/32. RabA2a, alongside the single Rab isoform of the subclade A3, shows a partial co-localization with both a VHA-a1 positive compartment and the PVC (Chow et al., 2008). Interestingly, RabA2a and RabA3 both also localize at the cell plate, suggesting a putative role in polarized secretion. Interestingly, an additional role for RabA2a in establishing apical polarity in *Arabidopsis* root tips was recently established through chemical genetic approaches (Li et al., 2017). Subgroup A4 also seems to be involved in polarized secretion. In support of this, Rab4b is found to localize at the tip of root hairs, but only during their growth (Preuss et al., 2004). Its TGN localization was shown to be dependent on actin polymerization and the RabA4b compartment does not co-fractionate with the Qa-SNARE AtSYP41 and the Qc-SNARE AtSYP51, both TGN markers (Preuss et al., 2004). This result underlines that the TGN is a structure composed of a multitude of different membrane types with potentially diverse functions. RabA4b is also tightly associated with PIP2 homeostasis through interactions with several PI4K isoforms and co-

localises with the PI4P phosphatase (Thole et al., 2008; Kang et al., 2011). The role of RabA4 in tip growth cells is further exemplified by the requirement for functional RabA4d for pollen tube growth (Szumlanski and Nielsen, 2009).

In general, the clade RabA fine-tunes polarised secretion and defines specific membrane subregions for vesicle delivery. RabA5c probably best illustrates this role of the RabA clade. This Rab was found to accumulate just below the PM at each of the 8 corners of plant cells (Kirchhelle et al., 2016). Specific inhibition of RabA5c induces perturbations in the cell geometry of the developing lateral organs (Kirchhelle et al., 2016). This default in anisotropic growth and cytokinesis occurs without disrupting default membrane trafficking. Recently, the same group further demonstrated by genetic, modelling, and pharmacological approaches that microtubules and cellulose anisotropy react to the loss of functional RabA5c in plant cells (Kirchhelle et al., 2019). Finally, we can speculate as to a more general role for Rabs in the regulation of cell wall deposition as many Rabs from the clade A show defects cell wall composition (Lunn et al., 2013).

SNARE PROTEINS

SNARE HISTORY AND DEFINITION

SNAP25 (synaptosomal-associated protein of 25kDa) was the first SNARE protein discovered (Oyler et al., 1989) and during the intervening years the concept of SNAREs was established and developed by Rothman *et al*. The N-ethylmaleimide-sensitive fusion protein (NSF) factor was identified first, followed by the soluble NSF attachment proteins (SNAPs) which are characterized as critical components of intracellular membrane fusion. Finally, an affinity purification procedure isolated the

first SNAP receptors (SNAREs) from bovine brain cells (Söllner et al., 1993) and the era of SNAREs had begun!

Initially, the concept of SNARE-mediated transport was centered on the concept that one SNARE was present at the transport vesicle (v-SNARE, v for vesicles) and one at the acceptor membrane (t-SNARE, t for target). SNAREs were then further divided according to the amino acids present in the hydrophobic heptad repeats in the center of the SNARE domain that engaged in membrane fusion. Using these criteria SNAREs were divided into four groups: Qa- (similar to syntaxin 1), Qb- (similar to the N-terminal half of SNAP25), Qc- (similar to the C-terminal half of SNAP25), and R-SNAREs. A functional SNARE complex for membrane fusion was determined to be generally formed from a Qa-Qb-Qc cis-SNARE complex on the target membrane and a R-SNARE (v-SNARE) on the transport vesicle to produce the functional fusion trans-SNARE complex (Fasshauer et al., 1998; Bock et al., 2001; Sutter et al., 2006) but this organization may not be the only one which can function in membrane fusion. The first SNAREs discovered in plants were the syntaxin homologue of a yeast pep12 mutant (Bassham et al., 1995) and the syntaxin-related Qa-SNARE AtSYP111 (KNOLLE) gene product involved in cytokinesis (Lukowitz et al., 1996). Since then, a huge number of plant SNAREs have been discovered and found to be critical players in numerous cellular trafficking pathways. To date, 65 SNAREs have been reported in *Arabidopsis* (Sanderfoot, 2007; Saito and Ueda, 2009; Kim and Brandizzi, 2012). This high number of SNAREs in *Arabidopsis* and plants in general, compared to the relatively smaller number (only 21) reported in mammalian cells, highlights the complexity and evolution of the endomembrane trafficking system in plants (Sanderfoot, 2007; Barlow and Dacks, 2018). The following chapters will cover SNAREs as key actors in many cellular processes of plant development.

The Concept of i-SNAREs

A very interesting concept for SNARE regulation emerged from the work of Rothman *et al*. A novel potential function of SNAREs, called i-SNAREs (for inhibitory or interfering SNAREs) was first described by Varlamov et al. (2004). The principle is that an i-SNARE will inhibit a fusion process by substituting for, or binding to, a subunit of a fusogenic SNARE complex to produce a non-fusogenic complex. Varlamov *et al*. discovered that certain subunits of the cis-Golgi SNARE complex function as i-SNAREs to inhibit fusion mediated by trans-Golgi SNARE complexes, and reciprocally (Varlamov et al., 2004).

