

Molecular characterisation of edge-directed trafficking
in *Arabidopsis*

Liam Elliott
Lincoln College
University of Oxford



A thesis submitted for the degree
of *Doctor of Philosophy*

Michaelmas Term 2019

Abstract

The complex organisation and spatio-temporally variable dynamics of the eukaryotic endomembrane system is underpinned by an equally complex array of molecular machinery. Whilst the organisation and dynamics of eukaryotic endomembranes have been observable for some time, detailed study of the molecular machinery associated with endomembranes has only become possible in recent decades. In plants, the organisation and dynamics of the endomembrane system is intimately connected to their plastic development and unique growth properties. It has been suggested that many of the specific requirements of plant growth and development are supported by unique features of post-Golgi trafficking, which differs substantially from that of mammals. In the model plant species *Arabidopsis thaliana*, the *trans*-Golgi network/early endosome functions as a post-Golgi sorting hub on which trafficking pathways to and from the cell surface converge. The function of the *trans*-Golgi network/early endosome is underpinned by a large number of molecular components, of which the Rab-A GTPases are a significant part. Rab GTPases cycle between membrane-associated and membrane-dissociated states and spatio-temporally organise the recruitment of other essential trafficking machinery to endomembranes. Recent work has shown that a member of the *Arabidopsis* Rab-A GTPases, RAB-A5c, localises to compartments at the geometric edges of cells in growing tissues as well as the *trans*-Golgi network/early endosome. Inhibition of RAB-A5c function produces defects in anisotropic growth that can be mimicked *in silico* through local perturbations in cell wall mechanical properties around cell geometric edges in finite element models of plant cells. This and other observations have led to the hypothesis that RAB-A5c defines a polarised pathway from the *trans*-Golgi network/early endosome to the cell surface via cell geometric edges, which possibly functions to modify the cell wall in growing tissues specifically around cell edges. The existence of such a trafficking pathway remains uncertain however in the absence of any identified pathway cargoes, which also limits possible further investigation of the organisation and functions of such a pathway.

In this thesis, I investigate possible interactors of RAB-A5c and components of its associated trafficking pathway identified through co-immunoprecipitation. This work identifies two Receptor-Like Proteins as possible direct interactors of RAB-A5c and components of its associated trafficking pathway. This identification confirms the existence of an edge-directed trafficking pathway in growing tissues of *Arabidopsis* and that cell geometric edges are a discrete spatial domain of the *Arabidopsis* plasma membrane. Moreover, localisation and trafficking of truncated variants of these Receptor-Like Proteins gives indications as to their possible functions and contributions of different protein domains to their trafficking. Two hypotheses for the function of these Receptor-Like Protein components of edge-directed trafficking are presented and will be the subject of future investigations. Finally, perturbation of the endomembrane and cytoskeletal systems challenges previously-held assumptions about the organisation of edge-directed trafficking and the suitability of techniques commonly used to investigate Rab GTPase function in plants.

Acknowledgements

The completion of this thesis owes much to many people.

I first thank all the staff who work to ensure that the Department of Plant Sciences and Interdisciplinary Biosciences DTP run smoothly, making day-to-day work much easier than it otherwise would be. I also thank the staff on all levels who made my time at Lincoln College so enjoyable.

Scientifically, I must thank all members of the lab past and present: Dr Monika Kalde, Dr Bernadette Gehl, Dr Jacob Siefert, Antoine Chevalier, Mary Fesenko, Dr Xinyu Zhang, Dr Niloufer Irani and Dr Charlotte Kirchhelle. I am particularly indebted to Monika Kalde for her work in developing the co-immunoprecipitation protocol that provides a foundation for this thesis, and to Bernadette Gehl for helping me orientate myself in the early days and teaching me many of the everyday techniques used in this thesis. I am also very grateful to Niloufer Irani for her constant and continuing advice and guidance, for the provision of several materials used in this thesis, and for her good humour. I also thank Caroline O'Brien and Sarah Rodgers for ensuring everything in the lab runs smoothly and Antoine Chevalier for his assistance in generating materials used for BiFC in this thesis.

I thank Professor Nico Geldner (University of Lausanne), Professor Karin Schumacher (University of Heidelberg) and Professor Christopher Grefen (University of Tuebingen) for the provision of published materials used in this thesis. I am also indebted to Dr Sebastian Wolf (University of Heidelberg) for the gift of CRISPR-Cas9 mutant lines used in this study, and for his openness to discussion and sharing of information.

Outside of the science, I thank everyone in Lincoln College MCR who has helped make the past 4 years fun and easy outside of the lab, as well as my coursemates on the Interdisciplinary Biosciences DTP. I'm particularly grateful to the residents of Green Street and the Emily Carr House for all the good times, and my family for their good-humoured support.

I owe much to Dr Mark Fricker and Professor Hugh Dickinson. Mark for his support over the past year and a half as well as useful advice and insights, and Hugh for his constant presence, stability, good humour and work to keep things going. I must also thank Dr Roni McGowan for ensuring things ran smoothly lately.

I also am deeply grateful to Professor Farhah Assaad (Technical University of Munich) for her support, encouragement and engagement in other areas of work. I further thank my examiners Professor Jenny Russinova (VIB-UGent) and Professor Lee Sweetlove for their insightful comments and discussions.

Above all, this thesis owes the most to two people.

Dr Charlotte Kirchhelle has been unwavering in her support of me and this work, for which I am all the more grateful given that it was never an official requirement of her to be so. She has provided invaluable and constant technical and scientific advice, as well as much moral support. Discussing, conducting and evolving this and other work surrounding this project with her has been an absolute joy and I could not have done it without her. A scientific 'big sister' if ever there was one.

Finally, this work was overshadowed by the death of its instigator and primary supervisor, Dr Ian Moore, in August 2018. Ian was a unique mix of kind, funny, loyal and generous with an outstanding intellect and sense of scientific integrity. His support, encouragement and sense of responsibility towards me and the rest of his lab never failed, even in his final weeks when it was clear that he had very little time left. Ian's strong scientific legacy speaks for itself, but I hope that his personal legacy will go on in those people he taught and supervised, and I will be forever grateful to be one of those.

Abbreviations

LECA	Last Eukaryotic Common Ancestor
ER	Endoplasmic Reticulum
TGN/EE	<i>trans</i> -Golgi Network/Early Endosome
PM	Plasma Membrane
GDI	Guanine nucleotide Displacement Inhibitor
GDI-DF	GDI-Displacement Factor
PRA1	PRENYLATED RAB ACCEPTOR 1
GEF	Guanine nucleotide Exchange Factor
VPS9a	VACUOLAR PROTEIN SORTING-ASSOCIATED PROTEIN 9a
MON1-CCZ1	MONENSIN SENSITIVITY PROTEIN 1- CALCIUM CAFFEINE ZINC SENSITIVITY 1
TRAPP	TRANSPORT PROTEIN PARTICLE
GAP	GTPase Activating Protein
CSC	Cellulose Synthase Complex
APGs	Arabinogalactan Protein Glycans
SYP	SYNTAXIN OF PLANTS
ECH	ECHIDNA
YIP4	YPT INTERACTING PROTEIN 4
PIN	PIN-FORMED
AUX1/LAX	AUXIN RESISTANT1/LIKE AUXIN RESISTANT1
ES16	Endosidin16
PEN3	PENETRATION3
NIP5;1	NOD26-LIKE INTRINSIC PROTEIN 5;1
Q	Glutamine
L	Leucine
N	Asparagine
I	Isoleucine
S	Serine
RPS5a	RIBOSOMAL PROTEIN 5a
DEX	Dexamethasone

DMSO	Dimethyl sulphoxide
NPSN12	NOVEL PLANT SNARE 12
CLASP	CLIP-ASSOCIATED PROTEIN
GCP2/3	GAMMA TUBULIN COMPLEX PROTEINS 2 and 3
RLK	Receptor-Like Kinase
WAK	Wall Associated Kinase
CrRLK1L	<i>Catharanthus roseus</i> RLK1-Like
THE1	THESEUS1
FER	FERONIA
ANX1/2	ANUXR1/2
RLP	Receptor-Like Protein
LRR	Leucine Rich Repeat
TMD	Transmembrane Domain
MAMP	Microbe Associated Molecular Pattern
PRR	Pathogen Recognition Receptor
NLP	Necrosis and ethylene-inducing peptide 1-Like Protein
SOBIR1	SUPPRESSOR OF BIR1,1
BAK1	BRI1-ASSOCIATED KINASE 1
Cf-9	CLADOPOSRIMUM FULVUM-9
Avr-9	AVIRULENCE9
CLV1	CLAVATA1
CLV2	CLAVATA2
CLV3	CLAVATA3
WUS	WUSCHEL
TMM	TOO MANY MOUTHS
EPF	EPIDERMAL PATTERNING FACTOR
RLP44	RECEPTOR-LIKE PROTEIN 44
RLP4	RECEPTOR-LIKE PROTEIN 4
RLP4-L	RECEPTOR-LIKE PROTEIN 4-LIKE
UBQ10	UBIQUITIN10
BLAST	Basic Local Alignment Search Tool

CIP1	COP1-INTERACTING PROTEIN 1
NAT6	NUCLEOBASE ASCORBATE TRANSPORTER 6
MLD	Malectin-Like Domain
ECD	Extracellular Domain
MCC	Mander's Correlation Coefficient
BFA	Brefeldin A
Wm	Wortmannin
Tyr A23	Tyrphostin A23
Tyr A51	Tyrphostin A51
ES9-17	Endosidin9-17
BiFC	Bimolecular Fluorescence Complementation
ConcA	Concanamycin A
PUF2	PLANT-UNIQUE RAB5 EFFECTOR 2
MS	Murashige and Skoog

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Chapter 1: Introduction

I have not yet made so full and satisfactory observations as I desire on this plant, which seems to be a subject that will afford abundance of information. But as far as I have had the opportunity to examine it, I have discovered with my Microscope very curious structures and contrivances.

Robert Hooke

Micrographia, or Some Physiological Description of Minute Bodies

1665, London

1.A: Dynamic endomembranes: An ongoing concern

It is over 350 years since Robert Hooke first prescribed the term 'cell' to the basic unit of eukaryotic life in his 1665 work *Micrographia* and yet, the fascination of scientists of many disciplines with this structure only continues to increase. Unbeknown to Hooke he was actually describing the delineating cell walls of *Quercus suber* and could not therefore observe the vast and complex ultrastructure of the eukaryotic cell, on which much attention has since been focussed since the initial observations of Hooke and those of his contemporary, Anton van Leeuwenhoek. This transition has not been a linear one. Despite Hooke's initial observation in 17th century, it was not until the early 19th century that 'cell theory' became established, compounded by Matthias Jakob Schleiden and Theodor Schwann working on plants and animals respectively (Schleiden, 1838; Schwann, 1839). Cell theory was subsequently augmented into its still-recognisable form by Rudolf Virchow (Virchow, 1855), illustrated by his conclusion 'Omnis cellula e cellula' – that all cells arise from other cells. Appreciation of the ultrastructure of cells gained momentum and significance following the establishment of cell theory. Robert Brown had, in 1833, already described the existence of the nucleus (Brown, 1833) and observations of other subcellular compartments followed throughout the 19th century. This burst of observation and interest in eukaryotic cellular and subcellular organisation was followed by a second surge coincident with the development of electron microscopy in the early to mid-20th century. We are now arguably living in a third age of investigation of cellular structure and function, fuelled by the progress made in the last 30 years in

the development and application of natural and synthetic fluorescent proteins to live cell imaging with confocal laser-scanning microscopy. This, and the emergence of genetically- and developmentally-tractable model species, has allowed for previously impossible investigation of subcellular structure and of its role in supporting functionality of individual cells and higher levels of organisation within multicellular organisms. Consequently, there is now much interest in how the function of subcellular compartments can influence and coordinate the growth and development of tissues and whole organs. This is particularly relevant when considering the morphogenesis of plants, which produce variable body plans from an invariant embryo. The model plant *Arabidopsis thaliana* is the most comprehensively understood plant species both in terms of its development and of the organisation of its subcellular endomembrane system.

The plastic development of *Arabidopsis*, and of other plants, integrates both biotic and abiotic stimuli. As an organising unit of such development, the endomembrane system of *Arabidopsis* is required to be similarly dynamic. The endomembrane system of *Arabidopsis*, like all eukaryotic endomembrane systems, consists of a series of intracellular membrane-bound compartments of distinct identities and functions interconnected by membrane trafficking pathways. Proper organisation and dynamic function of this endomembrane system is essential for growth and development of *Arabidopsis*, as demonstrated by the severe growth and development defects of mutants in important molecular components of the endomembrane system (Mayer et al., 1993; Assaad et al., 1996; Lauber et al., 1997; Geldner et al., 2003; Goh et al., 2007; Teh & Moore, 2007; Chow et al., 2008; Fendrych et al., 2010; Thellman et al., 2010; Gendre et al., 2011; Reyes et al., 2014; Gadeyne et al., 2014; Kriegel et al., 2015; Kirchhelle et al., 2016). A dynamic endomembrane system depends on two key factors. First is the establishment, maintenance and, when required, changes of membrane identity. Second is the co-ordination and regulation of vesicle trafficking between subcellular compartments (Derby & Gleeson, 2007; Segev, 2011; Barr, 2013; Noack & Jallais, 2017).

1.B: Controlling membrane identity: Small GTPases

Membrane identity is central to the function of all eukaryotic endomembrane systems, allowing for establishment and maintenance of discrete subcellular compartments. The mechanisms underpinning definition of membrane identity remain only partially understood in many cases, but broadly involves membrane-associated GTPases, variation in lipid composition and activity of other trafficking-associated proteins such as SNARE proteins, tethering factors and kinases (reviewed by Barr, 2013; Eisenberg-Bord et al., 2016; Noack & Jallais, 2017; Casares et al., 2019). Whilst permanent membrane identity is required in some cases to maintain discrimination between different endomembrane compartments in the face of constant membrane flux from other compartments of different identities, membrane identity is also required to be dynamic in other cases and is particularly important for endomembrane compartments whose identity is transient. A key example of dynamic membrane identity is the now well-supported cisternal-maturation model of Golgi apparatus function (reviewed by Glick & Luini, 2011) in which Golgi cisternae mature from *cis* to *trans* within individual Golgi bodies. Maintenance of discrete compartments within eukaryotic cells, as well as capability for dynamic changes in identity, provide a basis for trafficking between compartments in order to satisfy basic requirements of cellular function. Regulation and co-ordination of such vesicular trafficking between compartments is essential to maintain both cell and endomembrane compartment size and identity. Vesicular trafficking is therefore regulated at various points, including at the point of vesicle formation and at fusion with the target membrane.

Whilst many proteins are known to associate with the eukaryotic endomembrane system, small GTPases have emerged as central to both the maintenance of membrane identity and the regulation of vesicular trafficking. Small GTPases act as 'molecular switches', and are classically considered to cycle between an inactive, membrane-dissociated GDP-bound state and an active, membrane-associated GTP-bound state. Such cycling between membrane association and dissociation is regulated by numerous GTPase interactors, many of which are themselves key components of

membrane trafficking. Of the known groups of small GTPases, the Arf, Rho, and Rab GTPases are the best studied. Arf GTPases are particularly known to function in the formation of vesicle coats on membranes, and in the subsequent processes of budding and vesicle release, in yeast and mammalian cells but remain poorly characterised in plants (reviewed by Yorimitsu et al., 2014; Donaldson & Jackson, 2012). Rho GTPases interact with components of the actin cytoskeleton, connecting membrane dynamics and the organising cytoskeleton. As such, they have been particularly implicated in processes such as actin-driven cell migration in mammalian cells, cell adhesion and development of cell protrusions (reviewed by Hodge & Ridge, 2016). Finally, the Rab GTPases have emerged as key general regulators of membrane identity, recruiting other trafficking components to membranes in a spatio-temporally restricted manner.

Rab GTPases are found in all characterised eukaryotes and evidence has also been presented of the possible existence of Rab-like proteins in some prokaryotes (Surkont & Pereira-Leal., 2016). The human and *Arabidopsis* genomes encode similar numbers of Rab GTPases (60 and 57 respectively) despite the obvious morphological and evolutionary differences between higher plants and metazoans. Moreover, striking examples also exist of proliferation of large numbers of Rab GTPases in 'basal' organisms. For example, the genome of the protist *Entamoeba histolytica* encodes over 90 Rab GTPases of which 69 have only low similarity to Rab GTPases found elsewhere (Saito-Nakano et al., 2005). Despite this, some groups of Rab GTPases found in both plants and metazoans are believed to have been present in the Last Eukaryotic Common Ancestor (LECA), indicating ancient functions of major Rab groups (Diekmann et al., 2011; Elias et al., 2012; Klöpper et al., 2012). This is reinforced by ability of some Rab GTPases to complement Rab mutants in distant organisms (Haubruck et al., 1989; Palme et al., 1993; Bednarek et al., 1994; Fabry et al., 1995). In *Arabidopsis*, the 57 Rab GTPases are phylogenetically split into 8 clades termed Rab-A to Rab-H, with each clade related to one or more metazoan Rab GTPase clade (Figure 1.1; Rutherford & Moore., 2002).

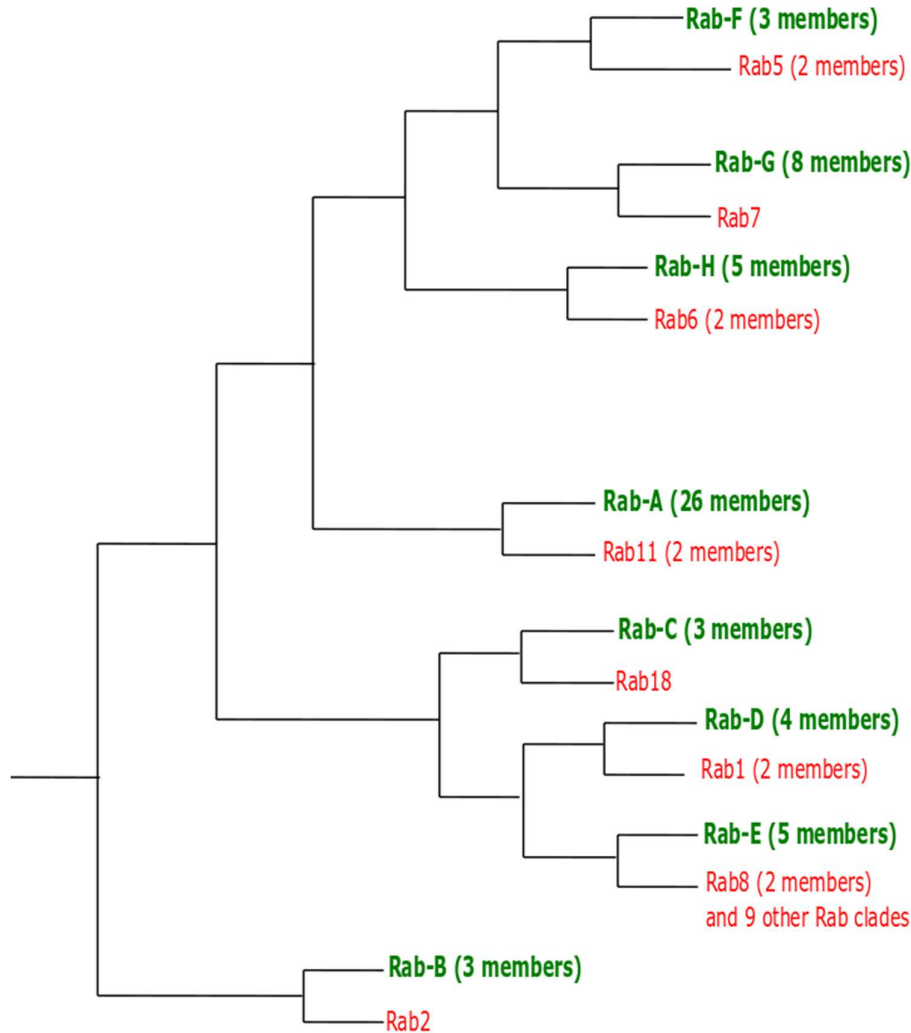


Figure 1.1: Simplified phylogeny of Arabidopsis Rab GTPase clades compared to mammalian clades. Green text = Arabidopsis clades. Red text = mammalian clades. Representative only, does not incorporate quantitative phylogenetic parameters. For comprehensive phylogeny, see Rutherford & Moore, 2002.

1.C: Rab GTPases in the *Arabidopsis* endomembrane system

In somatic cells of *Arabidopsis*, each major endomembrane compartment, and the pathways between them, is defined by one or more groups of Rab GTPases. In addition to being key regulators of membrane identity and vesicle trafficking, Rab GTPases can therefore provide useful targets for proteomic characterisation of particular endomembrane compartments and their associated trafficking pathways (Heard et al., 2015). In *Arabidopsis*, Rab-B and Rab-D GTPases function in

Endoplasmic Reticulum (ER)-to-Golgi traffic, whilst Rab-E and Rab-H GTPases are believed to define trafficking pathways directly between the Golgi and the plasma membrane (PM) (Moore et al., 1997; Batoko et al., 2000; Cheung et al., 2002; Zheng et al., 2005; Camacho et al., 2009; Johansen et al., 2009; Pinheiro et al., 2009; Mayers et al., 2017; He et al., 2018 – Figure 1.2a). Post-Golgi trafficking represents the intersection between the internal environment of the cell and the external environment in both the plant and mammalian endomembrane systems however, this is where some of the most prevalent differences in the organisation and functions of the plant and mammalian endomembrane systems exist. Moreover, it has been suggested that the phylogenetic differences between *Arabidopsis* Rab GTPases and mammalian Rab GTPases reflect such differences in post-Golgi trafficking requirements between plant and mammalian cells (Rutherford & Moore, 2002; Woollard & Moore, 2008). The first organelle in *Arabidopsis* post-Golgi trafficking is the *trans*-Golgi network/early endosome (TGN/EE). In mammalian cells, endocytic traffic from the PM to an early endosome feeds traffic to a distinct recycling endosome and late endosome, with secretory traffic to the cell surface arising directly from the Golgi. In *Arabidopsis* however, the *trans*-Golgi network/early endosome (TGN/EE) receives both exocytic traffic from the Golgi and endocytic traffic from the PM (Dettmer et al., 2006; Chow et al., 2008; Viotti et al., 2010 – Figure 1.2b), a striking difference to organisation of the mammalian endosome system. Members of the highly-expanded *Arabidopsis* Rab-A clade predominantly localise to the TGN/EE and are believed to define trafficking pathways from the TGN/EE to the cell surface (Preuss et al., 2004; Chow et al., 2008; Kang et al., 2011; Feraru et al., 2012; Qi et al., 2013; Asaoka et al., 2014; Berson et al., 2014; Kirchhelle et al., 2016). The TGN/EE itself is thought to derive via a maturation process from the *trans*-Golgi cisternae and both Golgi-associated and Golgi-independent TGN/EEs exist (Kang et al., 2011; Uemura et al., 2014). Trafficking from the TGN/EE also includes routes to late endosomes/pre-vacuolar compartments and eventually to the vacuole, which are defined and supported by Rab-F and Rab-G GTPases (Sohn et al., 2003; Kotzer et al., 2004; Lee et al., 2004; Ueda et al., 2004; Nielsen et al., 2008; Bottanelli et al., 2011, 2012; Cui et al., 2014; Singh et al., 2014; Ito et al., 2018; Rodriguez-

Furlan et al., 2019; Figure 1.2b & c). The *Arabidopsis* TGN/EE also supports specialised trafficking pathways involved in plant immune responses (reviewed by Elliott et al., 2020), as well as the biogenesis of a transient membrane compartment during cytokinesis, the cell plate (reviewed by McMichael & Bednarek, 2015; Smertenko et al., 2017). Proteomic dissection of the TGN/EE further confirms a complex molecular organisation underpinning this multi-functionality, highlighting abundances of both known trafficking components, including Rab GTPases, as well as many proteins of unknown function (Drakakaki et al., 2012; Heard et al., 2015).

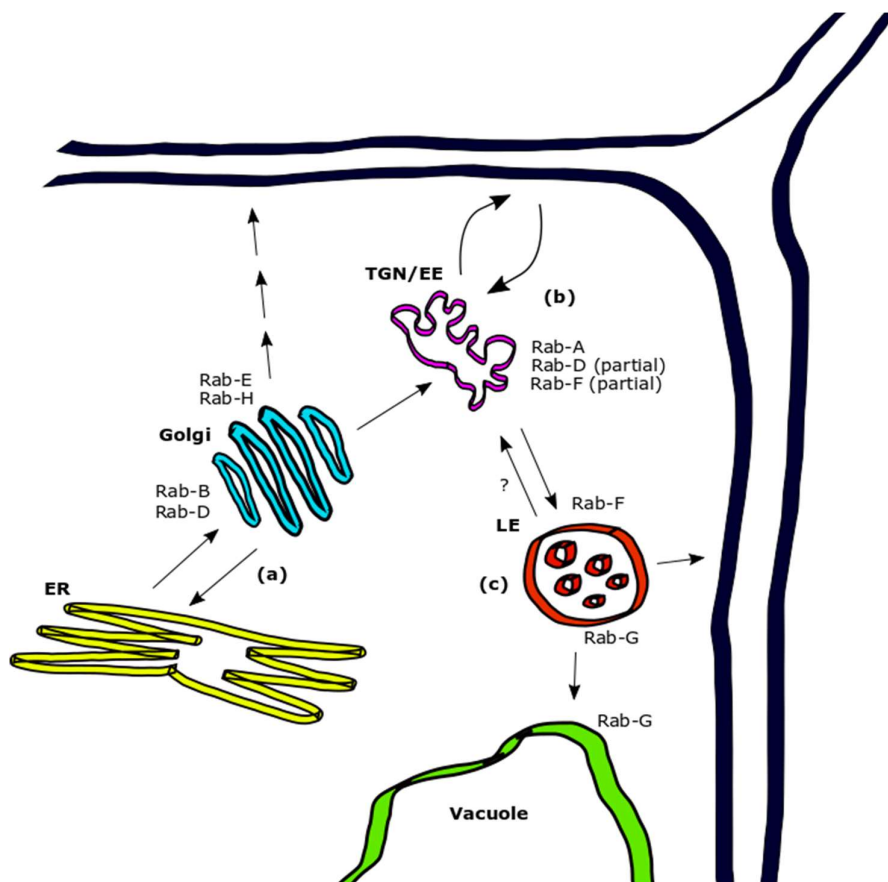


Figure 1.2: Organisation of the *Arabidopsis* endomembrane system and steady-state localisations of Rab GTPases. (a) Rab-D, and also likely Rab-B, GTPases localise to the Golgi and function in ER-to-Golgi traffic. Rab-E and Rab-H GTPases also localise predominantly to the Golgi and define independent trafficking pathways between the Golgi and PM. (b) Rab-A GTPases localise to the TGN/EE (and the PM in some cases), which receives both secretory and endocytic trafficking. Rab-F partially localises to the TGN/EE and supports trafficking to late endosomes. (c) Rab-F GTPases localise to populations of multivesicular late endosomes and are exchanged for Rab-G GTPases in late endosome maturation. Rab-G localises to both mature late endosomes and the tonoplast and functions in late trafficking steps to the vacuole.

1.D: Controlling Rab GTPase association with the endomembrane system

The role of Rab GTPases (and indeed, of other small GTPases) in dynamic spatio-temporal control of post-Golgi trafficking in plants cannot be discussed in detail without considering the many interacting factors of Rabs, several groups of which are critical for the controlled association of Rab GTPases with endomembranes (Behina & Munro., 2005; Grosshans et al., 2006; Barr., 2013).

Association of Rab GTPases in their cytosolic GDP-bound form with membranes is peripheral and transient, and is facilitated in most cases by post-translational geranylgeranylation of two conserved C-terminal cysteine residues. Binding of Guanine nucleotide Displacement Inhibitor (GDI) to these isoprenoid groups prevents peripheral association of GDP-bound Rab GTPases with membranes (Sasaki et al., 1990; Ullrich et al., 1993; Figure 1.3a). This is however counteracted by the presence of membrane-associated GDI-Displacement Factors (GDI-DF), which catalyse the removal of GDI from Rab GTPases and allow association of geranylgeranylated Rab GTPases with membranes (Dirac-Svejstrup et al., 1997; Sivars et al., 2003; Figure 1.3a). This rapid association and removal of Rab GTPases from membranes is often likened to ‘membrane sampling’. Despite the 57 Rab GTPases encoded by the *Arabidopsis* genome, only three Rab-GDI homologues are currently known in *Arabidopsis* (Ueda et al., 1996; Zárský et al., 1997; Ueda et al., 1998). PRENYLATED RAB ACCEPTOR 1 (PRA1) was the first identified Rab GDI-DF in yeast (Sivars et al., 2003) and, compared to the limited number of Rab-GDI homologues known in plants, the *Arabidopsis* PRA1 family has 19 members (Alvim Kamei et al., 2008). Whether these all possess GDI-DF activity is unknown however, some evidence of GDF activity from PRA1 proteins has been identified in rice (Bahk et al., 2009) and trafficking defects of certain proteins have been described for loss-of-function or overexpression of PRA1 members in both *Arabidopsis* and tomato (Lee et al., 2017; Pizarro et al., 2018). Stable and specific association of Rab GTPases following ‘membrane sampling’ is traditionally considered to be promoted by an additional group of Rab interactors – Guanine nucleotide Exchange Factors (GEFs). Rab GEFs catalyse an exchange reaction of GDP for GTP on Rab GTPases, resulting in GTP-bound Rabs which are resistant to membrane removal by GDI, thus triggering stable and in at least some

cases targeted membrane association (Barr, 2013; Blümer et al., 2013; Figure 1.3b). Some Rab GEFs have now been identified in *Arabidopsis*, including VACUOLAR PROTEIN SORTING-ASSOCIATED PROTEIN 9a (VPS9a) as a GEF for Rab-F GTPases and the dimeric MONENSIN SENSITIVITY PROTEIN 1-CALCIUM CAFFEINE ZINC SENSITIVITY 1 (MON1-CCZ1) complex for Rab-G GTPases (Vernoud et al., 2003; Nordmann et al., 2010; Cui et al., 2014). Evidence has also emerged supporting the TRANSPORT PROTEIN PARTICLE (TRAPP) II complex (a known Rab GEF in yeast and metazoans) as a possible GEF for Rab-A GTPases (Morozova et al., 2006; Qi et al., 2011; Qi & Zheng, 2011; Riedel et al., 2018; Kalde et al., 2019), as well as the STOMATAL CYTOKINESIS DEFECTIVE (SCD) complex and Golgi-localised LOSS OF TGN (LOT) for Rab-E and Rab-H respectively (Mayers et al., 2017; Jia et al., 2018). In order to support the dynamic membrane identity of the endomembrane system, stable association of Rab GTPases with membranes must also be reversible in a regulated manner. Removal of Rab GTPases from membranes is facilitated by Rab GTPase Activating Proteins (Rab GAPs), which promote hydrolysis of GTP to GDP and return the Rab to a GDP-bound form, which is sensitive to membrane removal by GDI (Figure 1.3c). GAP activity on plant Rab GTPases was first shown through isolated membrane fractions increasing GTPase activity of Rab GTPases *in vitro* (Anai et al., 1994) and since then 20 candidate GAPs have been identified in the *Arabidopsis* genome. However, none of these has been investigated in detail (Vernoud et al., 2003).

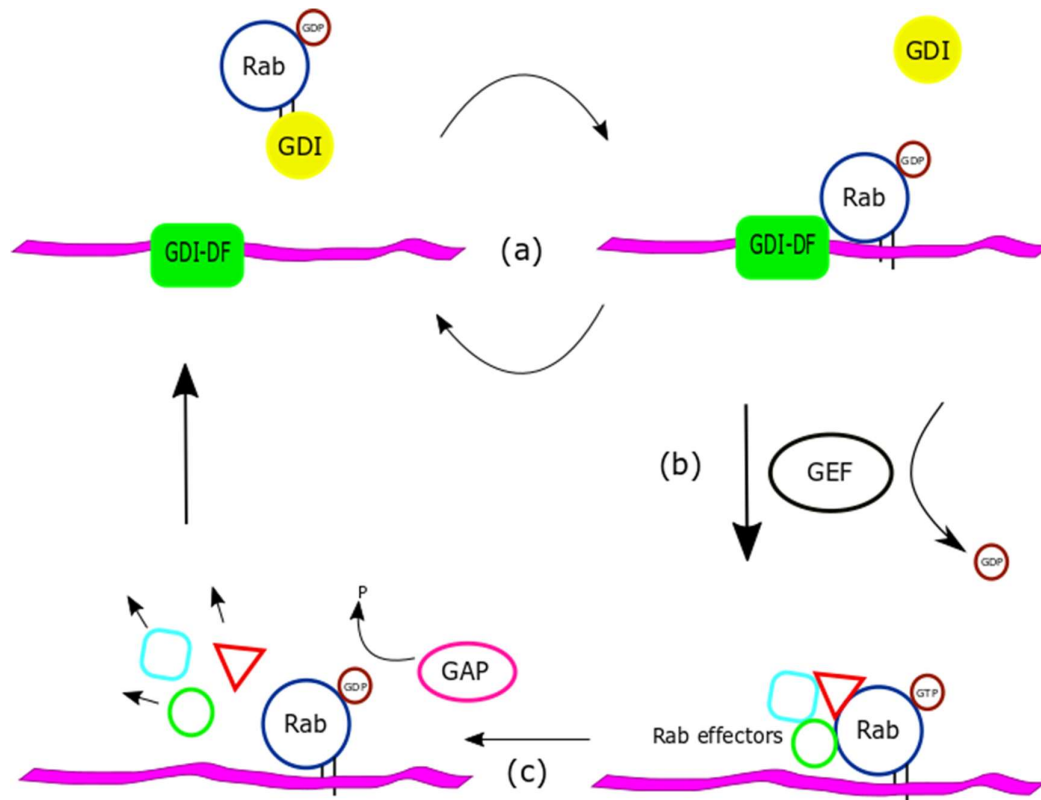


Figure 1.3 Regulation of Rab GTPase membrane association and dissociation in eukaryotes

(a) Cytosolic GDP-bound Rab GTPases bind a Guanine nucleotide Displacement Inhibitor (GDI) which prevents their peripheral membrane association via C-terminal isoprenoid modifications. GDI binding to Rab GTPases is displaced by activity of membrane-bound GDI-Displacement Factors (GDI-DFs). Counteracting functions of GDI and GDI-DFs cause GDP-bound Rab GTPases to transiently cycle on and off membranes in a manner referred to as 'membrane sampling' (b) Rab Guanine nucleotide Exchange Factors (GEFs) catalyse exchange of GDP for GTP, rendering the Rab insensitive to GDI activity. GTP-bound Rab GTPases can therefore stably associate with membranes and recruit effectors. (c) Rab GTPase Activating Proteins (GAPs) catalyse hydrolysis of GTP to GDP, rendering Rab GTPases sensitive to GDI activity and returning them to a 'sampling' state.

Controlled association and dissociation of Rab GTPases with membranes supports the recruitment of Rab effectors, which function in a variety of ways to support membrane identity and vesicular trafficking, to specific membranes in a spatio-temporally organised manner and is also important for proper trafficking of other proteins essential to cell function that are organised by Rab GTPases (Rab cargoes). Comparatively few plant Rab effectors and cargoes are known in detail however, with examples including lipid-modifying phosphatidylinositol 4-kinases (Preuss et al., 2004, 2006; Camacho et al., 2009), cell wall modulating factors (Ellinger et al., 2014), GEFs and regulatory factors

for other Rab GTPases (Cui et al., 2014; Ito et al., 2018), SNAREs (Ebine et al., 2011) and tethering complexes (Takemoto et al., 2018). Numerous other factors that are directly or indirectly influenced by Rab GTPases are known in yeast and metazoans, including kinesins, myosin subunits, other small GTPases and their interactors, and cytoskeleton-regulators (Seabra et al., 2004; Grosshans et al., 2006; McDonold & Fromme, 2014; Frémont et al., 2017). It seems likely that other such factors known to be functionally connected to Rab GTPases in yeast and metazoans also are linked to Rab GTPases in plants, but have yet to be described. In order to understand the functional significance of a specific Rab GTPase and of its associated endomembrane compartment, it is therefore essential to identify effectors and cargoes of the Rab GTPase. Traditional methods of identifying Rab interactors include yeast two-hybrid screens and heterologous complementation assays. More recently, proteomic-based studies have yielded high numbers of new candidates. However, validation work is required to confirm such candidates as genuine Rab effectors or cargoes (Gillingham et al., 2014; Heard et al., 2015).

Functional characterisation of Rab GTPases in *Arabidopsis* has been essential in mapping them to specific trafficking pathways and compartments in plants. Multiple methods have been employed in functional characterisation of Rab GTPases, including use of insertional knockout mutants of Rab GTPases or their regulatory interactors (Goh et al., 2007; Szumlanski & Nielsen, 2009; Asaoka et al., 2013; Lunn et al., 2013b; Ebine et al., 2014; He et al., 2018), as well as RNA-mediated suppression of Rab GTPases (Blanco et al., 2009; Lunn et al., 2013). Redundancy has however been demonstrated between members of some *Arabidopsis* Rab GTPases subclasses using combinations of insertional knockout mutants (Pinheiro et al., 2009). In order to circumvent such redundancy, dominant negative mutant variants have often been used in functional studies of Rab GTPases, especially in the case of larger Rab GTPase subclasses. Rab dominant negative mutants feature point mutations at highly-conserved residues and are believed to inhibit the function of wild-type GTPases by titrating away essential interaction factors and effectors (Olkonen & Stenmark, 1997). Expression of

dominant negative mutant variants have been widely used in *Arabidopsis* to investigate Rab GTPase function in trafficking and development, especially in the case of TGN/EE-localised Rab GTPases (Zheng et al., 2005; Chow et al., 2008; Pinheiro et al., 2009; Asaoka et al., 2013; Qi et al., 2013; Kirchhelle et al., 2016, 2019; Li et al., 2017).

1.E: Rab GTPases and the TGN/EE

In TGN/EE proteomes multiple *Arabidopsis* Rab GTPase clades are represented however, *in planta* localisation studies have identified members of the Rab-A and Rab-D clades as being the main Rab GTPase clade whose members localise to the TGN/EE *in planta* (Drakakaki et al., 2012; Kalde et al., 2019; reviewed by Elliott et al., 2020). Rab-D GTPases are represented by 4 members in *Arabidopsis* and localise to both Golgi bodies and the TGN/EE (Pinheiro et al., 2009 – Figure 1.2a &b). Despite some localisation to the TGN/EE, and their presence in TGN/EE proteomes, evidence suggests that Rab-D GTPases function in ER-to-Golgi traffic (Batoko et al., 2000; Zheng et al., 2005; Pinheiro et al., 2009; Drakakaki et al., 2012) and their role at the TGN/EE, if any, remains unclear. Members of the largest clade of *Arabidopsis* Rab GTPases, the Rab-A clade, predominantly or partially localise to the TGN/EE (Preuss et al., 2004; Chow et al., 2008; Kang et al., 2011; Feraru et al., 2012; Qi & Zheng, 2013; Asaoka et al., 2014; Berson et al., 2014; Kirchhelle et al., 2016 – Figure 1.2b). Several Rab-A GTPases do not overlap entirely with other TGN/EE markers and so label sub-domains of the TGN/EE, supporting a scenario in which the multifunctionality of the *Arabidopsis* TGN/EE may be underpinned by a multi-domain structure (Chow et al., 2008; Feraru et al., 2012; Qi & Zheng, 2013). 26 *Arabidopsis* Rab GTPases are members of the Rab-A clade, meaning that the Rab-A clade contains around half of all *Arabidopsis* Rab GTPases. The Rab-A clade is itself divided into 6 subclasses, Rab-A1 to Rab-A6, and its large size is in stark contrast its mammalian homologues, which number just 3 (Rutherford & Moore, 2002). The plant-specific diversification of the TGN/EE-localised Rab-A clade (Figure 1.1) has been suggested to reflect and support the multiple pathways that converge on it and their importance for plant growth and development (Woollard & Moore, 2008). Similarly, the

divergence of Rab8, Rab10 and related Rab GTPases in mammals (compared to the five members of the related Rab-E clade in *Arabidopsis*; Figure 1.1), which are involved in Golgi-to-PM transport, presumably reflects the reliance of mammalian cells on this secretory pathway (Rutherford & Moore, 2002). A unique function of the plant TGN/EE is its role in supporting the biogenesis of a transient endomembrane compartment during cytokinesis – the cell plate. Numerous Rab-A GTPases re-localise to the cell plate during cytokinesis, linking trafficking from the TGN/EE to the biogenesis of the cell plate (Chow et al., 2008; Asaoka et al., 2013; Qi & Zheng, 2013; Berson et al., 2014; Davis et al., 2016; Kirchhelle et al., 2016).

Of the diverse functions of the *Arabidopsis* TGN/EE, the most fundamental to plant growth and development is the organisation of protein and carbohydrate cargoes into polarised trafficking pathways to support various functions at the cell surface. Understanding of the molecular organisation and regulation of these pathways remains limited despite their importance in plant growth and development however, work has begun to functionally assign Rab GTPases and their interactors to these pathways.

The TGN/EE as a post-Golgi sorting hub for proteins and carbohydrates: Rab GTPases and the key molecular players

1.F: The TGN/EE and the cell wall

One of the foremost functions of post-Golgi traffic in plants is the biogenesis and maintenance of the plant cell wall. Understanding of trafficking of plant cell wall components is commonly split between cellulosic and non-cellulosic cell wall components. Deposition of cellulose, the primary component of the plant cell wall, is driven by delivery of biosynthetic cellulose synthase complexes (CSCs) to the PM. CSC presence in the Golgi has been known for some time (Haigler & Brown, 1986; Paredez et al., 2006) and it is believed that CSCs are delivered from Golgi at the cell periphery to the PM. Delivery

and positioning of CSCs at the PM, and in turn the arrangement of cellulose microfibrils in the cell wall, is underpinned by a combination of actin-driven Golgi movement in the cell periphery and spatial positioning of CSC insertion by cortical microtubules (Crowell et al., 2009; Gutierrez et al., 2009; Sampathkumar et al., 2013). Imaging and proteomic analyses have also identified populations of CSCs at the TGN/EE (Crowell et al., 2009; Gutierrez et al., 2009; Drakakaki et al., 2012). However, the role of the TGN/EE in CSC trafficking remains unclear and comparatively un-investigated. The vacuolar ATPase mutant *deetiolated3 (det3)*, which has increased TGN/EE pH, reduces CSC exocytosis to the cell surface. However, Golgi motility was also reduced in the *det3* mutant and so the extent to which reduced CSC exocytosis is a direct consequence of TGN/EE dysfunction in *det3* remains unclear (Luo et al., 2015). Recent work on the Rab-H GTPase RAB-H1b has also provided the first possible link between secretory Rab GTPases and cellulose deposition (He et al., 2018). RAB-H1b localises to both the Golgi and TGN/EE and is predominantly expressed in the hypocotyl (Johansen et al., 2009; He et al., 2018; Renna et al., 2018). A *rab-h1b* mutant background has reduced hypocotyl cell wall thickness and reduced content of several cell wall components, most prominently cellulose. CSC distribution is defective in *rab-h1b*, indicating a possible role for RAB-H1b in CSC exocytosis (He et al., 2018). Golgi morphology and motility was however also defective in the *rab-h1b* background, raising the possibility that defective Golgi function may be the source of these defects rather than a specific effect of RAB-H1b on CSC trafficking (He et al., 2018).

