A study of the vesicle trafficking pathways regulating polar auxin transport and root development

JULIEN AGNEESSENS

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Julien Jean Agneessens

Submitted for the qualification of Doctor of Philosophy

Department of Biosciences, Durham University

December 2023

Abstract

PIN proteins have a crucial role in the polar transport of the phytohormone auxin in plant tissues. In some cell types, PIN proteins have a polar distribution at the plasma membrane. Vesicle trafficking is essential to generate this polarity but also for the control of the abundance of PIN proteins at the plasma membrane trough their internalisation and secretion. Among the molecular regulators of PIN trafficking, VAMP714, a SNARE protein responsible for vesicle fusion, was shown to be essential for PIN polarity. The aim was to investigate this molecular mechanism in more detail. A protein-protein interaction approach has allowed the identification of PIN trafficking regulators interacting with VAMP714 in intracellular compartments, possibly acting as recycling "hubs" for PIN proteins.

Transcriptomic analysis of *vamp714* mutants showed a reduced expression of a broad range of cell wall related genes. Consistently, cell wall composition is altered in those mutants. Confocal images showed that VAMP714 is required for the proper localization of cellulose synthase machinery. Also, the consequences of the changes in cell wall on PIN mobility at the plasma membrane were investigated.

VAMP714 belongs to a family of four members, the VAMP71s. Unlike other VAMP71s that are tonoplast residents, VAMP714 mainly localizes to the Golgi. Comparisons of VAMP71 domains showed that only a small number of residues are responsible for the subcellular localization of VAMP714. In tissues and organs, different yet partly overlapping VAMP71 expression patterns were observed. The function of VAMP71s were also investigated and together with expression results indicate some level of sub-functionalization of VAMP71s.

Acknowledgments

First of all, I would like to thank Keith. I did not really realize the importance of the supervisor before I started this PhD. The levels of trust and support I got from you are unmatched. Your kindness always made me feel welcome, and over the years Durham has become a second home. What you have done for me is much more than "just" giving me the opportunity to pursue a PhD in your lab and I am delighted to know that my journey here has not come to an end.

How can one thank Keith without thanking Jen? Thanks for your help, your advice is always precious. It is also a pleasure to have a quick chat in the corridors with you.

I would like to thank the BBSRD, NLD DTP and Durham University for the funding and support of my studies.

My experience in the lab would have been very different without Pat and Johan. Pat, the material and the advices you gave me were so valuable and helped me greatly. As importantly, your positivity, your kindness and your motivation when it comes to research have been very inspiring. Johan, many times having a chat about my experimental work with you was very useful. You also make the life in the lab more entertaining. Dank u!

I would like to thank Xiaoyan for her help when I started the project and her availability when I had questions about her transgenic lines.

Cian, my labmate who became a housemate and a friend, we have been through so many crazy, funny and unforgettable moments. I had the privilege to witness you running a marathon under 3 hours. The dedication and the passion you put in your training are impressive and motivate me to give it all to reach my goals. More importantly, the way you welcomed me and made me feel comfortable in our lab is something I am really grateful for. Thanks buddy!

The lab members I have met over the years are all people I am very fond of and would deserve more than a quick "thank you" but I am afraid it would take pages to thank you all individually. Helen, Rodrigo, Fahad, Amy, May, George, Archie, Michael, Jialei, Mokhles, Qing, Wafa thank you for making my daily life in the lab so enjoyable.

We have also had visiting students and academics during my time in the lab who came from different continents and shared their culture and their delicious food with us. Thanks Jana, Arran, Tanzeela, Sumera, Maria, Alice, Haozhan, Eugenie and Alexandre. It was a pleasure having you in the lab.

Thanks to our neighbours from Lab1003 for their help when I was running low on enzymes or plants for dipping. Shraboni, Yuqi, Ashish, Joanna, Joey, Miguel, Uli, Merci, Ola, Dario thank you!

Thank you to the staff members for making research in the department possible. Thanks Tim and Joanne from the bioimaging facilities for your help and expertise. Thanks, Gavin, Caroline, Lucy, Gail and Abigail from the purchasing office. Thanks genomics facility team Bekky, Vicky, Gillian and the proteomics facility team Rachael and Adrian. Also thank you Emma and Avril from the support staff.

Thanks Stephen Fry from the University of Edinburgh for your help with the cell wall analyses and also to thank you to Chris and Marie from your group. I have heard you retired recently and wish you a happy retirement.

Thank you Weinbin for your help with the whole genome sequencing analysis.

Thank you also to my thesis committee member over the years, Tony, Marc, Heather, Peter. Your help and guidance were much appreciated.

Thanks Bekkie and Ollie for your friendship and your invitations to "come ‘round for tea". Thank you to my housemate Matt, I always enjoy our chats in the kitchen and I had never seen someone so passionate about rugby before I met you. I am looking forward to the next 6 nations. Many thanks to the friendly faces in the department often too polite to remind me that I talk too much.

Finally, a huge thank you to my family for their constant support. Thanks for sharing my interest or at least my enthusiasm for my research project.

To all of you thanks again for making my PhD such a great experience!

List of Abbreviations

½ MS	Half strength Murashige & Skoog media
ABA	Abscisic acid
AIR	Alcohol insoluble residues
Arabidopsis	*Arabidopsis thaliana*
AREs	Auxin responsive elemeny
ARF	Auxin responsive factor
BFA	Brefeldin A
BiFC	Bimolecular fluorescence complementation
BR	Brassinosteroids
CESA	Cellulose synthase
CSC	Cellulose synthase complex
Col-0	Columbia
CTL1	Choline transporter-like1
dH_2O	Distilled water
DN	Dominant negative
E. Coli	*Escherichia coli*
EDZ	Elongation and differentiation zone
EE	Early endosome
LE	Late endosome
ER	Endoplasmic reticulum
FRAP	Fluorescence recovery after photobleaching
FRET-FLIM microscopy	Förster resonance energy transfer and fluorescence lifetime imaging
GA	Gibberellic acid
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GUS	β-glucuronidase
IAA	Indole-3-acetic acid

IP-MS	Immunoprecipitation-Mass Spectrometry
LD	Longin domain
N. benthamina	*Nicotiana benthamiana*
PDs	Plasmodesmata
PM	Plasma membrane
PIN	PIN-FORMED
PPI	Protein-Protein Interaction
QC	Quiescent centre
SNARE	soluble N-ethylmaleimidesensitive factor attachment protein receptor
RAM	Root apical meristem
RFP	Red fluorescent protein
SYP	Syntaxin of Plants
T-DNA	transfer DNA
TLC	Thin layer chromatography
TGN	trans-Golgi network
TMD	transmembrane domain
TZ	Transition zone
VAMP	Vesicle-associated membrane protein
YFP	Yellow fluorescent protein
WT	Wild type

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Chapter 1: Introduction

It can be overwhelming to think that enzymatic reactions happening in an animal cell or, at a more macroscopic level every movement that animals make, are powered by energy that was once converted from solar energy into chemical energy (contained in energy rich biomolecules) by a photosynthetic organism. Therefore, plants have a central role in providing food for the world population. The finite land surface for growing crop plants and the rapid growth of the human population, that reached 8 billion people in November 2022 (https://www.un.org/en/global-issues/population#:~:text=Our%20growing%20population,and%202%20billion%20since%201998), raise the question of food safety and security, especially with predictions of increasing soil salinity levels due to global warming and over-irrigation (Hassani 2021, Azapagic 2021). The United Nations website estimates that already 1.5 billion people are living in areas where the soil is too salty to be fertile.

In the United Kingdom the thematic challenge of food safety and security has motivated the funding of research in the field of bioscience. UK research and innovation through the Biotechnology and Biological Sciences Research Council (BBSRC) are planning to spend £376 million in research in the next 5 years and a part of this funding will be used to address global challenges including food security and safety but also sustainable agriculture and farming (https://www.ukri.org/news/bbsrc-funds-new-research-at-leading-bioscience-institutes/) .

Fundamental research helps understanding how plants grow and develop and how they interact with their environment. Genetic and molecular studies of flowering plants are commonly carried out in the model organism *Arabidopsis thaliana*. The genome of this species has been sequenced and annotated and the development from the first fertilized cell to the mature plants is well characterized. Also, the short life cycle of this plant makes it easier to study the progeny of genetically modified or mutant lines.

The research activity conducted in our laboratory focuses on understanding the hormonal regulation of root development in stress and non-stress (if it exists!) situations. This allowed the identification of a regulator of the vesicle trafficking of PIN auxin transporters, VAMP714 which is the subject of this study.

1.1 Root formation during embryogenesis

Roots are the organ that allows structural support for the aerial part of the plant but also the uptake of water and nutrients necessary for plant growth (Petricka, Winter, and Benfey 2012; Overvoorde, Fukaki, and Beeckman 2010). Root formation initiates during embryogenesis (Scheres, Benfey, and Dolan 2002). After fertilization, the egg cell (now zygote) undergoes a carefully controlled set of horizontal and vertical divisions changing progressively the shape of the embryo and ending in the formation of a seedling-shaped embryo (Seefried et al. 2014) (Fig 1.1). The first division of the zygote is asymmetric and generates cells with a different cellular content that also divide following different patterns (Yi Zhang, Xu, and Dong 2023). Most of the organs originate from the apical cells but roots originate from both the apical (majority of the root) and the basal cell (root apical meristem)(Scheres, Benfey, and Dolan 2002).

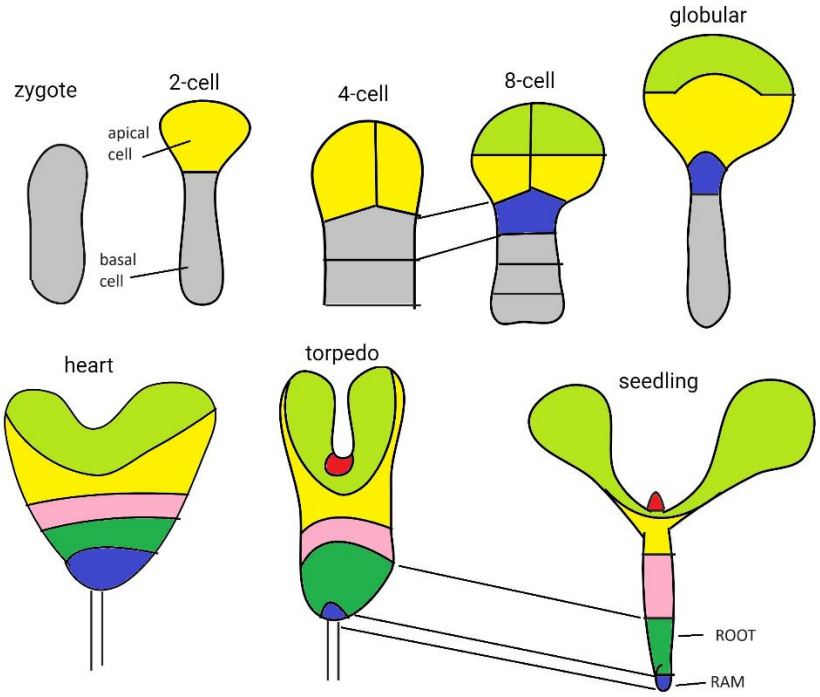


Fig. 1.1 Root formation during embryognesis. The colour code indicates the embryonic origin of plant organs. The first division of the zygote produces the apical (yellow) and the basal cell (grey) that have distinct shapes and cytoplasmic contents. The former generates most of the tissues present in seedlings through cell division and regional differentiation. Tissues that give rise to leaf (green) and root apical meristem (RAM, blue) appear at the 8-cell stage. Later, at the heart stage, embryonal parts that generates the central domain (yellow), the

hypocotyl (pink) and root (brown) are established. The shoot apical meristem is formed at the torpedo stage. Figure created with biorender.com and inspired by the figure from Kim, Dhar, and Lim (2017).

1.2 Plants are sessile organisms

Unlike animals, plants have a sessile lifestyle. As a consequence, they have acquired strategies for accessing nutrients, defending themselves and develop in ways that are very different from that observed in the animal kingdom. Among these strategies, plants have the ability to grow and develop thorough their life offering them a great level of plasticity (Cao and Li 2010; Scintu et al. 2023). This indeterminate growth is allowed by a group of stem cells that are maintained during post embryonic life, the meristems (Jürgens 2003). In the root, the meristem responsible for primary root growth (along the vertical axis) is called the root apical meristem (RAM) because it is located at the tip of the root (Scheres, Benfey, and Dolan 2002). The stem cell niche of the RAM is organized around a rarely dividing group of cells, the quiescent centre, that maintains the surrounding initials cells in their undifferentiated state (van den Berg et al. 1997) (Fig. 1.2). The initials generate the different cell types of the root by dividing along the vertical axis forming a file of cells originating from a same initial cell (Desvoyes, Echevarría, and Gutierrez 2021).

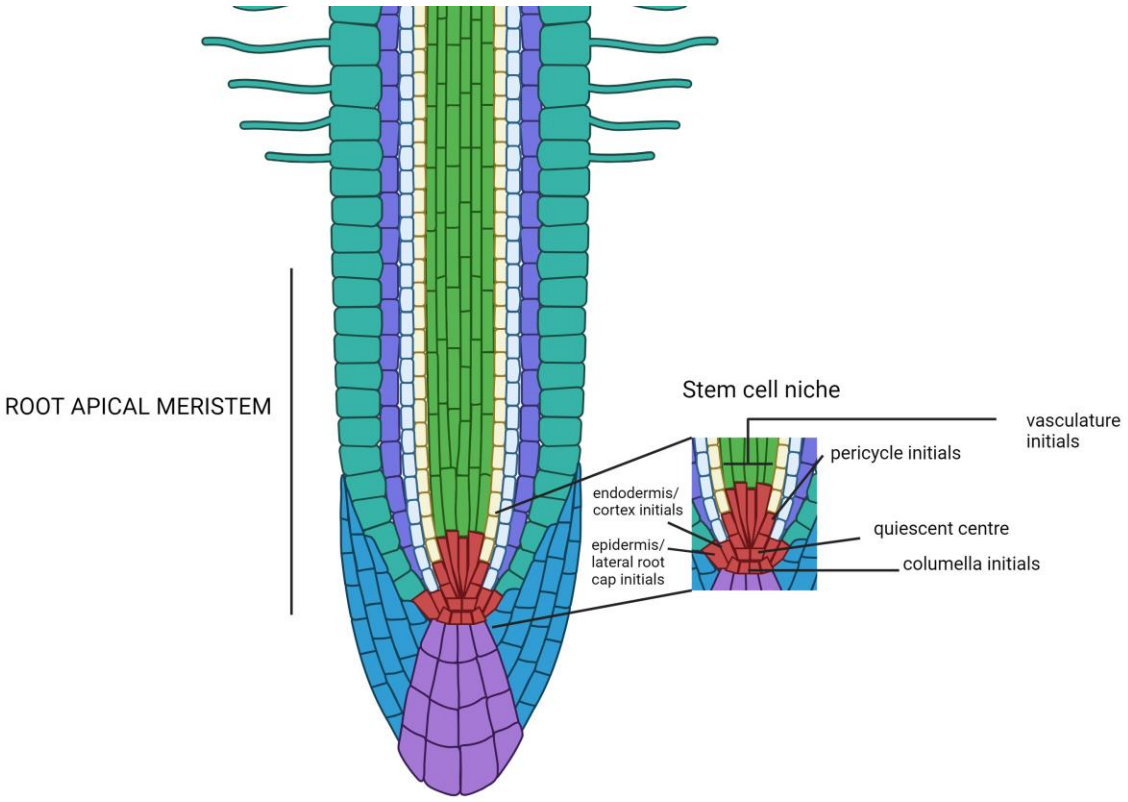


Fig. 1.2 Organisation of the Stem cell niche of the root apical meristem. Figure created with biorender.com.

Although meristems are responsible for providing cells for root growth, the other process that allows plant to grow, cell elongation, happens higher up in the root in the elongation zone (Beemster and Baskin 1998). Plants have also developed the ability to grow towards positive stimuli or away from negative stimuli. This ability is named tropism. Interestingly, the same stimulus can be positive for one organ and negative for another. For example, shoots grow towards the light whereas roots grow away from the light consistent with the functions of these organs (Silva-Navas et al. 2016).

1.3 Phytohormonal crosstalk during root development

Multicellular organisms like *Arabidopsis* require coordinated cellular activities. In plants this is achieved by chemical messengers occurring at low concentration, the phytohormones (Williams 2011). Roots are permanently exposed to signals from their surrounding environment that are perceived and integrated for an optimal response of the plant (Benková 2016). A substantial body of evidence indicates that hormones act as internal mediators in the interactions between plants and their environment (Benková 2016). The principal hormones are all involved with regulating root growth to a greater or lesser extent but not always in the

same tissues (Ubeda-Tomás, Beemster, and Bennett 2012). They influence the main elements of root growth, cell elongation and cell division. The nature of the crosstalk between plant hormones varies depending on which hormones are interacting. For example, auxin and cytokinin (CK) can have antagonistic effects on cell division and differentiation (Zhang et al. 2013) whereas ethylene and auxin have synergistic inhibitory effect on cell elongation (Alarcón, Lloret, and Salguero 2014); although an auxin-independent pathway for ethylene has also been identified (Rowe et al. 2016). Stress conditions also affect hormonal crosstalk. Indeed, in plants subjected to osmotic stress ABA inhibits auxin transport and overrides the effect of ethylene which can rescue the expression of PIN1 auxin transporters (Rowe et al. 2016). This suggests that hormonal crosstalk in root fine-tunes a response that integrates environmental signals and the internal developmental program.

Auxin is omnipresent in root development processes (Garay-Arroyo et al. 2012) and the other hormones almost always regulate root growth through the control of auxin transport and homeostasis. Gibberellin (Ubeda-Tomás et al. 2008) positively regulate RAM activity by promoting auxin transport while cytokinin negatively regulate RAM activity by repressing auxin biosynthesis and transport (Raines et al. 2016). In the elongation zone most hormones inhibit cell elongation excepted gibberellins (GAs), which promote elongation of cells through inhibition of the activity of DELLA proteins (Ubeda-Tomás et al. 2008). The transition zone between the RAM and the elongation zone is a hotspot for hormonal crosstalk (Kong et al. 2018; Li et al. 2020). This zone coincides with an auxin minimum and a cytokinin maximum. Cytokinin inhibits auxin via SHY2-mediated inhibition of auxin transport and promotion of auxin conjugation through ARR1. Brassinosteroids also act on SHY2 in the transition zone (Fig. 1.3). Repression of auxin response progressively drives the meristematic cells toward differentiation.

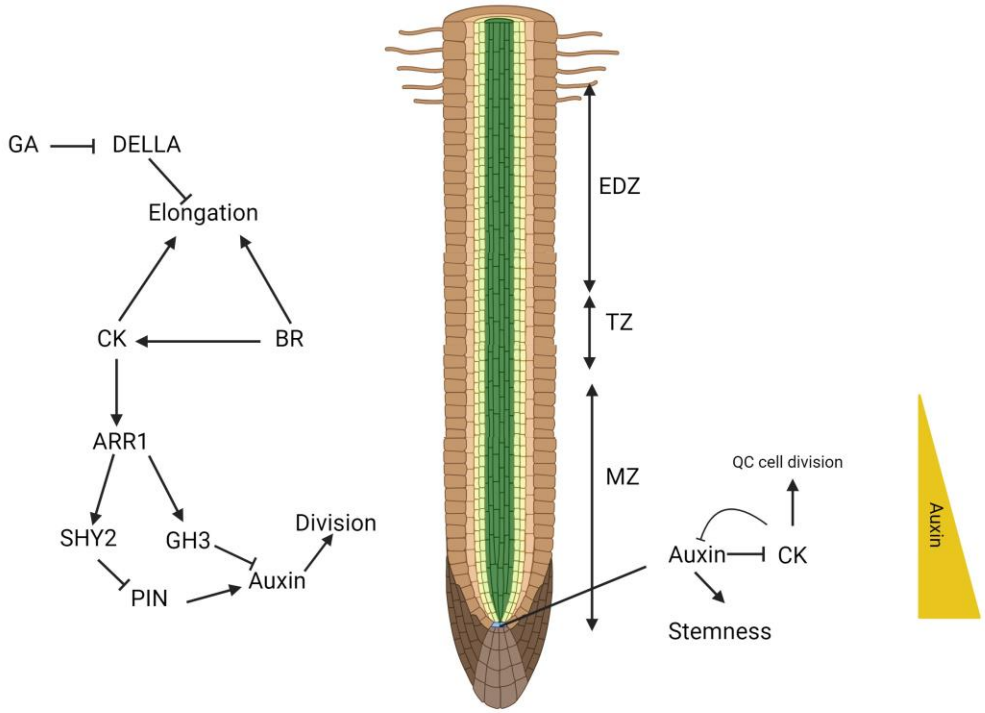


Fig. 1.3 Hormonal crosstalk in the root. The transition zone is a hotspot for hormonal crosstalk in *Arabidopsis* roots and coincide with an auxin minimum where cytokinin together with brassinosteroids promote cell differentiation and inhibit auxin regulated cell division. Cytokinin signaling protein ARR1 promotes auxin conjugation through GH3 therefore reducing levels of active IAA and inhibits PIN-mediated auxin transport through SHY2, a negative regulator in auxin signaling from the AUX/IAA family (see section 1.5). MZ meristematic zone, TZ transition zone, EDZ elongation and differentiation zone. Flathead= inhibition, Arrowhead= promotion. The triangles represent the auxin gradient. This figure was created with biorender.com and inspired by (Kong et al. 2018).

1.4 Effects of auxin on root growth and development

Indole-3-acetic acid (IAA) is the most abundant naturally occurring form of auxin (Guo et al. 2019; Bartel 1997). IAA is a small molecule composed of a flat hydrophobic ring, the indole group and a carboxyl group. IAA has a central role in the development of the root where it is involved in all the processes required for growth and architecture. A key component of the root organization is the distribution of auxin along the root longitudinal and radial axes, commonly referred as auxin gradients. Auxin gradients are established in the embryo where

they contribute to the formation of the apical-basal axis (Seefried et al. 2014). In the root of seedlings, the auxin maximum in the root tip spatially restricts the expression of *PLETHORA* (*PLT*) (Blilou et al. 2005) and *WUSHEL RELATED HOMEOBOX 5* (*WOX5*) (Burkart et al. 2022) transcription factor genes, keeping the QC and the surrounding stem cells undifferentiated. It has been shown that in turn PLT stabilises the auxin maximum by promoting auxin transport toward the root tip (Blilou et al. 2005). Auxin is also involved in the division of meristematic cells. Not all stem cells are dividing at the same rate. The QC divides infrequently in comparison to the initial cells. This is explained by the auxin promoting cell division of initial cells whereas WOX5 and BRAVO repress cell division while promoting cell stemness in the QC (Betegón-Putze et al. 2021). The progressive decrease in auxin concentrations moving upwards from the root apex defines the transition zone when auxin reaches a minimum where cytokinin and brassinosteroids initiate the elongation and differentiation processes (Kong et al. 2018).

Auxin flows also have an essential role in the gravitropic response. When placed horizontally roots bends toward gravity. Amyloplasts in the columella, also called statoliths, act as the gravity sensor. Indeed, starchless amyloplasts, which are less dense, showed an altered response to gravity (Su et al. 2017). When the root is placed vertically, the statoliths precipitate to the lower side of cell. This triggers a relocalization of PIN3 and PIN7 auxin efflux transporters to that side of the cell (Kleine-Vehn et al. 2010; Su et al. 2017) and therefore allows a downward flow of auxin at the root tip. Subsequently, an asymmetry in the auxin flow appears with more auxin building up at the lower side of root and less auxin at the higher side. This asymmetry of auxin levels is translated into an asymmetry of cell growth. High auxin levels are thought to inhibit root growth rapidly through alkalinization of the cell walls. Ultimately, the differential growth generates the bending of the root tip, so allowing the root to grow toward gravity.

Gravitropism is generated via changes in auxin flows but those changes do not modify the root zonation and organization. Therefore, zonation cannot be a direct reading of auxin gradients. An elegant study showed that the kinetics of zonation formation and gravitropism are different (Salvi et al. 2020). Stem cell niche maintenance requires the restriction of PLT to the root apex and that then slowly migrates upwards. The slowly established PLT gradient controls the zonation and is not sensitive to short time changes in auxin flow. On the other hand, root growth changes rapidly. This explains how these two processes, although regulated by the same molecule, can be separated.

Finally, auxin is essential for lateral root formation. If multiple phytohormones regulate this process it has been shown that local accumulation of auxin in the pericycle is necessary and sufficient for specification of founder cells. Indeed, interesting experiments show that auxin production and accumulation in single pericycle, activated through a Cre-Lox system, are sufficient to induce founder cell identity (Dubrovsky et al. 2008). Additionally, auxin regulates lateral root patterning and emergence (Lavenus et al. 2013).

1.5 Auxin signalling

The major signalling machinery of auxin signalling converts changes in auxin levels to changes in gene transcription (Leyser 2018). The core system is well described and consists of an auxin-dependent degradation of transcription repressors (Lavy and Estelle 2016). When auxin levels are low, the auxin responsive factors (ARFs) that bind to promoters of auxin-regulated genes are locked by a dimer of AUXIN/INDOLE 3-ACETIC ACID (Aux/IAA) repressor proteins. Aux/IAAs recruit the corepressor TOPLESS (TPL) that stabilizes the repressed state through chromatin modification. When auxin levels increase, auxin binds to the TRANSPORT INHIBITOR REPONSE1 (TIR1)/AUXIN SIGNALING F-BOX PROTEIN (AFB) receptors and Aux/IAAs which act as coreceptors. TIR1/AFB are part of SCF-type E3 ubiquitin protein ligase E3 complex that ubiquitinates Aux/IAAs leading to their degradation by the proteasome (Leyser 2018) (Fig. 1.4). This process releases the ARFs and allows the expression of auxin responsive genes. This process is rapid, whereby changes in gene expression have been measured between 3 and 5 minutes after auxin treatment (McClure et al. 1989). Interestingly, some of these genes are Aux/IAAs indicating the existence of a negative feedback loop. There are 23 known ARFs in *Arabidopsis* (Li et al. 2016) and 29 Aux/IAAs (Luo, Zhou, and Zhang 2018). There is evidence that ARFs have differential affinities for the Aux/IAAs (Cancé et al. 2022; Luo, Zhou, and Zhang 2018). Additionally, variation in activity of different combinations of ARF dimers and competition between activator and repressor ARFs for the same promoter are possible (Leyser 2018) and would contribute to the complexity of the system and might offer an explanation for the difference in auxin response between cells.

Responses that are independent of the TIR1/AFB system have been identified. They involve different auxin receptors and have specific responses. Also, auxin regulation of very fast processes such as changes in cytosolic Ca^{2+} suggest the existence of a non-transcriptional (Leyser 2018; Monshausen et al. 2011) auxin response.

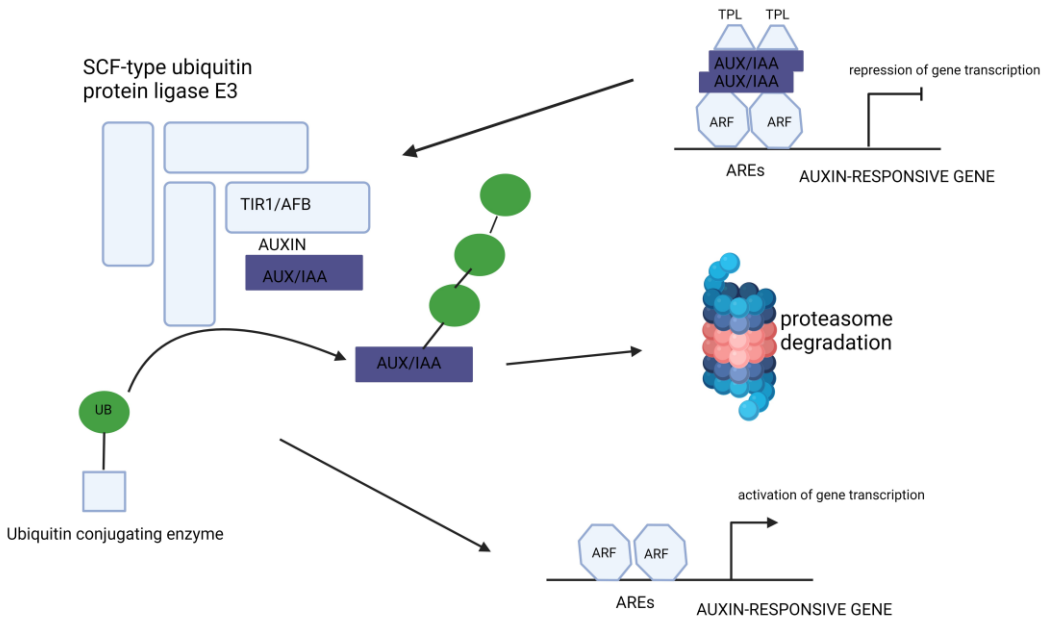


Fig. 1.4 Auxin signalling machinery. Auxin binding to TIR1/AFB and AUX/IAA induces the proteasome mediated degradation of AUX/IAA. Flat head inhibition, Arrow head promotion. ARES are the motif recognized by ARF within the promoter of auxin-responsive gene. Figure created with biorender.com and inspired by (Leyser 2018).

1.6 Auxin transport

1.6.1 Auxin flows in the root

Auxin flows in plant organs have been well described and reviewed (Křeček et al. 2009; Leyser 2006; Michniewicz, Brewer, and Friml 2007; Kepinski and Leyser 2005). Source organs like the root tip or the young leaves are the main contributors that feed these flows (Ljung et al. 2005; Overvoorde, Fukaki, and Beeckman 2010). A bulk transport of auxin from leaves to the root tip through the phloem has been proposed. This transport is rapid, 7 cm/h, in comparison to the cell-to-cell transport in other cells such as in the meristem (10 mm/h) (Overvoorde, Fukaki, and Beeckman 2010). However, these two types of transport are not mutually exclusive and could perfectly co-exist. The cell-to-to cell transport is better described and gives the orientation to the auxin flows. A family of auxin transporters, the PIN-FORMED (PIN) proteins, are responsible for the efflux of auxin from cells and play a crucial role in this polar auxin transport (PAT) (Fig. 1.5) (Blilou et al. 2005). In roots, auxin transport towards the tip in the stele is mediated by PIN1. Auxin is transported to the QC by

PIN4 (Friml et al. 2002) and redirected laterally by PIN3 and PIN7 (Peer et al. 2011). The role of PIN3 and PIN7 was elegantly investigated by activating auxin biosynthesis in the QC with a *WOX5* promoter and monitoring auxin accumulation after induction with a precursor (Blilou et al. 2005). In the epidermis, PIN2 is responsible for the shootward basipetal transport of auxin (Blilou et al. 2005). Reflux from the epidermis to the stele is mediated by auxin transporters (Fig. 1.5) (Peer et al. 2011) and auxin transport through plasmodesmata (Mellor et al. 2020).

Interestingly, the described auxin transport system is able to self-repair when the root is wounded (Leyser 2006). After tissue repairing, PAT reconnects the interrupted flows via what is commonly referred to as auxin canalization. In this process auxin increases, through positive feedback loops, its own polar transport creating narrow channels for an auxin flow. A similar canalization process has been described for vascular development in the Arabidopsis leaf (Scarpella, 2004; Scarpella et al. 2006).

The recent emergence of a next generation auxin biosensor is an exciting news for the study of auxin transport (Herud-Sikimić et al. 2021). Unlike former auxin biosensors for measuring the auxin response, this new system allows the direct auxin binding which ultimately leads to a measurable fluorescence resonance energy transfer (FRET) signal (Herud-Sikimić et al. 2021). More interestingly, auxin binding to this biosensor is reversible allowing in vivo visualization of auxin spatio-temporal distribution of auxin at single cell resolution. Purely in terms of auxin transport, this technology would permit to observe how different auxin transporters affect auxin accumulation in specific cell types by using mutants for PIN proteins for example. Therefore, this is a step forward in tackling the current challenge of real-time monitoring of auxin transport. However, current versions auxin biosensors are targeted either to the nucleus or the ER. Since cell-to-cell auxin transport requires auxin efflux to the apoplast, it would be interesting to develop an apoplastic auxin biosensors to visualize polar auxin transport (Are auxin levels higher at the basal cell wall in the stele in comparison to the apical sside? Or the other way around in the epidermis?)

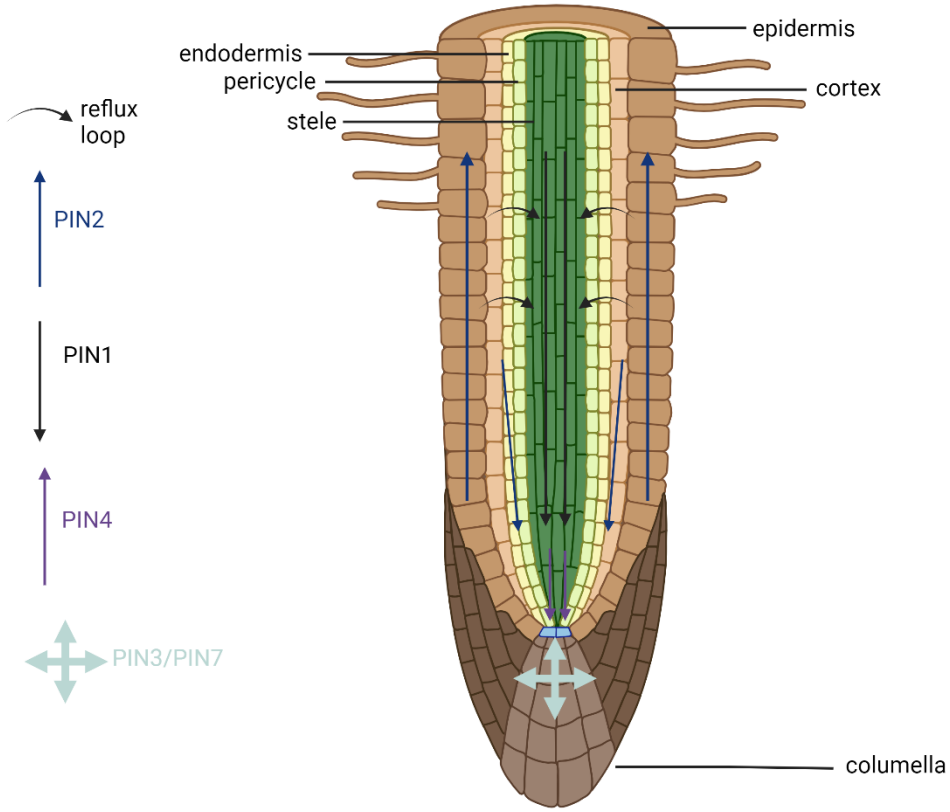


Fig. 1.5 PIN-mediated Auxin flows in the root. Auxin movement is sometimes referred to as "reverse fountain" (Swarup and Bennett 2003). The different PIN-mediated auxin transport plays role crucial in that pattern. PIN1 proteins that preferentially localize at the basal side in stele cells proteins are responsible for auxin transport towards the root tip. PIN4 proteins have a more restricted distribution in the stele and cortex around the stem cell niche and allow auxin flux in the QC. On the other hand, PIN3 and PIN7 mediate the efflux from the QC. Auxin is then redirect shootward by apically distributed PIN2 in the cortex. Reflux loop is the term describing the auxin diffusion from the outer layers of the root to the inner layers thereby connecting the shootward flow to the rootward auxin flow. Figure created with biorender.com and inspired by (Blilou et al. 2005)

1.6.2 Auxin transporters

Auxin influx into the cell and efflux from the cytosol to the extracellular space or to organelles is ensured by different kinds of auxin carriers. To date, 4 families of auxin transporters have been identified: PIN, PIN-LIKE (PLS), AUX/LAX, and ABCB (Balzan, Johal, and Carraro 2014). Recently, auxin transporters that derive from proteins that originally transport other substrates have also been reported (Geisler 2021). The only family that is exclusively dedicated to the influx of auxin is the AUX/LAX transporters. On the other

hand, PIN and PILS are efflux carriers and only mediate the efflux of auxin. ABCB are thought to mainly export auxin, but an import activity has also been observed (M. Geisler et al. 2017).

The auxin transport systems are also energetically distinct. ABCB transporters are pumps relying on the hydrolysis of ATP while AUX/LAX, PIN and PLS are passive transporters (Zwiewka et al. 2019). However, AUX/LAX are proton/auxin symporters unlike PIN and PLS, which are gradient-driven uniporter carriers (Zažímalová et al. 2010).

Currently, more than 30 auxin transporters have been identified in *Arabidopsis*, twice as many as the number known when the interesting review "Auxin transporters, why so many?"(Zažímalová et al. 2010) was published. Recently, Geisler in his retrospective addressed that question and hypothesized that it could be part of a general strategy for the adaptation to sessile life style (Geisler 2021). The initial question is interesting and the proposed answer plausible, but why so many? The answer to that question depends on what kind of transporters they are compared to. The number of other hormone transporters is smaller (Léran et al. 2020) while cation transporters belong to large families (Mäser et al. 2001; Grabov 2007). Aquaporins are another well characterized group of transporters and in the genome of Arabidopsis 38 genes with homology to aquaporins have been reported (Quigley et al. 2002). Interestingly, transport of water facilitated by aquaporins is, unlike auxin transport, not specific to plants, allowing comparisons with non-sessile organisms. There are 13 aquaporins in *Homo sapiens* (Azad et al. 2021) which is much smaller than the number of these proteins in plants. However, it would be overly simplifying to conclude that it is only because of the non-sessile life style of humans. Indeed, the mechanism of water transport at the whole organism scale are different in plant and animal kingdoms (McElrone, 2013). Both animal and plants have long-distance transport system for water which does not require aquaporins. However, the cell-to-cell water transport facilitated by aquaporins occurs on longer distances in plants than in animals (Ye, Holbrook et al. 2008) and across different cell types (Heinen, Ye, and Chaumont 2009) suggesting a higher need for the regulation of aquaporin-mediated water transport. Another factor that could account for the higher abundance of aquaporins in plants is the requirement for water fluxes across the tonoplast and the PM. In animals cells, aquaporins only localize at the PM but in plants cells a whole subfamily of aquaporins are tonoplast-resident proteins (TIPs) and coexist with the PM located aquaporins (PIPs) (Maurel et al. 2015).

Among the known transporters of auxin, PINs are by far the most studied and the best characterized. There are 8 PIN proteins in *Arabidopsis* divided in two subgroups - the canonical and the non-canonical PIN. The canonical group has 5 plasma membrane- (PM-) resident members characterized by a long hydrophilic loop; and the 3 ER resident non-canonical PINs with a short loop (Křeček et al. 2009). The particularity of PINs is the polar localization at the PM of some the family members in specific tissues (Kleine-Vehn et al. 2009).

The mechanism by which PINs transport auxin remained poorly understood for a couple of decades after their identification in the late 1990s (Gälweiler et al. 1998). The structure of PIN proteins was mostly known through topology studies. PINs consist in 2 repeats of 5 transmembrane domains (TMDs) connected by a hydrophilic loop (Křeček et al. 2009). The difference in the loop size between canonical and non-canonical suggests that it could be involved in the sorting of PIN to their target membrane. Indeed, swapping experiments have shown that hydrophilic loop is a determinant of the correct subcellular localization but not the only one since TMDs also affect membrane targeting (Zhang et al. 2020).

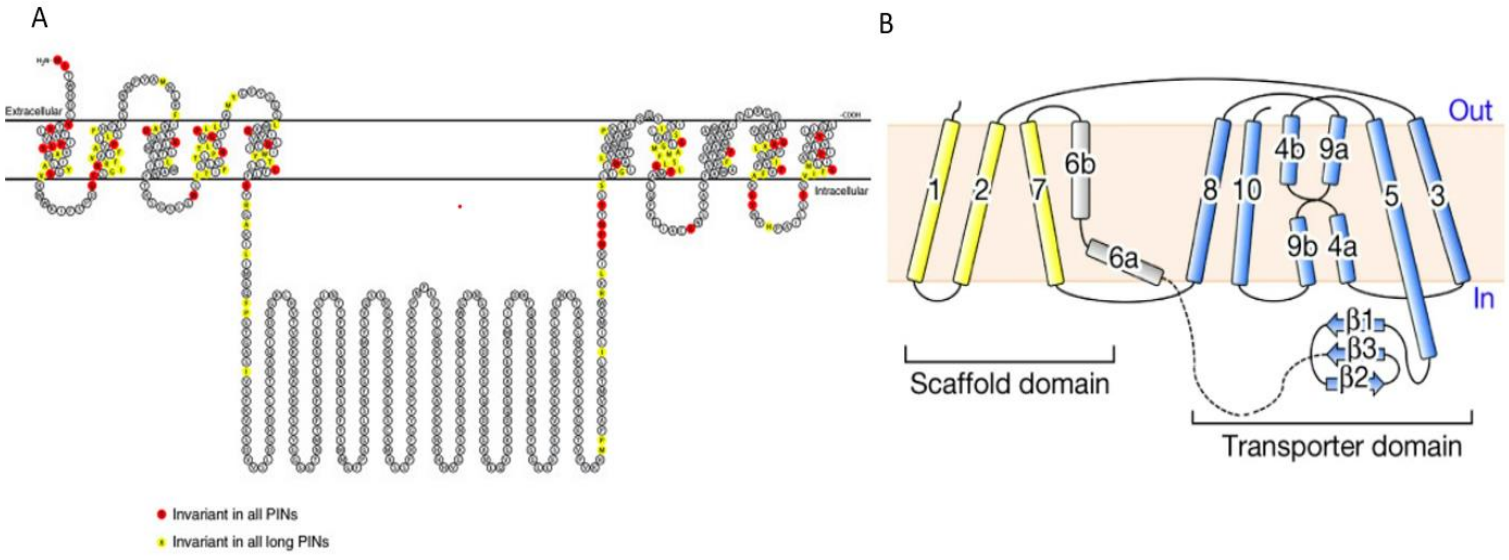


Fig. 1.6 Comparison of PIN structure from topological and Cryo-EM studies. Former model of auxin structure (A) taken from (Křeček et al. 2009), current model of PIN structure taken from (Yang et al. 2022).

Recent advances in cryo-EM have allowed long-awaited insights in the structure and mechanism of PIN proteins (Ung et al. 2022; Yang et al. 2022). Both canonical and non-canonical PIN structures are available and both have a scaffolding and a binding domain (Ung et al. 2022; Yang et al. 2022) (Fig. 1.6). The proposed mechanism for PIN-mediated auxin efflux is the so-called elevator mechanism (Fig.1.7). In the inward open conformation when IAA is not bound to PIN proteins, the transporters are facing the cytosol. Upon binding of IAA, the conformation of PIN changes, inducing the rotation of the protein to the outward open conformation which allows IAA release at the non-cytosolic side. Then, the empty IAA binding site leads to the return to the inward open conformation. The described mechanism is characteristic of acid/sodium symporters and Na/H^+ antiporter that have the same fold as PINs (Ung et al. 2022; Joshi and Napier 2023). A support site that is present in symporters and antiporters for the binding of ions and a site that could mediate proton translocation are also present in PIN proteins but different assays showed no effect of sodium ions or protons on PIN activity, and PIN proteins are currently thought to be uniporters (Ung et al. 2022).

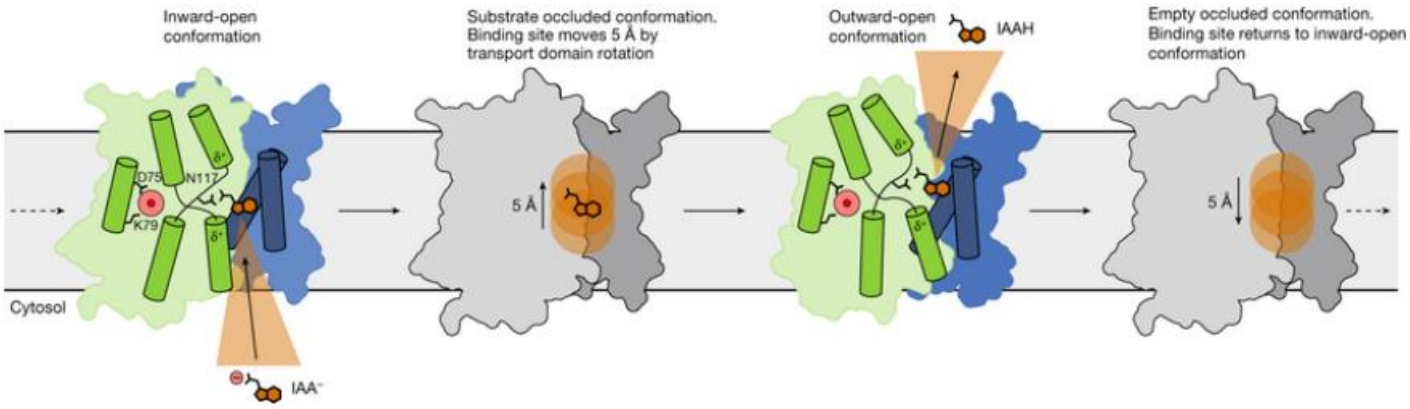


Fig. 1.7 Proposed mechanism of PIN-mediated auxin transport on the basis of the protein structure.
Figure taken from (Ung et al. 2022)

1.6.3 Chemiosmotic hypothesis

The chemiosmotic hypothesis was proposed in the 1970s (Raven 1975) but still is widely accepted as a general explanation of the cell-to-cell transport of auxin. IAA is a weak acid due to its carboxyl group (pKa = 4.75), therefore it is present in its protonated IAAH and deprotonated form IAA^-. The apoplast has a pH of approximately 5.5 (Fig. 1.8), and the protonated form is able to simply diffuse across the membrane because it is a small neutral protein. The influx of the charged deprotonated form is facilitated by influx carriers AUX/LAX. Inside the cell, the pH is neutral (pH = 7) and IAA is almost entirely in its deprotonated version (Fig. 1.8). Because of the charge, auxin is trapped in the cell and relies on transporters for its export. In tissues where PINs have a polar distribution, auxin can then mainly get out of cell at one side, so giving direction to the auxin flow. Therefore, the chemiosmotic hypothesis and PIN localization account for the directional auxin streams (Michniewicz, Brewer, and Friml 2007).

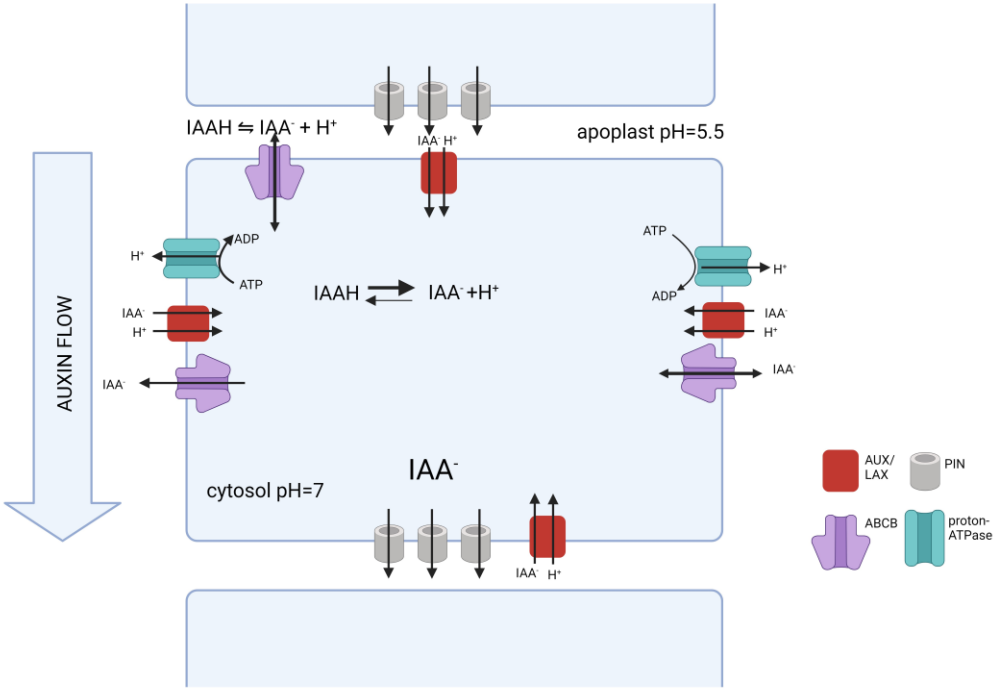


Fig. 1.8 Chemiosmotic hypothesis. Figure created with biorender.