In plants, the first possibility of such a regulatory function of SNAREs was suggested by Foresti and coworkers (2006), they observed that the trafficking from the PVC to the lytic vacuole was inhibited by the overexpression of the syntaxin AtSYP21. Subsequently, De Benedictis *et al.* (2013) demonstrated that the Qc-SNAREs AtSYP51 and AtSYP52 localize to both the TGN and the tonoplast, and further established that they act either as t-SNAREs for membrane fusion when present in the TGN/PVC compartments or as i-SNAREs when accumulated at the tonoplast. A review detailing the main aspects of the i-SNARE concept has been published on plants (Di Sansebastiano, 2013). Recently, Chung et al. (2018) showed that AtMEMB12 overexpression resulted in the accumulation of the antimicrobial protein PR1 (Pathogenesis-Related Gene 1) in intracellular membranes, consistent with AtMEMB12 knockout mutants demonstrating increased resistance to the bacterial pathogen Pst (*Pseudomonas syringae* pv. tomato), as the absence of AtMEMB12 stimulates the exocytosis of PR1 (Zhang et al., 2011; Chung et al., 2018). This is in agreement with the suggestion that AtMEMB12 may be a negative regulator for PR1 secretion. It was proposed that AtMEMB12 could be involved in retrograde trafficking

394 from the Golgi back to the ER, and therefore PR1 could be recycled to the ER and
395 not be secreted (Zhang et al., 2011). Chung et al. have then demonstrated that
396 AtBET12 interacts with AtMEMB12 (Chung et al., 2018).
397 The effect of SNAREs overexpression on PR1 may result either from the titration of
398 critical SNARE partners disrupting SNARE machinery homeostasis or from
399 overexpressed SNAREs acting as an i-SNARE in the early secretory pathway and
400 therefore preventing the secretion of PR1-containing vesicles. Also, the general
401 anterograde transport pathway was not perturbed by AtBET12, suggesting a
402 potential role of AtBET12 in « specifically » regulating pathogenesis-related protein
403 secretion and plant immunity.
404 Further work will be required to characterize further i-SNARE activity in plants, and
405 determine whether the targeting of non-fusogenic SNAREs to specific compartments
406 could tightly regulate protein trafficking in response to various environmental and
407 stress conditions.
408

409 **SNARES IN THE SECRETORY PATHWAY**

410 The involvement of SNAREs at different steps of the secretory pathway (ER-Golgi
411 interface and anterograde/retrograde trafficking, TGN and post-Golgi trafficking, the
412 plasma membrane and cytokinesis) will be discussed alongside new roles and
413 concepts concerning their functions and regulation.
414 A non-exhaustive distribution of SNAREs in the various compartments of the
415 secretory pathway is given in Figure 3.
416

417 **SNAREs in Anterograde ER to Golgi Trafficking**

Compared to other protein families that function within the plant transport machinery, relatively little is known about the involvement of SNAREs at the ER-Golgi interface. The first studies detailing the role of SNAREs in the anterograde transport from the ER to the Golgi were developed through a collaboration between the teams of Dr. P. Moreau and Professor C. Hawes (Chatre et al., 2005). By the heterologous expression of *Arabidopsis* SNARE proteins in tobacco leaf epidermal cells, the ER/Golgi localisation of the R-SNARE AtSec22 and the Golgi localisation of the Qa-SNARE AtSYP31 (~AtSed5), the Qb-SNARE AtMemb11 and the Qc-SNARE AtBET11 (~AtBS14a) were identified. Overexpression of these SNAREs and especially the R-SNARE AtSec22 and the Qb-SNARE AtMemb11, resulted in both a Golgi membrane marker (ERD2) and a secretory soluble marker (secYFP) becoming retained in the ER network (Chatre et al., 2005), indicating their involvement in the ER-Golgi anterograde transport. Bubeck *et al.* (2008) also demonstrated that overexpression of the Qa-SNARE AtSYP31 and the Qb-SNARE AtMemb11 impaired ER to Golgi trafficking. The overexpression-dependent inhibition of the ER to Golgi trafficking of several markers was either due to the titration/trapping of partners of these SNAREs or was a consequence of i-SNARE activity as discussed above.

The co-localization of AtSec22 and the GTPase Sar1 at punctae on the ER membrane is indicative of partial localization of these proteins to ER-export sites (Chatre et al., 2005). Subsequently, it was demonstrated that the loss of the function of AtSec22 leads to the fragmentation of the Golgi in pollen and impaired gametophyte development, and retention in the ER of the plasma membrane syntaxin AtSYP124, demonstrating the critical role of AtSec22 in ER-Golgi trafficking (El-Kasmi et al., 2011).

Furthermore, expressing an ER-blocked version of AtSYP31 in transgenic tobacco plants affected plant growth (Melser et al., 2009). Finally, the Qc-SNAREs AtBET11 and AtBET12 could also be required for the polar elongation of pollen tubes and embryo development (Bolaños-Villegas et al., 2015).

The concept of 'Golgi entry core compartment' (GECCO)

Many questions remain about the dynamics of the ER-Golgi interface and interactions between the ER-export sites and the cis-Golgi, how the stacked cisternae of the Golgi are formed and maintained, and which molecular mechanisms are involved.