Whilst the relationship between the TGN/EE and cellulose deposition remains unclear, its role in secretion of non-cellulosic cell wall components is better characterised. Pectin and xyloglucan epitopes have been identified in vesicles clustered at the *trans*-Golgi face and TGN in clover and sycamore cells (Lynch and Staehelin, 1992; Zhang and Staehelin, 1992). More recently, immunolabelling has also identified xyloglucans and pectins in *Arabidopsis* TGN/EE compartments (Stierhof and El Kasmi, 2010; Kang et al., 2011). Xyloglucans specifically have been immunolocalised TGN/EE compartments that were also labelled by the Rab-A GTPase Rab-A4b and which eventually

fuse with the plasma membrane (Kang et al., 2011). Furthermore, RNA-mediated inhibition of Rab-A orthologues in tomato has also been linked to defective fruit cell wall composition, particularly in non-cellulosic wall components, and reduced secretion of hydrolytic enzymes (Lu et al., 2001; Lunn et al., 2013). Excitingly, a method for glycomic analyses of subcellular compartments defined by a particular target protein has recently been described (Wilkop et al., 2019). Using this method, isolated SYNTAXIN OF PLANTS 61 (SYP61)-labelled TGN/EE compartments were found to contain diverse non-cellulosic carbohydrates (Wilkop et al., 2019). This method may provide a powerful future tool for comparing glycomic composition of different TGN/EE subdomains and Rab GTPase-defined secretory pathways from the TGN/EE.

Recent characterisation of *Arabidopsis* TGN/EE molecular components has identified supporting players in secretion of non-cellulosic cell wall components from the TGN/EE with possible links to Rab GTPases. One such component is the ECHIDNA(ECH)-YPT INTERACTING PROTEIN 4 (YIP4) complex. YIP proteins are interactors of Rab GTPases in yeast, and in *Arabidopsis* both *ech* and *yip4a yip4b* double mutants are defective in cell elongation and secretion of pectin and xyloglucan (Gendre et al., 2011; Gendre et al., 2013; McFarlane et al., 2013). Moreover, the TGN/EE-localised ECH-YIP4 and TRAPP II (a possible GEF for Rab-A GTPases) complexes function in partially overlapping secretory routes from the TGN/EE to the cell wall. Cell-wall labelling Arabinogalactan Protein Glycans (AGPs) label the cell surface in both *ech* and TRAPP II *trs120* single knockout mutants, but accumulate intracellularly in an *ech trs120-5* double mutant background (Ravikumar et al., 2018). YIP4 members also interact with TGN/EE-localised TGNap1 and a *tnap1* mutant is, like *ech*, defective in seed coat mucilage secretion (Renna et al., 2018). Interestingly, TGNap1 was also shown to interact with RAB-H1b. Association between TGNap1 and RAB-H1b may provide support for Rab-H function in secretion of non-cellulosic wall components as well as a possible role in trafficking of CSCs (He et al., 2018; Renna et al., 2018). As well as supporting delivery of cell wall components, some recently described TGN/EE molecular components also function in the establishment and maintenance of

polar subcellular domains, which is another key function of the *Arabidopsis* TGN/EE (reviewed by Nakamura & Grebe, 2018; Elliott & Kirchhelle, 2019).

1.F: The TGN/EE and polarity

The maintenance of polar domains in *Arabidopsis* cells is essential for growth and development at both the cellular, tissue and whole-body scale. The surface of *Arabidopsis* root epidermal cells can broadly be divided into three discrete geometric regions: faces, edges (the point where two faces meet) and vertices (the point where three edges meet; Figure 1.4). Faces of the PM are further divided into apical (shootward) and basal (rootward) facial domains, as well as outer (soil-facing) and inner lateral domains (Figure 1.4). Such discrete geometric regions have been recognised for some time (reviewed by Elliott & Kirchhelle, 2019) however, it has only been more recently appreciated that they are defined by polar localisation of proteins and other subcellular components. The TGN/EE is increasingly appreciated as essential for establishment and maintenance of polarised localisation of proteins to these discrete subcellular domains (Qi et al., 2011; Feraru et al., 2012; Kirchhelle et al., 2016; Li et al., 2017; reviewed by Nakamura & Grebe, 2018; Elliott & Kirchhelle, 2019).

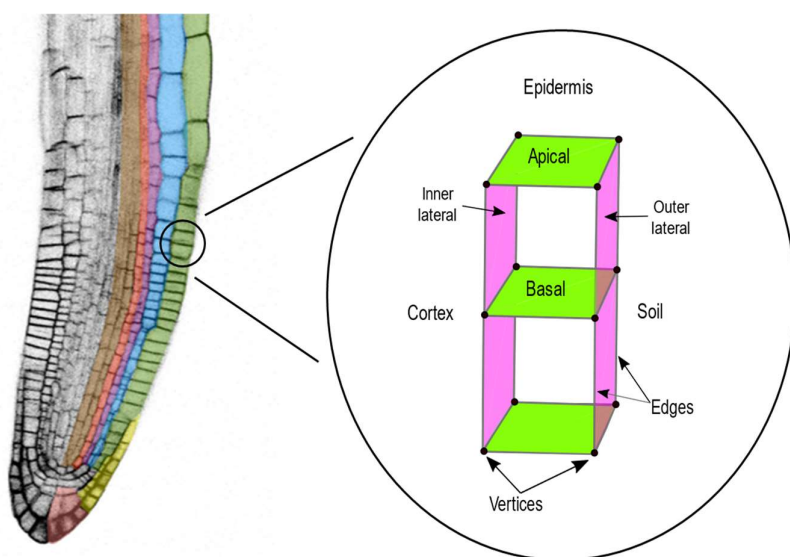


Figure 1.4: Geometric domains of *Arabidopsis* root epidermal cells.
Adapted from Elliott & Kirchhelle, 2019.

The TGN/EE is known to support the establishment and maintenance of polar PM domains, to which a variety of proteins localise in a polarised manner. Among the most intensely studied of these proteins are auxin transporters, notably PIN-FORMED (PIN) efflux transporters and AUXIN RESISTANT1/LIKE AUXIN RESISTANT1 (AUX1/LAX) influx transporters. PIN and AUX1/LAX transporters localise to the apical or basal faces of the *Arabidopsis* root cell PM, and establish tissue-wide auxin gradients essential for growth (reviewed by Zhou & Luo, 2018; Luo et al., 2018). Polar localisation of PIN proteins is proposed to be maintained by a combination of spatially-confined endocytosis and polarised recycling from the TGN/EE to the PM (Kleine-Vehn et al., 2009; Kitakura et al., 2011; Kleine-Vehn et al., 2011; Li et al., 2017; Glanc et al., 2018). The identity and function of the trafficking regulators underpinning this polarity remain largely unknown however, Rab GTPases and their interactors have been implicated (Qi et al., 2011; Feraru et al., 2012; Li et al., 2017).

Recently, the chemical agent Endosidin 16 (ES16) was found to disrupt polar recycling of apically-localised PIN2, but not of basal PIN1 (Li et al., 2017). ES16 also disrupts the localisation of laterally-polarised proteins, indicating that polarisation of basally-localised proteins may be maintained by a separate pathway to non-basal proteins. One target of ES16 is the TGN/EE-localised Rab-A GTPase RAB-A2a, and expression of a dominant-negative RAB-A2a mutant variant caused intracellular accumulation of apically-localised PIN2 and disrupted its recycling, but had no effect on trafficking of basally-localised PIN1 (Li et al., 2017). However, dominant-negative mutant variants of TGN/EE-localised RAB-A1b disrupt recycling of both PIN1 and PIN2 (Feraru et al., 2012), indicating that a subset of Rab-A GTPases may function in specific trafficking to polar domains whereas others may function more broadly. Interestingly, mutants of the TGN/EE-localised TRAPP complex, a proposed Rab-A GEF, also disrupt polarised trafficking in a selective manner. Mutants in TRAPP subunits disrupt polarity of apical PIN2 but not of basal PIN1 (Qi et al., 2011). However, RNA-mediated suppression of BET5, a core TRAPP subunit thought to be shared by all TRAPP complex variants, disrupts localisation of both PIN1 and PIN2, indicating that like Rab-A GTPases, different TRAPP

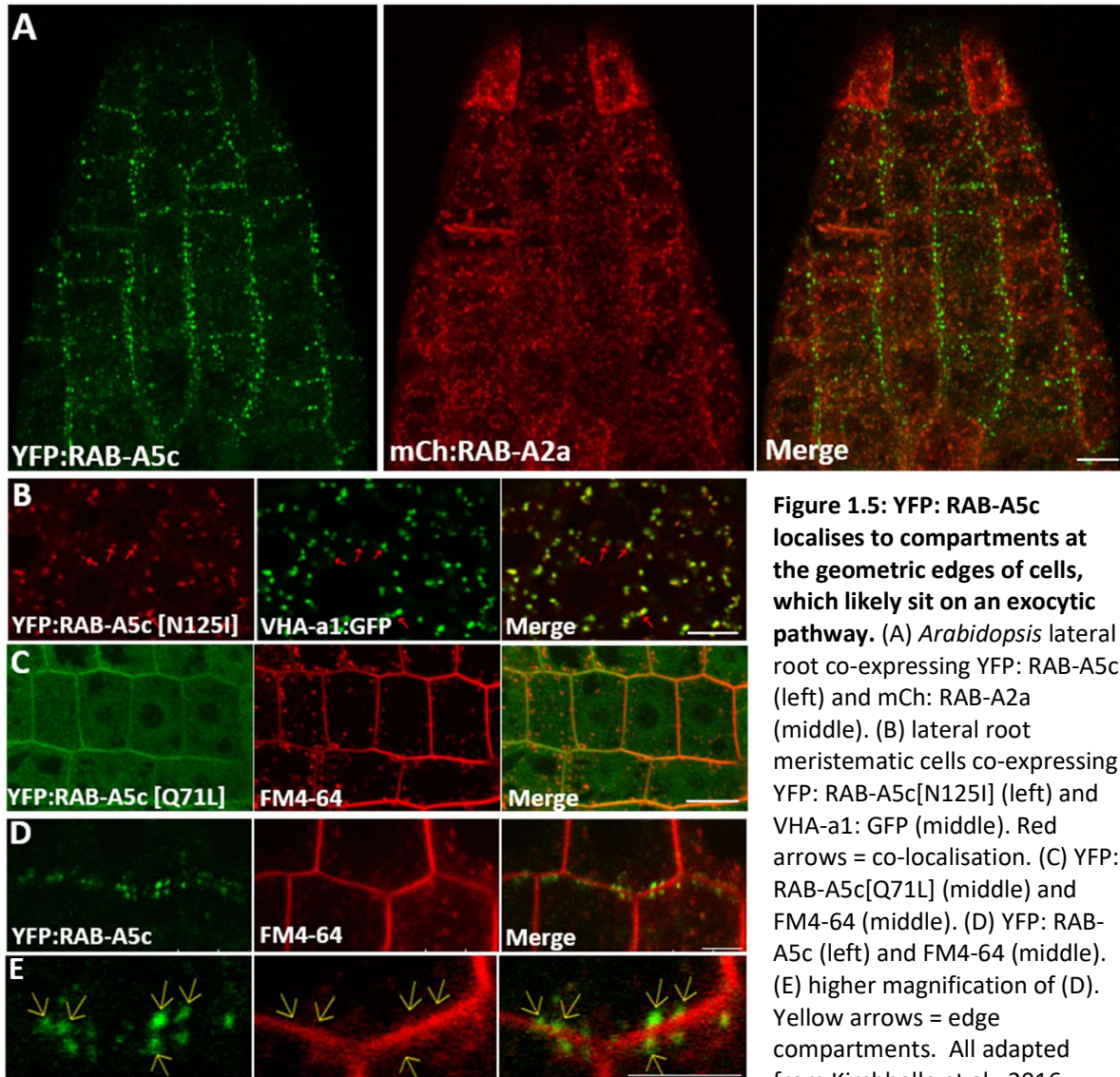
complex variants may have different roles in polarised trafficking from the TGN/EE to PM (Zhang et al., 2018).

Polarisation to outer and inner lateral domains of the PM in *Arabidopsis* root cells is well-described for proteins involved in interaction with the extracellular environment, particularly nutrient transporters and pathogen-defence proteins (Miwa et al., 2007; Strader & Bartel, 2009; Ruzicka et al., 2010; Takano et al., 2010; Barberon et al., 2014; Khare et al., 2017). Components of the post-Golgi traffic-regulating exocyst complex EXO84b and EXO70A1 are also polarised to the outer lateral surface of *Arabidopsis* root epidermal cells (Fendrych et al., 2013). Moreover, exocyst component *exo84b* mutants disrupt polarisation of outer-polarised PENETRATION3 (PEN3 – Strader & Bartel, 2009) and NOD26-LIKE INTRINSIC PROTEIN 5;1 (NIP5;1-Takano et al., 2010), and EXO84b polar localisation is itself sensitive to the actin-disrupting *act7* mutant (Mao et al., 2016). Exocyst is a known effector and interactor of Rab GTPases in yeast and metazoans. However, this has yet to be demonstrated in plants, and so it is unclear which Rab GTPases may contribute to polarisation in the outer-inner lateral axis in plants. Endocytic traffic has also been implicated in maintenance of lateral polarity in the PM. Mutants in the ADAPTOR PROTEIN 2 (AP2) complex of clathrin-mediated endocytosis reduce the lateral polarity of NIP5;1, with threonine phosphorylation at the N-terminus of NIP5;1 essential for polarity-maintaining endocytosis (Wang et al., 2017). Similarly, AP2 - dependent endocytosis is also required for polar localisation of the borate transporter BOR1 to the inner lateral domain of *Arabidopsis* root cells (Takano et al., 2010; Yoshinari et al., 2019).

1.G: RAB-A5c and the TGN/EE: a new polarised trafficking pathway?

As well as underpinning the polarisation of discrete plasma membrane facial domains, recent work has identified the TGN/EE as the likely origin of a previously unrecognised polarised trafficking pathway to another of the discrete geometric regions of plant cells – the cell edge. The Rab-A GTPase RAB-A5c localises to previously unidentified membrane compartments at the geometric edges of cells in developing primordia of *Arabidopsis* (Figure 1.5A; Kirchhelle et al., 2016).

Interestingly, RAB-A5c-labelled cell edge compartments are absent in mature tissues but are present in young root, leaf and floral primordia (Kirchhelle et al., 2016). This identification of edge-localised membrane compartments in *Arabidopsis* supports historic observations of vesicles at cell edges in electron micrographs of *Azolla* and *Adiantum* ferns (Gunning et al., 1978; Panteris et al., 1993). Using lateral roots as a model growing tissue, evidence suggests that *Arabidopsis* RAB-A5c cell-edge compartments derive from the TGN/EE and eventually fuse with the PM. The mutant variant YFP: RAB-A5c[Q71L], which has a Glutamine (Q) to Leucine (L) substitution, is predicted to have reduced GTPase activity and can be considered to be constitutively active (Olkkonen & Stenmark, 1997; Ueda et al., 2001; Sohn et al., 2003; Chow et al., 2008). YFP: RAB-A5c[Q71L] partially labels the PM in lateral root epidermal cells (Figure 1.5C; Kirchhelle et al., 2016). By contrast, the mutant variant YFP: RAB-A5c[N125I], which has an Asparagine (N) to Isoleucine (I) substitution, is predicted to have reduced affinity for nucleotide binding, and a stabilised interaction with the Rab GEF at the point of membrane association (Schmitt et al., 1996; Jones et al., 1995; Olkkonen & Stenmark, 1997; Batoko et al., 2000). YFP: RAB-A5c[N125I] does not localise to cell edge compartments and instead labels the TGN/EE (Figure 1.5B; Kirchhelle et al., 2016). This evidence supports a scenario in which RAB-A5c cell-edge compartments in growing tissues arise from the TGN/EE and traffic to the cell geometric edges, with RAB-A5c losing membrane-association shortly before or after fusion with the plasma membrane (Kirchhelle et al., 2016). Moreover, cell-edge compartments labelled by YFP: RAB-A5c do not accumulate the endocytic tracer FM4-64, even after prolonged treatment (Figure 1.5D; Kirchhelle et al., 2016), and YFP: RAB-A5c itself also faintly labels the TGN/EE (Kirchhelle et al., 2016). Interestingly, YFP: RAB-A5c is not uniformly localised to all cell edges in lateral roots but is significantly enriched at longitudinal cell edges compared to transverse cell edges (Kirchhelle et al., 2019). The identification of the unique cell-edge localisation of membrane-associated RAB-A5c indicates that cells in developing primordia of *Arabidopsis* contain a trafficking pathway from the TGN/EE to their geometric edges. This putative trafficking pathway has been termed 'edge-directed trafficking'.



In order to investigate the function of RAB-A5c from its unique cell edge localisation, the dominant negative RAB-A5c[N125I] mutant was expressed in a spatio-temporally controlled manner. The pOp/LhGR system (Moore et al., 1998; Craft et al., 2005) is widely used to conditionally control gene expression in a spatio-temporally restricted manner through application of the corticosteroid Dexamethasone (DEX). LhGR is a DEX-inducible chimeric transcription factor that binds to the pOp promoter, which is upstream of the gene-of-interest, upon activation and drives transcription of the

gene-of-interest in a spatio-temporally restricted manner. RAB-A5c[N125I] conditional expression from the *Arabidopsis* RIBOSOMAL PROTEIN 5a (AtRPS5a) promoter in the pOp/LhGR system (termed AtRPS5a>DEX>A5c[N125I]; Kirchhelle et al., 2016) was used to investigate the effects of RAB-A5c functional inhibition at both the cell and tissue scales. Germination of AtRPS5a>DEX>[N125I] plants on DEX resulted in severely stunted root and shoot growth (Figure 1.6A; Kirchhelle et al., 2016). To investigate these defects more closely at the cellular level, the PM marker YFP: NOVEL PLANT SNARE 12 (YFP: NPSN12) was co-expressed with AtRPS5a>DEX>A5c[N125I] to observe changes in cell geometry. Using specifically developed imaging chambers, lateral roots of plants co-expressing AtRPS5a>DEX>[N125I] and YFP: NPSN12 were monitored over extended periods following induction of RAB-A5c[N125I] expression (Kirchhelle et al., 2016; Kirchhelle & Moore, 2017).

Expression of RAB-A5c[N125I] resulted in cytokinesis defects similar to those produced by functional inhibition of other members of the Rab-A clade (Figure 1.6B; Chow et al., 2008; Qi & Zheng, 2013; Kirchhelle et al., 2016) however, it also resulted in highly abnormal cell geometries in young primary and lateral roots. Particularly, cells in the elongation zone of lateral roots 24-48 hours after the induction of RAB-A5c[N125I] expression were significantly increased in radial expansion relative to longitudinal expansion compared to wild-type lateral roots, with many cells rupturing after 72 hours of RAB-A5c[N125I] expression (Figure 1.6C & D). These defects in lateral root cell expansion therefore represent a transition from the usual anisotropic (unidirectional) growth of such cells to almost isotropic growth upon inhibition of RAB-A5c function. Moreover, these defects in cell expansion appeared to be independent of cytokinesis defects (Kirchhelle et al., 2016), indicating a specific role for RAB-A5c in the maintenance of anisotropic growth in *Arabidopsis* lateral roots.

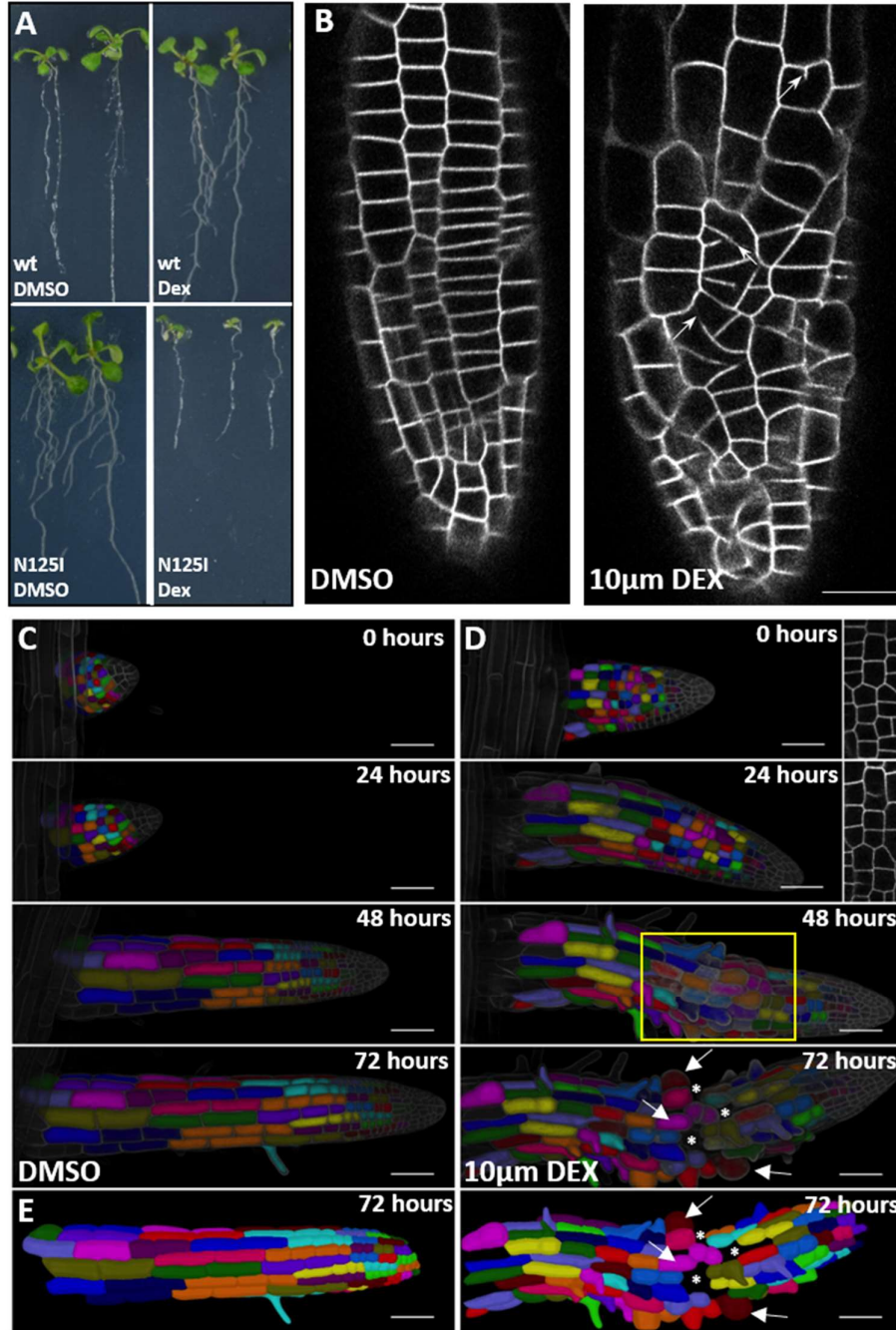


Figure 1.6: Expression of dominant-negative RAB-A5c[N125I] causes defects in anisotropic cell growth in *Arabidopsis* lateral roots. (A) 10 day old Col-0 or AtRPS5a>DEX>A5c[N125I] seedlings grown on 20µm DEX or equivalent volume Dimethyl sulphoxide (DMSO). (B) *Arabidopsis* lateral roots co-expressing YFP: NPSN12 and AtRPS5a>DEX>A5c[N125I]. 20µm DEX or equivalent volume DMSO, 48 hours. (C & D) 3D-segmented *Arabidopsis* lateral roots co-expressing YFP: NPSN12 and AtRPS5a>DEX>A5c[N125I] after transfer to 20µm DEX or equivalent volume DMSO. Yellow box = elongation zone cells, * = ruptured cells, white arrows = abnormal cell geometries (E) Duplicate of (C&D) at 72 hours without YFP: NPSN12 outline. All adapted from Kirchhelle et al., 2016.

Inhibition of primary root growth caused by expression of RAB-A5c[N125I] is partially suppressed by co-expression of YFP: RAB-A5c in a dose-dependent manner, indicating that RAB-A5c[N125I] acts to specifically inhibit RAB-A5c. Moreover, expression of RAB-A5c[N125I] does not disrupt trafficking of PIN2: GFP or YFP: NPSN12, nor does it disrupt secretion of secreted (sec)GFP or endocytic traffic of YFP: NPSN12 and FM4-64, suggesting that RAB-A5c[N125I] expression does not disrupt bulk secretory or endocytic trafficking (Kirchhelle et al., 2016). RAB-A5c localisation to compartments at cell geometric edges in growing tissues is reminiscent of the known localisation of microtubule-associated CLIP-ASSOCIATED PROTEIN (CLASP) and GAMMA TUBULIN COMPLEX PROTEINS 2 and 3 (GCP2/3) to cell edges in primary and lateral root meristematic cells, from where they contribute to organisation of the cortical microtubule array (Ambrose et al., 2011, Ambrose & Wasteneys, 2011; Le & Ambrose, 2018). Despite also localising to the highly-spatially confined cell edge domain, neither CLASP: GFP nor GCP2: GFP overlap substantially with YFP: RAB-A5c (Kirchhelle et al., 2016, 2019). Moreover, YFP: RAB-A5c remains edge-localised in *clasp-1* and *spr3* (a GCP2 mutant) mutant backgrounds and both CLASP: GFP and GCP2: GFP remain edge-localised after expression of RAB-A5c[N125I], indicating that RAB-A5c localisation to cell edges is independent of CLASP and GCP2 and *vice versa* (Kirchhelle et al., 2016, 2019).

Inhibition of RAB-A5c function through expression of RAB-A5c[N125I] therefore indicates that cell geometric edges may be important subcellular domains in the maintenance of anisotropic growth and cell geometry in lateral roots, independently of microtubule organisation. Strikingly, *in silico* 2D finite-element linear-elastic modelling of a simplified lateral root epidermis also demonstrated that localised softening of cell edges around vertices can produce swelling under turgor similar to that seen in lateral roots expressing RAB-A5c[N125I] (Kirchhelle et al., 2016), indicating that disruption of cell edge mechanical properties could theoretically translate into plant growth defects. YFP: RAB-A5c has further been shown to associate with cortical microtubules and expression of RAB-A5c[N125I] results in increased anisotropy of cortical microtubules and cellulose synthase complex trajectories

in lateral roots (Kirchhelle et al., 2019). This is surprising as anisotropic (highly ordered) cortical microtubule arrays are generally associated with anisotropic growth, whereas expression of RAB-A5c[N125I] causes increasingly isotropic cell growth in lateral roots. *In silico* and genetic work indicates that increased microtubule anisotropy upon expression of RAB-A5c[N125I] may be a compensatory mechanism to attempt to counteract the cell swelling caused by inhibition of RAB-A5c function. Kirchhelle and colleagues therefore propose that RAB-A5c facilitates localised modification of the cell wall around cell geometric edges in growing tissues of *Arabidopsis* through defining and regulating an edge-directed trafficking pathway from the TGN/EE to the PM via cell edge compartments, and that this local modification of the cell wall around cell edges is required to maintain anisotropic growth of individual cells in *Arabidopsis* lateral roots (Kirchhelle et al., 2016, 2019). RAB-A5c therefore functions from a unique subcellular localisation in growing tissues of *Arabidopsis* to support cell anisotropic growth, and may define an edge-directed trafficking pathway as a route for spatially-restricted modification of the cell wall in growing tissues.

1.G: Integrating cues from the cell wall

Post-Golgi trafficking in plants therefore supports a possible polar trafficking pathway for local modification of the cell wall via cell geometric edges, in addition to other suspected polar trafficking pathways to the cell wall and polar trafficking in plant immunity (reviewed by Elliott & Kirchhelle, 2019; Elliott et al., 2020). Dynamic maintenance of such polarisation during plant growth, development and immune responses invariably requires integration of intrinsic and extrinsic cues. Whilst the mechanisms by which such cues influence endomembrane trafficking pathways remain unclear, cell-surface signalling modules are beginning to be characterised in detail as the first point of perception of intrinsic and extrinsic cues in plants. One site of perception of particular importance during plant growth, development and immune responses is the cell wall, and key to such cell wall-based signalling modules in plants are Receptor-Like Kinases (RLKs), Receptor Like Proteins (RLPs) and Wall-Associated Kinases (WAKs). Several such kinases have been linked to sensing of abundance

of certain cell wall components and sensing of cell wall integrity, particularly members of the *Catharanthus roseus* RLK1-Like (CrRLK1L) group of *Arabidopsis* RLKs. THESEUS1 (THE1) is a sensor of cell wall cellulose content and the other CrRLK1L members FERONIA (FER) and ANUXR1 and 2 (ANX1/2) have been implicated in sensing of cell wall integrity (Hematy et al., 2007; Boisson-Dernier et al., 2009; Miyazaki et al., 2009; Guo et al., 2009; Deslauriers and Larsen, 2010; Feng et al., 2018; reviewed by Wolf, 2017). Moreover, some WAKs are known to bind pectin *in vitro* and RNAi-mediated suppression of WAK4 results in severe growth defects, suggesting that WAKs function as part of a signalling module responding to pectin integrity or abundance in the cell wall (Lally et al., 2001; Decreux and Messiaen, 2005; Kohorn et al., 2009; reviewed by Kohorn & Kohorn, 2012). In addition, the diverse Leucine-Rich Repeat RLP family is known to support the functions of RLKs in *Arabidopsis* and other plants in perception of intrinsic and extrinsic cues at the cell surface. RLPs typically consist of an extracellular region with Leucine-Rich Repeat (LRR) regions, a transmembrane domain (TMD) and a short intracellular cytoplasmic tail (Wang et al., 2008; Jamieson et al., 2018). Extracellular LRR regions consist of 23 to 25 amino acids and are believed to function in receptor ligand perception. The majority of RLPs contain two blocks of LRR regions in their extracellular domains, separated by an island domain (Wang et al., 2008). LRR regions are also a conserved feature of RLKs however, unlike RLKs, RLPs do not contain an intracellular kinase domain or other domains known to be involved in signalling (Wang et al., 2008). Whilst RLP sequences are plant-specific, they have been described as analogous to Toll-Like Receptors in metazoans, which are structurally similar and also consist of an extracellular domain with LRRs and a short cytoplasmic tail (Jamieson et al., 2018).

Through association with RLKs, RLPs that have been functionally characterised can be broadly divided into two categories: those that function in plant immunity and those that function in plant development. Some of the earliest and most detailed characterisation of RLPs and their associated RLK come from studies of roles for RLPs in plant immune responses. Perception of pathogens by

plants is often mediated by recognition of microbe associated molecular patterns (MAMPs), which are conserved small molecules essential for pathogen survival, by pathogen recognition receptors (PRRs). RLPs involved in plant immunity can either act as PRRs themselves or functionally associate with them. *Arabidopsis* RLP23, for example, binds conserved pathogenic Necrosis and ethylene-inducing peptide 1-like proteins (NLPs; Albert et al., 2015). RLP23 associates constitutively with the RLK SUPPRESSOR OF BIR1-1 (SOBIR1) and, upon binding to NLPs, interacts with a second RLK, BRI1-ASSOCIATED KINASE-1 (BAK1). Such examples of RLPs acting as direct PRRs are not confined to *Arabidopsis*. In rice, the RLP OsCEBiP binds the fungal cell wall component chitin, and functions with the RLK OsCERK1 in subsequent immune responses (Kaku et al., 2006; Shimizu et al., 2010; Shinya et al., 2012) and in tomato, the RLP SIEIX2 is important in resistance to fungal pathogens through direct perception of fungal xylanases (Ron & Anvi, 2004; Bar et al., 2010). In addition to acting directly as PRRs in plant immunity, some RLPs do not directly perceive MAMPs, but still function in pathogen-triggered immune responses. For example, mutants in the tomato RLP CLADOPOSIUM FULVUM-9 (Cf-9) are susceptible to the fungus *Cladosporium fulvum* due to defective recognition of the pathogen effector AVIRULENCE9 (Ar9) (Jones et al., 1994). Cf-9 does not however seem to directly bind Avr9 (Luderer et al., 2001) indicating that Cf-9 may function alongside a separate Avr9-binding protein. Several other examples of RLPs implicated in pathogen perception in *Arabidopsis* and potato have yet to be shown to bind directly a MAMP or pathogen effector (Yang et al., 2012; Jehle et al., 2013; Du et al., 2015).

RLP function is also an essential component of plant development, which is illustrated by some well-studied examples. In *Arabidopsis*, the RLP CLAVATA2 (RLP10/CLV2) is essential for the maintenance of meristem size (Kayes & Clark, 1998; Jeong et al., 1999). CLV2 functions alongside the RLK CLAVATA1 (and other RLKs) to perceive the intercellular differentiation-promoting peptide CLAVATA3 (CLV3), repressing expression of WUSCHEL (WUS), which promotes of stem cell identity in shoot meristems (Kayes & Clark, 1998; Jeong et al., 1999; Schoof et al., 2000; Rojo et al., 2002; Lenhard et al., 2003; reviewed by Pan et al., 2016). Expression of CLV3 by stem cells in the shoot

meristem represses expression of WUS in the generative quiescent centre. This results in fewer stem cells being produced and reduced CLV3 expression, thus providing a feedback loop to maintain meristem size and proper development. *Arabidopsis* RLP17, better known as TOO MANY MOUTHS (TMM), also functions in the maintenance of organ patterning, controlling stomatal patterning in leaves. TMM associates with ERECTA RLKs to perceive EPIDERMAL PATTERNING FACTOR (EPFs) peptides produced by stomatal precursor cells. Position-dependent perception of EPFs by TMM and associated RLKs initiates a signalling cascade that represses SPEECHLESS, a positive regulator of stomatal lineage initiation (Yang & Sack, 1995; Nadeau & Sack, 2002; Lee et al., 2012; Meng et al., 2015; reviewed by Le et al., 2014). A further recently-described RLP with roles in growth and development through acting as a cell-wall integrity sensor is RLP44. RLP44 associates with the well-characterised RLK BAK1, which in turn forms a receptor complex with the brassinosteroid receptor BRASSINOSTEROID INSENSITIVE 1 (BRI1; Wolf et al., 2014). Brassinosteroid signalling is known to function in cell wall synthesis and modification, and an RLP44 mutant suppresses brassinosteroid-mediated compensatory responses caused by expression of the pectin methylesterase inhibitor PMEox (Wolf et al., 2014). Over-expression of RLP44 did not alter sensitivity of plants to application of the brassinosteroid epi-brassinolide in contrast to overexpression of the brassinosteroid receptor BRI1, which rendered plants more sensitive. Moreover, plants over-expressing RLP44 had very similar phenotypes to those over-expressing BRI1. Taken together, these results indicate that RLP44 functions as part of an RLP44-BAK1-BRI1 receptor complex sensing cell wall perturbations, but does not itself directly perceive brassinosteroids (Wolf et al., 2014). Recently, RLP44 and BRI1 have also been implicated in brassinosteroid-independent specification of root vasculature identity through interaction with PHYTOSULFOKINE RECEPTOR-1 (PSKR1), supporting other observations that the same RLP can function differently in different tissues/cell types (Holzwardt et al., 2018, 2020). Interestingly, proper C-terminal phosphorylation of RLP44 appears to be required for the interaction of RLP44 with BAK1 but not for interaction with PSKR1, providing the first mechanistic insights into how the differential functions of RLP44 are co-ordinated (Gomez et al., 2019).

As post-Golgi trafficking and its spatio-temporal control is known to support both biogenesis of the cell wall and trafficking responses to plant pathogens (reviewed by Elliott et al., 2020), cell-surface signalling modules such as those involving RLKs and RLPs are therefore strong candidates for integration of intrinsic and extrinsic cues into the spatio-temporal regulation of such post-Golgi trafficking pathways in plants however, no definitive links have yet been identified. RAB-A5c has been suggested to define a dynamic route for polar cell wall modification around cell edges in growing tissues of *Arabidopsis* (Kirchhelle et al., 2016, 2019). This is presumably in response to specific biochemical or mechanical requirements in the cell wall around cell geometric edges in growing tissues however, how these requirements are perceived and how a possible edge-directed trafficking pathway from the TGN/EE may be controlled spatio-temporally to respond to such extrinsic and intrinsic cues remains unknown. Presumably, this and other possible examples of polar trafficking to the cell wall and polar trafficking responses to plant pathogens may themselves require polar establishment or maintenance of cell surface/cell wall-based signalling modules to perceive polar cues. However, polarised trafficking or maintenance of cell surface/cell wall-based signalling modules has not yet been reported, meaning that there are no specific known candidates for possible integration of cues into, and co-ordination of, polar trafficking pathways such as edge-directed trafficking. This and other questions remain regarding the organisation and function of RAB-A5c-associated trafficking in *Arabidopsis*, and work in this thesis will begin to address these.

1.H: Open questions

Many questions remain regarding the function and composition of a possible edge-directed trafficking pathway in *Arabidopsis*, as well as the significance of cell geometric edges in general as an emerging discrete cellular domain in plant cells. The most fundamental of these is does RAB-A5c define an edge-directed trafficking pathway in growing tissues of *Arabidopsis*? Identification of effectors and cargoes of RAB-A5c and additional possible pathway components is likely to be crucial

to confirming this. A second question is how RAB-A5c, amongst the related wider Rab-A clade, specifically defines cell edge compartments and a possible edge-directed trafficking pathway. Whether this is due to localisation-regulating protein sequences within RAB-A5c, regulatory activity of specific interactors of RAB-A5c or other factors such as membrane compartment lipid composition (or possibly a combination of these) remains unclear. A third open question is the identity and nature of interactors of RAB-A5c and protein components of its associated trafficking pathway. Identification of such proteins is likely to be essential in furthering understanding of the spatio-temporal control of possible edge-directed trafficking, and how it may integrate intrinsic and extrinsic cues to function in control of growth anisotropy in *Arabidopsis*. A final question is whether RAB-A5c does indeed define a route for local modification of the cell wall via cell geometric edges as suggested by Kirchhelle and colleagues (Kirchhelle et al., 2016, 2019). As already discussed, identification of Rab cargoes and effectors is essential in understanding Rab GTPase function, as well as the function of the associated trafficking pathway/membrane compartment. Identification of interactors of RAB-A5c and additional molecular components of cell edge compartments will therefore assist with addressing each of these four open questions, as well as moving towards greater molecular understanding of plant cell geometric edges.

1.1: Work pre-dating this project

Work pre-dating this project began the process of identifying of interactors of YFP: RAB-A5c and possible additional components of cell edge compartments through a co-immunoprecipitation and proteomic analyses targeted against YFP: RAB-A5c. This was part of a wider undertaking to develop a co-immunoprecipitation protocol effective against Rab GTPases, which are only transiently membrane-associated. Other co-immunoprecipitation studies of Rab GTPases have in the past yielded large numbers of protein hits as possible Rab interactors or effectors (Gillingham et al., 2014; Heard et al., 2015). Validation of such large numbers of candidate interactors is however virtually impossible. Enriching protein hits for small numbers of possible specific interactors of Rab GTPases is

therefore desirable in proteomic investigation of Rab interactomes where the focus is on validation and functional investigation of candidate interactors. To achieve this, co-immunoprecipitation against YFP: RAB-A5c was accompanied by identical experiments targeting TGN/EE-localised YFP: RAB-A2a (Chow et al., 2008) and tonoplast-localised YFP: RAB-G3f (Geldner et al., 2009) respectively to allow for comparative ranking of candidate interactors, and identification of those most likely to be specific interactors of YFP: RAB-A5c (for full details of the co-immunoprecipitation protocol and candidate interactor ranking process, see Appendix 1). The result of the ranking process was a ranked list of 314 protein hits as possible specific interactors of YFP: RAB-A5c. This list of YFP: RAB-A5c interactors provided a starting point for further investigation into the molecular composition of cell edge compartments and their associated trafficking pathway in *Arabidopsis*.

1.J: Project aims & Summary

The main aims of my project were:

- To validate or dismiss identified putative interactors of YFP: RAB-A5c as possible effectors, cargoes or regulators of RAB-A5c, or additional components of cell edge compartments
- To use identified interactors of YFP: RAB-A5c to confirm or refute whether RAB-A5c defines a polarised trafficking pathway to the cell surface
- To investigate the specific function of identified interactors of YFP: RAB-A5c in edge-directed trafficking
- To use confirmed interactors of YFP: RAB-A5c and possible novel components of cell edge compartments as tools for further investigation of the spatio-temporal dynamics and function(s) of RAB-A5c and its associated trafficking pathway

In this thesis, I investigate and characterise the subcellular localisation of proteomically-identified candidate interactors of YFP: RAB-A5c. Two candidate interactors of YFP: RAB-A5c, RECEPTOR-LIKE PROTEIN 4 (RLP4) and a previously unrecognised RLP, RECEPTOR-LIKE PROTEIN 4-LIKE (RLP4-L), are

found to localise to cell edge compartments, the first proteins other than Rab-A5 GTPases identified as doing so. Investigation of the trafficking of fluorescently-tagged RLP4 and RLP4-L confirms that RAB-A5c defines a polar edge-directed trafficking pathway to the cell surface and demonstrates that cell geometric edges are also polar domains of the *Arabidopsis* PM. Investigation of subcellular localisation and trafficking of fluorescently-tagged truncated variants of RLP4 and RLP4-L indicates the contributions of different protein domains to their trafficking and suggests possible functions for these RLPs in edge-directed trafficking. Two functional hypotheses are presented for RLP4 and RLP4-L and these will be the subject of future investigations. Finally, perturbation of the trafficking of known and newly-identified components of edge-directed trafficking question previously-held assumptions about this trafficking pathway, and raises significant questions regarding techniques commonly used to investigate Rab GTPase-associated trafficking pathways.