1.6.4 Symplastic auxin transport

Auxin is a small molecule that is able to diffuse through pores that connect the cytosol of adjacent cells, the plasmodesmata (PDs). What makes symplastic transport of auxin particularly interesting is that it has theoretically all the elements for a directional auxin transport that does not directly require the activity of auxin transporters. Indeed, not only can auxin flow from one cell to the neighbouring cells but also a gating system exists to open and close PDs with callose plugs. Therefore, closure of PDs at specific sides of the cell would result in a directional flow of auxin. Recent studies combining experimental and mathematical modelling indicate that it is not a fictional scenario. Genetic evidence comes from the study of *gsl8*, a loss of function mutant of *GLUCAN SYNTHASE LIKE 8* which regulates callose synthesis (Han et al. 2014). In hypocotyl phototropism, callose deposition is more important at the shaded side, allowing the accumulation of auxin. Disruption of callose deposition through the inhibition of GSL8 activity prevents the formation of an auxin gradient in the hypocotyl and no bending can be observed (Han et al. 2014). In leaves, deposition of callose at PDs connecting cells in the transverse direction was higher than in cells with PDs connecting cells in the longitudinal direction (Mellor et al. 2020). This creates a quicker auxin flow in the longitudinal direction in vascular tissues connecting the root tip to the petiole, and was shown to play a role in shade avoidance (Mellor et al. 2020).

Interestingly, the ability of auxin to promote and reinforce is own transport by acting on its own transporter polarity and activity has also been reported for symplastic transport of auxin, although it could be tissue-specific (Band 2021). Another similarity with the carrier-mediated auxin transport is the difference in the abundance of plasmodesmata in some tissues resulting in differential auxin conductivity. Models have predicted that this differential abundance of PDs is important for the auxin distribution at the apex of the root (Band 2021).

It has been proposed that auxin transporters would only account for auxin sink formation at PM subdomains for symplastic transport which would change the paradigm of cell-to-cell auxin transport (Geisler 2021) since it was previously thought that auxin was transported from the cells to the apoplasm, through PIN proteins and ABCB transporters, and then from the apoplasm to neighbouring cells via AUX/LAX transporters. Nonetheless, both carrier-mediated and symplatic transport systems are believed to be interdependent rather than independent and add another level of plasticity in the control of auxin gradients (Band 2021; Mellor et al. 2020).

1.7 Cellular basis of PIN populations at the PM

Biological membranes separate cellular compartments with different properties (Sallese, Pulvirenti, and Luini 2006). These properties are constantly adjusted by transporters located in the membranes of the different organelles or at the PM (in other words, in the endomembrane system) (Sallese, Pulvirenti, and Luini 2006). This process is essential for the homeostasis of the cell. Different factors regulate a population of transporters at their target membrane (Fig. 1.9). Abundance of transporters can be regulated via de novo synthesis of the proteins, the recycling from an internal pool of proteins to the plasma membrane (Geldner et al. 2001), the endocytosis of PM located transporters inside the cell (Kitakura et al. 2011) and through protein degradation (Kleine-Vehn et al. 2008). Modification of the activity of transporters is another type of regulation (Bassukas, Xiao, and Schwechheimer 2022). Finally, lipid composition of membrane domains and cell walls have been reported to maintain transporters within a specific region of the membrane (Feraru et al. 2011; Kleine-Vehn et al. 2011). Interestingly, some biological processes like tropism or cell elongation seem to favour a type of regulation over the others possibly because of the kinetics of these different processes. In the case of the auxin transporter PINs, another aspect needs to be regulated, the protein polar distribution at the PM.

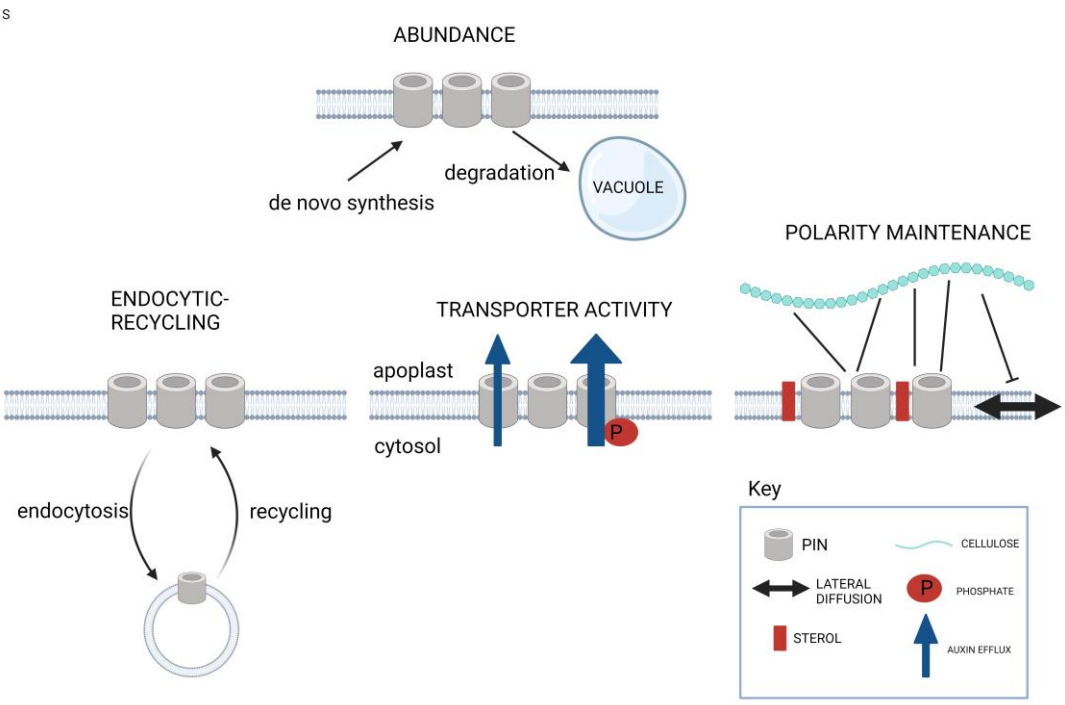


Fig. 1.9 Processes regulating PIN populations at the PM. Figure created with biorender.com

1.7.1 Vesicle trafficking of PIN proteins

It is widely accepted that membrane proteins are transported to the different compartments of the endomembrane system in small membrane enclosed vesicles. Membrane proteins are synthesised at the ER. From the ER most proteins follow a default route, the secretory pathway, in the anterograde direction (Bassham et al. 2008) - first to the Golgi and then to the PM. From the PM/extracellular environment proteins are recruited by another default route, the endocytic pathway, and transported in the retrograde direction to endosomes and to the vacuole (Bassham et al. 2008). In addition to the default routes, specialised pathways from the ER exist for chloroplast, mitochondrion and peroxisome sorting (Bassham et al. 2008; Agaoua et al. 2021).

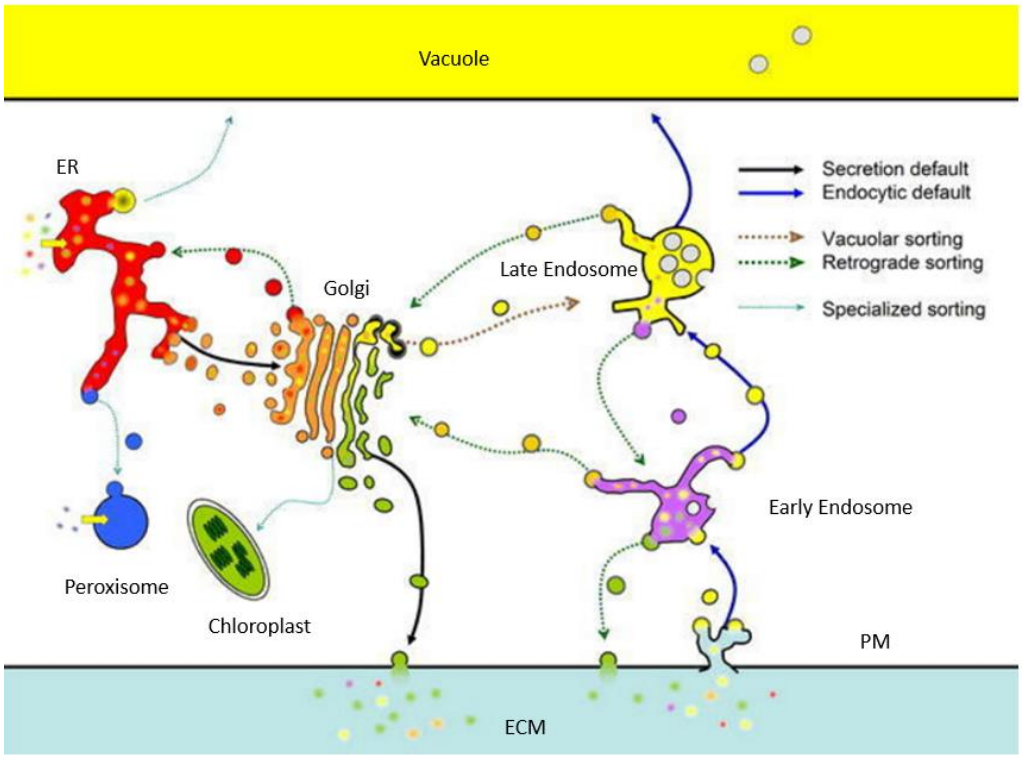


Fig. 1.10 Pathways of the vesicle trafficking in Arabidopsis. By default, newly synthesised at the ER are transported to the plasma membrane or the extra cellular matrix (ECM) (secretion, black arrows) whereas endocytosed protein at the PM are transported to the vacuole (endocytosis blue arrows). Alternative routes coexist with those default pathways. Retrograde sorting acts as a reversed secretion pathway (dashed green arrors). Vacuolar proteins that do not follow the endocytic pathway are sorted to the vacuole from the Golgi (via late endosomes) through the vacuolar sorting (dashed brown arrows). Specialized sorting pathway allow transport from the ER to various organelles (dashed cyan arrows). Figure taken from (Bassham et al. 2008)

Vesicle budding at the site of a donor membrane is due to a category of proteins called the coat-GTPases (Singh et al. 2018). Coat-GTPases recruit proteins transported in the vesicles, usually referred to as cargo proteins or cargos, and an element of the vesicle cage involved in the vesicle formation, the coat. Coat-GTPases are only active in their GTP-bound conformation. This activation is ensured by GTPase EXCHANGE FACTORS (GEF) and inactivation is mediated by GTPase Activation Protein (GAP) (Nielsen 2020; Vernoud et al. 2003). Vesicle transport to the target membrane occurs in association with the cytoskeleton (Kotchoni et al. 2009). Vesicles are then tethered at their target membrane by tethering factors, docking factors and Rab-GTPases (Bassham et al. 2008). Like coat GTPase the activity of Rab-GTPases is regulated by GEFs and GAPs. Rab-GTPases recruit the tethering and docking factor and also the soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNARE) proteins for vesicle fusion. Vesicle fusion is the last event of vesicle trafficking and involves the formation of a tetrameric SNARE complex consisting in 3 Q-SNAREs at the target membrane (also called t-SNAREs) and a vesicle associated R-SNARE (v-SNARE).

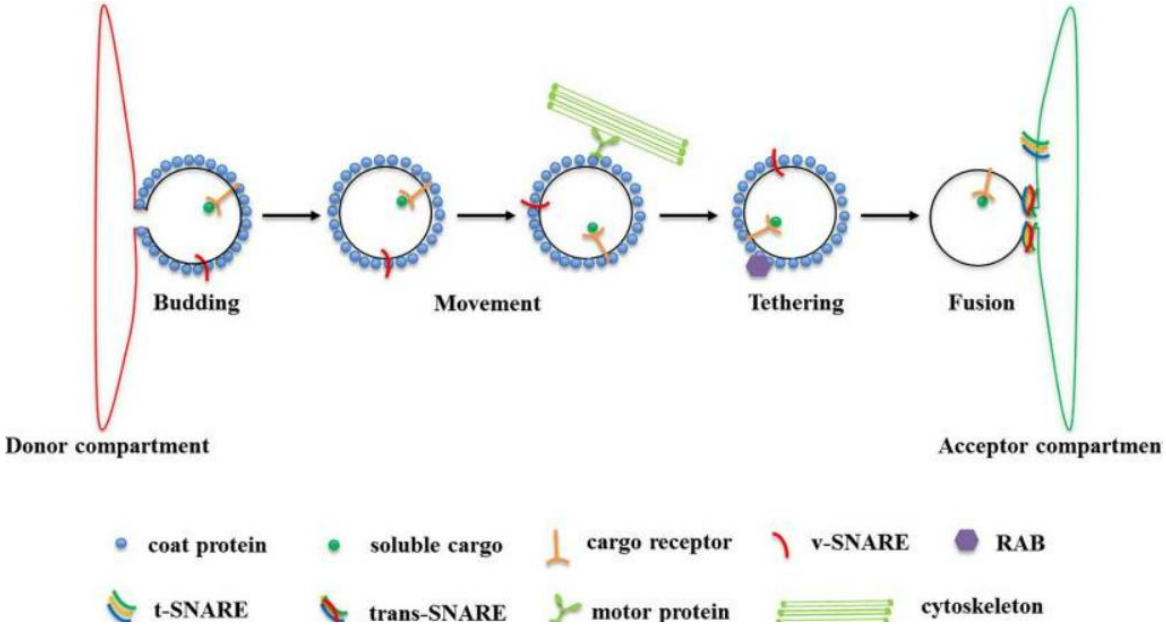


Fig. 1.11 Events of the vesicle trafficking. Vesicle formation or "budding" at a donor compartment is mediated by coat proteins that are also involved in cargo recruitment. Vesicles en route to their target membrane are moving along actin filaments. This is followed by the tethering of the vesicle to the acceptor compartment where the fusion of the uncoated vesicle mediated by SNARE proteins occurs. Figure taken from (Gu et al. 2020).

Early stages of PIN trafficking are not yet fully described but GNOM-LIKE was reported to control basal polarity of PIN1 independently of the endocytic recycling pathway, and together with GNOM through the regulation of the early trafficking (Doyle et al. 2015), possibly the ER-to-Golgi transport, of PIN transporters. Post-Golgi trafficking of PINs on the contrary has been the subject of numerous studies. The use of brefeldin A (BFA), an inhibitor of protein recycling, has been crucial in the study of PIN trafficking. In cells expressing PIN1, BFA treatment induces an intracellular accumulation of PIN proteins that is rapidly reversible following BFA withdrawal (Geldner et al. 2001). Also, inhibition of protein synthesis does not change the intracellular accumulation of PIN indicating that it is caused by protein cycling between different compartments of the cell rather than cytoplasmic accumulation of newly synthesised proteins. This cycling involves the cytoskeleton, since treatment with cytochalasin D (cytD) and Latrunculin B (latB) actin polymerization inhibitors prevents the relocalization of PIN1 at the PM after BFA wash out. Use of BFA allowed the identification of GNOM, a coat-GTPase GEF from the ARF-GEF family, which is a molecular target of BFA. GNOM is now known to be essential for the basal localization of PIN proteins. Several regulators of PIN cycling have been identified and unsurprisingly they are mostly components of the vesicle trafficking machinery. Endocytosis of PIN is inhibited when clathrin, the core compenent of the endocytic coat machinery is impaired. Recycling of PIN from Trans Golgi Network (TGN)/Early Endosomes (EE) to the PM is ensured by GNOM, ARF-GEFs, (Geldner et al. 2003a), tethering factors, the exocyst (Drdová et al. 2013) , Rab-GTPAses, BEX5 (Feraru et al. 2012), Rab-GEFs, VAN4 (Naramoto, Nodzyłski, et al. 2014) and SNAREs, VAMP714 (Gu et al. 2021).

To date, only two molecular determinants of PIN polarity *per se* have been identified: GNOM and PINOID which are respectively responsible for basal and apical localization of PINs (Richter et al. 2010; Kleine-Vehn et al. 2009). Disruption of the activity of these proteins causes the transcytosis of PINs from their WT localization to another side of the cell. The molecular mechanism for the "decision making" of PIN polarity remains elusive. It has been proposed that phosphorylation by PID would act as molecular switch for the recruitment by an ARF-GEF independent of GNOM (still unidentified) mediating the apical trafficking (Kleine-Vehn et al. 2009) while GNOM regulates the basal targeting of non-phosphorylated PIN.

1.7.2 Regulation of PIN activity

Identification of mutants with impaired PAT but no effect on PIN polarization led to the development of auxin efflux measurement assays (Zourelidou et al. 2014a). Such assays are mainly conducted in *Xenopus laevis* oocytes. Interestingly, when PIN1 is expressed alone the auxin efflux measured is similar to the background level (Zourelidou et al. 2014b). However, when PIN1 was co-expressed with the kinase D6PK, an efflux of auxin was measurable, indicating that D6PK is an activator of PIN proteins. The confirmation that the regulation of PIN by D6PK is due to its kinase activity came with use of a dead variant of the protein that is unable to promote auxin efflux (Zourelidou et al. 2014b). DP6K kinase activity was shown to happen at the basal side of the PM (Zourelidou et al. 2014b). Phosphorylation sites of D6PK and other kinases regulating PIN activity, including PID which is also essential for PIN polarity, have been identified and are located in the hydrophilic loop of PIN proteins (Bassukas, Xiao, and Schwechheimer 2022). Interestingly, phospholipids modulate PIN activity in a process involving PDK1 and D6PK, suggesting a potential role of lipidic environment for regulating PIN activity (Tan et al. 2020).

Since phosphorylation of PIN is key for regulation of PIN-mediated efflux it would be interesting to investigate the effect of other post-translational modifications using the already existing assays of PIN activity.

1.7.3 PIN clustering and polarity maintenance

PIN efflux carriers are mainly observed in the centre of polar domains where they have a severely reduced lateral mobility. Within their domain PINs localize in non-mobile clusters with a diameter of 100-200 nm (Kleine-Vehn et al. 2011). Sterols are thought to contribute to clustering of PIN and pharmacological disruption of sterols reduces PIN polar distribution (Kleine-Vehn et al. 2011). Clustering of PINs in specific locations characterized by a specific lipid composition within the PM could therefore be a way to maintain the polarity of PIN proteins.

An elegant screening system to find genes that restore the gravitropic response in PIN2:PIN1-HA transgenic lines allowed the identification of CELLULOSE SYNTHASE 3 (CESA3) as a regulator of PIN polarity (Feraru et al. 2011). Chemical inhibition of cellulose and protoplasting were shown to have a dramatic effect on PIN polar distribution. Fluorescence

recovery after photobleaching experiments showed that this can be explained by the role of cellulose in reducing PIN lateral mobility (Feraru et al. 2011; Martinière et al. 2012a).

PIN maintenance in their polar domain is essential for their polar localization, as models have shown that polar exocytosis alone cannot account for PIN polarity (Kleine-Vehn et al. 2011).

1.7.4 PIN degradation

Protein turnover is required for cellular integrity and is regulated by protein biosynthesis and degradation (Rodriguez-Furlan, Minina, and Hicks 2019). Importance of protein degradation in the clearance of malfunctioning PIN is intuitive but it is conceptually more difficult to understand why this type of regulation would be selected over protein relocalization or modulation of PIN activity that has a supposedly lower energy cost. The decision to recycle or degrade proteins is poorly understood but it seems that environmental conditions may help in choosing between regulation mechanisms (Wawrzyńska and Sirko 2020). Indeed, during the gravitropic response of root, epidermal cells of the upper and lower side of the root have different levels of PIN2 in their vacuoles resulting in differences in PIN2 degradation (Kleine-Vehn et al. 2008; Baster et al. 2013). Vacuolar accumulation of PIN2 is higher at the upper side, increasing their degradation, which reduces the intensity of the auxin flow at that side that causes the root bending. Interestingly VPS29 retromer subunit is able rescue PIN2 of vacuolar targeting in multi vesicular bodies (MVB) and allow the auxin efflux carriers to cycle back to the PM (Kleine-Vehn et al. 2008).

1.8 VAMP714, PIN polarity regulation by SNAREs

Among the proteins regulating PIN vesicle trafficking, VAMP714 was identified in a genetic screen for root growth regulators using activation tagging in our laboratory (Gu et al. 2021) (Fig.1. 12 A). The enhancer cassette inserted in the genome between two coding regions in a dwarf mutant that was isolated. The gene overexpressed codes for a SNARE, a protein that mediates the fusion of vesicle at a target membrane.

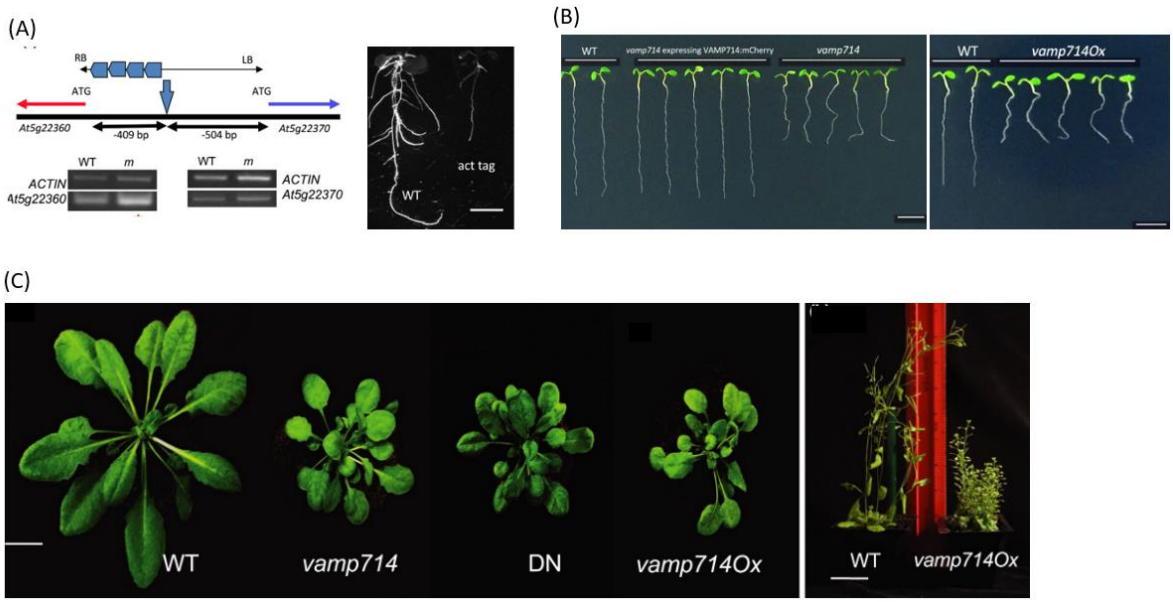


Fig. 1.12 Identification of VAMP714, a regulator of root growth. (A) Activation tagging screening allowed the identification of a mutant for the *VAMP714* gene; (B) the knockout mutant displays short roots which can be rescued by the expression of VAMP714:mCherry under the control of its native promoter. An overexpressor line *vamp714Ox* displays a similar phenotype. Aerial parts of independent VAMP714 missexpressors also differ phenotypically from WT plants. They are characterized by abnormally shaped leaves (C left) and the mature plants are dwarf (C right). DN dominant negative, *vamp714Ox* VAMP714 overexpressor. Figure taken from (Gu et al. 2021)

Different mutants misexpressing VAMP714 show multiple auxin-related phenotypes and are rescued by complementation with VAMP714 (Fig. 1.12 B and C). Establishment of auxin gradients in the root is disrupted in these mutants suggesting altered PAT or auxin biosynthesis (Gu et al. 2021). Due to the predicted protein function, the former option was investigated. In lines misexpressing VAMP714, PIN proteins fail to localize in their polar domain at the PM (Fig. 1.13 B). VAMP714 was also observed in the same vesicle moving toward the PM (Fig.1.13 A). Together, these results indicate that VAMP714 is essential for PIN polar localization likely by mediating the fusion of PIN-containing vesicles at the PM. PIN proteins cycle between intracellular compartments to the PM, suggesting that VAMP714 is also involved in PIN recycling.

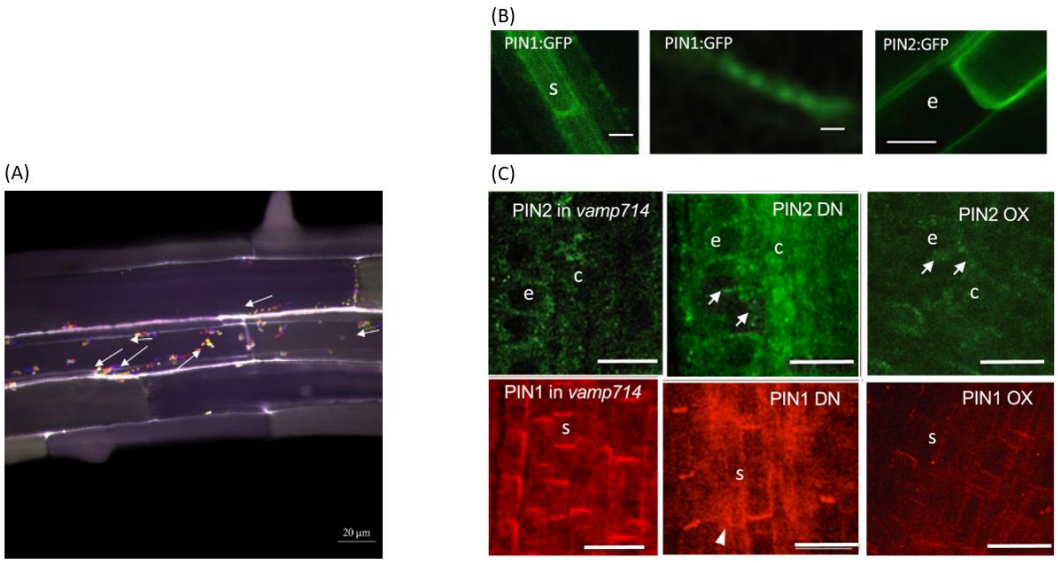


Fig. 1.13 VAMP714 is essential for PIN polar localization. (A) VAMP714 vesicle (white arrows) moving toward the PM; (B) Disruption of VAMP714 activity causes the mislocalization of PIN1 at the basal side in the stele (s) and PIN2 at the apical side in the epidermis (e). (C) VAMP714 DN dominant negative, VAMP714 OX overexpressor; arrow heads show impaired polar localization. Figure taken from Gu et al. (2021).

Auxin transport has been the subject of a substantial number of publications, some of which are truly fascinating and inspiring, and involve a large number of parameters, one of them being VAMP714. As importantly if not more so, efforts have been made to connect these parameters to build a complete picture of auxin transport. This picture obviously still has gaps but the emergence of new tools allowing the visualization of auxin transport (Herud-Sikimić et al. 2021) are likely to help filling them, at least partly. Although not used in this study, modelling is an unmatched tool to predict the contribution of individual parameters to a global system especially when combined with experimental data.

Project aims

The present study aimed to contribute to the elaboration of a bigger picture of auxin transport regulation mediated by VAMP714 by i) connecting the different regulators of PIN trafficking; ii) investigating the link between PIN trafficking and PIN polarity maintenance; and iii) studying the similarities and differences within a family of auxin induced SNAREs, the VAMP71s. The approach has been to identify putative VAMP714 protein interactors, investigate the link between VAMP714, PINs and cell wall composition, and investigate the functions of related VAMP71 family members.

Chapter 2: Materials and methods

2.1 Materials

2.1.1 Chemicals

The chemicals used were obtained from Merck (Gillingham, UK), Fisher Scientific Ltd (Loughborough) or Melford laboratories Ltd (Ipswich, UK) unless otherwise stated.

2.1.2 Plant material

Arabidopsis thaliana wild type (WT) seeds of the Columbia ecotype (Col-0) were obtained from lab stocks. All the mutants and reporter lines are in Col-0 background. *Nicotiana benthamiana* plants were obtained from departmental stocks (Durham University, UK).

2.1.2.1 Reporter lines

The *ProVAMP711:GUS*, *ProVAMP712:GUS* and *35S:CTL1:GFP* reporter lines were generated through promoter and gene cloning and transformation of *Arabidopsis* as described in sections 2.3 and 2.4. *ProVAMP714:VAMP714:GFP*, *ProVAMP714:VAMP714:mCherry* and *ProPIN2:PIN2:GFP* were obtained from Dr. Xiaoyan Gu (Durham University). *ProCESA3:GFP:CESA3* courtesy of Dr. Samantha Vernhettes (Institut Jean-Pierre Bourgin, Versailles, France). *ProPIN1:PIN1:GFP* and *ProPIN2:PIN2:GFP* lines were obtained from lab stocks but were originally given to us respectively by Eva Benkova (Benkova, 2003) and Ben Scheres (Wageningen University, the Netherlands).

2.1.2.2 Mutant lines

T-DNA insertion lines *vamp712* (GABI_054H03), *vamp713* (SALK_032706), *vamp714* (SALK_005917 and GABI_844B05) and *ctl1* (SALK_065853C) were all ordered from the Nottingham Arabidopsis Stock Centre (NASC, https://arabidopsis.info/).

2.1.2.3 Progeny of genetic crosses

Progeny of crossing: *vamp714* x *ProCESA3:GFP:CESA3*, *vamp714* x 35S:CTL1:GFP, *ctl1* x *ProVAMP714:VAMP714:mCherry* and *vamp712* x *vamp713*.

2.1.3 Genotyping of T-DNA insertion lines

Before usage, T-DNA insertion lines were genotyped in order to determine whether or not the plants were homozygous for the mutation. The process of genotyping consists in running in parallel two PCR reactions with two different sets of primers using genomic DNA as a template and then running the samples on an agarose gel. One set of primers contains two gene specific primers flanking the T-DNA insertion site referred as LP and RP and the other set of primers contains a T-DNA specific primer referred as border primer and the RP (Fig. 2.1A). Provided that the PCR reactions work, WT plants that do not carry a T-DNA should only display the LP+RP band, homozygous plants would only display the border+RP band, and the heterozygous plants would display the 2 bands (Fig. 2.1B).

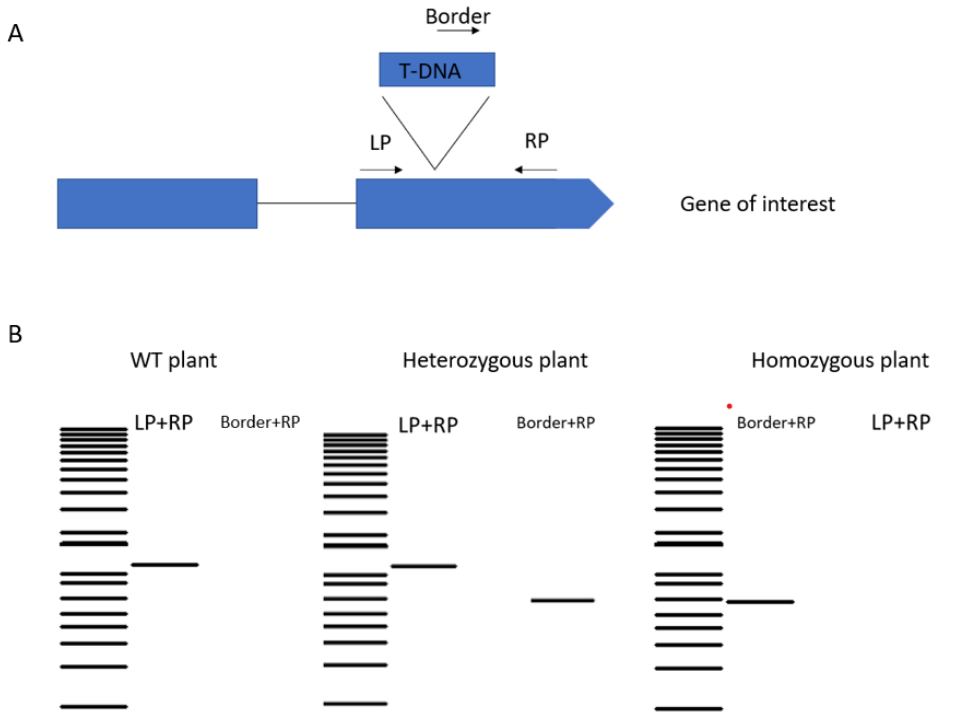


Fig. 2.1 Genotyping procedure. T-DNA insertion site and primer design (A). The three possible outcomes of the genotyping as displayed on an agarose gel when a set of primers containing the T-DNA specific primer (Border+ RP) and a set of primers containing only gene specific primers (LP+RP) flanking the T-DNA are used (B).

2.1.4 Genetic crosses

Unopened floral buds were opened and the flower emasculated by gently removing the immature anthers using fine forceps and keeping the pistil. Floral buds and flowers around the emasculated buds were removed to avoid undesired crossing. This was done by eye but the work was double-checked under a Zeiss STEMI SV8 dissecting stereomicroscope (Carl Zeiss, Cambridge, UK). Then, mature anthers from a pollen donor plant were gently brushed on the sigma of emasculated plants. Crossed flowers were labelled with tape and placed back in a growth chamber or a greenhouse. Mature siliques were collected before senescence.

2.2 Plant tissue culture

2.2.1 Seed sterilization

In order to grow plants in sterile conditions, seeds were incubated in a solution of 5% sodium hypochlorite with 0.02% Triton X-100 for 15 minutes. The seeds were then washed several times with sterile deionised water in a laminar flow hood.

2.2.2 Culture media

Plants were grown on half strength Murashige and Skoog medium (½ MS) (Murashige & Skoog, 1962). 2.2g of MS medium (Sigma) was weighed and solubilized in 1 L water and then the solution was brought to a pH of 5.7 with 1M KOH, a concentration of 10g/L of agar was used to make up solid ½ MS media.

2.2.2.1 Treatment with chemicals

Plant treated with choline chlorite were grown for 7 days after germination (D.A.G) on solid ½ MS and transferred to a Petri dish containing liquid ½ MS with 1 mM choline chlorite. Control plants were transferred to a Petri dish containing only liquid ½ MS. The plants were then immediately used for microscopy.

The plant treated with the cellulose inhibitor isoxaben were germinated on ½ MS with 2 to 12 pM isoxaben.

2.2.3 Plant growth conditions

Prior to their exposure to growth conditions, seeds were stratified at 4°C for 3 days to obtain synchronized germination. All the seedlings were grown in 10x10 cm² square Petri dishes (Sarstedt, Numbrecht Germany) each containing 50 ml of ½ MS agar and sealed with Micropore™ tape. Plates with stratified seeds were transferred to a growth cabinet under long day conditions, 8h of darkness and 16h of light at a temperature of 22°C and light intensity of 3000 lux. In order to avoid differences in light exposure plates were kept in the same part of the growth cabinet.

For growth on soil, 14 day-old plants were transferred from Petri dishes to pots filled with F2S Seed & Modular and Sand Compost (ICL professional horticulture) consisting mostly of Sphagnum moss peat and grown in long day conditions at 22°C in a growth room or in a greenhouse.

2.3 Cloning

2.3.1 Total RNA extraction

In order to isolate RNA from *Arabidopsis* 20 mg of 7 days old seedlings were flash frozen and ground into a fine powder. Then the RNA was extracted using the ReliaPrep™ tissue miniprep system (Promega) and following the provider's instructions. Briefly, ground tissues were transferred to a microcentrifuge tube and sheared in 500 µl of lysis buffer by pipetting the solution up and down 10 times trough a P200 pipette tip and then centrifuged at 14000 x *g* for 3 minutes. The supernatant was then transferred to a new microcentrifuge tube and mixed with 170 µl of Isopropanol by vortexing for 5 sec. Then, the solution was transferred to a

column and centrifuged 1 minute at 14000 x *g* so that the RNA could bind the membrane. The membrane was washed with 500 µl of a wash solution and left to incubate in a solution containing DNaseI for 15 minutes. Then, the membrane was washed once with a column wash solution and twice with an RNA wash solution. Finally, the RNA was eluted using 30 µl of RNase free water and stored at -80°C.

The purity and the amount of the RNA were measured using a NANODROP1000 spectrophotometer (ThermoFisher Scientific, Hemel Hempstead, UK).

2.3.2 cDNA synthesis

Typically, 400 ng total RNA was used as template for the reverse transcription into cDNA. The reaction was completed using UltraScript™ cDNA Synthesis Kit and primed with Oligo(dT). Reagents were mixed in a PCR tube as follows

Reagent	**20 µl reaction**
5x cDNA synthesis mix	**4 µl**
20x Ultrascript™ RT	**1 µl (200 units)**
Total RNA	**400 ng**
ddH20	Up to 20 µl final volume

The tubes were then incubated 10 minutes at 42°C and 10 minutes at 85°C in a thermocycler. The samples are placed on ice after incubation and used for PCR or kept at -20°C.

2.3.3 Polymerase chain reaction (PCR)

The cDNA was used as a template to amplify the coding sequence of the genes of interest. The widely used Gateway® (ThermoFisher Scientific, Hemel Hempstead, UK) cloning system was chosen to carry out gene cloning. The system is based on recombination reactions that bacteriophage λ uses to insert or excise DNA sequences in and out of the *Escherichia coli* (*E. coli*) genome (Fig. 2.2).

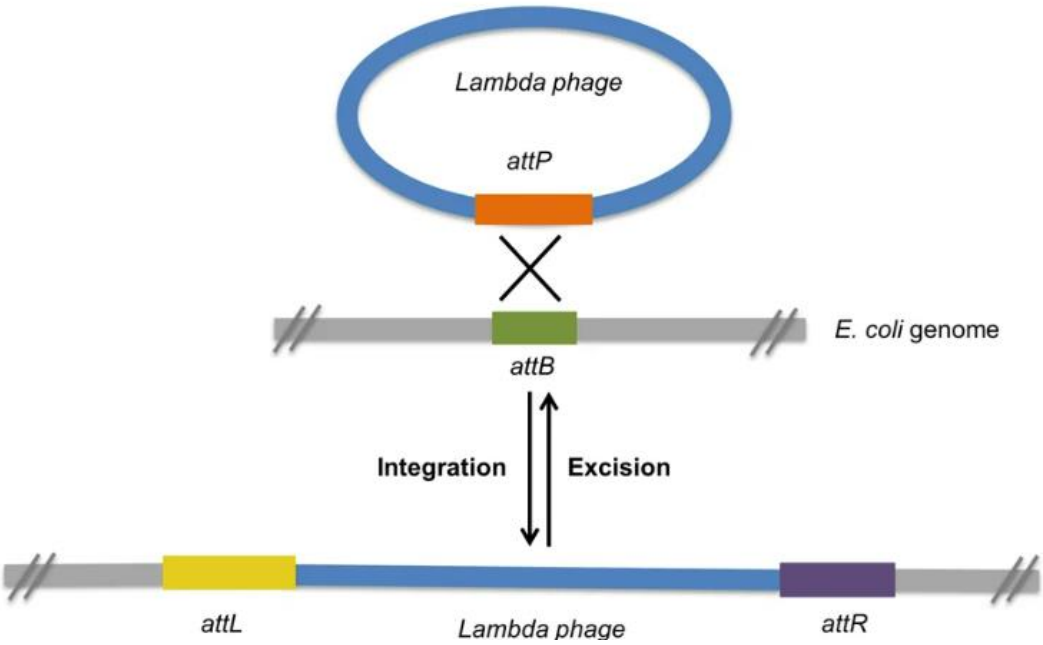


Fig. 2.2 Gateway cloning. Representation of the bacteriophage λ DNA recombination system involving *att* sites. Illustration taken from Addgene website. https://blog.addgene.org/plasmids-101-gateway-cloning

To be able to use the Gateway system the sequences of interest need to be amplified using primers carrying attB sites. Primers were designed to amplify specifically the coding sequence but the stop codon was omitted for C-terminal fusion with a fluorescent protein tag and were synthesised by IDT (https://eu.idtdna.com/pages, Leuven Belgium). A Tm calculator tool was used to make sure the difference in melting temperature was no bigger than 5°C. A table containing the cloning primers can be found in Appendix I. The reactions were conducted with VerFi and HiFi Polymerases (PCRBIOSYSTEMS, London, UK) and using the following mixes

HiFi

Reagents	**50 µl reaction**
5X PCRBIO reaction buffer	**10 µl**
Forward primer (10 µM)	**2µl**
Reverse primer (10 µM)	**2 µl**
HiFi polymerase (2 units/µl)	**0.5 µl**
cDNA template	**2 µl**
ddH_2O	**33.5 µl**

VeriFI

Reagents	50 µl reaction
Forward primer (10 µM)	**2 µl**
Reverse primer (10 µM)	**2 µl**
cDNA template	**2 µl**
2X VeriFI mix	**25 µl**
ddH_2O	**19 µl**

PCR tubes containing the mix were then spun briefly and incubated in a thermocycler in the following conditions

Cycles	Temperature	Time	Note
1	**95°C**	1 min	Initial denaturing
35	**95°C**	15 sec	Denaturing
	55°C	15 sec	Annealing
	72°C	1 min/kb	Extension
1	**72°C**	5 min	Final extension
1	**4°C**	Infinite hold	Refrigerate

2.3.4 Gel electrophoresis

The PCR samples were run using gel electrophoresis to separate DNA fragments according to their length. The gels were made up of 1% (w/v) agarose dissolved in 1x TAE buffer (dilution of 50X TAE stock, 242g TRIS-BASE, 100 ml of 0.5M EDTA (pH8) and 57.1 ml of glacial acetic acid in a final volume of 1L topped up with dH_2O). Ethidium bromide was added to the gel at a concentration of 1 ug/ml to allow visualization of DNA on a gel doc. The length of the PCR fragments was determined by comparison with a generuler 1 kb DNA ladder or a generuler 100 bp DNA ladder (Thermofisher scientific). Samples were loaded on the gel after mixing with tritrack loading dye (Thermofisher scientific) and run in an electrophoresis tank at 120 V for 20 minutes. Finally, gels were visualized on a Syngene InGenius LHR Gel Doc and GeneSnap software.

2.3.5 PCR product purification

The PCR products were purified to remove the primers and the polymerase. If no undesired band was on the gel in addition to the band of interest, PCR fragments were purified using the Monarch® PCR & DNA CleanUP kit (NEB). When non-specific bands were detected alongside the band of interest the latter was purified from the gel using the GeneJET Gel Extraction Kit (Thermofisher scientific).

Purified DNA was then quantified using a NANODRP1000 spectrophotometer.

2.3.6 BP Reaction

AttB flanked PCR products were cloned in the donor vector pDONR207 or PDONR™/Zeo using BP clonase II enzyme mix (Fig. 2.3). Vector map and sequences can be found in Appendix II. The components of the reaction were mixed as follows and incubated overnight at 25°C.

Reagents	Volume
PCR product	**3 µl**
pdonr207/pDONR/Zeo (150ng/µl)	**1 µl**
BP clonase II enzyme mix	**1 µl**

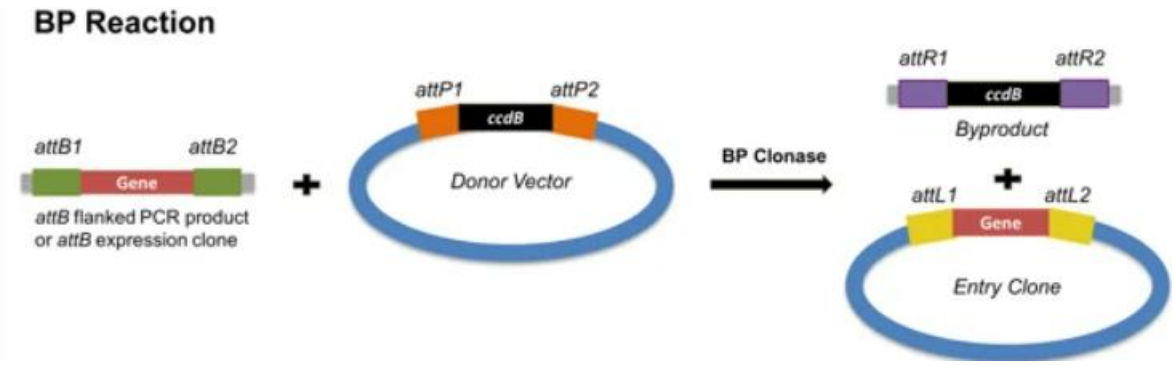


Fig. 2.3 BP reaction. Visual representation of the BP reaction. AttB flanked genes are cloned in the region flanked by attP sites by the BP clonase enzymes. The clone obtained is referred as Entry clone. Image taken from Addgene website. https://blog.addgene.org/plasmids-101-gateway-cloning

2.3.7 Transformation of *E. coli*

The entire volume of the BP reaction was mixed with 50 µl of *DH5α* competent cells in a microcentrifuge tube and incubated on ice for 30 minutes. The mixture was then incubated at 42°C for 45 sec. The bacteria were subsequently placed back on ice for two minutes. A volume of 950 µl of LB medium (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L Sodium Chloride) (Bertani, 1951) was added in sterile conditions and the samples were incubated at 37°C while shaking at a rate of 200 rpm for an hour. A volume of 150 l µl of bacteria was spread on LB with 1% agar (w/v) and 50 µg/ml gentamycin. For pDONR™/Zeo low salt (5 g/L) LB was used as recommended by the provider and 100 ug/ml of zeocin was used for the selection of transformed bacteria.

2.3.8 Colony PCR

A sample of bacteria from 4 colonies was collected from the plates using a tip and the colonies were given a number. The bacteria were then mixed in 20 µl of sterile dH_2O and 3 µl were used as a template for a PCR in order to detect the presence of the insert in the backbone vectors. Two positive colonies were selected to be cultured overnight in liquid LB with appropriate antibiotics.

2.3.9 Plasmid DNA extraction

The isolation of plasmid DNA was completed by using the alkaline lysis method (Birnboim, 1979). 1.5 ml of bacteria cultured in 5 ml of liquid LB overnight on a shaker at 220 rpm was transferred to a microcentrifuge tube and centrifuged at 14000 rpm for one minute to pellet the cells. The LB was discarded and the pellet resuspended by pipetting in 350 µl of resuspension buffer (Qiagen). Then, 350 µl of lysis buffer (Qiagen) were added, the tubes were gently inverted and the samples were incubated 5 minutes at room temperature. The lysis was stopped by adding 400 µl of neutralization solution (Qiagen) and gently mixing by inverting the tubes. The samples were then centrifugated at 14000 rpm for 10 minutes. 700 µl of supernatant were transferred to a new tube and mixed with an equal volume of isopropanol and incubated on ice for 5 minutes. The samples were then centrifuged at 14000 rpm for 5 minutes to pellet the DNA. The pellet was subsequently washed with 500 µl with 70% ethanol. The ethanol was discarded and the open tubes were placed on a thermoblock for 5

minutes at 65°C to evaporate remaining ethanol. Finally, the DNA was resuspended in sterile dH_2O.

2.3.10 Sequencing

The sequencing of plasmids and PCR products was carried out by DBS genomics (Durham, UK). DNA samples (150 ng/µl for plasmid and 100 ng/µl for PCR products) were sent with sequencing primers (3.2 pm/µl) . Generally, around 1 kb of DNA sequence was readable and fragments of more than 1kb were sequenced using multiple overlapping primers in order to obtain the whole sequence. The results were opened using a plasmid editor (https://jorgensen.biology.utah.edu/) and blasted using ncbi nucleotide blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_SPEC=GeoBlast&PAGE_TYPE=BlastSearch). The sequences were also aligned to a reference sequence to confirm their integrity.

2.3.11 LR reaction

Genes of interest were cloned in destination vectors PMDC83GFP and PMDC83mCherry (Fig. 2.4) (see Appendix II) for Carboxyl-terminal fusion with a fluorescent tag whereas the genes with Amino-terminal tag were cloned in PMDC43GFP and PMDC43mcherry or PMDC43YFPN and PMDC43YFPC (see Appendix II) using the LR reaction. For transcriptional reporters, promoters of were cloned in pBGWFS7 (see Appendix II). The different components were mixed as followed and incubated at 25°C for 4 hours.