In tobacco BY-2 cells treated with BFA, the formation of small punctate structures loaded with proteins originating from the cis-most cisternae of the Golgi were observed. These structures were found adjacent to the ER-export sites, and act as scaffolds for Golgi regeneration after BFA washout (Ito et al., 2012). Then, using SCLIM 3D time-lapse observations (super-resolution confocal live imaging microscopy), it was found that a *trans*-Golgi marker was transported through this compartment during Golgi regeneration, indicating that the *cis*-most cisternae of the Golgi receive cargo directly from the ER and likely the ER export sites. This compartment was termed the 'Golgi entry core compartment' (GECCO), and is formed independently of the COPII and COPI machinery, and interestingly resembles the ERGIC (ER-Golgi Intermediate Compartment) identified in mammalian cells (Ito et al., 2018a). In addition, it was found that the Qa-SNARE AtSYP31, a *cis*-Golgi marker that localises to GECCO upon BFA treatment, is normally resident of the *cis*-most cisternae of the Golgi (Ito et al., 2018b). Therefore, as this SNARE was found to play a role in ER to Golgi trafficking (Chatre et al., 2005; Bubeck et al., 2008), it may

be possible that its function at the ER-Golgi interface is linked to the molecular mechanisms required for the de novo formation of the first Golgi cisternae.

In the future, as discussed above for the SNARE complexes, it would be of interest to determine which other SNAREs and partners are present in the GECCO to understand further membrane dynamics and cargo trafficking at the ER-Golgi interface.

SNAREs in Retrograde Golgi to ER Trafficking

Even less is known of the role of SNAREs in retrograde Golgi to ER trafficking. The overexpression of the Qa-SNARE AtSYP81 was found to inhibit both anterograde and retrograde transport between the ER and the Golgi, and its localisation to the subdomains of the ER physically separated from the Golgi suggested that they may correspond to ER import sites (Bubeck et al., 2008).

Furthermore, the Qc-SNARE AtSYP72 is located at punctae in the ER, suggesting a localization that is compatible with a role in retrograde transport (Lerich et al., 2012).

What about SNARE Complexes at the ER-Golgi interface?

Few attempts have been made to identify putative SNARE complexes at the ER-Golgi interface. Tai and Banfield (2001) determined that AtBET11 and AtBET12 could form different complexes in vitro with the yeast ER (Sec22) and Golgi (Bos1, Gos1, Sed5 and YKT6) SNAREs. Sec22, Gos1 and Sed5 (AtSYP31 in *Arabidopsis*) were the most abundant SNAREs observed in these complexes. However, after attempting interactomics of Qa-SNAREs after immunoprecipitations performed on transgenic plants expressing fluorescent constructs, Fujiwara *et al.* (2014) were unable to detect SNARE proteins that could interact with the Qa-SNAREs AtSYP31 or AtSYP32, most

likely due suboptimal expression of the transgenic proteins (Fujiwara et al., 2014). Therefore, identifying the endogenous SNARE complexes involved in trafficking at the ER-Golgi interface might prove challenging.

In addition, nothing has yet been published on putative partners of the Qa-SNARE AtSYP81. SNARE complexes at the ER-Golgi interface require further attention in the future in order to understand the overall dynamics and regulation of protein trafficking at this interface.

ER SNAREs and new aspects of their Regulation and Function

SNAREs (throughout cells, not those solely localized to the ER) are tail-anchored proteins through their C-terminal hydrophobic domain, and it is only recently that a SNARE, Qc-SNARE AtSYP72, was shown to be integrated into the ER membrane via the GET (Guided Entry of Tail-anchored proteins) system (Srivastava et al., 2017). Xing *et al.* (2017) have further analyzed some components of the GET (Guided Entry of Tail-anchor) pathway in *Arabidopsis*, and reduced root hair elongation in detected in defective lines, probably corresponding to reduced amounts of nascent SNAREs, and reduced growth phenotypes in overexpressing lines, suggesting a strong regulatory role of the GET pathway in SNARE biogenesis and cellular homeostasis (Xing et al., 2017).

An interesting discovery is the « unexpected » role of a SNARE in ER interaction with the cytoskeleton. ER streaming and remodelling is highly dependent on membrane-cytoskeleton interactions, Cao *et al.* (2016) have identified that Qc-SNARE AtSYP73, bearing actin-binding domains, actively anchors the ER membrane to actin filaments. Loss of AtSYP73 function affects the morphology of the ER network, ER streaming, and plant growth, as is observed for myosin-XI mutants (Cao et al., 2016).

Finally, of the ER-Golgi SNAREs, the Qb-SNARE AtMemb11 was found to interact with the GDP-bound form of ARF1 in the Golgi (Marais et al., 2015). The disturbance of the anterograde pathway caused by overexpression of AtMemb11 (Chatre et al., 2005; Bubeck et al., 2008) may be either directly related to the role of AtMemb11 in a SNARE complex or indirectly as a result of the titration of Arf1. AtMemb11 (mostly localized at the cis-Golgi cisternae) could, therefore, function both as a SNARE for membrane fusion and as a regulator of Arf1 for modulating the COPI machinery. However, it is not known whether AtMemb11 is a member of a SNARE complex at the cis-Golgi apparatus.

GOLGI/TGN: SNAREs at the Hub of Protein Sorting

The TGN is a tubular/vesicular organelle that can be considered as the central point for sorting of secretory and vacuolar cargos. Therefore, membrane trafficking is strongly regulated at the TGN which is enriched with several families of SNAREs.