Chapter 2: Subcellular localisation and dependence on RAB-A5c function identify RLP4 and RLP4-L as components of a RAB-A5c-defined edge-directed trafficking pathway in *Arabidopsis*

2.A: Introduction

For initial investigation of candidate interactors of YFP: RAB-A5c in *Arabidopsis*, a method for identifying candidates of particular interest for investigation was required. Given the unique nature of RAB-A5c's localisation to cell edge compartments, identification of such localisation in candidate interactors would be a strong initial indication that such candidates likely function in a RAB-A5c-associated trafficking pathway. I therefore decided to introduce fluorescent protein fusions of candidate interactors into *Arabidopsis* in order to screen for localisation to cell edge compartments in lateral roots using live-cell confocal imaging. Identification of localisation to cell edge compartments of any candidate interactors would highlight such interactors as likely being of particular interest in further investigation of the dynamics and function of RAB-A5c-associated trafficking in *Arabidopsis*.

It was first necessary to restrict the list of candidate interactors of YFP:RAB-A5c to a manageable number for investigation, prioritising those likely to be of greatest interest. The comparative ranking process outlined in Appendix 1 had ranked 314 YFP:RAB-A5c candidate interactors in relation to their abundance in proteomes of YFP: RAB-A2a and YFP: RAB-G3f. However, this method did itself not produce any obvious cut-off point to base investigation on. On inspection of the highest-ranking interactors, I noticed that a putative plant TRAPP complex component, AtBET5, appeared at position 23 (once higher-ranking contaminant candidates were removed – see Appendix 1, Figure 1). TRAPP II is, as already discussed, a known GEF for Rab GTPases in yeast and metazoans (Morozova et al., 2006; Riedel et al., 2018) and has been functionally placed upstream of some Rab-A GTPases in plants (Qi et al., 2011; Qi & Zheng, 2011; Kalde et al., 2019). A TRAPP complex component is therefore an unsurprising hit in a Rab-A GTPase interactor proteome. Interactions between TRAPP

complexes and Rab GTPases are predicted to occur between core-TRAPP components and the GTPase (Cai et al., 2008; Thomas et al., 2019). AtBET5 is a core-TRAPP component of TRAPP complexes in yeast and metazoans, and binary interaction data in *Arabidopsis* supports its placement as a core component of plant TRAPP complexes (Kim et al., 2006; Yip et al., 2010; Tan et al., 2013; Taussig et al., 2013; Kalde et al., 2019). The placement of a protein with likely connections to Rab GTPases at number 23 on the interactor list of YFP:RAB-A5c candidate interactors gives special interest to the candidate interactors ranking above this. I therefore decided to investigate further the top-ranking 22 candidate interactors of YFP: RAB-A5c (23 candidates excluding the top-ranking candidate which was RAB-A5c itself; Appendix 1, Figure 1), up to and including the TRAPP component AtBET5, as possible specific interactors of RAB-A5c and as additional components of cell edge compartments.

2.B: Generation of fluorescent protein fusions of YFP: RAB-A5c candidate interactors

For initial screening for cell edge localisation of YFP: RAB-A5c candidate interactors using fluorescent protein fusions, I expressed candidate interactors as N- or C-terminal fluorescent protein fusions depending on functional annotations from existing literature and basic protein structure prediction software. For the majority of candidate interactors, pUB-DEST vectors (Grefen et al., 2010) containing the UBIQUITIN10 (UBQ10, pUB in vectors) promoter were selected for their *in planta* expression with fluorescent protein fusions. For full details of candidate cloning procedure and vector selection see Chapter 7 (Materials & Methods). Three candidate interactors, the putative lectin At1g52000, COP1-INTERACTING PROTEIN 1 (CIP1, At5g41790) and NUCLEOBASE ASCORBATE TRANSPORTER 6 (NAT6, At5g62890), could not be amplified via PCR despite several attempts including nested PCR. These candidate interactors could therefore not be investigated at this stage. All other candidate interactors were cloned from genomic DNA and fluorescent protein fusion constructs transformed into *Arabidopsis* through *Agrobacterium*-mediated transformation (Clough & Brent, 1998).

Plants co-expressing fluorescent fusions of candidate interactors with YFP: RAB-A5c were examined for co-localisation of candidates with YFP: RAB-A5c at cell edge compartments in lateral roots. Plants were first imaged at the T1 generation for initial indications and subsequently at T2 & T3 generations for final confirmation and acquisition of images used in this thesis. Generations deriving from at least two independent T1 transformation events were examined for each candidate in order to ensure reproducibility of candidate interactor localisation patterns.

2.C: Localisations of candidate interactors of YFP: RAB-A5c

Of the 21 candidate interactors transformed into *Arabidopsis* lines expressing proA5c:: YFP: RAB-A5c (YFP: RAB-A5c), 9 gave no appreciable fluorescence in lateral roots or other tissues, leaving 12 candidates for investigation as possible interactors of YFP: RAB-A5c. Localisation of these candidate interactors was examined in lateral root meristematic cells due to clear localisation to cell edges of YFP: RAB-A5c in lateral root meristematic cells. Encouraging, many of these candidate interactors were found to localise to the cell surface either at the PM or in the apoplast, conducive to possible interactions between these proteins and RAB-A5c at the cell surface or dependence of these candidates for trafficking to the cell surface on RAB-A5c (Figure 2.1, summarised by Table 1; for expanded figure see Appendix 2, Figure 1). The subcellular localisation of several uncharacterised proteins was also revealed. Perhaps the most interesting of these is At4g18070. pUB:: At4g18070: RFP strikingly labels what is likely the tonoplast (Figure 2.1 & Appendix 2, Figure 1) however it has no predicted TMD, indicating that it may associate with the tonoplast peripherally. At4g18070 also has a very unusual predicted protein structure, with a predicted sequence of 16 consecutive serine residues towards its N-terminus. These unusual features and its striking localisation to the tonoplast make At4g18070 an interesting candidate for possible future investigation. Although many candidate interactors of YFP: RAB-A5c were found to localise to the cell surface, 3 of these stood out as likely being of particular interest. pRAB-A5b:: YFP: RAB-A5b, pUB:: RLP4: RFP, pUB:: RLP4-L: RFP

each localised to cell edge compartments, highlighting these as strong candidates for possible interaction with, or functional relationship to, RAB-A5c. The localisations of these 3 candidates were therefore examined in greater detail.

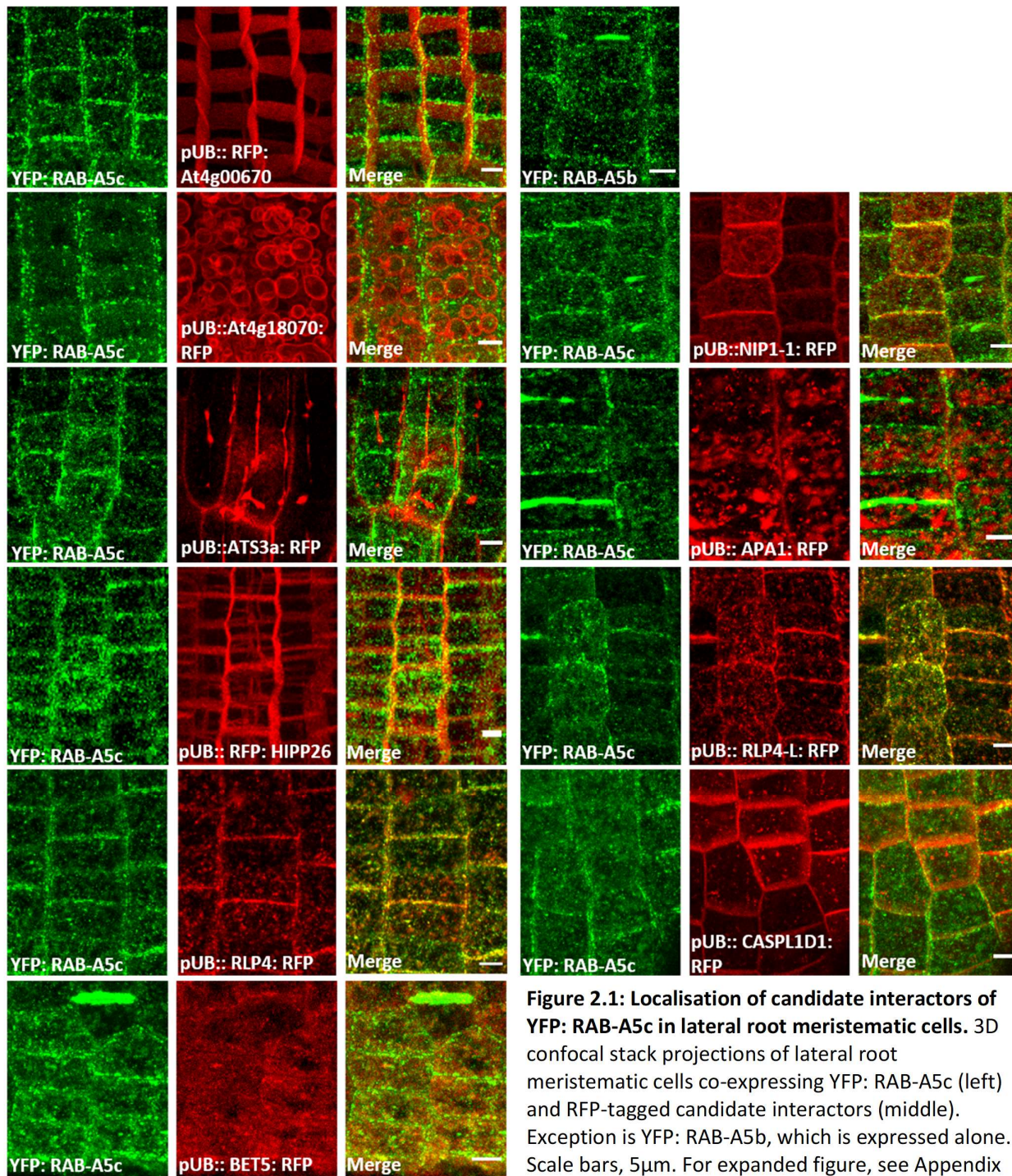


Figure 2.1: Localisation of candidate interactors of YFP: RAB-A5c in lateral root meristematic cells. 3D confocal stack projections of lateral root meristematic cells co-expressing YFP: RAB-A5c (left) and RFP-tagged candidate interactors (middle). Exception is YFP: RAB-A5b, which is expressed alone. Scale bars, 5µm. For expanded figure, see Appendix 2, Figure 1. All from 10 day old T2 seedlings.

Table 1: Expression and localisation of candidate interactors of YFP: RAB-A5c in *Arabidopsis* lateral roots

<u>Candidate Interactor</u>	<u>N- or C-terminal fusion</u>	<u>Fluorescence? (Y/N)</u>	<u>Localisation in lateral roots</u>
At3g15356	C-terminal	N	
At1g52000	C-terminal	N	
At4g31330	Both generated	N	
At4g04460	C-terminal	N	
At4g00670	N-terminal	Y	PM
RAB-A5b (At3g07410)	N-terminal	Y	Cell edge compartments and other intracellular compartments
At4g18070	C-terminal	Y	Tonoplast?
SUC1 (At1g71880)	C-terminal	N	
GLL23 (At1g54010)	C-terminal	N	
NIP1-1 (At4g19030)	C-terminal	Y	PM and endoplasmic reticulum (?)
ATS3a (At2g41475)	C-terminal	N	Extracellular
HIPP26 (At4g38580)	N-terminal	Y	PM
AtMSL9 (At5g19520)	C-terminal	N	
APA1 (At1g11910)	C-terminal	Y	Vacuolar and extracellular (?)
At1g25570 ('RLP4-L')	C-terminal	Y	Cell edge compartments, PM and other intracellular compartments
GLL22 (At1g54000)	C-terminal	N	
RLP4 (At1g28340)	C-terminal	Y	Cell edge compartments, PM and other intracellular compartments
CASPL1D1 (At4g15610)	C-terminal	Y	PM and intracellular compartments
At5g35735	Both generated	Y (but only of N-terminal)	PM and intracellular compartments
OCT1 (At1g73220)	C-terminal	N	
AtBET5 (AT1G51160)	C-terminal	Y	Intracellular compartments and cytosol

2.D: YFP: RAB-A5b labels compartments at cell geometric edges

RAB-A5c is just one of 5 GTPases that make up the Rab-A5 subclass in *Arabidopsis* (Rutherford & Moore, 2002). It has been postulated that members of this subclass act redundantly (Kirchhelle et al., 2016). However, it has until now been unclear whether any other Rab-A5 GTPases also localise to compartments at cell geometric edges like RAB-A5c. RAB-A5b and its endogenous promoter were therefore introduced into the vector pBINPLUS (as described in Chow et al., 2008) to give pA5b::YFP: RAB-A5b. When introduced into the Col-0 background through *Agrobacterium*-mediated transformation (Clough & Brent, 1998), pA5b::YFP: RAB-A5b localised to compartments at the both transverse and longitudinal geometric edges of lateral root meristematic cells (Figure 2.2A, B, E, white arrows). Like YFP: RAB-A5c and other Rab-A GTPases, pA5b::YFP: RAB-A5b also labelled the cell plate during cytokinesis, and was noticeably confined to the leading edge (Figure 2.2C & D). YFP: RAB-A5c co-localises with microtubules of the PPB at cell edges prior to cytokinesis (Kirchhelle et al., 2019). No structures resembling the PPB were seen to be labelled by pA5b::YFP: RAB-A5b here however, co-expression with a tubulin marker will be required to definitively investigate this.

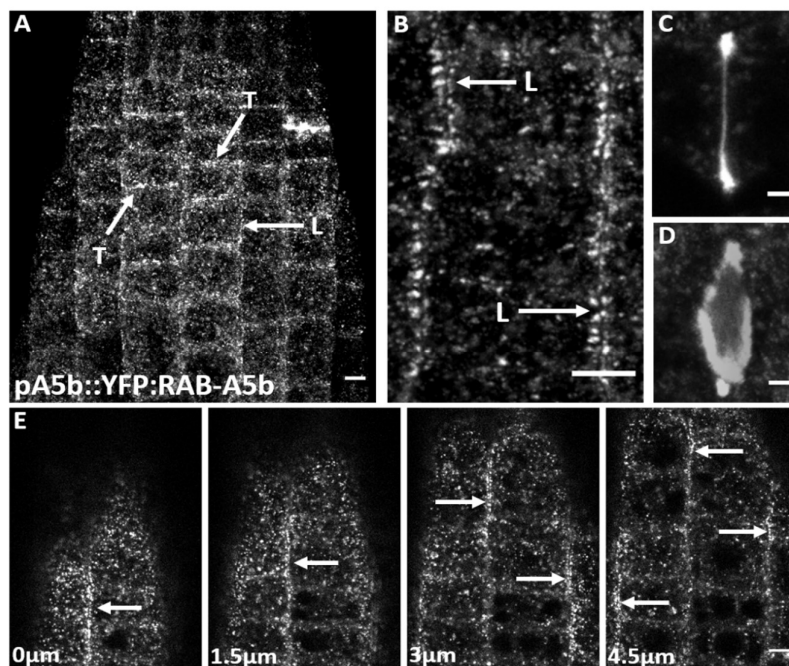


Figure 2.2: pA5b::YFP: RAB-A5b labels compartments at cell geometric edges (white arrows, T= transverse edge, L= longitudinal edge) in lateral roots (A,B) 3D confocal stack projections of lateral roots expressing YFP: RAB-A5b. (C, D) confocal slice (C) and rotated 3D confocal projection (D) during cytokinesis. (E) successive confocal slices of lateral root meristematic cells expressing YFP: RAB-A5b. 1.5 μ m intervals. Scale bars, 5 μ m. All from 10 day old T2 seedlings.

2.E: RLP4: RFP and RLP4-L: RFP co-localise with YFP: RAB-A5c at cell edge compartments when expressed from the UBQ10 promoter

Whilst RLP4 has previously been annotated as part of the Receptor-Like Protein family of cell surface receptors in *Arabidopsis* (Wang et al., 2008; Jamieson et al., 2018), RLP4-L has not previously been described as part of this family of proteins. Basic Local Alignment Search Tool (BLAST; www.uniprot.org & www.arabidopsis.org) searches indicate that RLP4-L has the highest protein-sequence homology with RLP4 of any *Arabidopsis* protein (Pairwise Identity = 34.7%, Pairwise Similarity = 50.2%; see Appendix 2, Figure 2) and basic structural predictions also indicate that the two proteins have similar protein domains and domain arrangements. Both have predicted extracellular Malectin-Like Domains and like other RLPs, LRR regions, a TMD and a short intracellular tail (Appendix 2, Figure 2). RLP4 contains three LRR regions and, unlike for the majority of RLPs, these are not separated by an island domain (Wang et al., 2008). Basic alignment indicates that RLP4-L shares this arrangement (Appendix 2, Figure 2). In addition to belonging to a minority of RLPs that lack an LRR-separating island domain, the presence of an extracellular Malectin-Like Domain makes RLP4 and newly-identified RLP4-L unique among *Arabidopsis* RLPs (Wang et al., 2008; Jamieson et al., 2018). Transcriptome data indicates that RLP4 is widely-expressed in *Arabidopsis* and is supported by GUS-based expression profiling, indicating activity of the RLP4 promoter in roots, lateral roots and leaves of young *Arabidopsis* plants (Wang et al., 2008; Sultana et al., 2019). However, expression analyses have not yet been performed for RLP4-L.

When expressed from the UBQ10 promoter, both C-terminally tagged pUB:: RLP4: RFP and pUB:: RLP4-L: RFP co-localised with YFP: RAB-A5c cell edge compartments at both transverse and longitudinal cell edges of lateral root meristematic cells (Figure 2.3A & B, white arrows). Moreover, both pUB:: RLP4: RFP and pUB:: RLP4-L: RFP also localised to the PM in lateral roots (Figures 2.3B, yellow arrows). In addition to localising to both cell edge compartments and the PM, pUB:: RLP4: RFP and pUB:: RLP4-L: RFP also labelled additional intracellular compartments (Figures 2.3A & B).

Kirchhelle and colleagues showed that YFP: RAB-A5c was significantly enriched at the outer surface of lateral root meristematic cells and attenuated with increased depth from this surface (Kirchhelle et al., 2016). Initial observations indicate that this is also the case for pUB:: RLP4: RFP and pUB:: RLP4-L: RFP localisation to cell edge compartments and the PM, which seemed to be enriched towards to outer surface and to visibly attenuate with depth (Figure 2.3C & D). Interestingly, RLP4: RFP and RLP4-L: RFP labelling of the PM also seemed to follow this pattern (Figure 2.3C & D). RLP4: RFP and RLP4-L: RFP, when expressed from the UBQ10 promoter, are therefore the first non-GTPase proteins identified as capable of localising to YFP: RAB-A5c-labelled cell edge compartments.

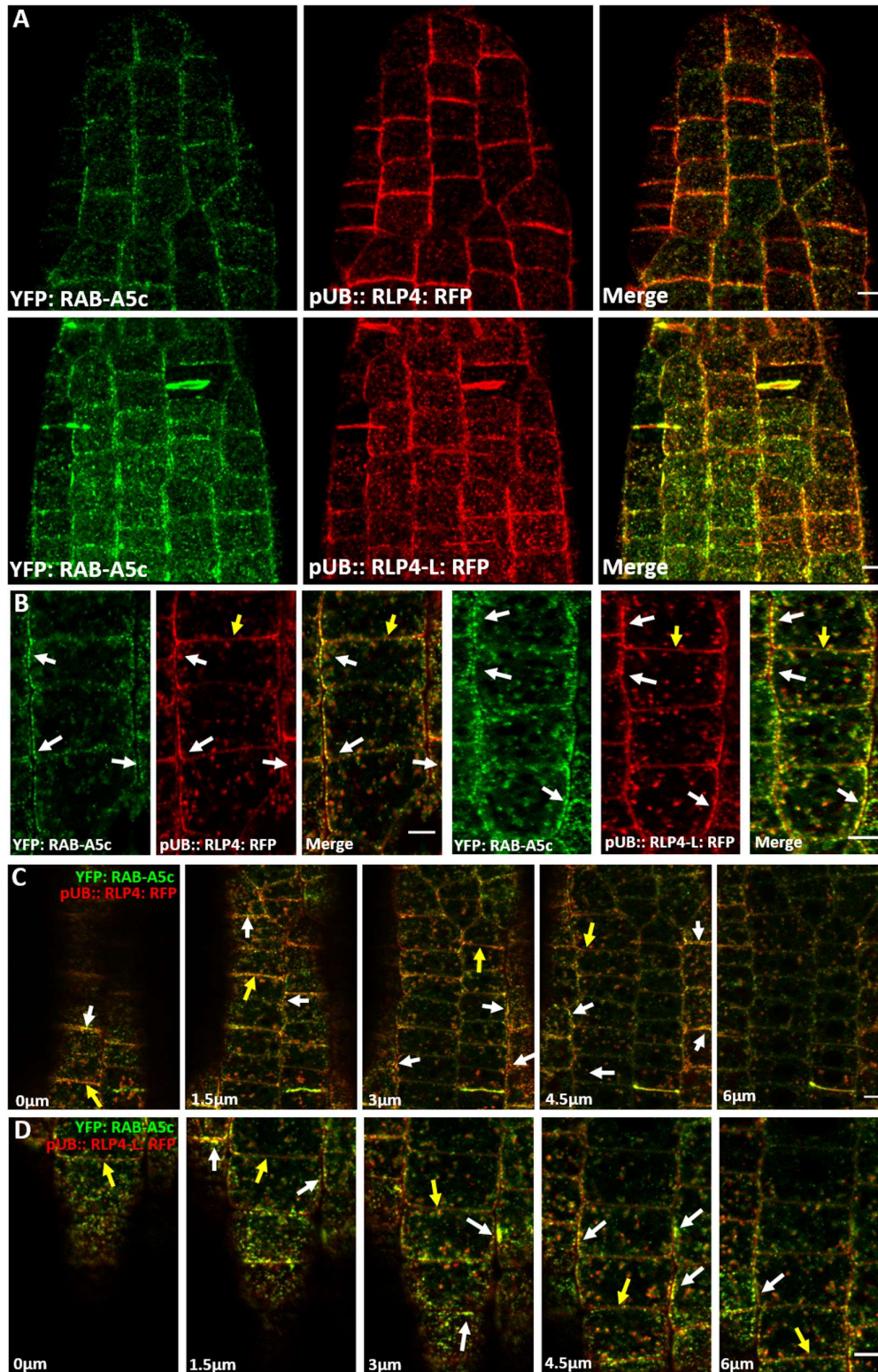


Figure 2.3: pUB:: RLP4: RFP and pUB:: RLP4-L: RFP co-localises with YFP: RAB-A5c at cell edge compartments (white arrows) and also labels the PM (yellow arrows). (A,) 3D confocal stack projections of lateral roots co-expressing YFP: RAB-A5c (left) and pUB:: RLP4: RFP or pUB:: RLP4-L: RFP (middle). (B) confocal slice of lateral root meristematic cells. (C,D) successive confocal slices of lateral roots, 1.5μm intervals. Scale bars, 5μm. All from 10 day old T2 seedlings.

2.F: RLP4: RFP and RLP4-L: RFP localisation to cell edge compartments is reproducible from their endogenous promoters

Expression of pUB:: RLP4: RFP and pUB:: RLP4-L: RFP is driven by the commonly-used UBQ10 promoter, which may result in transgene over-expression. Over-expression of transgenes can result in expression in aberrant tissues/cell types, or ectopic localisations to additional subcellular structures. I therefore sought to replicate the localisation of RLP4: RFP and RLP4-L: RFP to cell edge compartments through expression from their endogenous promoters. The UBQ10 promoter was therefore removed from pUB-DEST vectors and the promoters and 5'-UTR regions of RLP4 and RLP4-L (defined as 2.6kb upstream and 1.6kb upstream of the start codon respectively) inserted directly upstream of their coding sequences, giving pRLP4:: RLP4: RFP/GFP and pRLP4L:: RLP4-L: RFP/GFP. When expressed in the YFP: RAB-A5c background, both pRLP4:: RLP4: RFP and pRLP4L:: RLP4-L: RFP also co-localised with YFP: RAB-A5c at cell edge compartments (white arrows) in young lateral roots, as well as labelling the PM (yellow arrows) and other intracellular compartments (Figures 2.4A & B). In addition to labelling cell edge compartments, YFP: RAB-A5c also associates with cortical microtubules and co-localises with the PPB at cell edges prior to cytokinesis, and like other Rab-A GTPases also re-localises to the cell plate during cytokinesis (Kirchhelle et al., 2016, 2019). Both pRLP4:: RLP4: RFP and pRLP4L:: RLP4-L: RFP co-localised with YFP: RAB-A5c at the growing cell plate during cytokinesis (Figure 2.4C). In addition, pRLP4:: RLP4: RFP and pRLP4L:: RLP4-L: RFP also co-localised with YFP: RAB-A5c at cell edges during what appeared to be formation of the PPB (Figure 2.4D).

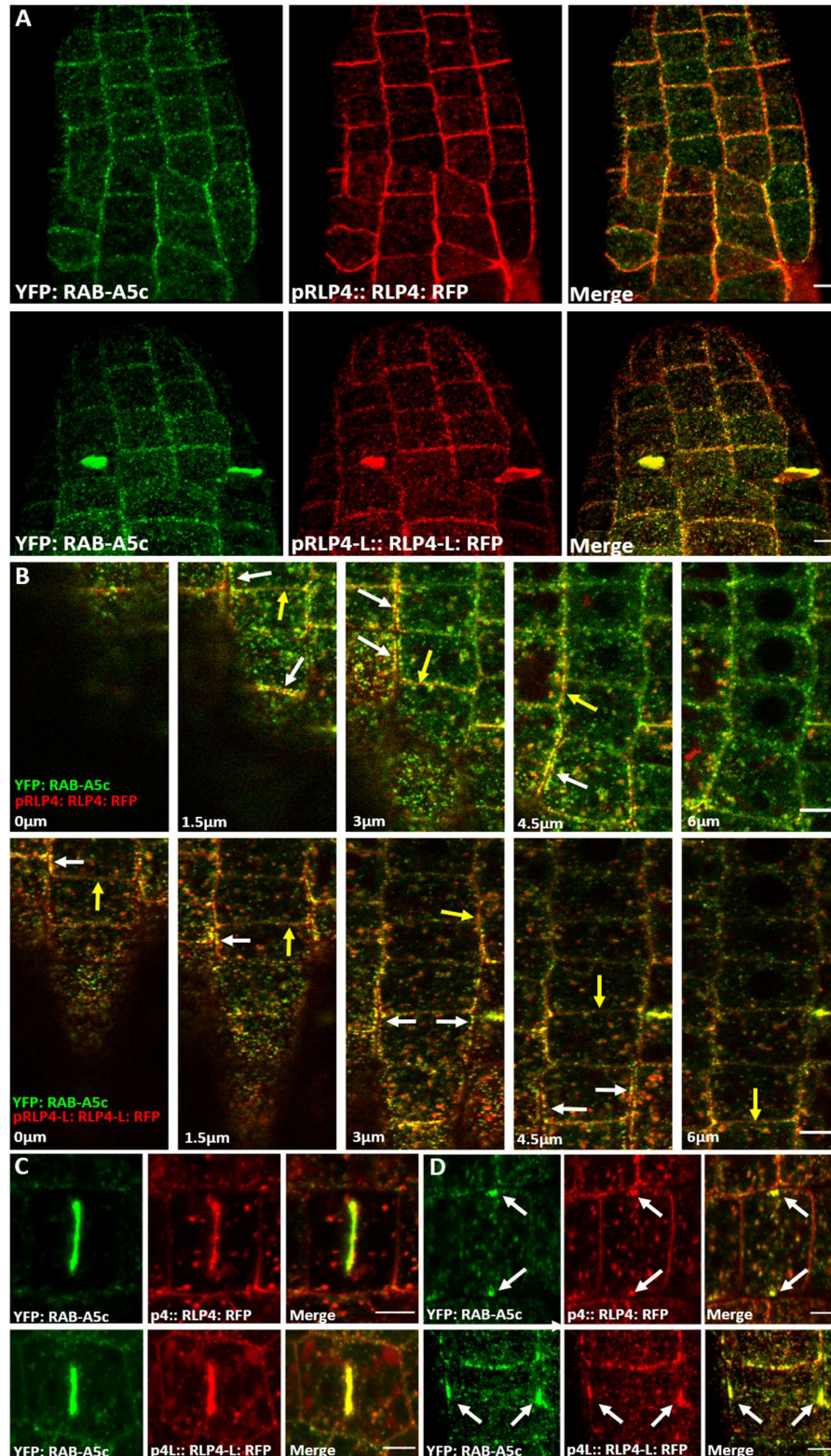


Figure 2.4: pRFP4:: RFP and pRFP4L:: RFP co-localise with YFP: RAB-A5c at cell edge compartments and label the PM (A) 3D confocal stack projections of lateral roots co-expressing YFP: RAB-A5c (left) and pRFP4:: RFP or pRFP4L:: RFP (middle). (B) successive confocal slices of lateral root meristematic cells, 1.5 μm intervals. (C, D) Confocal slices of cells during cytokinesis (C) and at pre-prophase (D, white arrows = probable PPB). Scale bars, 5 μm. All from 10 day old T2 seedlings.

Expression of pRLP4:: RLP4: RFP and pRLP4L:: RLP4-L: RFP in a YFP: RAB-A5c-expressing background still however constitutes over-expression, as endogenous RLP4 and RLP4-L are also present. I therefore transformed pRLP4:: RLP4: GFP and pRLP4L:: RLP4-L: GFP into knockout mutant plants of RLP4 and RLP4-L respectively generated using CRISPR-Cas9 (*rlp4* and *rlp4l* - provided by Ann-Kathrin Schuerholz & Sebastian Wolf, University of Heidelberg; see Chapter 7, Figure 7.1 for details). pRLP4:: RLP4: GFP and pRLP4L:: RLP4-L: GFP also localised to cell edge compartments (white arrows) and the PM (yellow arrows) in the *rlp4* and *rlp4l* backgrounds respectively (Figure 2.5A & B). Localisation of pRLP4:: RLP4: GFP and pRLP4L:: RLP4-L: GFP in *rlp4* and *rlp4l* backgrounds to cell edge compartments and PM also appeared to attenuate with increased depth from the outer surface (Figure 2.5A & B). Localisation of RLP4: RFP/GFP and RLP4-L: RFP/GFP to cell edge compartments is therefore not an artefact of expression from the UBQ10 promoter. Expression of pRLP4:: RLP4: GFP and pRLP4L:: RLP4-L: GFP in *rlp4* and *rlp4l* backgrounds respectively also confirmed that a) RLP4 and RLP4-L localisation to cell edge compartments is independent of the specific fluorescent protein variant used and b) that cell edge compartment localisation of fluorescently-tagged RLP4 and RLP4-L is also visible in the absence of YFP: RAB-A5c.

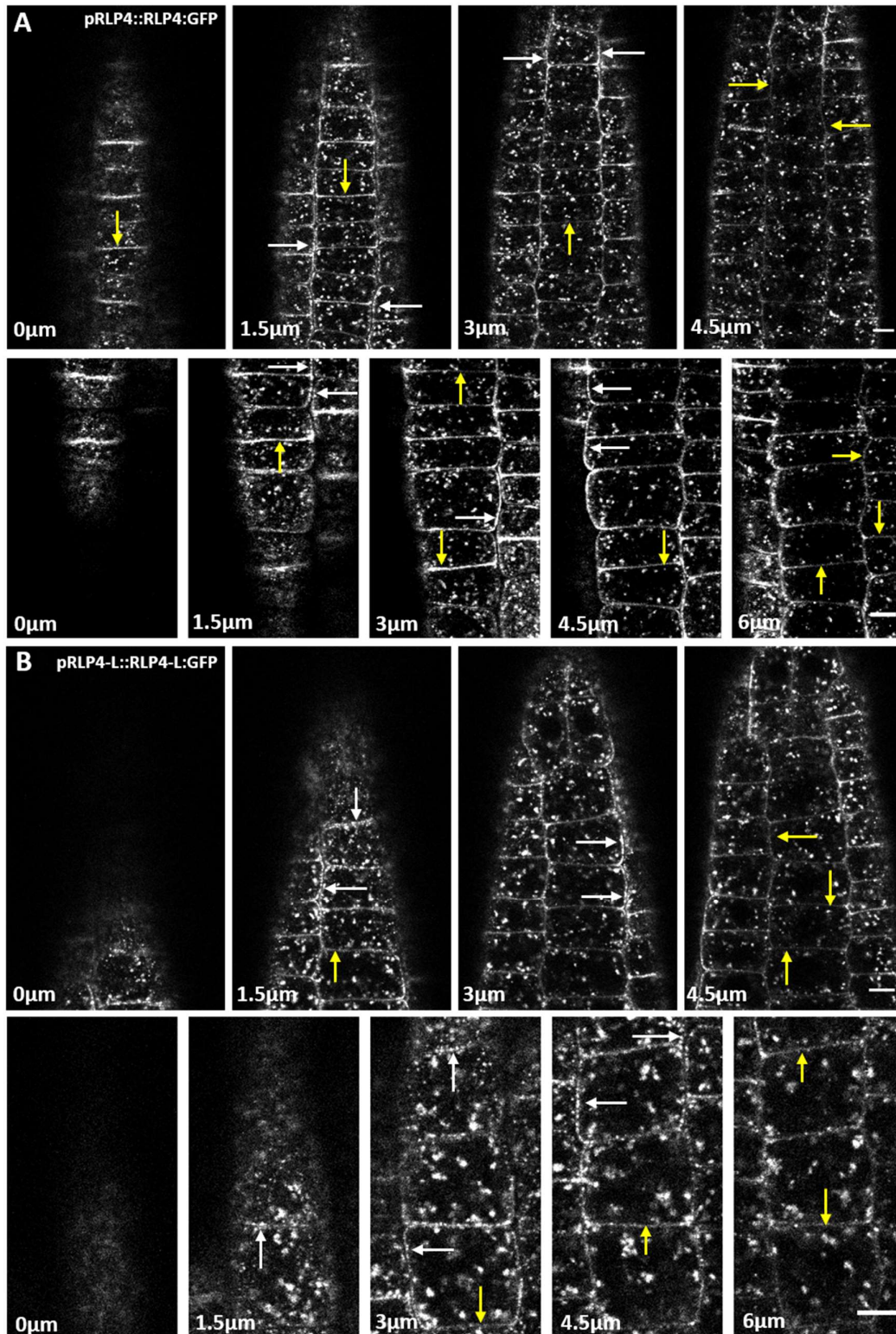


Figure 2.5: pRLP4:: RLP4: GFP and pRLP4L:: RLP4-L: GFP label cell edge compartments (white arrows) and the PM (yellow arrows) in *rlp4* and *rlp4l* knockout backgrounds respectively. (A, B) sequential confocal slices of lateral root meristematic cells expressing pRLP4:: RLP4: GFP (A) or pRLP4L:: RLP4-L: GFP (B) in *rlp4* and *rlp4l* backgrounds respectively. 1.5 μm intervals. Scale bars, 5 μm. All from 10 day old T2 seedlings.

2.G: Fluorescently-tagged RLP4 and RLP4-L localisation is independent of fluorophore positioning

pUB-driven and pRLP4/pRLP4L-driven fluorescent protein fusions of RLP4 and RLP4-L were designed as C-terminal fusions due to the presence of a signal peptide at the N-terminus of both proteins. C-terminally tagged variants of other *Arabidopsis* cell surface receptors cannot however complement mutant backgrounds of these receptors, indicating that C-terminal tagging of cell-surface receptors is in at least some cases disruptive to their function (Ntoukakis et al., 2011; Hurst et al., 2018). In anticipation of possible future functional characterisation of RLP4 and RLP4-L, as well as to investigate whether trafficking of RLP4 and RLP4-L to cell edge compartments is affected by fluorophore positioning, I fused RLP4 and RLP4-L with fluorescent proteins at their N-termini. When transformed into YFP: RAB-A5c plants, both N-terminally tagged pUB:: secRFP: RLP4 and pUB:: secRFP: RLP4-L co-localised with YFP: RAB-A5c at cell edge compartments and also labelled the PM, as well as intracellular compartments (Figure 2.6). pUB:: secRFP: RLP4 and pUB:: secRFP: RLP4-L both also co-localised with YFP: RAB-A5c at what appears to be the PBB at cell edges (Figure 2.6C & D). This confirms that the localisation pattern of fluorescently-tagged RLP4 and RLP4-L is independent of fluorophore N- or C-terminal positioning.

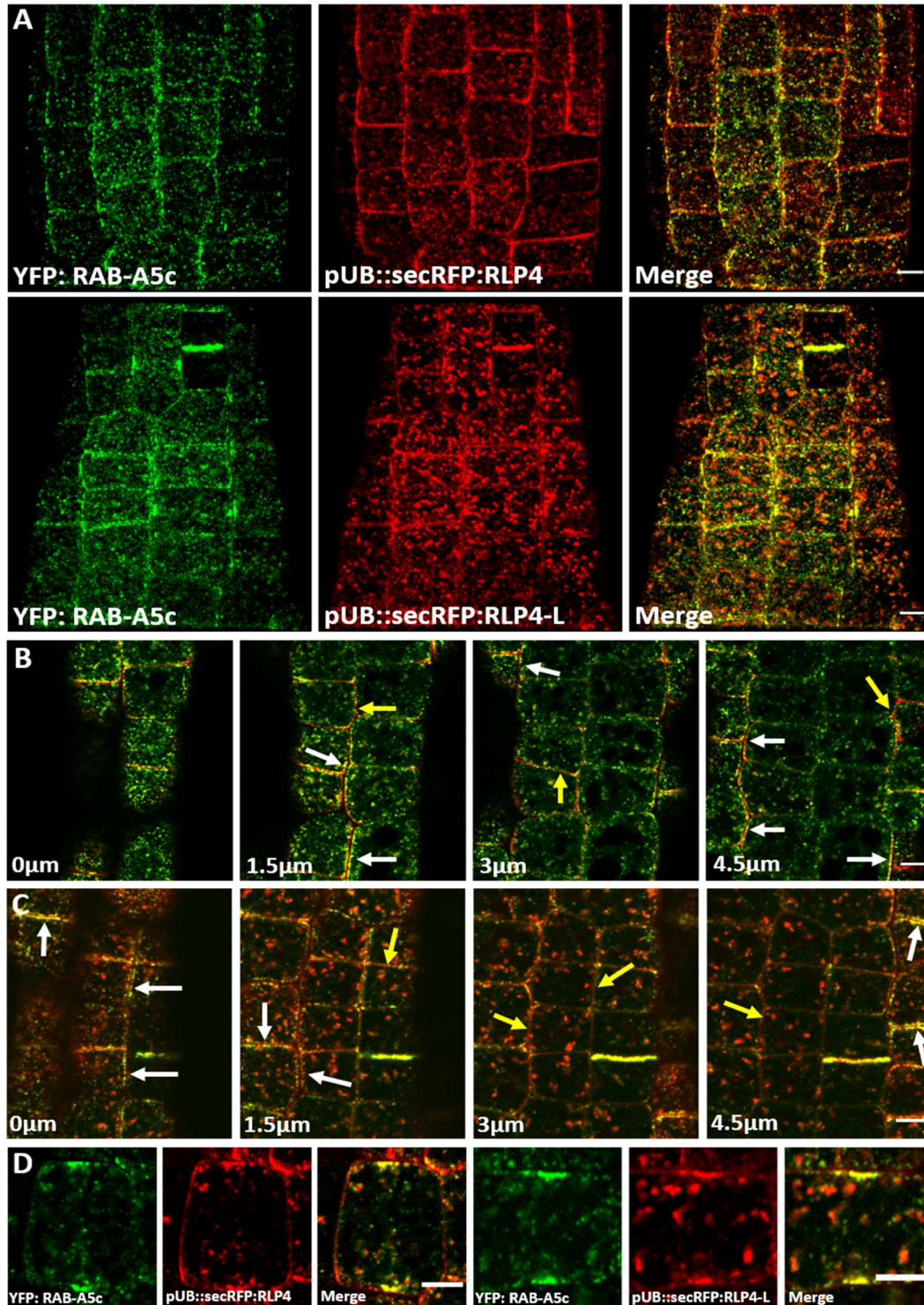
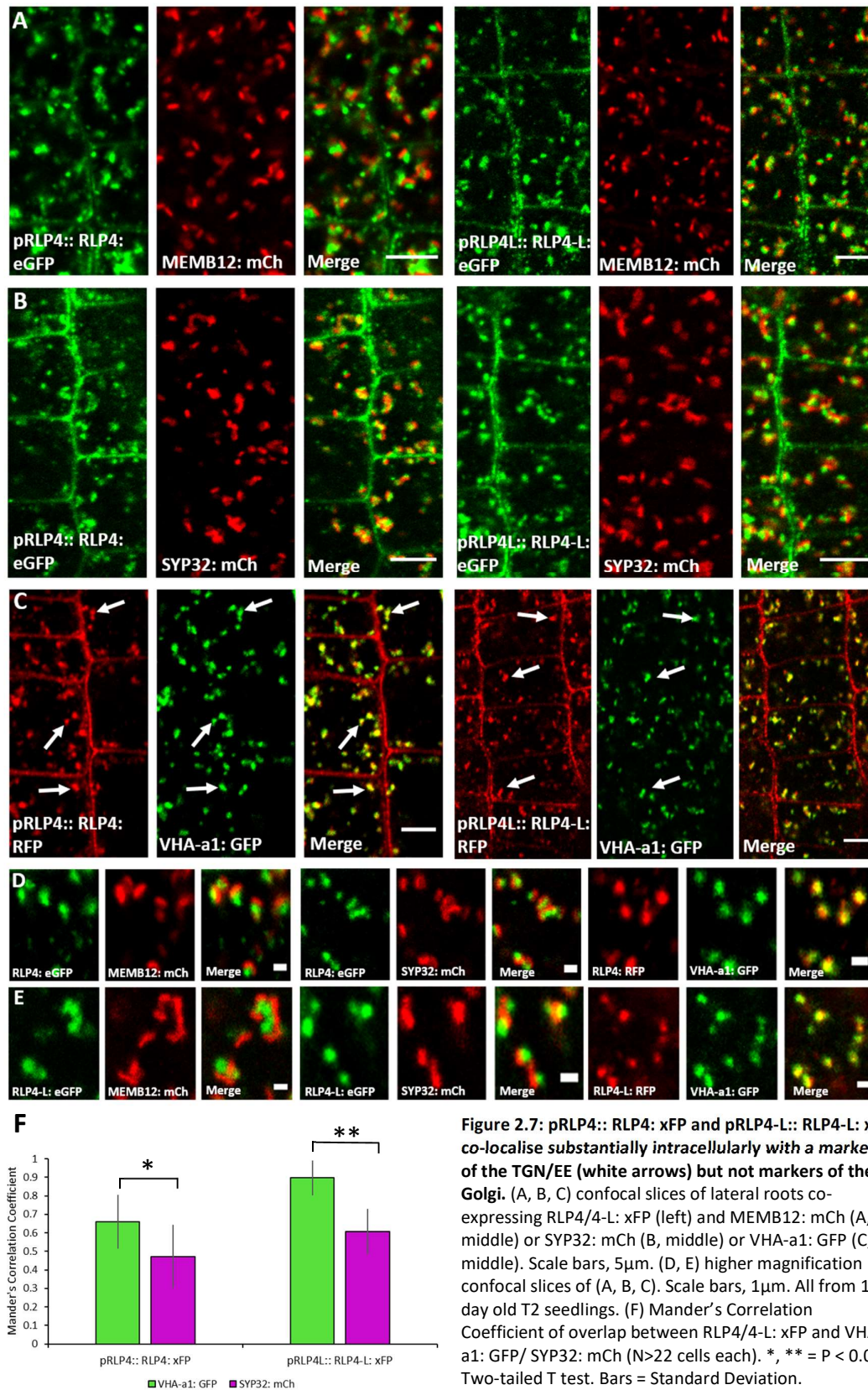


Figure 2.6: pUB:: secRFP: RLP4 and pUB:: secRFP: RLP4-L co-localise with YFP: RAB-A5c at cell edge compartments (white arrows) and also label the PM (yellow arrows). (A) 3D confocal stack projections of lateral roots co-expressing YFP: RAB-A5c (left) and pUB:: secRFP: RLP4 (middle) or pUB:: secRFP: RLP4-L (middle). (B, C) successive confocal slices of lateral roots co-expressing YFP: RAB-A5c (green) and pUB:: secRFP: RLP4 (B, red) or pUB:: secRFP: RLP4-L (C, red). 1.5μm intervals. (D) confocal slice of cells at pre-prophase co-expressing YFP: RAB-A5c (left) and pUB:: secRFP: RLP4 (middle) or pUB:: secRFP: RLP4-L (middle). Scale bars, 5μm. All from 10 day old T2 seedlings.