Reagents	**Volume**
Entry clone (150ng/µl)	**2 µl**
Destination vector (150ng/µl)	**2 µl**
LR clonase II enzyme mix	**1 µl**

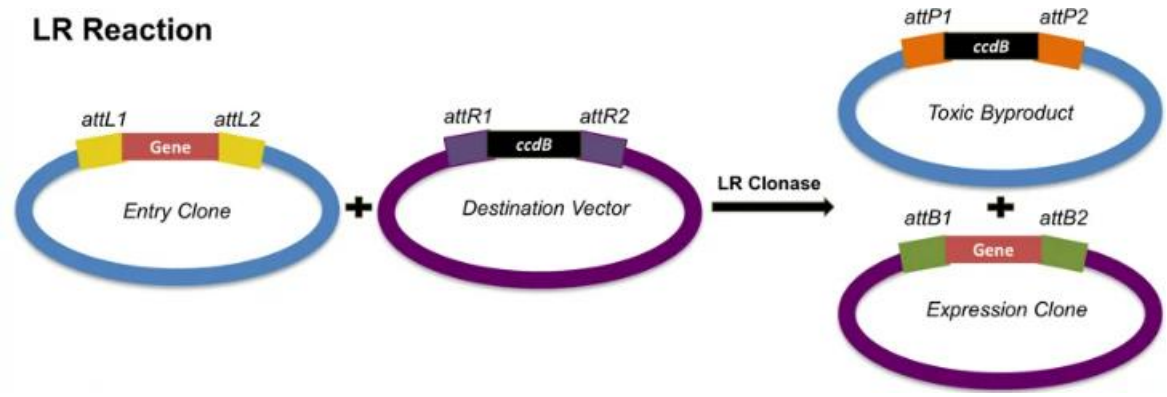


Fig. 2.4 LR reaction. The attL flanked sequence of the Entry cloned was clone in the destination vectors by the LR clonase enzymes. The clone obtained is the expression vector used for plant transformation. Image taken from Addgene website. https://blog.addgene.org/plasmids-101-gateway-cloning

The whole reaction volume was used for the transformation of *E.coli* and the expression plasmid was extracted and sequenced as explained previously.

2.3.12 Agrotransformation

The strain GV3101 of *Agrobacterium tumefaciens* was used to transform the genome of plants. Expression vectors were inserted in chemically competent cells by heat shock. A volume of 50 µl of competent cells were mixed with 1 µg of plasmid DNA and incubated on ice for 5 minutes. The bacteria were then flash frozen and kept in liquid nitrogen for 5 minutes. The frozen samples were transferred to a heat block et 37°C for 5 minutes, 200 µl of LB were added and the samples were incubated at 28°C for 2 hours. Eventually, the whole volume was spread a petri dish containing LB agar and the appropriate antibiotics (Rifampicin 50 µg/ml and Gentamycin 50 µg/ml for GV3101 vectors and Kanamycin 50 µg/ml or Spectinomycin 50 µg/ml respectively for PMDC vectors and pBGWFS7). The plates were incubated for 2 days at 28°C to obtain colonies.

2.4 Plant transformation

2.4.1 Transient gene expression in *Nicotiana benthamiana*

Typically, 2- to 3-week-old plants were selected for transfection. 5 ml of Agrobacteria expressing the sequences of interest were cultured overnight and centrifuged at 3500 x *g* for 10 minutes to pellet the cells. The LB was discarded and the cells were washed three times by resuspension in 1ml of infiltration buffer (5 mg/ml glucose, 50 mM MES (pH 5.7), 2 mM

$Na_3PO_4 \cdot 12H_2O$ and 0.1 mM acetosyringone) and centrifugation at 3500 x *g*. The samples were then diluted 10 times with infiltration buffer and the OD_{600} was measured. Samples were diluted again in infiltration buffer to reach an OD_{600} of 0.01 to 0.1. Agrobacteria containing the constructs were then mixed in a 1:1 volume ratio with agrobacteria expressing P19 protein for higher transfection efficiency and infiltrated in the leaves with a 1 ml syringe through the abaxial surface. When 2 constructs were coexpressed, bacteria were mixed in a 1:1:1 volume ratio prior to infiltration.

Plants were then placed back in a growth cabinet for 3 days to allow the expression of the transgenes before monitoring.

2.4.2 Stable transformation of *Arabidopsis:* Floral dipping

Arabidopsis plants were grown to the stage where they started producing many flowers but before the formation of siliques. *Agrobacterium* cells were grown overnight at 28°C in 5 ml of LB+antibotics and the whole volume was transferred in a flask containing 500 ml of LB+antibiotics. The cells were the aliquoted in 50 ml Falcon tubes and centrifuged at 3500 x *g* for 30 min. The pellet was resuspended in 500 ml of dH_2O with 25 g of sucrose and 250 µl of Silwett L-77. Flowers of *Arabidopsis* plants were dipped in that solution for 1 minute then kept in the dark and wrapped in a plastic bag overnight to keep high humidity. The next day the bag was removed and plants were allowed to grow in normal conditions. The dipping was typically repeated once or twice with a 7-day separation.

When plants were fully mature, they were allowed to dry and the seeds were collected for selection of transformants. Hygromycin resistant plants were screened on plates containing 50 µg/ml hygromycin and basta (Glufosinate Ammonium) resistant plants were screened on soil by spraying the herbicide (Kurtail, 120 mg/L) on seedlings. At least two independent transformants were used for phenotypical characterization and GUS staining assays.

2.5 Protein-Protein interactions

2.5.1 Microscopy

2.5.1.1 Förster resonance energy transfer and fluorescence lifetime imaging microscopy (FRET-FLIM)

Transfected *N. benthamina* were collected and squares of approximatively 0.5 x 0.5 cm^2 were cut from the leaves. The samples were placed on a slide so the abaxial surface faces the objective of the microscope. The samples were kept in water under a coverslip and used for confocal microscopy. Samples were observed on a LEICA TCS SP5 microscope with GFP and RFP filters using a HCX PL APO CS 63x/1.2 water lens. When a cell coexpressing the two constructs was observed an image was captured with the Confocal LAS AF software using the same conditions between experiments (Argon laser 22%, 488 channel 33% for GFP and 596 channel 33% for mCherry/ Gain 850, offset -2, pinhole 115um). A FLIM wizard in the software was used to access SymPhotime software and to proceed to the FLIM analyses. Advanced laser 488 was switched on to measure the lifetime of GFP in FLIM settings and acquired until reaching 10 000 counts option and 512x512 format were selected in the acquisition menu. The output curves were fitted to different exponential decay models and the fit with the χ^2 closest to 1 was selected to calculate the lifetime. In both control and interaction measurement experiments the lifetime of the donor (GFP) was measured. The measurements were repeated at least 4 times for statistical analyses.

2.5.1.2 Bimolecular Fluorescence Complementation (BiFC)

N. benthamiana leaf samples were prepared as previously described and observed on a LEICA TCS SP5 with the HCX PL APO CS 63x/1.2 water lens or on a Zeiss 800 confocal microscope using the C-APOCHROMAT 40x/1.2 water lens. The 488 laser was used (33% on the SP5 and 1% on the Zeiss 800, excitation, 500 ± 10 nm; emission, 535 ± 15 nm). Different combinations were tested:

Expression	Fluorescence	Purpose
Protein1YFPC+protein2YFPN	Only if interaction	Interaction measurement
Protein1YFPN+protein2YFPC	Only if interaction	Interaction measurement
Protein1YFPN+protein1YFPC	Yes	Positive control
Protein1YFPN	No	Negative control
Protein1YFPC	No	Negative control
Protein2YFPN	No	Negative control
Protein2YFPC	No	Negative control

YFPN and YFPC are respectively the N-terminal half and C-terminal half of the YFP that will form the whole YFP protein if the 2 halves are close enough (less than 10nm) (Hu et al., 2002).

2.5.2 Co-immunoprecipitation

2.5.2.1 Protein extraction

Proteins were extracted from 2 g of transfected *N. benthamina* leaves in non-denaturing conditions. The leaves were flash frozen and ground into a fine powder using a mortar and a pestle in a 4°C room. The powder was transferred to a 50 ml tube and 3 ml of extraction buffer were added (50 mM Tris/HCl pH7.5, 150 mM NaCl, 15 mM $MgCl_2$, 10% glycerol (v/v), 1 mM EDTA, 10 mM DTT, 0.5% (v/v) Nonidet P-40 (Sigma), 1X protease inhibitor cocktail (Thermofisher scientific, 100X stock)) and the samples were vortexed for 1 minute. The mixture was incubated on a roller shaker for 30 minutes and then centrifuged 15 minutes at 4°C to pellet the debris. The clean supernatant was transferred to a new tube and stored at -20°C. Prior their loading on a protein gel, the samples were boiled at 95°C in 1:1 ratio with SDS-sample buffer (120mM Tris/HCl pH 6.8, 20 % glycerol, 4 % SDS, 0.04 % bromophenol blue, 10 % ß-mercaptoethanol)

2.5.2.2 Western blotting

Acrylamide gels (1.5 mm thickness) were casted between two glass plates using the Mini-PROTEAN® Tetra Handcast Systems (Bio-Rad) using the following components.

Resolving gel (2 gels 10% Acrylamide)

Components	Volume
dH_2O	5.9 ml
Acrylamide/Bis (30%)	5 ml
1.5M Tris pH8.8	3.7 5ml
SDS 10%	150 µl
APS 10%	150 µl
TEMED	15 µl

The acrylamide was allowed to polymerize for 15 min at RT.

Stacking gel (2 gels 4% Acrylamide)

Components	Volume
dH_2O	6 ml
Acrylamide/Bis (30%)	1.33 ml
1.5M Tris pH8.8	2.5 ml
SDS 10% (w/v)	100 µl
Ammonium persulfate 10% (w/v)	100 µl
TEMED	10 µl

The acrylamide was allowed to polymerize for 15 min at RT.

Cast gels were placed into an electrophoresis tank with the middle part filled to the top with 1x SDS-running buffer (3.03 g/L Tris base, 14.4 g/L glycine and 1 g/L SDS) and the rest of the tank was three-quaters full. Samples were then loaded on the gels alongside a page ruler protein ladder (Thermofisher scientific) and run 1h 30 min at 70 V and 1 h at 120 V.

The gel was then carefully taken out of the glass plates and placed onto a nitrocellulose membrane (Amersham Protran Premium 0.45 NC) within a sandwich as displayed in Fig. 2.5, and inserted into a plastic cassette. The sandwich was exposed to a voltage of 15 V overnight in a tank filled with transfer buffer to allow migration of the proteins from the gel to the membrane.

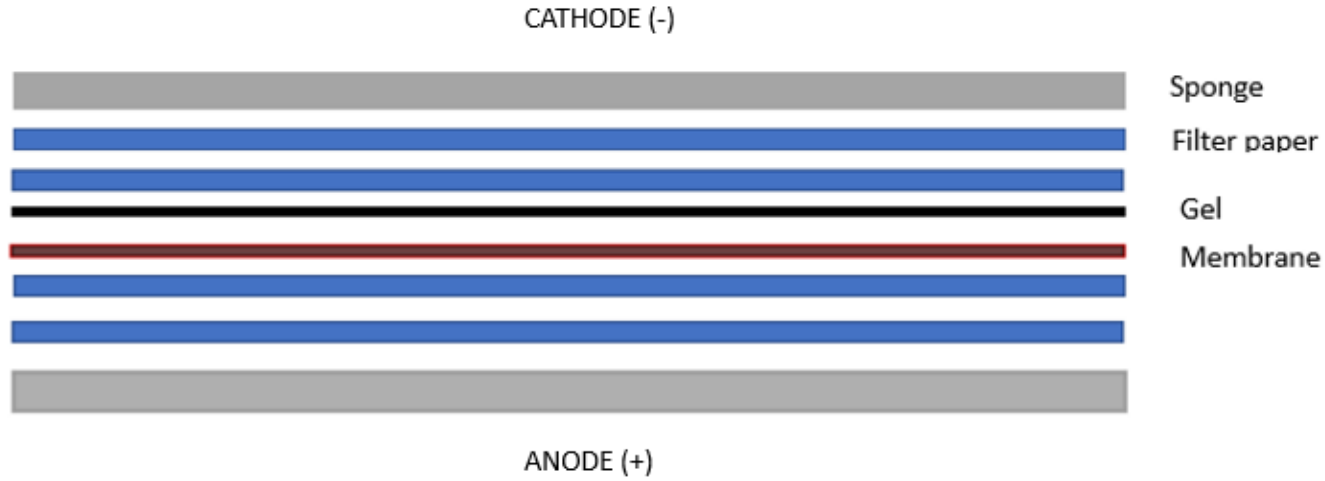


Fig. 2.5 Protein transferred. Negatively charged proteins from the Acrylamide gel migrating towards the anode and blotting the nitrocellulose membrane.

The blotted membrane was incubated a few seconds in Ponceau solution (1 g/L Ponceau S tetrasodium salt in 19:1 water:acetic acid) for protein visualisation and destained with TBS-T

(20 mM Tris,150 mM NaCl and 0.1% (v/v) Tween20). If proteins were detected the membrane was blocked with TBS-T+5% milk for an hour. Then, the membrane was incubated for 5 h with the primary antibody (1:1000 mCherry polyclonal antibody, proteintech) in TBS-T+5% milk at with gentle shaking. The membrane was subsequently washed 4 times for 4 minutes with TBS-T. The membrane was then incubated 1 hour at RT with a secondary antibody (1:2500 HRP conjugated chicken anti-rabbit, Agrisera). After 4 more washes the membrane was covered with chemiluminescent ECL substrate (Biorad) and inserted in an iBrightFL1000 (Themofisher scientific) imager for visualization.

2.5.2.3 RFP-trap

In order to precipitate a network of interaction proteins the ChromoTek RFP-Trap® magnetic agarose method was selected. *N. benthamiana* plants were transfected with 35S:VAMP714:mCherry constructs and the proteins were extracted as explained previously. A 35S:mCherry control was also selected for mass spectrometry analysis.

A volume of 25 µl of magnetic beads was transferred to a microcentrifuge tube and 500 µl of dilution buffer (10 mM Tris/Cl pH 7.5, 150 mM NaCl, 0.5 mM EDTA) was added. The buffer was pipetted out while the tubes were on a magnet for bead separation. Then, 1.5 ml of protein extract (input) were added and the samples were incubated for 1 h at 4°C on a end-to-end shaker. Beads were separated using a magnet and 50 µl of the supernatant was collected as the flowthrough, the rest was discarded. 500 µl of ice-cold wash buffer (10 mM Tris/Cl pH 7.5, 150 mM NaCl, 0.05 % Nonidet™ P40 Substitute, 0.5 mM EDTA) were added and discarded after separation of the beads on a magnet. This operation was repeated three times and the tube was changed before the last wash. The samples were boiled in SDS-sample buffer to allow protein detachment from the beads and beads were discarded after separation on the magnet (eluate). The input, the flowthrough and the eluate were used for western blotting to ensure that the immunoprecipitation worked.

For mass spec elution from the beads was carried out using in a solution of tetraethylammonium bromide 50 mM pH 8.5 with 10% SDS.

2.6 Cell wall composition analysis

2.6.1 Alcohol insoluble residues (AIR)

Prior to all cell wall analyses, samples were dried using the AIR method. This has the advantage of removing most of the inorganic salt low molecular weight metabolites and pigments that could interfere with some assays. Typically, 50 mg of fresh material was weighed on an analytical balance and incubated in 80% ethanol at 55°C overnight (Fry, 2001). The ethanol was removed and the samples were air dried overnight with acetone. The cell wall enriched material was weighed and used for downstream analysis.

2.6.2 Cellulose quantification, Updegraff method

Cellulose was quantified using a colorimetric method developed by Updegraff (Updegraff, 1969). 1-2 mg of AIR was weighed and dissolved in acetic nitric reagent (8:2:1 acetic acid:nitric acid:H_2O) at 98°C for 10 minutes. Samples were then centrifugated at 14000 rpm for 10 minutes and most of the supernatant was removed. 1 ml of dH_2O was added to the samples and they were centrifugated for 10 minutes at 14000 rpm. Half of the supernatant was discarded, 1ml of acetone was added and the samples were spun for 5 minutes. Then, 1 ml of supernatant was removed and replaced by 1 ml of acetone and the samples were again spun for 5 minutes. The samples were left to dry overnight. Samples were then mixed by vortexing in 100 µl of 67% sulphuric acid. 400 µl of dH_2O was added as well as 1 ml of anthrone (1% (w/v) in concentrated sulfuric acid) while the tubes were on ice. Eventually, the samples were boiled on a heatblock for 5 minutes and the OD_{620} was measured with a spectrophotometer. The quantification was achieved by comparing the OD_{620} values of the samples to those of a glucose standard curve (50 nM, 100 nM, 150 nM, 200 nM, 300 nM of glucose were used with 3 replicates each to generate the curve).

2.6.3 Matrix polysaccharides analysis, thin layer chromatography (TLC)

Matrix polysaccharides were hydrolysed with trifluoroacetic acid (TFA) to obtain monosaccharides that were run on a TLC plate. AIR was weighed on an analytical balance and resuspended in 2 M TFA in order to have a concentration of 0.5% (w/v). The samples were then incubated at 120°C for 1 hour. The solution was centrifuged 10 minutes at 14000 rpm. The supernatant was collected and dried in a speedvac for 4 hours and dissolved in dH_2O to obtain 1% (w/v). The hydrolysate was loaded on a TLC silica plate (TLC Silica gel

60 F_{254},merck) and run in ethyl acetate/pyridine/aceticacid/water (EPAW; 6:3:1:1) for 2 hours. Then the plate was allowed to dry 2 hours and placed back in EPAW. The plate was allowed to dry again overnight. Sugars were stained with thymol and allowed to dry for 1 hour. The plate was then transferred to an oven and incubated at 104.5°C for 10 minutes for visualization.

2.7 Fluorescence recovery after photobleaching (FRAP)

FRAP measurements were done with a Zeiss 800 confocal microscope. Images of the root tip of plants expressing PIN2:GFP were taken using the 488 laser and eGFP spectrum using Zen blue software https://www.zeiss.com/microscopy/en/products/software/zeiss-zen.html. Epidermal cells in the root apical meristem (Fig. 2.6) were chosen for the measurements since PIN2 has polar localization in the epidermis and so is easy to visualise and photobleach. The central part was selected as PIN2 is also concentrated in centre of the polar domain, also referred as the superpolar domain, where its lateral diffusion is decreased (Kleine-Vehn, 2011). A rectangle was drawn around the region of interest and the laser power was set to 100%. A two-minute-long time series was then performed. FRAP analyses were done on Fiji using create spectrum jru v1, combine all trajectories jru v1, normalize trajectories jru v1, batch FRAP jru v1 and average trajectories plugins developed by Stowers (https://research.stowers.org/imagejplugins/ImageJ_tutorial2.html).

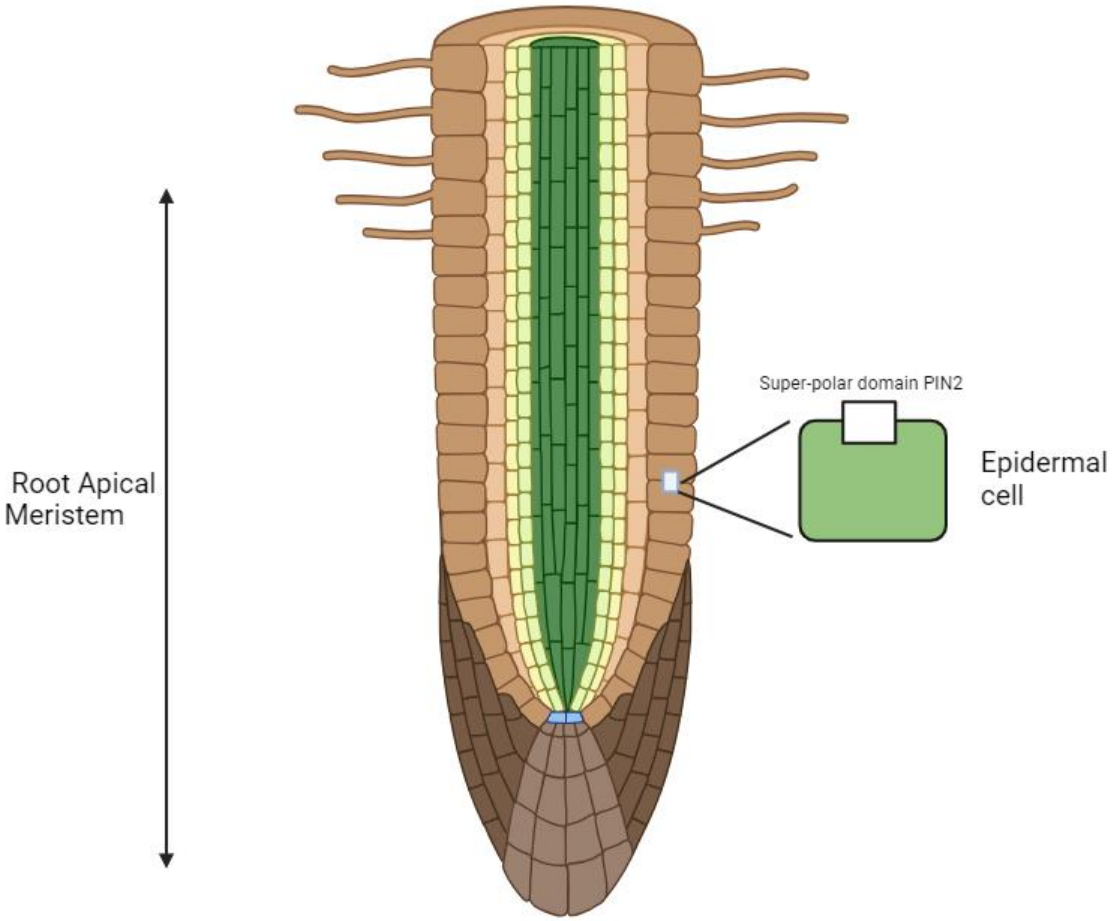


Fig. 2.6 Selected tissues and cells for FRAP. All the bleached membranes belonged to epidermal cells in the root apical meristem of plant expressing PIN2:GFP. The central part of the polar domain (Super-polar domain) was chosen for FRAP analysis.

2.8 GUS staining

The reporter gene *GUS* (*uidA*, encoding beta-glucuronidase) was used for the localization of promoter activity in transgenic plants. Activity of the enzyme is localised by incubating tissues in X-Glucuronide which is colourless but is converted to a blue precipitate in tissues expressing the GUS enzyme.

Seedling were covered by X-gluc (1 mM) freshly mixed in a solution of 100 mM sodium phosphate (pH 7), 10 mM EDTA, 0.1% (v/v) Triton X-100 and incubated overnight at 37°C.

The seedlings were then placed on a slide in dH_2O and a cover slip was placed on top. Images were acquired with an Axioskop 50 (Zeiss) light microscope.

2.9 Gravitropism assays

Seedlings were grown for 4 days on ½ MS plates and turned to a 90° angle. The bending angle towards the direction of gravity was measured after 24 h for at least 10 seedlings.

2.10 RNA-sequencing

Plants were grown for 7 days after germination on standard ½ MS plates in a growth cabinet under the previously described conditions. Seedlings were grown at the same time and close to each other to get the light exposure as similar as possible.Total RNA from three seedlings was extracted as explained in section 2.3.1 for each biological replicate. A total of 3 biological replicates was used for each genotype. The RNA samples were shipped on dry ice to Novogene (Cambridge, UK) who assessed the quality of the samples, prepared the cDNA library and carried out the sequencing. The RNA-seq workflow is presented in Fig. 2.7. Novogene also provided a bioinformatics analysis of Differently Expressed Genes (DEGs), Gene Ontology (GO), KEGG pathways, Single Nucleotide Polymorphisms (SNP) and a Protein-Protein Interaction analysis (PPI).

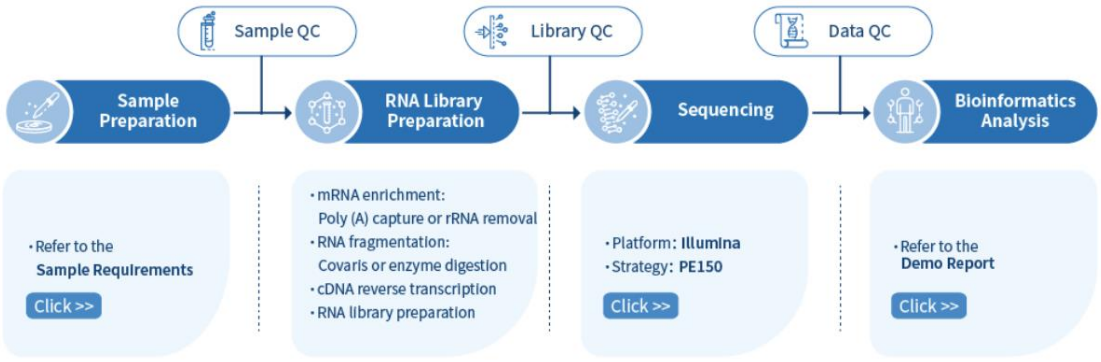


Fig 2.7 Novogene workflow for RNA-seq analysis.

2.11 Whole genomic DNA sequencing

Genomic DNA of mutant plants was purified using the CTAB method, as follows.

Leaf material was weighed and flash frozen with liquid nitrogen. The tissues were then ground into a fine powder and transferred to a microcentrifuge tube and 300 µl of pre-

warmed (60°C) CTAB buffer (hexadecyltrimethylammonium bromide 2% (w/v), NaCl 1.4 M, EDTA 20mM (pH 8), Tris-Cl 100 mM (pH8)) were added per 100 mg of plant material. 50 ng/ml of RNase A was added to the solution and the samples were mixed with a vortex mixer. The tubes were then incubated 1 hour at 60°C and inverted periodically. After being allowed to cool down at RT, 300 µl of chloroform was added to the samples and the tubes were mixed by vortexing. The samples were then centrifugated 10 minutes at 20 000 x *g* to separate phases. The upper fraction was transferred to a new tube and centrifugated again to increase the purity. An equal volume of isopropanol was then added to the samples to allow DNA precipitation and they were incubated for an hour at -20°C. The tubes were centrifugated at 15 minutes at 20 000 x *g* and the pellet was washed with 1 ml of 70% ethanol. After air drying, the pellet was resuspended in sterile dH2O. Purity and integrity of the samples were assessed by Nanodrop measurements and running the DNA on an agarose gel.

The whole genome sequencing was carried out by Novogene using Illumina NovaSeq X Plus /NovaSeq6000.

The results were analysed to find T-DNA copies in the genome of *vamp712* mutants. NGS whole genome sequencing data were downloaded from Novogene. The names of the samples were v7122 and V7122, which were independent plants of the same knockout genotype for the *VAMP712* gene AT2G25340 grown from seeds of the same generation. They were both presumed homozygous plants from the gabikat T-DNA line GABI_054H03 and contained T-DNA from the vector pAC106. The T-DNA sequence was obtained from GenBank entry AJ537513.1. *Arabidopsis thaliana* genome sequence TAIR10 was downloaded from ensemblplants (https://plants.ensembl.org/index.html). Sequence reads were trimmed using trimmomatic (version 0.39, https://github.com/usadellab/Trimmomatic). Sequence reads were mapped to TAIR10 genome and the T-DNA sequence using bwa-mem (version 0.7.17, https://github.com/lh3/bwa) and sorted using samtools (version 1.9, https://github.com/samtools). Sequence coverage was obtained using mosdepth (version 0.3.3, https://github.com/brentp/mosdepth) and summarised using multiqc (version 1.14, https://github.com/ewels/MultiQC). T-DNA insertions were identified using TDNAscan (https://github.com/BCH-RC/TDNAscan). Sequence alignments were viewed using IGV (version 2.16.1, https://software.broadinstitute.org/software/igv/

Chapter 3: Vesicle trafficking and PIN polar localization: unravelling a network of interacting proteins

3.1 Introduction

Vesicle trafficking is a key element in the control of PIN auxin transporter localization and polarity in plant cells. Indeed, the endocytic recycling of PINs, which is the mechanism that generates polar distribution of these auxin transporters in specific tissues, is regulated by proteins of the vesicle trafficking machinery (Geldner et al. 2003b; Kleine-Vehn et al. 2009). PIN-mediated auxin transport and its regulation is a popular field of research given the importance of auxin gradient establishment in numerous biological processes. Proteins, lipids, cytoskeleton and cell wall components have been identified as major actors of vesicle trafficking underlying the complexity of the regulation of PIN populations at the plasma membrane. Published review articles (Adamowski and Friml 2015; Luschnig and Vert 2014) have helped progress towards understanding the big picture of PIN regulation but many questions remain unanswered. A striking example is how little is known about the relationship between the known molecular actors of PIN vesicle trafficking.

In our laboratory, former members and myself have characterized VAMP714, a R-SNARE protein as a regulator of PIN mediated polar auxin transport required for proper plant root growth and development (Gu et al. 2021). This chapter aims to study the relationship and possible the cooperation between VAMP714 and other components of the vesicle trafficking machinery involved in maintaining the integrity of the root apical meristem.

3.2 *N. benthamiana* as a system for orthologous protein expression

Proper subcellular localization is essential for optimal activity of proteins. Therefore, in *vivo* analyses were preferred to *in vitro* approaches in order to visualize and measure protein interactions, and can be observed in their biological context. In this study, the transient and orthologous expression of the proteins of interest was carried out in *N. benthamiana*. The main motivations to use this system were the efficiency of transfection, the large size of the leaf epidermal cells that facilitates subcellular studies and the rapidity of the process. Even though most marker proteins of *Arabidopsis* seem to conserve their subcellular localization, some variation in *Arabidopsis* proteins have also been observed, pointing to the limitations of the system (Youjun Zhang et al. 2020). A good practice is to compare localization of the protein of interest in *N. benthamiana* and *Arabidopsi*s where possible. Transient expression of VAMP174:mCherry under the 35S promoter in leaf epidermal cells of *N. benthamiana* showed that the fusion proteins localize in the Golgi/TGN (Fig. 3.1 A, central and right panels (thin arrows)), the plasma membrane (Fig. 3.1 A) and in reticulate structures typical of the endoplasmic reticulum (ER) (Fig. 3.1 A, left panel (thick arrow)). Interestingly, ePlant online tool for subcellular localization information showed that the protein localizes to those compartments in *Arabidopsis* (Fig. 3.1 B). This is also in agreement with co-localization experiments of VAMP714:GFP with the Golgi marker ST and ER marker HDEL performed in *Arabidopsis* (Gu et al. 2021) (Fig. 3.1 C).

Together, these results show that VAMP714 conserves its localization in *N. benthamina* and the reliability of transient expression for *in vivo* protein-protein interaction (PPI) measurement purposes. It also indicates at least a partial conservation in the protein sorting machineries in plant species but further analysis of this is beyond the scope of this study.

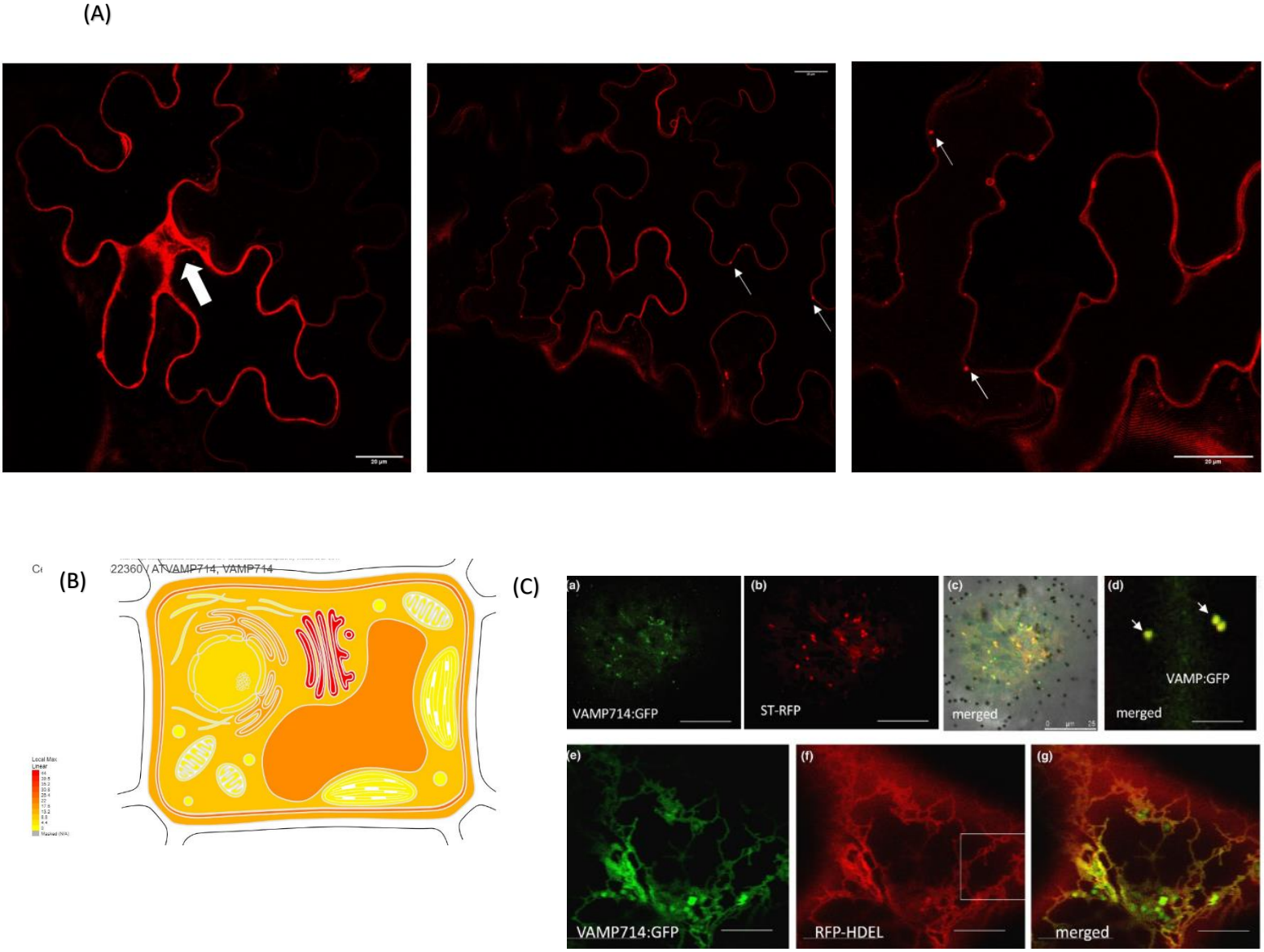


Fig. 3.1 Expression of VAMP714 in *N. benthamiana*. (A) Fusion protein VAMP714:mCherrry localizes in multiple compartments within the cells, the thick arrow head indicates expression in reticulate structure (likely ER), and thin arrows designate Golgi/TGN. (B) Predicted expression of VAMP714 according to ePlant (https://bar.utoronto.ca/eplant/). (C) Co-localization of VAMP714:GFP and Golgi marker ST and ER marker in *Arabidopsis*; the arrows designate the merged fluorescence in the Golgi.

3.3 Selection of candidate interactors

The approach chosen to study the relationship between VAMP714 on the one hand and regulators of PIN vesicle trafficking on the other hand is the analysis of protein-protein interactions (PPI). Numerous proteins involved the vesicle trafficking of PIN have been characterized in the literature but this list was narrowed down to genes that like *VAMP714* are

involved in root growth and development. The first search for candidates was completed by using online tools for PPI predictions. Among the potential interactors only SYP22, a Qa-SNARE, has been previously linked to PIN mediated auxin transport. It has been demonstrated that SYP22 is required for PIN1 polar localization in leaf vascular cells (Shirakawa et al. 2009) but it has no known effect on root development so the candidate was not studied further.

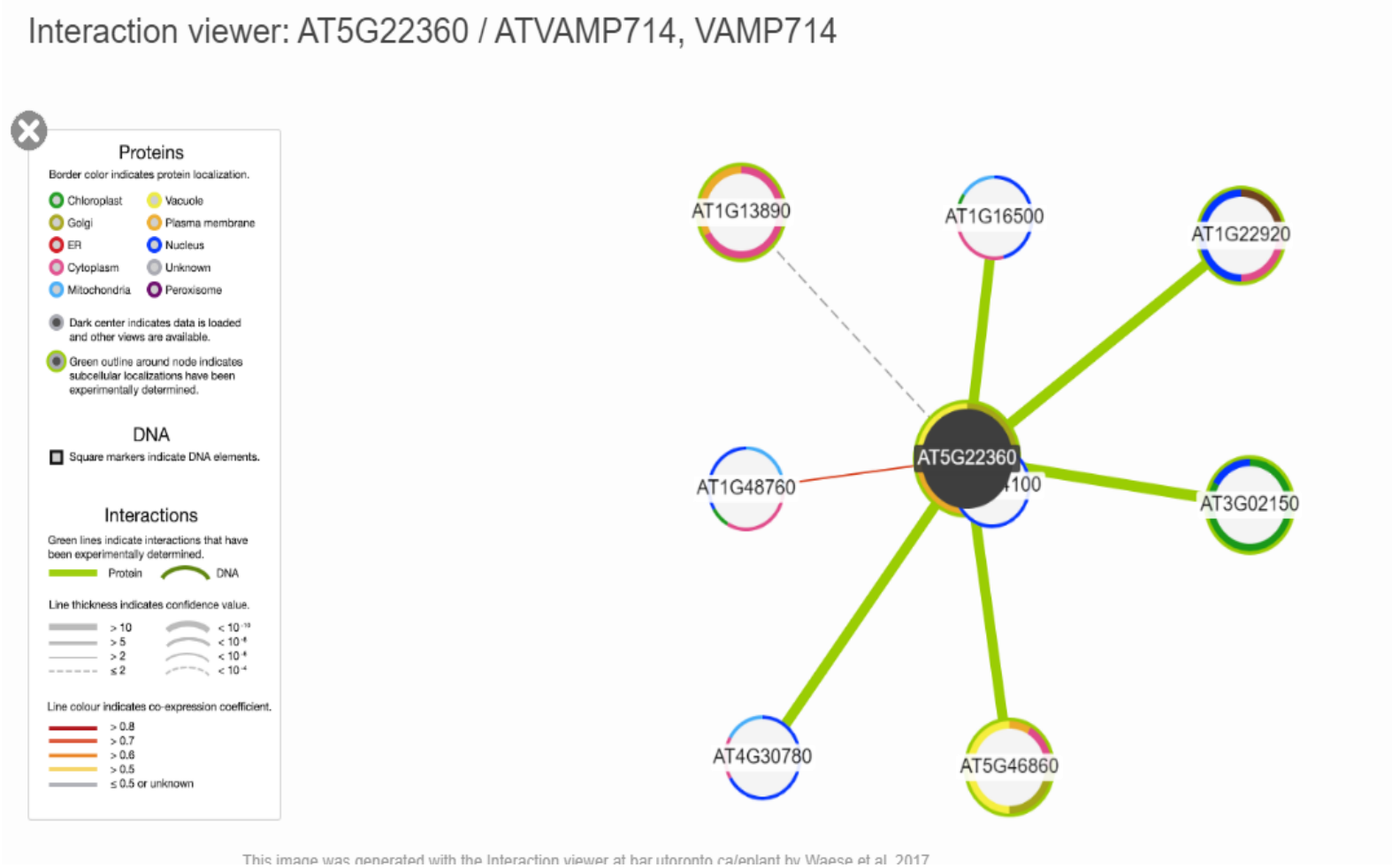


Fig. 3.2 Known and predicted interactors of VAMP714 according to ePlant. A complete legend is present on the left-hand side of the figure. The border colour of the circle containing the gene encoding for VAMP714 (centre) and its interactors indicate the subcellular localization of these proteins. Th green lines connecting the circles indicate published and experimentally determined interactions whereas lines of a different colour are predicted interactions. The thickness and colour of the latter indicate respectively their confidence value and co-expression coefficient. Overlapping circles represent DNA-protein interactions. A complete legend is present on the left-hand side of the figure.

An alternative approach was to use available proteomics data. A large proteomic experiment on *Arabidopsis* seedlings published in 2015 (Heard et al. 2015) used 7 markers of specific cell compartments that also are vesicle trafficking proteins in an attempt to dissect the different trafficking routes. In that experiment, VAMP714 was pulled down together with ARA5, ARA7 and RABG3f (Heard et al. 2015) (Fig 3.3). Interestingly, in a published PhD

thesis (Postma, 2018) studying Rab GTPases in the context of plant immunity, VAMP714 was also pulled down with ARA5 and ARA7. Rab5 GTPases (that have two homologs in *Arabidopsis*, ARA7 and Rha1) are crucial in PIN endocytic recycling and have been shown to allow endocytosis of vesicles carrying PIN proteins (though this is now a retracted article, and so conclusions should be made with caution and require further verification). Indeed, in

Bait	Prey	PreyGene	Spec	SpecSum	AvgSpec	NumReplic	ctrlCounts	AvgP	MaxP	TopoAvgP	TopoMaxP	SaintScore	FoldChang	BFDR	boosted_by
RFP-RABF2b/ARA7	AT5G22360	AT5G22360	0|0|2	2	0.67	3	0|0|0|0|0|0	0.33	0.98	0.33	0.98	0.33	6.67	0.4	
YFP-RABD2a/ARA5	AT5G22360	AT5G22360	0|1|5	6	2	3	0|0|0|0|0|0	0.33	1	0.33	1	0.33	20	0.31	
YFP-RABG3f	AT5G22360	AT5G22360	0|3|0	3	1	3	0|0|0|0|0|0	0.33	1	0.33	1	0.33	10	0.36	
YFP-VAMP711	AT5G22360	AT5G22360	4|0|3	7	2.33	3	0|0|0|0|0|0	0.67	1	0.67	1	0.67	23.33	0.13	

Fig. 3.3 Immunoprecipitation of VAMP714 (AT522360) with markers of cell compartments (Heard et al. 2015)

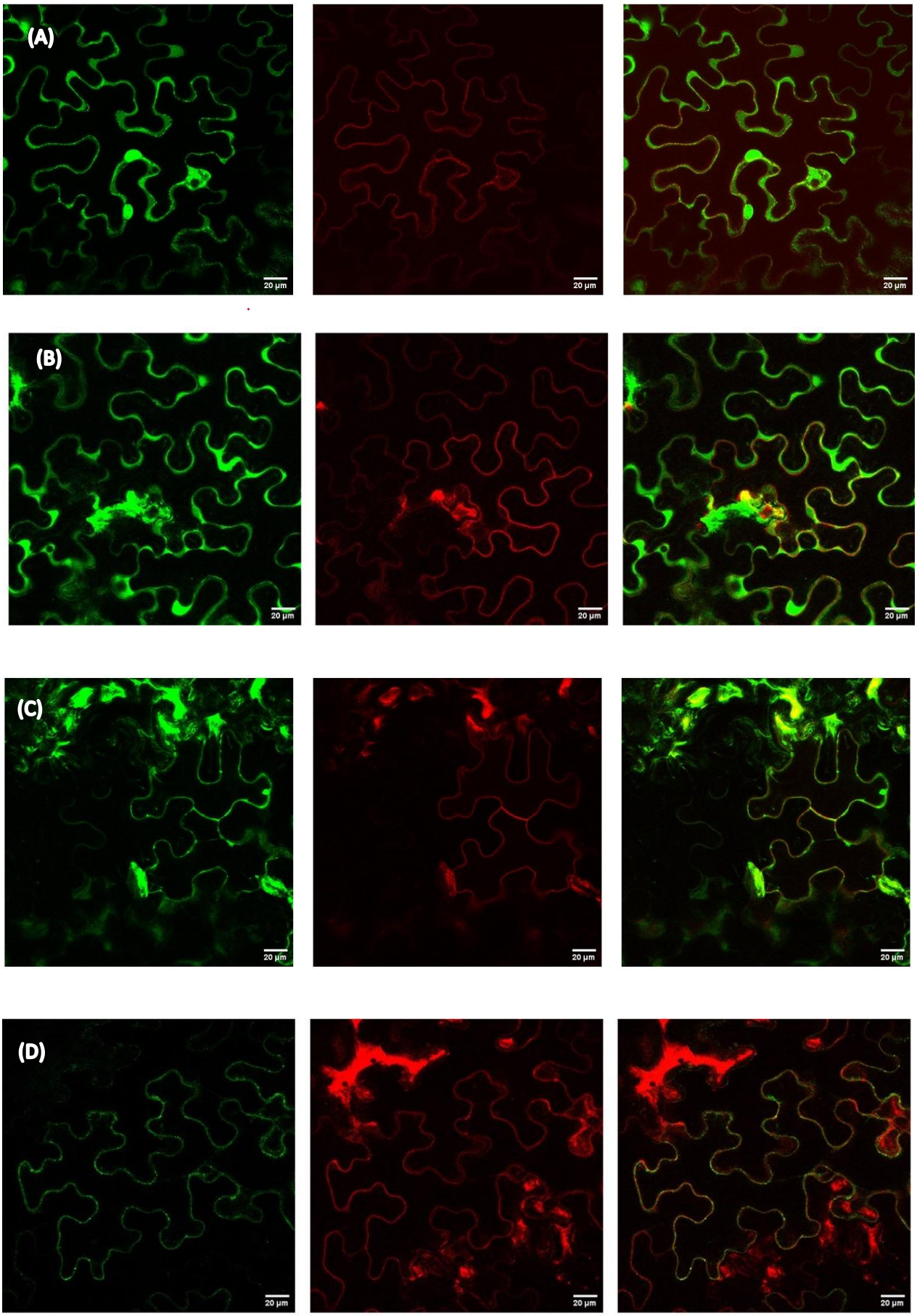
an ARA7 dominant negative mutant, PIN1 and PIN2 lose their polarity in root cells. ARA7 was therefore selected as a candidate for PPI.

A final approach was based purely on knowledge from the literature. Candidates must meet the following criteria: i) being associated with vesicle trafficking of PIN proteins, ii) expression in the root apical meristem, iii) sub-cellular localization in Golgi/TGN or endosomes or at the plasma membrane and iv) similar phenotype as *vamp714* when mutated. Six candidates where selected: VPS29 (Jaillais et al. 2007), BEX5 (Feraru et al. 2012), GNOM (Geldner et al. 2003b), VAN4 , CTL1 , and EXO70A1. Interestingly, those candidates have different functions in the trafficking machinery making them complementary.

3.4 VAMP714 co-localizes with candidate interactors

Physical interactions between proteins requires that the interactors are very close to each other, typically at a distance of 10 nm or less (Yen et al. 2014). As the cells are composed of various compartments separated from each other by biological membranes, it is also essential for interacting proteins to have at least the interacting motifs within the same cellular compartment. Therefore, colocalization of candidate interactors is a prerequisite for PPI studies. All the candidates tagged with GFP were used together with VAMP714:mCherry for co-infiltration of *N. benthamiana* epidermal cells and the subcellular localization of the fusion proteins was observed. In order to assess protein colocalization, fluorescent signals in cells expressing both tagged proteins were merged. As expected, the protein of interest displayed various expression patterns. Indeed, whereas VPS29 and BEX5 (Fig. 3.4 A and B, left panel) display a more diffuse expression pattern, ARA7 and EXO70A1 (Fig. 3.4 E and F,

left panel) localize in discrete punctate compartments. As for CTL1 and VAN4 (Fig. 3.4 C and D), they exhibit a hybrid expression pattern dominated by punctate structures but with observable continuous expression at the periphery of the cell. Interestingly, VAMP714 co-localizes, at least partly, with candidate interactors in the different compartments they are expressed in (Fig. 3.4 right panel).