Sanderfoot *et al.* (2001) have identified a functional separation between the Qa-SNAREs of the AtSYP4 family, which reside mainly in the trans-Golgi network, and the Qa-SNAREs of the AtSYP2 family, found predominantly in the pre-vacuolar compartment (Sanderfoot et al., 2001). They also found that the Qc-SNARE AtSYP61 is a resident of the TGN and can form complexes with the Qb-SNARE AtVTI12 and either of the Qa-SNAREs: AtSYP41 or AtSYP42. Surprisingly, the interactomics of Qa-SNAREs only revealed the Qa-SNARE AtSYP43 (Fujiwara et al., 2014). In addition, Chen *et al.* (2005) have determined, by using a liposome fusion assay, that the Qa-SNARE AtSYP41 and the Qc-SNARE AtSYP61 are likely to function in independent vesicle fusion reactions with the Qb-SNARE AtVTI12 (Chen et al., 2005). Chen et al. (2005) also identified that the R-SNARES YKT61 and

YKT62 are essential for membrane fusion mediated by either the Qa-SNARE AtSYP41 or the Qc-SNARE AtSYP61, which determined the first SNARE complexes which could be required for membrane fusion at the TGN. The identification of these different possible SNARE complexes supports the concept of a sorting station located in the TGN.

In an extensive study of the AtSYP4 family (using *syp42syp43* double mutant, tracking protein cargos such as secGFP and 12S globulin), Uemura et al. (2012) demonstrated that the Qa-SNARE members of the AtSYP4 group were involved in multiple transport pathways (specifically the secretory pathway, vacuolar transport pathway, and perhaps the retrieval pathway from the late endosomes/pre-vacuolar compartment to the TGN) (Uemura et al., 2012).

Interestingly, they identified that the polar plasma membrane localization of the auxin efflux carrier PIN2-GFP was not disturbed but that its vacuolar transport for degradation was impaired (Uemura et al., 2012). These results provide evidence for the complexity of regulation of protein transport by SNAREs (and its partners) at both the level of cargo specificity and transport pathways targeted.

Breakthrough: Isolation of SYP61 TGN-derived Vesicles

A key development in our understanding of the function of the TGN was the immune-isolation and subsequent proteomic analysis of AtSYP61 TGN-derived vesicles (Drakakaki et al., 2012). Drakakaki et al. (2012) were able to identify 145 proteins that were specific to the SYP61 TGN-derived vesicles without contamination by known pre-vacolar markers AtSYP21 or AtSYP51.

Among the proteins identified, analysis of the AtSYP61 proteome identified numerous proteins that function in cellular transport machinery and cargos of high interest for further analysis:

- The Qa-SNAREs AtSYP41 and AtSYP43, the Qb SNARE VTI12, corroborating possible SYP41/SYP61/VTI12 and SYP43/SYP61/VTI12 SNARE complexes at the TGN as described earlier, but confirmed by interactomics of Qa-SNAREs only for the Qa-SNARE AtSYP43 (Fujiwara et al., 2014). These apparent discrepancies probably just reveal how difficult it is to visualize these protein complexes as a function of the technology used and the nature and developmental stage of the material used…

- The plasma membrane SNAREs such as the Qa-SNARE AtSYP121 (PEN1), the Qb/Qc SNARE AtSNAP33 and the R-SNARE AtVAMP722 were also identified (SNAREs facilitating the secretion of cell wall components to the plasma membrane in response to pathogen attack) together with AtVPS45 and several VSRs (VSR3, VSR4 and VSR7), suggesting that the AtSYP61 compartment is highly involved in segregating the secretory and vacuolar pathways.

- GTPases such as AtRABD2a and AtRABD2b were abundantly present and two YIPs (YPT-interacting proteins) were identified in the AtSYP61 proteome. As already indicated above, YIP4a and YIP4b were demonstrated to be critical trafficking components in ROP-dependent root hair formation (Gendre et al., 2019).

- The AtSYP61 proteome contained a Trs120 homolog, a member of the TRAPPII complex possibly involved in cytokinesis. It also included four orthologs of TRAPPI complex subunits. TRAPPI being associated with ER to Golgi transport, the presence of components of TRAPPI in AtSYP61 vesicles suggests that plant TRAPPI might be involved in vesicle fusion at the TGN rather than in ER to Golgi transport, without excluding the possibility of ER-TGN contacts.

590 - Several CESA (Cellulose synthase A) complexes were identified in the SYP61
591 proteome. The analysis with the specific inhibitor endosidin 1 revealed that trafficking
592 of CESA complexes can be TGN dependent. Recently, co-localization of CESA3 and
593 AtSYP61 was found to be enhanced upon CESTRIN (CESA trafficking inhibitors)
594 treatment, corroborating the requirement of the AtSYP61 compartment in CESA
595 complexes trafficking (Worden et al., 2015).
596 - The SYP61 proteome also contained the protein ECHIDNA. It has been shown that
597 ECHIDNA is required for the TGN-mediated trafficking of the auxin influx carrier
598 AUX1 to the plasma membrane, whereas its involvement in the transport of the auxin
599 influx carrier LAX3 or the auxin efflux carrier PIN3 was minor. Trafficking defects of
600 AUX1 in *ech* mutants were correlated with perturbation of secretory vesicle formation
601 at the TGN (Boutté et al., 2013).
602 This AtSYP61/ECHIDNA compartment of the TGN was also shown to be enriched
603 with sphingolipids carrying α-hydroxylated acyl-chains of at least 24 carbon atoms,
604 critical for the polar secretory sorting of the auxin carrier PIN2 to the apical
605 membrane of *Arabidopsis* root epithelial cells. A disturbance of the tubulo-vesicular
606 structure of the TGN was observed, revealing that, together with the specific proteins
607 identified in the cellular transport machinery, these specific sphingolipids govern the
608 morphology and dynamics of this TGN-subdomain.
609 The TGN-localized coiled-coil protein TNO1 is a putative tethering factor that
610 interacts with the Qa-SNARE SYP41 and is required for TGN localization of the Qc-
611 SNARE SYP61. Interestingly, the TGN was disrupted and vesicle formation from the
612 Golgi cisternae was affected in a *tno1* mutant, and these defects were rescued by
613 overexpression of either the Qa-SNARE SYP41 or the Qc-SNARE SYP61. These