2.H: Fluorescently-tagged RLP4 and RLP4-L also label the TGN/EE in lateral roots

All fluorescent protein fusions of RLP4 and RLP4-L investigated so far localised to intracellular compartments distinct from cell edge compartments and the PM. YFP: RAB-A5c partially labels the TGN/EE and may define an edge-directed trafficking pathway from the TGN/EE to the cell surface (Kirchhelle et al., 2016, 2019). I therefore hypothesised that fluorescently-tagged RLP4 and RLP4-L also intracellularly label the TGN/EE. In order to test this, pRLP4:: RLP4: RFP/GFP and pRLP4L:: RLP4-L: RFP/GFP were transformed into plants expressing published markers of either the TGN/EE (VHA-a1: GFP; Dettmer et al., 2006) and the Golgi (MEMB12: mCh & SYP32: mCh; Geldner et al., 2009) . Structures labelled by pRLP4:: RLP4: GFP and pRLP4L:: RLP4-L: GFP were closely associated with the Golgi markers SYP32: mCh and MEMB12: mCh (Figure 2.7A &B) but were morphologically distinct from these upon close inspection (Figure 2.7D &E). Structures labelled by pRLP4:: RLP4: RFP and pRLP4L:: RLP4-L: RFP did however visually overlap well with, and were morphologically similar-to, structures labelled by the TGN/EE-marker VHA-a1: GFP (Figure 2.7C, D & E), indicating a degree of steady-state accumulation of fluorescently-tagged RLP4 and RLP4-L at the TGN/EE. Moreover, Mander's Correlation Coefficients indicated that structures labelled by pRLP4:: RLP4: xFP and pRLP4L:: RLP4-L: xFP overlapped with TGN/EE-localised VHA-a1: GFP to a significantly greater degree than with Golgi-localised SYP32: mCh (Figure 2.7F).

The lipophilic endocytic tracer dye FM4-64 marks the endocytic route from the PM and so is commonly used to confirm localisation to the TGN/EE, which functions as an early endosome in *Arabidopsis* (Dettmer et al., 2006; Chow et al., 2008; Viotti et al., 2010). Intracellular compartments labelled by pRLP4:: RLP4: GFP and pRLP4L:: RLP4-L: GFP in lateral roots also accumulated FM4-64 (Figure 2.8), providing further evidence that fluorescently-tagged RLP4 and RLP4 partially label the TGN/EE.



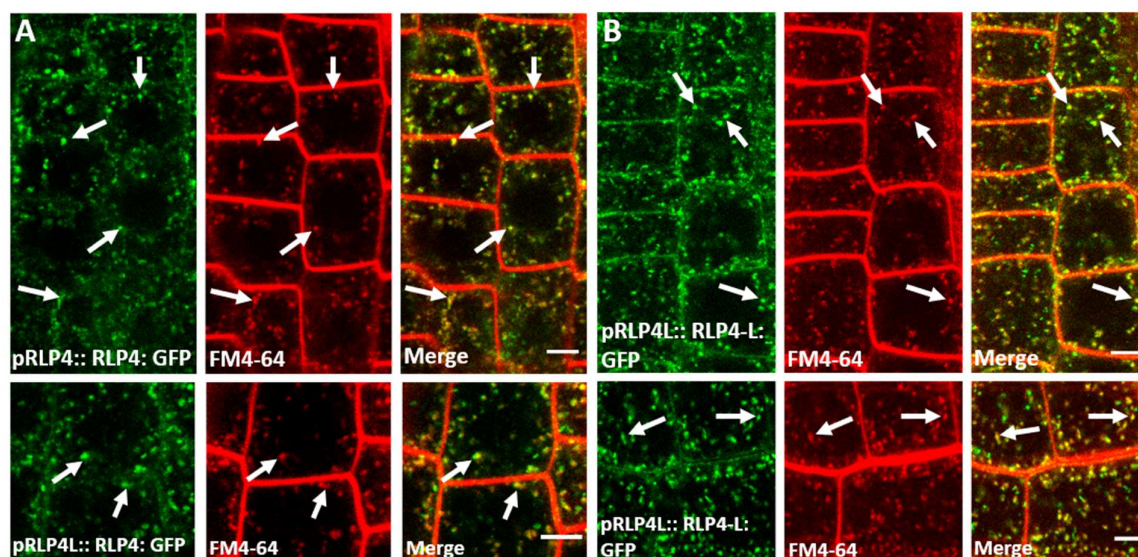


Figure 2.8: pRLP4:: RLP4: eGFP and pRLP4L:: RLP4-L: eGFP label intracellular structures that accumulated the endocytic tracer dye FM4-64 (white arrows). (A, B) confocal slices of lateral root meristematic cells expressing pRLP4:: RLP4: GFP (A) or pRLP4L:: RLP4-L: GFP (B). 50μm FM4-64, 30 minutes. Scale bars, 5μm. All from 10 day old T2 seedlings.

2.1: Investigating possible functional relationships between YFP: RAB-A5c and candidate interactors

Taken together, the co-localisation approach revealed that numerous candidate interactors of YFP: RAB-A5c identified by co-immunoprecipitation predominately localise to areas of the cell surface, raising the possibilities that such candidate interactors may depend on RAB-A5c for delivery to the PM or the apoplast, or that these cell-surface localised candidate interactors may be required for proper localisation of RAB-A5c. Excitingly, fluorescent proteins fusions of three candidate interactors, the Rab-A5 GTPase RAB-A5b and the Receptor-Like Proteins RLP4 and RLP4-L also localise to cell edge compartments. The near-unique nature of localisation to cell edge compartments particularly indicates a functional relationship between these candidate interactors and RAB-A5c. I therefore decided to investigate possible functional dependence of both edge-localised and non-edge-localised candidate interactors on RAB-A5c for their trafficking and localisation.

2.J: Insertional mutant knockouts of edge-localised GTPases do not abolish edge-localisation of

RLP4: YFP and RLP4-L: YFP.

As already discussed, a variety of methods have been reported for investigating functional dependence of trafficking and other processes on Rab GTPase activity, including use of insertional knockout mutants, expression of dominant-negative mutant variants and RNA-mediated suppression of Rab GTPases. An insertional SAIL knockout mutant of *rab-a5c* (SAIL_119_B07) is phenotypically wild-type, and it has previously been assumed that this lack of phenotypic perturbation is due to redundancy within the Rab-A5 subclass (Kirchhelle et al., 2016; Kirchhelle, 2016. DPhil Thesis). This is supported by the observation here that YFP: RAB-A5b also labels compartments at cell geometric edges (Figure 2.2). The identification of fluorescently-tagged RLP4 and RLP4-L as also localising to cell edge compartments presents the first opportunity to visualise trafficking to these compartments in the absence of fluorophore-tagged Rab-A5 GTPases. I therefore used this to demonstrate that trafficking to cell edge compartments is not abolished by insertional knockout mutants of edge-localised Rab-A5 GTPases.

In order to investigate whether trafficking to cell edge compartments remains functional in SAIL knockout mutants of edge-localised Rab-A5 members, I transformed fluorescent protein fusions of RLP4 and RLP4-L into *rab-a5b rab-a5c* double SAIL knockout mutant plants, which are also phenotypically wild-type-like (*rab-a5b* = SAIL_886_G06, transcriptional null mutant; see Chapter 7, Figure 7.2 for characterisation). pRLP4:: RLP4: YFP and pRLP4L:: RLP4-L: YFP still localised to cell edge compartments and the PM in the *rab-a5b rab-a5c* background (Figure 2.9A, B & C), attenuating with increased distance from the outer surface (Figure 2.9B). This demonstrates that cell edge compartments are still present in the transcriptional absence of edge-localised RAB-A5c and RAB-A5b, and that trafficking of fluorescently-tagged RLP4 and RLP4-L to cell edge compartments and the PM also continues in their absence.

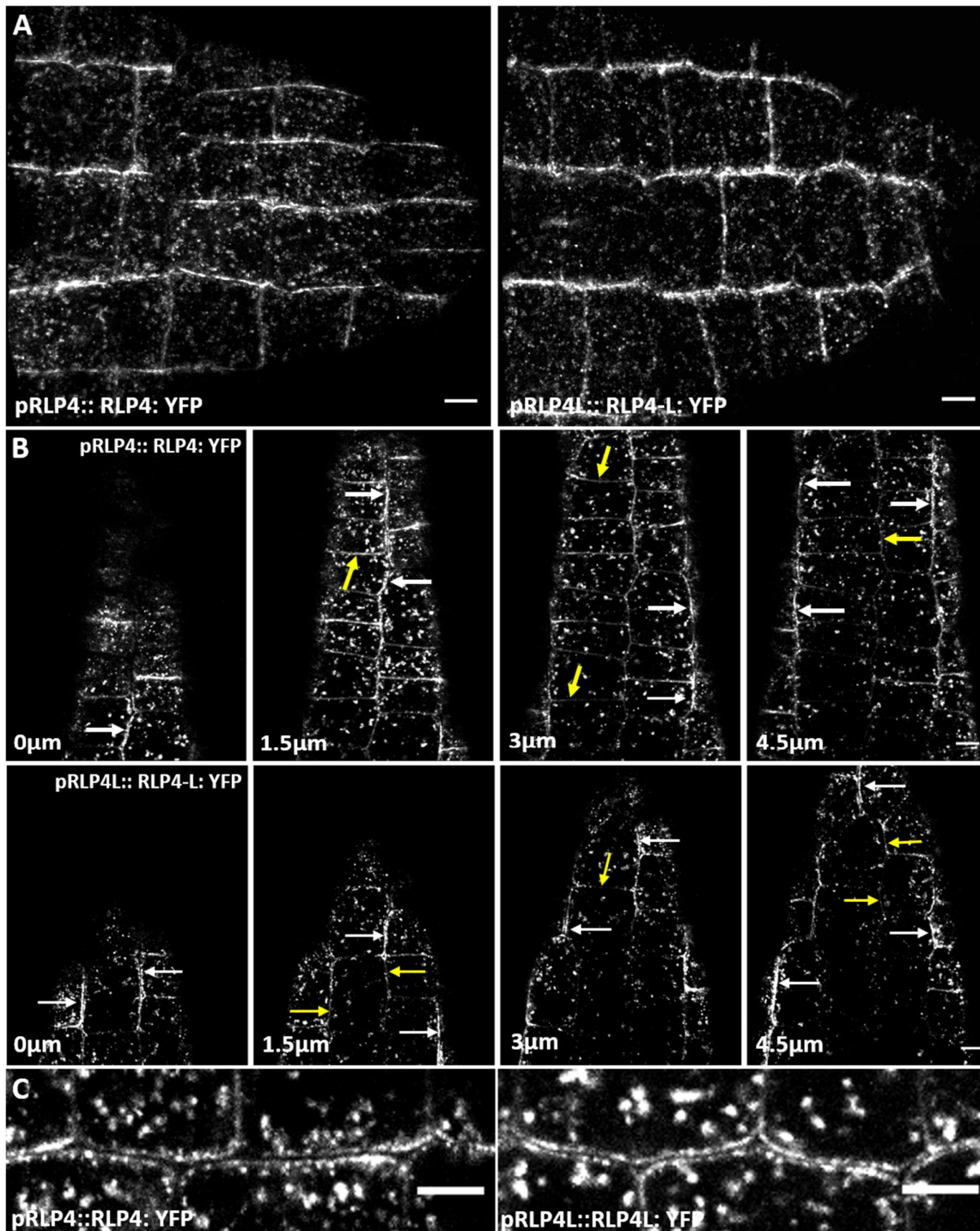


Figure 2.9: pRLP4::RLP4:YFP and pRLP4L::RLP4-L:YFP label cell edge compartments (white arrows) and the PM (yellow arrows) in a *rab-a5b, rab-a5c* knockout background . (A) 3D confocal stack projections of lateral roots expressing pRLP4::RLP4:YFP or pRLP4L::RLP4-L:YFP. (B) successive confocal slices of lateral roots expressing pRLP4::RLP4:YFP or pRLP4L::RLP4-L:YFP, 1.5μm intervals. (C) high resolution confocal slices of lateral root meristematic cells expressing pRLP4::RLP4:YFP or pRLP4L::RLP4-L:YFP. Scale bars, 5μm. All from 10 day old T2 seedlings.

The possibility that localisation to cell edge compartments may be a wider feature of the Rab-A5 subclass and the fact that trafficking of fluorescently-tagged RLP4 and RLP4-L continues in the transcriptional absence of RAB-A5c and RAB-A5b supports previous assumptions of redundancy within the Rab-A5 subclass. Kirchhelle and colleagues used expression of dominant-negative RAB-A5c[N125I] to investigate possible functions for RAB-A5c and to circumvent such possible redundancy. I therefore investigated whether expression of dominant-negative RAB-A5c[N125I] could disrupt the localisations of fluorescently-tagged candidate interactors of YFP: RAB-A5c.

2.K: Investigating YFP: RAB-A5c candidate interactor dependence on RAB-A5c function through expression of dominant-negative RAB-A5c[N125I]

For initial characterisation of the effects of RAB-A5c[N125I] expression (Kirchhelle et al., 2016), a variety of *AtRPS5a>DEX>A5c[N125I]* lines were generated with varying phenotypic severity, presumably corresponding to varying expression levels of the RAB-A5c[N125I] protein. For use in this study, an *AtRPS5a>DEX>A5c[N125I]* line with a strong phenotype upon DEX induction (here termed *AtRPS5a>DEX>A5c[N125I].1*, described by Kirchhelle et al., 2016) and another with a weaker phenotype were selected (*AtRPS5a>DEX>A5c[N125I].2*; Figure 2.10A). Interestingly, YFP: RAB-A5c in *AtRPS5a>DEX>A5c[N125I].1* backgrounds was found to be mis-localised from cell edge compartments to intracellular compartments and the cytosol even in the absence of DEX (Figure 2.10B). This indicates that *AtRPS5a>DEX>A5c[N125I].1* lines express RAB-A5c[N125I] without application of DEX. *AtRPS5a>DEX>A5c[N125I].1* lines are phenotypically wild-type in the absence of DEX (Kirchhelle et al., 2016), indicating that the expression level of RAB-A5c[N125I] without DEX is insufficient to cause defects in cytokinesis and cell geometry. When co-expressed with *AtRPS5a>DEX>A5c[N125I].2* however, YFP: RAB-A5c remained edge-restricted in the absence of DEX, and only mis-localised to intracellular compartments and the cytosol upon treatment with DEX (Figure 2.10C).

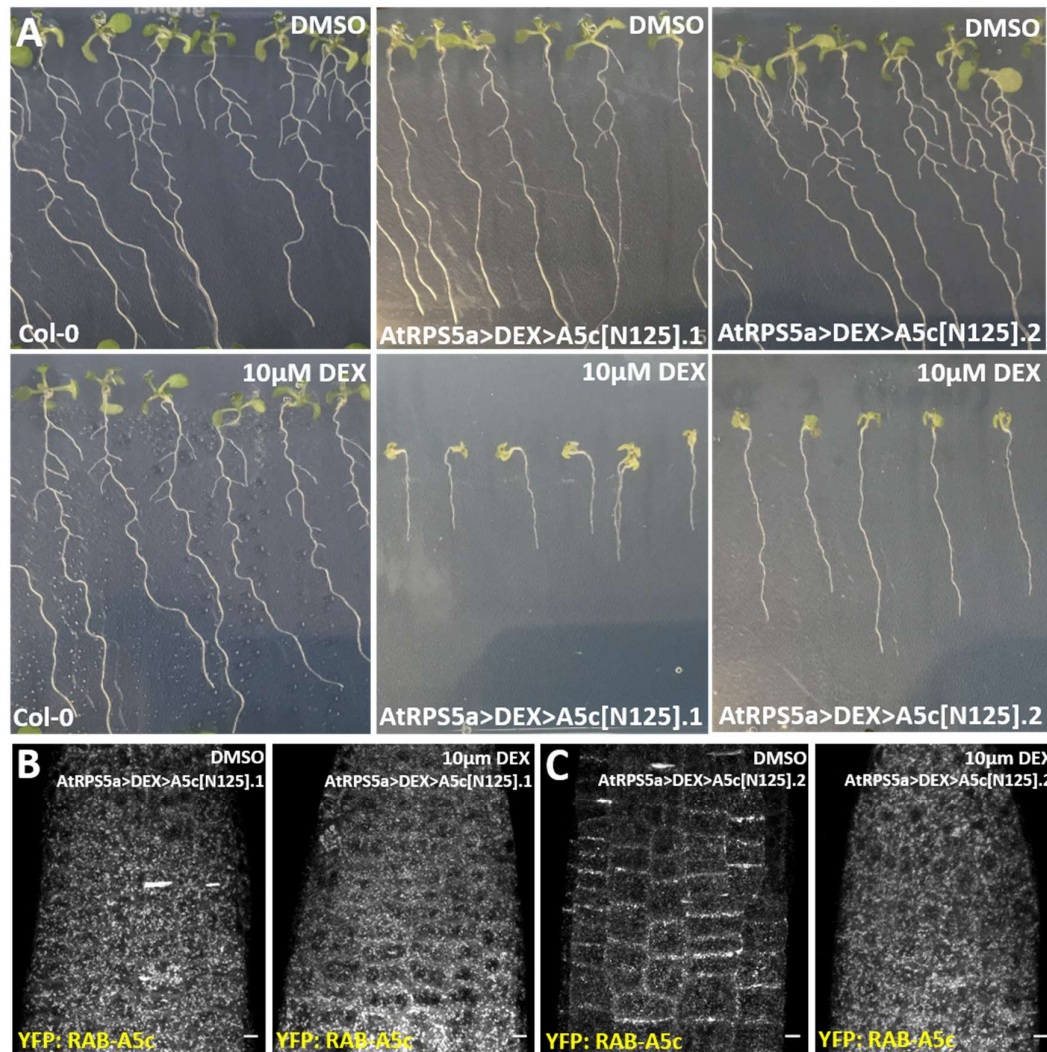


Figure 2.10: YFP: RAB-A5c is mis-localised by *AtRPS5a>DEX>A5c[N125I].1* in the absence of DEX but not by *AtRPS5a>DEX>A5c[N125I].2*. (A) Col-0, *AtRPS5a>DEX>A5c[N125I].1* and *AtRPS5a>DEX>A5c[N125I].2* seedlings germinated and grown on 10µm DEX for 10 days. (B) Lateral roots co-expressing *AtRPS5a>DEX>A5c[N125I].1* and YFP: RAB-A5c. (C) Lateral roots co-expressing *AtRPS5a>DEX>A5c[N125I].2* and YFP: RAB-A5c. 10µm DEX or equivalent volume Dimethyl sulphoxide (DMSO), 72 hours. Scale bars, 5µm. All from 10 day old T3 seedlings.

The majority of candidate interactors of YFP: RAB-A5c, despite not labelling cell edge compartments, localised to the cell surface, raising the possibility that they may depend on RAB-A5c function for this localisation, or be interactors of RAB-A5c at the cell surface. The strongly phenotypic *AtRPS5a>DEX>A5c[N125I].1* line was therefore used to investigate the possible dependence of candidate interactors that did not localise to YFP: RAB-A5c-labelled cell edge compartments on RAB-

A5c function. Plants co-expressing YFP: RAB-A5c and fluorescently-tagged candidate interactors were therefore crossed with *AtRPS5a>DEX>A5c[N125I].1* plants. As described by Kirchhelle and colleagues, lateral roots after 72 hours of DEX-induced *AtRPS5a>DEX>A5c[N125I].1* expression had substantial defects in cytokinesis and cell geometry (Kirchhelle et al., 2016). In these triple transgenic lines co-expressing YFP: RAB-A5c, *AtRPS5a>DEX>A5c[N125I].1* and fluorescently-tagged candidate interactors, 72 hours of *AtRPS5a>DEX>A5c[N125I].1* expression did not however noticeably affect the localisation of the majority of candidate interactors of YFP: RAB-A5c, despite YFP: RAB-A5c being mis-localised (Figure 2.11; for full images see Appendix 2, Figure 3). Interestingly, an exception to this was the *Arabidopsis* TRAPP component AtBET5. pUB:: BET5: RFP in the absence of *AtRPS5a>DEX>A5c[N125I].1* localised predominantly to the cytosol and to some discernible intracellular compartments, similar to the described localisations of other *Arabidopsis* TRAPP components (Qi et al., 2011; Ravikumar et al., 2018). After 72 hours of *AtRPS5a>DEX>A5c[N125I].1* expression however, pUB:: BET5: RFP localisation appeared to shifted towards intracellular compartments compared to in the absence of *AtRPS5a>DEX>A5c[N125I].1* (Figure 2.11, bottom; for full image see Appendix 2, Figure 3 & Appendix 2, Figure 4 for additional images).

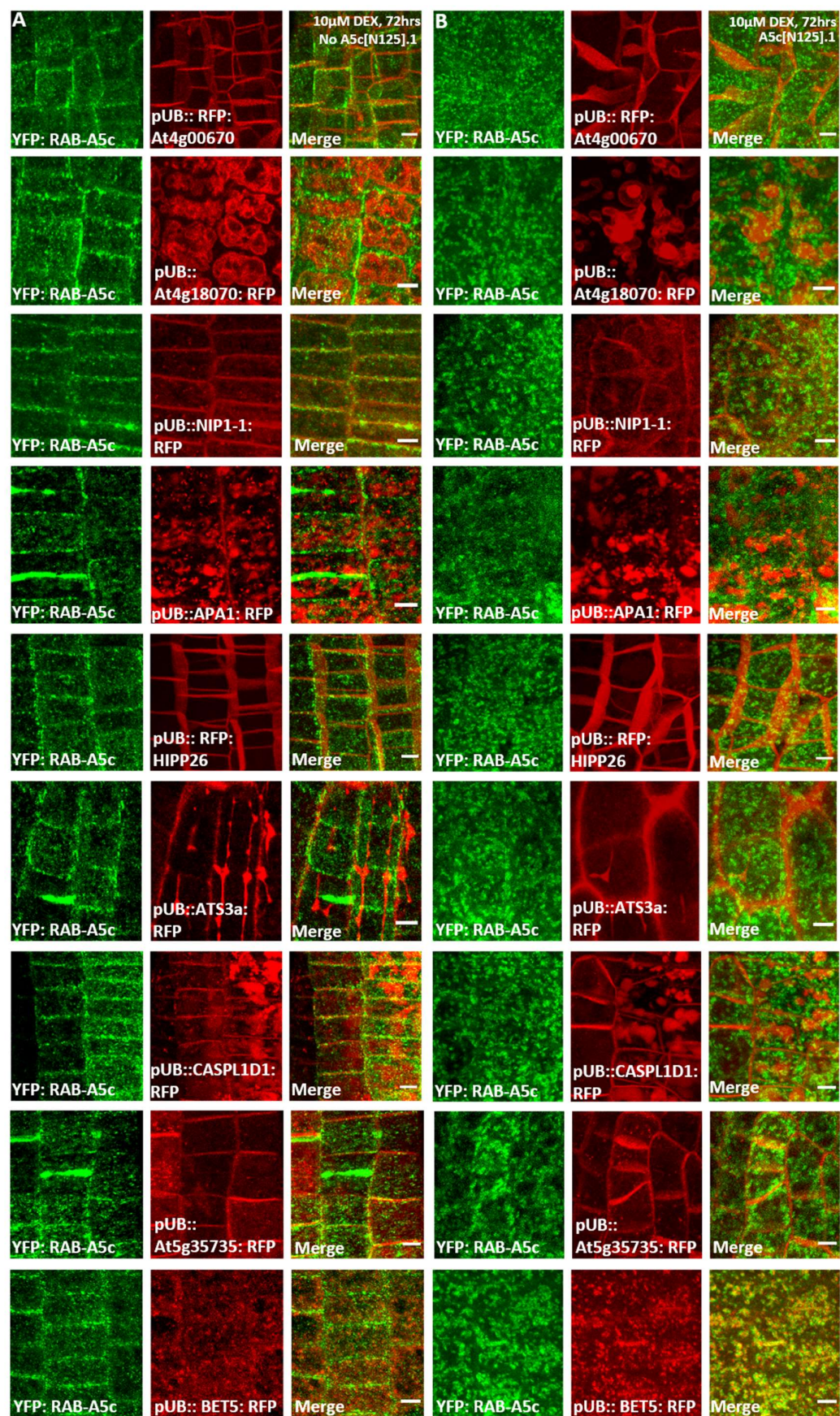


Figure 2.11: Expression of *AtRPS5a>DEX>A5c[N125I].1* does not substantially alter the localisation patterns of initially considered candidate interactors of YFP: RAB-A5c. (A) cropped 3D confocal stack projections of lateral root cells expressing co-expressing YFP: RAB-A5c and candidate interactors. 10µM DEX, 72 hours. (B) cropped 3D confocal stack projections of lateral root cells co-expressing YFP: RAB-A5c, candidate interactors and *AtRPS5a>DEX>A5c[N125I].1*. 10µM DEX, 72 hours. All from 10 day old T2 seedlings. Scale bars 5µm.

2.L: Edge-localisation of RLP4: RFP and RLP4-L: RFP is disrupted by expression of RAB-A5c[N125I]

For candidate interactors that did not localise to cell edge compartments, the strongly-phenotypic *AtRPS5a>DEX>A5c[N125I].1* was used to test for dependence of candidate localisation on RAB-A5c function. *AtRPS5a>DEX>A5c[N125I].1* lines have disrupted YFP: RAB-A5c localisation even in the absence of DEX however, indicating a low-level of RAB-A5c[N125I] expression even in the absence of DEX (Figure 2.10). For candidate interactors of YFP: RAB-A5c identified as localising to cell edge compartments, the alternative *AtRPS5a>DEX>A5c[N125I].2* line (which does not mis-localise YFP: RAB-A5c in the absence of DEX; Figure 2.10) was crossed into backgrounds expressing YFP: RAB-A5c and pUB:: RLP4: RFP or pUB:: RLP4-L: RFP to investigate their dependence on RAB-A5c function. This gave triple transgenic lines co-expressing YFP: RAB-A5c, *AtRPS5a>DEX>A5c[N125I].2* and pUB:: RLP4/4-L: RFP.

In the absence of DEX, both YFP: RAB-A5c and pUB:: RLP4: RFP/pUB:: RLP4-L: RFP remained at cell edge compartments in the *AtRPS5a>DEX>A5c[N125I].2* background supporting the conclusion that the possible auto-expression of RAB-A5c[N125I] in *AtRPS5a>DEX>A5c[N125I].1* is absent or very low in *AtRPS5a>DEX>A5c[N125I].2* lines (Figure 2.10, 2.12 & 2.13). After 72 hours of *AtRPS5a>DEX>A5c[N125I].2* expression, the localisations of YFP: RAB-A5c, pUB:: RLP4: RFP and pUB:: RLP4-L: RFP differed markedly from those of DMSO-treated controls (Figures 2.12 & 2.13). Edge compartment localisation of pUB:: RLP4: RFP and pUB:: RLP4-L: RFP was abolished after 72 hours of *AtRPS5a>DEX>A5c[N125I].2* expression, with their localisation to the PM also noticeably reduced. Instead, intracellular localisation of pUB:: RLP4: RFP and pUB:: RLP4-L: RFP predominated upon

expression of AtRPS5a>DEX>A5c[N125I].2, compared to their dramatic enrichment at cell edges in the controls (Figures 2.12 & 2.13). This removal from the cell edge by expression of AtRPS5a>DEX>A5c[N125I].2 was also reproducible for fluorescent protein fusions of RLP4 and RLP4-L expressed from their endogenous promoters (Appendix 2, Figure 5). YFP: RAB-A5c localisation to cell edge compartments was abolished in favour of localisation to intracellular compartments and the cytosol after 72 hours of AtRPS5a>DEX>A5c[N125I].2 expression similar to the disruption already seen for AtRPS5a>DEX>A5c[N125I].1 expression (Figures 2.12 and 2.13). After 72 hours of AtRPS5a>DEX>A5c[N125I].2 expression, lateral roots did not have the extensive cytokinesis and cell swelling defects produced by the equivalent period of AtRPS5a>DEX>A5c[N125I].1 expression (Kirchhelle et al., 2016), supporting observations that expression of AtRPS5a>DEX>A5c[N125I].2 from germination had a less detrimental effect on growth and development than AtRPS5a>DEX>A5c[N125I].1 expression from germination (Figure 2.10A).

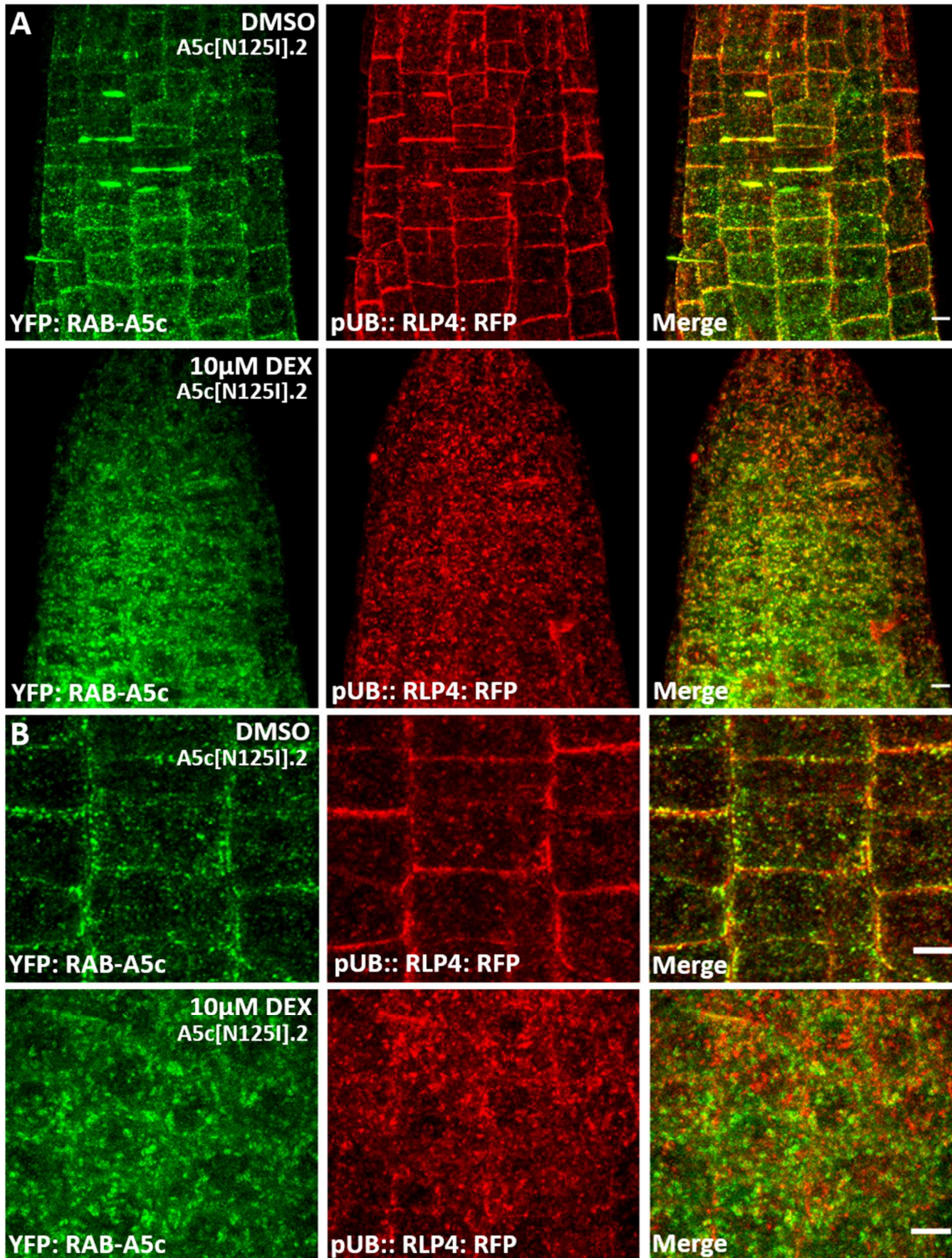


Figure 2.12: Expression of *AtRPS5a*>*DEX*>[N125I].2 disrupts localisation of pUB:: RLP4: RFP in lateral roots. (A) 3D confocal stack projections of lateral roots co-expressing YFP: RAB-A5c (left) and pUB:: RLP4: RFP (middle) and *AtRPS5a*>*DEX*>[N125I].2. (B) higher magnification 3D projection of lateral root meristematic cells. 10μM DEX or equivalent volume DMSO, 72 hours. All from 10 day old F3 seedlings. Scale bars, 5μm.

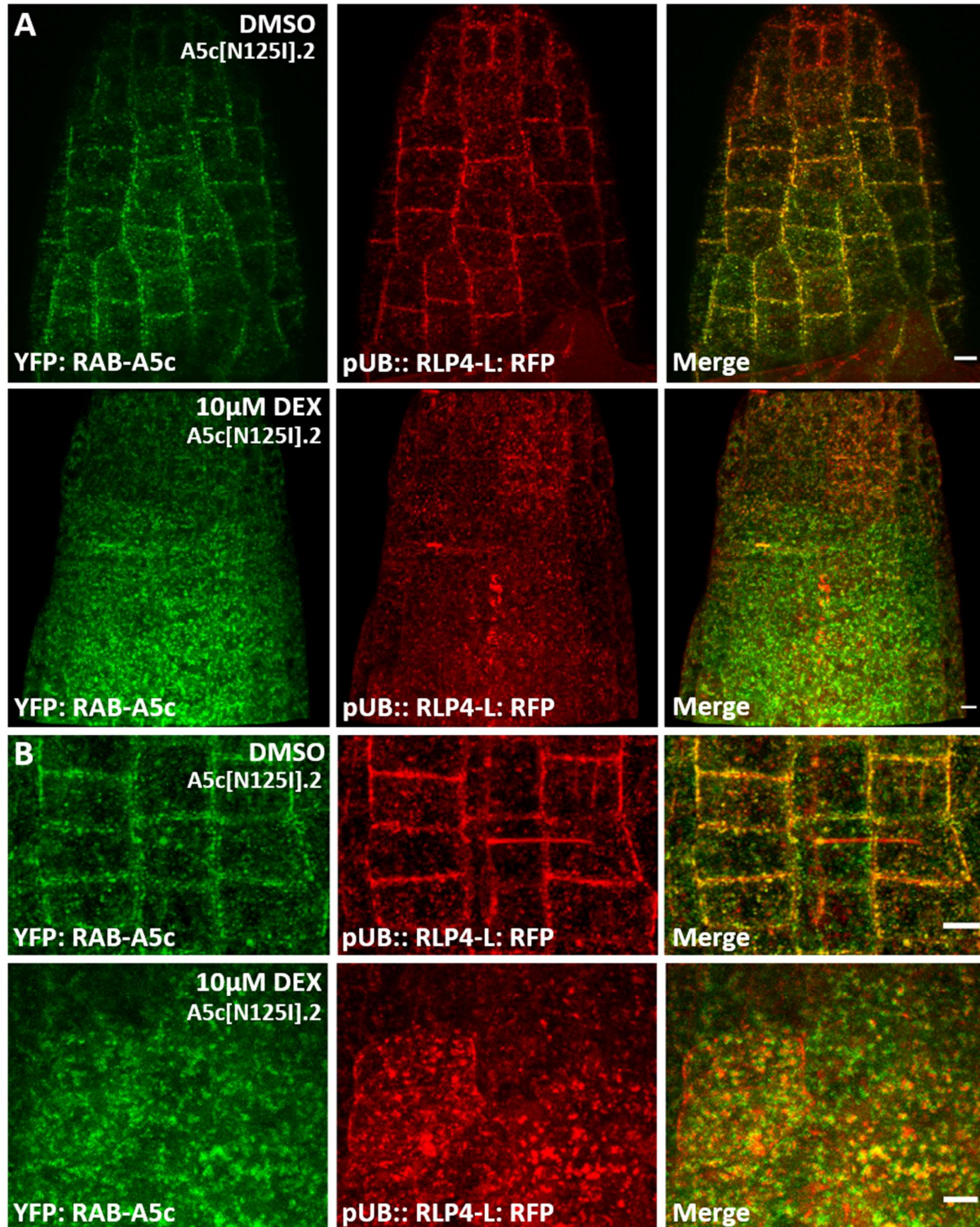


Figure 2.13: Expression of AtRPS5a>DEX>[N125I].2 disrupts localisation of pUB:: RLP4-L: RFP in lateral roots (A) 3D confocal stack projections of lateral roots co-expressing YFP: RAB-A5c (left) and pUB:: RLP4-L: RFP (middle) and AtRPS5a>DEX>[N125I].2. (B) higher magnification 3D projection of lateral root meristematic cells. 10µM DEX or equivalent volume DMSO, 72 hours. All from 10 day old F3 seedlings. Scale bars, 5µm.

2.M: YFP: RAB-A5b localisation is disrupted by expression of RAB-A5c[N125I]

YFP: RAB-A5b also localises to compartments at the geometric edges of cells in *Arabidopsis* lateral roots (Figure 2.2). This supports a scenario in which the unusual localisation to cell geometric edges of RAB-A5c may be a wider feature of the Rab-A5 subclass, possibly requiring shared interaction factors. When co-expressed with AtRPS5a>DEX>A5c[N125I].2, YFP: RAB-A5b localisation to cell edge compartments was abolished after 72 hours and instead shifted to intracellular compartments and the cytosol in a manner similar YFP: RAB-A5c disruption upon RAB-A5c[N125I] expression (Figure 2.14). Disruption of YFP: RAB-A5b localisation supports the assumption that RAB-A5c[N125I] expression also inhibits the function of other possibly edge-localised GTPases, which may be a broad feature of the Rab-A5 subclass, and that known edge-localisation of Rab GTPases is possibly supported by common interaction factors. Whether edge-localisation is also a feature of the remaining three uncharacterised *Arabidopsis* Rab-A5 GTPases remains unknown.

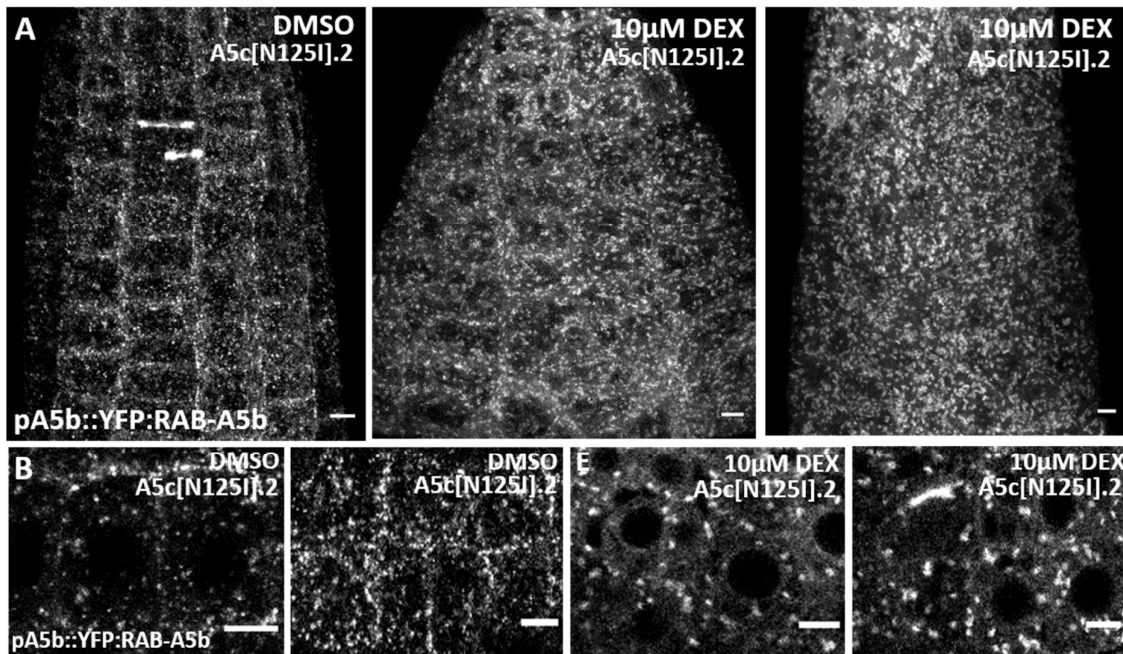


Figure 2.14: Expression of AtRPS5a>DEX>A5c[N125I].2 disrupts pA5b:: YFP: RAB-A5b localisation in lateral roots (A) 3D confocal stack projections of lateral roots co-expressing YFP: RAB-A5b and AtRPS5a>DEX>A5c[N125I].2. (B) higher magnification confocal slices of lateral root meristematic cells. 10µM DEX or equivalent volume DMSO, 72 hours. All from 10 day old T2 seedlings. Scale bars, 5µm.