(A)
(B)
(C)
(D)
20 µm

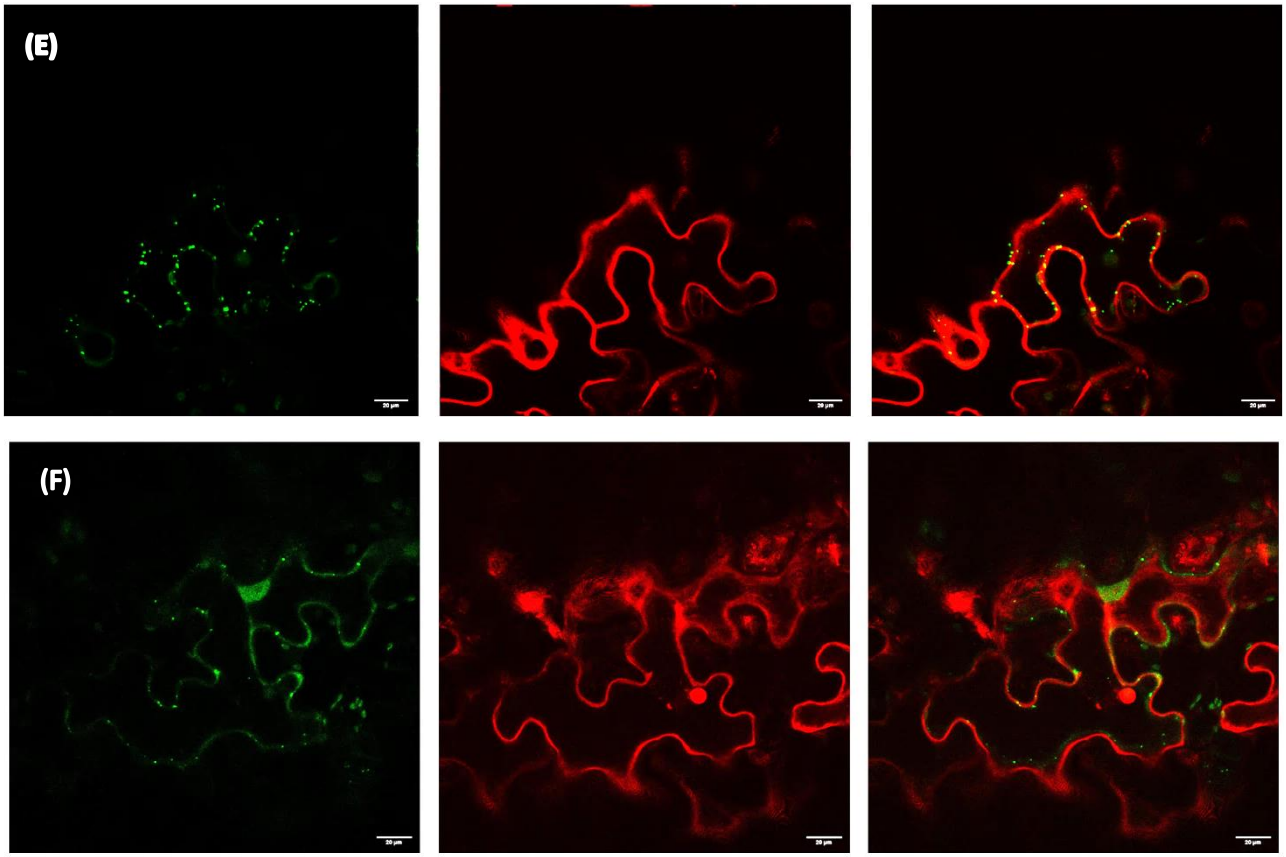


Fig. 3.4 VAMP714 co-localizes with candidate interactors. *N. benthamiana* laves were co-infiltrated with VAMP714:mCherry (middle column) and candidate fused to GFP, respectively VPS29:GFP (A), BEX5:GFP (B), VAN4:GFP (C), CTL1:GFP (D), ARA7:GFP (E), and EXO70A1:GFP (left column). Merged signal is represented in the right column.

3.5 PPI measurements

3.5.1 Experimental design

Subcellular localization of candidate interactors together with their molecular function suggest that they could interact with VAMP714. In order to investigate the possible PPI, two independent microscopy-based techniques were used: Förster resonance energy transfer and fluorescence lifetime imaging microscopy (FRET-FLIM), and bimolecular fluorescence complementation (BiFC). In the present study, knowing the topology of the proteins is crucial to choose the appropriate fusion with the fluorescent tag for successful PPI measurements. VAMP714 is an integral protein with its N-terminus and C-terminus facing respectively the cytosol and the cell wall. Apart from CTL1 which is an integral protein, all the candidates are

membrane-associated rather than inside the membrane and face the cytosolic side of their target membrane. Therefore, an N-terminally tagged version of VAMP714 was generated. This differs from previous studies where the C-terminus of the protein was tagged. This is because signal peptides located at the N-terminus of integral proteins may be cleaved. However, the N-terminally tagged VAMP714 did not display a different subcellular localization in comparison with its C-terminally tagged version. Candidate interactors were tagged at their N-terminus for Rab-GTPases to not disrupt their C-terminal anchor and for the remaining proteins both C-terminal and N-terminal versions were generated.

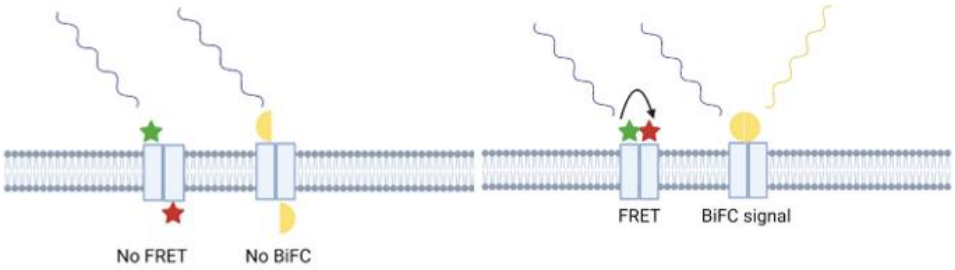


Fig. 3.5 Importance of fluorescent tag position for confocal microscopy experiments. This figure was created with biorender.

3.5.2 VAMP714 interacts with CTL1 and ARA7 in FRET-FLIM analyses

Numerous PPI methods have been and keep being developed in parallel with technological progress. In this study, microscopic analyses were primarily used since they allow us to observe where proteins interact *in vivo* and in which cellular compartment the interaction occurs. Fluorescent tags have a central role in microscopy-based methods because they allow the visualisation of the proteins. Here, FRET-FLIM was selected to carry out the PPI measurements. This technique takes advantage of the transfer of energy between 2 fluorophores at a short distance from each other, which generally happens with the fluorescent tags of interacting proteins. FRET-FLIM differs from FRET because the lifetime of a fluorophore is measured rather than the signal intensity of the acceptor fluorophore. An advantage of measuring the lifetime is that it is independent of the fluorophore concentration. Also, FRET-FLIM can be used when there is a smaller overlap in the emission spectrum of the donor and the excitation spectrum of the acceptor fluorophore (Tramier et al. 2006).

The fluorescence decay of GFP was measured by time-correlated single photon counting after excitation with a FLIM laser in *N. benthamiana* epidermal cells co-expressing VAMP714:mCherry and candidate:GFP. The lifetime of the donor (candidate:GFP) in the absence of VAMP714:mCherry was also measured as a control.

The lifetime measured for the candidate:GFP only was 2.6 ± 0.2 ns for all the candidates, which corresponds to the lifetime of GFP (usually in a range between 2.4 and 2.6 ns). In the presence of VAMP714:mCherry most of the candidate proteins did not show any sign of interaction as the lifetime changed from 0.1 ns or less. The absence of interaction is exemplified by VPS29:GFP but the results for other proteins are described in the Appendix III.

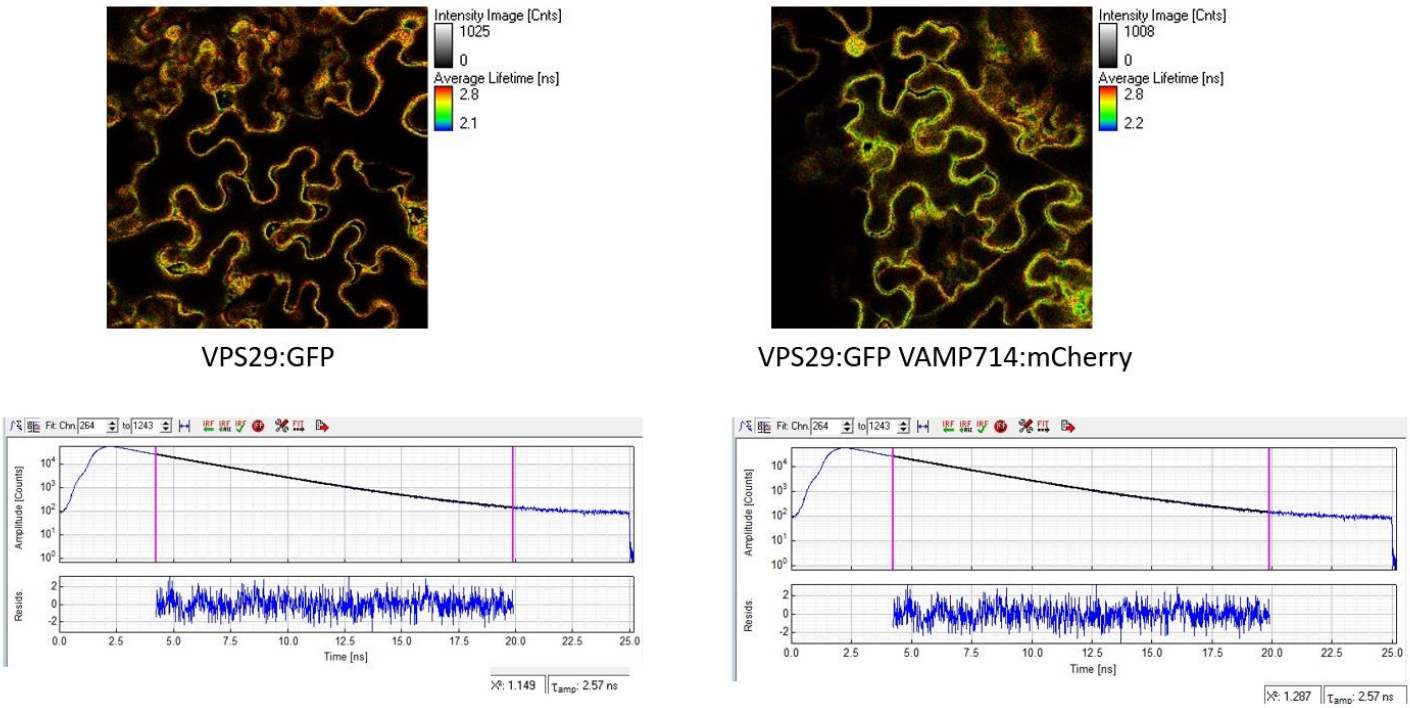


Fig. 3.6 VPS29 does not interact with VAMP714 in FRET-FLIM analysis. The beatmap shows a uniform distribution of signal with a life time around 2.6 ns (colour code) and the fluorescence decay of VPS29:GFP is similar in the presence of VAMP714.

Interestingly, when CTL1:GFP is expressed together with VAMP714:mCherry the lifetime shifts from 2.6 ± 0.02ns to 2.31 ± 0.03ns (Fig . 3.7 B), represented by a shift of the frequency of counts to the left (Fig. 3.7 A bell curves). Measurements were repeated 4 times for

statistical comparisons (non-parametric *t*-test) (Fig. 3.7 C). It has been shown by direct linking of RFP to GFP that a lifetime reduction of around 0.3 ns (Wang et al. 2014) is indicative of protein proximity suggesting that CTL1 and VAMP714 interact. More interestingly, the beatmap (spatial distribution of lifetime measurements in the cell) (Fig. 3.7 A) shows areas with strong shift in the lifetime (see colour code Fig. 3.7 A, right) as compared to the CTL1:GFP cells where the lifetime of GFP is uniform (Fig. 3.7 A, left).

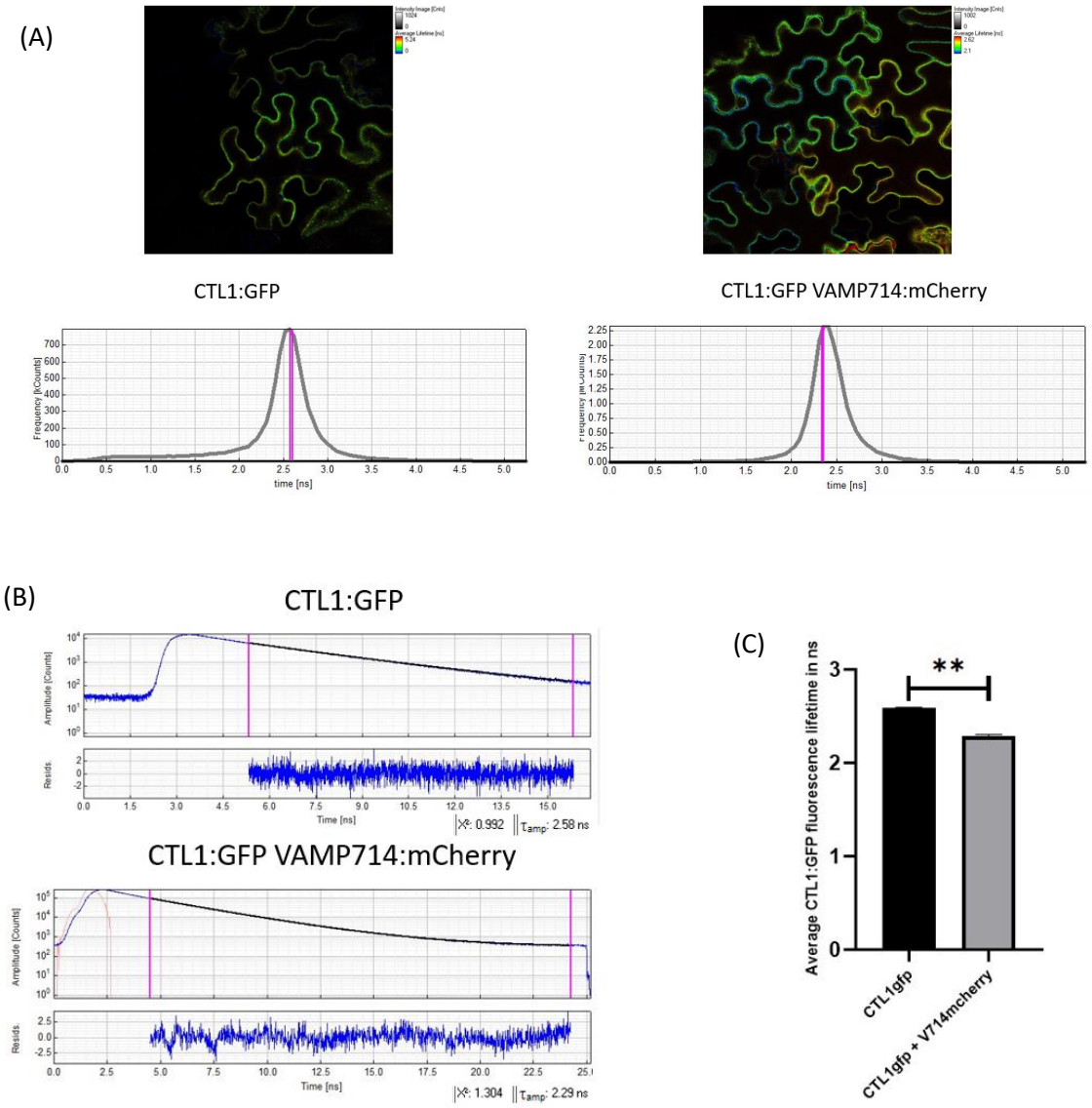


Fig. 3.7 CTL1 interacts with VAMP714 in FRET-FLIM experiments. Visual representation of fluorescent signal lifetime in the cell shows a change in some part of the cell when CTL1:GFP and VAMP714:mCherry are co-expressed leading to a shift in GFP lifetime that can be observed in the distribution of photon counts over time (A), and by comparing decay curves (B), the difference was significant in a non-parametric *t*-test (N=4, **P<0.01).

Also interestingly, the RAB5-GTPase ARA7:GFP also had a significant decrease in its lifetime when expressed alongside VAMP714:mCherry. Indeed, the lifetime of ARA7:GFP was measured in a 2.44 ± 0.02 ns range and dropped to 2.10 ± 0.03 ns when in presence of VAMP714:mCherry. Note that in order to increase the overall amount of fluorescent signal the FLIM measurements were carried out on parts of the cell containing the fluorescent punctate structures.

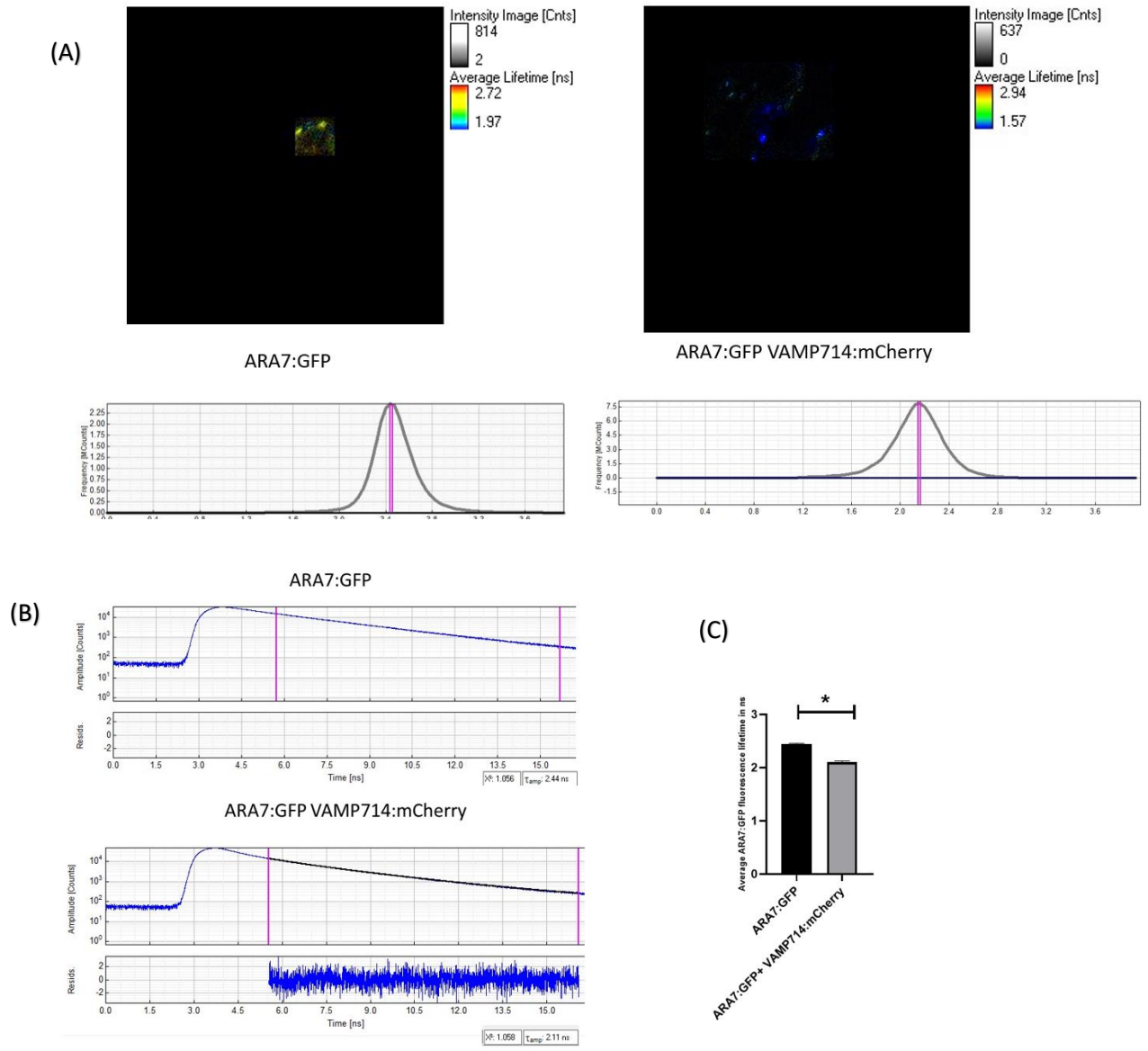


Fig. 3.8 FLIM-FRET measurements indicate an interaction between ARA7 and VAMP714. Change of lifetime in a restricted part of the cell show a change of lifetime within punctate structures (A). The decrease in lifetime was also observable in the photon counts distribution (A) and the decay curves (B). Lifetime of ARA7:GFP alone was significantly longer than ARA7:GFP in presence of VAMP714:mCherry in a non-parametric *t*-test (N=4, *P<0.0286).

3. 5. 3 VAMP714 also interacts with CTL1 and ARA7 in BiFC analyses

In order to confirm the interaction measured with FRET-FLIM, candidates were used in BiFC experiments. This straightforward technique also provides information on the subcellular location of the interaction. However, it is known to generate false positives and therefore caution should be taken in order to avoid known pitfalls. Published articles provide advice on how to perform these experiments in the best possible conditions, which involves choosing appropriate controls (Kudla and Bock 2016; Horstman et al. 2014). In the present study, VAMP714-YFPn and VAMP714-YFPc expressed together was chosen as the positive control (Fig. 3.9 left panel). Candidate interactors fused to only one of the YFP fragment is considered as an inappropriate control but were still used to ensure that the separate fragments do not fluoresce when attached to the protein of interest. Most appropriate controls described in the literature involve the mutation of interaction sites. However, this is only applicable to verify the interactions between proteins with known interaction domains. Here, another appropriate approach was used which consists of showing that there are proteins that localize to the same compartments as VAMP714 but do not interact with it. This was done using VPS29. Indeed, it was previously shown that VPS29 co-localizes with VAMP714. However, when co-expressed VPS29 and VAMP714 tagged with complementary YFP fragments do not display any fluorescent signal (Fig. 3.9 middle and right panels).

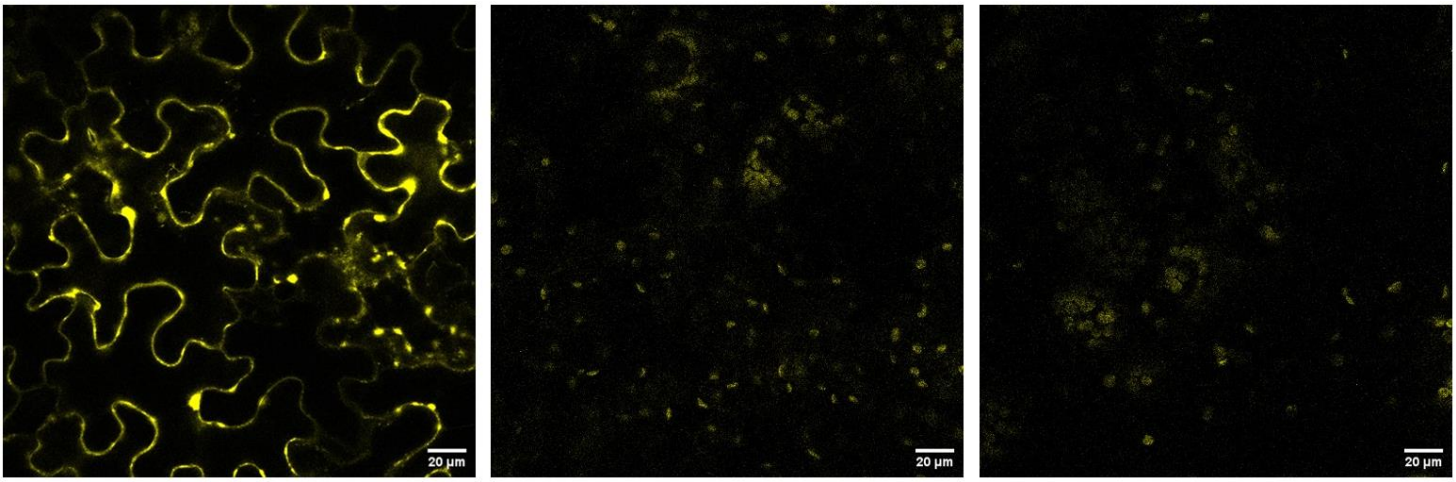


Fig. 3.9 BiFC controls. VAMP714 tagged with complementary YFP fragments was used as a positive control (left panel) and VPS29 which did not show an interaction (regardless the YFP fragments combinations) was chosen for the negative control (middle and right panels).

Interestingly, CTL1 and VAMP714 that were found to interact in FRET-FLIM experiment also showed a fluorescent signal when co-expressed with complementary YFP fragments (Fig. 3.10, upper panel). Note that the signal is lost when the same proteins are fused to non-complimentary fragments (Fig. 3.10, lower panel). The fluorescence is observed in punctate structures suggesting that the interaction occurs in specific cell compartments.

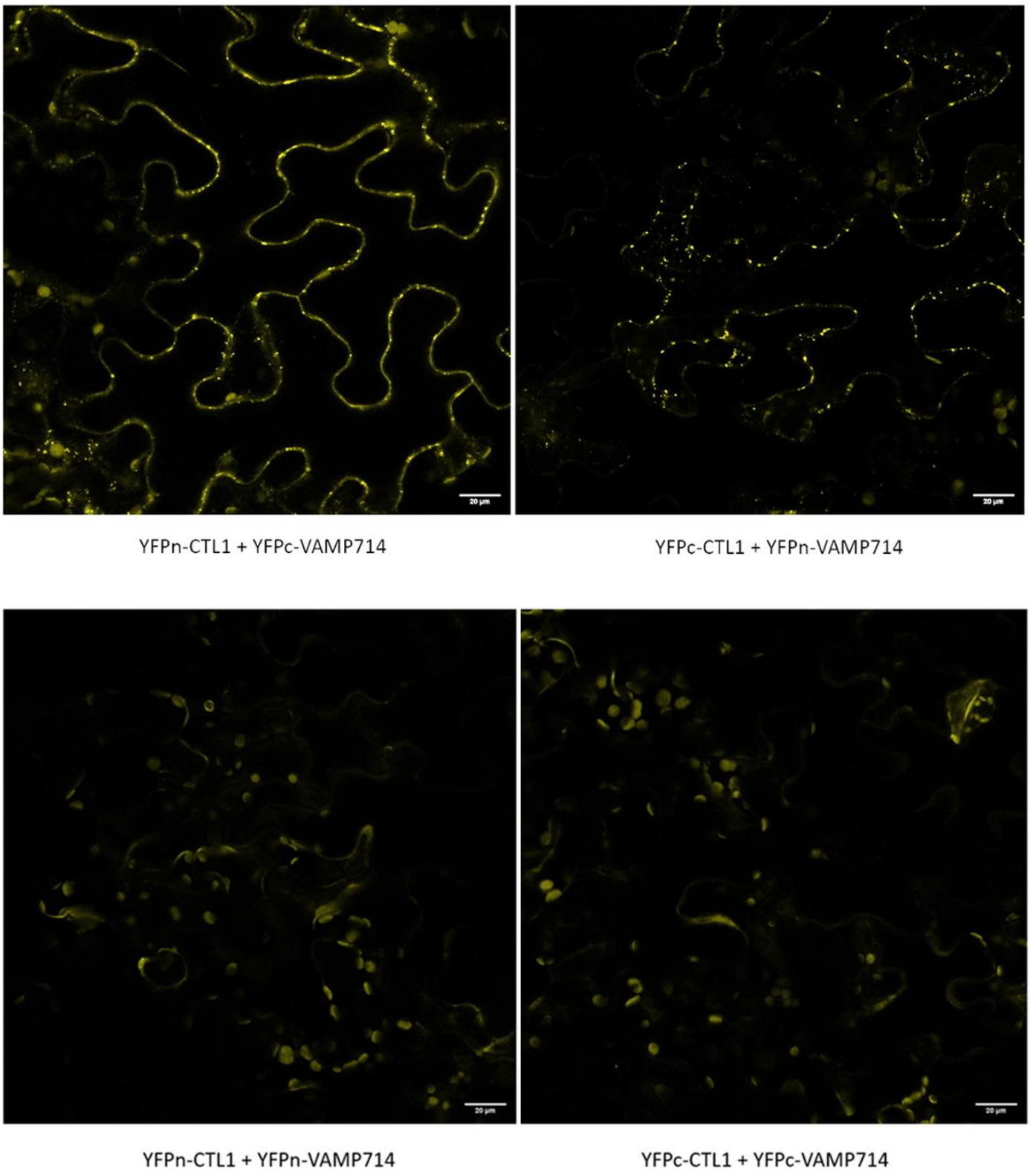


Fig. 3.10 VAMP714 and CTL1 interact within a specific cell compartment. The different combinations showing an interaction are presented in the upper panel and the negative controls are presented in the lower panel.

Furthermore, ARA7 that was shown to interact with VAMP714 through FRET-FLIM measurements also interacts in the BiFC analysis (Fig. 3.11). As observed for CTL1, the interaction occurs in punctate compartments and the signal was lost when non-complementary fragments of YFP were used as a tag for VAMP714 and ARA7.

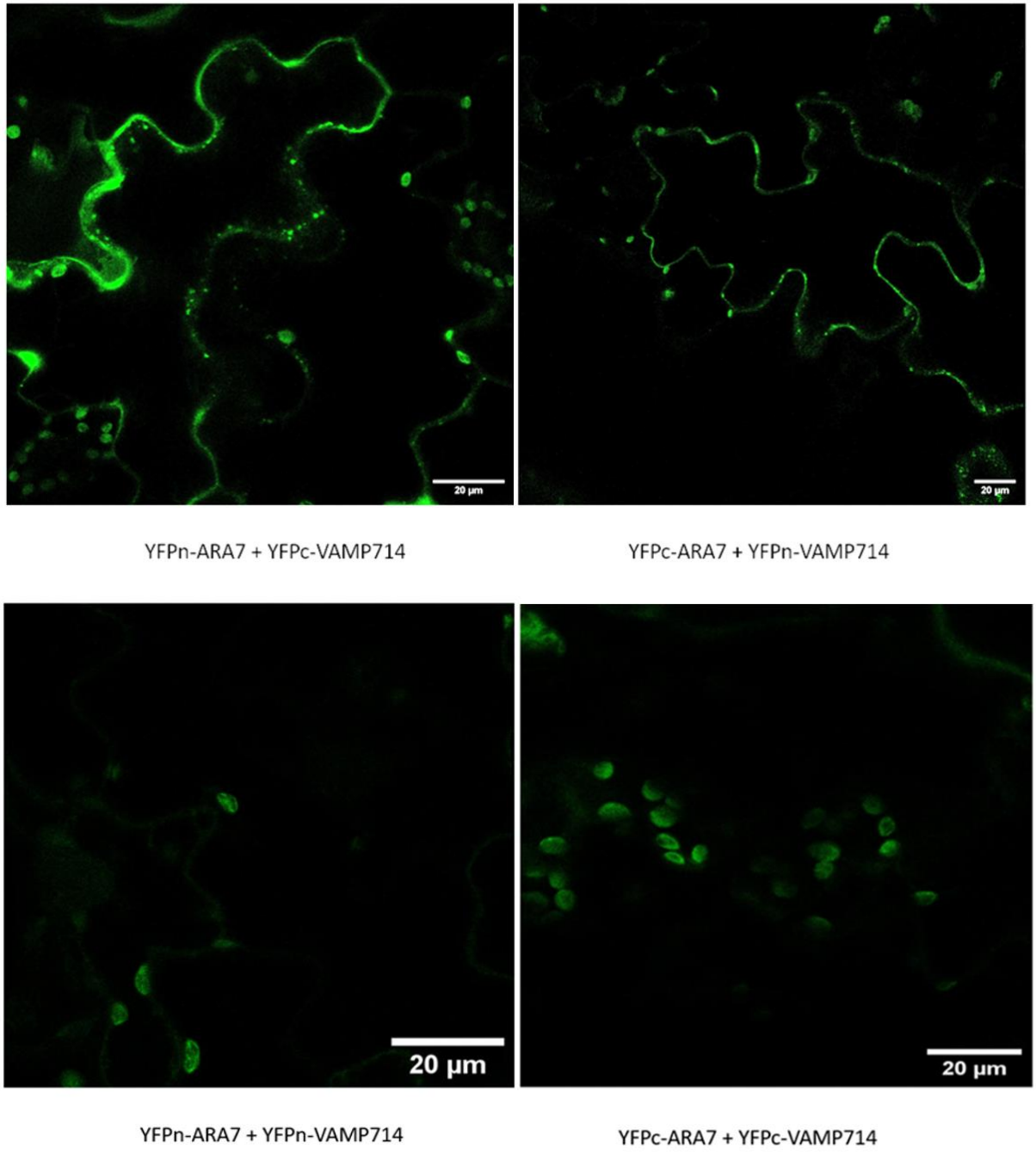


Fig. 3.11 BiFC assay shows an interaction between ARA7 and VAMP714. The different combinations showing an interaction are presented in the upper panel and the negative controls are presented in the lower panel.

The exocyst subunit EX070A1 was not tested in FRET-FLIM experiments but displays a fluorescent signal in BiFC analyses (Fig. 3.12). Here again, the interaction takes place in discrete and punctate cell compartments.

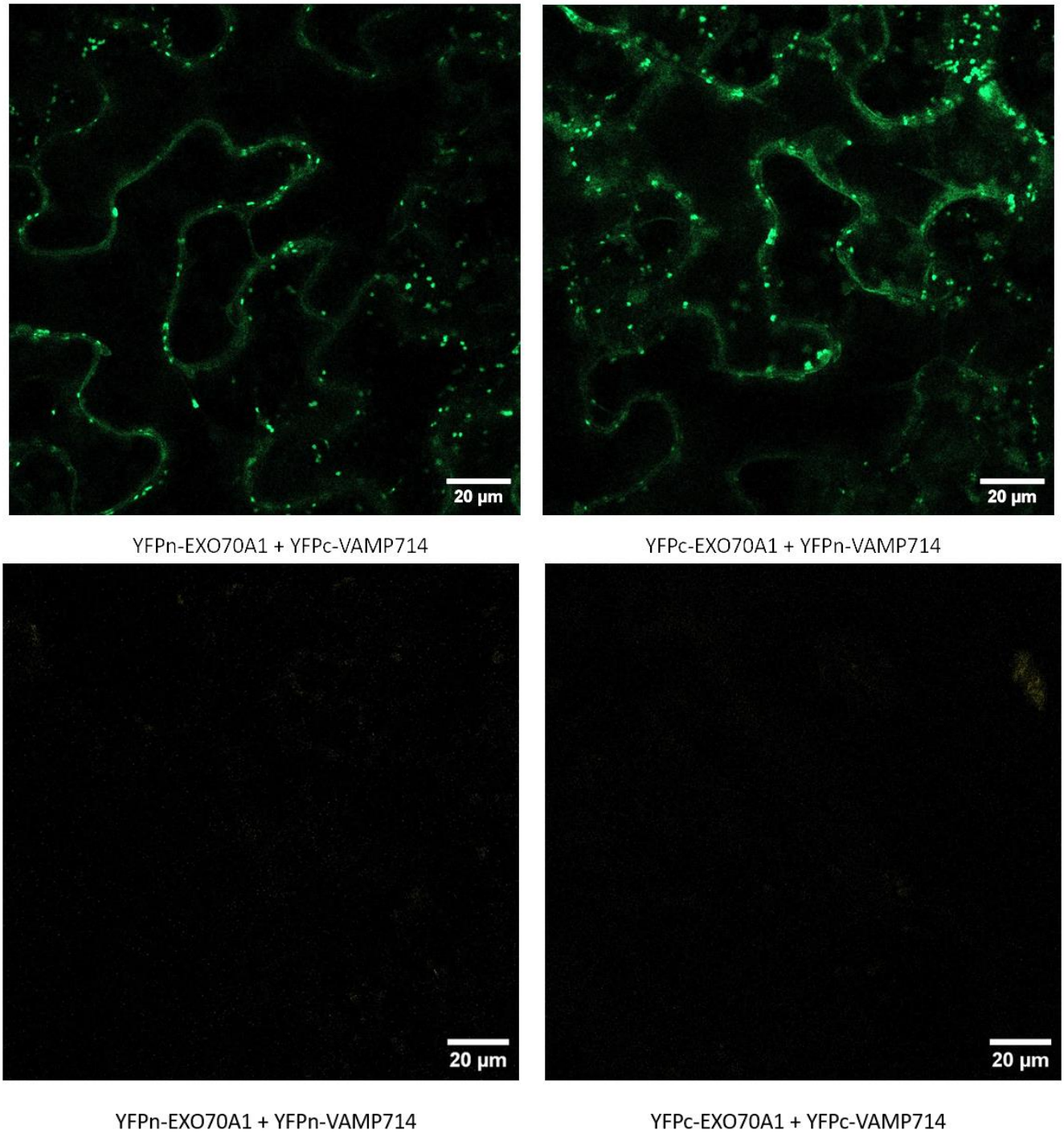


Fig. 3.12 EX070A1 and VAMP714 interact in BiFC assay. The different combinations showing an interaction are presented in the upper panel and negative controls are presented in the lower panel.

Finally, a BiFC interaction was observed when BEX5 and VAMP714 were expressed together (Fig. 3.13). The fluorescent pattern was similar to those observed for other candidate interactors.

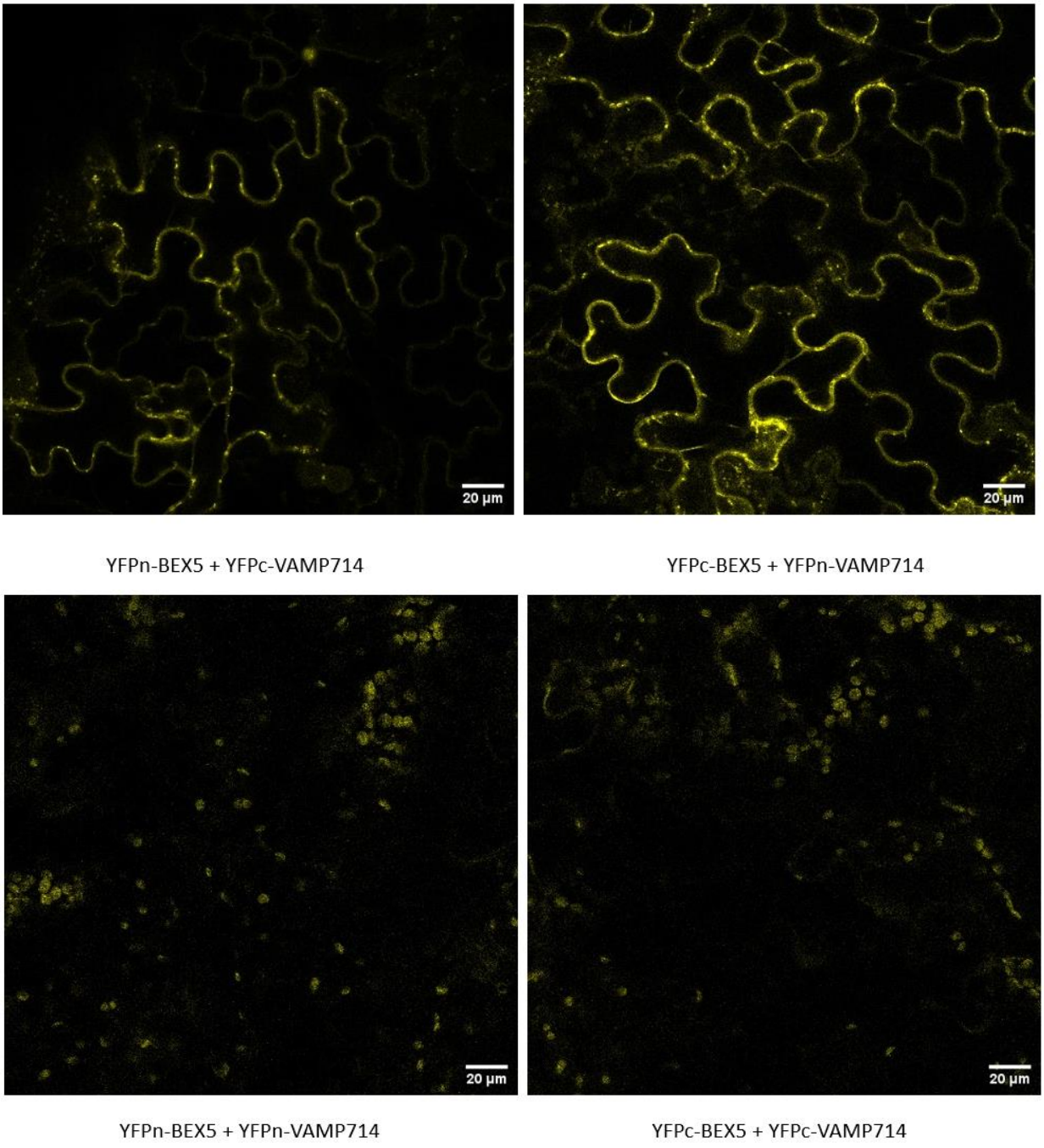


Fig. 3.13 BEX5 and VAMP714 interact in BiFC assay. The different combinations showing an interaction are presented in the upper panel and negative controls are presented in the lower panel.

3.5.4 Summary of PPI analysis

In order to better understand the relationship between the different regulators of PIN vesicle trafficking, the candidate proteins were tested for PPI. Here above, only the positive interactions were presented for easier reading. However, with the exception of EXO70A1 FRET-FLIM measurements, all candidate interactors were tested with two methods and the outcomes are summarized in Table. 3.1. Of the four candidates identified as interactors of VAMP714 in BiFC assays, only two interacted in FRET-FLIM measurements. This could be explained because the angle of the fluorophore is critical for FRET success. In order to allow an optimal energy transfer, the fluorophores need to be aligned in parallel (Strotmann and Stahl 2022). Such a parameter being difficult to control could explain the differences in results between PPI measurement methods. On the other hand, BiFC experiments tend to give false positives when not carried out with care. Here, the fluorescence was observed in specific cellular compartments which suggests that complementation of free complementary fragments of YFP is not likely to be responsible for the signal (soluble YFP localizes in the cytosol). However, close proximity between proteins within small-sized organelles could explain this signal but that would be in contradiction with the absence of signal for proteins that colocalize with VAMP714, i.e. VPS29 used as a negative control. Because it is good practice to validate PPI with independent methods, CTL1 and ARA7 are the candidates retained as interactors of VAMP714. Also, note that both methods used are indicative of very close proximity between two proteins rather than a strict measure of a physical interaction.

Name	FRET-FLIM	BiFC	Function
VPS29	NO	NO	Component of the retromer complex
BEX5	NO	YES	Rab-GTPase
VAN4	NO	NO	Rab-GEF
GNOM	NO	NO	ARF-GEF
EXO70A1	Not tested	YES	Exocyst subunit
CTL1	YES	YES	Choline transporter
ARA7	YES	YES	Rab5-GTPase

Table 3.1 Summary of the PPI experiments of VAMP714 with the different candidate interactors.

3.6 VAMP714 interactors also interact with each other in a specific subcellular compartment

The function of ARA7 and CTL1 and their interaction with VAMP714 within similar

structures suggest that they could also interact with each other. Indeed, CTL1 and ARA7 displayed a BiFC signal when co-expressed in *N. benthamiana* epidermal cells. This signal was concentrated in the same kind of compartment previously observed in the interaction with VAMP714 (Fig. 3.14, upper). Even though the interaction with EXO70A1 has not been tested with FRET-FLIM, the protein was selected for interaction with ARA7 since the two proteins have been co-precipitated in a proteomic experiment in *Arabidopsis* (Heard et al. 2015). Here again, a signal was detected in punctate structures (Fig. 3.14 lower). At least one combination of the controls and the interactions are represented in Fig. 3. 14 (additional combinations are presented in Appendix 3).

The nature of the compartment in which the interaction occurs is not known with certainty. While ARA7 is commonly used as a marker of late endosomes and prevacuolar compartments (PVC), it has also been shown that the TGN marker VHA-a1 is present in ARA7 proteome (Heard et al. 2015). As for CTL1 and VAMP714, they have a broad expression pattern, respectively in the TGN, PM and PVC and Golgi, PM and ER. However, taken together the data suggest that regulators of PIN trafficking interacting with VAMP714 also interact with each other in the same compartment post-Golgi trafficking.

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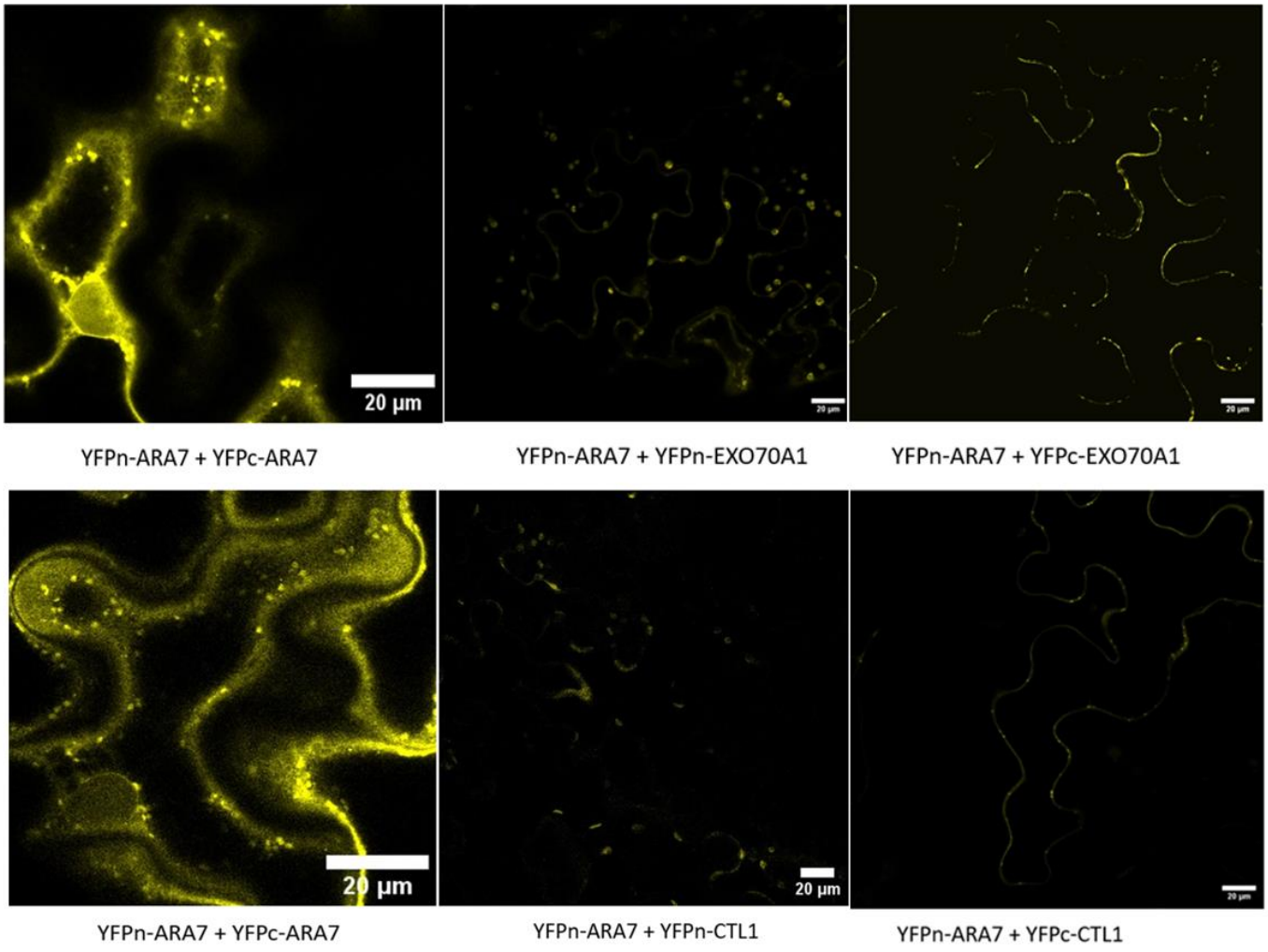


Fig. 3. 14 BiFC assays show an interaction between CTL1/EXO70A1 and ARA7 and in punctate structures. The upper panel shows the fluorescent signal observed for the positive control, the negative control and the interaction of CTL1 with ARA7. The lower panel represents the fluorescent signal observed for the positive control, the negative control and the interaction between EXO70A1 with ARA7.