614 results supported the implication of the tethering factor TNO1 in vesicle fusion at the
615 TGN (Yang et al., 2019).
616 Finally, SCYL2A/B are clathrin-binding proteins which interact with the Qb-SNAREs
617 AtVTI11 and AtVTI12, and CHC1, supporting the notion that SCYL2A/B proteins are
618 involved in clathrin-mediated vesicle trafficking (Jung et al., 2017). Root hair tip-
619 localized proteins such as ROP2, RHD2, RABA4B, SYP123, and CSLD3, are critical
620 for proper root hair tip growth, but only CSLD3 was mis-localized in the root hairs of
621 *scyl2b* mutants, supporting the conclusion that SCYL2B to some extent mediates the
622 tip localization of CSLD3 (Cellulose synthase-like protein D3) in root hairs. Therefore,
623 SCYL2B may act as a component of clathrin-mediated vesicle membrane trafficking
624 that regulates secretory processes mediated by TGN and PVC in the process of root
625 hair tip growth (Jung et al., 2017).
626 As highlighted by Renna and Brandizzi (2020), the numerous tools developed
627 (reverse genetics, proteomics, chemical inhibitors, super-resolution live-cell imaging)
628 together with lipidomics (Wattelet-Boyer et al., 2016) are now available to unravel the
629 different functions of the TGN, to determine all the key actors and functional
630 machineries in the different subdomains involved in cargo sorting and transport, and
631 finally to investigate the homeostatic regulation of the TGN in various environmental
632 conditions.
633
634 ***SNAREs at the Golgi-released independent TGN (GI-TGN)***
635 The Golgi-released independent TGN (GI-TGN) is a TGN-derived compartment,
636 released from the Golgi-associated TGN (GA-TGN), which has been only described
637 in plant cells thus far (Kang et al., 2011; Uemura et al., 2014).

Further work has subsequently revealed a tight association between the GI-TGN and the secretory R-SNAREs AtVAMP721 and AtVAMP722, but to a lesser extent AtVAMP727 (vacuolar pathway) was determined (Uemura et al., 2019). The GI-TGNs were shown to mediate the transport of the R-SNARE AtVAMP721 to the plasma membrane, and the Qa-SNAREs of the AtSYP4 group may be involved in the recycling of AtVAMP721 between the plasma membrane and late endosomes, in addition to the transport from the GA-TGN compartment to the plasma membrane via the GI-TGN compartment, through undetermined mechanisms (Uemura et al., 2019). The GI-TGN is proposed to function as a transit compartment between the Golgi and the plasma membrane, and in this model, the GA-TGN could mature into the GI-TGN and then into secretory vesicles through the increase of the concentration of AtVAMP721-dependent components of the secretory pathway (Uemura et al., 2019).

SNAREs in Plasma Membrane Biogenesis, Cytokinesis, and Symbiosis

A tremendous amount of research has been performed recently, focused on post-Golgi trafficking to the plasma membrane and the implication of SNAREs. These studies have revealed the multitude of SNARE complexes that can be formed and attempts to identify the features linked to the specificity of SNARE functions in constitutive exocytosis, cytokinesis, or innate immunity were reached. As discussed earlier, the possibility that SYP121 and SYP122 drive independent secretory events were proposed (Rehman et al., 2008). Then, Professor G. Jürgens et al. demonstrated that the Qa-SNAREs AtSYP111 and AtSYP121 are not switchable in their respective functions in cytokinesis and innate immunity (Reichardt et al., 2011). In addition, they determined that the Qa-SNARE AtSYP132 could replace the Qa-SNARE AtSYP111, indicating that AtSYP132 could be more related to membrane

fusion in constitutive exocytosis. The strict specificity observed between the Qa-SNAREs AtSYP111 and AtSYP121 may suggest the involvement of different (specific) SNARE complexes. In a recent interactomic study of Qa-SNAREs, Fujiwara et al (2014) identified the Qc-SNARE AtSYP71 and the R-SNARE AtVAMP721 as partners of the Qa-SNARE AtSYP121 but unfortunately were not able to elucidate a SNARE interactant for the Qa-SNARE AtSYP111 (Fujiwara et al., 2014). This may be explained by the insufficient expression of this SNARE linked to its normally specific expression during cytokinesis. In addition, they identified several SNAREs for the Qa-SNARE AtSYP132 (the Qb-SNAREs AtNPSN11 and AtNPSN13, and again the Qc-SNARE AtSYP71 and the R-SNARE AtVAMP721). Using this approach, Fujiwara et al. also determined that the Qa-SNARE AtSYP122 could interact with the same SNAREs interacting with the Qa-SNARE AtSYP132.