2.N: Summary & Discussion

Investigation of proteomically-identified candidate interactors of YFP: RAB-A5c has revealed that a substantial proportion of these candidates localise to the cell surface, indicating that comparative ranking against YFP: RAB-A2a and YFP: RAB-G3f proteomes in the original co-immunoprecipitation protocol to reduce contamination from other subcellular regions have been at least partially successful. Whilst the majority of these candidate interactors did not localise to YFP: RAB-A5c-labelled cell edge compartments, disruption of RAB-A5c function through expression of dominant-negative RAB-A5c[N125I] was used to test whether localisation of such candidate interactors could still depend on RAB-A5c function. The lack of disruption of the majority of candidate interactors of YFP: RAB-A5c by RAB-A5c[N125I] expression, and their absence from cell edge compartments, has allowed for significant refinement of candidate interactors likely to be of significant further interest. An exception to this is the *Arabidopsis* TRAPP component AtBET5. Localisation of pUB:: BET5: RFP, despite not labelling YFP: RAB-A5c-labelled cell edge compartments, was possibly disrupted by expression of RAB-A5c[N125I], appearing to be shifted towards intracellular compartments from a predominantly cytosolic localisation pattern in the absence of RAB-A5c[N125I] expression. In *Arabidopsis*, the TRAPP II complex (of which AtBET5 is thought to be a component) has been placed functionally upstream of Rab-A1 and Rab-A2 GTPases (Qi et al 2011, Qi & Zheng, 2011; Kalde et al., 2019). These indications of functional association between Rab-A GTPases and TRAPP components was the predominant reason why AtBET5 was selected as the lowest-ranking candidate interactor of YFP: RAB-A5c for further investigation. This possible disruption of pUB:: BET5: RFP localisation by RAB-A5c[N125I] expression highlights possible functional interactions between *Arabidopsis* TRAPP complexes and other Rab-A GTPase subclasses and merits further investigation.

Proteomic identification and subsequent investigation of candidate YFP: RAB-A5c interactors has also identified three additional proteins capable of localisation to cell edge compartments. The first of these was the Rab-A5 GTPase RAB-A5b. YFP: RAB-A5b also localises to compartments at cell

geometric edges, indicating that cell edge localisation may be a broader property of Rab-A5 subclass members. The observation that a second member of the Rab-A5 subclass of Rab-A GTPases also localises to cell edge compartments, as well as the fact that RLP4: YFP and RLP4-L: YFP continue to label cell edge compartments and the PM in a *rab-a5b rab-a5bc* background, supports previous speculation that the lack of disruption to cytokinesis, anisotropic growth and cell geometry in knockout *rab-a5* SAIL mutants may be due to redundancy within the Rab-A5 subclass (Kirchhelle et al., 2016; Kirchhelle, 2016. DPhil Thesis). Moreover, disruption of YFP: RAB-A5b localisation by expression of dominant-negative RAB-A5c[N125I] supports a scenario in which detrimental effects of RAB-A5c[N125I] on cell growth and geometry are a result of broader functional inhibition of the Rab-A5 subclass, and indicates that Rab-A5 GTPase function may be supported by common regulatory interaction factors.

In addition to RAB-A5c's close relative RAB-A5b, the Receptor-Like Protein RLP4 and its closest relative RLP4-L also localise to cell edge compartments. RLP4: RFP and RLP4-L: RFP co-localise with YFP: RAB-A5c at cell edge compartments at both transverse and longitudinal cell edges of lateral root meristematic cells, and also label the PM. Localisation of fluorescent protein fusions of RLP4 and RLP4-L to cell edge compartments is independent of fluorescent protein variant, fluorescent protein tag positioning and also occurs when fluorescently-tagged RLP4 and RLP4-L are expressed from their endogenous promoter sequences. Previous work has provided evidence that RAB-A5c defines an edge-directed trafficking pathway from the TGN/EE to the cell surface, and that such a trafficking pathway may provide a route for local modification of the cell wall around cell edges (Kirchhelle et al., 2016, 2019). Localisation of fluorescently-tagged RLP4 and RLP4-L to the TGN/EE, cell edge compartments and the PM, and disruption of their cell surface localisation by RAB-A5c[N125I] expression now provides firm evidence of an edge-directed trafficking pathway within *Arabidopsis* lateral roots, of which RLP4 and RLP4-L are the first known non-GTPase components.

Previously, the lack of definitive demonstration of an edge-directed trafficking pathway in growing tissues and the absence of known cargoes of this pathway limited investigations into the organisation and function of RAB-A5c and its associated trafficking pathway. The identification of RLP4 and RLP4-L provides an exciting opportunity for further investigation of both the organisation and possible functions of edge-directed trafficking in the maintenance of anisotropic growth and cell geometry in growing tissues of *Arabidopsis*.

Chapter 3: An image analysis pipeline quantitatively identifies RLP4:

RFP and RLP4-L: RFP as polarised within the PM of *Arabidopsis* lateral root epidermal cells

3.A: Introduction

As the first identified non-GTPase components of edge-directed trafficking, RLP4 and RLP4-L demonstrate that edge-directed trafficking is a novel route to the PM in lateral root epidermal cells of *Arabidopsis*. The striking localisation of fluorescently-tagged RAB-A5c, RLP4 and RLP4-L to cell edge compartments and fluorescently-tagged RLP4 and RLP4-L to the PM indicates this trafficking pathway may be a polarised route to the cell surface. Polarity of proteins within the PM of *Arabidopsis* root epidermal cells is well-described and is broadly split into apically-, basally- and laterally-polarised proteins. Polarisation in the apical-basal axis is well-described for auxin transporters, whose asymmetric distribution establishes and maintains tissue-wide auxin gradients essential for plant patterning and development (reviewed by Luo et al., 2018; Zhou & Luo, 2018). Polarity in the outer (soil-side) and inner lateral axis often involves proteins functionally related to interaction with the external environment, such as nutrient transporters and pathogen-response proteins (reviewed by Nakamura & Grebe, 2018). Some of the trafficking pathways supporting polarisation in the *Arabidopsis* PM are beginning to be understood in molecular detail and may involve Rab GTPases, or their effectors (Fendrych et al., 2013; Mao et al., 2016; Li et al., 2017). Whilst polarisation to cell edges has been described for membrane compartments and cytoskeleton organisers (Ambrose et al., 2011, Ambrose & Wasteneys, 2011; Kirchhelle et al., 2016; reviewed by Elliott & Kirchhelle, 2019), polarisation to cell edges within the PM has not been demonstrated. The localisation of RLP4: RFP and RLP4-L: RFP to cell edge compartments raises the question of whether these proteins are also edge-polarised within the PM. Initial imaging of fluorescently-tagged RLP4 and RLP4-L in lateral root epidermal cells indicated that neither of these proteins uniformly labelled

the cell surface. Polarisation of other proteins within the PM of *Arabidopsis* root epidermal cells is strongly connected to their function, and so characterisation of any polarised distribution of fluorescently-tagged RLP4 and RLP4-L in the PM may be of particular relevance to any future functional characterisations of RLP4 and RLP4-L. I therefore quantitatively investigated the distribution of pUB::RLP4:RFP and pUB::RLP4-L:RFP within the PM of *Arabidopsis* lateral root epidermal cells.

3.B: Developing a method for quantifying pUB:: RLP4: RFP and pUB:: RLP4-L: RFP distribution in the PM

In order to quantitatively analyse the distributions of pUB:: RLP4: RFP and pUB:: RLP4-L: RFP in the PM of *Arabidopsis* lateral root epidermal cells, I first crossed plants expressing pUB:: RLP4: RFP and pUB:: RLP4-L: RFP with plants expressing YFP: NPSN12 from the UBQ10 promoter (Geldner et al., 2009), which is uniformly distributed across the PM of *Arabidopsis* root and lateral root cells (Geldner et al., 2009; Kirchhelle et al., 2016, 2019). YFP: NPSN12 was selected for use both to compare pUB:: RLP4: RFP and pUB:: RLP4-L: RFP distribution in the PM to, and as a proxy for PM position during quantification. When compared to YFP: NPSN12 in both 3D and orthogonal projections, both pUB:: RLP4: RFP and pUB:: RLP4-L: RFP were clearly unevenly distributed in the PM of *Arabidopsis* lateral root epidermal cells. YFP: NPSN12 is evenly spread across the PM in all directions with increased depth from the outer surface of lateral root epidermal cells. pUB:: RLP4: RFP and pUB:: RLP4-L: RFP are strikingly different to this, appearing to attenuate with increased distance from the outer surface, indicating that pUB:: RLP4: RFP and pUB:: RLP4-L: RFP are polarised towards the outer lateral surface of *Arabidopsis* lateral root epidermal cells (Figure 3.1A, B & C, yellow arrows). The identification of pUB:: RLP4: RFP and pUB:: RLP4-L: RFP as components of an edge-directed trafficking pathway to the cell surface also indicates a scenario in which RLP4 and RLP4-L are deposited at the PM via highly spatially-restricted cell edge compartments. It is possible therefore that RLP4 and RLP4-L are edge-polarised within the PM itself as a consequence of spatially-restricted delivery to the PM via cell edges. If this is the case, pUB:: RLP4: RFP and pUB:: RLP4-L: RFP intensity at the outer surface of lateral root epidermal cells should attenuate relative to YFP: NPSN12 with increased distance from cell edges. This was difficult to determine visually due to the thinness of the outer lateral surface however, orthogonal projections indicated that pUB:: RLP4: RFP and pUB:: RLP4-L: RFP intensity at the outer lateral surface was strong at crosswalls (Figure 3.1B, blue arrows) but weak and uneven away from edges compared to YFP: NPSN12 (Figure 3.1B, white arrows). I therefore developed a method to quantify the distribution of pUB:: RLP4: RFP and pUB::

RLP4-L: RFP at the outer lateral surface of *Arabidopsis* lateral root epidermal cells relative to YFP:

NPSN12 at different distances from cell edges.

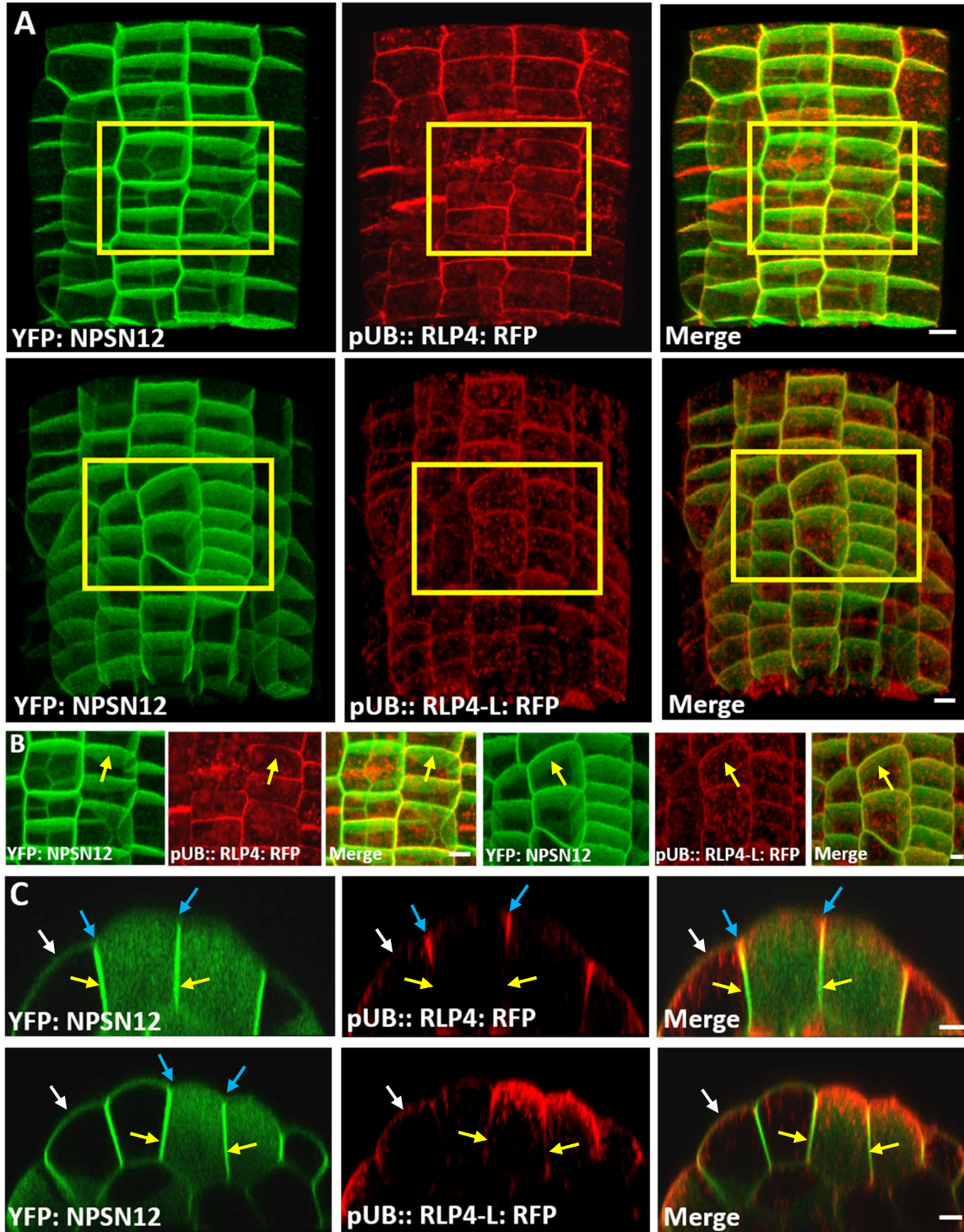


Figure 3.1: pUB:: RLP4: RFP and pUB:: RLP4-L: RFP are unevenly distributed at the PM compared to YFP: NPSN12. (A) 3D projections of Arabidopsis lateral roots co-expressing YFP: NPSN12 (left) and pUB:: RLP4: RFP (middle, top) or pUB:: RLP4-L: RFP (middle, bottom). (B) Higher magnifications of yellow boxed regions in (A). (C) Orthogonal projection of YFP: NPSN12 (left) and pUB:: RLP4: RFP (middle, top) or pUB:: RLP4-L: RFP (middle, bottom). White arrows = outer lateral surface. Blue arrows = crosswalls at surface. Yellow arrows= crosswalls at depth. All from 10 day old F3 seedlings. Scale bars, 5μm.

In order to investigate pUB:: RLP4: RFP and pUB:: RLP4-L: RFP distribution at the PM compared to uniformly-distributed YFP: NPSN12, I used MorphographX (Barbier de Reuille et al., 2015) to develop a pipeline for segmentation of individual cells in lines co-expressing YFP: NPSN12 and pUB:: RLP4/RLP4-L: RFP and subsequent quantitative analysis of pUB:: RLP4: RFP and pUB:: RLP4-L: RFP intensity relative to YFP:NPSN12 intensity in different regions of the PM.

It was first necessary to prepare YFP: NPSN12, RLP4: RFP and RLP4-L: RFP image channels for quantification. YFP: NPSN12 is exclusively localised to the PM (Geldner et al., 2009; Kirchhelle et al., 2016, 2019) in *Arabidopsis* roots and lateral roots however, both pUB:: RLP4: RFP and pUB:: RLP4-L: RFP localise intracellularly to the TGN/EE and cell edge compartments. Quantification of pUB:: RLP4: RFP and pUB:: RLP4-L: RFP distribution within the PM required the removal of these compartments from the images in order to avoid any contribution of compartments positioned close to the cell surface to quantification of pUB:: RLP4: RFP and pUB:: RLP4-L: RFP distribution in the PM. In order to remove compartments labelled by pUB:: RLP4: RFP and pUB:: RLP4-L: RFP from images, cell volumes of Gaussian-blurred YFP: NPSN12 images were segmented using a 3D auto-seeded watershed algorithm, and a 3D mesh was generated based on the segmented voxel data using a Marching Cube Surface function (Figure 3.2A). This 3D mesh was then shrunk in all directions by both 0.25µm and 0.5µm (Figure 3.2B). The 0.25µm shrunk mesh or 0.5µm shrunk mesh were then subtracted from the RLP4/RLP4-L: RFP channel (Figure 3.2C). This removed all intracellular signal more than 0.25µm or 0.5µm away from the PM (Figure 3.3). These 0.25µm and 0.5µm values were chosen to match values used in 2D finite element models by Kirchhelle and colleagues, based on an average distance of cell edge compartments from the PM of 0.5µm seen in electron micrographs (Kirchhelle et al., 2016). 0.25µm shrunk meshes are therefore intended to be a more stringent method for compartment removal, and 0.5µm shrunk meshes a less stringent method. For details of parameters used in tools for generating the 3D watersheds, see Chapter 7 (Materials & Methods).

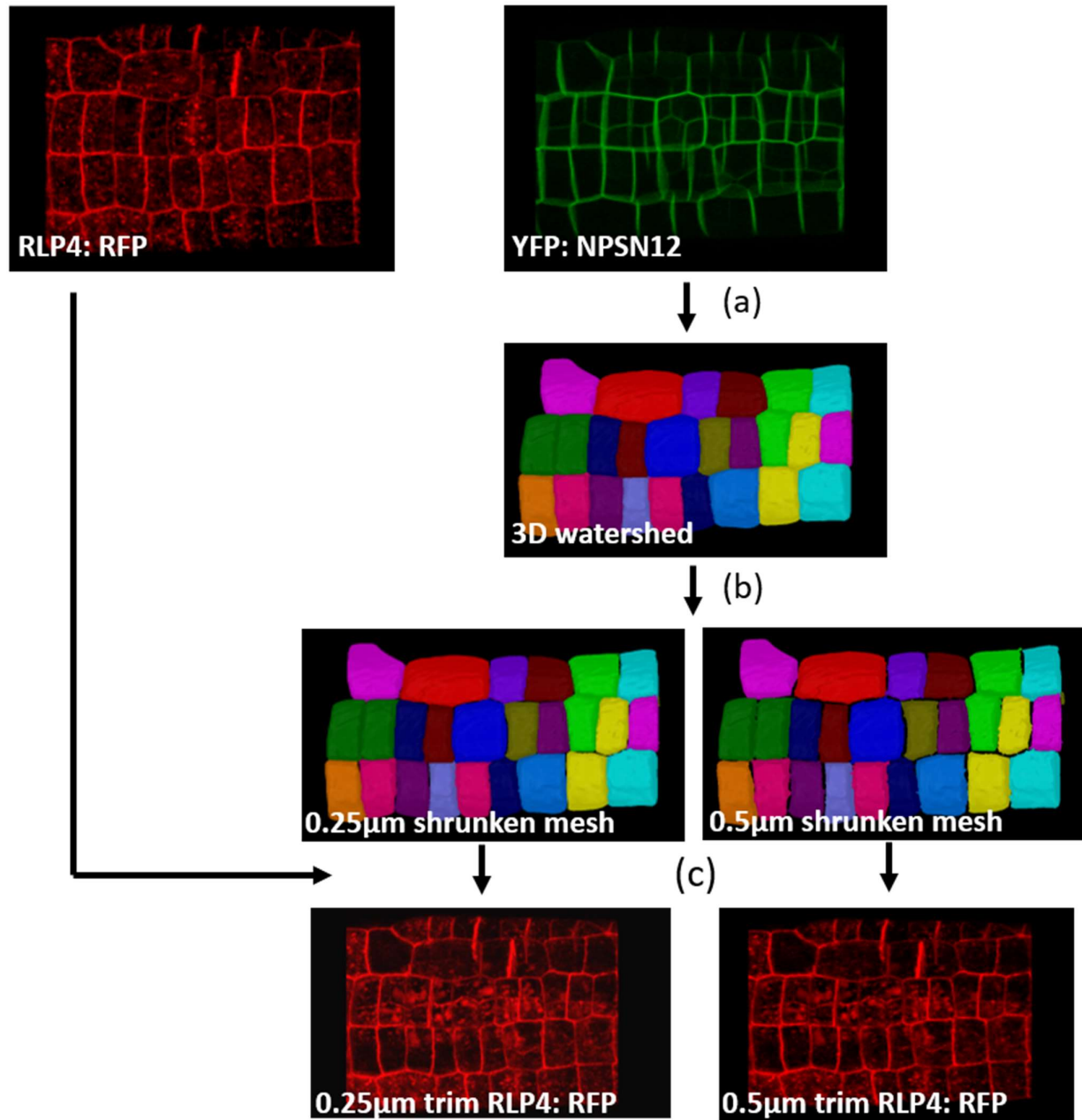


Figure 3.2: Pipeline for removing intracellular compartments from RLP4: RFP (and RLP4-L: RFP) images. (a) YFP: NPSN12 images are Gaussian blurred and 3D watershed used to outline cell volumes. Cell volumes below the organ surface and cells not entirely captured by the image were removed manually (b) 3D cell volumes were converted to a 3D mesh and this mesh shrunk in all directions by 0.25µm and 0.5µm respectively. (c) 0.25µm and 0.5µm shrunken 3D meshes were then subtracted from RLP4/4-L: RFP images to remove intracellular signal.

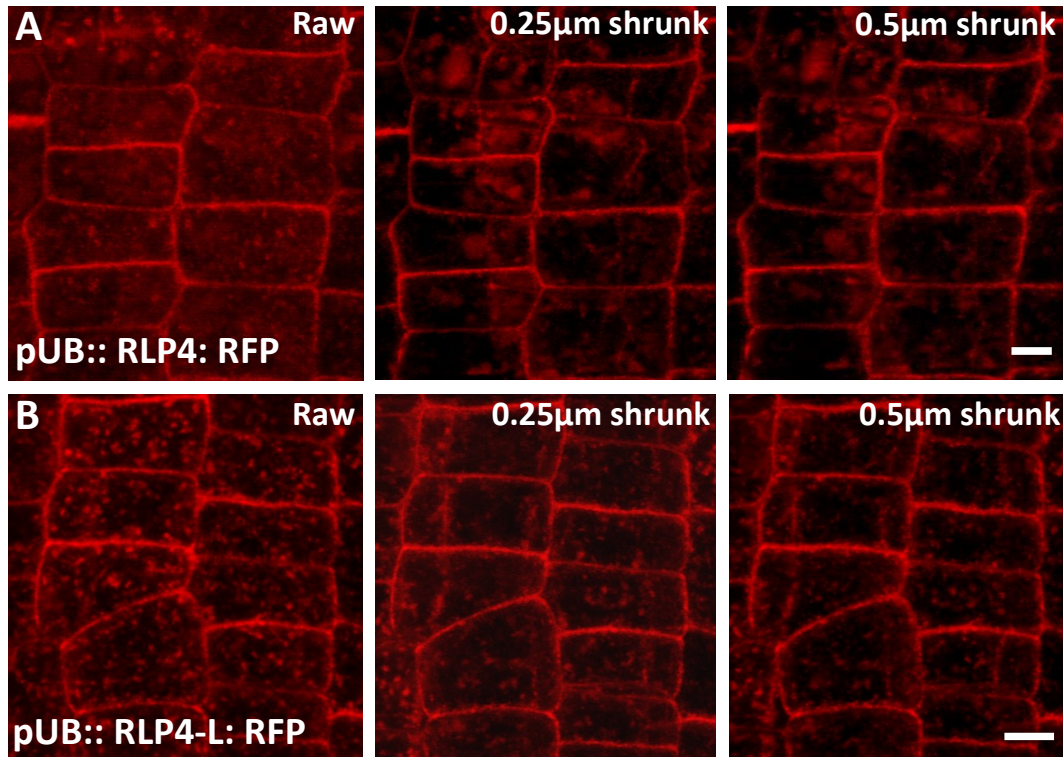


Figure 3.3: Removal of intracellular compartments from RLP4: RFP and RLP4-L: RFP using 3D shrunk meshes. (A) 3D projections of lateral root meristematic cells expressing pUB:: RLP4: RFP with raw (left), 0.25 μ m shrunk (middle) and 0.5 μ m shrunk (right) images. (B) pUB:: RLP4-L: RFP. All from 10 day old F3 seedlings. Scale bars, 5 μ m.

It was then necessary to use YFP: NPSN12 to produce a 2D mesh delineating the surface of the imaged lateral root cells. A 2D mesh was used instead of a 3D mesh as the former allows annotation and numerical identification of cell edges as reference points during the analysis. A Gaussian blur filter was applied to YFP: NPSN12 images, and Edge Detect and Marching Cube Surface functions used to produce a 2D surface outline of the lateral root (Figure 3.4A). This was projected onto the YFP: NPSN12 root and cell surface outline and optimally fitted to the surface through manual adjustment of edge detection parameters (Figure 3.4B). Watershedding was then used to numerically identify individual cells and cell outlines (Figure 3.4C). Identities of cells that were not entirely captured in the image or for which the 2D surface did not sufficiently follow the actual curvature of the root were merged to avoid inaccurate annotation of cell edges (Figure 3.4C, yellow arrows), and imperfect cell outlines on the 2D mesh adjusted manually. For details of parameters used in tools for generating the 2D mesh, see Chapter 7 (Materials & Methods).

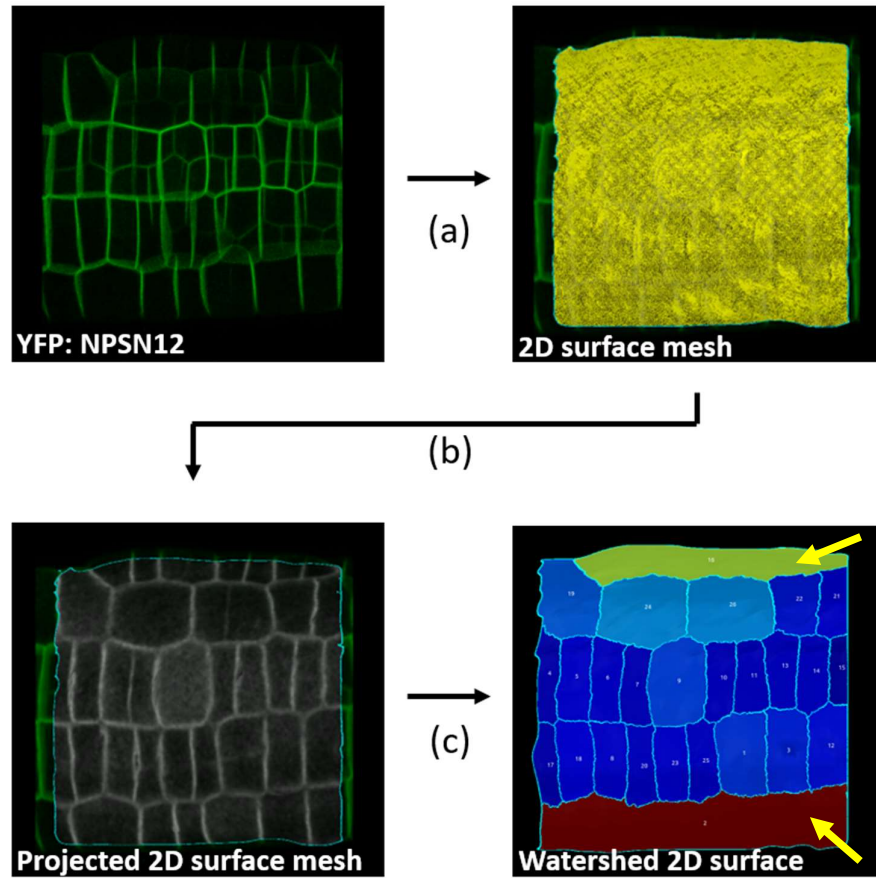


Figure 3.4: Pipeline for generating a 2D surface mesh based on YFP: NPSN12 cell outlines
(a) YFP: NPSN12 images are Gaussian blurred and a 2D surface mesh generated using edge detect and marching cube surface functions. This mesh was refined using smooth and subdivide functions (b) Cell outlines were projected onto the 2D surface mesh, which was then watersheded give individual cell and edge identities. (c) Cells not entirely captured on the image or where the 2D surface did not accurately follow were merged (yellow arrows).

3.C: pUB:: RLP4: RFP and pUB:: RLP4-L: RFP define edge-restricted domains within the PM

In order to investigate the possibility that pUB:: RLP4: RFP and pUB:: RLP4-L: RFP are enriched around cell edges compared to the adjacent cell face at the outer lateral surface of *Arabidopsis* lateral root epidermal cells, pUB:: RLP4: RFP and pUB:: RLP4-L: RFP distribution at the cell surface were quantified relative to that of YFP: NPSN12. Using a 2D surface mesh and trimmed RLP4/4-L: RFP images (as described above), absolute YFP: NPSN12 intensity and absolute pUB:: RLP4/4-L: RFP

intensity at the outer lateral PM surface were measured in regions 0.5µm , 1µm, 1.5µm and 2µm away from the adjacent cell edges respectively (Figure 3.4). YFP: NPSN12 and pUB:: RLP4/4-L: RFP intensities per 0-0.5µm, 0.5-1µm, 1 -1.5µm and 1.5 -2µm border area were then calculated and pUB:: RLP4/4-L: RFP intensity normalised against that of YFP: NPSN12 for each border area. Each value for 0.5-1µm, 1 -1.5µm and 1.5 -2µm border areas was then normalised against the equivalent value for the 0-0.5µm border area to remove variation in absolute RFP intensity (populations segregating for pUB:: RLP4: RFP and pUB:: RLP4-L: RFP were used).

In 0.25µm trimmed images, both pUB:: RLP4: RFP and pUB:: RLP4-L: RFP significantly attenuated relative to YFP: NPSN12 with increased distance from cell edges at the outer lateral surface of *Arabidopsis* root epidermal cells (Figure 3.5A). Interestingly, pUB:: RLP4: RFP attenuated to a significantly greater degree relative to YFP: NPSN12 than pUB:: RLP4-L: RFP at all distances away from the cell edge (Figure 3.5A). In 0.5µm trimmed images, attenuation of pUB:: RLP4: RFP relative to YFP: NPSN12 was slightly reduced compared to 0.25µm trimmed images however, attenuation of pUB:: RLP4-L: RFP relative to YFP: NPSN12 was abolished in 0.5µm trimmed images (Figure 3.5B). As already noted, pUB:: RLP4-L: RFP visibly localised to intracellular compartments to a greater degree than pUB:: RLP4: RFP (Figure 3.1 & 3.3), indicating that a 0.5µm trim is insufficient to entirely remove these intracellular compartments, at least in the case of pUB:: RLP4-L: RFP. I therefore decided to use only 0.25µm trimmed images for further analyses. In 0.25µm trimmed images, pUB:: RLP4: RFP and pUB:: RLP4-L: RFP are therefore significantly enriched closer to cell geometric edges at the outer lateral surface of *Arabidopsis* lateral root epidermal cells, albeit to differing degrees.

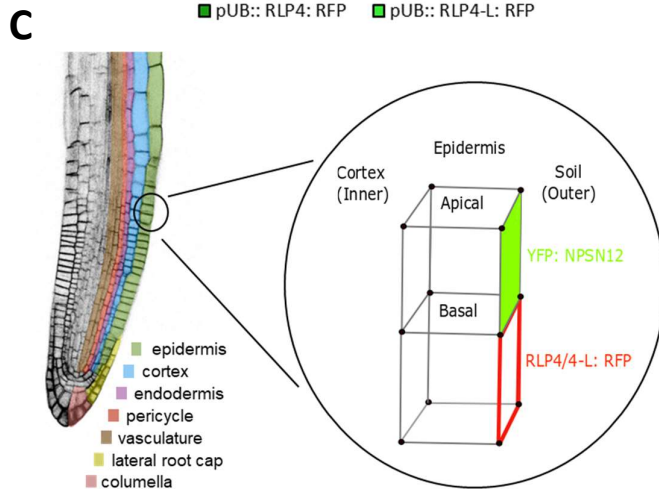
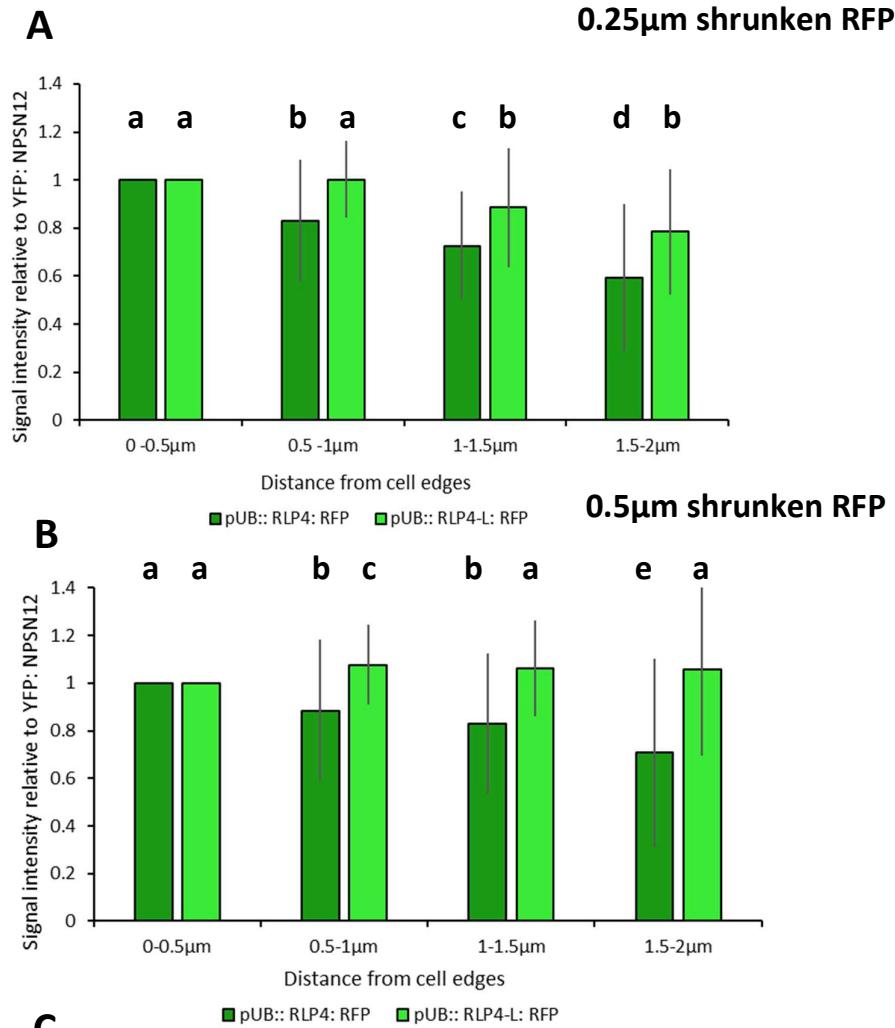


Figure 3.5: RLP4: RFP and RLP4-L: RFP attenuate relative to YFP: NPSN12 with increased distance from cell edges at the outer lateral surface of *Arabidopsis* lateral root epidermal cells. (A) RLP4/4-L: RFP intensity relative to YFP: NPSN12 at 0.5-1 μ m, 1-1.5 μ m and 1.5-2 μ m from cell edges relative to the value at 0-0.5 μ m from the cell edge. 0.25 μ m trimmed RFP images. (B) 0.5 μ m trimmed RFP images. Bars = Standard Deviation. N = over 75 cells each. Two-way ANOVA and Tukey post-hoc Test: same letter indicates no significant difference ($p > 0.01$), different letter indicates a significant difference ($p < 0.01$). (C) Schematic representation of RLP4/4-L: RFP edge-enrichment in lateral root epidermal cells. Adapted from Elliott & Kirchhelle, 2019.

3.D: pUB:: RLP4: RFP and pUB:: RLP4-L: RFP are polarised to the outer lateral surface of lateral root epidermal cells

Initial imaging of pUB:: RLP4: RFP and pUB:: RLP4-L: RFP indicated that the PM-localised proportion of both of these proteins attenuate with increased depth from the outer lateral surface (Figure 3.3). YFP: RAB-A5c is significantly enriched at outer surface sections of *Arabidopsis* lateral root meristematic cells compared to medial sections (Kirchhelle et al., 2016), supporting the possibility that edge-directed trafficking components RLP4 and RLP4-L are also polarised in a similar manner at the cell surface. In order to investigate possible polarisation of fluorescently-tagged RLP4 and RLP4-L in the outer-inner lateral axis, pUB:: RLP4: RFP and pUB:: RLP4-L: RFP intensities relative to that of YFP: NPSN12 were quantified at different depths from the outer lateral surface. 2D meshes generated for YFP: NPSN12 images were projected at depths of 0-2µm, 2-4µm and 4-6µm from the outer lateral surface. YFP: NPSN12 and pUB:: RLP4/4-L: RFP intensity at a 0-0.5µm border width from cell edges at each depth were measured and pUB:: RLP4/4-L: RFP intensity normalised against YFP: NPSN12 intensity. Each value for 2-4µm and 4- 6µm was then normalised against the equivalent 0- 2µm surface value. Both pUB:: RLP4: RFP and pUB:: RLP4-L: RFP attenuated significantly relative to YFP: NPSN12, to similar degrees, with increased distance from the outer lateral surface in 0.25µm trimmed images (Figure 3.6). pUB:: RLP4: RFP and pUB:: RLP4-L: RFP are therefore polarised towards the outer lateral surface of *Arabidopsis* lateral root epidermal cells, presumably reflecting delivery from YFP: RAB-A5c labelled cell edge compartments, which are enriched at outer lateral sections compared to medial sections of lateral root epidermal cells (Kirchhelle et al., 2016).

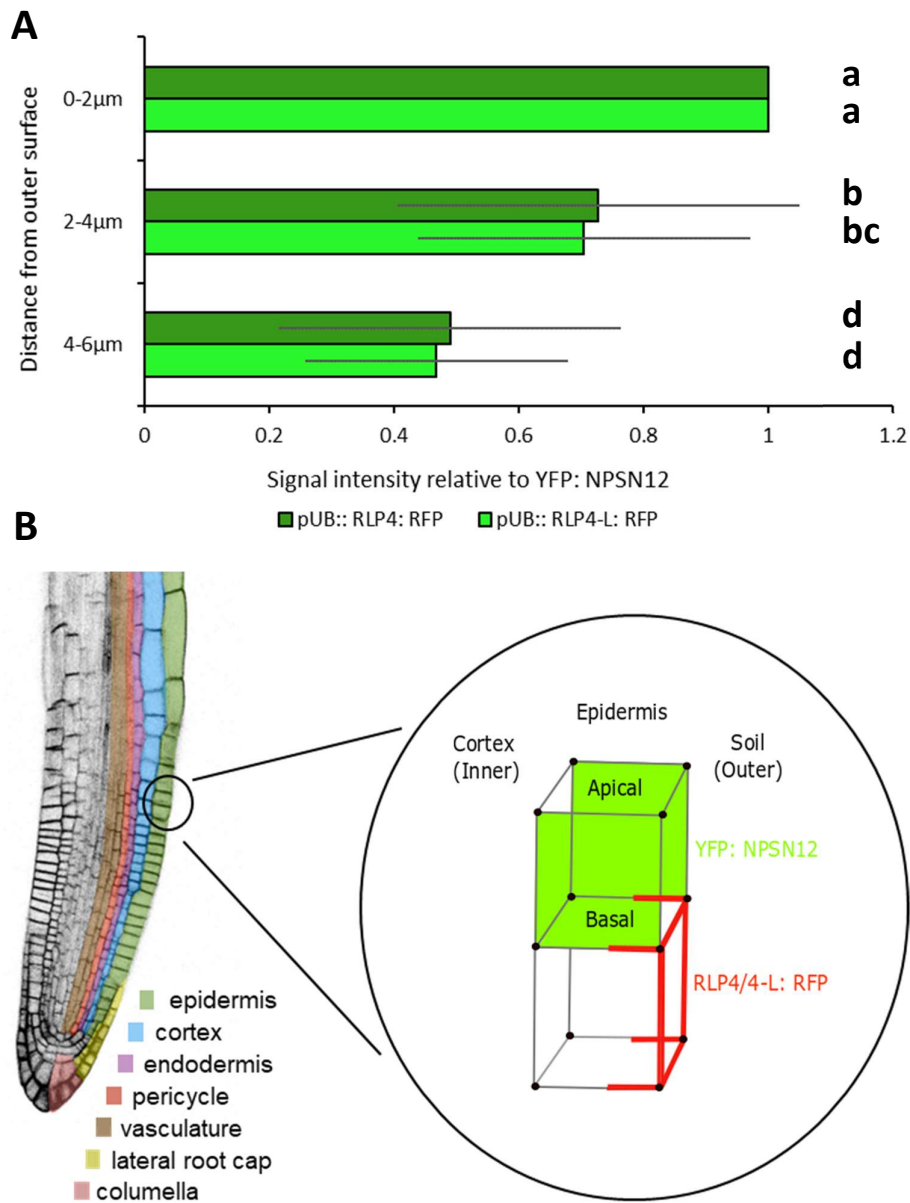


Figure 3.6: RLP4: RFP and RLP4-L: RFP attenuate relative to YFP: NPSN12 with increased distance from the outer lateral surface of *Arabidopsis* lateral root epidermal cells. (A) RLP4: RFP and RLP4-L: RFP intensity relative to YFP: NPSN12 at 2-4µm and 4-6µm from the outer lateral surface, relative to intensity 0-2µm from the outer lateral surface. 0.25µm shrunk RFP images. Bars = Standard Deviation. N = over 75 cells each. Two-way ANOVA and Tukey post-hoc Test: same letter indicates no significant difference ($p > 0.01$), different letter indicates a significant difference ($p < 0.01$). (B) Schematic representation of RLP4/4-L: RFP polarisation in the Inner-Outer axis of lateral root epidermal cells. Adapted from Elliott & Kirchhelle, 2019.