3.7 VAMP714 colocalizes but does not interact with its cargo PIN1 in FRET FLIM measurements

So far, this research has focused on finding interactions between regulators of PIN vesicle trafficking and other proteins of interest**.** Surprisingly, where effects of trafficking regulators on PIN cycling have been investigated, only a few studies have investigated the interaction of those regulators with their cargo. In previous work, it was hypothesized that VAMP714 and PIN are present in the same vesicles allowing the proper localization of the auxin efflux

carriers (Gu et al. 2021). That hypothesis was based on the observation that VAMP714 localizes in moving vesicles and behaves like PIN in response to trafficking inhibitors BrefeldinA and LatrunclinB. In order to further examine this possibility, VAMP714 and PIN1 were used for PPI measurements. When co-expressed in *N. benthamiana* epidermal cells the two proteins colocalize in membranous structures both inside and at the periphery of the cells (Fig. 3.15).

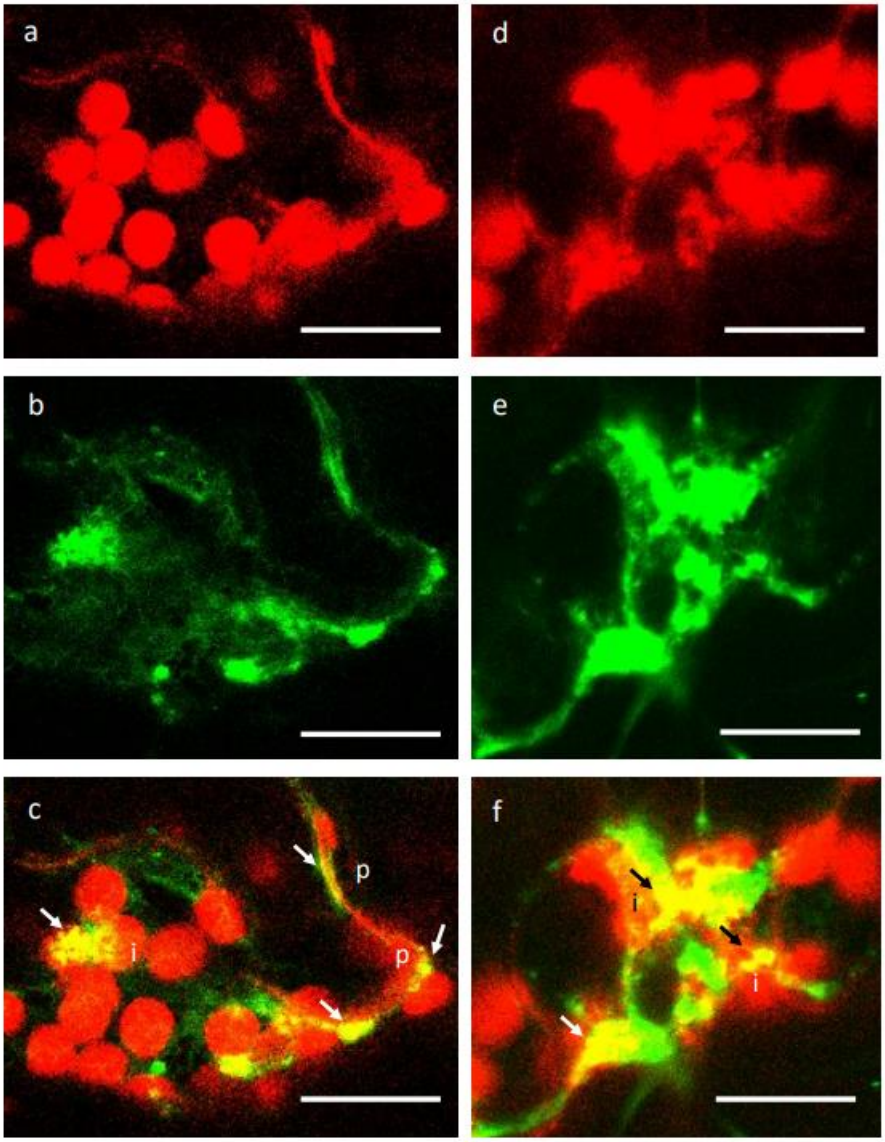


Fig. 3.15 VAMP714 and PIN1 colocalize in membranous structures inside and at the periphery of the cell. Regions of cells expressing VAMP714:mCherry are represented in the upper panels while the same regions expressing PIN1:GFP are represented in the middle panels. Merged images are displayed in the bottom panels. Scale bars 25 μm, arrows show the colocalization, i: internal membranes, p: membrane at the periphery of the cell.

However, FRET-FLIM measurements indicate that PIN1 and VAMP714 do not interact. Indeed, no important changes could be observed when VAMP714:mCherry was expressed together with PIN1:GFP (Fig. 3.16). The topology of the proteins did not allow to carry out BiFC assays to further investigate the interaction between VAMP714 and PIN1.

PIN1 is thought to be a cargo of vesicles containing VAMP714 (Gu et al. 2021). Proteomic experiment on the SYP61 SNARE have shown that it interacts with its cargo (Drakakaki et al. 2012). Therefore, it would be interesting to use alternative PPI techniques. However, it is possible that SNAREs do not interact with all their cargos.

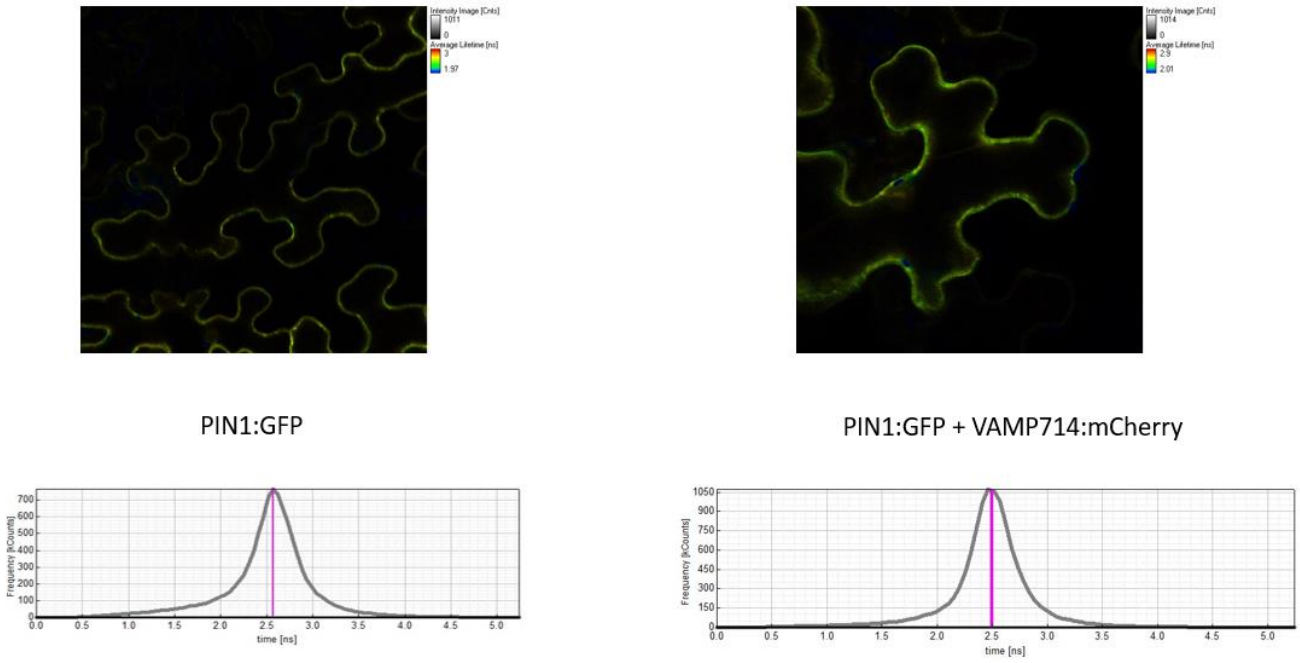


Fig. 3.16 PIN1 and VAMP714 do not interact in FLIM-FRET measurements. No difference in lifetime was observed by comparing the beatmap of the lifetime (upper panel) or the distribution of photon counts between PIN1:GFP alone and PIN1:GFP co-expressed with VAMP714:mCherry (lower panel).

3.8 Choline regulates VAMP714 subcellular localization in the meristematic zone of the root

The identification of interactors of VAMP714 raises the question of why these proteins are interacting. It is possible that ARA7 and potentially EXO70A1 are part of a complex of proteins that together with VAMP714 help in targeting the appropriate cellular compartment for vesicle fusion. Indeed, such complexes where tethering factors Rab-GTPase and SNAREs interact together have already been described (Shi et al. 2023). However, the reason for the

interaction between VAMP714 and choline transporter CTL1 could be more subtle. The role of CTL1 in trafficking of PIN (Y. Wang et al. 2017) but also ion transporters (Gao et al. 2017) has been demonstrated in the literature but the mechanism by which this happens remains poorly understood. Nonetheless, choline transport mediated by CTL1 appears to have a central role in its function in regulating vesicle trafficking. It is thought that the loss of function mutation of *CTL1* leads to an increase of choline in the cytosol which blocks the activity of the phosolipase D that normally cleaves the choline head of phospholipids (Gao et al. 2017). This results in a change in the abundance of choline bound phospholipid facing the cytosol and could be the reason of the vesicle trafficking defects observed in *ctl1*. The disruption of vesicle trafficking in response to an increased concentration of cytosolic choline supports this hypothesis (Gao et al. 2017). Here I investigate if choline treatment affects VAMP714 localization. 7 day-old *Arabidopsis* seedlings transgenic for proVAMP714::VAMP714:GFP were treated with 1 mM of choline for 1h. Strikingly, this resulted in an accumulation of VAMP714 in aggregates in the meristematic zone of the root suggesting that choline homeostasis is required for proper VAMP714 localization (Fig. 3.17).

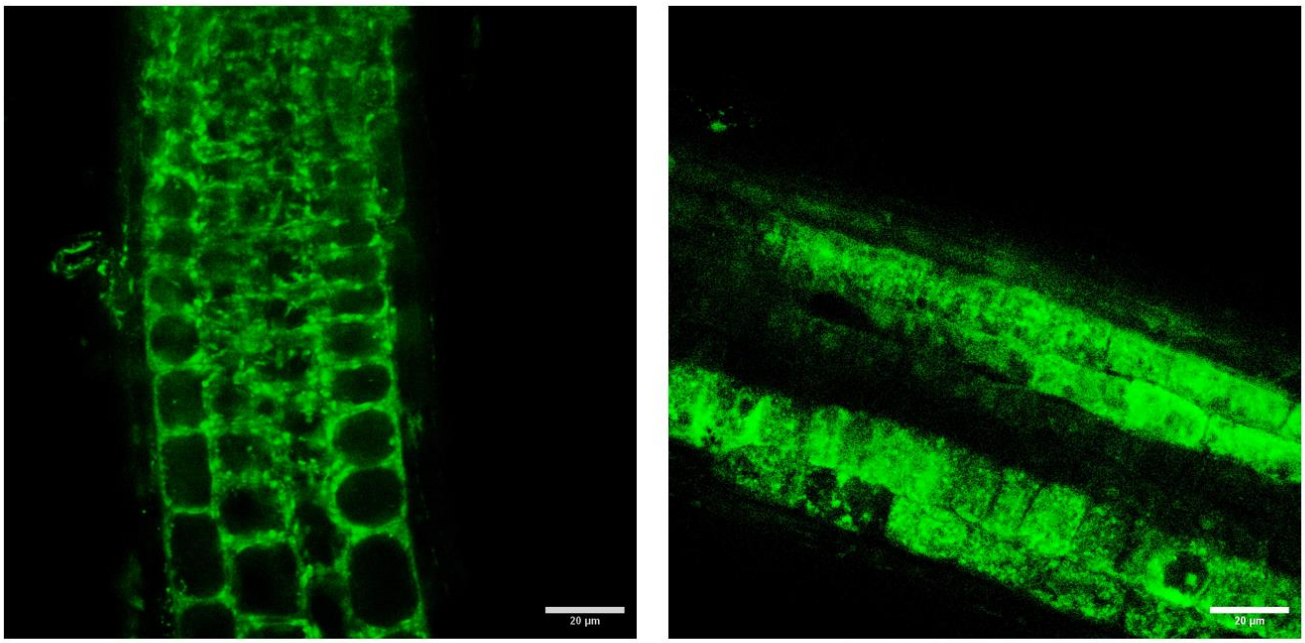


Fig. 3.17 Choline treatment disrupts VAMP714 localization in cells of the root meristem. The image was taken in the root apical meristem just below the root tip (the images are upside down, in other words the root tip is located above the image on the left-hand side and in the upper left corner of the image on the right-hand side). Non-treated *Arabidopsis* roots expressing VAMP714:GFP (left) compared to plants from the same line incubated with 1 mM choline for one hour (right).

In order to determine if this mechanism is to some extent specific to certain proteins and especially to auxin transport proteins, plant expressing tagged versions of the auxin efflux carriers PIN1 and PIN2 were treated with 1 mM choline. Interestingly, choline had no apparent effect on PIN2 distribution (Fig. 3.18 upper panel), while intracellular abundance of PIN1 was increased following choline treatment (Fig. 3.18 lower panel). This is consistent with previous observation that PIN1 and PIN3 recycling in the stele of the meristematic zone decreased drastically in *ctl1* plants whereas PIN2, mainly present in the epidermis, was not affect by the mutation (Feraru et al. 2011). This suggests at least a partial difference in the mechanism by which PINs are regulated and this difference is tissue specific.

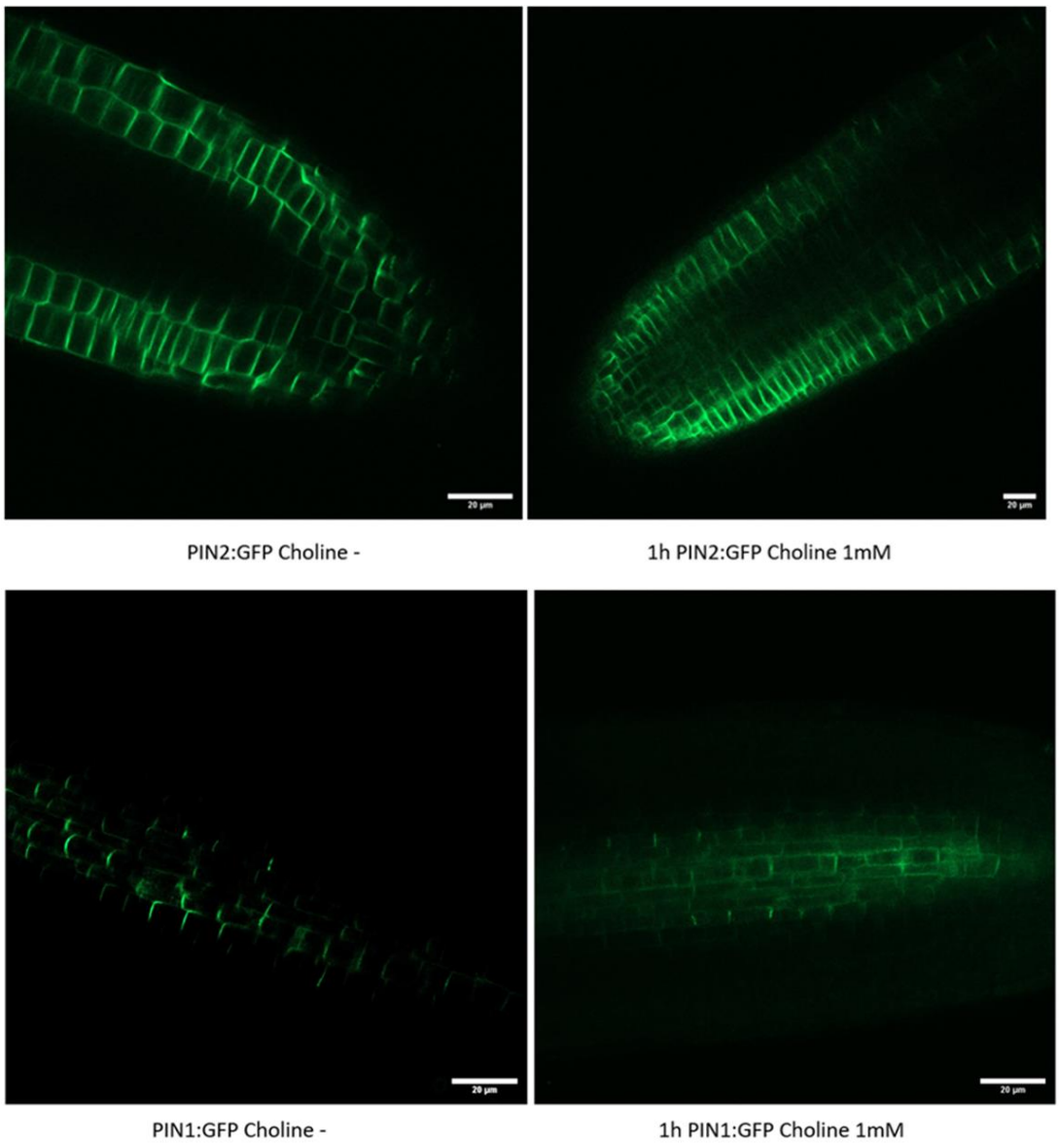


Fig. 3.18 PIN transporters respond differently to choline treatment. The upper panel represents the expression of PIN2:GFP with (left) or without (right) choline treatment. The lower panel shows the effect of choline on PIN1:GFP distribution with (left) or without (right) choline treatment.

3.9 Isolation of VAMP714 proteome

3.9.1 Immunoprecipitation of VAMP714

For the purpose of isolating proteins that physically bind VAMP714 in a complementary approach to microscopy based PPI, VAMP714:mCherry was immuniprecipitated using nanobody-covered beads. To achieve this, the tagged protein was expressed in *N. benthamiana* based on the assumption that the trafficking machinery of *Arabidopsis* is similar to that in tobacco plants. If it would be naïve to exclude potential species-specific differences, examples from the literature are showing no "propensity for aberrant localization of proteins" in Arabidopsis vs *N. benthamiana* comparisons (Bally et al. 2018). The advantage of using *N. benthamiana* is that it is easier to extract a large amount of protein. mCherry-specific antibodies were first tested on total protein extracts to ensure the presence of the tagged protein (Fig. 3.19 A). A 50 kDa band was observed which correspond to the combined VAMP714 (25 kDa) and mCherry (26 kDa). The proteins were then extracted in non-denaturing conditions for pull-down. VAMP714 was identified in the eluate fraction proving the protein binding to the beads (Fig. 319 C). A negative control for mass spectrometry (MS), the mCherry tag, was also precipitated and tested on a western blot (Fig. 3.19 B).

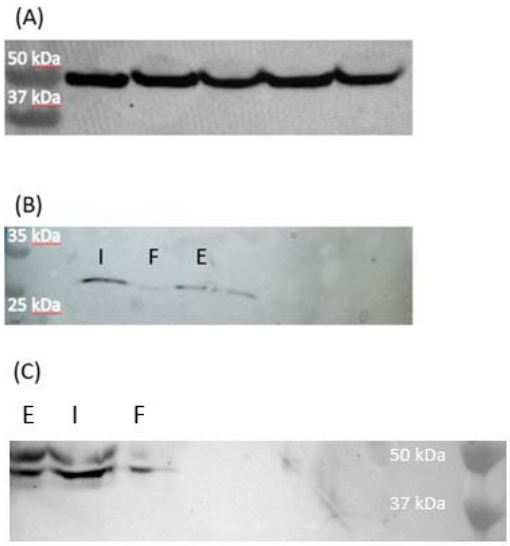


Fig. 3. 19 Immunoprecipitation of VAMP714:mCherry and the MS control mCherry. VAMP714:mCherry was identified on a western blot, the different bands represent different amounts (from left to right, 2 samples of 50 µg, one sample of 30 µg, and 2 samples of 40 µg of total protein extract) of the same protein (A), negative control for mass spectrometry, mCherry (B) , and the protein of interest, VAMP714:mCherry (C), were immunoprecipitated. E eluate, F flowthrough and I input.

3.9.2 Mass spectrometry

The immuno-precipitated VAMP714:mCherry and mCherry tag samples (3 repeats each) were used for LC (liquid chromatography)-MS. The mCherry control was used as a filter to remove proteins precipitated because of the binding to the tag from the VAMP714:mCherry proteome. Despite being precipitated in the same conditions most samples showed contamination in their chromatogram (Fig. 3.20).

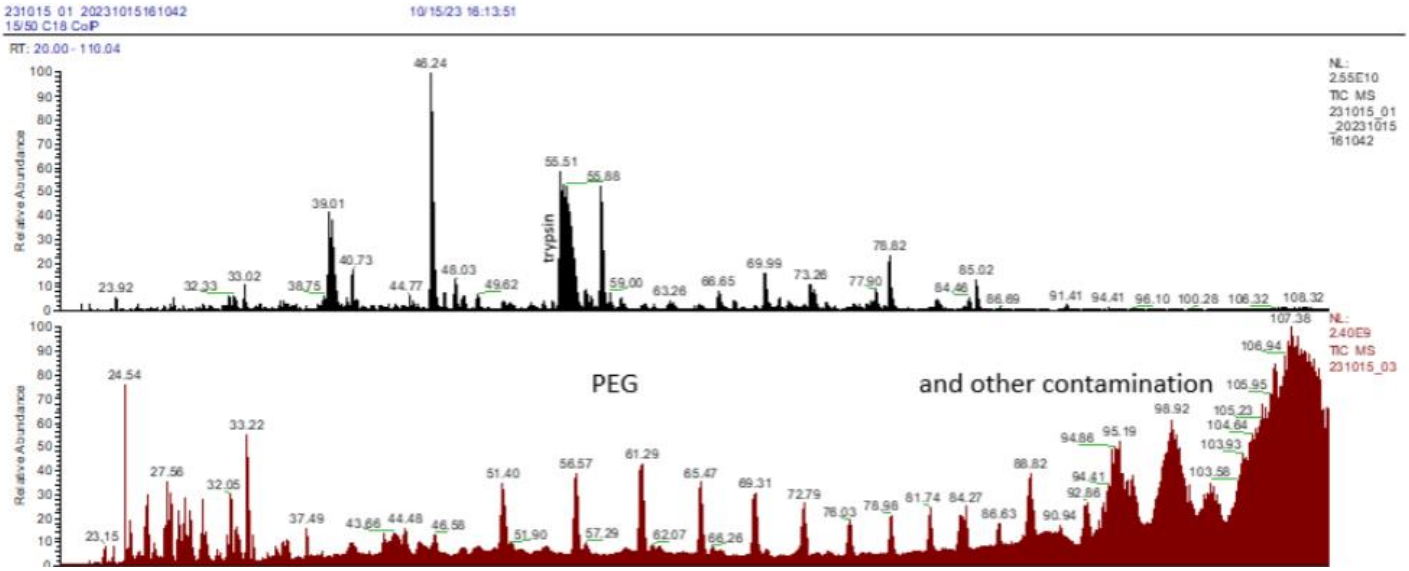


Fig. 3.20 Chromatogram analysis show contamination in precipitated samples. A chromatogram of a non-contaminated VAMP714:mCherry sample (upper panel) compared to a contaminated VAMP714:mCherry sample (lower panel). PEG, polyethylene glycol.

Both VAMP714:mCherry and mCherry baits were found in the proteome of VAMP714:mCherry probably because of the degradation of the fusion protein. Therefore, the tag cannot be used as a filter to identify VAMP714-specific interactors. However, VAMP714:mCherry proteome was still used to look for vesicle trafficking proteins. Surprisingly, few of those proteins were identified. Neither CTL1 nor ARA7 were found. Also, in the top 40 of the most abundant proteins, many chloroplastic proteins were identified (Fig. 3.21) which is not consistent with subcellular of the VAMP714. Because the samples were extracted from *N. benthamiana* leaves rich in plastids, it could explain a certain level of contamination with plastidic proteins. All together, these results suggest that the VAMP714 proteome was not isolated successfully.

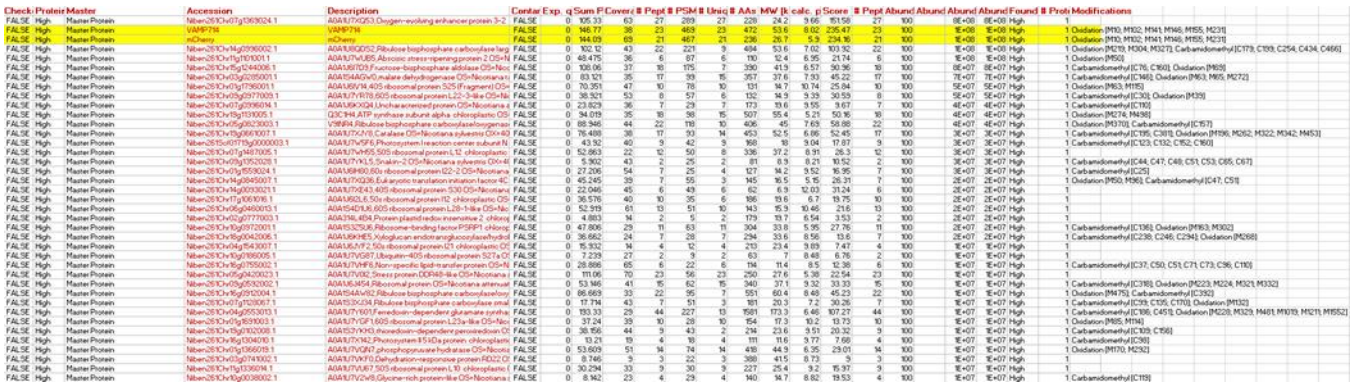


Fig. 3.21 Top 40 of the most abundant proteins precipitated with VAMP714:mCherry. Proteins that do not belong to *N. benthamiana* are highlighted in yellow.

3.9 Summary

This chapter aimed to identify proteins that could belong to the same vesicle trafficking pathway of PIN proteins as VAMP714. It was hypothesized that proteins of a same pathway are likely to physically interact with each other. Therefore, a list of candidate interactors involved in PIN trafficking and expressed in the same tissues and cellular compartments as VAMP714 were selected. Candidate proteins from *Arabidopsis* were expressed transiently in *N. benthamiana* and all showed at least partial overlaps in their subcellular localization. PPI measurements using FRET-FLIM and BiFC allowed to retain ARA7 and CTL1 as VAMP714 interactors. ARA7 is a RAB5-GTPase involved in PM membrane endocytosis and endosomal trafficking pathways whereas CTL1 is a TGN-located choline transporter and regulator of vesicle trafficking. Interestingly, observed interactions occur in yet-to-be-determined punctate subcellular compartments. Moreover, ARA7 and CTL1 also interact together in similar compartments in BiFC experiments. This suggests that VAMP714 and its interactors regulate PIN recycling from internal cellular compartments which is consistent with the previous observation that VAMP714 cycles between internal membranous compartments and the PM in PIN-containing vesicles. However, the current PPI results do not show an interaction between VAMP714 and its cargo PIN1. Unlike ARA7, CTL1 is not a canonical vesicle trafficking machinery component despite being associated with vesicle transport (likely through the regulation of membrane composition). Therefore CTL1-VAMP714 relationship was subject to further investigations. The results indicate a role choline in VAMP714 localization at the root tip. Proteomic analysis did not allow to identify vesicle trafficking

interactors of VAMP714 but this is likely the consequence of technical issues in the immune-precipitation step.

Chapter 4: VAMP714 mediated trafficking and cell wall integrity

4.1 Introduction

All plant cells are all encased by cell walls. Plant cell walls provide structural support for the plants but also protection against biotic (Sato, Kadota, and Shirasu 2019) and abiotic stresses (Gall et al. 2015). The composition of cell walls varies during growth and the development to allow different cell types to fulfil their function (Somssich et al., 2016). In plant roots, the different zones are characterized by cell walls with different physical properties (Somssich et al., 2016). A well-illustrated example is the difference between the flexible cell wall of the elongation zone and the rigid cell wall of the mature cells in the differentiation zone. Interestingly, while hemicelluloses and pectin are synthesized in the Golgi and then secreted (Sinclair et al., 2018), cellulose is produced by large cellulose synthase complexes subject to trafficking to perform their function at the plasma membrane (Bashline et al., 2014). This suggests that vesicle trafficking machinery has a central role in the regulation of cell wall composition. Indeed, it has been shown SNAREs, EXOCYST complex and RAB-GTPases mediate cell wall deposition (Sinclair et al., 2018). More interestingly, cellulose allows the maintenance of PINs in their polar domain, showing a previously poorly studied link between cell wall and auxin-regulated processes such as gravitropism (Feraru et al. 2011). This chapter aims to investigate if VAMP714 plays a role in the trafficking of cell wall material, how this happens and does it affect PIN stability at the plasma membrane?

4.2 Transcriptomic and proteomic data link VAMP714 and cell wall regulation

"Omics" studies represent a popular approach to understand biological changes in single cells, tissues or organisms at a large scale. Here, the transcriptome of 7 days old seedlings of Columbia (Col-0) wild-type plants was compared with the transcriptome of *vamp714* plants grown in the same conditions using RNA-sequencing. Among the genes of interest, cell wall regulating genes that were differentially regulated were all downregulated. Cell wall organization and biogenesis was one of the most represented group of genes in the biological process (BP) gene ontology category with 68 downregulated genes (Fig. 4.1). A reduced level of transcripts was measured for all the genes involved in the regulation of all the of primary cell wall components namely cellulose, hemicelluloses and pectin (list of differentially regulate genes available in Appendix IV). While the effect of the mutation of the *VAMP714*

gene on the transcription of cell wall related genes is very likely to be indirect, the trend observed suggests a requirement for VAMP714 in cell wall homeostasis.

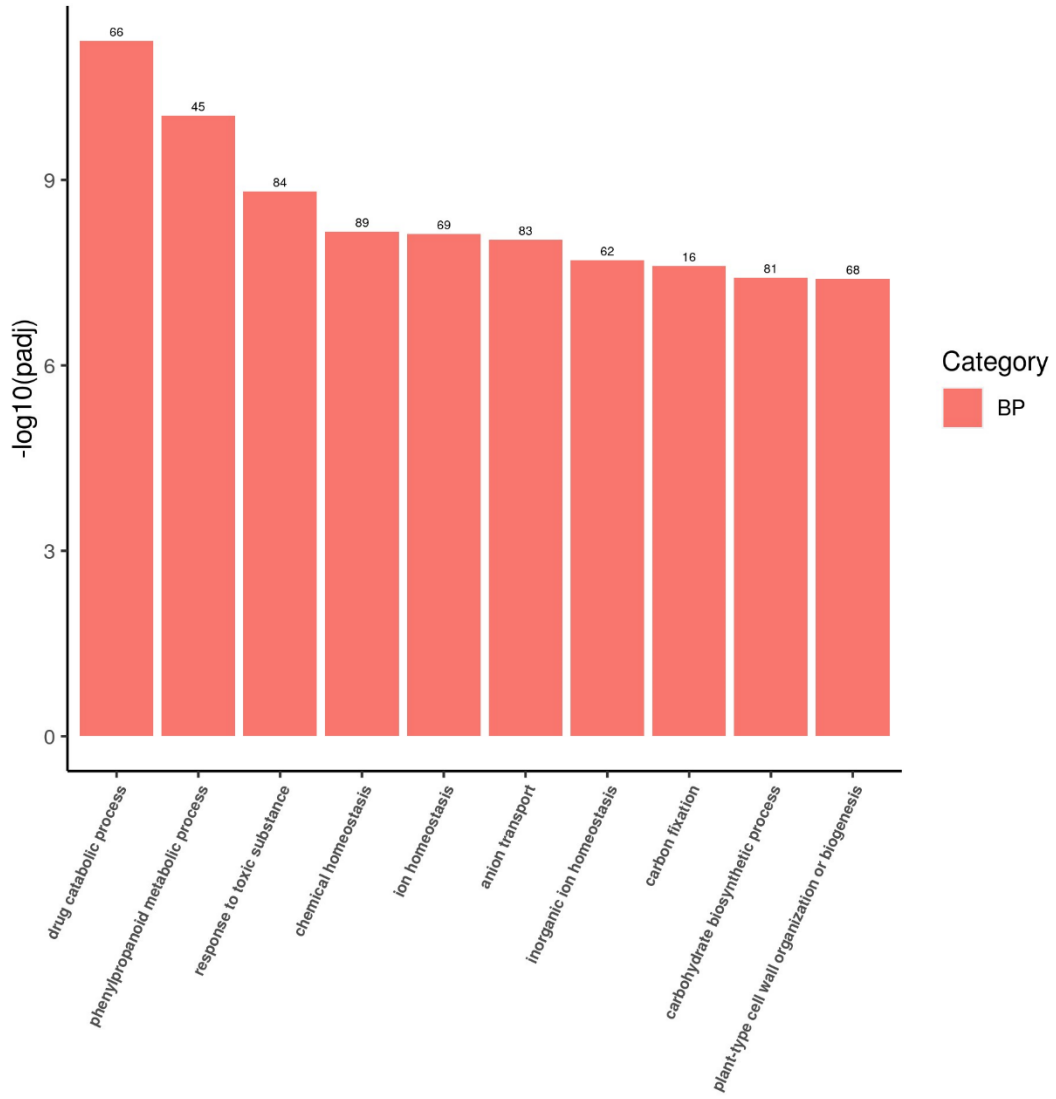


Fig. 4.1 RNA-seq results show a downregulation of cell wall-related genes in 7 day-old *vamp714* plants when compared to Col-0. BP category stands for biological process. The p-values are adjusted (values greater than the cut-off 0.05 were trimmed) and are represented in a base 10 logarithm form for clarity.

A proteomics experiment carried out at the Sainsbury lab in Norwich by Heard et al. (2015) and later confirmed by Postma (2018) further indicates a link between VAMP714 and the cell wall by showing that VAMP714 was precipitated together with cellulose synthases CESA3, CESA1 and CESA6 in ARA7-specific compartments. CESA1 and CESA3 have been shown to always occupy the same position in the cellulose synthase complex and have no

redundancy with other CESAs (Desprez et al. 2007). CESA3 is particularly interesting since it contributes to the maintenance of PIN polarity (Feraru et al. 2011). More interestingly, ARA7 was previously shown (see Chapter 3) to interact with VAMP714 in a specific cellular compartment, possibly the same compartment that also contains those CESAs.

Taken together the proteomic and transcriptomic data suggest that VAMP714 could be a molecular actor of the vesicle trafficking-mediated regulation of cell wall compositon.

Bait	Prey	PreyGene	Spec	SpecSum	AvgSpec	NumReplic	ctrlCounts	AvgP	MaxP	TopoAvgP	TopoMaxP	SaintScore	FoldChange	BFDR
RFP-RABF2b/ARA7	AT5G05170	AT5G05170	6|15|13	34	11.33	3	3|1|1|2|1	0.98	1	0.98	1	0.98	8.5	0
RFP-RABF2b/ARA7	AT5G64740	AT5G64740	2|3|6	11	3.67	3	2|2|1|2|0	0.46	0.95	0.46	0.95	0.46	2.75	0.24
RFP-RABF2b/ARA7	AT4G32410	AT4G32410	3|11|11	25	8.33	3	0|2|2|1|1	0.82	1	0.82	1	0.82	7.14	0.02

Fig. 4.2 Cellulose synthases immunoprecipitated with RFP-ARA7. CESA3 (AT5G05170), CESA6 (AT5G4740) and CESA1 (AT4G32410) are present in ARA7 proteome.

4.3 VAMP714 mutation has no apparent effect on the dry mass

Fractionation of the alcohol insoluble residue (AIR) is typically used prior to cell wall (CW) analysis (Fangel et al. 2021). This process consists of treating plants with ethanol or methanol/chloroform to remove nucleic acids, small free saccharides, lipids and pigments to provide dry samples enriched in cell wall polysaccharides and structural proteins (Fangel et al. 2021). Here, it was reasoned that if VAMP714 is a positive regulator of cell wall composition the weight difference could be measured in the AIR fraction when *vamp714* plants are compared to wild type plants. Similar comparisons are available in the literature (Yuenyong et al. 2019) that also looked at the change in fresh weight. According to these data the dry weight of wild type seedlings is approximately 10% of the fresh weight whereas our measurements indicate that the dry fraction represents approximately 6% of the fresh weight. The comparison in the AIR weight obtained from 50 mg of fresh 10 day-old seedlings showed no significant difference between the genotypes (Fig. 4.3). A suprisingly high dry weight for the *vamp714* mutants was seen

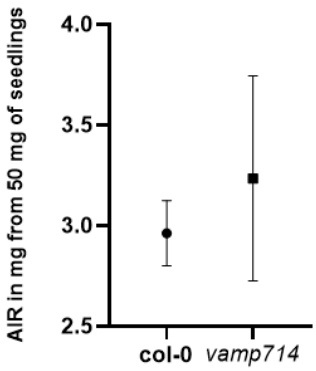


Fig. 4.3 There is no significant difference in dry weight between Col-0 and *vamp714* mutants. N=4

4.4 VAMP714 is involved in cellulose deposition in the cell wall

Cellulose is the main structural component of the cell wall and accounts for most of its mechanical strength (Rongpipi et al. 2019). The chemical composition of cellulose consists in molecules of glucose linked to each other by β-1,4-glycosidic bonds (Rongpipi et al. 2019). In addition to the covalent bounds, hydrogen bonds and Van der Waals forces between the chains of glucose give cellulose a crystal-like structure named cellulose microfibril. This crystalline structure makes cellulose harder to decompose than other polysaccharides (Sumarno et al. 2021). The Updegraff method is commonly used for the quantification of glucose content (Updegraff 1969). This simple and robust method is a colorimetric assay using the carbohydrate condensing agent anthrone. A nitric-acetic reagent is used to remove hemicelluloses and lignin from the AIR (Dampanaboina et al. 2021) and the cellulose is subsequently hydrolysed into glucose with sulphuric acid (Fig. 4.4 A). Because remaining free glucose can interfere with the measurements, the last wash of the AIR was used to detect the presence of free glucose but no measurable levels of glucose were detected (data not shown). Interestingly, disruption of VAMP714 expression (in the *vamp714* mutant) leads to a significant reduction cellulose content (Fig. 4.4 C).

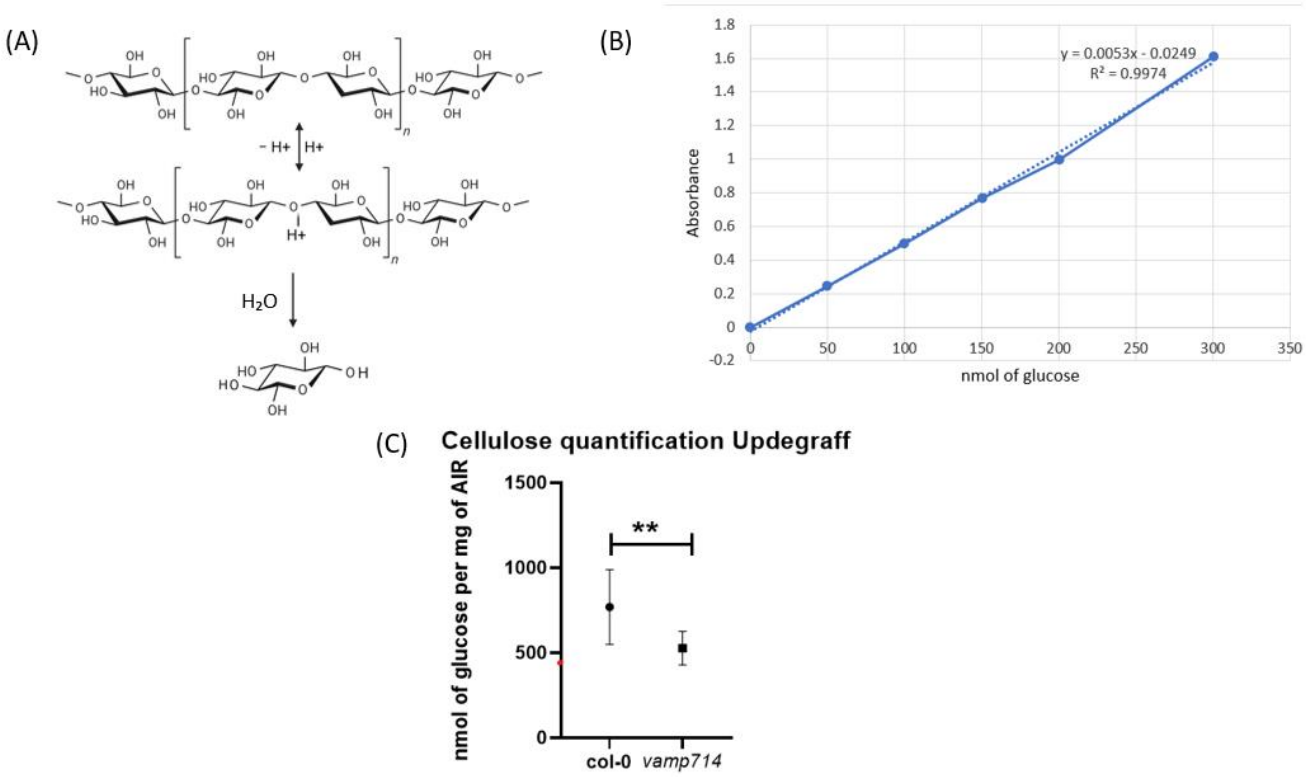


Fig. 4.4 Cellulose content is reduced in the *vamp714* mutants (A) Cellulose was hydrolysed in acidic conditions with sulphuric acid to generate monomers of glucose. (B) Unknown concentration of glucose in the samples was determined using a standard curve of known glucose concentrations and measuring the 620 nm absorbance in the presence of anthrone. (C) *vamp714* has a reduced amount of cellulose when compared to Col-0 in a Mann-whitney test. N=6, ** pval<0.01.

The amount measured in nmol of glucose per mg of AIR was converted to µg mg^{-1} (µg per mg of AIR) as usually presented in the literature. The median amount of cellulose in wild type plants of 117 µg mg^{-1} dropped to 100 µg mg^{-1} in the *vamp714* mutant corresponding to a 15% decrease in cellulose content. As a comparison, a similar value of 114 µg mg^{-1} of cellulose in wild type plants was measured for *Arabidopsis* leaf tissues (Rui and Anderson 2016). These results indicate a role of VAMP714 in controlling cellulose accumulation in the cell wall.

4. 5 VAMP714 disruption is associated with reduced hemicellulose content

Analysis of the *vamp714* transcriptome indicates a large diversity of cell wall components downregulated in response to mutation of the *VAMP714* gene (Fig. 4.5). The most affected group of genes is involved in the secondary cell wall formation (17 out of 68 downregulated genes). Interestingly, a large number of cell wall-resident proteins playing a role in cell wall

integrity and reinforcement, the extensins, and in cell wall loosening, the expansins, was detected.

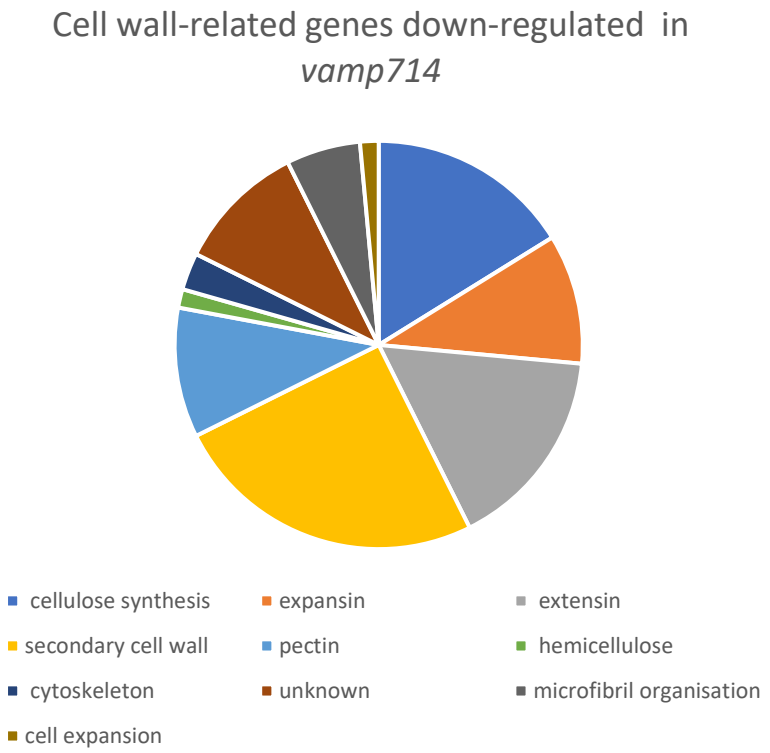


Fig. 4.5 Cell wall related genes downregulated in *vamp714* encode for proteins regulating a broad range of cell wall components.

The most affected gene function linked to the primary cell wall is associated with cellulose but pectins and hemicelluloses-related genes are also downregulated. The changes in cellulose due to the mutation of *VAMP714* raises the question of the effects of the mutation on the other components the primary cell wall. Cellulose microfibrils are embedded in a matrix of hemicellulosic and pectinic polysaccharides commonly referred as matrix polysaccharides (Sandhu et al. 2009). In order to evaluate their composition, matrix polysaccharides are usually broken down to monosaccharides that can be separated on a thin-layer chromatography (TLC) plate using enzymatic or acidic hydrolysis. Here, a monosaccharide fingerprint was obtained by hydrolysis of matrix polysaccharides with 2 M trifluoroacetic acid. 2.5 µl of hydrolysate (1% AIR (w/v)) was loaded on a TLC plate and migrated in EPAW solvent. For both 5 weeks old rosette samples and 10 days-old whole seedlings the fingerprint of WT samples was compared to *vamp714* samples (Fig. 4.6). Most monosaccharides did not show any observable differences. However, glucose level in both leaves and seedlings were lower for the *vamp714* plants. Slightly lower levels of galactose were also measured in leaves but not in seedlings suggesting a difference in matrix

polysaccharides composition between tissues or upon tissue maturation. A dominant negative transgenic of VAMP714 was also tested but proven later to be nonfunctional. The levels of monosaccharides in these plants were similar to those of the WT samples.