Further studies devoted to understanding the requirement of SNARE complexes and their regulation in cytokinesis revealed several critical points: (i) two distinct complexes (Qa-SNARE AtSYP111/Qb,c-SNARE AtSNAP33/R-SNARE AtVAMP721,722 and Qa-SNARE AtSYP111/Qb-SNARE AtNPSN11/Qc-SNARE SYP71/AtVAMP721,722) were found to associate to drive membrane fusion (El Kasmi et al., 2013); (ii) cytokinesis still occurs in Qa-SNARE AtSYP111 mutant embryos. Park et al (2018) identified cytokinesis defects in a Qa-SNARE AtSYP132 mutant and defined an additional SNARE complex involving the Qa-SNARE AtSYP132 and the same partners shared with the Qa-SNARE AtSYP111, with overlapping and non-overlapping functions of these two complexes (Park et al., 2018); (iii) Karnahl et al (2018) have evidenced that the Sec1/Munc18 (SM) regulatory proteins of SNARE complexes AtSEC11/KEULE (Karnik et al., 2015; Karnahl et al., 2018; Zhang et al., 2019) and its paralog AtSEC1B are respectively

predominantly involved in cytokinesis and secretion regulation; (iv) Interestingly, it was determined that after stopping vesicle formation at the TGN, cis-SNARE complexes were found to accumulate in the early secretory pathway, strongly suggesting that these inactive cis-SNARE complexes are already formed in the ER and transferred as such to the plasma membrane for better efficiency of cytokinesis and directional growth (Karnahl et al., 2017).

Pollen tube growth requires the action of several SNAREs. Three Qa-SNAREs (AtSYP124, AtSYP125 and AtSYP131) are considered as pollen-specific. Slane et al (2017) have shown that they can have overlapping functions in pollen development since only the triple mutant presented a strong gametophytic defect (Slane et al., 2017). This result supports a functional redundancy within members of a given SNARE gene family but a functional specificity of its members has not to been ruled-out. Although Silva et al (2010) determined that syntaxins alone do not provide the level of specificity required for apical growth, they have identified a specific distribution of SYP124 mediating an exocytic flow occurring in the flanks of the pollen tube apex and that the syntaxins of the SYP1 family have a different distribution in the pollen tube.

Interestingly, Li et al (2019) have discovered a Tomosyn protein that can bind, through its C-terminal R-SNARE like motif several Qa-SNAREs, and act as a negative regulator of secretion to control pollen development (Li et al., 2019).

Lastly, Pan et al (2016) have discovered an unexpected maturation of the gene of the Qa-SNARE SYP132 in *Medicago trunculata* (Pan et al., 2016). Effectively, it has been shown that it undergoes alternative cleavage and polyadenylation during the transcription process, which produces two isoforms of the SNARE: MtSYP132A (A for alternative) and MtSYP132C (C for canonical). MtSYP132A is localised to the

symbiosome membrane and participates in the maturation of symbiosomes, whereas MtSYP132C is the major form in non-nodulated roots and is involved in classical secretory functions, unrelated to symbiosis. They concluded that the presence of SYP132A in angiosperms strongly correlates with the establishment of arbuscular mycorrhizal symbiosis.

SNAREs and other protein families are largely involved in plant cell-microbe interactions (Nathalie Leborgne-Castel and Bouhidel, 2014). A massive remodeling of the host cell PM is required for the formation, and maintenance, of a perimicrobial membrane which will have a unique protein composition and therefore identity. Challenges for the future will be to identify the trafficking pathways and machineries involved in the different types of interactions, and to determine how pathogens affect the protein composition of the host cell PM and consequently plant defense/immunity (Nathalie Leborgne-Castel and Bouhidel, 2014).

SNAREs in Ion Transport Regulation

Years ago, Professor M. Blatt et al. established a link between the Qa-SNARE AtSYP121, the plasma membrane KAT1 K^+-channel activity, and stomatal control (Eisenach et al., 2012). Grefen et al (2015) have further evidenced that the Qa-SNARE AtSYP121 interacts with a specific domain of KAT1 (the voltage sensor domain) which confers a voltage-dependent control of secretion (Grefen et al., 2015). In addition, Zhang et al (2015, 2017) discovered that the R-SNARE AtVAMP721 also interacts with KAT1 but also with another K^+-channel, KC1, indicating a tight regulation between the K^+-channels and the AtSYP121/AtVAMP721 SNARE complex (Zhang et al., 2015; Zhang et al., 2017). Finally, Waghmare et al (2019) determined that the Qb,c-SNARE AtSNAP33 stabilizes the AtSYP121/AtVAMP721 SNARE

complex after priming of the Qa-SNARE AtSYP121, through its interaction with K^+-channel (Waghmare et al., 2019). It was suggested that this binding could correspond to a primary state for the initiation of the secretory/fusion machinery for exocytosis.

Although the single mutants of AtSYP121 and AtSYP122 did not show any phenotype, revealing a high redundancy, Waghmare et al (2018) have identified some cargos specific for both SNAREs through a proteomic approach, suggesting that each Qa-SNARE may be engaged to some extent in different pathways according to the nature of some cargos (Waghmare et al., 2018). It has been demonstrated previously that the Qa-SNARE AtSYP121 is involved in the delivery of the aquaporin PIP2;5 in maize and the aquaporin PIP2;7 in *Arabidopsis* to the plasma membrane, implying this SNARE functions in the regulation of plasma membrane water permeability and cell osmotic homeostasis (Besserer et al., 2012; Hachez et al., 2014). Water and nutrient uptake in root hairs has been shown to involve the Qa-SNAREs AtSYP123 and AtSYP132 which interact with the R-SNAREs AtVAMP721,722,724 at the tip (Ichikawa et al., 2014). The Qa-SNARE AtSYP132 was also found to regulate the transport of and as a consequence the activity of H^+-ATPase at the plasma membrane and this in an auxin-dependent manner (Xia et al., 2019). Modulation of plasma membrane H^+-ATPases AHA1 and AHA2 activities were found to involve the R-SNARE AtVAMP711 during drought stress since its deletion increased H^+-ATPase activity and slowed down stomatal closure in response to both abscisic acid and drought treatments (Xue et al., 2018).