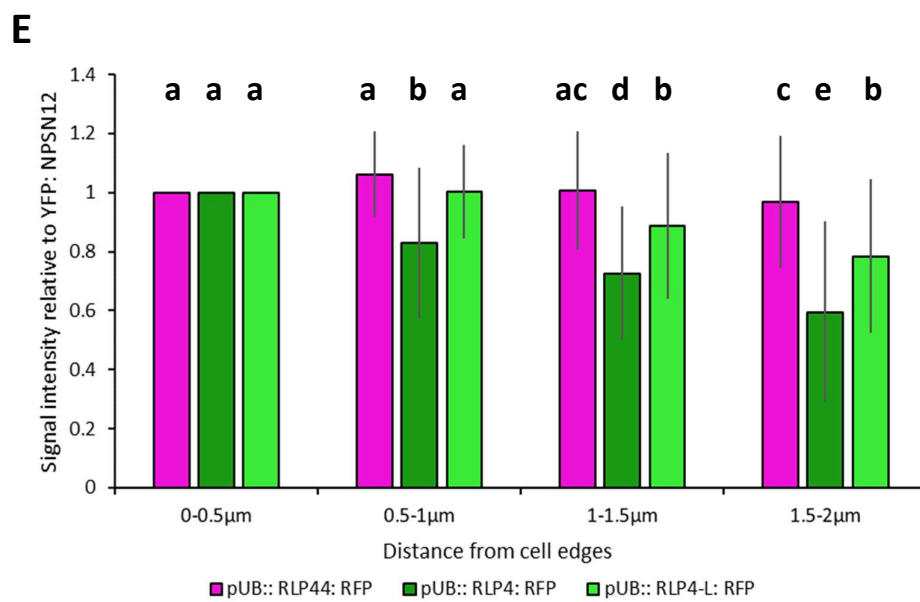
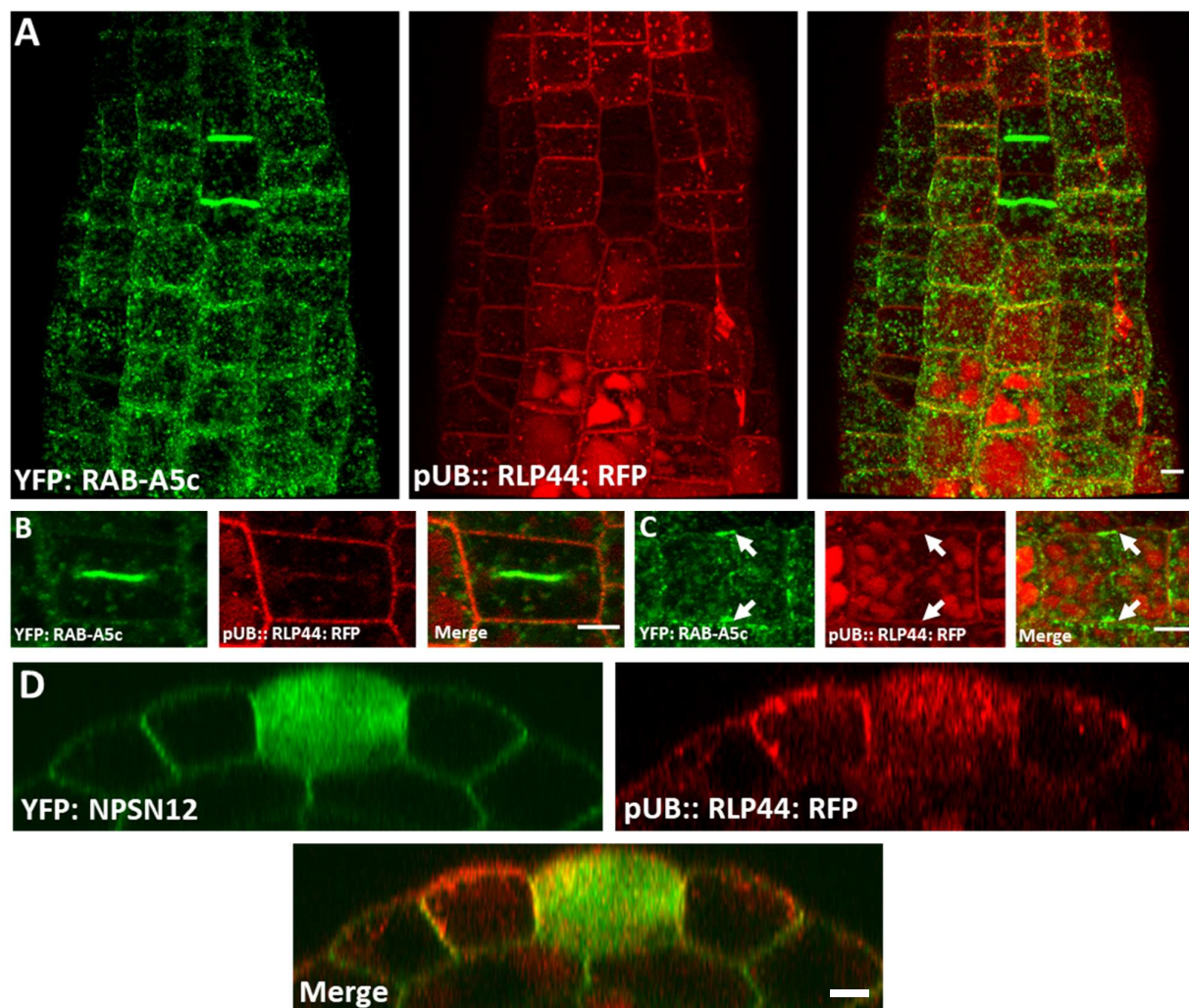
3.E: pUB:: RLP44: RFP distribution in the PM of lateral root epidermal cells is different from that of

pUB:: RLP4: RFP and pUB:: RLP4-L: RFP

Quantification of the polarisation of pUB:: RLP4: RFP and pUB:: RLP4-L: RFP in the PM of *Arabidopsis* lateral root epidermal cells has shown that these proteins are asymmetrically distributed and are polarised towards the outer lateral surface, where they are edge-enriched. It is unclear however, how unique this polarisation pattern is within the diversity of *Arabidopsis* RLPs and RLKs, as these receptor families are seldom characterised through such an approach. I therefore decided to investigate the polarisation of another *Arabidopsis* RLP in order to preliminarily assess how unique the polarised PM distribution of pUB:: RLP4: RFP and pUB:: RLP4-L: RFP in *Arabidopsis* lateral root epidermal cells may be.

RAB-A5c and its associated trafficking pathway have been proposed to function in local modification of the cell wall via cell edges (Kirchhelle et al., 2016, 2019) and it is possible that edge-directed trafficking cargoes RLP4 and RLP4-L support such a function. In order to compare the distribution of the possibly cell wall-functioning RLP4 and RLP4-L with another RLP, I selected RLP44 for further investigation. RLP44 contains a similar number of LRR regions to RLP4 and RLP4-L (four compared to RLP4's three) and, also like RLP4 and RLP4-L, does not contain an LRR-separating island domain (Wang et al., 2008). RLP44 does not however contain an extracellular MLD and is consequently smaller than RLP4 and RLP4-L. It is also not the most closely related RLP to RLP4/RLP4-L (Wang et al., 2008) but was selected largely due to its known functions in plant development, including as a component of brassinosteroid-mediated cell wall perturbation sensing, as well as the fact that some of the factors controlling its functional and subcellular distribution are beginning to be characterised (Wolf et al., 2014; Holzward et al., 2018; Gómez et al., 2019). RLP44 has previously been reported to localise to the PM and intracellular compartments in primary root meristematic cells (Wolf et al., 2014). In order to first confirm that RLP44 does not label cell edge compartments, I introduced the full-length RLP44 sequence into the pUB-DEST vector pUBC-RFP-DEST (Grefen et al., 2010) to

generate pUB:: RLP44: RFP and transformed this into plants expressing YFP: RAB-A5c or YFP: NPSN12. In lateral roots, pUB:: RLP44: RFP did not co-localise with YFP: RAB-A5c at cell edge compartments or at the edges of the PPB, instead labelling the PM and intracellular compartments in a manner similar to that reported for RLP44: GFP localisation in primary root (Figure 3.7A, B & C; Wolf et al., 2014). Quantitative analysis confirmed that pUB:: RLP44: RFP attenuated with increased distance from cell edges relative YFP: NPSN12 at the outer lateral surface to a significantly lesser degree than either pUB:: RLP4: RFP or pUB:: RLP4-L: RFP, and is therefore comparatively uniformly distributed across the cell surface (Figure 3.7D & E). Co-expression of pUB:: RLP44: RFP and YFP: NPSN12 also indicated that pUB:: RLP44: RFP did not appear to attenuate substantially with increased distance away from the outer surface. Whilst pUB:: RLP44: RFP did attenuate relative to YFP: NPSN12 with increased distance from the outer lateral surface, it did so to a significantly lesser degree than either pUB:: RLP4: RFP or pUB:: RLP4-L: RFP between 0-2 μ m and 4-6 μ m from the outer lateral surface. (Figure 3.7D & F). Quantitative analysis of pUB:: RLP44: RFP distribution therefore indicate that cell wall-functioning RLP44 is uniformly distributed across the cell surface compared to pUB:: RLP4: RFP and pUB:: RLP4-L: RFP.



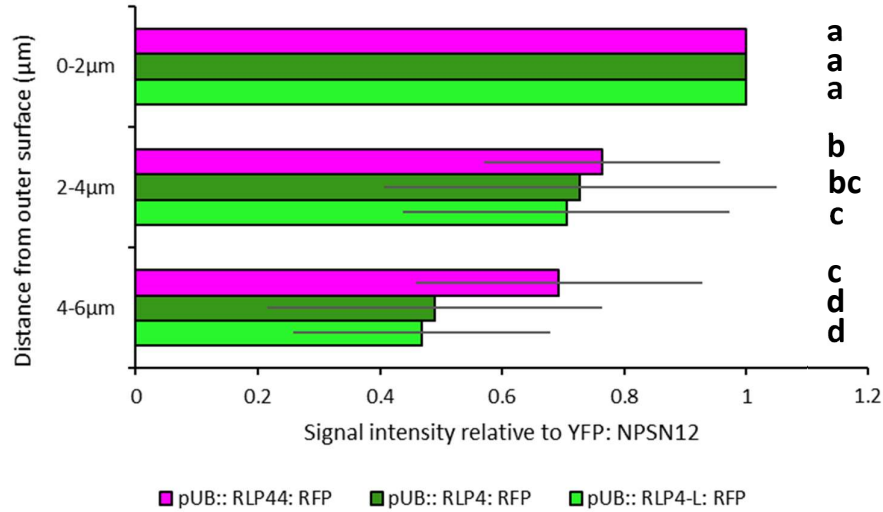
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Figure 3.7: RLP44: RFP has a different subcellular localisation pattern to RLP4: RFP and RLP4-L: RFP.

(A) 3D projection of lateral root co-expressing YFP: RAB-A5c (left) and pUB:: RLP44: RFP (middle). (B,C) confocal slices of lateral root cells during cytokinesis (B) and pre-prophase (C). (D) orthogonal projection of lateral root meristematic cells. All from 10 day old T2 seedlings. Scale bars, 5µm. (E) RLP4: RFP, RLP4-L: RFP (duplicate of data from Chapter 3, Figure 3.5A) and RLP44: RFP intensity relative to YFP: NPSN12 at 0.5-1µm, 1-1.5µm and 1.5-2µm from cell edges relative to the value at 0-0.5µm from the cell edge. (F) RLP4: RFP and RLP4-L: RFP intensity (duplicate of data from Figure 3.6) and RLP44: RFP relative to YFP: NPSN12 at 2-4µm and 4-6µm from the outer lateral surface, relative to intensity 0-2µm from the outer lateral surface. 0.25µm trimmed RFP images. Bars = Standard Deviation. Two-way ANOVA and Tukey post-hoc Test: same letter indicates no significant difference ($p > 0.01$), different letter indicates a significant difference ($p < 0.01$). N = over 75 cells.

3.F: Summary & Discussion

Quantification of pUB:: RLP4: RFP and pUB:: RLP4-L: RFP distribution within the PM of *Arabidopsis*

lateral root epidermal cells has confirmed that neither of these proteins are evenly distributed at the cell surface. As well as labelling cell edge compartments, pUB:: RLP4: RFP and pUB:: RLP4-L: RFP are edge-enriched within the PM and attenuate significantly relative to YFP: NPSN12 at the outer lateral surface with increased distance from adjacent cell edges. Interestingly, pUB:: RLP4-L: RFP attenuates to a significantly lesser degree than pUB:: RLP4: RFP with increased distance from cell edges at the outer surface however, any possible significance of this remains unclear. pUB:: RLP4: RFP and pUB:: RLP4-L: RFP are also enriched at the outer lateral surface of *Arabidopsis* lateral root epidermal cells

compared to medial sections, attenuating significantly relative to YFP: NPSN12 with increased depth from the outer surface. YFP: RAB-A5c is also enriched at the outer lateral surface (Kirchhelle et al., 2016), indicating that edge-directed trafficking is polarised towards this PM domain, which is reflected in the polarisation of pUB:: RLP4: RFP and pUB:: RLP4-L: RFP in the PM (Figure 3.8). Despite the quantification presented here, the extent of enrichment of YFP: RAB-A5c, RLP4: RFP and RLP4-L: RFP at cell edges at the inner lateral face of *Arabidopsis* lateral root epidermal cells remains unclear (Figure 3.8). This was not quantified here using the method described above due to difficulty in accurately separating the inner lateral face of epidermal cells from cortical cells below using this method.

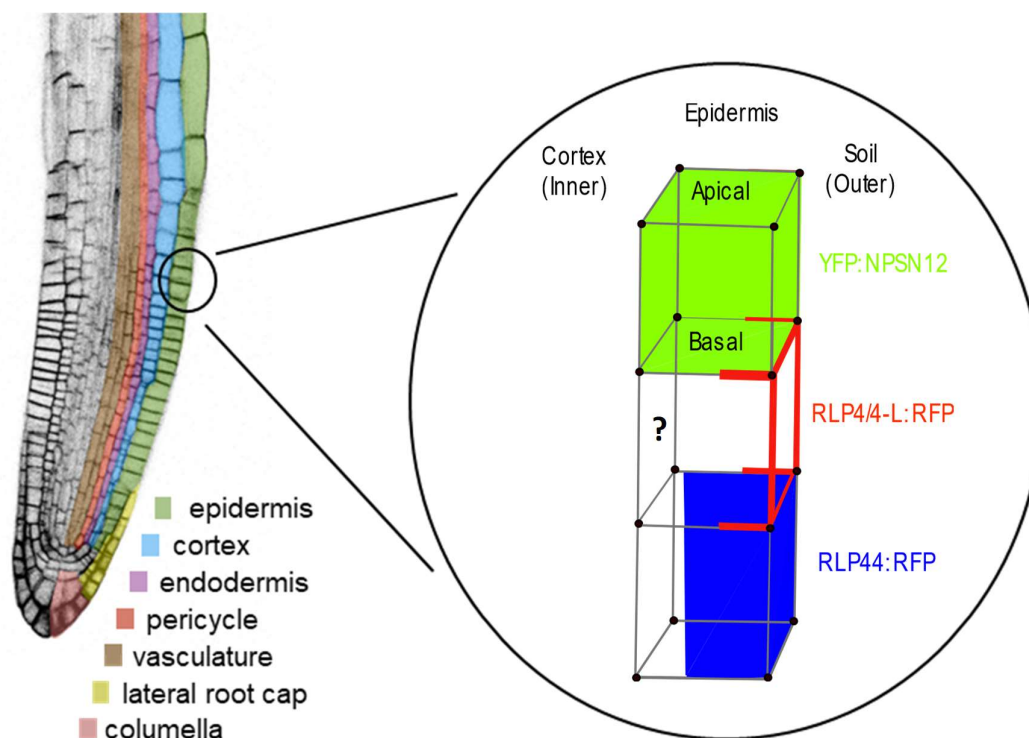


Figure 3.8 Comparative polarisations of YFP: NPSN12, RLP4/4-L: RFP and RLP44: RFP within the PM of *Arabidopsis* lateral root epidermal cells. Adapted from Elliott & Kirchhelle, 2019.

Fluorescently-tagged RLP4 and RLP4-L are therefore the first quantifiably edge-enriched proteins to be described in the *Arabidopsis* PM. The quantification pipeline described here however could be used as a method for assessing edge-restriction of other proteins which may also be edge-polarised within the PM, and investigation of any future candidate edge-polarised PM proteins (Yoshida et al., 2019, Elliott & Kirchhelle, 2019). It could also be used to further quantify the distribution of pUB:: RLP4: RFP and pUB:: RLP4-L: RFP across lateral roots. YFP: RAB-A5c is significantly enriched at longitudinal cell edges compared to transverse cell edges in lateral root meristematic cells, a pattern which is disrupted in mutants of edge-localised CLASP and GCP2 microtubule-associated proteins (Kirchhelle et al., 2019). It has been suggested that this localisation pattern may be associated with differential activity of RAB-A5c at different cell edges. Whether or not RLP4 and RLP4-L, as components of edge-directed trafficking, are also enriched at longitudinal edges compared to transverse ones could be determined using the quantification pipeline presented here and will be the subject of future investigation. This and functional characterisation of RLP4 and RLP4-L will help investigate the interesting possibility that different cell edges may be associated with differential trafficking in growing tissues (Kirchhelle et al., 2019).

The quantification pipeline presented here has also demonstrated that pUB:: RLP44: RFP is more uniformly distributed in the PM of *Arabidopsis* lateral root epidermal cells than pUB:: RLP4: RFP or pUB:: RLP4-L: RFP (Figure 3.7 & 3.8). This also confirms that polarisation patterns of pUB:: RLP4: RFP and pUB:: RLP4-L: RFP are not a universal feature of *Arabidopsis* RLPs. Polarisation of PM proteins to different domains of the cell surface is often linked to their function, and so the localisation differences between pUB:: RLP44: RFP and pUB:: RLP4/4-L: RFP at the cell surface indicates that the unique polarisations of the latter may link to a specialised function at the cell surface for RLP4 and RLP4-L compared to other uniformly-distributed cell wall-functioning proteins. The quantified differences in pUB:: RLP44: RFP distribution relative to YFP: NPSN12, compared to pUB:: RLP4/4-L: RFP relative to YFP: NPSN12 also confirm that differences between pUB:: RLP4/4-L: RFP and YFP:

NPSN12 distribution in the PM are not due to differences in emission properties between YFP and RFP, and are likely of biological origin.

Chapter 4: Subcellular localisation and trafficking of fluorescently-tagged truncated variants of RLP4 and RLP4-L indicate their possible functions in edge-directed trafficking

4.A: Introduction

The identification of RLP4 and RLP4-L as components of edge-directed trafficking raises the possibility that these Receptor-Like Proteins may contribute to the function of this highly polarised trafficking pathway. Expression of dominant negative RAB-A5c[N125I] causes specific defects in anisotropic growth and cell geometry in *Arabidopsis* lateral roots (Kirchhelle et al., 2016). Evidence presented here supports the assumption that RAB-A5c[N125I] expression disrupts an edge-directed trafficking pathway, linking the defects in anisotropic growth produced by RAB-A5c[N125I] expression to disruption of a RAB-A5c-mediated edge-directed trafficking pathway to the cell surface. As the only known non-GTPase components of edge-directed trafficking, I hypothesise that RLP4 and RLP4-L likely support some aspect of the as-yet unknown function of RAB-A5c and edge-directed trafficking in maintenance of anisotropic growth in *Arabidopsis*. In order to investigate possible functions for RLP4 and RLP4-L, this chapter focusses on beginning functional characterisation these proteins. RLP4 and RLP4-L share structural features typical of RLPs, consisting of a LRR-containing extracellular region, a TMD and a short intracellular tail region which does not encode a kinase domain. RLP4 and RLP4-L are unique among RLPs however as they each feature a Malectin-Like Domain (MLD) in their extracellular regions (Figure 4.1; Jamieson et al., 2018).

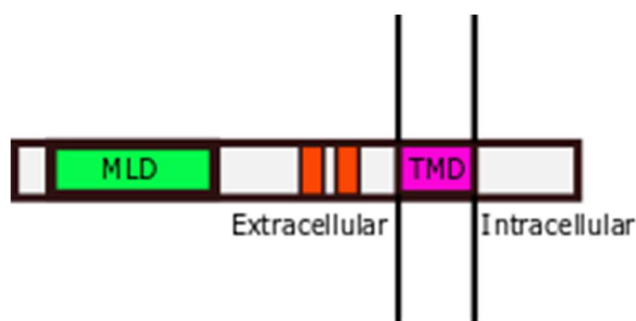


Figure 4.1: Simplified structure of RLP4 and RLP4-L. MLD = Malectin-Like Domain, TMD = Transmembrane Domain, Red bars = Leucine Rich Repeats.

MLDs have been identified as putative cell-wall binding domains in plants however, whether they may do this directly or through interaction with other cell-wall binding proteins remains unclear, as does the possible functional significance of MLDs in plants (Feng et al., 2018; Lin et al., 2018; Du et al., 2018; Moussu et al., 2018; Dünser et al., 2019). Based on the protein sequences of RLP4 and RLP4-L, I postulate that the extracellular domains (ECDs) of RLP4 and RLP4-L may fulfil a function at the cell surface, possibly through association with the cell wall, whilst the intracellular regions may function in trafficking of these proteins to cell geometric edges. To investigate this, I first examined the trafficking dynamics and subcellular behaviour of the individual protein regions of these RLPs, generating fluorescently-tagged truncated variants of RLP4 and RLP4-L and expressing these *in planta*. Three truncations of RLP4 and RLP4-L were generated, based largely on the possible extracellular-intracellular functional division within these proteins (Figure 4.2).

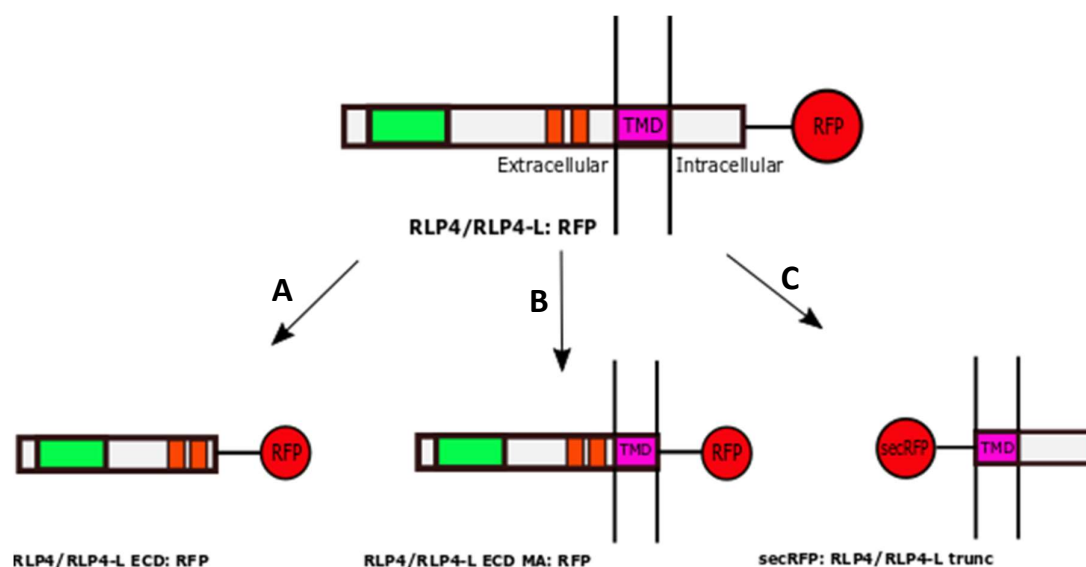


Figure 4.2: Fluorescently-tagged truncated variants of RLP4 and RLP4-L.

- (A) RLP4/RLP4-L ECD: RFP: lacking transmembrane domain and intracellular regions
- (B) RLP4/RLP4-L ECD MA: RFP: lacking endogenous intracellular regions
- (C) secRFP: RLP4/RLP4-L trunc: lacking endogenous extracellular regions

4.B: Fluorescently-tagged ECDs of RLP4 and RLP4-L associate with the cell wall in lateral roots

As already described, RLP4 and RLP4-L contain predicted ECDs, a single C-terminal TMD and a short intracellular tail. The ECDs of RLP4 and RLP4-L both contain MLDs, followed by a series of LRR regions before their TMDs. MLDs may facilitate binding to cell wall carbohydrates either through direct binding or indirectly through interactions with other cell-wall associated proteins (Feng et al., 2018; Lin et al., 2018; Dünser et al., 2019). In order to investigate whether the ECDs of associate with the *Arabidopsis* cell wall following deposition at the PM through edge-directed trafficking, I expressed fluorescent protein fusions of the ECDs of RLP4 and RLP4-L stably in *Arabidopsis*. The ECDs of RLP4 and RLP4-L (defined as ending at the last amino acid before their predicted TMDs) were introduced into pUB-RFP-DEST (Grefen et al., 2010) to generate pUB:: RLP4 ECD: RFP (RLP4 ECD: RFP; Figure 4.2) and pUB:: RLP4-L ECD: RFP (RLP4-L ECD: RFP; Figure 4.2). When transformed into plants expressing YFP: RAB-A5c, both RLP4 ECD: RFP and RLP4-L ECD: RFP labelled the extracellular regions between cells in young lateral roots (Figure 4.3A & B). Both protein fusions appeared to be particularly enriched at the outer and inner cell edges and vertices of the extracellular spaces (Figure 4.3D). This pattern was however indistinguishable from that of secreted RFP, pUB:: secRFP (secRFP), in young lateral roots, indicating that this distribution pattern within the extracellular space is a consequence of the geometry of this space rather than a specific property of the ECDs of RLP4 and RLP4-L (Figure 4.3C & D).

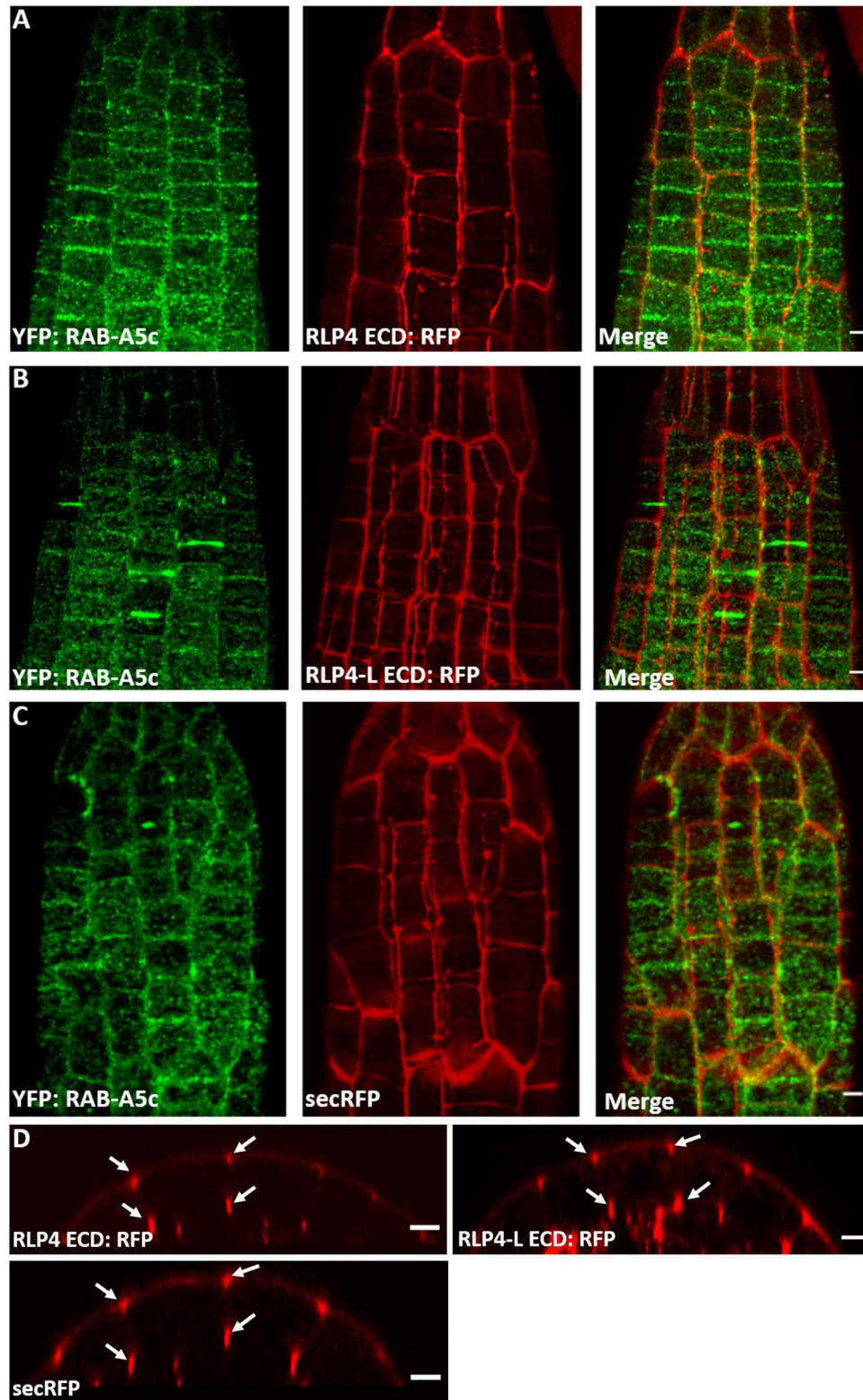
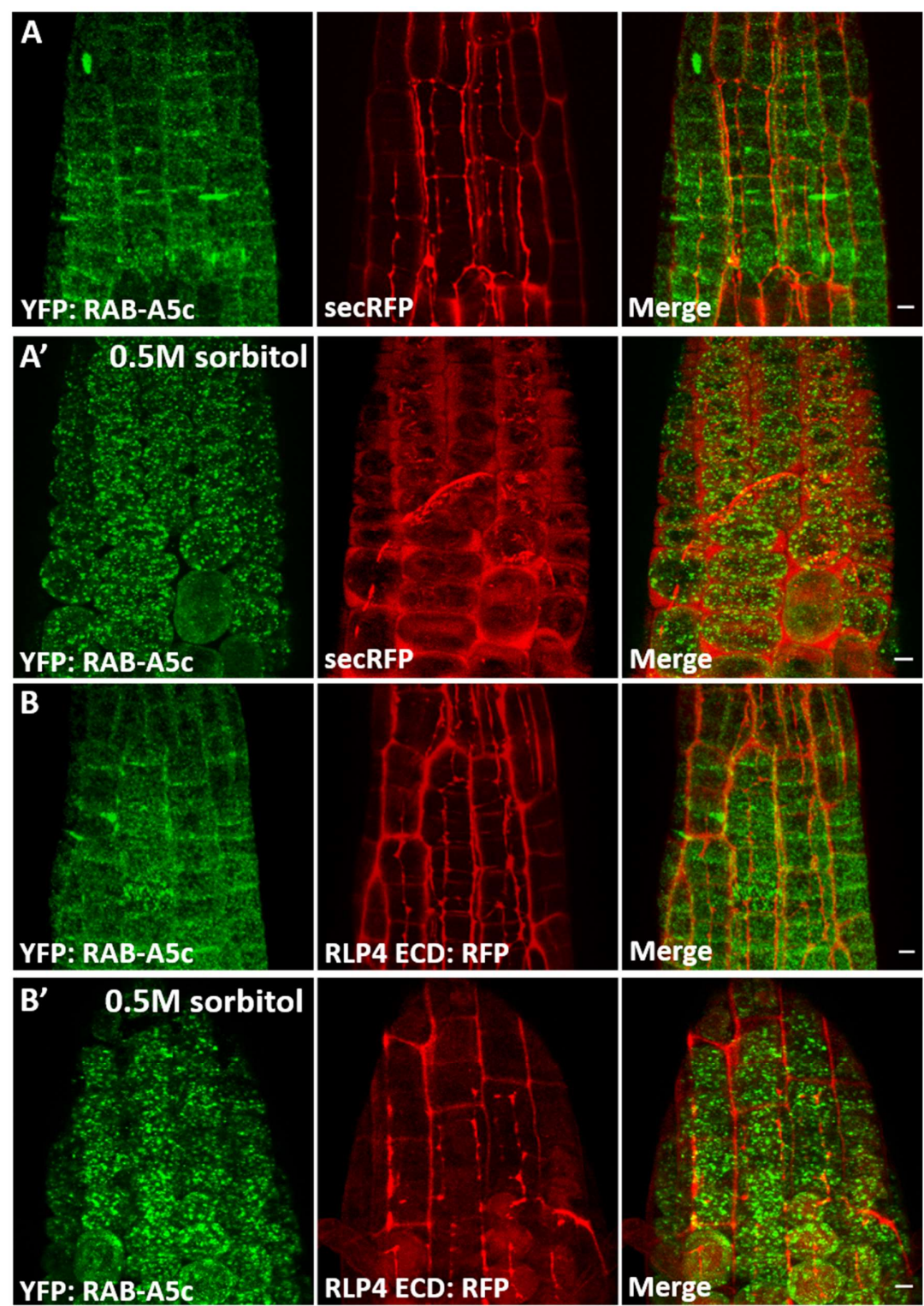


Figure 4.3: Fluorophore-tagged ECDs of RLP4 and RLP4-L localise to the extracellular region between cells in lateral roots. (A, B,C) 3D confocal stack projections of lateral roots co-expressing YFP: RAB-A5c (left), RLP4 ECD: RFP (middle, A) or RLP4-L ECD: RFP (middle, B) or secRFP (middle, C). (D) orthogonal projections. White arrows = outer and inner vertices. All from 10 day old T2 seedlings. Scale bars 5µm.

In order to test whether fluorescently-tagged ECDs of RLP4 and RLP4-L physically associate with the cell wall in the extracellular space of lateral roots, I plasmolysed lateral root cells in plants expressing RLP4 ECD: RFP and RLP4-L ECD: RFP. Plasmolysis is widely used to test the association of plant extracellular proteins with the cell wall, with non-cell wall binding proteins spreading evenly into the intercellular spaces created by the plasmolysis. Cell wall binding proteins, by contrast, remain in the space delineated by the cell wall (Lertpiriyapong & Sung, 2003; Ambrose et al., 2013; Vaddepalli et al., 2017). Lateral root cells were therefore plasmolysed using 0.5M sorbitol for 30 minutes. For this experiment, longer lateral roots of over 800µm were examined as younger, shorter laterals otherwise used in this thesis showed only limited plasmolysis. This may be due to smaller cell size in these lateral roots, or to the presence of the recently-described protective cuticle of young lateral roots in *Arabidopsis* (Berhin et al., 2019). In plants expressing secRFP, RFP signal in lateral roots filled the intracellular spaces produced by plasmolysis, differing dramatically from its localisation in non-plasmolysed lateral roots expressing secRFP (Figure 4.4A vs. A' & Figure 4.5A). This was noticeably different to plants expressing RLP4 ECD: RFP and RLP4-L ECD: RFP however, in which RFP signal did not uniformly re-distribute into the spaces produced by plasmolysis. RLP4 ECD: RFP and RLP4-L ECD: RFP remained sharply delineated between cells after plasmolysis, indicating that they remain associated with the cell wall after plasmolysis (Figure 4.4B vs. B' & C vs. C'; Figure 4.5B & C). Fluorescently-tagged ECDs of RLP4 and RLP4-L are therefore capable of cell-wall binding in *Arabidopsis* lateral roots, whereas an extracellular fluorophore control is not. Interestingly, as well as remaining cell-wall associated after plasmolysis, RLP4 ECD: RFP and RLP4-L ECD: RFP seemed to remain polarised to outer cell edges and corners around lateral root cells (Figure 4.5B & C). Whether this localisation after plasmolysis reflects restriction of a specific cell wall component at these regions with which RLP4 ECD: RFP and RLP4-L ECD: RFP associate, or whether the cell wall as a whole is particularly enriched at these regions, remains unknown. Localisation of YFP: RAB-A5c was also noticeably disrupted by the plasmolysis, re-localising to large intracellular compartments (Figure 4.4

and 4.5). Whether this is a consequence of the loss of geometric edges due to the plasmolysis or due to disruption of the endomembrane/cytoskeletal systems is unclear.



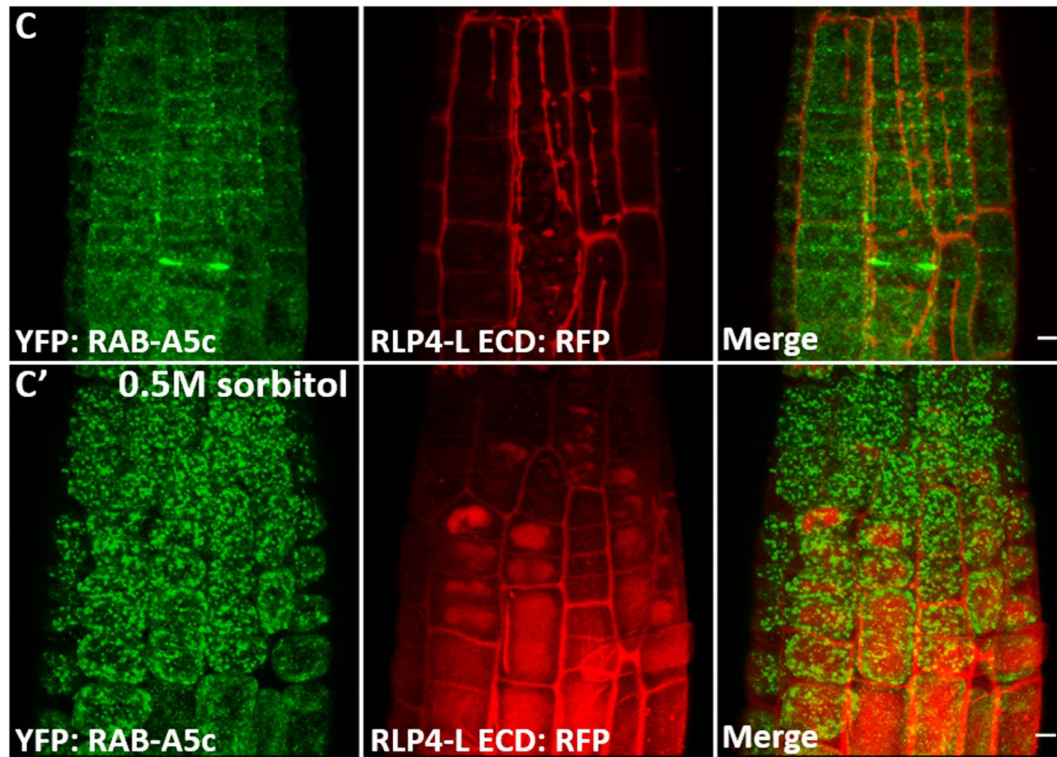


Figure 4.4: The ECDs of RLP4 and RLP4-L remains associated with the cell wall in lateral roots after cell plasmolysis. (A, B, C) 3D confocal stack projections of lateral root co-expressing YFP: RAB-A5c (left) and secRFP (A, middle) or RLP4: ECD (B, middle) or RLP4-L: ECD. (C, middle) (A', B', C') 3D confocal stack projections of plasmolysed lateral roots. 0.5M sorbitol, 30 minutes. All from 10 day old T2 seedlings. Scale bars 5 μ m.

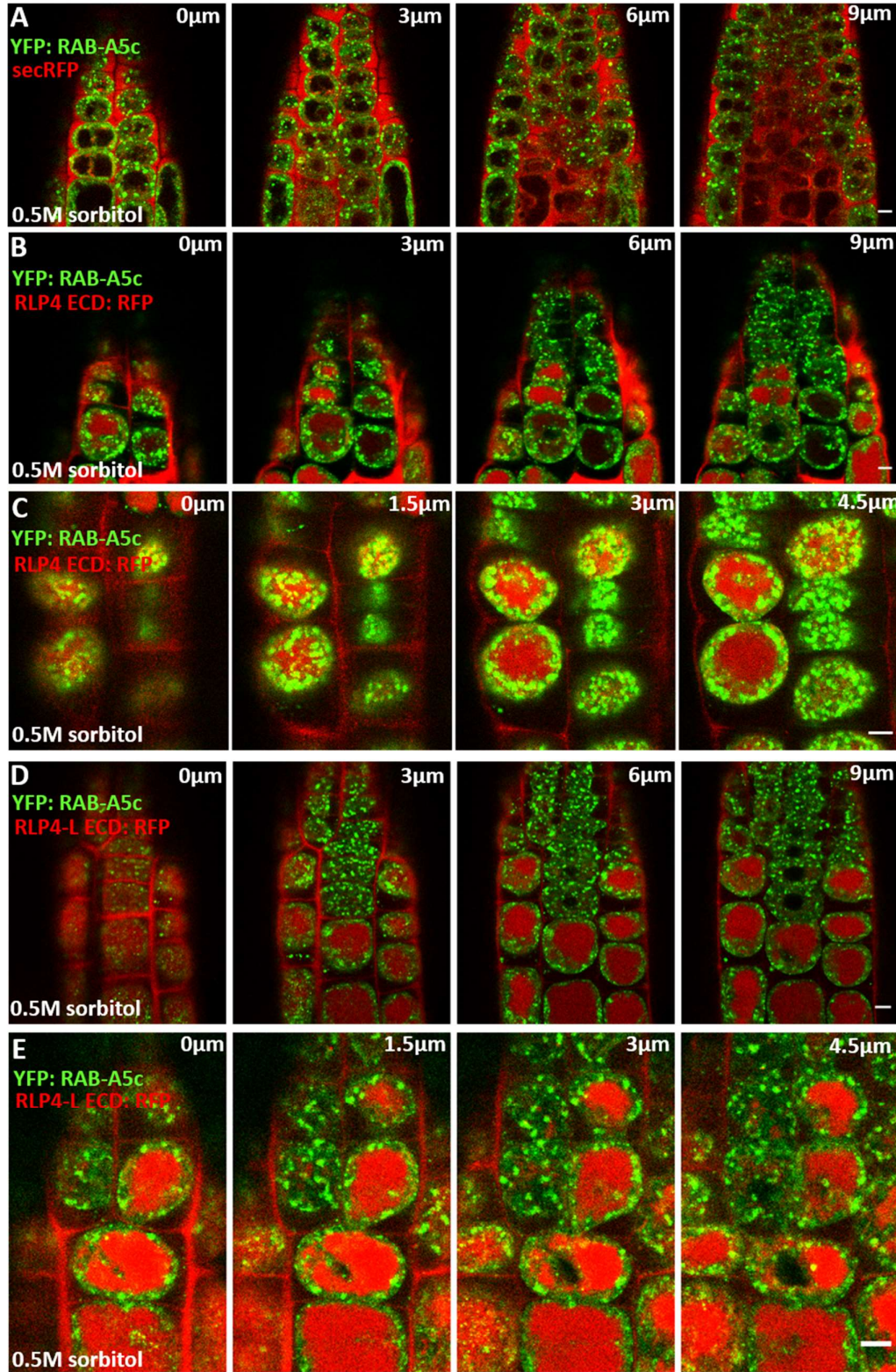


Figure 4.5: The ECDs of RLP4 and RLP4-L remain associated with the cell wall in lateral roots in a polarised manner (white arrows) after cell plasmolysis. (A) sequential confocal slices of lateral root cells co-expressing YFP: RAB-A5c (green) and secRFP (red). (B, D) YFP: RAB-A5c (green) and RLP4 ECD: RFP (red, B) or RLP4-L: ECD: RFP (red, D). (C, E) higher magnification confocal slices of separate roots. 0.5M sorbitol, 30 minutes. All from 10 day old T2 seedlings. Scale bars 5μm. 3μm or 1.5μm intervals.

4.C: Fluorescently-tagged membrane-associated ECDs of RLP4 and RLP4-L are polarised within lateral epidermal root cells but do not label cell edge compartments

The cell-wall binding ECDs expressed as RLP4 ECD: RFP and RLP4-L ECD: RFP are not membrane-associated, in contrast to those of full-length RLP4 and RLP4-L. It is therefore assumed that these truncations are secreted uniformly, as they lack membrane association and the intracellular tail that presumably interacts with cytoplasmic trafficking components. In order to test this assumption that the intracellular tails of RLP4 and RLP4-L are the domains responsible for RLP4 and RLP4-L's polarised entry into edge-directed trafficking through interaction with trafficking machinery, and the possibility that interactions via their ECDs may also contribute to entry into this pathway, I expressed fluorescently-tagged membrane-associated ECDs of RLP4 and RLP4-L, lacking their intracellular tails, *in planta*.