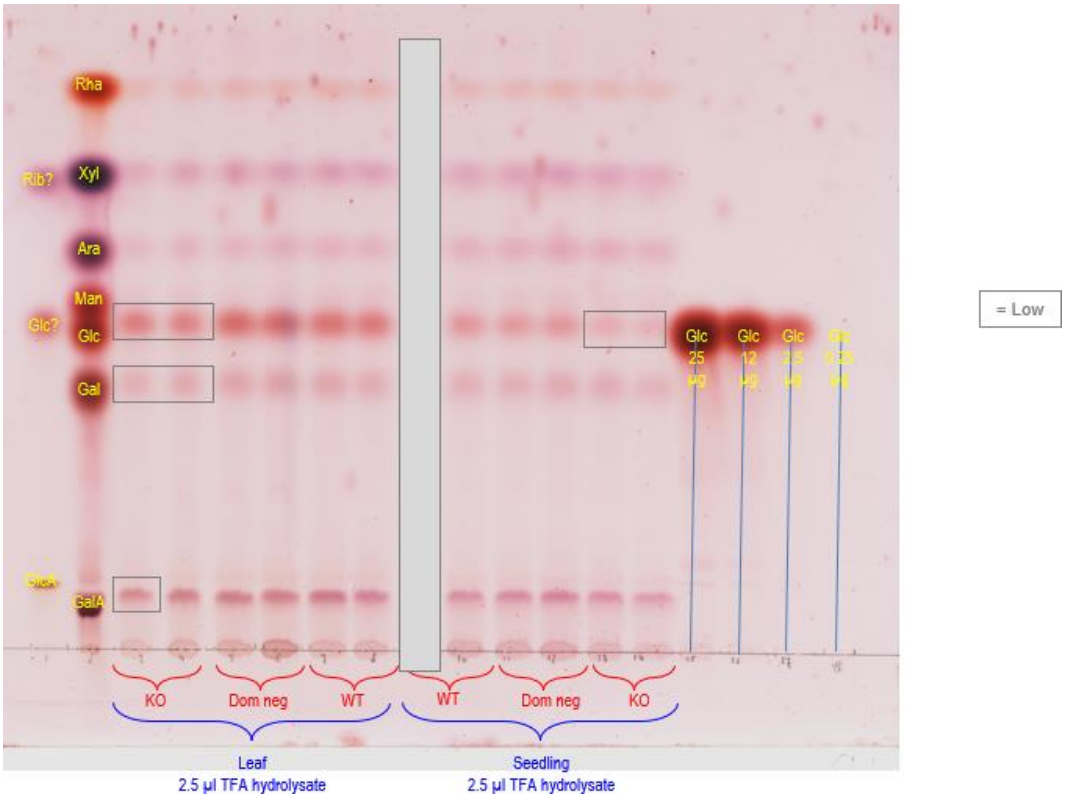


Fig. 4.6 TLC of TFA hydrolysates of matrix polysaccharides shows reduced levels of glucose in leaves and whole seedlings of *vamp714* mutants. GalA = D-Galacturonic acid, Gal= Galactose, Glc = Glucose, Man = Mannose, Ara = Arabinose, Xyl = Xylose and Rha = Rhamnose.

A gradient of glucose was run on the TLC to allow semiquantitative analysis of glucose changes in the different genotypes. The glucose gradient was used to produce a standard curve that was fitted the most accurately using a logarithmic approximation. The amount of glucose in WT leaves was approximately 80 µg per mg of AIR compared to 58 µg mg^{-1} in *vamp714* leaf samples. Lower levels were detected in seedlings with 34 µg mg^{-1} and 22 µg mg^{-1} respectively for WT and *vamp714* plants. Interestingly, glucose units are present in hemicelluloses but not in pectins suggesting that VAMP714 regulates hemicellulose content of cell walls but not the abundance of pectins.

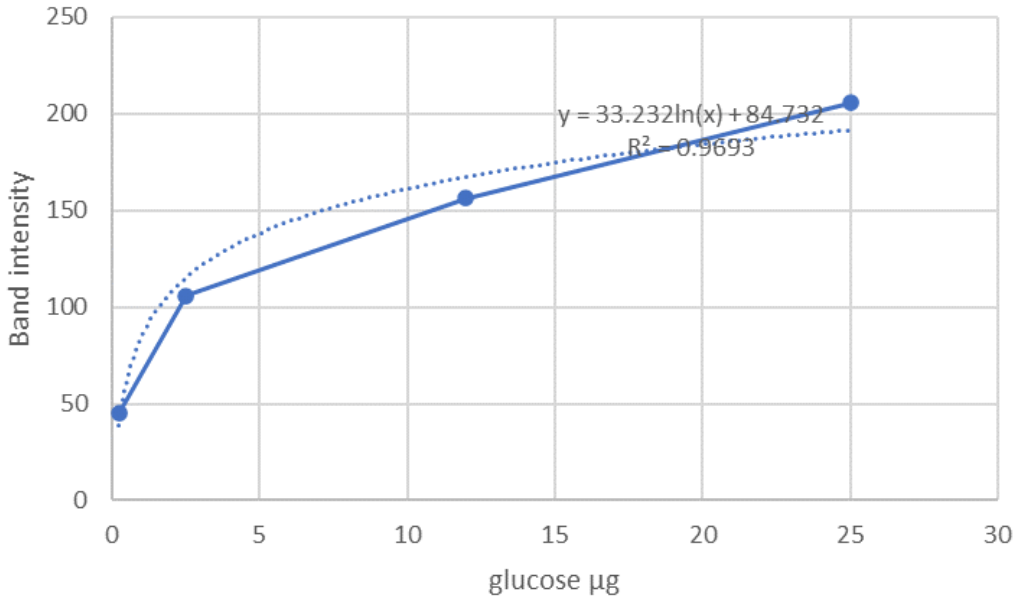
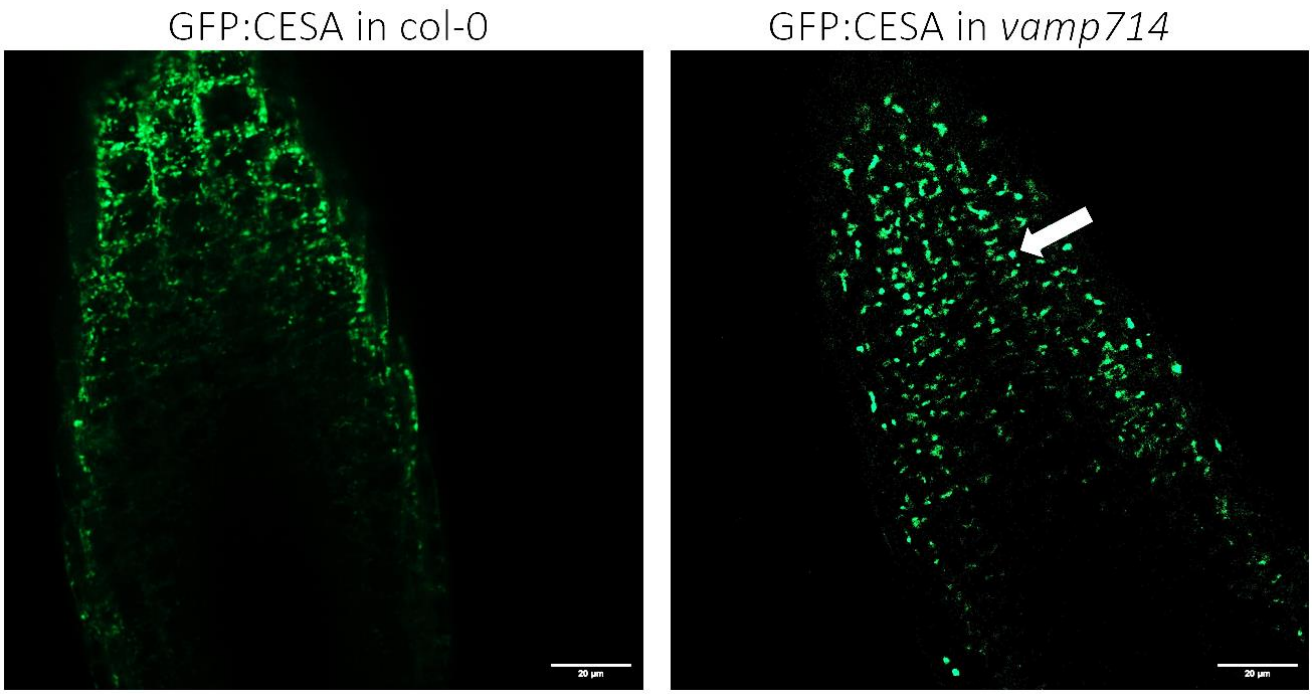


Fig. 4.7 Semiquantitative determination of glucose content from TLC. The equation was used for computations and the best fit (R^2) was obtain with a logarithmic function.

4.6 VAMP714 is required for proper localisation of CESA3

VAMP714 is a regulator of root growth through the control of PIN-mediated auxin transport, allowing establishment of auxin gradients at the root tip (Gu et al. 2021). Auxin controls the expression of numerous genes, and crosstalk between cell wall related genes and auxin homeostasis genes has already been demonstrated (Lehman and Sanguinet 2019). The reduced levels of cell wall components measured could then explain the reduced abundance of the cell wall biosynthetic machinery. However, due to the function of VAMP714 in vesicle trafficking, the role of the protein in the trafficking of the CSC component CESA3 was investigated. Plants expressing GFP:CESA3 were crossed with *vamp714* to obtain plants expressing GFP:CESA3 in the mutant background. Interestingly, a disruption of VAMP714 activity leaded to a dramatic change in the localization of the cellulose synthase. A reduced number of punctate structures containing CESA3 was observed in the mutant and the protein accumulates in larger compartments (Fig 4.8 white arrow). While the effects of VAMP714 on CESA3 abundance cannot be ruled out, these results indicate a role of VAMP714 in trafficking of cellulose synthases.

(A)



(B)

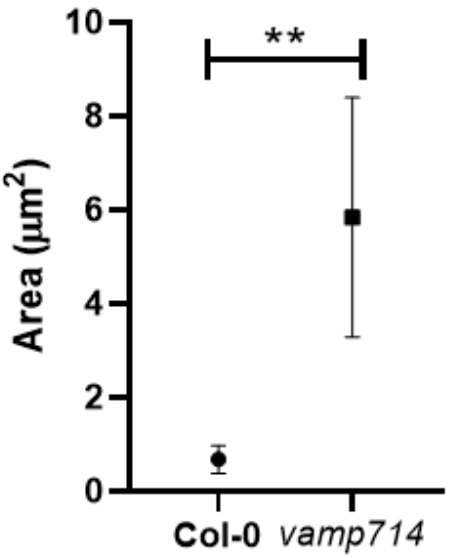


Fig. 4.8 VAMP714 is essential for correct localization of CESA3. (A) GFP:CESA3 in WT background (left) and in the *vamp714* background (right) at the root apex. The tip of the root is located at upper side of the image. The arrow indicates enlarged punctate structures associated with GFP:CESA3 in the mutant. (B) Size of the punctate structures was measured using Fiji by drawing the outline and using area measurement tool to determine the area within that perimeter. The difference between the size of the fluorescent compartments in the different genetic backgrounds was then compared using a Mann-Whitney test N=6 ** pval <0.01.

4.7 VAMP714 is essential for PIN2 population dynamics at the plasma membrane

A key aspect in the regulation of the cell-to-cell transport of auxin in tissues is the control of the polarity and the abundance of PIN proteins at the PM (Adamowski and Friml 2015). This control occurs through endocytosis and recycling of PIN respectively from and toward the PM (Fig.4.9) (Kleine-Vehn et al. 2011; Geldner et al. 2003b). However, PIN recycling alone is not sufficient for PIN polarity (Kleine-Vehn et al. 2011). Indeed, together with a polar trafficking to the PM, PIN efflux carriers need to remain in their polar domain. The maintenance of the polarity is largely influenced by cell walls (Feraru et al. 2011). Indeed, both pharmacological and genetic disruptions of the cell wall integrity cause a loss of PIN polarity (Feraru et al. 2011). Fluorescence recovery after photobleaching (FRAP) experiments and clustering measurements of PIN2 have shown that the cell wall maintains PIN polarity by retaining these auxin transporters in domains with reduced lateral diffusion (Feraru et al. 2011; Martinière et al. 2012b). Here, FRAP experiments were conducted to investigate if VAMP714 affects PIN polarity maintenance. Recovery of fluorescence can occur through recycling of PIN from internal compartments or through lateral diffusion. In order to study the effect on the latter, the recovery was measured for 2 minutes, which is not long enough to allow protein recycling (Feraru et al. 2011).

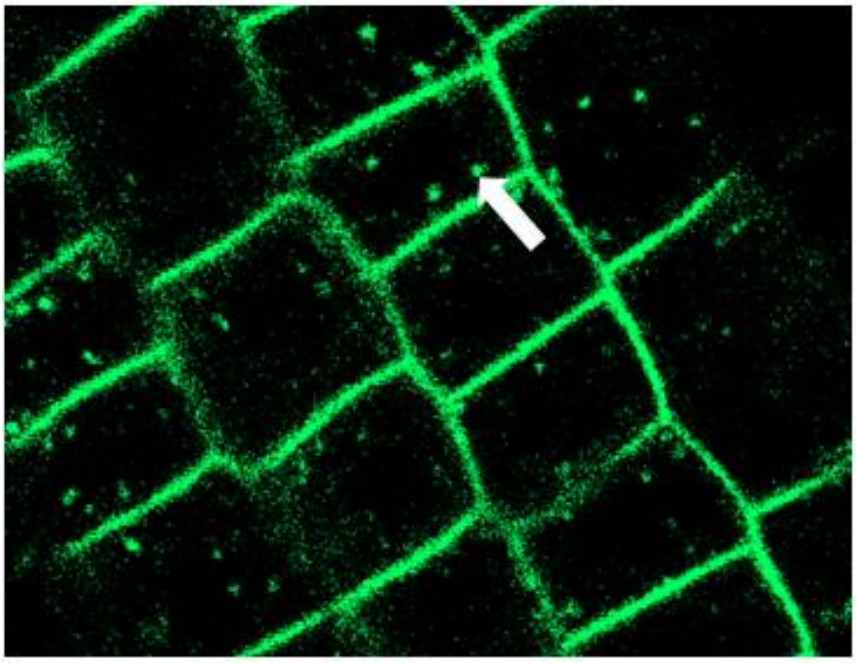
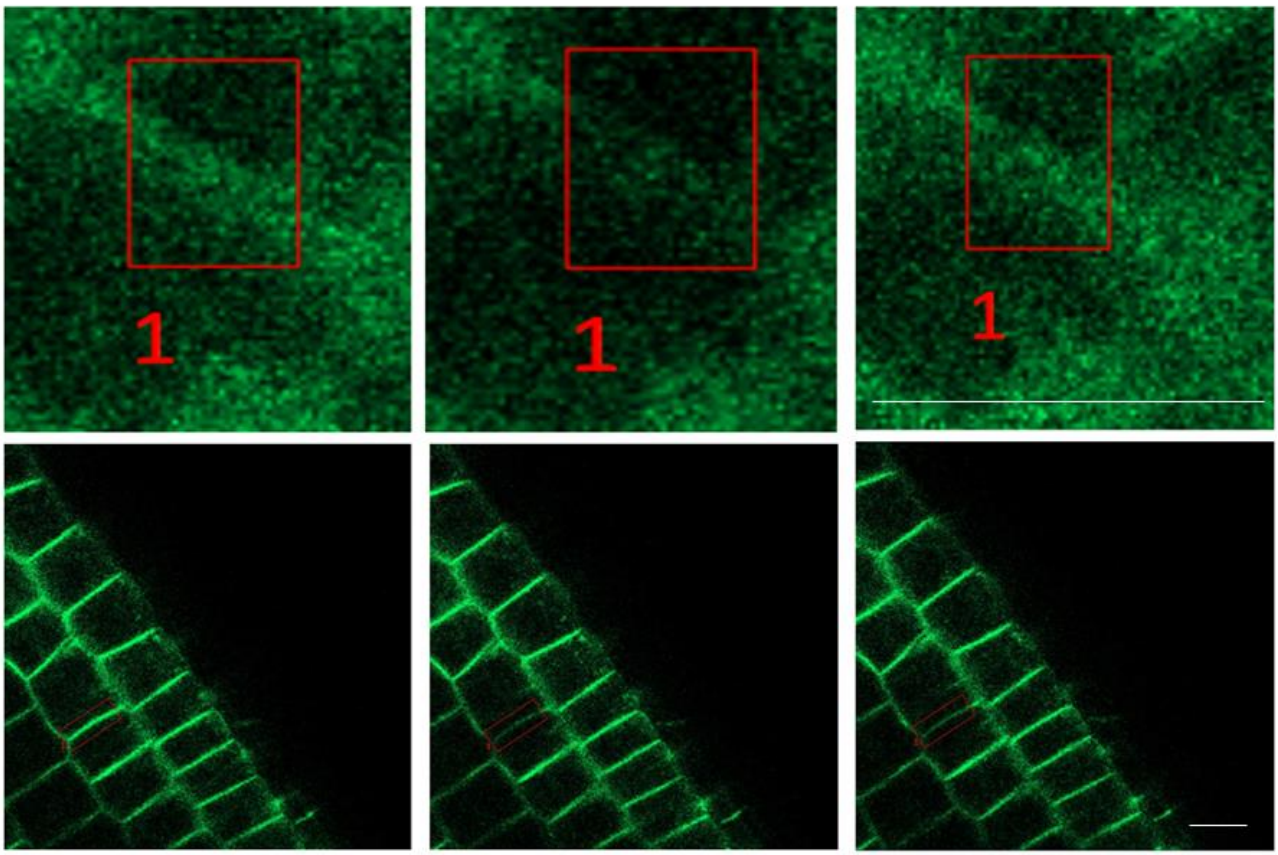


Fig. 4.9 PIN2 is recycled from intracellular compartments to the PM in epidermal cells of the root. The white arrow indicates intracellular PIN2.

As expected, the recovery of PIN2:GFP fluorescence was very weak in WT plants, around 10% of the prebleaching intensity (Fig. 4.10 B). This is consistent with previous FRAP measurements and confirms the low mobility of PIN2 proteins at the plasma membrane (Martinière et al. 2012b). Interestingly, when the plants were treated with 12 pM of the cellulose synthase inhibitor isoxaben, the recovery increased to reach 20% of the prebleaching intensity (Fig. 4.10 B). This is lower than the 30% observed In proPIN2*::PIN2:GFP* treated with 2 pM isoxaben plants by Feraru (Feraru et al. 2011), but still indicates that cellulose plays a role in the control of the lateral diffusion of PIN2. The strongest recovery was observed in PIN2:GFP expressed in the *vamp714* background (95% of the prebleaching intensity), more than recovery of the mobile PM protein LTI6b (75%) (Martinière et al. 2012b). However, in these plants PIN2 localization is altered due to the mutation (Gu et al. 2021). Therefore, recovery could happen through diffusion of vesicles containing PIN2 in the cytoplasm. It is also possible that both the lateral diffusion at PM and the PIN2 vesicles account for the observed recovery. Together, the results are rather a confirmation that the PIN2 population at PM is regulated by VAMP714 rather than indicating its involvement in maintaining PIN2 polarity.

(A)



(B)

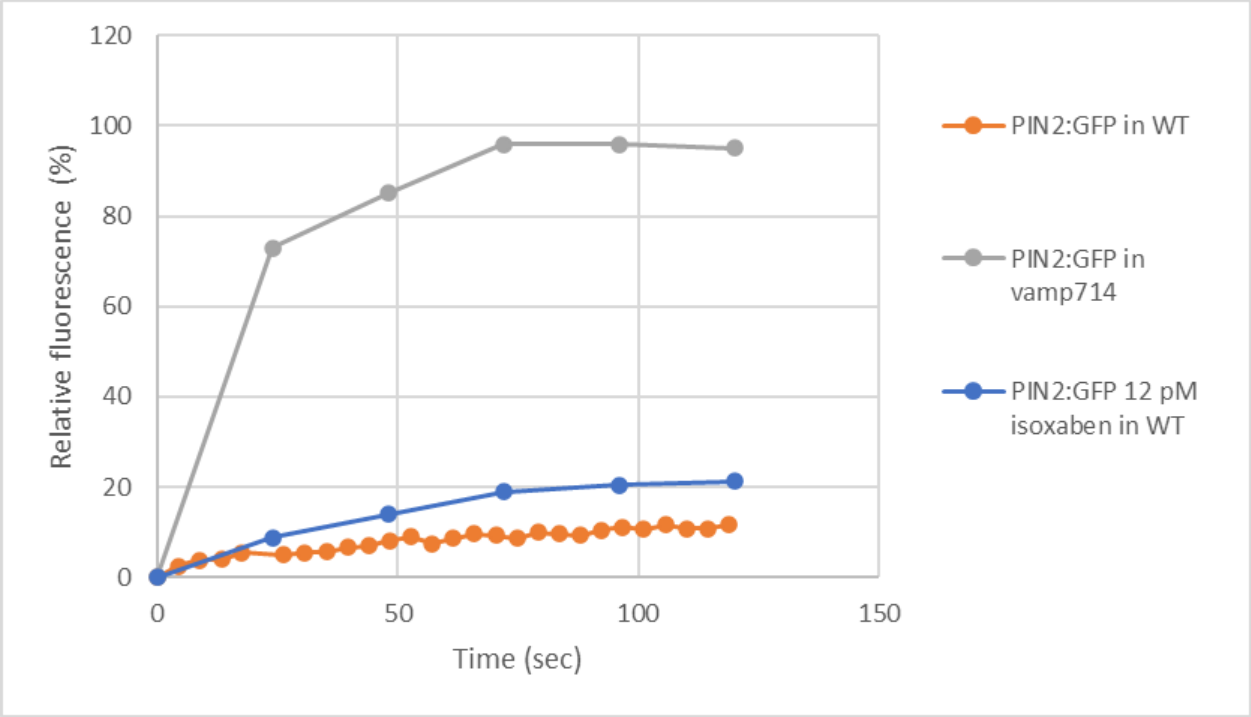


Fig. 4.10 VAMP714 disruption increases the recovery of PIN2:GFP after photobleaching. (A)The upper panel shows the region of bleached in the *vamp714* background before (left), during (middle) and 2 min after bleaching (right). The lower panel represents PIN2:GFP in a WT background. White scale bar=10µm. (B) Monitored recovery over a 2 min time frame in the two backgrounds and using 12 pM of the cellulose synthase inhibitor isoxaben. The fluorescence is presented relative to the fluorescence level measured prior bleaching.

4.8 Summary

The experiments in this chapter aimed to investigate the role of VAMP714 in the trafficking of cell wall components. This was motivated by transcriptomic results indicating a significant downregulation of enzymes controlling cell wall composition in *vamp714* and proteomics data from the literature showing physical proximity between VAMP714 and the CSC. Firstly, effects of knockout mutation of VAMP714 on cell wall components was investigated. In comparison to WT plants, cellulose content in *vamp714* mutants was significantly reduced (15% lower on average) indicating the potential role of VAMP714 in cellulose deposition. Acidic hydrolysis of matrix polysaccharides generated lower glucose in *vamp714* plants as compared to WT plants. As glucose is only present is only present in one matrix

polysaccharide, hemicellulose, these results suggest a that VAMP714 could regulate hemicellulose accumulation in the cell wall. The main motivation behind the study VAMP714-mediated cell wall regulation was to look into the possible link between vesicle trafficking of PIN proteins and PIN polarity maintenance, two key elements in polar distribution of PIN proteins. This was achieved by expressing the CSC subunit GFP:CESA3, regulator of PIN polarity maintenance, in the *vamp714* background. Images obtained indicate that CESA3 fails to localize properly in that background suggesting that VAMP714 is required for the trafficking of CSC components. CESA3 regulates PIN polarity through the reduction of their lateral diffusion of these proteins at the PM. Therefore, effects of VAMP714 on the mobility of PIN2:GFP mobility was measured using FRAP experiments. In the *vamp714 plants*, the mobility of PIN2:GFP is dramatically increased but this could be another consequence of VAMP714's role in vesicle trafficking masking the effect of the mutation on the lateral diffusion of PIN rather than an actual role in PIN maintenance.

Chapter 5: Characterization of the VAMP71 family of R-SNAREs

5.1 Introduction

A recent study of the phylogeny of SNAREs indicates the presence of 64 genes encoding for SNARE proteins in the *Arabidopsis* genome (Gu et al. 2020). Among the 3 clades of R-SNAREs containing a well conserved arginine residue (R) in their SNARE domain, VAMP714 was shown to belong to a small clade comprising VAMP711-14, also sometimes referred as VAMP7C, and containing only four members. VAMP711 and VAMP712 (Leshem et al. 2006; Xue et al. 2018) and recently VAMP714 (Tang et al. 2022) involvement in salt or drought stress has been studied and VAMP714 role in polar auxin transport is thoroughly described (Gu et al. 2021). Interestingly, all VAMP7C members are induced by auxin. Despite available publications, VAMP711-13 still remain poorly characterized.

The objective of this chapter is to contribute to a better characterization of *VAMP71* genes by investigating the phenotypes of the mutants, their expression and their potential role in auxin transport.

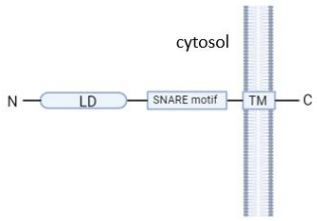
5.2 VAMP714 belongs to a unique subclade

R-SNAREs are divided in 3 major subfamilies present in all the Viridiplantae: the Sec22-, YKT6- and VAMP7-like R-SNAREs (Gu et al. 2020). Duplication events in *Arabidopsis* within the 2 family of VAMP7-like, VAMP71s and VAMP72s, generated respectively 4 and 8 members. R-SNAREs in plants are typically composed of a N-terminal domain that contains the Longin Domain (LD), a coiled-coil SNARE motif, and a transmembrane domain (TM) (Fig. 5.1 A), although the latter is lacking in the YKT6 (Lipka et al. 2007). In VAMP7-like, the role of the LD and TM in the subcellular localization of the proteins was investigated by swapping domains of VAMPs with different subcellular localization (Uemura et al. 2005). This showed that the LD is essential for the vacuole localization of VAMPs but not enough to prevent completely the native localization of chimeric proteins containing the vacuolar LD, with the rest of the protein belonging to non-vacuolar resident SNAREs.

Among the VAMP71s, 3 members, VAMP711, VAMP712 and VAMP713, localize to the tonoplast, whereas VAMP714 localizes to the Golgi. Swapping VAMP714 LD with VAMP711 in VAMP714 LD is sufficient for the tonoplast targeting of the chimeric protein

(Uemura et al., 2005). Therefore, the primary structures of VAMP71 LDs were compared. Similarity in amino acid sequences is high, above 95%, between the VAMP711-13 but drops below 80% when VAMP711-13 are compared to VAMP714 (Fig. 5.1 B), which is consistent with the differences observed in the subcellular localization of the proteins. A multiple alignment of the family members was done in order to find residues that are specific to VAMP714 LD at a given position (Fig. 5.1 C). The reasoning being that if residues are different within the VAMP711-13 they cannot account for the localization of the protein since they are all tonoplast residents. Interestingly, only 4 out of 115 residues fulfilled these criteria (Fig. 5.1 C red frames). This suggests that a small number of amino acids is responsible for Golgi targeting of VAMP714 which could be confirmed by site-directed mutagenesis.

(A)



(B)

	VAMP711	VAMP712	VAMP713	VAMP714
VAMP711	100	95.6	97.4	78.4
VAMP712	95.6	100	94.8	77.6
VAMP713	97.4	94.8	100	79.3
VAMP714	78.4	77.6	79.3	100

(C)

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VAMP714     VYAVVARGTVVLAEFSAVTGNTGAVVRRILEKLSPEISDERLCFSQDRYIFHILRSDGLT      60
VAMP713     IFALVARGTVVLSEFSATSTNASSISKQILEKLPGNDSDSHMSYSQDRYIFHVKRTDGLT      60
VAMP711     LYALVARGTVVLSEFTATSTNASTIAKQILEKVPGD-NDSNVSYSQDRYVFHVKRTDGLT      59
VAMP712     LYALVARGTVVLAELSTTSTNASTIAKQILEKIPGN-GDSHVSYSQDRYVFHVKRTDGLT      59
            ::*:********:*:::.:*:.:: ::****: : .*..:.:*****:**:*:****

VAMP714     FLCMANDTFGRRVPFSYLEEIHMRFMKNYGKVAHNAPAYAMNDEFFSRVLHQQME-   115
VAMP713     VLCMADETAGRNIPFAFLDDIHQRFVKTYGRAIHSAQAYSMNDEF-SRVLSQQMEF   115
VAMP711     VLCMAEETAGRRIPFAFLEDIHQRFVRTYGRAVHTALAYAMNEEF-SRVLSQQIDY   114
VAMP712     VLCMADEDAGRRIPFSFLEDIHQRFVRTYGRAIHSAQAYAMNDEF-SRVLNQQIEY   114
            .****::  **.:**::*::** **::.**:. *.* **:**:** **** **::
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Fig. 5.1 Primary sequence comparison of the LD of VAMP71s. (A) Domains of the VAMP71s, (B) similarity in the LDs (in percent) after pairwise alignments using Needleman-Wunsch algorithm,.(C) Multiple alignment of the VAMP71 LDs, using Clustal Omega. Positions of residues that are different in VAMP714 but similar in VAMP711-13 are highlighted by red frames. * identical, : conservative replacement,. Semiconservative replacement.

Although the LD is sufficient for organelle targeting, it is not the sole domain responsible for R-SNARE localization (Uemura et al. 2005) . Therefore, other domains were also used for primary sequence comparisons. Pairwise comparisons of the synaptobrevin domain (containing the SNARE motif) of VAMP71s show a similar trend to the one observed for the LDs. Indeed, high similarity, around 90%, was observed between VAMP711-13 and a lower similarity was observed between them and VAMP714. Here again, very few residues, only two, were both specific to VAMP714 at a given position while similar in the other VAMP71s.

(A)

	VAMP711	VAMP712	VAMP713	VAMP714
VAMP711	100	90.9	93.2	79.5
VAMP712	90.9	100	86.4	78.4
VAMP713	93.2	86.4	100	73.9
VAMP714	79.5	78.4	73.9	100

(B)

```
VAMP714     PSVDTLNRVRGEVSEIRSVMVENIEKIMERGDRIELLVDKTATMQDSSFHFRKQSKRLRR      60
VAMP713     PNADRMSRIKGEMSQVRNVMIENIDKVLDRGERLELLVDKTENMQGNTFRFRKQARRYRT      60
VAMP711     PNADRINRIKGEMNQVRGVMIENIDKVLDRGERLELLVDKTANMQGNTFRFRKQARRFRS      60
VAMP712     PNADTISRIKGEMNQVRDVMIENIDNILDRGERLELLVDKTANMQGNTFRFRKQTRRFNN      60
            *..* :.*::**:.::*.**:***:::::**:*:******* .**..:*:****::* .

VAMP714     ALWMKNAKLLVLLTCLIVFLLYIIIASF       88
VAMP713     IMWWRNVKLTIALILVLALVVYIAMAFV       88
VAMP711     NVWWRNCKLTVLLILLLLVIIYIAVAFL       88
VAMP712     TVWWRNCKLTLLLILVLLVIIYIGVAFA       88
             :* :* ** : *  :: .::** :*
```

Fig. 5.2 Primary sequence comparison of the synaptobrevin domains of VAMP71s. (A) Percentage similarity in the synaptobrevin domain after pairwise alignments using Needleman-Wunsch algorithm. (B) Multiple alignments of the VAMP71 synaptobrevin domains using Clustal Omega. Positions of residues that are different in VAMP714 but similar in VAMP711-13 are highlighted by red frames. * = identical, : = conservative replacement, . = semiconservative replacement.

Finally, the TM sequences of VAMP71s were analysed. Interestingly, the pattern was different from the pattern observed for other domains. VAMP711 and VAMP712 are still highly similar but different from VAMP713 and VAMP714. Also, a surprisingly low similarity was observed between VAMP714 and VAMP713. A multiple alignment of the VAMP71s shows that VAMP713 fails to align properly with the rest of the members. The difference between the TM of VAMP713 and VAMP7111-12 suggests that the TM is not involved in tonoplast targeting which has been shown with chimeric VAMP71s (Uemura et al. 2005). Because of the nature of the TM, it is possible that a core of hydrophobic residues is essential for membrane anchoring whereas other residues "buried" in the membrane bilayer are not subject to a high pressure of selection, allowing bigger differences in the primary sequence.

(A)

	VAMP711	VAMP712	VAMP713	VAMP714
VAMP711	100	90.9	68	60.9
VAMP712	90.9	100	60	60.9
VAMP713	68	60	100	31
VAMP714	60.9	60.9	31	100

(B)

```
VAMP714      ----VLLTCLIVFLLYIIIASFCGGI 22
VAMP711      ---TVLLILLLLVIIYIAVAFLCHG- 22
VAMP712      ---TLLLILVLLVIIYIGVAFACHG- 22
VAMP713      NVKTIALILVLALVVYIAMAFV---- 22
                 : *  :: .::** :*
```

Fig. 5.3 Primary sequence comparison of TM of VAMP71s. (A) Percentage similarity in the TM domain after pairwise alignments using Needleman-Wunsch algorithm. (B) Multiple alignments of the VAMP71 TM domains using Clustal Omega. * = identical, : = conservative replacement, . = semiconservative replacement.

Phylogeny studies of VAMP71s using both domain comparisons (Gu et al. 2020; Uemura et al. 2004) and whole-protein sequence show that VAMP714 forms a subclade within the VAMP71 family. This is consistent with the differences in subcellular localization and protein functions of VAMP714 observed in the literature (Uemura et al. 2005). Taken together, the analysis of the primary sequence results allowed identification of potential key residues in the LD and the synaptobrevin domain that make VAMP714 a somewhat unique VAMP71 protein.

5.3 VAMP71s have different but partly overlapping expression patterns

SNAREs are functionally redundant proteins. Indeed, many cases loss of function mutation of individual genes encoding for SNAREs does not generate obvious phenotypes in non-stress conditions (Waghmare et al. 2018). However, it seems that there are different degrees of overlap in their functions. Promoter swapping experiments showed that SYP21 and SYP22 are not only redundant but interchangeable (Uemura et al. 2010) while KNOLLE and SYP32 have overlapping function in cytokinesis in embryos but also non-overlapping function in secretion and cellularization of the endosperm (Park et al. 2018). Analysis of *syp121* and *syp122* proteomes have shown that while SYP121 and SYP122 mostly have similar cargoes, some proteins are specific to SYP121 and SYP122-mediated trafficking (Waghmare et al. 2018). Recent phylogeny studies suggest that duplicated SNAREs in *Arabidopsis* are more

likely to be subject to sub-functionalization to different tissues than neo-functionalization since no association is observed between genetic and expression distance (Gu et al. 2020). VAMP711-13 are very similar/almost identical proteins as described in the previous section. It was therefore hypothesized that if these proteins have specific functions, it is likely to be due to some level of tissue sub-functionalization. To investigate this possibility, the expression of the VAMP71s was analysed on the eplant online tool (Fig. 5.4) that gives a visual representation of the expression data from the ATGenExpress project (Kilian et al. 2007). An advantage of this tool is that all the expression measurements are represented using the same scale, making comparisons possible between different genes (Waese et al. 2017). A striking difference between *VAMP711-12* and *VAMP713-VAMP714* is that high levels of expression of *VAMP711-12* genes is specific to one or a small number of tissues while *VAMP713-VAMP714* genes have similar levels of expression in various tissues and organs (Fig. 5.4). On an absolute scale, it is interesting to note that the most abundant VAMP71 is VAMP711. Very high levels of expression of the gene in mature pollen make its expression look low in other parts of the plant but it is the most expressed VAMP71 in the majority of tissues and organs. In contrast, absolute levels of VAMP713 are low but similar across a broad range of tissues and organs. Together, the observed expression patterns of VAMP71s support the hypothesis of tissue sub-functionalization, albeit incomplete specialization since overlaps exist in the expression of these R-SNAREs.

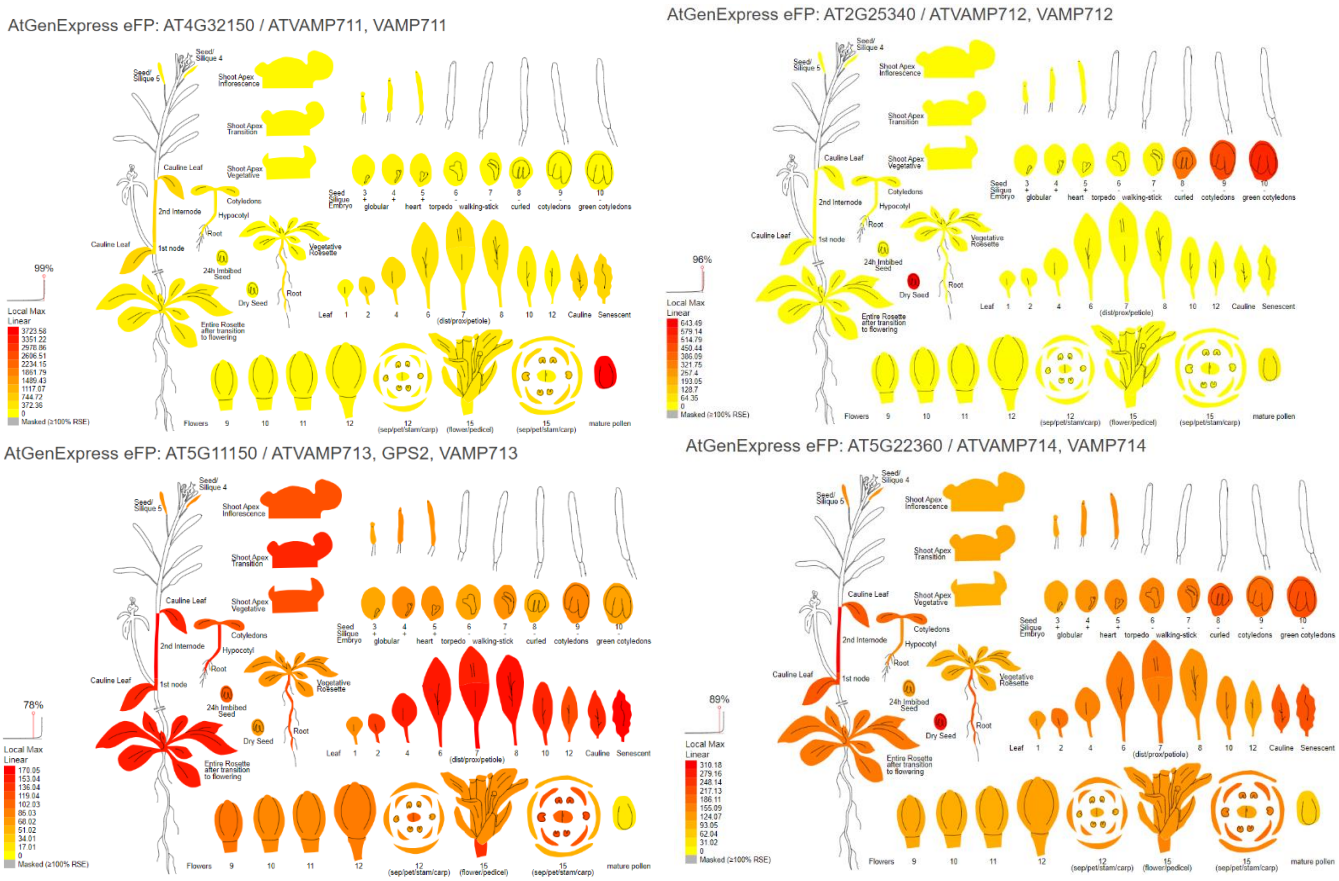

Fig. 5.4 Expression of VAMP71s genes. Comparison of the transcriptional expression of VAMP71 using the eplant eFP viewer tool using AtGenExpress data set (Kilian et al. 2007).

To further investigate gene expression of VAMP71s in seedlings, promoter::β-Glucuronidase (GUS) reporter lines were generated. To do so, the 2kb sequence upstream the start codon was cloned in a plasmid (pBGWFS7) containing the GUS enzyme coding sequencing. An advantage of these reporters of promoter activity is that it offers visual and spatial information of transcriptional expression of genes of interest. To facilitate whole seedling observation, seedlings were observed 4 days after germination. However, gene expression was monitored over a 2-week time frame to detect temporal changes in gene expression patterns. *VAMP711* is expressed in a large portion of the root, the root tip being the only non-stained region (Fig. 5.5 right panel). The staining was carried out on at least 2 independent lines (which had an identical expression pattern) and compared to available online root expression data (Fig.5.5 left panel). This showed a decreased expression at the root tip which is consistent with the GUS-staining. A non-negligible difference is the predicted expression in the hypocotyl that couldn't be observed with the staining even in older seedlings (data not

shown). Interestingly, VAMP711 is also expressed in root hairs and lateral root primordia (Fig. 5.5 lower panel).

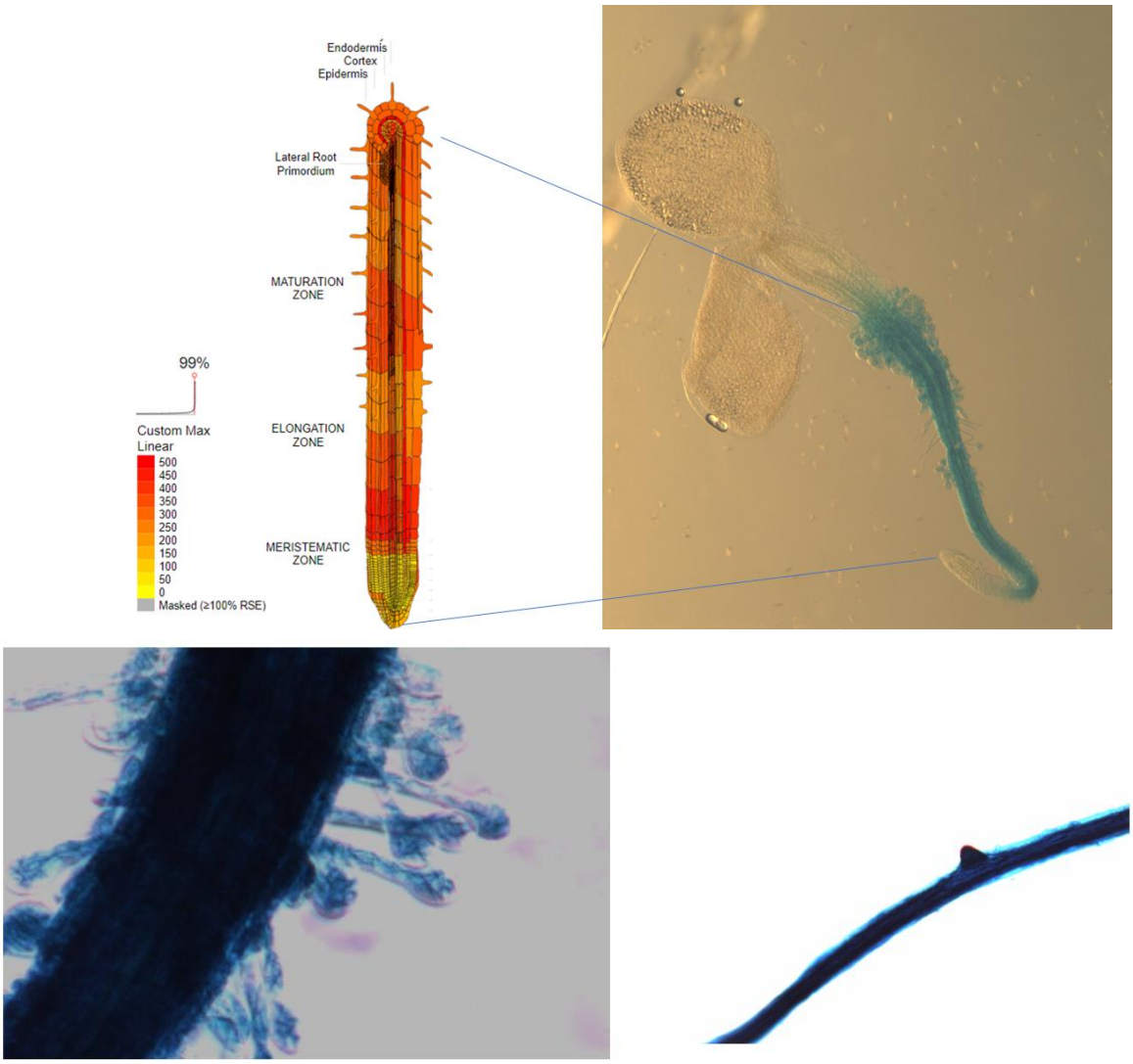

Fig. 5.5 Expression of *VAMP711* in Arabidopsis seedlings. GUS-staining of 4-days old *proVAMP711:GUS* seedlings (upper right) was compared to predicted root expression on eplant (upper left). Expression in root hairs (left) and root primordia (right) are highlighted in the lower panel.

VAMP712 was predicted to have a very low expression in seedlings and its expression is highly specific to dry seeds (Fig. 5.4). Consistent with this, very low GUS staining was observed in the reporter lines (Fig. 5.6). However, faint expression is detectable in the hypocotyl and appears stronger in older seedlings (Fig. 5.6 lower panel).

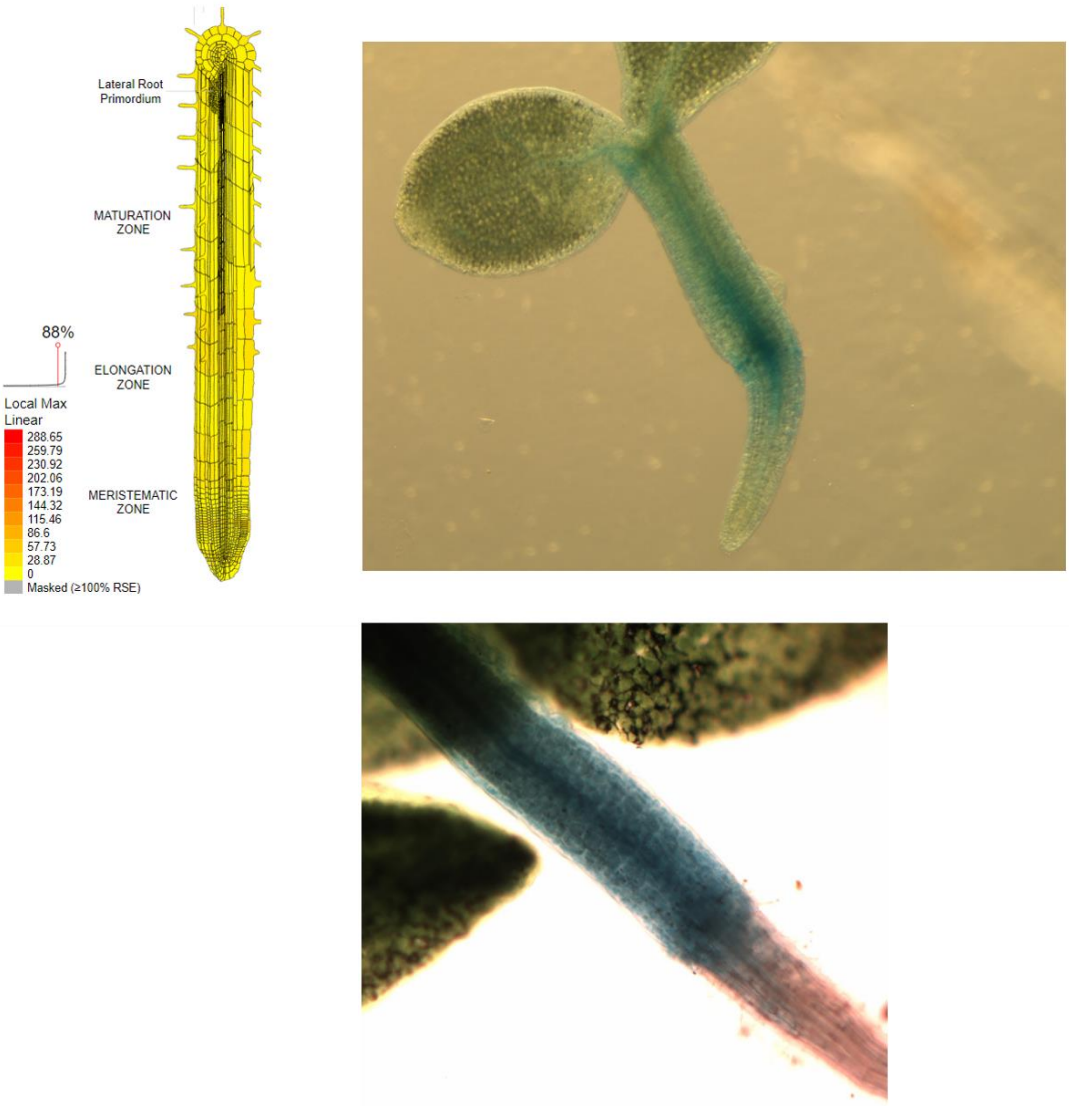

Fig. 5.6 Expression of *VAMP712* in Arabidopsis seedlings. GUS staining of 4-days old *proVAMP712:GUS* seedlings (upper right) was compared to predicted root expression on eplant (upper left). Expression in the hypocotyl of a 7-days old seedlings is presented in the lower panel.