SNAREs in Pathogen Defense

762 Another interesting feature is how pathogens differently take over the host early
763 secretory pathway for their own purposes, as illustrated by the following examples.
764 The pathogen *Colletotrichum orbiculare* develops hyphae inside the host cucumber
765 (*Cucumis sativus*) by using the CsSec22-dependent secretory pathway to secrete
766 biotrophy effectors toward the interface between the pathogen and the host (Irieda et
767 al., 2014). On the contrary, during the infection of tobacco (*Nicotiana benthamiana*)
768 by the Turnip mosaic virus, overexpression of the R-SNARE NbSec22 blocked the
769 early secretory pathway but enhanced the movement of replication vesicles,
770 indicating that these vesicles bypassed the Golgi and used an alternative route (pre-
771 vacuolar/multi-vesicular bodies) for virus propagation (Cabanillas et al., 2018).
772 Recently, Sasvary et al (2018) have studied the replication of the Tomato bushy stunt
773 virus (TBSV) in tobacco and yeast (Sasvari et al., 2018). They have found in yeast
774 that the viral replication protein p33 can interact with Use1p, the syntaxin Ufe1p and
775 its plant ortholog AtSyp81. In tobacco, the replication of TBSV RNA had an efficiency
776 of only 25% in the Nb*SYP81* knockdown plants whereas that of the *Tobacco mosaic*
777 *virus* had an efficiency of 70%, suggesting that SYP81 (required for retrograde
778 protein transport in plants) was to some extent specifically required for tombusvirus
779 RNA replication and accumulation in plants, but the retrograde transport pathway
780 was shown not to be required in yeast.
781 Finally, they could propose in yeast a model on an assembly hub role of the yeast
782 Ufe1 and Use1 SNARE proteins at specific subdomains of the ER for the formation of
783 the TBSV replication compartment (Sasvari et al., 2018). Such a model with SYP81
784 and other SNAREs/partners may also be functional in plant cells.
785 Through investigation of the powdery mildew fungus pathogen *Blumeria graminis* f.
786 sp. *hordei*, and after reconstruction of 3-D images, Uemura et al. (2019) showed that

787 the Qa-SNARE AtSYP43 and the R-SNARE AtVAMP721 accumulated at specific
788 sites of fungal ingress. It was also shown that the Qa-SNAREs of AtSYP4 group and
789 the R-SNARE VAMP721 are involved in the secretion of cell wall-modification
790 enzymes, which are known to be crucial for plant growth and pathogen defense.
791 Plasma membrane SNAREs are also known to be critical for pathogen defense. The
792 Qa-SNARE AtSYP121 (PEN1), the Qb/Qc SNARE AtSNAP33 and the R-SNARE
793 AtVAMP721/722 are involved in the response to ascomycete and oomycete
794 pathogens. Yun et al (2013) have shown that the infected plant prioritizes the use of
795 the R-SNARE AtVAMP721/722 and partners for secretion linked to the defense
796 pathway instead of secretion linked to plant growth (Yun et al., 2013). Kim et al
797 (2014) have shown that the powdery mildew resistance protein RPW8.2 is
798 transported to the plant-fungal interface through AtVAMP721 vesicles and that
799 defense is highly decreased in the absence of this R-SNARE (Kim et al., 2014).
800 It was then shown that synaptotagmin 1 deletion increased the resistance to the
801 pathogen *Golovinomyces orontii*, and that the fungus induced interactions between
802 the Qa-SNARE AtSYP121 and synaptotagmin 1 and inhibition of the formation of the
803 AtSYP121 SNARE complex (Kim et al., 2016).
804 Brassinosteroid-induced genes control several aspects of plant development and
805 especially stress and pathogen response. It was proposed that the SNARE complex
806 containing the Qa-SNARE AtSYP22 and the R-SNARE AtVAMP727 drives the
807 transport to the plasma membrane of the brassinosteroid receptor BRI1 (Zhang et al
808 2019), and regulates plant resistance to pathogens by controlling the amount of BRI1
809 reaching/accumulating at the plasma membrane (Zhu et al., 2019). Cao et al (2019)
810 have also shown in rice that the Qa-SNARE OsSYP121 accumulates at pathogen
811 penetration sites can interact with the Qb,c-SNARE OsSNAP32 and the R-SNARE

OsVAMP714/724 and contributes to host resistance to rice blast induced by the fungal pathogen *Magnaporthe oryzae* (Cao et al., 2019).

Finally, lipid signaling through phospholipase Dδ has been found to regulate plant innate immunity (Xing et al., 2019). Following pathogen attack, it was shown that the phospholipase Dδ is secreted via an AtVAMP721/722-mediated secretory process and led to a focused production of phosphatidic acid which activates reactive oxygen species and jasmonic acid signaling pathways (Xing et al., 2019). Therefore, we can imagine how many molecular interactions and different levels of regulation we still have to discover to understand all these mechanisms…

CONCLUSIONS AND EXPECTATIONS FOR THE FUTURE…

A first important conclusion concerning Rabs and SNAREs is the multi-combinatorial possibilities and specificities which are offered by the number of actors that evolution produced for both protein families. This originates, of course, from the increased needs of devoted machineries to many aspects of plant development and plant responses to their environment.