In order to express the membrane-associated ECDs of RLP4 and RLP4-L *in planta*, sequences encoding the ECD and subsequent TMD of RLP4 and RLP4-L respectively were introduced into pUBC-RFP-DEST (Grefen et al., 2010) to generate pUB:: RLP4 ECD MA: RFP (RLP4 ECD MA: RFP; Figure 4.2) and pUB:: RLP4-L ECD MA: RFP (RLP4-L ECD MA: RFP; Figure 4.2). When transformed into plants expressing YFP: RAB-A5c, neither RLP4 ECD MA: RFP nor RLP4-L ECD MA: RFP substantially labelled cell edge compartments in lateral roots (Figure 4.6A & B), nor did they appear to co-localise with YFP: RAB-A5c at what is likely to be the edges of the PPB (Figure 4.6D). Instead, RLP4 ECD MA: RFP and RLP4-L ECD MA: RFP both labelled the PM, as well as some intracellular compartments (Figure 4.6A & B). The membrane-associated ECDs of RLP4 and RLP4-L are therefore insufficient to visibly localise to YFP: RAB-A5c cell edge compartments alone.

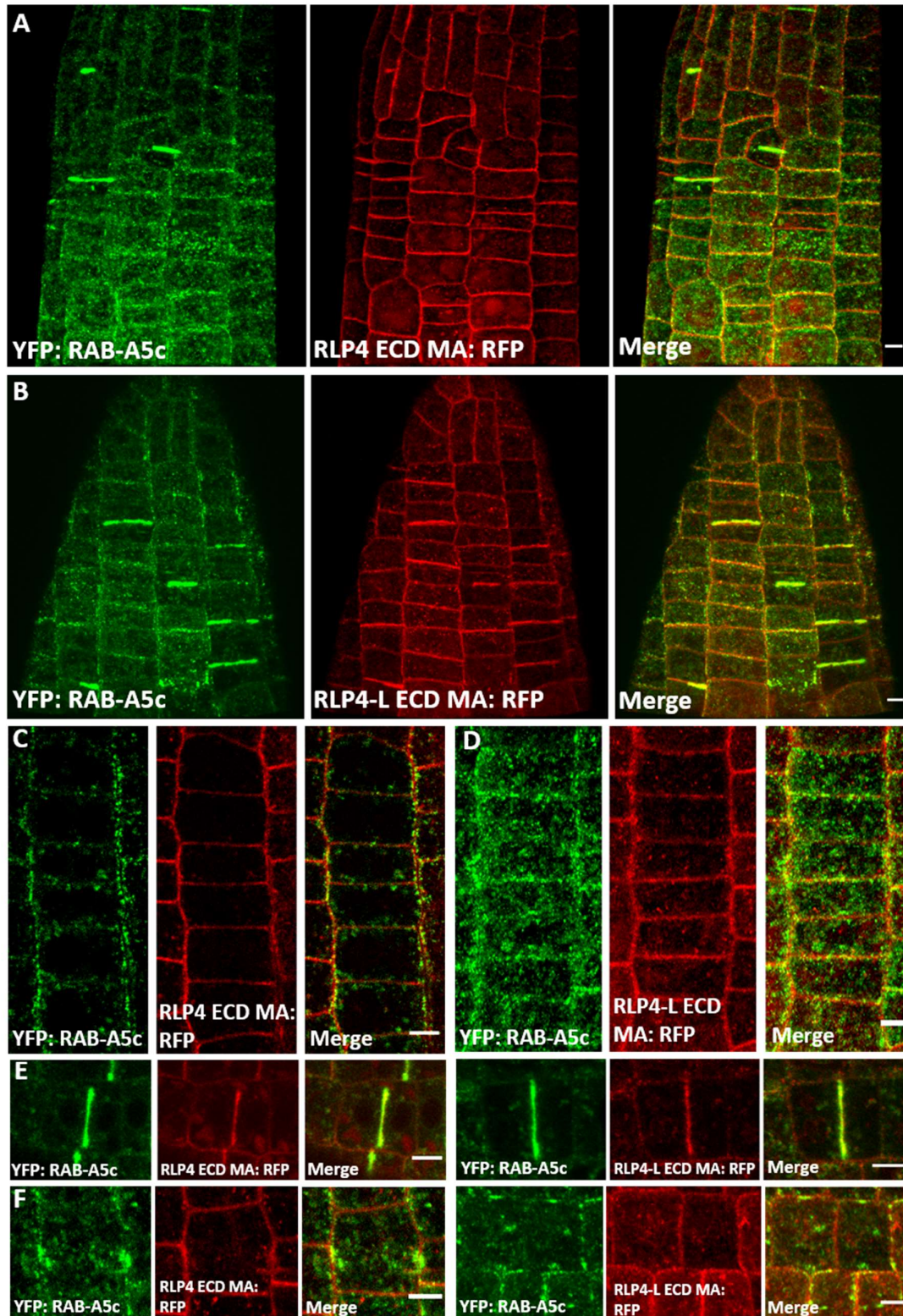


Figure 4.6: Membrane-bound ECDs of RLP4 do not label cell edge compartments. (A,B) 3D confocal stack projections of lateral roots co-expressing YFP: RAB-A5c (left) and RLP4 ECD MA: RFP (A, middle) or RLP4-L ECD MA: RFP (B, middle). (C,D) higher magnification images of lateral root meristematic cells. (E,F) confocal slice and 3D stack projection of meristematic cells co-expressing during cytokinesis (E) and pre-prophase (F). All from 10 day old T2 seedlings. Scale bars, 5µm.

Simple protein topology predicts that the C-terminal domains of RLP4 and RLP4-L form short intracellular tails, which would be predicted to be the site of interaction with trafficking-organising machinery. If the ECDs of RLP4 and RLP4-L do not contribute to trafficking of these proteins, it would be assumed that as RLP4 ECD MA: RFP or RLP4-L ECD MA: RFP should be secreted to the PM in a uniform manner through bulk secretory traffic. Strikingly however, RLP4 ECD MA: RFP does not uniformly label the PM and appears to be polarised towards the outer surface compared to evenly-distributed RFP: NPSN12 (Figure 4.7A & C, white arrows vs. yellow arrows; Geldner et al., 2009). RLP4-L ECD MA: RFP also appears to be polarised to the outer surface however, this is less striking than for RLP4 ECD MA: RFP (Figure 4.7B & C, white arrows vs. yellow arrows).

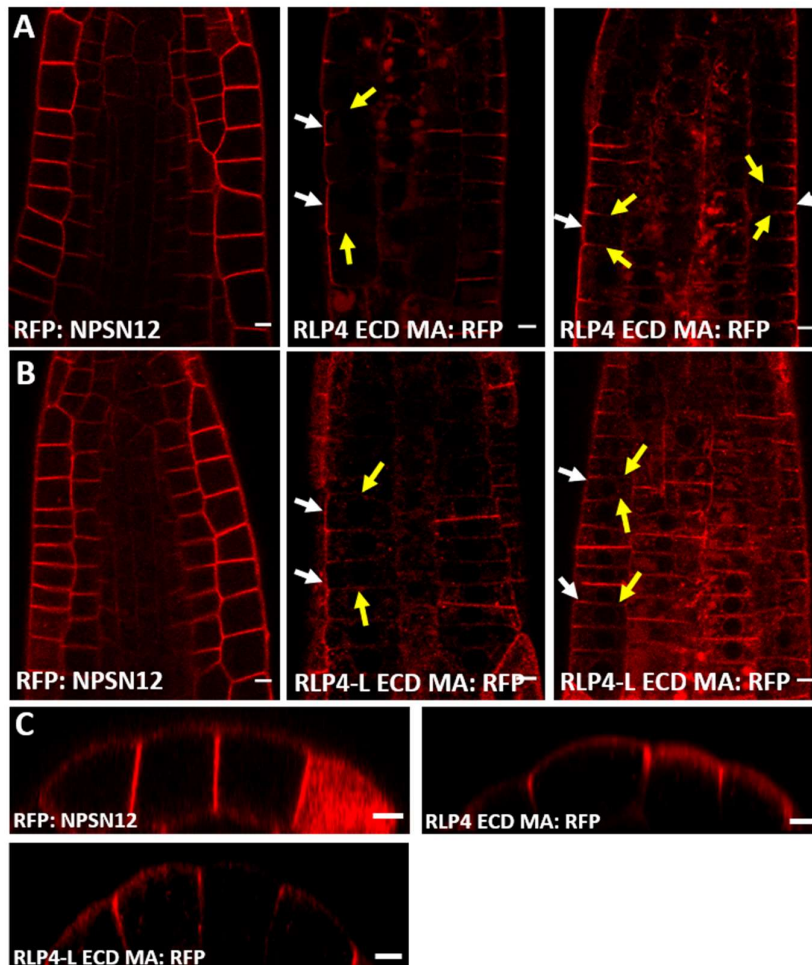


Figure 4.7: Membrane-bound ECDs of RLP4 and RLP4-L are not evenly distributed at the PM. (A, B, C) confocal slices or orthogonal projections of lateral root meristematic cells expressing RFP: NPSN12 or RLP4 ECD MA: RFP (A, C) or RLP4-L ECD MA: RFP (B, C). All from 10 day old T2 seedlings. Scale bars, 5μm. White arrows = outer surface, yellow arrows = depth away from outer surface.

Fluorescently-tagged membrane associated ECDs of RLP4 and RLP4-L are therefore insufficient for steady-state localisation to cell edge compartments. Thus, intracellular domains of RLP4 and RLP4-L may play key roles in their entry into edge-directed trafficking. I therefore investigated the trafficking dynamics of the intracellular domains of RLP4 and RLP4-L

4.D: The ECDs of RLP4 and RLP4-L are required for their localisation to cell edge compartments

In the absence of the ECD, the remaining regions of RLP4 and RLP4-L consist of a single TMD and a short intracellular tail, which is presumably the site of interaction with trafficking machinery. I therefore fluorescently-tagged truncated variants of RLP4 and RLP4-L lacking their ECDs: pUB::secRFP: RLP4 trunc (secRFP: RLP4 trunc; Figure 4.2) and pUB::secRFP: RLP4-L trunc (secRFP: RLP4-L trunc; Figure 4.2) and transformed these into plants expressing YFP: RAB-A5c.

Interestingly, both secRFP: RLP4 trunc and secRFP: RLP4-L trunc exclusively labelled intracellular structures in lateral roots (Figure 4.8). In contrast to full-length RLP4 and RLP4-L fluorescent protein fusions, co-localisation with YFP: RAB-A5c at cell edge compartments and labelling of the PM was not seen for either secRFP: RLP4 trunc or secRFP: RLP4-L trunc (Figure 4.8). Cases of partial co-localisation between secRFP: RLP4 trunc and secRFP: RLP4-L trunc with intracellular structures labelled by YFP: RAB-A5c were however observed (Figure 4.8C & D, white arrows). Moreover, secRFP: RLP4 trunc or secRFP: RLP4-L trunc did not co-localise with YFP: RAB-A5c at the cell plate during cytokinesis, nor did they co-localise with YFP: RAB-A5c at what are likely the edges of PPBs (Figure 4.8E & F). C-terminally tagged RLP4 and RLP4-L localise to the TGN/EE (Chapter 2, Figure 2.7), indicating that the structures labelled by secRFP: RLP4 trunc or secRFP: RLP4-L trunc may also be TGN/EEs. Full-length N-terminally-tagged RLP4 and RLP4-L (pUB: secRFP: RLP4 and pUB: secRFP: RLP4-L; Chapter 2, Figure 2.6) also intracellularly co-localise with the TGN/EE marker VHA-a1: GFP, but not the Golgi-marker ST: YFP (Appendix 2, Figure 8).

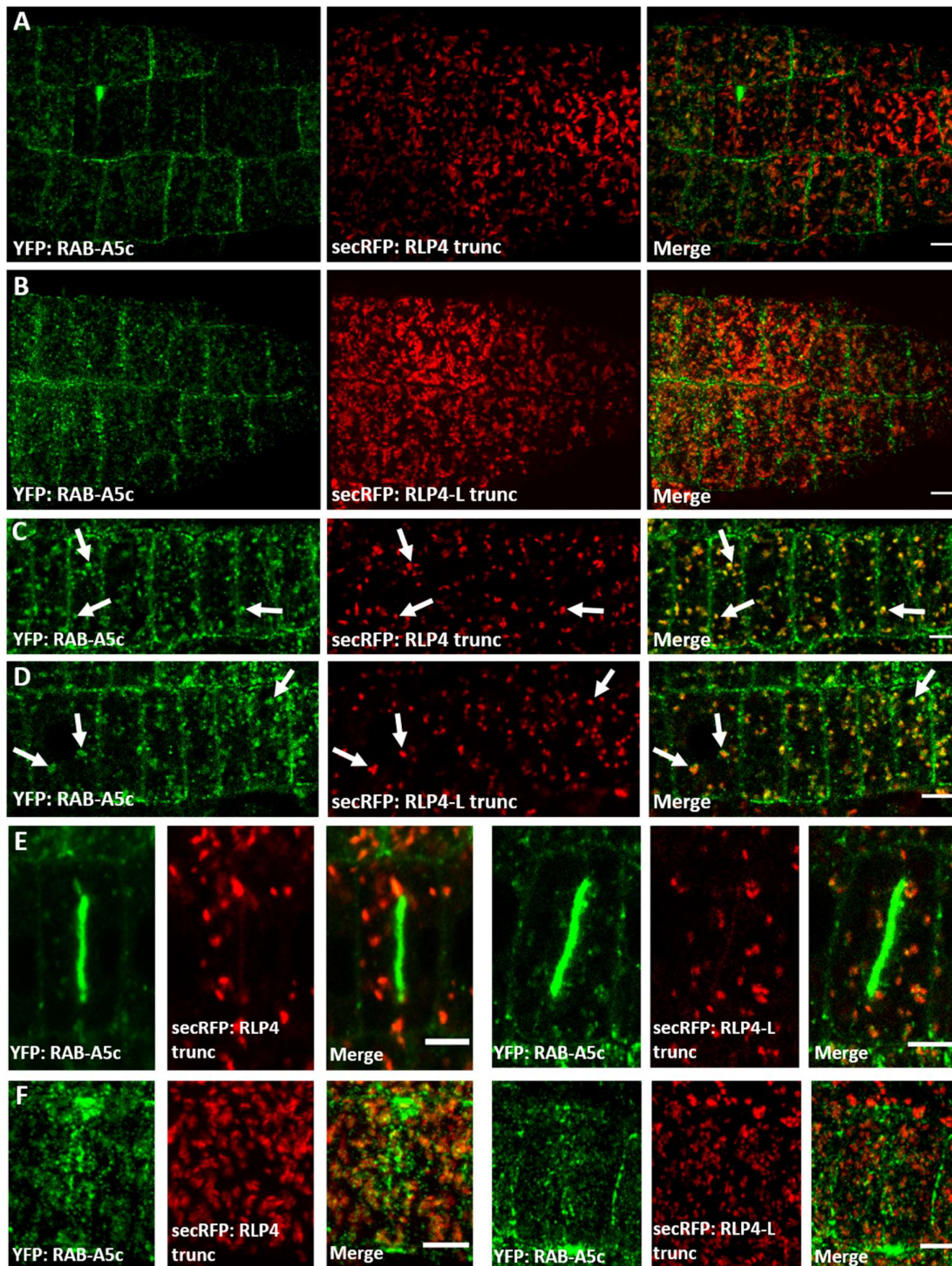
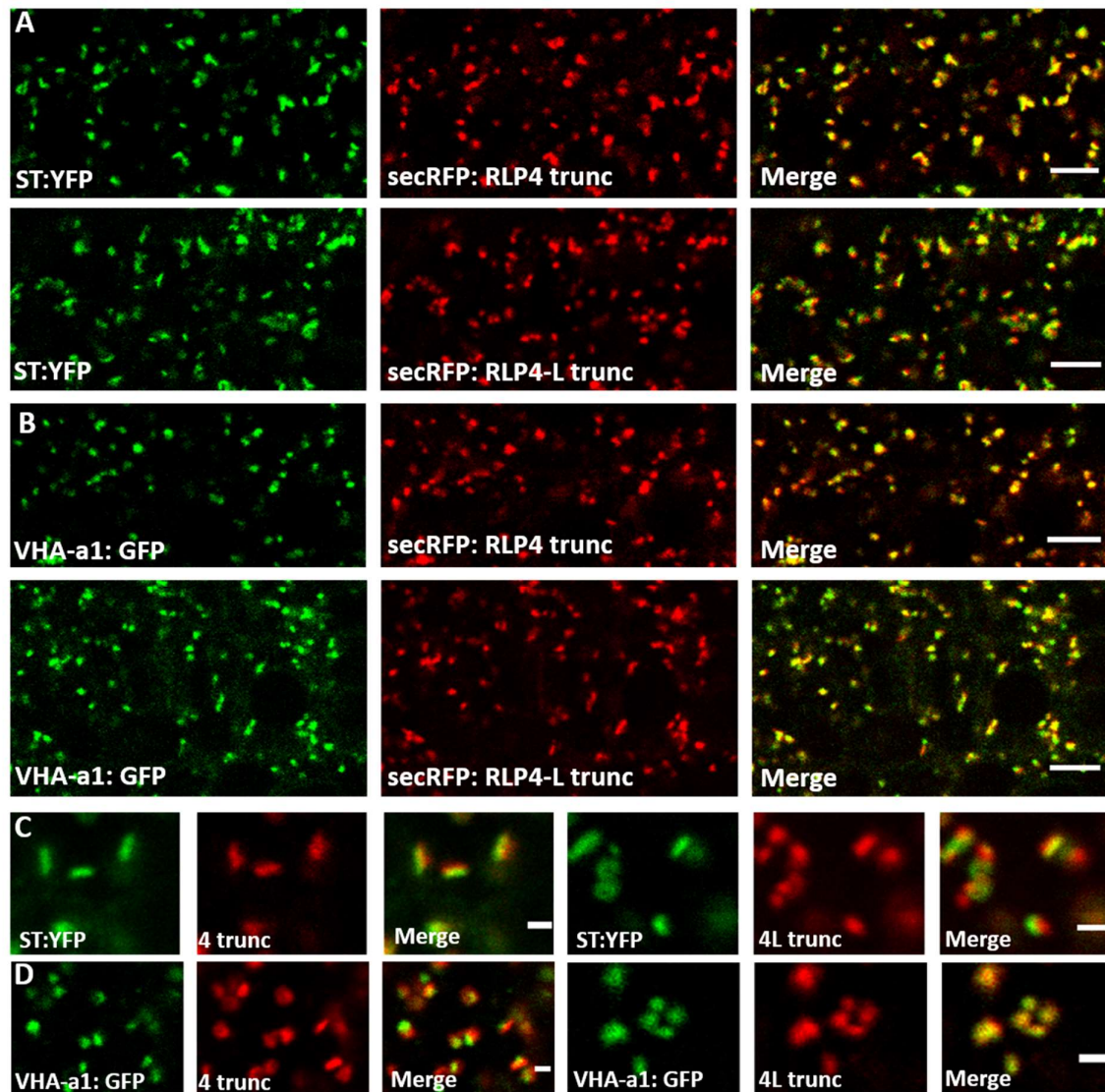


Figure 4.8: RLP4 and RLP4-L truncations lacking their ECDs do not localise to cell edge compartments and instead label intracellular structures (white arrows). (A, B) 3D confocal stack projections of lateral roots co-expressing YFP: RAB-A5c (left) and secRFP: RLP4 trunc (A, middle) or secRFP: RLP4-L trunc (B, middle). (C, D) higher magnification confocal slices of lateral root meristematic cells. (E, F) lateral root meristematic cells during cytokinesis (E) and pre-prophase (F). All from 10 day old T2 seedlings. Scale bars, 5µm.

In order to identify if the intracellular compartments to which secRFP: RLP4 trunc and secRFP: RLP4-L trunc exclusively localise are also TGN/EEs, I also transformed secRFP: RLP4 trunc and secRFP: RLP4-L trunc into plants expressing markers of the TGN/EE or the Golgi. The structures labelled by secRFP: RLP4 trunc and secRFP: RLP4-L trunc seemed to closely associate with both Golgi labelled by ST: YFP and TGN/EEs labelled by VHA-a1:GFP (Figure 4.9A & B). Upon closer inspection, subtle differences were noticed between secRFP: RLP4 trunc and secRFP: RLP4-L trunc localisation. secRFP: RLP4 trunc seemed to overlap to a greater degree with ST: YFP than VHA-a1: GFP, whilst secRFP: RLP4-L seemed to overlap with VHA-a1: GFP more than ST: YFP (Figure 4.9C & D). Quantification of co-localisation between secRFP: RLP4 trunc and secRFP: RLP4-L trunc and these markers supported this observation. Mander's Correlation Coefficient (MCC) values indicate that both secRFP: RLP4 trunc and secRFP: RLP4-L trunc overlap to high degrees with ST: YFP and VHA-a1: GFP (Figure 4.9E). secRFP: RLP4 trunc overlapped with ST: YFP to a greater degree than secRFP: RLP4-L trunc (MCC: 0.673 vs. 0.565; Figure 4.9E) whilst secRFP: RLP4-L overlapped with VHA-a1: GFP to a greater degree than secRFP: RLP4 (MCC: 0.732 vs. 0.639; Figure 4.9E). Neither of the differences between secRFP: RLP4 trunc and secRFP: RLP4-L trunc overlap with ST: YFP or VHA-a1: GFP were statistically significant (Figure 4.9E) however, supporting a scenario in which secRFP: RLP4-L label both the Golgi and TGN/EE but possibly to partially differing degrees. Fluorescently-tagged RLP4/RLP4-L truncated variants lacking their ECD therefore do not localise to cell edge compartments or the PM and instead predominately label the Golgi and TGN/EE, visually appearing to arrest in the secretory pathway at the Golgi and TGN/EE. The localisation of these fluorescently-tagged RLP truncated variants indicates that both RLP4 and RLP4-L also require their ECDs, as well as their intracellular tails, for steady-state localisation to cell edge compartments in *Arabidopsis* lateral roots.



E

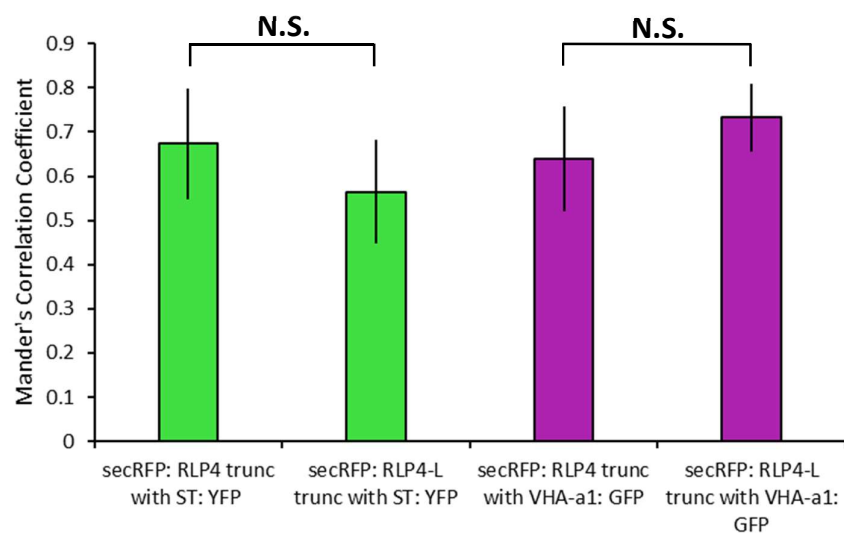


Figure 4.9: RLP4 and RLP4-L truncations lacking their ECDs localise to the TGN/EE and Golgi. (A, B) confocal slices of lateral roots meristematic cells co-expressing ST: YFP (A, left) or VHA-a1: GFP (B, left) and secRFP: RLP4 trunc (middle) or secRFP: RLP4-L trunc (middle). (C, D) higher magnification images. All from 10 day old F2 seedlings. Scale bars 5µm (A, B) and 1µm (C, D). (E) Mander's Correlation Coefficient of overlap between secRFP: RLP4 trunc and secRFP: RLP4-L trunc with ST: YFP or VHA-a1: GFP (N>50 cells each). N.S. = Not Significant, $P > 0.01$. Two-tailed T-test. Bars = Standard Deviation.

4.E: The ECDs of RLP4 and RLP4-L may stabilise their presence at the cell surface

In order to further test the dual-localisation of secRFP: RLP4 and secRFP: RLP4-L to the Golgi and TGN/EE, I treated lines those lines with the mycotoxin Brefeldin A (BFA), which inhibits protein recycling from the TGN/EE and causes formation of large TGN/EE-derived membrane aggregates known as 'BFA bodies'. Treatment with BFA can be used to discriminate between localisation of proteins to the Golgi and TGN/EE, with proteins that localise to the TGN/EE labelling the core of BFA bodies and Golgi-localised proteins clustering around the periphery of BFA bodies (Grebe et al., 2003; Robinson et al., 2008; Chow et al., 2008; Uemura et al., 2014; Naramoto et al., 2014). When lines co-expressing YFP: RAB-A5c and secRFP: RLP4 trunc or secRFP: RLP4-L trunc were treated with BFA, YFP: RAB-A5c labelled the core of BFA bodies (Figure 4.10B, B' & D, D'). secRFP: RLP4 trunc and secRFP: RLP4-L trunc also labelled the core of BFA bodies as well as more peripheral compartments, further indicating that these truncations localise to both the Golgi and TGN/EE (Figure 4.10B, B' & D, D'). Strikingly however, treatment of plants expressing secRFP: RLP4-L trunc with BFA also caused re-localisation of secRFP: RLP4-L trunc to the PM in lateral roots (Figure 4.10B & B', white arrows). Re-localisation of secRFP: RLP4 to the PM in lateral roots could also be produced by treatment of plants with BFA, but to a seemingly lesser extent than for secRFP: RLP4-L trunc (Figure 4.10D & D', white arrows). Some aspect of BFA's inhibitory effect on post-Golgi trafficking therefore seems capable of partially re-localising fluorescently-tagged truncated variants of RLP4 and RLP4-L from exclusively intracellular localisation at the Golgi and TGN/EE, to the PM.

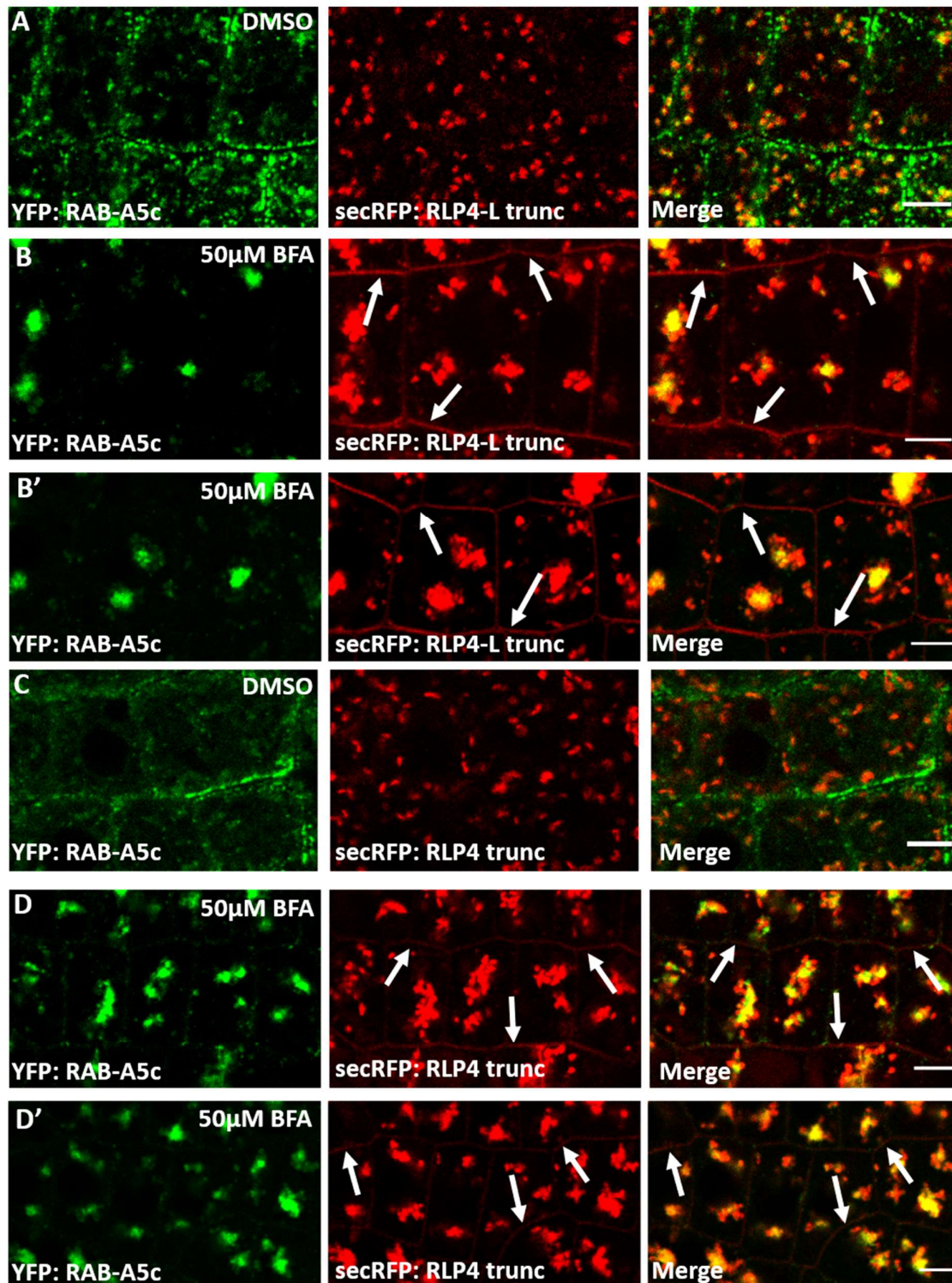


Figure 4.10: Treatment with BFA re-localises secRFP: RLP4 trunc and secRFP: RLP4-L trunc to the PM (white arrows). (A,B,B',C,D,D') confocal slices of lateral root meristematic cells co-expressing YFP: RAB-A5c and secRFP: RLP4 or secRFP: RLP4-L. 50 μM BFA, 30 mins (B, B', D, D') or equivalent volume DMSO (A,C). All from 10 day old T2 seedlings. Scale bars 5 μm.

The re-localisation of secRFP: RLP4-L trunc and secRFP: RLP4 trunc to the PM after treatment with BFA is surprising. BFA is traditionally thought to inhibit secretory recycling traffic from the TGN/EE in *Arabidopsis* through targeting BFA-sensitive GEFs of Arf GTPases, including the vesicle trafficking regulator GNOM, resulting in accumulation of recycling secretory proteins in large TGN/EE-derived BFA bodies (Geldner et al., 2003; Robinson et al., 2008). BFA therefore typically removes or reduces proteins cycling between the PM and internal membranes from the PM in favour of internalisation (Geldner et al., 2001; Teh and Moore, 2007; Geldner et al., 2007; Chow et al., 2008). This is strikingly in contrast to the re-localisation to the PM of secRFP: RLP4 trunc and secRFP: RLP4-L trunc produced by treatment with BFA. One possible explanation for this phenomenon is that treatment with BFA inhibits some aspect of trafficking that promotes retention of secRFP: RLP4 trunc and secRFP: RLP4-L trunc in Golgi and TGN/EE, and that BFA treatment allows release of these into the secretory pathway. An alternative explanation is that secRFP: RLP4 trunc and secRFP: RLP4-L trunc are actually not arrested at the Golgi and TGN/EE but are instead actually cycling rapidly between the late secretory pathway and the PM. Despite largely being considered as an inhibitor of secretory recycling traffic, BFA is known to disrupt internalisation of some PM proteins, supporting the involvement of BFA-sensitive ARF-GEF targets in some cases of endocytosis (Uhlir-Hansen & Yanagishita, 1995; Teh & Moore, 2007; Naramoto et al., 2010; Irani et al., 2012). BFA is also visibly destructive to the endomembrane system, causing spatio-temporal reorganisation of the Golgi, TGN/EE and late endosomes in *Arabidopsis* root cells. It is therefore not unreasonable to expect that disruption of endocytic trafficking may also be an off-target effect of BFA on the plant endomembrane system, in addition to known roles for BFA-sensitive targets in endocytosis (Naramoto et al., 2010; Irani et al., 2012).

In order to attempt to discriminate between possible scenarios for why BFA re-localises secRFP: RLP4 trunc and secRFP: RLP4-L trunc to the PM, I chose to examine whether known inhibitors of endocytosis could also re-localise secRFP: RLP4/RLP4-L trunc to the PM in *Arabidopsis* lateral roots. Multiple pharmacological agents are widely used as endocytosis inhibitors in *Arabidopsis* roots (Irani

& Russinova, 2009). Wortmannin is one such inhibitor that targets phosphoinositide kinases, and is known to cause characteristic swelling of late endosomes in *Arabidopsis* root cells, as well as defects in endocytosis in a dose-dependent manner (Emans et al., 2002; Park et al., 2003; Jallais et al., 2008; Robinson et al., 2008; Wang et al., 2009; Niemes et al., 2010; Takáč et al., 2012). A second commonly-used endocytosis inhibitor in *Arabidopsis* is the tyrosine analogue Tyrphostin A23. Tyrphostin A23 in mammalian cells is believed to inhibit endocytosis through disrupting the interaction of the AP-2 adaptor complex with endocytic cargoes (Banbury et al., 2003), and in *Arabidopsis* disrupts PM localisation of endocytic machinery (Van Damme et al., 2011; Di Rubbo et al., 2013; Fan et al., 2013; Wang et al., 2016). Tyrphostin A23 has therefore been used to inhibit endocytic trafficking of various *Arabidopsis* PM proteins (Barberon et al., 2011; Beck et al., 2012; Irani et al., 2012; Smith et al., 2014). Interestingly, treatment with both Wortmannin and Tyrphostin A23 also resulted in partial re-localisation of secRFP: RLP4-L trunc to the PM in lines co-expressing YFP: RAB-A5c and secRFP: RLP4-L trunc (Figure 4.11B, B' & D, D', white arrows. Note: DMSO was used as a control for Wortmannin treatments but for Tyrphostin A23 treatments, its inactive analogue Tyrphostin A51 was used as a control). In some cases, striking loss of YFP: RAB-A5c cell edge compartments in favour of localisation of YFP: RAB-A5c to the PM in lateral roots was seen after treatment Wortmannin or Tyrphostin A23 (Figure 4.11B' & D', yellow arrows). This was however only sometimes seen and in many cases did not occur even when re-localisation of secRFP: RLP4-L trunc to the PM was seen. Interestingly, treatment with Wortmannin and Tyrphostin A23 did not re-localise secRFP: RLP4 trunc to the PM (Figure 4.12).

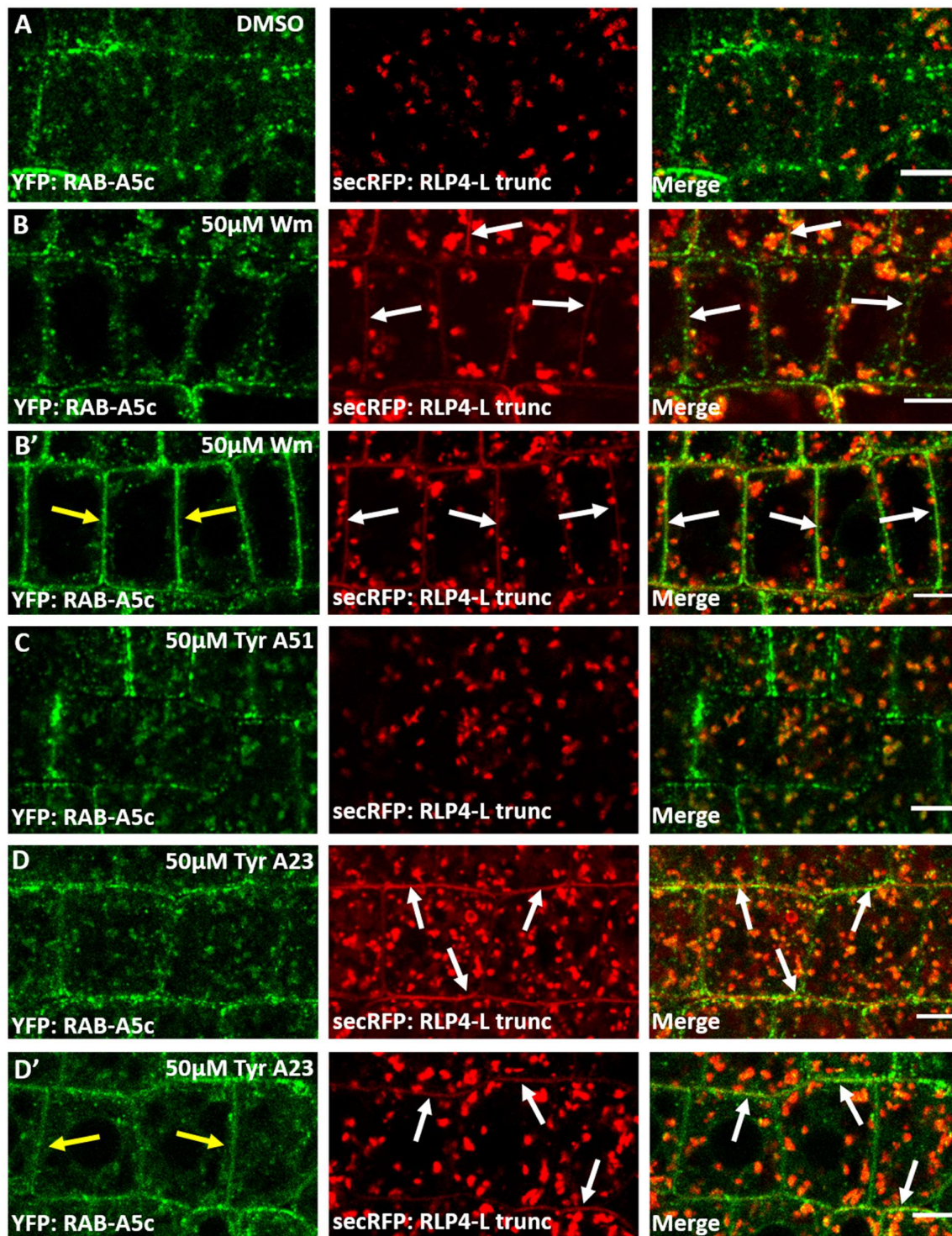


Figure 4.11: Treatment with Wortmannin (Wm) and Tyrphostin A23 (Tyr A23) re-localises secRFP::RLP4-L trunc to the PM (white arrows) and also sometimes YFP: RAB-A5c to the PM (yellow arrows). (A, B, B', C, D, D') confocal slices of lateral root cells co-expressing YFP: RAB-A5c (left) and secRFP: RLP4-L trunc (middle). (A, B, B') 50μM wortmannin, 90 mins or equivalent volume DMSO. (C, D, D') 50μM Tyrphostin A23 or Tyrphostin A51, 90 mins. All from 10 day old T2 seedlings. Scale bar 5μm.

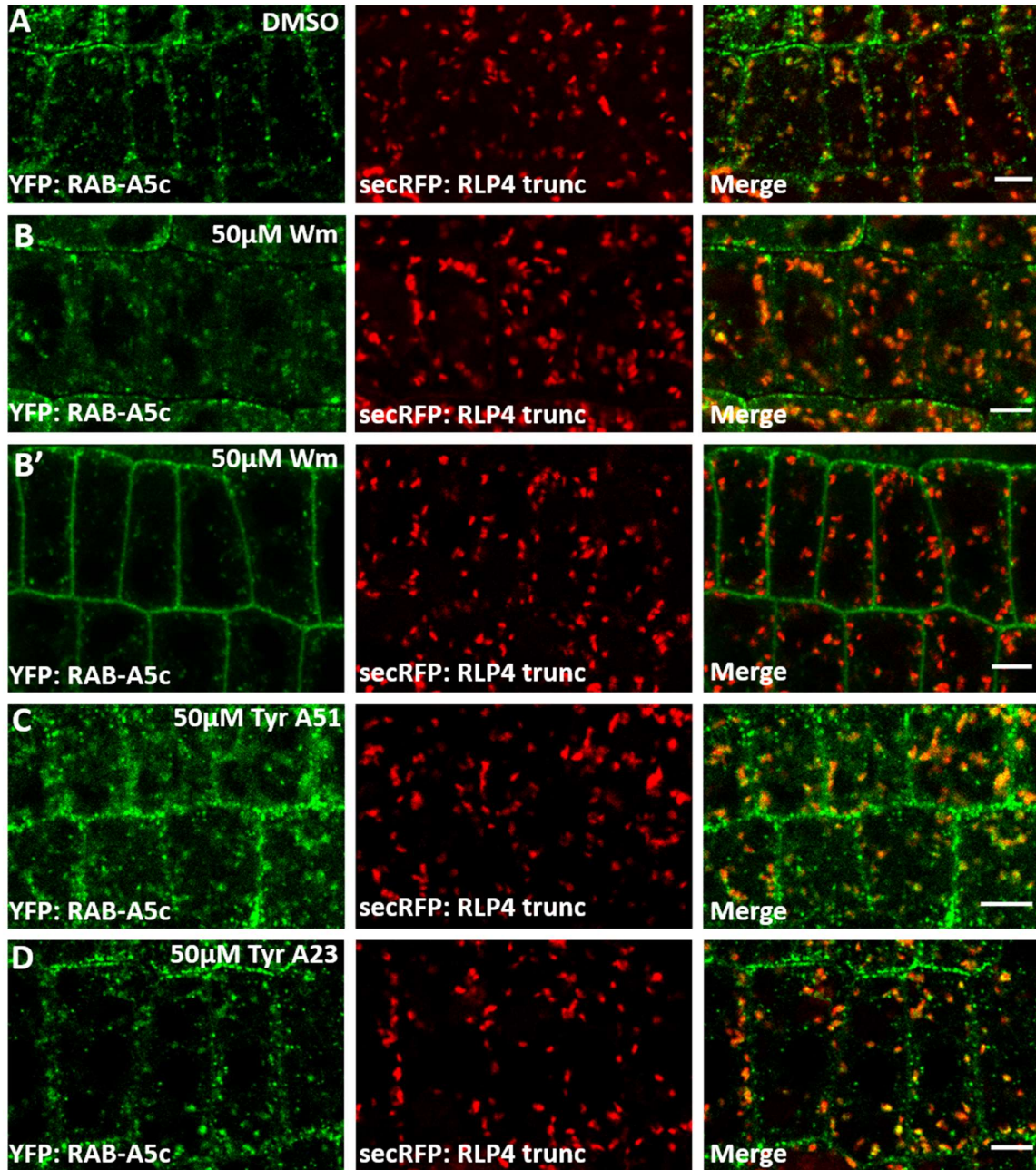


Figure 4.12: Treatment with Wortmannin (Wm) and Tyrphostin A23 (Tyr A23) does not re-localise secRFP:: RLP4 trunc to the PM (white arrows). (A, B, B', C, D) confocal slices of lateral root cells co-expressing YFP: RAB-A5c (left) and secRFP: RLP4-L trunc (middle). (A, B, B') 50 μM wortmannin, 90 mins or equivalent volume DMSO. (C, D) 50 μM Tyrphostin A51 or Tyrphostin A23, 90 mins. All from 10 day old T2 seedlings. Scale bar 5 μm.