The expression of *VAMP714* was studied in a previous analysis and shows that the gene is expressed in vascular tissues in both root and aerial organs (Gu et al. 2021). The expression of *VAMP714* in root was compared to predicted expression and shows similar results, although some predicted fluctuations in expression along the root axis are not obvious in the stained roots (Fig. 5.7).

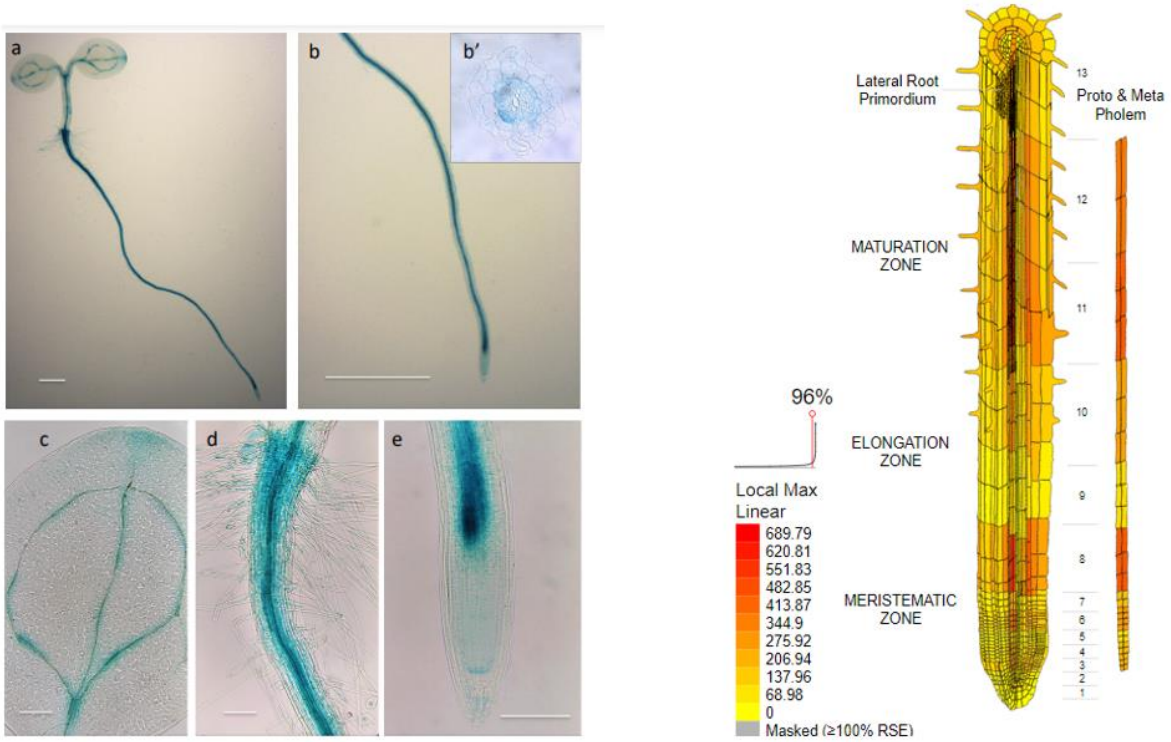

Fig. 5.7 Expression of *VAMP714* in Arabidopsis seedlings. GUS-staining of 4-days old *proVAMP714:GUS* seedlings (left) obtained from (Gu et al. 2021) was compared to predicted root expression on eplant (right). Expression in vascular in different organs re presented in the lower panel.

All together the comparison of expression of *VAMP71* genes showed differences in expression patterns in seedlings, whole plants and across developmental stages. However, overlaps are also observed. For example, *VAMP711* and *VAMP714* are expressed on the same regions along the longitudinal axis of the root but in different tissues across the radial axis in seedlings. This suggest that VAMP71s may have been subject to some level of subfunctionaliztion.

5.4 Single *vamp711-13* mutants do not exhibit obvious phenotypes

Available studies on VAMP71s have made use of lines with disrupted expression of the proteins (Leshem et al. 2006; Xue et al. 2019; 2018). However, the lines used in these articles were different and some lines were knockdown plants (Leshem et al. 2006) that are at risk of silencing down the generations. In order to avoid this problem, *knockout* plants were obtained. For *vamp712* and *vamp713* the T-DNA insertion lines GK-054H03 and SALK_032706C were obtained from NASC. In both cases, T-DNA insertion in an exon was chosen to ensure the suppression of activity of the protein encoded by these genes. The homozygous status of the mutation was confirmed by genotyping using combinations of T-

DNA and gene specific primers (Fig. 5.8). VAMP711 was knocked out using CRISPR technology. In addition to the *knockout* mutation, an overexpressor of *VAMP711* was also generated for phenotypic analysis.

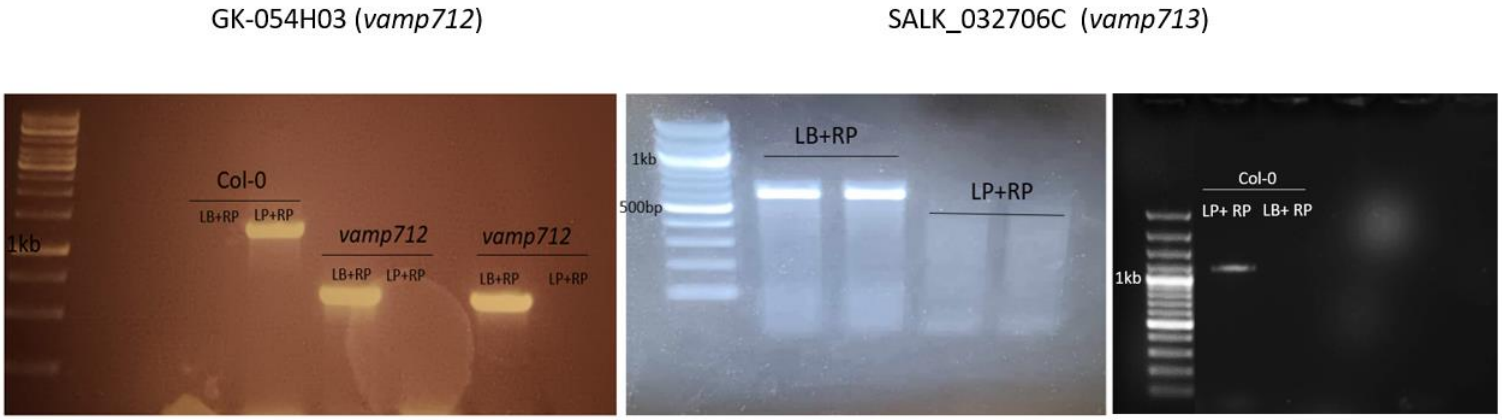

Fig 5.8 Genotyping of *vamp71* T-DNA insertion lines. A T-DNA specific (LB+RP) set of primers and a gDNA specific (LP+RP) set of primers were used to confirm the homozygous status of the *vamp712* (left) and *vamp713* (right, Col-0 control on a separate gel) T-DNA insertion mutants.

VAMP71s have been studied in the context of resistance to stress. Disruption of VAMP71 genes has been proposed to increase salt resistance by maintaining vacuole integrity (Leshem et al. 2006) and also to increase resistance to high-pH stress (Xue et al. 2019). However, VAMP711 is required for drought stress resistance in an ABA mediated process (Xue et al. 2018; Leshem et al. 2010). In the absence of stress, on the other hand, no phenotypes have been observed and previously studied single and multiple mutants have been characterized as undistinguishable from WT plants (Leshem et al. 2006; Xue et al. 2019). Nonetheless, the different misexpressors were analysed and compared to WT plants. Consistent with previous observations, no obvious differences were observed in *vamp71s*. However, the *VAMP711* overexpressor (35S promoter driven expression of *VAMP711*), *VAMP711Ox*, shows a dwarf phenotype (Fig. 5.9). A previously described *VAMP711* overexpressor using a weaker *UBQ10* promoter does not have a phenotype (Xue et al. 2018) suggesting a potential importance of the *VAMP711* expression levels for the modified activity of the protein. An alternative explanation could be the site of insertion of the T-DNA containing the overexpressing gene cassette but that possibility can be ruled because the independent transformants have the same phenotype (data not shown).

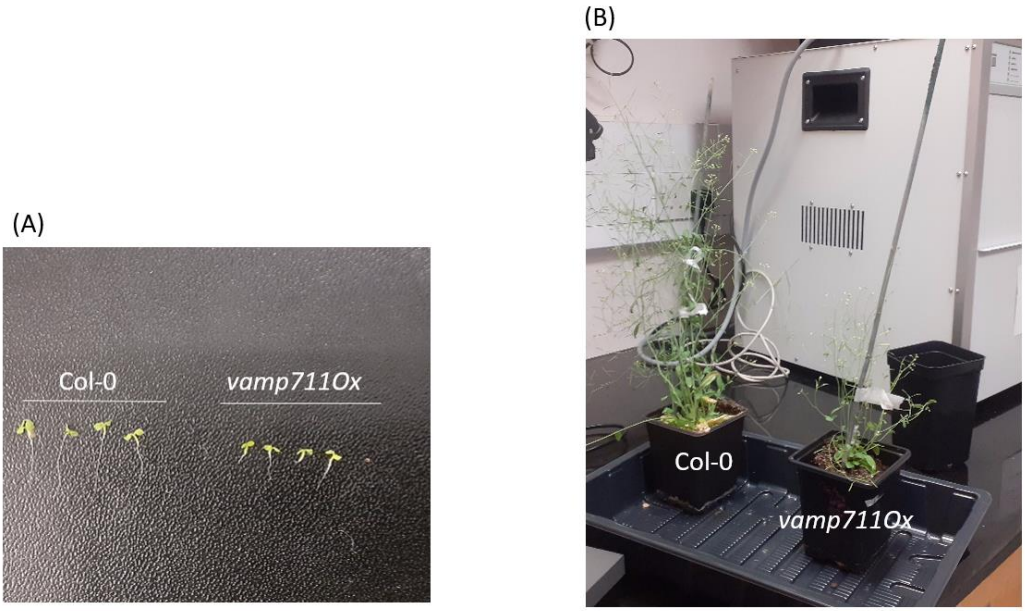

Fig 5.9 Characterization of *vamp711Ox* lines. Seedlings overexpressing VAMP711 have shorter roots than WT plants (A) and mature plants have short stems.

The *vamp712* mutants were further analysed because of the detection of a small proportion of plant displaying a phenotype. Indeed, the overall root architecture of these plants was different to wildtype (Fig. 5.10 upper panel). Loops in the roots and an increased amount of root hairs were also observed in the mutant. In the aerial organs, leaf size and shape were altered in the *vamp712* plants (Fig. 5.10 lower panel).

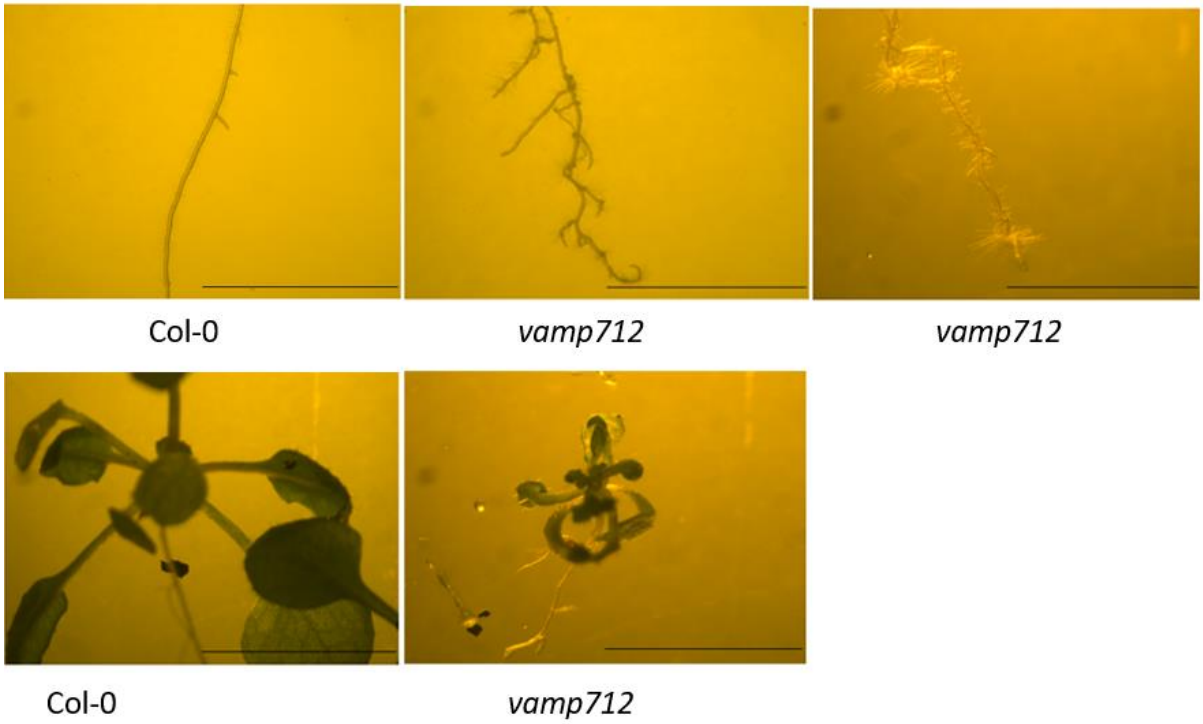

Fig 5.10 Some vamp712 show altered root and leaf development. Root tip of WT plants (upper left) as compared to *vamp712* (upper right). Defects in leaf size and shape were also observed when vamp712 mutants (lower right) are compared to WT plants (lower right). Scale bar: 1cm

There are several possible explanations for the observed phenotypic variation between individual *vamp712* plants. In terms of genetics, it could be due to one or more additional T-DNA insertion events or by the presence of point mutations. Therefore, genomic DNA of two independent *vamp712* plants, one from the progeny of the plants displaying a phenotype and one plant displaying no phenotype, was extracted and used for whole-genome sequencing. Raw data were processed as described in Section 2. 11. In both plants only one insertion was detected in the whole genome and the insertion occurred in the *VAMP172* gene (Fig. 5.11 B). The possibility of multiple T-DNA insertions can therefore be ruled out. However, it is still possible that point mutations are responsible for the phenotype observed in some of the plants but it is more likely to be a combination of mutations rather than a single mutation. Indeed, the frequency of the plants with a phenotype was low, around 2%, much lower than the ratio predicted by Mendelian heredity for a single gene. Alternatively, microenvironmental differences may also have caused the difference in phenotype.

(A)

Sample Name	≥ 1X	≥ 5X	≥ 10X	≥ 30X	Mean Cov.	Median
S39_S40_combined	100.00%	99.00%	87.00%	2.00%	21.4X	15.0X
S39_V7122	99.00%	83.00%	30.00%	1.00%	11.0X	8.0X
S40_v7122	99.00%	79.00%	25.00%	1.00%	10.4X	7.0X

(B)

Fig. 5.11 *vamp712* mutants carry a single T-DNA. The reads coverage of two *vamp712* mutants (one with and one without observable phenotype) after whole DNA sequencing at different levels of depth (nX) (A) a T-DNA scan indicated the presence of one T-DNA in *VAMP712* gene highlighted with IGV software (coloured sequence) for both plants (B)

Taken together, the phenotypical characterization of the *vamp711-13* loss-of-function mutants shows no observable differences with WT plants when grown in standard lab conditions for *Arabidopsis*. This is consistent with the results of previous studies. This suggests redundancy between VAMP71s or tonoplast R-SNAREs in general. However, roles of VAMP71s, mostly studied in stress conditions, are not redundant in several abiotic stress responses (Xue et al. 2019; Leshem et al. 2010). For example, all VAMP71s increase resistance to high-pH treatment but a triple mutant *vamp711-vamp712-vamp714* shows an enhanced resistance in comparison to single mutants (Xue et al. 2019). This suggests a complex balance in overlapping and specific functions between VAMP71 proteins. It is possible that a subfunctionalization exists between these proteins but its effects are only observable under stress conditions.

5.5 VAMP712 and VAMP713 are involved in root gravitropic response

All *VAMP71* genes are transcriptionally induced following a treatment with 100 µM IAA (Gu et al. 2021) suggesting that these proteins could be involved in auxin-regulated biological

processes. The role of VAMP714 in polar auxin transport has already been shown to be essential in the gravitropic response and maintaining the root apical meristem (Gu et al. 2021). Interestingly, VAMP713 has also been studied in the context of gravitropism. Indeed, in *vamp713* mutants in the Wassilewskija ecotype of *Arabidopsis*, also called *gravity persistent signal* (gps2), inflorescence stems do not bend properly once place vertically after cold gravistimulation (Nadella et al. 2006). This was correlated with abnormal expression pattern of the auxin responsive gene *IAA2*. Since gravitropism is a dynamic process involving a redirection of the auxin flow (Band et al. 2012), a root gravitropism assay was conducted in order to investigate the role of VAMP71s in auxin distribution. The plants were grown vertically for 4 days on plates that were then rotated by 90°, and the external angle between the root and the horizontal axis corresponding to the root growth axis was measured after 24 h (Fig. 5.12 B). Interestingly, different levels of bending were observed. Some *vamp713* mutants showed an increased bending (Figure 5.12 A middle panel) but this was not detected in *vamp712* or WT plants. The percentage of plants with a low degree of bending was higher in the *vamp712* than in the other genotypes (Fig. 5.12 C). This suggests that *vamp712* plants have a delayed response to gravitropism. Longer exposure to gravity results in a normal growth of the root towards gravity (data not shown) indicating that VAMP71s are involved in the early stage of the gravitropism during bending at the root apex. Interestingly, mutation of the *VAMP713* gene causes a "hyper bending" of the inflorescence stem after cold gravistimulation (Nadella et al. 2006), also observed in the root suggesting a potential role of VAMP713 in "stopping" the bending for appropriate growth towards gravity.

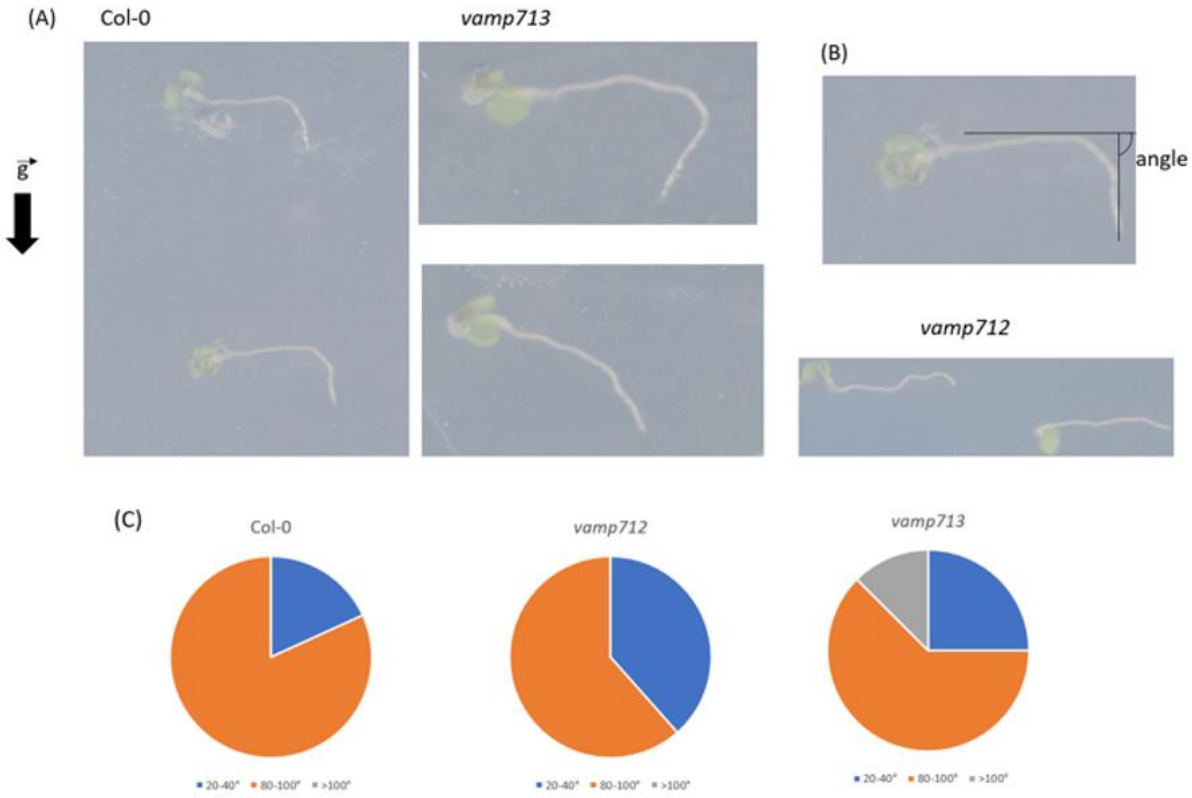

Fig. 5.12 vamp712 and vamp173 have a reduced response to gravitropic stimuli. Mutant with an abnormal root bending were observed in both vamp713 mutants (A) the bending angle was measured from the horizontal axis to to the external side of the root (B) the pie charts indicate the proportion of different bending angles in the studied genotypes (C). Black arrow= direction of gravity after rotation of the plants.

5.6 VAMP712 contributes to hypocotyl growth during etiolation

Because VAMP712 is expressed in the hypocotyl of *Arabidopsis* seedlings, which is known to elongate when seedlings are germinating in the dark (referred to as etiolation), the length of 7-day-old etiolated WT and *vamp712* plants were compared. A short hypocotyl phenotype was observed in *vamp712* mutants (Fig. 5.13). This suggests that VAMP712 has a role in the hypocotyl growth in the dark. Polar auxin transport has been shown to be reduced in the dark (Liu, Cohen, and Gardner 2011) and does not affect the elongation of the hypocotyl during etiolation (Jensen, Hangarter, and Estelle 1998). Therefore, the observed effect is not due to auxin transport. VAMP712 is a tonoplastic protein and is involved in maintaining vacuole homeostasis which could contribute to hypocotyl elongation in the dark. Interestingly, the tonoplatic transporter INT1 is required for hypocotyl growth in a sucrose-dependent way (Strobl et al. 2018). Surprisingly, INT1 is thought to affect cell elongation through the control

of cell wall composition (Strobl et al. 2018). However, the relationship between vacuolar transport of inositol and cell wall composition is not yet understood.

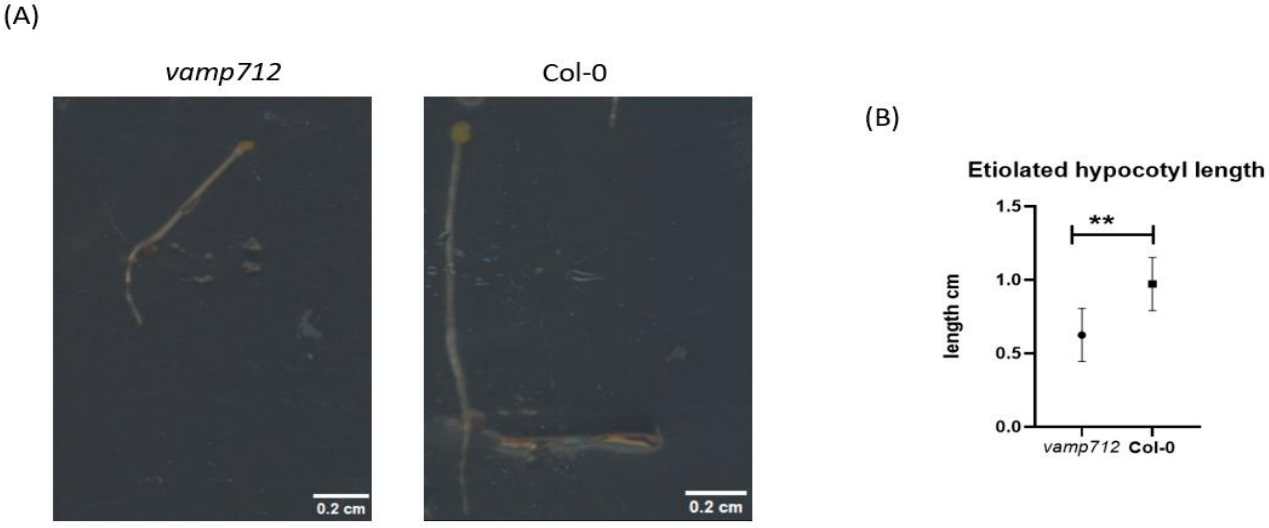

Fig. 5.13 Dark-grown vamp712 seedlings have short hypocotyls. Etiolated hypocotyl of a *vamp712* and a WT 7-day-old seedling (A), a significant reduction of the hypocotyl was measured in *vamp712* plants. ** pval=0.0003, Mann-Whitney test.

5.7 Summary

VAMP714 is a SNARE of the VAMP71 family but the subcellular localization of VAMP714 is different from the other VAMP71 members. This was previously demonstrated using domain swapping experiments. The primary sequence of VAMP71 proteins is accessible online allowing comparative studies. Domain comparisons have shown that the primary structure elements accounting for the difference in the localization of VAMP71s can be narrowed down to a small number of residues.

VAMP71s have different transcriptional expression patterns in seedlings and during the life cycle of *Arabidopsis* as indicated by expression databases and GUS reporter lines. This is could be due to some level of sub-functionalization in VAMP71s. This hypothesis was investigated by phenotypical characterization of *vamp71* knockout mutants. In regular conditions for lab-grown seedlings, *vamp711-713* mutants have no observable phenotype. However, mutations in *VAMP713* and *VAMP712* genes have observable effects on the

gravitropic response of roots and the elongation of etiolated hypocotyls. Taken together these results indicate if there is sub-functionalization in VAMP71s, it is mainly manifesting in response to external stimuli but in most situations, these proteins have an important overlap in their function.

Chapter 6: Discussion

6.1 An intracellular hub for PIN cycling

PINs were shown to rapidly cycle from intracellular compartments to the PM in the early 2000s (Geldner et al. 2001; 2003). Since then, numerous regulators of this phenomenon have been identified and found to localize to a wide range of cellular compartments. PIN proteins need to be recruited to vesicles that are going to follow secretory or endocytic pathways and transit in various compartments, which probably accounts for the large number of PIN trafficking regulators. Cellular compartments in plants are highly complex and distinct from other eukaryotic organisms (Geldner et al. 2009). An example illustrating this complexity is the compartmentalization within the TGN and where subdomains are responsible for sorting to different destinations. Indeed, Vesicle-Associated Membrane Protein 721 (VAMP721), AP complex-1 (AP1), and clathrin together form a specific subdomain for secretory trafficking, whereas VAMP727 and AP4 form another subdomain for vacuolar trafficking (Gao and Chao 2022; Shimizu et al. 2021). Subcellular localization of proteins is usually determined by using compartment markers fused to fluorescent tags. However, proteomic experiments using the most common markers of cellular compartments have shown that markers have overlapping localization (eg ARA5 was found in the proteome of ARA7) (Heard et al. 2015). This suggests the existence of a continuum between partly discrete compartments. If the role of TGN in vesicle recycling is well characterised, the endosomal pathways remain matter for further research. However, it has been shown that Rab5-GTPases reside in early and late endosomal compartments and participate in endocytosis and vacuolar targeting (Ito et al. 2016). The question is, from which compartments are proteins and especially PINs recycled to the PM? It is largely believed that recycling originates from the TGN that acts like an early endosome, receiving endocytosed PINs and sending them back to the PM. Within the TGN not only do both vacuolar and PM targeting pathways coexist, but it is also believed that they are independent secretion pathways. Indeed, PIN2 and PIN3 secretion is independent of Echidna TGN protein but not AUX1 (Elliott et al. 2020). Mutation of the BEX5/RabA1B small GTPase essential for PIN recycling disrupts the organization and the function of the TGN (Feraru et al. 2012), underlying the importance of the organelle in this process. In most studies TGN is referred as TGN/EE where no distinction is made between the TGN and the early endosomes, making it difficult to know if there is an EE specific recycling process. However, there is a type of endosomal recycling that is mentioned in the literature, the

rescuing of PIN from the vacuole sorting by the retromer in the late endosome for recycling to the PM (Jaillais et al. 2007). This pathway has been strongly contested by Robinson (2015) who considers that there is a lack of experimental evidence to support LE recycling, especially because there are no electron microscopy images of such a phenomenon.

VAMP714 interacts with ARA7 which is involved in retrograde trafficking for endocytosis; with CTL1 responsible for recycling of PIN; and possibly EXO70A1, a tethering factor shown to regulate PIN exocytosis. The interaction between these proteins occurs in small punctate structures but their nature has not been determined. Here, I propose that VAMP714 is transiting in cycling hubs in what I term the "TGN/EE" continuum where PIN endocytic and exocytic machinery meet and where the transition from one to the other occurs (Fig. 6.1).

Challenges and future work: Vesicle trafficking routes are different from metabolic pathways because it involves a diversity of compartments. Because of the essential role of vesicle trafficking in eukaryotes, mutations of its components are often lethal or have no visible effects due to functional redundancy (Zwiewka and Friml 2012). Therefore, classical genetic approaches to investigate the hub hypothesis are limited. Indeed, in both *ctl1* and *vamp714* mutant seedling roots, auxin gradients are severely disrupted and seeing a cumulative phenotype for auxin transport in the *ctl1 vamp714* double mutant is unlikely. It would then be concluded that the proteins are part of the same pathway even if they are not. Fortunately, it does not mean that the hypothesis cannot be challenged experimentally. An inducible ARA7 dominant negative protein has already been used to study the endocytosis of cargo proteins (Beck et al. 2012; Ito et al. 2016). It would be interesting to visualize the effect of this dominant negative in plants expressing in *ProVAMP714:VAMP714:mcherry* and if it affects the interaction of VAMP714 with CTL1 in punctate structures. Finally, colocalization between interacting proteins and TGN/EE would be essential to determine the compartment(s) where these interactions occur.

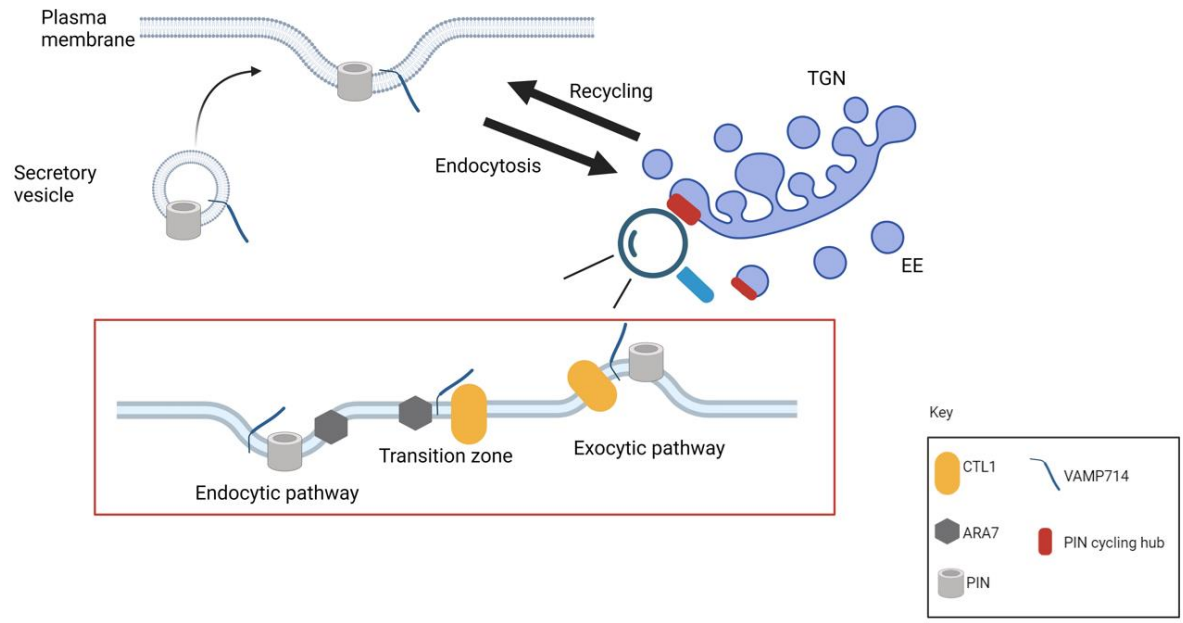

Fig. 6.1 Hub hypothesis. VAMP714, ARA7 and CTL1 interact in cycling hubs in the TGN/EE.

6.2 CTL1, at the crossroad of lipid homeostasis and vesicle trafficking

The story of CTL1 is interesting because, within a year, it was identified in 3 different screens by 3 different research groups (Kraner et al. 2017; Gao et al. 2017; Wang et al. 2017). CTL1 also called CHER1 (less likely to be mistaken with CHITINASE-LIKE protein 1, which is also CTL1) and encodes for a choline transporter involved in plasmodesmata maturation (Kraner, Müller, and Sonnewald 2017), ion homeostasis (Gao et al. 2017) and PIN-mediated auxin transport (Wang et al. 2017). In the last two of these processes, CTL1 is found to regulate vesicle trafficking of transporter proteins. This indicates that choline homeostasis is somehow associated with vesicle trafficking. Even though there is a limited amount of information on choline metabolism, the studies available are informative on choline effects on vesicle trafficking. The first key piece of information is that CTL1-mediated trafficking is tissue-specific. Indeed, mutation of *CTL1* disrupts PIN1 and PIN3 trafficking in the stele but not PIN2 in the epidermis (Wang et al. 2017). This cannot be explained by the expression of *CTL1* in the stele only since it has been shown that *CTL1* is expressed in epidermal cells in the RAM (Wang et al. 2017). Furthermore, CTL1 is essential for the recycling of ion transporters in the root epidermis (Gao et al. 2017). The second interesting point is that choline also regulates endocytosis. Indeed, in *ctl1* plants or plants treated with choline, internalization of the membrane dye FM4-64 is repressed (Gao et al. 2017).

A model for CTL1 effects on membrane composition was imagined by Gao et al. 2017 where they hypothesised that mutation of the *CTL1* gene causes an increase of cytosolic choline, which inhibits choline hydrolysis from phospholipids by phospholipase D at the layer facing the cytosol. This results in an asymmetry in lipid composition between the layer facing the cytosol and the layer facing the lumen of the TGN/EE. This asymmetry is thought to affect vesicle trafficking but the mechanism is not yet known.

In the present study, CTL1 was shown to interact with VAMP714. Also, choline treatment represses the correct subcellular localization of VAMP714. To my knowledge, this is the first example of a link between choline homeostasis and a protein of the trafficking machinery. Here, I hypothesise that CTL1 activity modulation can create a local change in membrane composition allowing the recruitment of VAMP714 and PIN proteins in exocytic vesicles.

Current data indicate that VAMP714 and CTL1 have overlapping but not identical functions in PIN trafficking. Whereas VAMP714 is essential for both PIN1 and PIN2 polarity, CTL1 and more generally choline regulate the trafficking of PIN1 but not PIN2. This suggests that VAMP714 mediates PIN trafficking through pathways that are different and tissue specific (Fig. 6.2). Because VAMP714 is likely to be involved solely in the fusion of PIN carrying vesicles, there might be a factor that is yet to be discovered and responsible for VAMP714 recruitment in epidermal cells.

Future work: In order to investigate the tissue-specific role of CTL1 in PIN recycling, it would be interesting to carry out a PPI experiment between VAMP714 and CTL1 in their native context. That could demonstrate whether the VAMP714-CTL1 association is stele-specific.

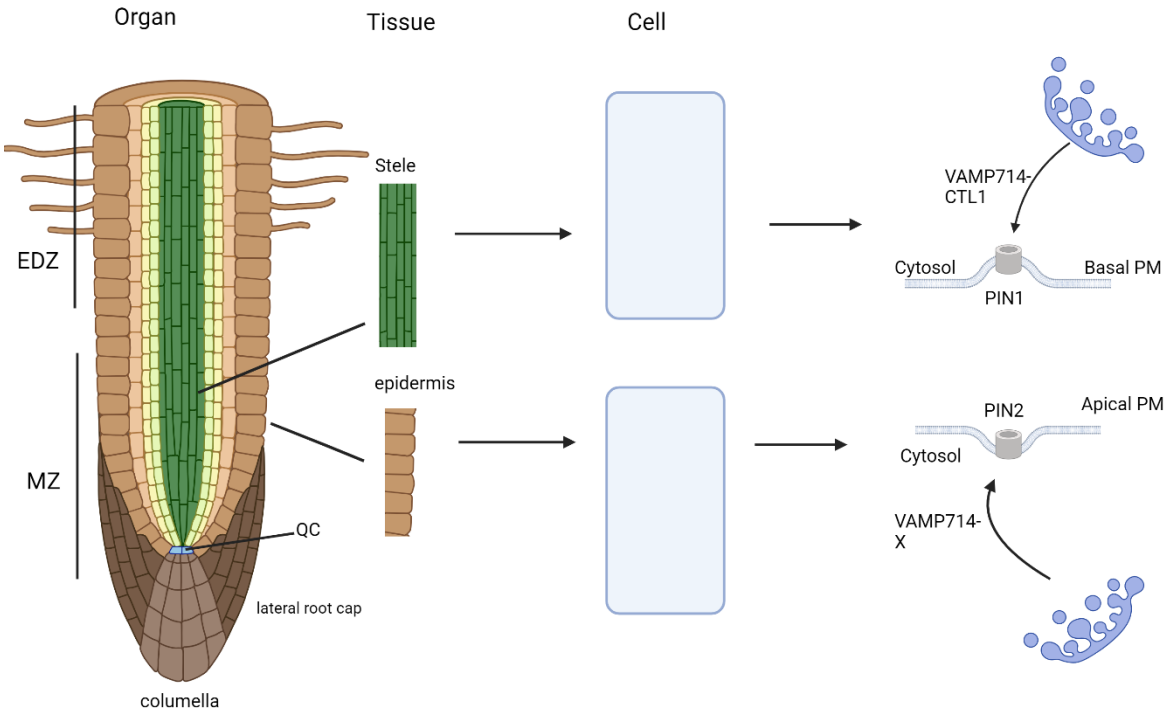

Fig. 6.2 Putative VAMP714-mediated pathways for PIN polar localization. MZ meristematic zone, EDZ elongation and differentiation zone, QC quiescent centre.

6.3 Fusion events during PIN cycling

Experiments conducted so far suggest that PINs en route to the PM are 'travelling' in the same vesicles as VAMP714. In this process VAMP714 is the 'key' that would open the 'door' of the PM for PIN proteins. Because of the function of VAMP714 interactor ARA7 in endocytosis, it is possible that VAMP714 is endocytosed together with PIN. Endocytic vesicles fuse with the TGN/EE which adds another fusion event to VAMP714 journey in the endomembrane system. Is VAMP714 involved in that process? Currently, there is not enough information to answer this question. Using the key-door analogy, VAMP714 could be a key for different doors (PM and TGN/EE) or another SNARE is endocytosed with PIN and mediates the fusion at the TGN/EE in a one key-one door scenario. Experimentally, this would be difficult to evaluate. However, some hints could come from the study of the VAMP714 SNARE complex. SNAREs do not work alone but form tetrameric complexes that mediate membrane fusion (Sutton et al. 1998). Also, it is common for SNAREs to reside in specific compartments. Therefore, using VAMP714 as a bait for IP-MS could allow us to look for PM and TGN/EE SNAREs in the VAMP714 proteome. That kind of experiment has been

conducted with Qa-SNAREs of *Arabidopsis* as baits (Fujiwara et al. 2014). VAMP714 was shown to interact with the vacuole resident SYP22 PVC/Vacuole resident Qa-SNARE but not with PM or TGN SNAREs. However, some interaction might have been missed due to the low expression level or the transient nature of the interaction. Furthermore, some of the Qa SNAREs did not have detectable interactors, suggesting that the IP-MS does not allow the capture all the interactors (Fujiwara et al. 2014).

6.4 VAMP714 and PIN polarity

VAMP714 is required for the polar localization of PIN1 and PIN2 respectively in their basal and apical domains (Gu et al. 2021). However, there is no evidence that VAMP714 is involved in the 'decision making' of PIN polarity and therefore it is likely that it acts downstream of GNOM in the stele and PID in the epidermis. PID is easy to accommodate with the proposed model of VAMP714-mediated cycling since it phosphorylates PIN at the PM and the phosphorylation is the signal for the recruitment of ARF-GEF of the apical trafficking that also recruits VAMP714 in recycling vesicles. Basal targeting is more difficult to explain because VAMP714 do not seem to interact with GNOM. GNOM was initially thought to localize at the TGN/EE (Geldner et al. 2003b) because of its role in protein recycling but a more advanced investigation has shown that it actually predominantly localizes at the Golgi apparatus (Naramoto, Otegui, et al. 2014; Naramoto et al. 2010) and can be detected in low abundance at the PM. In this article, it is proposed that GNOM regulates PIN trafficking indirectly (Fig. 6.3). This would explain why the VAMP714 PPI complex does not interact with GNOM. However, VAMP714 is also a Golgi protein, suggesting the possible existence of separate domains within the Golgi.

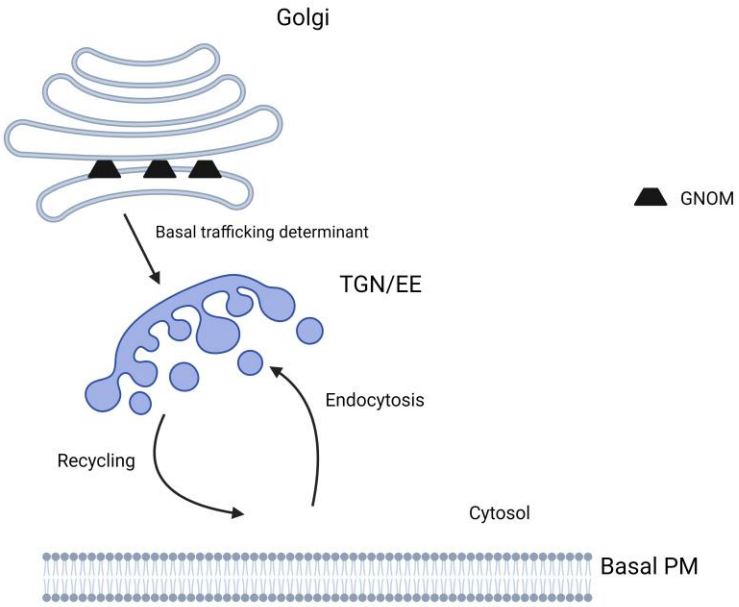

Fig. 6.3 Suggested indirect regulation of PIN basal polarity by GNOM. Inspired by (Naramoto, Otegui, et al. 2014)

6.5 All in all, just another brick in the cell wall

Hemicelluloses and pectins are synthesised in the Golgi and cellulose synthesis occurs at the PM through cellulose synthase (CESA) complexes (CSCs) (Sinclair, Rosquete, and Drakakaki 2018). Since vesicle trafficking is the main transport system between the different intracellular compartments and between intracellular compartments and the extracellular environment, it is likely to have a central role in the regulation of cell wall composition. The main difference in the trafficking of primary cell wall components is that vesicles transport the matrix polysaccharides to the cell wall whereas the cellulose biosynthesis machinery is subject to vesicle trafficking rather than cellulose itself. However, in both cases the TGN was shown to be a platform for cell wall sorting (Sinclair, Rosquete, and Drakakaki 2018; Hoffmann et al. 2021). Similarly to what is observed for transporter proteins, CSCs were shown to be internalized and recycled to the PM and this cycling regulates cellulose synthesis (Zhu and McFarlane 2022; Bashline, Li, and Gu 2014). This process is known to depend on CSC association with microtubules (MTs) but only a small number of canonical trafficking proteins have been linked to CSC transport (Sinclair, Rosquete, and Drakakaki 2018).

Nonetheless, some CESAs have been identified in the proteomes of markers of the endomembrane system, giving hints about the routes for CSC secretion (Drakakaki et al. 2012; Heard et al. 2015). Another interesting "omic" approach, the glycomic, using SYP61 SNARE as a bait to isolate TGN vesicle-specific polysaccharides, has provided understanding of the secretion of cell wall polysaccharides (Wilkop et al. 2019). It appears that matrix polysaccharide deposition in the cell walls is mediated at least partly by SYP61 vesicles.

In *vamp714* mutants, a broad range of genes involved in cell wall composition and organisation are downregulated. Because auxin gradients are impaired in those plants the auxin response is also affected (Gu et al. 2021). Cell wall genes are auxin responsive (Majda and Robert 2018) and the defects in auxin transport in *vamp714* mutants could account for the measured changes in the expression of these genes. However, the impaired localization of GFP:CESA3 in the *vamp714* mutant background suggests that VAMP714 is required for CSC trafficking. Futhermore, CESA3 and other CESAs were found in the proteome of ARA7 together with VAMP714 (Heard et al. 2015), indicating that VAMP714 and CSC components overlap in their routes in the endomembrane system. Cell wall components, and particularly cellulose, are essential for the maintenance of PIN polarity (Feraru et al. 2011). This is explained by the inhibitory effects of cell walls on PIN lateral diffusion. Although disrupting *VAMP714* expression severely increases the fluorescence intensity recovery after photobleaching of PIN2, the percentage of recovery seems too high as it is bigger than the recovery measured for mobile proteins (Martinière et al. 2012a). This could be because of the free-floating vesicles containing PIN2 that are blocked in the cytoplasm since VAMP714 is unable to perform its function. Given the effect of CESA3 on PIN lateral diffusion has been demonstrated (Feraru et al. 2011) it is likely that the mutation of VAMP714, through its effect on the trafficking of CESA3, has an effect on PIN mobility but this effect is masked due to aberrant PIN localization. Therefore, I hypothesise that VAMP714 regulates PIN trafficking and maintenance at the PM (Fig. 6.4).

Challenges and future work:

Models using parameters controlling PIN distribution at the PM indicate that polar targeting of PINs is not sufficient to explain PIN polarity (Kleine-Vehn et al. 2011), and that lateral diffusion is an important factor for polarity maintenance (Łangowski et al. 2016). Cell walls contribute to the decrease of the lateral diffusion which prevents PINs from escaping their

polar domain. The association between PINs and the cell wall is not fully understood yet but could involve the Hetchian strands that connect the PM to the cell walls (Łangowski et al. 2016). In order to further investigate the role of VAMP714 on PIN mobility, it would be interesting to observe PIN distribution within their domain. Indeed, PIN abundance across their polar domain is not homogenous (Kleine-Vehn et al. 2011), and changes in lateral diffusion rates would increase the homogeneity of PIN distribution. However, the distribution of PINs could also be affected by VAMP714-mediated PIN trafficking. The solution to this problem might then come from the measurement of the diffusion of PIN at single-molecule level.

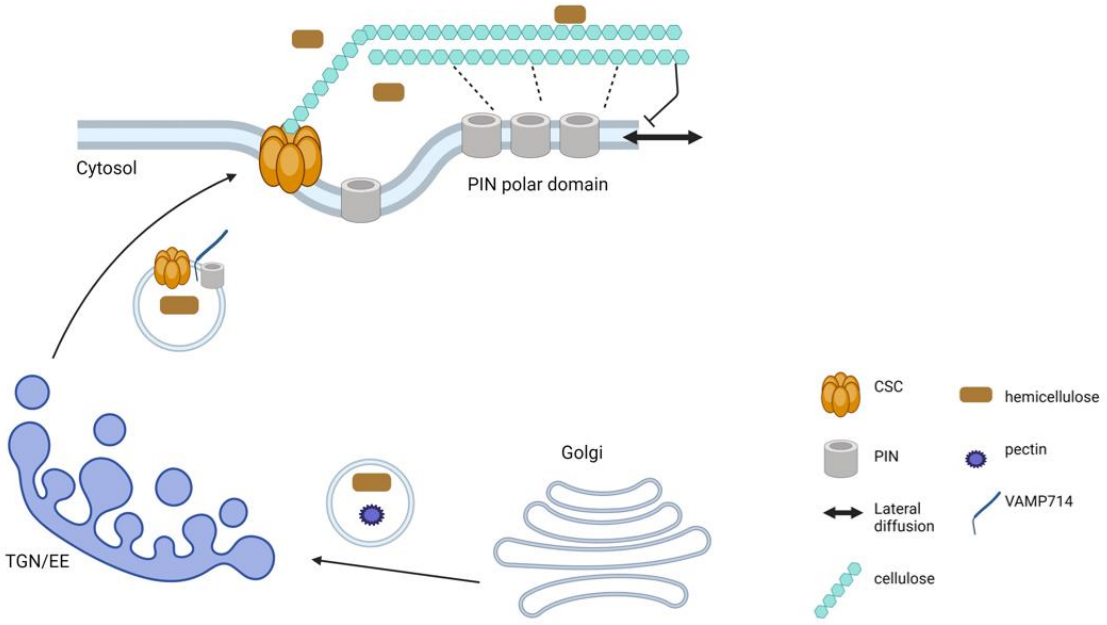

Fig. 6.4 Hypothetical model of a coordinate trafficking of PIN and CSCs contributing to maintain the polarity of PIN delivered at the PM in VAMP714 vesicles.