At the molecular and mechanistic level, this effectively implies a multitude of protein interactions, of regulation steps, signaling pathways, without excluding the lipid partners. Regarding SNAREs, more and more interactants are discovered such as QUIRKY (a member of the MCTP protein family having multiple C2 domains and transmembrane domains) which has recently been shown to interact with the Qa-SNARE AtSYP121 to sustain florigen transport in *Arabidopsis* (Liu et al., 2019).

In the case of Rab proteins, the high number of isoforms must be associated with the complexity of secretion processes in plants and especially regarding post Golgi

836 trafficking as it is highlighted in the recent review by Elliott et al. (2020). In addition,
837 Rabs are also surrounded by many regulating partners. For example, 20 RabGAP
838 and 19 PRA1 isoforms are present in the *Arabidopsis* genome. They are respectively
839 involved in Rab inactivation and Rab membrane targeting. As a consequence, it
840 would be particularly challenging to describe the combinatorial interactions that must
841 exist in cells on a one by one basis. New interactomic approaches that rely on
842 proximity labelling may be used to shed light on this complexity in plants (Del Olmo et
843 al., 2019; Gillingham et al., 2019).
844 Furthermore, the development of optogenetic approaches offers new avenues to by-
845 pass the genetic plasticity often observed with Rab knock out or overexpressor lines.
846 Indeed, optogenetically controlled oligomerisation was used to inactivate Rab
847 function in cells within minutes (Nguyen et al., 2016). These approaches are
848 promising for plant Rab biology and will help to further understand their precise
849 function in development or plant defense (Banerjee and Mitra, 2020).
850 Moreover, the involvement of many protein families (tethering factors, SNAREs, small
851 GTPases, adaptor proteins, ECHIDNA and so forth) in Golgi-plasma membrane (and
852 vacuolar) trafficking pathways highlights the requirement for a multiplicity of GI-TGN
853 subdomains and derived transport vesicles which must be engaged in numerous
854 transport pathways for numerous different cargos. A recent example is given by De
855 Caroli et al. (2020) on CesA6 and PGIP2 trafficking involving distinct subpopulations
856 of TGN-related endosomes.
857 The challenge for the future will therefore be to identify the respective protein
858 assemblies and to decipher the corresponding molecular mechanisms and their
859 regulation, which are associated with each potential pathway in this huge network of
860 membranes/organelles exchanges.

Finally, a new protein family may also deserve our future attention, the proteins which have been called phytolongins and correspond to non-SNARE longins. Phytolongins contain a typical SNARE-like longin domain but lack the central SNARE domain (for membrane fusion) which is replaced by a "PhyL domain" of unknown function, and the 4 proteins of this family are located along the secretory pathway : Phyl2.1 and Phy2.2 in the ER, Phyl1.2 in the Golgi, Phyl1.1 at the plasma membrane and to a lesser extent in post-Golgi compartments (de Marcos Lousa et al., 2016). These proteins, according to their cellular location and the absence of a « fusion domain », could regulate the SNARE complexes by interacting through their longin domain, could be involved in ER and Golgi structure, could participate along the secretory pathway and at the cell surface to plant responses to various stimuli.

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Legends to Figures

Figure 1: Schematic diagram of Rab cycle.
(i) Rab becomes prenylated at their C-terminus by an association between the Rab GeranylGeranyl Transferases (RGGT) and the Rab Escort Protein (REP). Before reaching the target vesicle, Rab is maintained in the cytoplasm through the action of REP to mask the Rab geranylgeranyl moiety. (ii) Membrane association is mediated by GDI displacement factor (GDF) belonging to the family of PRA1/YIP. REP protein or GDI protein are removed and Rab prenylated tail is inserted into the vesicle bilayer. (iii) Upon trigger, GDP/GTP exchange factors (GEF) mediate Rab conformation change. Once activated, Rab interacts with effector proteins such as tethering factors needed for vesicle docking with acceptor membrane. (iv) Rab inactivation is achieved by GTPase-activating proteins (GAP). They help Rab to hydrolyse GTP and turn them back to their inactive conformation. Then, Rab dissociate from the membrane and form a cytoplasmic complex with GDI proteins.

Figure 2: Localisation of the different clades of Rab involved in secretion.
Rab B and D are associated with ER to Golgi anterograde trafficking. Rab H participates to retrograde trafficking. Rab A and E are involved in the post Golgi trafficking.
ER: Endoplasmic reticulum, ERES: Endoplasmic reticulum exist site, GA-TGN: Golgi associated trans Golgi network, GI-TGN: Golgi independent trans Golgi network, PM: plasma membrane.

Figure 3: Localisation of the different SNAREs involved in secretion or other functions.
In blue SNAREs exclusively or mainly associated with the ER, in green SNAREs located in the Golgi and for some of them involved in ER-Golgi exchanges. In orange SNAREs located at the TGN compartments and are involved in different

steps/aspects of post Golgi trafficking. In red, SNAREs either present at the plasma membrane and/or engaged in trafficking to this membrane (SYP71 can have a dual-location between the ER and the PM, Suwastika et al., 2008).
For details on their location and functions, please refer to the text in the different chapters.
ER: Endoplasmic reticulum, ERES: Endoplasmic reticulum exist site, GA-TGN: Golgi associated trans Golgi network, GI-TGN: Golgi independent trans Golgi network, PM: plasma membrane.

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