6.6 Symplastic auxin transport

Identification of mutants with an impaired callose-dependent gating of PD causing defects in auxin transporters (Han et al. 2014) has highlighted the physiological relevance of symplastic auxin transport. CTL1 was shown to be required for PD maturation and for the proper localization of plasmodesmata callose-binding proteins (PDCB1 and PDCB2) (Kraner, Müller, and Sonnewald 2017). In his review lipids at the crossroad, Boutté estimates that it is likely that CTL1 regulates both PIN and PD-mediate auxin transport (Boutté 2018). Interestingly, the callose synthesis machinery is very similar to the CSCs and is active at the

PM (B. Wang, Andargie, and Fang 2022). VAMP714 is an interactor of CTL1 but the role of this SNARE in callose deposition has not been investigated. Therefore, maturation of PD could be observed in *vamp714* mutants with an electron microscope and a callose staining assay would indicate if VAMP714 and CTL1 also are associated in the callose synthesis machinery trafficking.

6.7 VAMP714, the ugly duckling of VAMP71s family

VAMP714 belongs to the VAMP71 family consisting of 4 members, VAMP711, VAMP712, VAMP713 and VAMP714. Whereas VAMP711-713 mainly localize to the tonoplast, VAMP714 localizes principally at the Golgi. Domain swapping experiments have shown that login domains of R-SNARE are crucial for their localization (Uemura, Sato, and Takeyasu 2005). Overall homology and similarity in the primary structure of VAMP71s is high but VAMP714 amino acid sequence differs slightly from the other members and VAMP714 forms its own clade. This is very likely to explain the unique function of VAMP714 within its family and the lack of redundancy that accounts for the phenotype of the *vamp714* single mutant, which is a rare occurrence in the world of SNAREs. Excitingly, the high similarity in VAMP71 login domains allowed the identification of a small number of residues specific to VAMP714. These residues make good targets for site-directed mutations. Mutations of individual of VAMP714-specific residues in the longin domain into VAMP711 residues and then different combination of mutated residues would allow to identify the amino acids/motifs responsible for VAMP714 localization.

A more ambitious project would be to determine the specificity of SNARE domains in VAMP71s. Expressing VAMP711 with a VAMP714 longin domain results in the localization of VAMP711 at the Golgi (Uemura, Sato, and Takeyasu 2005). Expressing this hybrid protein under the control of the VAMP714 promoter in a *vamp714* background could potentially rescue the phenotype of these mutants. If this not enough it is likely to be due to VAMP714 specific association with Q-SNAREs in SNARE complexes. Because the SNARE domains of VAMP71s are also highly similar it can be used for further site-directed mutagenesis, but this time to crack the SNARE association code of VAMP71s.

Given the central role of post-translational modifications in PIN polarity it is possible that they regulate the localization of other membrane proteins including SNAREs. Informatic analysis predicted that VAMP714 has two sumoylation sites and both are located in the longin domain (data not shown). This suggests a possible role of post-translational

modifications in VAMP714 sorting. Comparing post- translational modification profiles in VAMP71s would be a first informative step to test this hypothesis.

6.8 Sub-functionalization in VAMP71s

The number of SNAREs has increased in parallel with the rise of multicellularity in plants and most families of SNAREs appeared in multicellular plants (Sanderfoot 2007). This is consistent with the role of SNAREs in intracellular communication, a key aspect of multicellularity. The most recent phylogenetic study of SNAREs in Arabidopsis looked into the phylogenic relationships between these proteins (Gu et al. 2020). The lack of relationship between the genetic and expression distance supports the hypothesis that specialization in SNARE functions happen through sub-functionalization rather than neofunctionalization (Gu et al. 2020). Reporter lines and available online expression data indicate that VAMP71s have partly overlapping but mostly different expression patterns in time and space. VAMP711-713 have no obvious phenotype in non-stress conditions but were shown to be associated with different abiotic stress responses (Xue et al. 2018; Leshem et al. 2006; Xue et al. 2019). Interestingly, individual VAMP71s have non-redundant effects on alkaline stress resistance. This could be because VAMP71s belong to independent vacuolar sorting pathways but it is more likely to be the consequence of the different VAMP71 expression patterns. Promoter swapping experiments would help to further investigate the viability this hypothesis.

"Who is looking for auxin will find auxin" - if this is not already a phrase it should be, given the omnipresence of this phytohormone in biological processes in plants. VAMP71s are all induced in response to auxin (Gu et al. 2021). Apart from *vamp714*, no *vamp71* mutants display obvious auxin-related defects. However, in certain situations, like the gravitropic response, the root of *vamp713* mutants shows a hyper-bending. Another study showed that the *vamp713* mutation also altered the gravitropic response of inflorescences after exposure to cold (Nadella et al. 2006). Interestingly, vacuolar targeting of PINs for degradation is promoted at the upper side of the root during the root bending in response to gravitropic stimulation (Kleine-Vehn et al. 2008). Therefore, it is tempting to think that VAMP713 is involved in PIN vacuolar sorting. Because of the role of both auxin and VAMP71s in stress it is also possible that VAMP71s mediate auxin responses under stress conditions. Also, auxin regulates vacuolar morphology in a SNARE-dependent manner (Löfke et al.,2015.). During drought stress, VAMP711 mediates ABA-induced stomatal closure (Xue et al. 2018).

Together, these results suggest that phytohormones regulate stress response through VAMP71-dependent pathways.

6.9 Evolution of VAMP71s

The most ancestral protein related to VAMP71s according to available phylogenetic data is found in *Chlamydomonas reinhardtii*, a unicellular green alga. However, the function of this protein has not been studied yet. A single VAMP71 member is found in the common liverwort, *Marchantia polymorpha* and localizes at the tonoplast (Kanazawa et al. 2016). The ancestral vascular plant *Selaginella moellendorffii* has 4 VAMP71 orthologs. In angiosperms, the number of VAMP71s varies greatly between different species suggesting that independent duplication events account for the number of VAMP71 members in these species.

VAMP714 proteins form their own clade in the VAMP71 family. Interestingly, in rice OsVAMP714 localizes to the chloroplast where it has a non-redundant function with the highly homologous vacuolar OsVAMP711 (Sugano et al. 2016). This suggest that VAMP714 has evolved to contribute to non-vacuolar pathways making it a somewhat unique VAMP71.

6.10 Conclusion

In this study, the R-SNARE VAMP714 was shown to interact with other regulators of PIN trafficking machinery. Interestingly, VAMP714 also regulates the trafficking of CESA3, that was shown to contribute to PIN polarity maintenance. I would therefore suggest that there has been a selection in *Arabidopsis* for vesicle trafficking of PIN auxin transporters together with regulatory elements of PIN polarity to stabilize auxin flows in the plant. Within the VAMP71 family VAMP714 has acquired a function in post-Golgi trafficking that differentiates it from other members involved in vacuolar sorting. At a structural level, the differences between VAMP714 and other VAMP71 are subtle which opens the way to exciting studies on the structural determinants of VAMP714 specificity.

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Appendix I Cloning primers

Primer sequence 5'->3'	Forward primer	Reverse primer
VAMP714 N-TAG	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCGATTGTCTATGCTG	GGGGACCACTTTGTACAAGAAAGCTGGGTTTAAGATCTGCATGATGGTAA
VPS29 N-TAG	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTATGGTGCTGGTATTGGCA	GGGGACCACTTTGTACAAGAAAGCTGGGTCTACGGACCAGAGCTGGT
BEX5 N-TAG	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCAGGGTACAGAGTG	GGGGACCACTTTGTACAAGAAAGCTGGGTGATTTGAGCAGCACCCGAG
CTL1 N-TAG	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAGAGGACCTTTAGGA	GGGGACCACTTTGTACAAGAAAGCTGGGTGGTGAGTAAGACTCTGAACC
EXO70A1 N-TAG	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCTGTTGATAGCAGA	GGGGACCACTTTGTACAAGAAAGCTGGGTGCCGGCGTGGTTCATTCAT
GNOM N-TAG	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGGTCGCCTAAAGTTG	GGGGACCACTTTGTACAAGAAAGCTGGGTGCGAACCAGTTGTGTTTTC
PIN1C-TAG	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGATTACGGCGGCGGAC	GGGGACCACTTTGTACAAGAAAGCTGGGTCTAGACCCAAGAGAATGTAGTAGAG
ARA7 N-TAG	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGCTGCAGCTGGAAAC	GGGGACCACTTTGTACAAGAAAGCTGGGT CTAAGCACAACAAGATGAGCTC

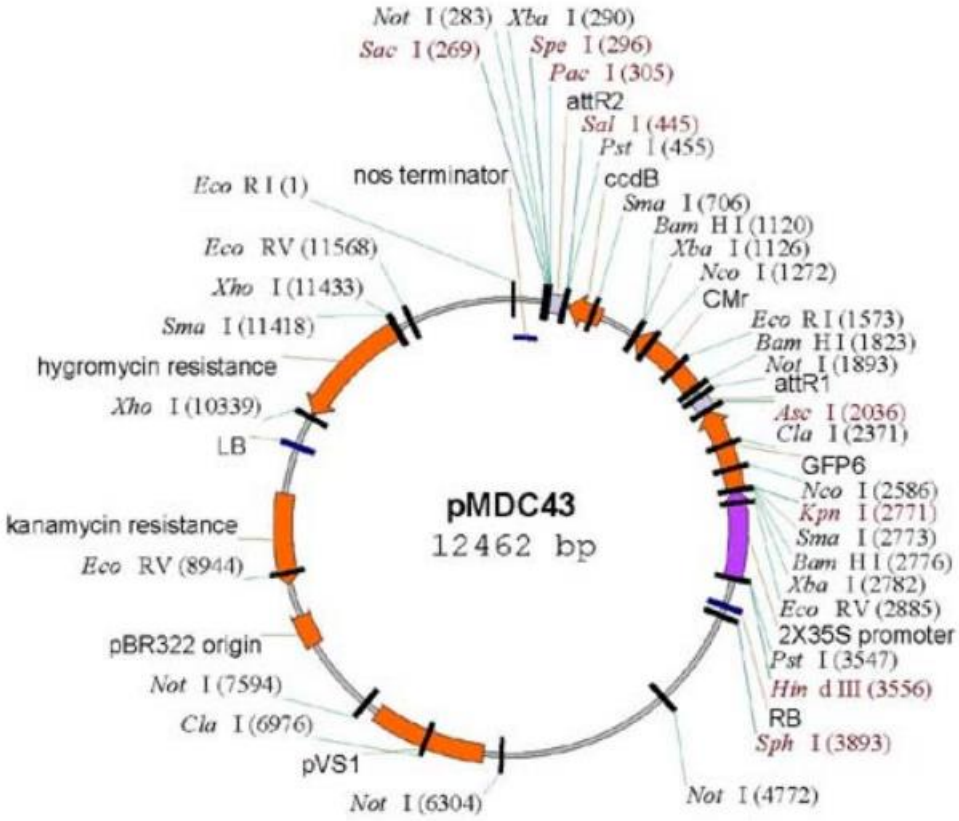
Promoter VAMP712	GGGGACAAGTTTGTACAAAAAAGCAGGCTTATTAAGCTATGTACCACCA	GGGGACCACTTTGTACAAGAAAGCTGGGTTCGGAAGAAATACTAAATAAATG
Promoter VAMP711	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTTGATTTTGGAAGGACAA	GGGGACCACTTTGTACAAGAAAGCTGGGT CTGGTCCTTCTTTTGCCT
VAMP711 OVER EXPRESSOR	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATGGCGATTCTGTACGCC	GGGGACCACTTTGTACAAGAAAGCTGGGTTTAAATGCAAGATGGTAGAGTAGG
VAMP712 OVER EXPRESSOR	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATGTCGATATTATACGCGT	GGGGACCACTTTGTACAAGAAAGCTGGGTTTAAACGCAAGAGGGTAG

Table S1 primers used for GATEWAY® cloning.

Appendix II Vector maps and sequences

Vector maps and sequences

1. PMDC43 GFP



Sequence

cgtaatcatggtcatagctgtttcctgtgtgaaattgttatccgctcacaattccacacaacatacgagccggaagcataaagtgtaaagc
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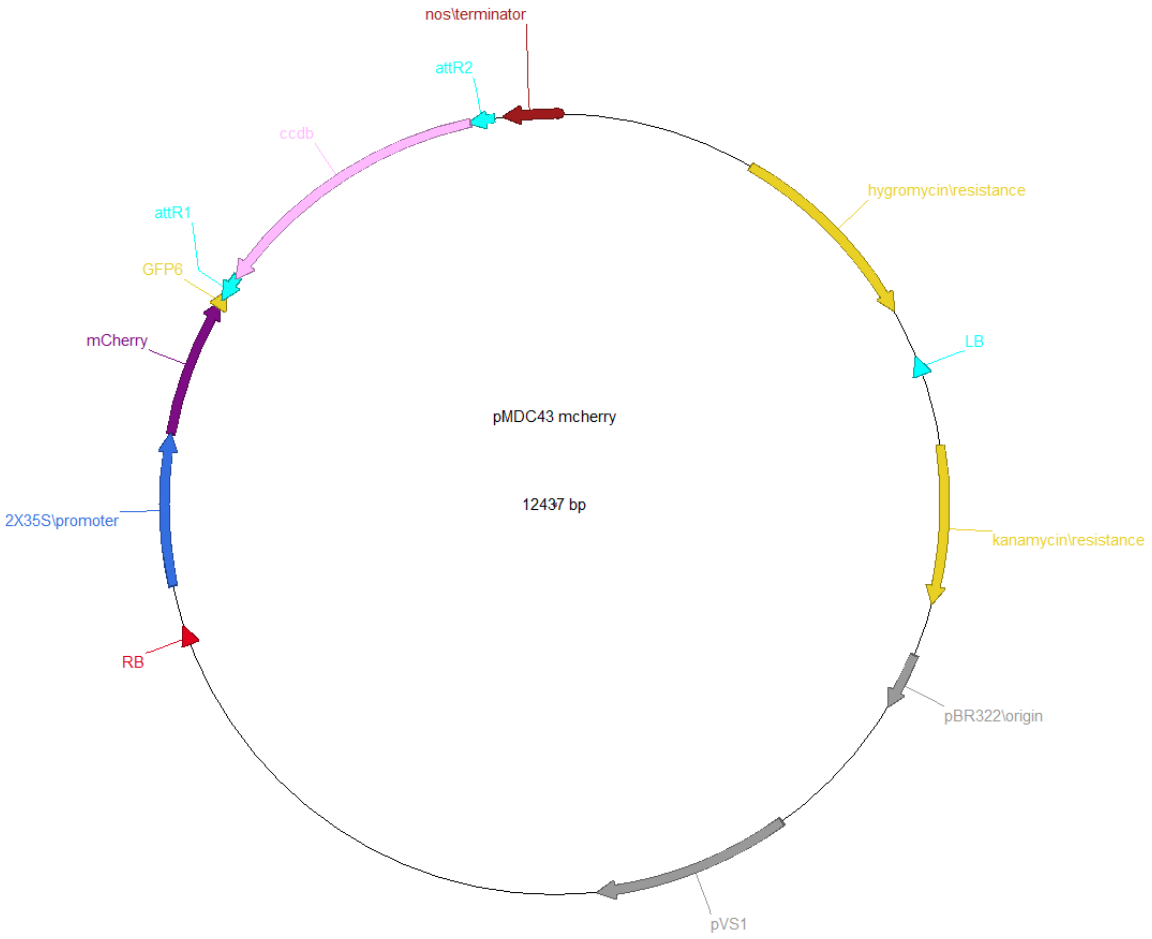

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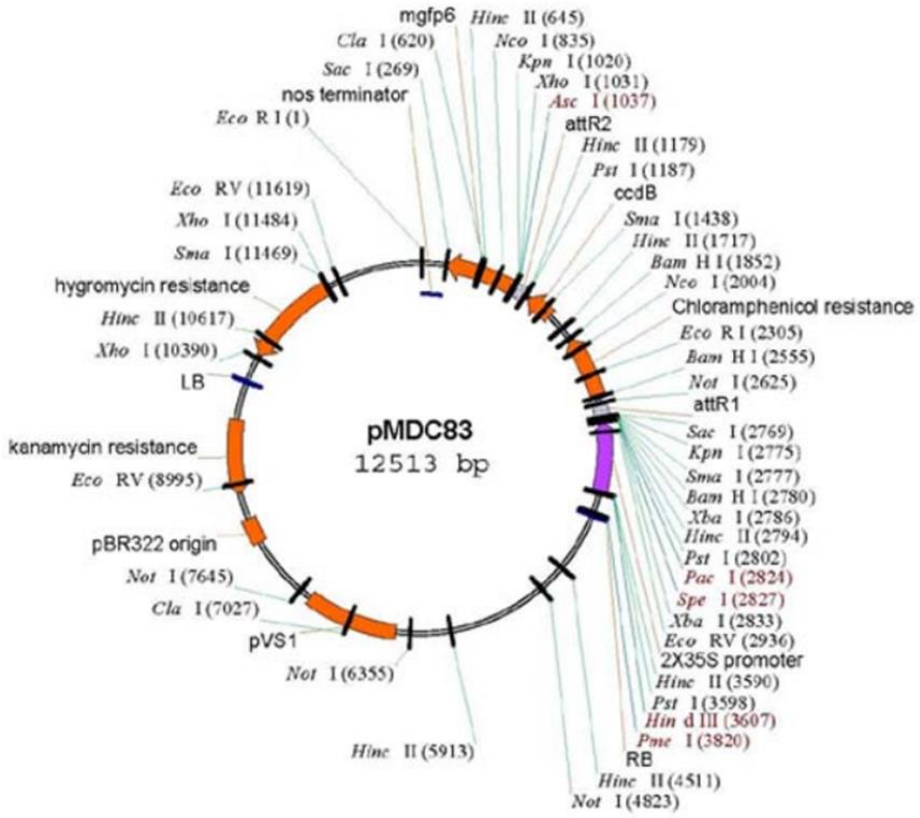

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4. PMDC83 mCherry

Map identicat to PMDC83 GFP but the mGFP6 sequence was replaced by mCherry.

Sequence

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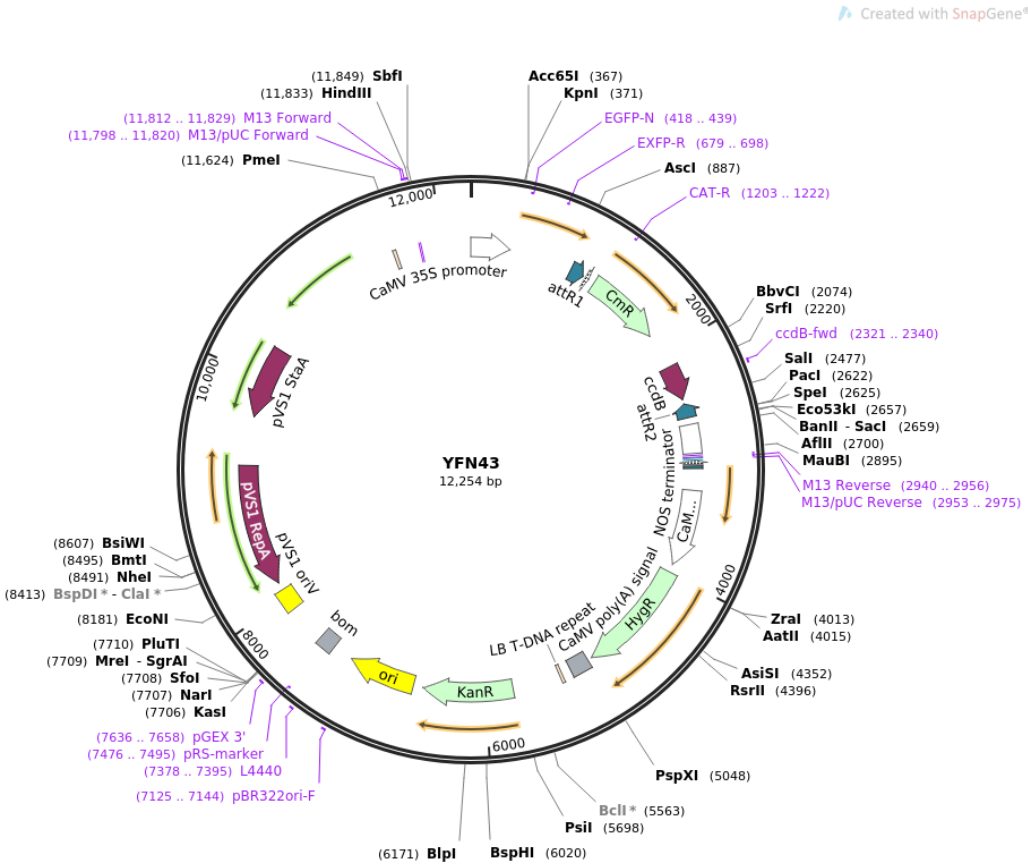

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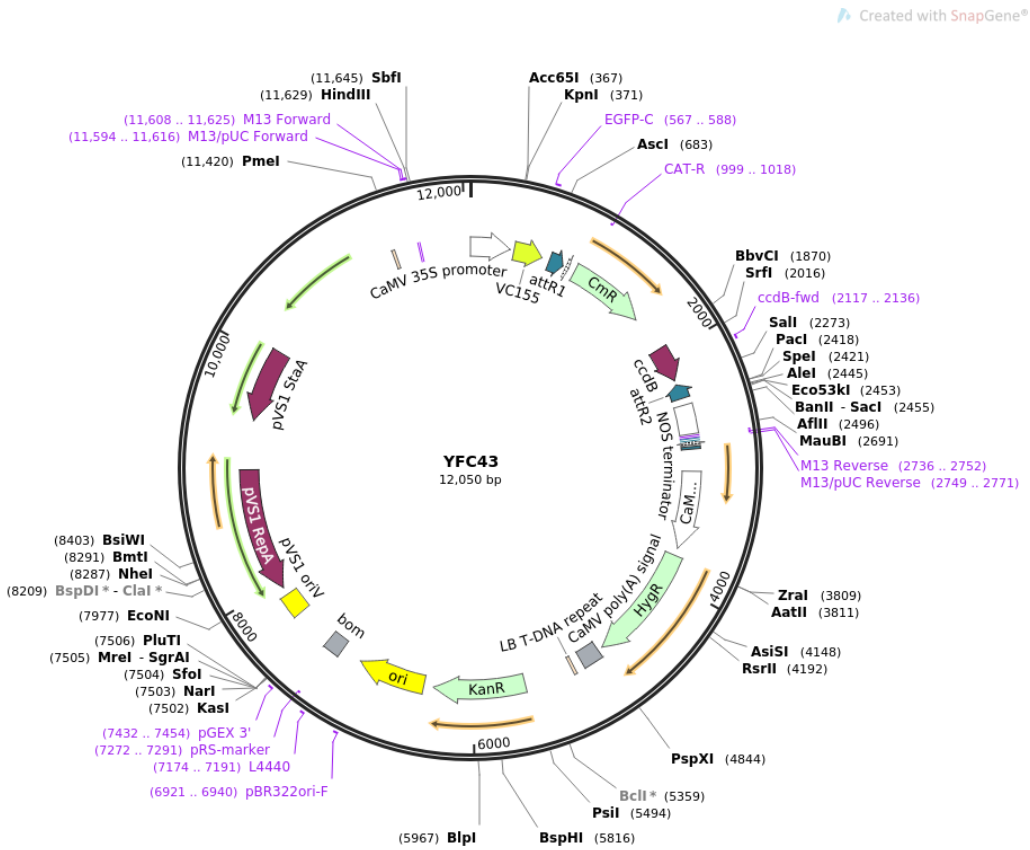

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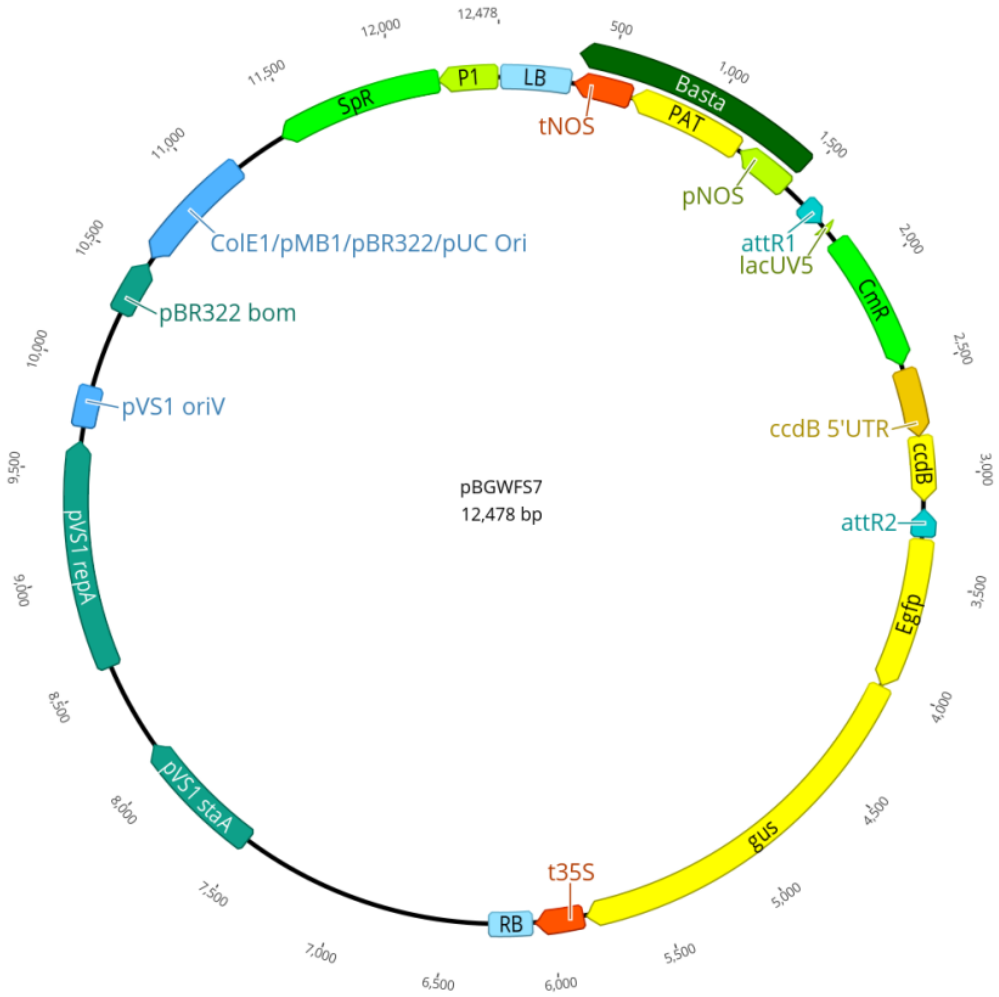

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Appendix III protein- protein interaction measurements

1. FRET-FLIM measurement of non-interacting proteins

1.1 BEX5

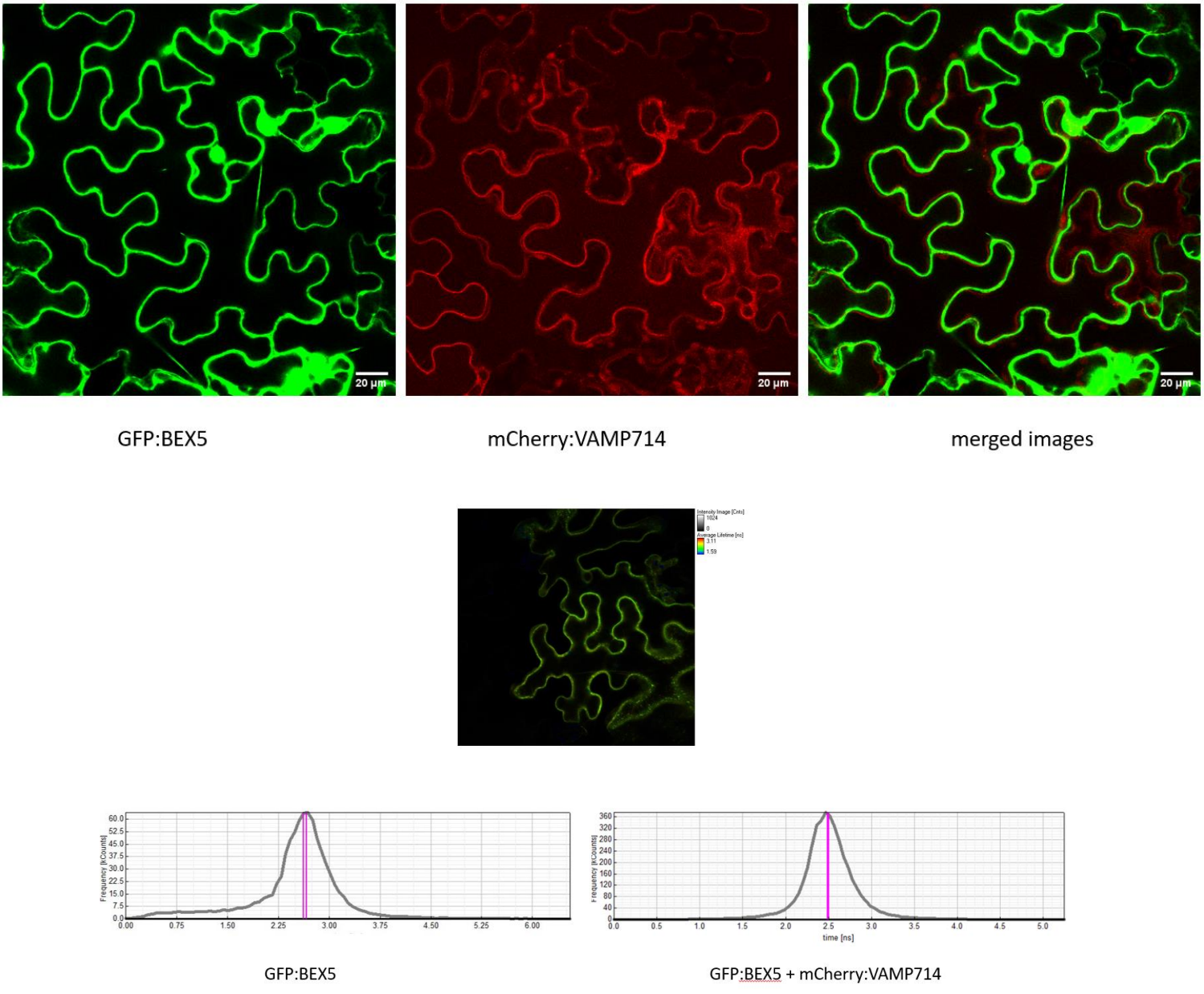
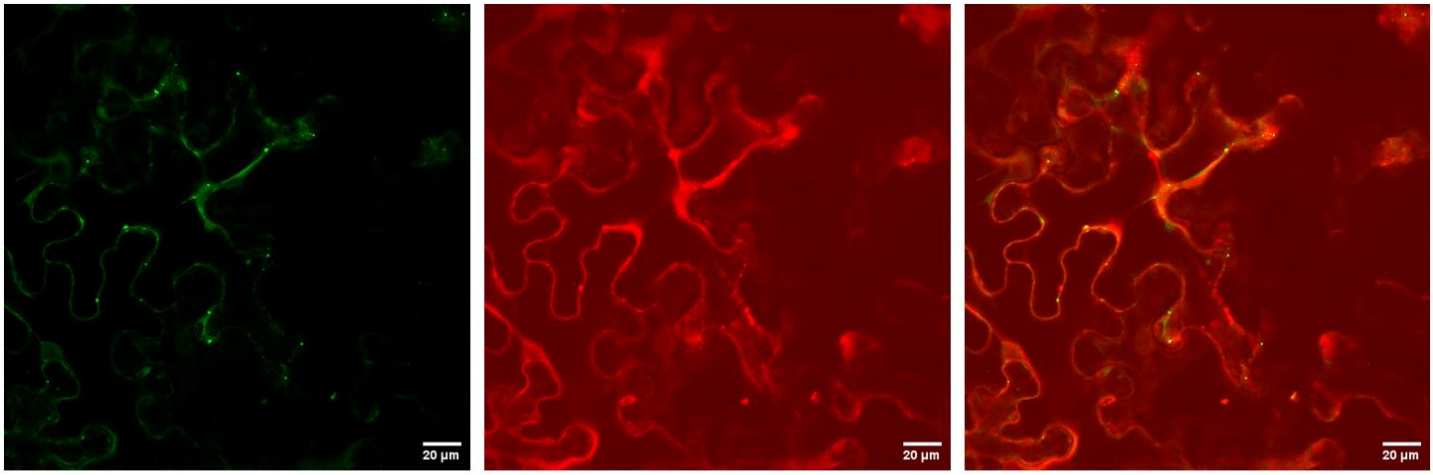
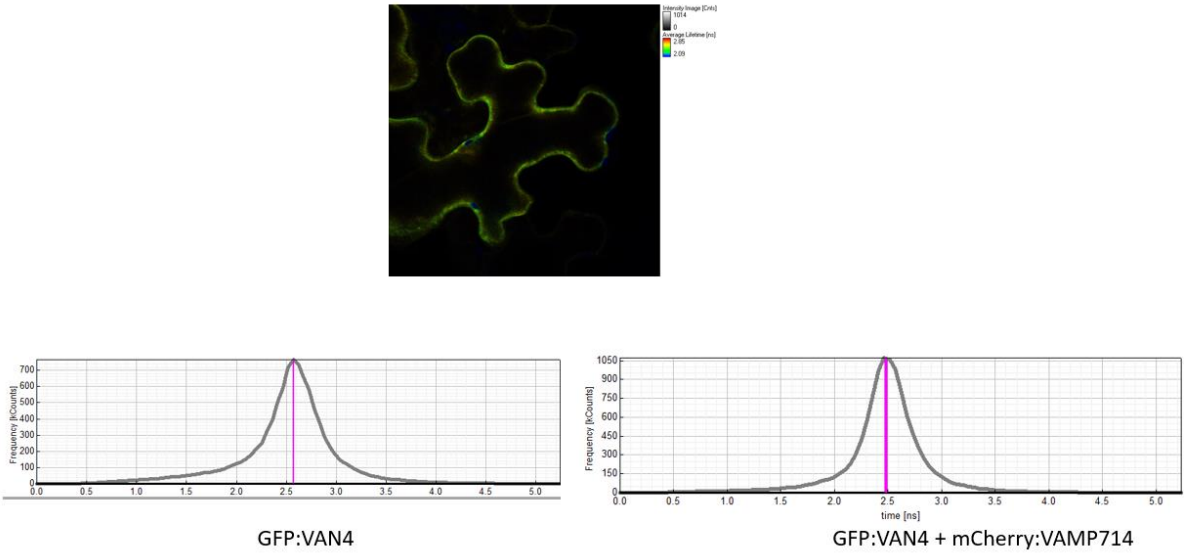


Fig. S1 BEX5 does not interact with VAMP714 in FRET-FLIM analysis. VAMP714 colocalizes with BEX5 on a large portion of the cell (upper panel). However, no change in GFP lifetime can be observed when GFP:BEX5 and mCherry:VAMP714 are co-expressed. Consistently, the the colour-code of the GFP:BEX5 beatmap is not affected in the presence of VAMP714.

1.2 VAN4



GFP:VAN4 mCherry:VAMP714 GFP:VAN4 + mCherry:VAMP714



GFP:VAN4 GFP:VAN4 + mCherry:VAMP714

Fig. S2 VAN4 does not interact with VAMP714 in FRET-FLIM analysis. VAMP714 colocalizes with VAN4 (upper panel). However, no change in GFP lifetime can be observed when GFP:VAN4 and mCherry:VAMP714 are expressed within the same cell. Consistently, the colour-code of the GFP:VAN4 beatmap is not affected in the presence of VAMP714.

1.3 GNOM

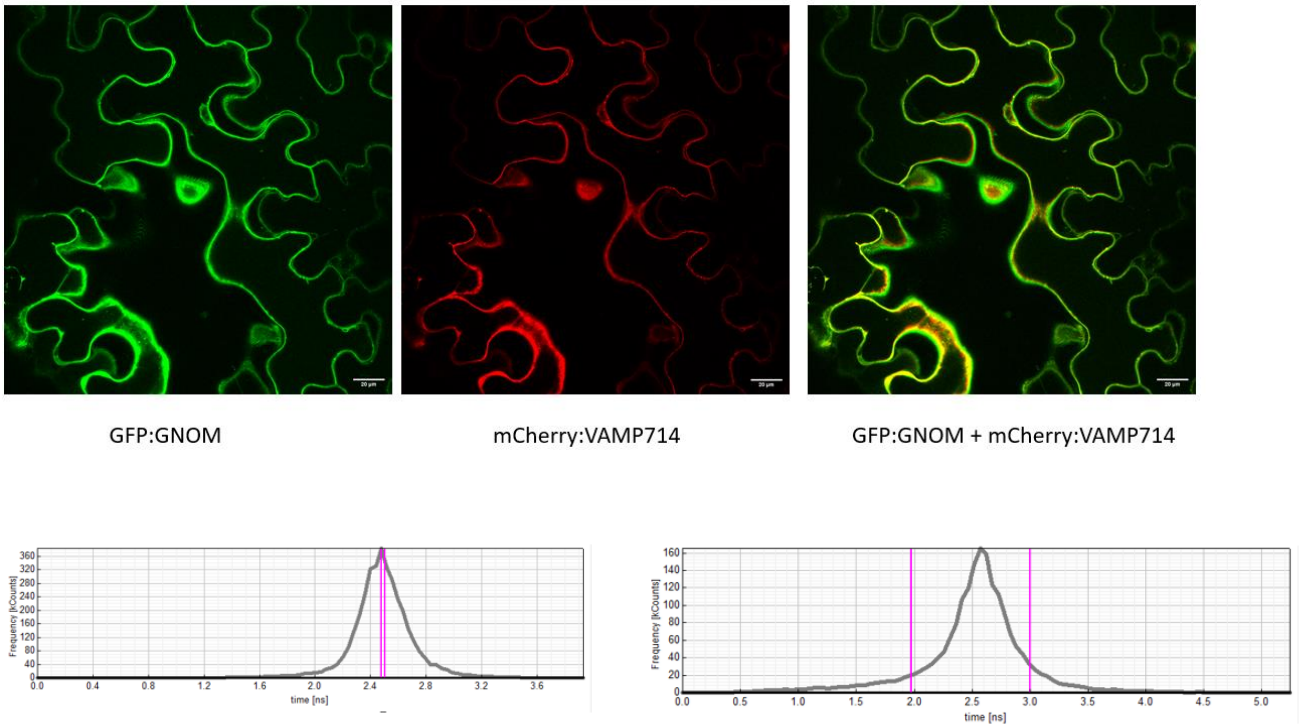


Fig. S3 GNOM does not interact with VAMP714 in FRET-FLIM analysis. VAMP714 colocalizes with GNOM (upper panel). However, no change in GFP lifetime can be observed when GFP:GNOM and mCherry:VAMP714 co-expressed.

2. Non-interacting proteins BiFC

2.1 VAN4

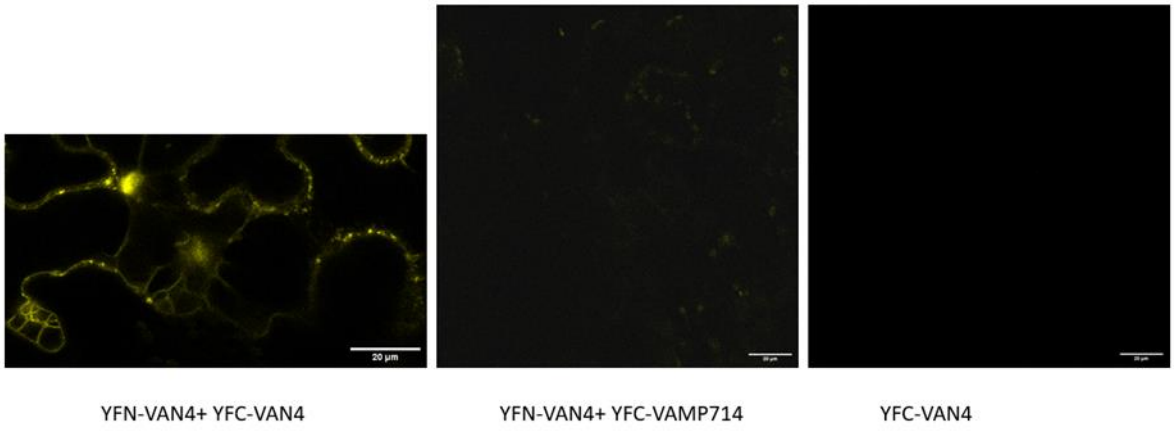


Fig. S4 VAN4 does not interact with VAMP714 in BiFC assays. When VAN4 is expressed with complementary YFP fragments, a fluorescent signal is observed. Nonetheless, when VAN4 and VAMP714 are expressed with complementary YFP fragments no signal is measured which is also the case of the negative control where YFC-VAN4 is expressed alone.

2.2 GNOM

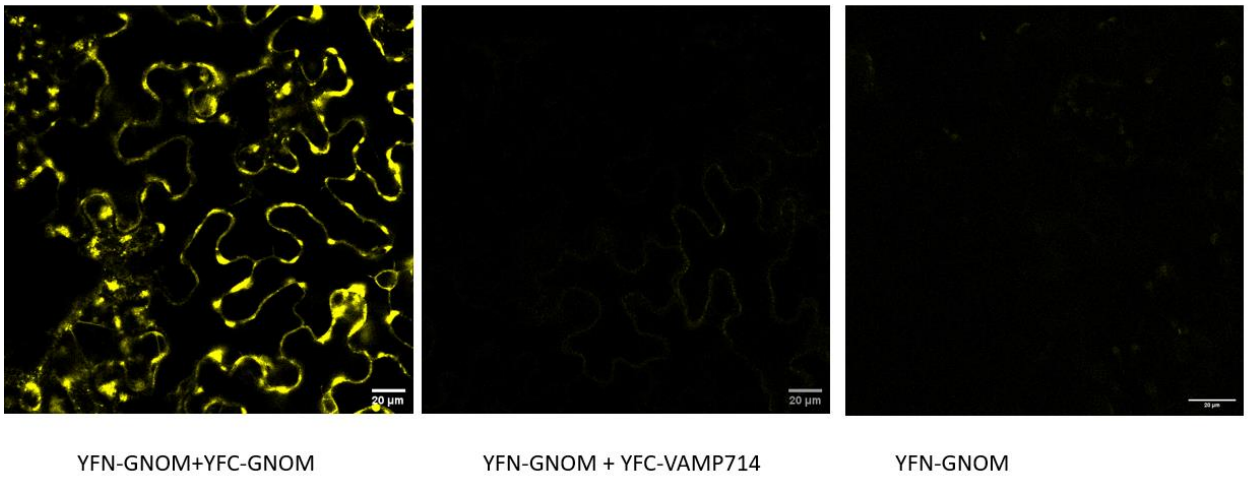


Fig. S5 GNOM does not interact with VAMP714 in BiFC assays. When GNOM is expressed with complementary YFP fragments, a fluorescent signal is observed. However, when GNOM and VAMP714 are expressed with complementary YFP fragments no signal is measured which is also the case of the negative control where GNOM is expressed with only a portion of the YFP tag.

Appendix IV List of significantly downregulated cell wall genes

gene ID	gene name/function	log2FoldChange
AT2G32540	cslb04	-2.959704513
AT1G69530	expansin1	-1.871561492
AT4G26690	GDBDL3 CELLULOSE AND PECTIN LINKING	-1.35507854
AT1G55850	CSIE1 CELLLULOSE	-1.006942967
AT1G26770	EXPASIN 10	-1.771832958
AT4G18780	CESA8	-3.555254918
AT4G00750	unknown	-1.562934999
AT2G28110	IRX7	-2.599208148
AT2G32530	CSLB03	-2.290437454
AT3G13870	gtpase cytoskeletton trafficking	-1.029078709
AT4G17880	secondary cell wall	-1.516910415
AT1G03870	unkown	-1.263127961
AT5G04960	pectin	-3.208815768
AT5G15490	pectin	-1.544030129
AT5G49660	unknown	-1.604561459
AT5G35190	unknown	-4.813828454
AT5G16910	CSLD3	-1.194949178
AT5G09870	CESA5	-1.306260738
AT1G23720	extensin15	-4.88203986
AT1G12560	expansin7	-4.719496589
AT1G43790	vascular tissue dev	-4.990240169
AT2G45750	unknown	-2.70911401
novel		-2.56323617
AT1G62980	expansin18	-3.264995169
AT2G38080	secondary cell wall	-1.976648564
AT4G33450	secondary cell wall	-7.018208487
AT1G55850	CSLE1	-1.006942967
AT5G17420	CESA7	-1.628295074
AT2G32610	CSLB1	-7.219464948
AT1G3380	LIGNIN	-1.98884732
AT3G28550	EXTENSIN 16	-3.348311279
AT1G28470	secondary cell wall	-3.025614823
AT3G54590	EXTENsin2	-2.982062614
AT2G24980	extensin6	-2.466370051
AT1G79620	secondary cell wall	-1.098510028
AT5G44030	secondary cell wall cellulose synthase	-1.130883289
AT4G13390	extensin12	-3.341272495
AT4G08410	extensin8	-2.796106532
AT4G09990	hemicellulose	-3.251721028
AT4G01630	expansin 19	-3.117945128
AT5G48920	secondry cell wall	-4.167194303
AT2G37090	secondary cell wall hemicellulose	-4.144175003
AT3G47380	pectin	-1.999534805
AT4G08400	extensin7	-3.949858229
AT1G62990	secondary cell wall	-1.369592689
AT1G09790	microfibril organisation	-5.88081271
AT3G12977	cell expansion	-2.260336531
AT5G16600	lignin secondary cell wall	-1.405769943
AT1G65680	beta-expansin	-6.002804842
AT3G26610	pectin cell elongation	-2.848958563
AT5G67210	hemicellulose secondary cell wall	-1.181845674
AT1G17950	pectin secondary cell wall	-2.028837419
AT4G01750	RHAMNOGALACTURONAN pectin	-1.247216838
AT3G54580	extensin17	-2.464787187
AT4G02270	unknown	-1.92864943
AT3G16860	microfibril organisation	-1.345484363
AT2G32620	cslb02	-6.418903728
AT3G18660	hemicellulose secondary cell wall	-2.84016124
AT5G06630	extensin9	-2.380631482
AT5G06640	extensin10	-1.75435695
AT1G48100	pectin cell expansion	-1.003724241
AT5G01360	secondary cell wall	-1.594323323
AT4G22680	lignin secondary cell wall	-5.318162456
AT5G60950	microfibril organisation	-1.407558947
AT5G49270	microfibril organisation	-4.725464614
AT3G55500	expnasin 16	-2.119077906
AT4G17220	cytoskeleton	-1.963071332
AT2G21140	unknown	-1.98251474

Table S2 List of the cell wall gene downregulated in 7-day-old *vamp714* seedlings. The gene significantly downregulated in the cell wall Biological Process (BP) GO category are listed and were used to determine the effect of VAMP714 on the different components of the cell walls.