Golgi and TGN/EE-localised secRFP: RLP4 trunc and secRFP: RLP4-L trunc can therefore be re-localised to the PM by the ARF-GEF inhibitor BFA. Re-localisation of secRFP: RLP4-L trunc to the PM following treatment with the endocytosis inhibitors Wortmannin and Tyrphostin A23 indicates that the re-localisation of secRFP: RLP4-L trunc and secRFP: RLP4 trunc to the PM produced by BFA may be due to disruption of endocytic trafficking as a secondary effect of the dramatic re-organisation of the endomembrane system in *Arabidopsis* root cells caused by BFA. Moreover, re-localisation of secRFP: RLP4-L trunc to the PM by endocytosis-inhibiting Wortmannin and Tyrphostin A23 (Figure 4.11) indicates that, despite being steady-state localised to the Golgi and TGN/EE, secRFP: RLP4-L trunc does enter secretory traffic to the PM but is rapidly removed from the PM by endocytosis. secRFP: RLP4-L trunc therefore only becomes visible at the PM upon inhibition of endocytosis, whereby it presumably continues to be secreted to the PM but its rate of removal is reduced. Re-localisation of secRFP: RLP4 trunc to the PM after treatment with BFA was noticeably less robust than for secRFP: RLP4-L trunc (Figure 4.10B & B' vs. D & D'). Moreover, secRFP: RLP4 trunc did not re-localise to the PM after treatment with either Wortmannin or Tyrphostin A23 (Figure 4.12). It was also noted that secRFP: RLP4 trunc-expressing lines were universally dimmer than those of the RLP4-L truncation. It is possible that the less robust re-localisation of secRFP: RLP4 trunc to the PM during BFA treatment and its lack of response to treatment with Wortmannin or Tyrphostin A23 is due to lower expression level, and that insufficient amounts of secRFP: RLP4 trunc accumulate at the PM upon disruption of endocytosis to be robustly visible. It is also notable that earlier imaging and co-localisation analyses indicated that secRFP: RLP4 trunc localisation may be shifted towards the Golgi compared to secRFP: RLP4-L trunc (Figure 4.9), possibly reducing the recycling population of secRFP: RLP4 trunc at the TGN/EE. Whether this is possible shift towards the Golgi of secRFP: RLP4 trunc compared to secRFP: RLP4-L trunc is also due to its lower expression level is unclear. It is also possible that the differences between secRFP: RLP4 trunc and secRFP: RLP4-L trunc localisation and responses to inhibitors of membrane trafficking are due to different trafficking properties of these

truncations, such as interactions with distinct trafficking machinery components, and this remains an open question.

4.F: Fluorescently-tagged truncated RLP44 behaves differently to equivalent truncations of RLP4 and RLP4-L

Investigating the trafficking dynamics and subcellular behaviour of individual protein domains of RLP4 and RLP4-L has given indications as to the possible functions of these proteins in edge-directed trafficking, and the contributions of their protein domains to their trafficking. It is unclear however, how unique the behaviour of the individual protein domains of RLP4 and RLP4-L are within the diversity of *Arabidopsis* RLPs. I previously demonstrated that pUB:: RLP4: RFP and pUB:: RLP4-L: RFP polarisation in the PM is not shared by pUB:: RLP44: RFP , indicating that the striking polarisation of fluorescently-tagged RLP4 and RLP4-L is not a universal feature of *Arabidopsis* RLPs and may support specialised functions. One of the most intriguing observations in this chapter is that truncated variants of fluorescently-tagged RLP4 and RLP4-L lacking their ECDs curiously both labelled the Golgi and TGN/EE, and no longer localised to cell edge compartments or the PM (Figures 4.8 & 4.9). Treatment with pharmacological inhibitors of post-Golgi trafficking and endocytosis partially re-localises these truncations to the PM, indicating that the ECDs of RLP4 and RLP4-L may be required for their stable localisation to the cell surface. In order to investigate whether other RLPs may behave similarly in the absence of their ECDs, I generated an equivalent truncation construct for RLP44 – pUB:: secRFP: RLP44 trunc (secRFP: RLP44 trunc)- and transformed this into plants expressing YFP: RAB-A5c or YFP: NPSN12. In lateral roots, secRFP: RLP44 trunc strikingly, and almost exclusively, labelled the PM (Figure 4.13). A fluorescently-tagged RLP44 truncation lacking its ECD is therefore capable of stable localisation to the PM, whereas equivalent truncations of RLP4 and RLP4-L are not.

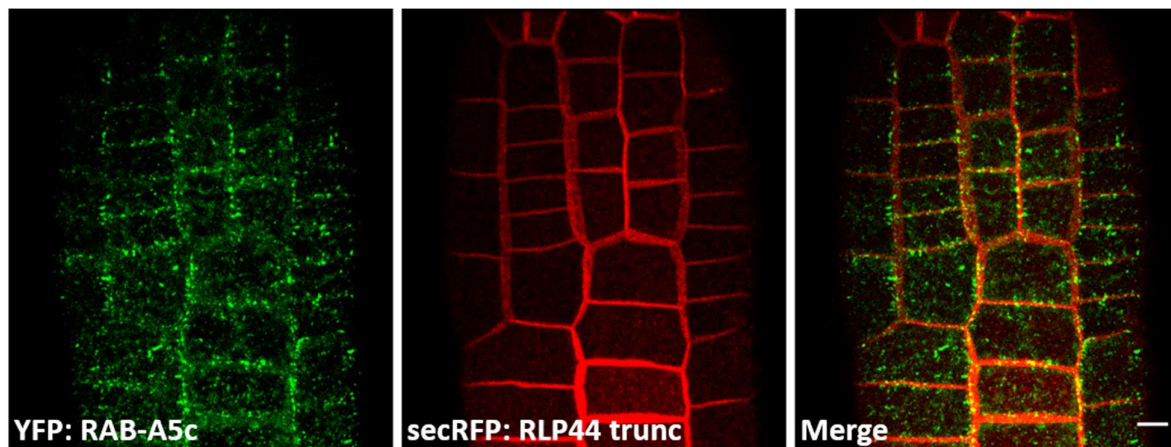


Figure 4.13: RLP44 trunc: RFP localises to the PM in *Arabidopsis* lateral roots. 3D projection of lateral root from a 10 day old T2 seedling co-expressing secRFP: RLP44 trunc and YFP: RAB-A5c. Scale bar, 5µm.

4.G: Transient BiFC assays indicate a possible direct interaction between fluorescently-tagged RAB-A5c and RLP4/RLP4-L at the TGN/EE

The disruption of RLP4 and RLP4-L localisation by expression of RAB-A5c[N125I] supports a functional relationship between RAB-A5c and these Receptor-Like Proteins in edge-directed trafficking. When developing functional hypotheses for RLP4 and RLP4-L function in edge-directed trafficking based on the observations made in this chapter, it is important to consider whether the functional relationship between RAB-A5c, RLP4 and RLP4-L may be due to a direct or indirect physical interaction between these proteins. I therefore chose to use Bimolecular Fluorescence Complementation (BiFC) to investigate the nature of the interaction between fluorescently-tagged RAB-A5c and RLP4/RLP4-L. BiFC involves tagging proteins of interest with split fluorophore variants, which unite and produce fluorescence upon very close proximity between the proteins of interest. Advantages of BiFC include that it allows live-cell imaging of protein-protein interactions *in planta* and can give indications as to the location of such interactions within the cells. BiFC is most often performed using transient expression in *Arabidopsis* protoplasts or in leaf epidermal cells of *Nicotiana tabacum*/*Nicotiana benthamiana*. I chose to use transient expression in *N. tabacum*.

Attempts to express full length pUB:: RLP4: RFP and pUB:: RLP4-L: RFP in *N. tabacum* produced no fluorescence, indicating that fluorescently-tagged full-length RLP4 and RLP4-L may be unstable when expressed in *N. tabacum*. I therefore transiently expressed split YFP-tagged truncated variants of RLP4 and RLP4-L lacking their ECD, pUB:: secRFP: RLP4 trunc: n/cYFP (secRFP: RLP4 trunc: n/cYFP) and pUB:: secRFP: RLP4-L trunc: n/cYFP (secRFP: RLP4-L trunc: n/cYFP) in *N. tabacum* (Figure 4.14). One advantage provided by this approach is that RLP trunc localisation could be visualised through secRFP independently of YFP signal produced by any possible protein-protein interaction.

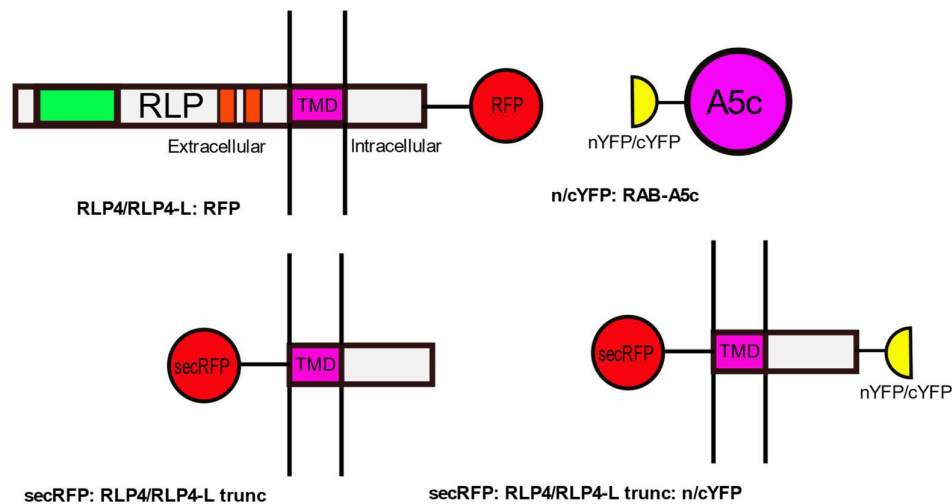


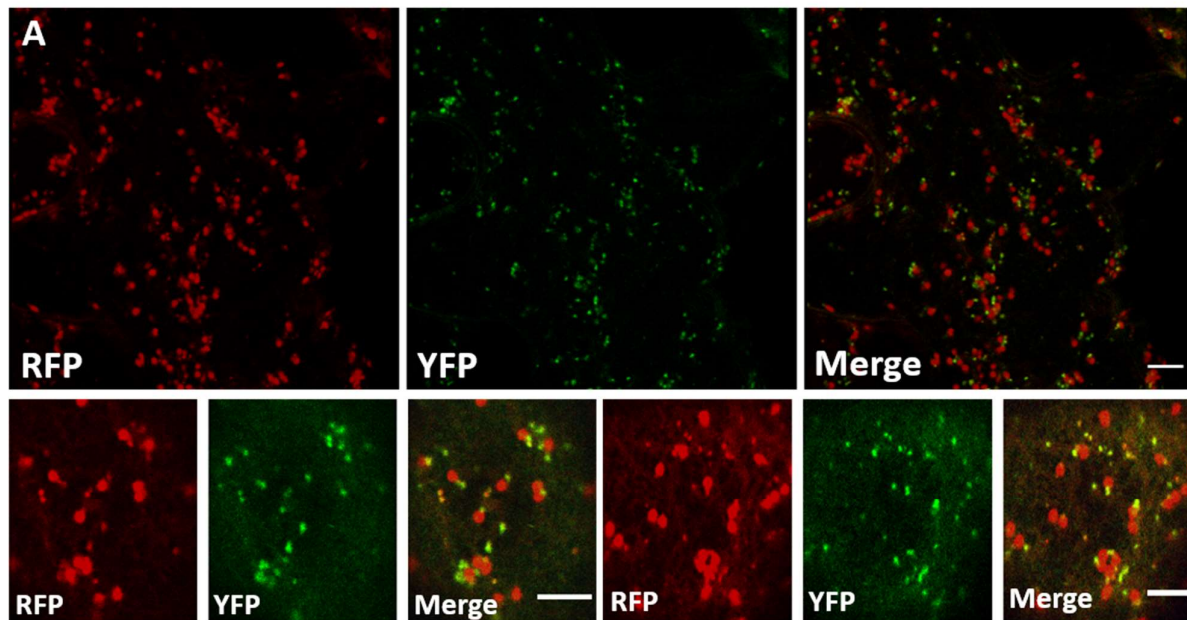
Figure 4.14: Schematic of RLP4/RLP4-L truncations used in transient BiFC assays in *N. tabacum*

When co-expressed with p35s:: c/nYFP: RAB-A5c (c/nYFP: RAB-A5c) in *N. tabacum*, RFP signal from truncated RLP4/4-L variants secRFP: RLP4/RLP4-L: n/cYFP labelled a series of intracellular compartments of varying sizes but not the PM of leaf epidermal cells (Figures 4.15 & 4.16). YFP signal was also present, indicating a possible direct interaction between the truncated RLP4/4-L variants secRFP: RLP4/RLP4-L: n/cYFP and c/nYFP: RAB-A5c. Interestingly, this YFP signal labelled smaller intracellular compartments that co-localised with a subset of the compartments labelled by

the RFP signal from secRFP: RLP4/RLP4-L trunc: n/cYFP (Figures 4. 15 & 4.16, white arrows). YFP signal was also noticeably absent from larger intracellular compartments labelled by secRFP: RLP4/RLP4-L trunc: n/cYFP (Figures 4.15 & 4.16). The possible direct interactions between truncated RLP4/4-L variants secRFP: RLP4/RLP4-L trunc: n/cYFP and c/nYFP: RAB-A5c therefore seems to occur at only a specific subset of the compartments labelled by truncated RLP4/4-L in leaf epidermal cells of *N.tabacum*.

To control for possible fluorophore cleavage and spontaneous YFP assembly, truncated RLP4/4-L and RAB-A5c BiFC constructs were co-expressed with free split-YFP variants. When co-expressed with p35S:: c/nYFP, secRFP: RLP4/RLP4-L trunc: n/cYFP gave no appreciable YFP fluorescence (Figure 4.17A & B). YFP fluorescence was however detected when c/nYFP: RAB-A5c were co-expressed with pUB:: n/cYFP (Figure 4.17C). This YFP fluorescence was however noticeably abundant in the cytosol, unlike that in the BiFC assays, which was exclusively compartmental. It is therefore possible that YFP variant cleavage from secRFP: RLP4/RLP4-L trunc: n/cYFP contributes to the YFP signal seen in BiFC assays between RAB-A5c and truncated RLP4/4-L variants, or that a cytosolic pool of n/cYFP: RAB-A5c can interact with free p35S:: c/nYFP. The differences between YFP localisation in these controls (Figure 4.17C) and the BiFC assays (Figures 4.15 and 4.16) indicate however that this cannot completely account for the YFP signal and pattern seen in BiFC assays. Whilst some caution must be exercised, BiFC assays indicate a possible direct interaction between RAB-A5c and truncated RLP4 and RLP4-L when expressed in *N.tabacum* leaf epidermal cells.

secRFP: RLP4 trunc: cYFP + nYFP: RAB-A5c



secRFP: RLP4 trunc: nYFP + cYFP: RAB-A5c

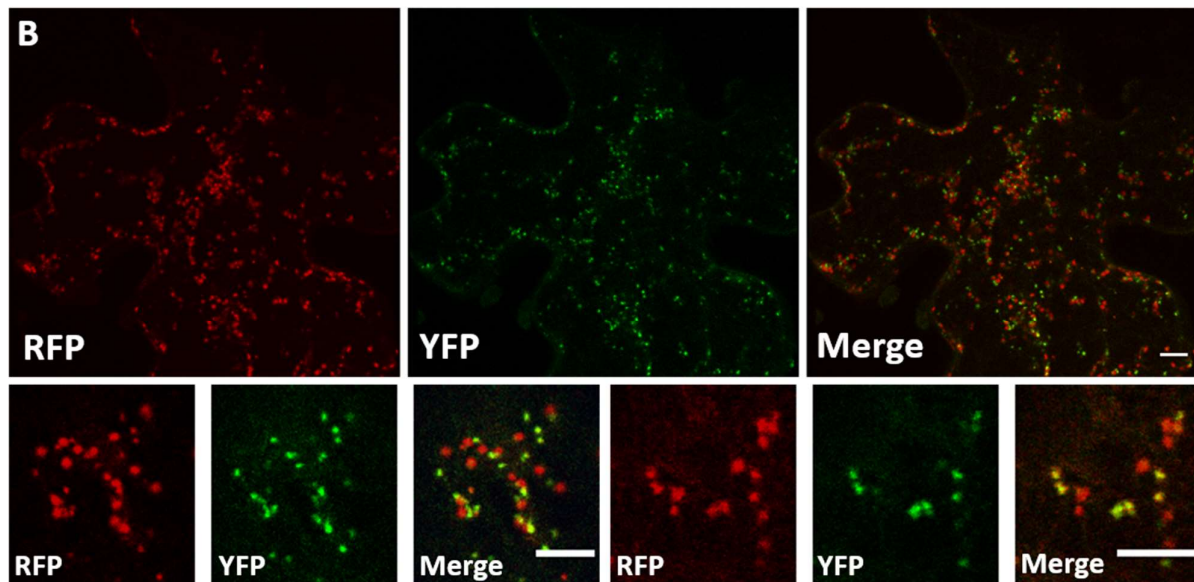
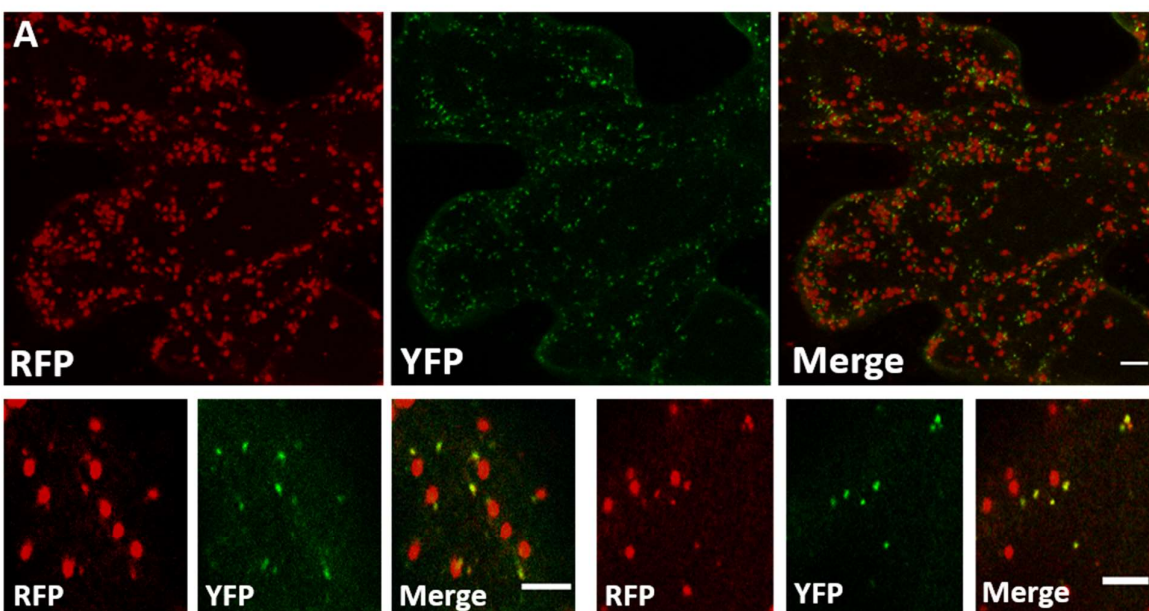


Figure 4.15: Transient BiFC assays in *N. tabacum* leaf epidermal cells indicate a possible direct interaction between RAB-A5c and truncated RLP4 at discrete subcellular foci. (A) 3D confocal stack projections and confocal slices of *N. tabacum* leaf epidermal cells co-expressing secRFP: RLP4 trunc: cYFP and nYFP: RAB-A5c. (B) 3D confocal stack projections and confocal slices of *N. tabacum* leaf epidermal cells co-expressing secRFP: RLP4 trunc: nYFP and cYFP: RAB-A5c. Infiltrated at O.D. 0.1, 48 hours. Scale bars, 5µm.

secRFP: RLP4-L trunc: cYFP + nYFP: RAB-A5c



secRFP: RLP4-L trunc: nYFP + cYFP: RAB-A5c

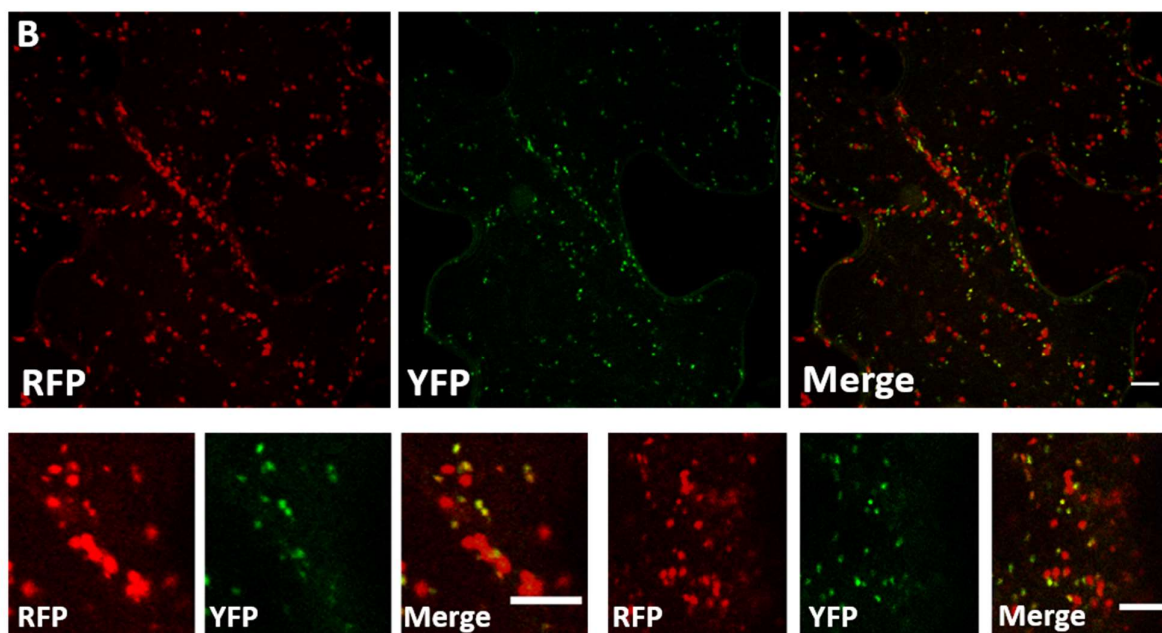


Figure 4.16: Transient BiFC assays in *N. tabacum* leaf epidermal cells indicate a possible direct interaction between RAB-A5c and truncated RLP4-L at discrete subcellular foci. (A) 3D confocal stack projections and confocal slices of *N. tabacum* leaf epidermal cells co-expressing secRFP: RLP4-L trunc: cYFP and nYFP: RAB-A5c. (B) 3D confocal stack projections and confocal slices of *N. tabacum* leaf epidermal cells co-expressing secRFP: RLP4-L trunc: nYFP and cYFP: RAB-A5c. Infiltrated at O.D. 0.1, 48 hours. Scale bars, 5µm.

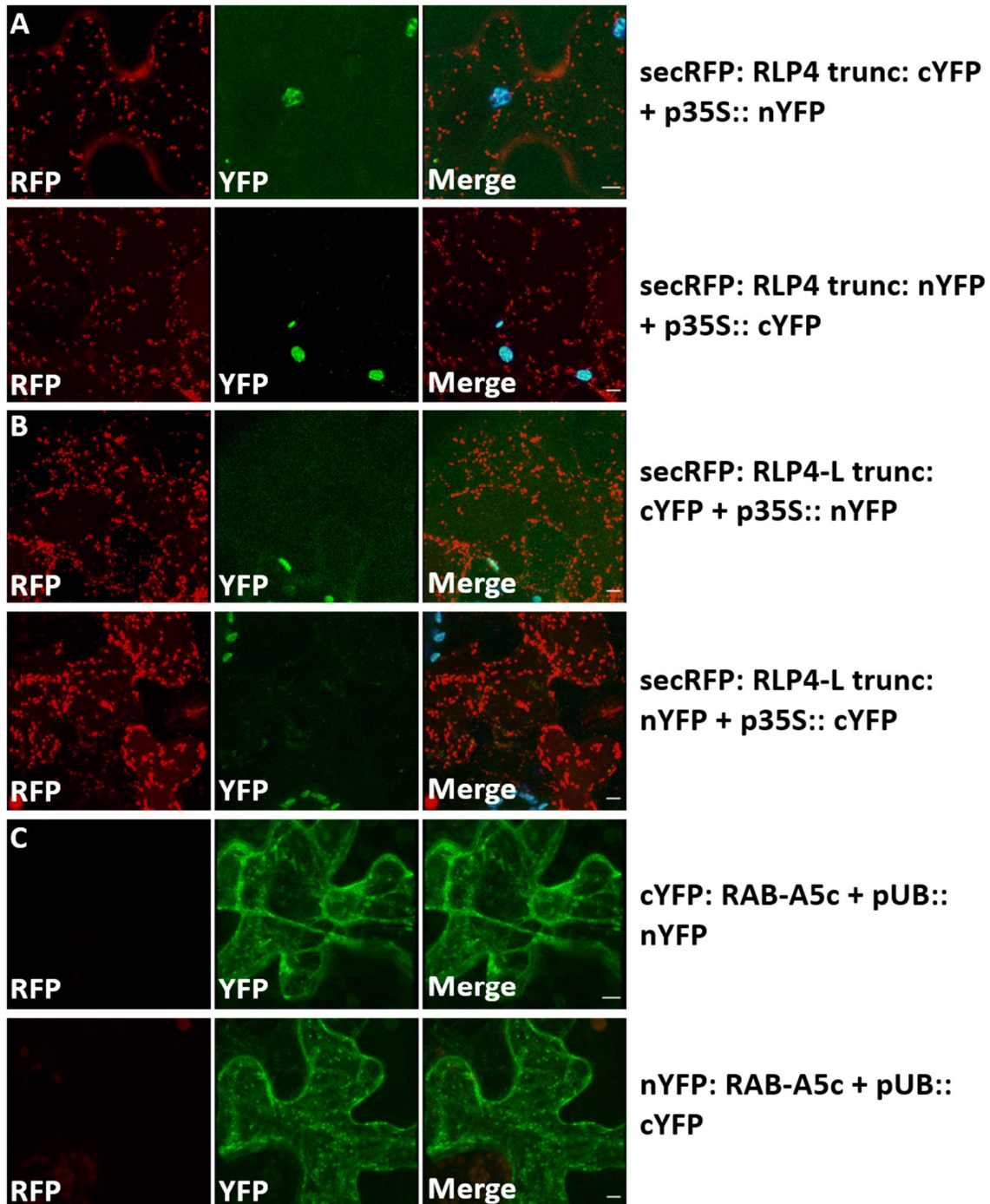


Figure 4.17: Control infiltrations for transient BiFC assays in *N. tabacum* leaf epidermal cells between RAB-A5c and truncated RLP4 & RLP4-L. (A) 3D confocal stack projections of *N. tabacum* leaf epidermal cells co-expressing secRFP: RLP4 trunc: cYFP/nYFP and p35S:: nYFP/cYFP. (B) 3D confocal stack projections co-expressing secRFP: RLP4-L trunc: cYFP/nYFP and p35S:: nYFP/cYFP. (C) 3D confocal stack projections co-expressing cYFP/nYFP: RAB-A5c and pUB:: nYFP/cYFP. Blue/green large compartments = chloroplast autofluorescence. Infiltrated at O.D. 0.1, 48 hours. Scale bars, 5µm.

Interestingly, truncated RLP4/4-L variants secRFP: RLP4/RLP4-L trunc: n/cYFP both labelled intracellular compartments of varying sizes when co-expressed with c/nYFP: RAB-A5c, with the YFP signal indicative of a direct interaction co-localising with a subset of these compartments. When co-expressed in *N.tabacum* with markers of the TGN/EE and Golgi, larger compartments labelled by secRFP: RLP4/RLP4-L trunc co-localised with the Golgi-maker ST: YFP (Figure 4.18A). By contrast, the smaller subset of compartments labelled by secRFP: RLP4/RLP4-L trunc co-localised with YFP: RAB-A2a, a marker of TGN/EE identity in *Arabidopsis*, which did not label the larger compartments (Figure 4.18B). This indicates that the possible direct interaction between split YFP-tagged RAB-A5c and truncated RLP4/4-L may occur specifically at compartments with TGN/EE identity, despite the truncated RLP4/4-L variants secRFP: RLP4/RLP4-L trunc also labelling additional compartments in *N.tabacum* leaf epidermal cells.

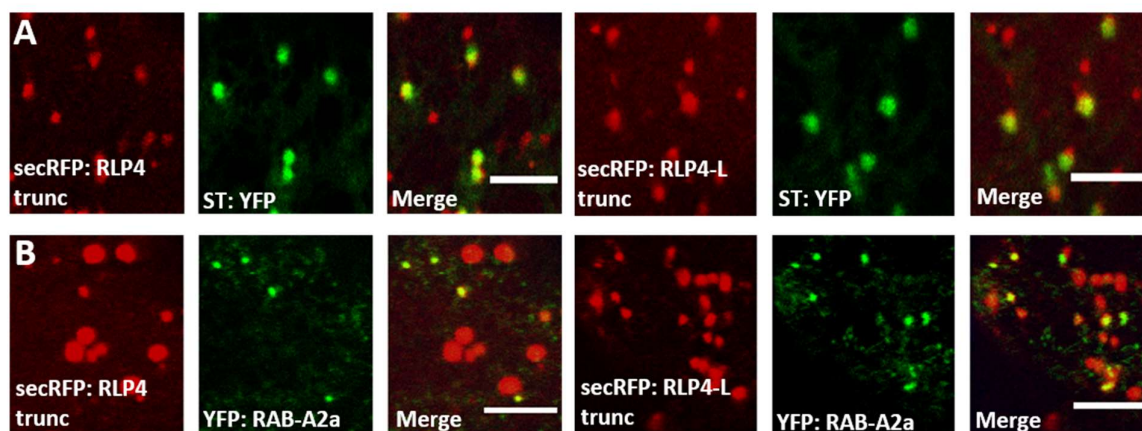


Figure 4.18: Truncated variants of RLP4 and RLP4-L label compartments which co-localise with Golgi and TGN/EE markers when transiently expressed in *N.tabacum* leaf epidermal cells. (A, B) confocal slices of *N.tabacum* leaf epidermal cells co-expressing secRFP: RLP4 trunc (left) or secRFP: RLP4-L trunc (left) and ST: YFP (middle). (B) confocal slices co-expressing secRFP: RLP4 trunc (left) or secRFP: RLP4-L trunc (left) and YFP: RAB-A2a (middle). Infiltrated at O.D. 0.1, 48 hours. Scale bars, 5µm.

4.H: Summary & Discussion

In this chapter, I have shown that fluorescently-tagged ECDs of RLP4 and RLP4-L associate with the cell wall in *Arabidopsis* lateral roots, and truncations of RLP4 and RLP4-L lacking ECDs localise to the Golgi and TGN/EE. Fluorescently-tagged truncated RLP4-L, and to a lesser extent truncated RLP4, re-localise to the PM upon possible disruption of endocytosis, indicating that they may cycle rapidly between the cell surface and late secretory compartments rather than arresting within the secretory pathway. Fluorescently-tagged truncations of RLP4 and RLP4-L lacking their intracellular domains also do not label cell edge compartments, indicating that the full-length RLP4 and RLP4-L sequences are required for their steady-state localisation to cell edge compartments. Fluorescently-tagged membrane-associated ECDs of RLP4 and RLP4-L, whilst lacking their intracellular domains, are however polarised within the PM despite not localising to YFP: RAB-A5c-labelled cell edge compartments. Moreover, transient BiFC assays indicate that split YFP-tagged RAB-A5c may be capable of direct interaction with the split YFP-tagged intracellular domains of RLP4 and RLP4-L, and that this interaction may specifically occur at the TGN/EE.

One of the most interesting observations made in this chapter is that RLP4 ECD MA: RFP and RLP4-L ECD MA: RFP, which lack the endogenous intracellular domains of RLP4 and RLP4-L, are capable of polarisation within the PM despite lacking their intracellular domains. Two possible scenarios may explain these observations. The first of these is that RLP4 ECD MA: RFP and RLP4-L ECD MA: RFP are secreted in a polarised manner to the PM. Such a scenario would assume that RLP4 and RLP4-L's N-terminal sequences are at least partially capable of organising RLP4 ECD MA: RFP and RLP4-L ECD MA: RFP into a polarised trafficking pathway, possibly through interaction with other intact trafficking cargoes. Despite not labelling cell edge compartments it is possible that such a polarised trafficking pathway is edge-directed trafficking. The labelling of cell edge compartments by full length fluorescently-tagged RLP4 and RLP4-L presumably reflects a level of retention of these proteins in cell edge compartments before trafficking to the PM. It is possible that the truncated

RLP4 ECD MA: RFP and RLP4-L ECD MA: RFP proteins may be secreted via edge-directed trafficking but rapidly transit through cell edge compartments and, as a consequence, do not visibly label them. Alternatively, RLP4 ECD MA: RFP and RLP4-L ECD MA: RFP may not enter edge-directed trafficking or another polarised pathway and instead enter bulk secretory traffic, but later selectively associate with an unidentified polarised factor in the PM or cell wall. This scenario would assume asymmetric removal of RLP4 ECD MA: RFP and RLP4-L ECD MA: RFP from the PM due to association with such a polarised factor. In order to investigate whether polarisation of RLP4 ECD MA: RFP and RLP4-L ECD MA: RFP in the PM is due to trafficking via edge-directed trafficking or association with a RAB-A5c-dependent cell-surface factor, RLP4 ECD MA: RFP and RLP4-L ECD MA: RFP will be introduced into backgrounds expressing RAB-A5c[N125I] in future work.

If the apparent polarisation of RLP4 ECD MA: RFP and RLP4-L ECD MA RFP in the PM is due to these truncations being uniformly secreted to the PM and associating with a polarised factor in the PM, one would assume that this polarisation is maintained by differential endocytosis across the cell face. As RLP4 ECD MA: RFP and RLP4-L ECD MA RFP lack their endogenous intracellular domains, it would be expected that differential endocytosis of these protein truncations across cell faces may instead depend on interactions at their ECDs, possibly with an as-yet unidentified polarised factor in the PM or cell wall. Given that the fluorescently-tagged domains of RLP4 and RLP4-L associate with the *Arabidopsis* cell wall, it is tempting to speculate that such an unidentified polarised factor that may maintain RLP4 ECD MA: RFP and RLP4-L ECD MA RFP at the PM in an polarised manner may be a polarised cell wall component through which RLP4 and RLP4-L associate via their ECDs. Full-length pUB:: RLP4: RFP and pUB:: RLP4-L: RFP are edge-restricted within the PM compared to evenly-distributed YFP: NPSN12, and are also polarised toward to outer lateral surface. Whether RLP4 ECD MA: RFP and RLP4-L ECD MA RFP are also edge-restricted is unclear however, they do appear to be enriched at the outer lateral surface, albeit to differing degrees. Quantitative investigation of whether RLP4 ECD MA: RFP and RLP4-L ECD MA RFP are also edge-restricted and the degree to which they are laterally-polarised has been initiated by introducing RLP4 ECD MA: RFP and RLP4-L

ECD MA RFP into backgrounds expressing YFP: NPSN12. If RLP4 ECD MA: RFP and RLP4-L ECD MA RFP are indeed quantifiably edge-restricted compared to YFP: NPSN12, this raises the interesting possibility that the ECDs of RLP4 and RLP4-L associate with a cell wall component that is both edge-restricted and polarised to the outer lateral surface of *Arabidopsis* lateral root cells. In support of this, RLP4 ECD: RFP and RLP4-L ECD: RFP remain associated with the cell wall after plasmolysis of *Arabidopsis* lateral roots whereas secRFP does not. Cell-wall associated RLP4 ECD: RFP and RLP4-L ECD: RFP remain polarised after plasmolysis, appearing enriched at the edges and corners of the outer lateral surface. It is unclear however whether polarisation is a specific property of the ECDs of RLP4 and RLP4-L, and therefore indicative of association with a polarised cell wall component, or also a feature of other cell-wall associated proteins after plasmolysis. Uniquely amongst RLPs, both RLP4 and RLP4-L contain Malectin-Like Domains (MLDs) in their ECDs, providing a candidate domain through which interaction of RLP4 and RLP4-L with the cell wall may occur.

Malectin is highly conserved in metazoans and is a di-glucose binding protein, recognising specific glycans in the endoplasmic reticulum of *Xenopus* (Schallus et al., 2008). MLDs in plants consist of two adjacent protein domains each with limited homology to Malectin and are found in many RLKs, including those implicated in sensing of cell wall composition and integrity such as THE1, FER and ANX1/2 (Hematy et al., 2007; Boisson-Dernier et al., 2009; Miyazaki et al., 2009; Guo et al., 2009; Deslauriers and Larsen, 2010; Feng et al., 2018; reviewed by Wolf, 2017). The MLD of FER binds the pectin-component polygalacturonic acid *in vitro* (Feng et al., 2018) however, other work has however questioned whether MLDs are direct carbohydrate-binding protein domains in plants. Crystal structures of the ECDs of CrRLK1 proteins ANX1/2 show that the MLDs of these proteins do not have the diglucose-binding structural regions present in metazoan Malectin, nor did they contain known carbohydrate-interacting structures found in bacterial carbohydrate-binding proteins (Du et al., 2018; Moussu et al., 2018). Moreover, the ANX1 ECD could not be found to bind any commercially-available cell wall carbohydrates *in vitro* (Moussu et al., 2018). Both crystal structures for ANX1 and ANX2 MLDs identify a cleft between the two Malectin-like regions within the MLD, and

suggest based on its properties that this may be a binding site for a proteinaceous ligand, rather than a carbohydrate ligand (Du et al., 2018; Moussu et al., 2018). Moussu and colleagues do however state that it is possible that MLDs contain an as-yet unknown carbohydrate binding structural site acquired by plants, and this remains an open question. They also specifically note the presence of mobile loop regions in the MLD of ANX1, and that known carbohydrate-binding regions of microbial carbohydrate-binding proteins also contain such mobile loops (Moussu et al., 2018). The MLD-containing ECD of FER also interacts with LRR-extensins, forming a module that controls vacuolar expansion in growing cells (Dünser et al., 2019). In this case, the authors propose that the cell-wall binding properties of FER's ECD may arise from interaction with these cell-wall binding expansins at the MLD cleft, rather than through direct MLD carbohydrate-binding (Dünser et al., 2019). Whether some, or all, MLDs directly bind carbohydrates or bind other directly cell-wall binding proteins therefore remains an open question. Investigation of the cell wall binding properties of the ECDs and MLDs of RLP4 and RLP4-L and the *in vivo* distribution of major cell wall components in *Arabidopsis* lateral root epidermal cells will be the subject of future investigation.

secRFP: RLP4 trunc and secRFP: RLP4-L trunc, which lack the endogenous ECDs of RLP4 and RLP4-L, do not localise to cell edge compartments or PM, instead labelling the TGN/EE and Golgi to differing degrees. Treatment with BFA and pharmacological inhibitors of endocytosis partially re-localises secRFP: RLP4-L trunc to the PM, whilst treatment with BFA also partially re-localises secRFP: RLP4 trunc to the PM. This supports a scenario in which, rather than arresting at the TGN/EE and Golgi, secRFP: RLP4 trunc and secRFP: RLP4-L trunc may cycle rapidly between the PM and late secretory compartments but only become visible at the PM upon inhibition of endocytosis. This indicates that the ECDs of RLP4 and RLP4-L are required for their stable presence at the cell surface and that the cell-wall binding ECDs of RLP4 and RLP4-L may modulate the rate of endocytosis of these proteins from the PM. Such a negative effect on internalisation of the ECDs of RLP4 and RLP4-L aligns well with the possibility that RLP4/RLP4-L ECD MA: RFP may be maintained at the PM in a polarised manner through association with a polarised factor in the PM or cell wall through the ECDs of RLP4

and RLP4-L. Whilst both secRFP: RLP4 trunc and secRFP: RLP4-L trunc are exclusively intracellularly-localised compared to full-length RLP4 and RLP4-L, their localisation patterns differed subtly. secRFP: RLP4-L trunc seems to localise more to the TGN/EE, whilst secRFP: RLP4 trunc is shifted towards the Golgi. The reason for this difference remains unknown and may reflect as-yet unknown differences in trafficking of RLP4 and RLP4-L. I also noticed however that lines expressing secRFP: RLP4 trunc were universally dimmer than those expressing secRFP: RLP4-L trunc, raising the possibility that the slightly differential localisation patterns of secRFP: RLP4 trunc and secRFP: RLP4-L trunc is a consequence of this. Moreover, the suitability of Tyrphostin A23 as an inhibitor of endocytosis has been questioned recently by work indicating that its inhibitory effects are a secondary consequence of cytoplasmic acidification (Dejonghe et al., 2016). Endosidin9-17 (ES9-17) has however recently been identified as a specific inhibitor of clathrin-mediated endocytosis, which targets the clathrin heavy chain without causing cytoplasmic acidification (Dejonghe et al., 2019). Whether ES9-17 is suitable for use in *Arabidopsis* lateral roots and if it can also trigger re-localisation of RLP4-L trunc: RFP to the PM remains to be seen.

Interestingly, BiFC assays indicated a possible direct interaction *in planta* between split YFP-tagged RAB-A5c and C-terminal regions of RLP4/RLP4-L that may occur specifically at the TGN/EE, despite secRFP: RLP4 trunc and secRFP: RLP4-L trunc also labelling other endomembrane compartments in *N. tabacum*. Criticisms of BiFC include a high rate of false positive indications of direct interactions and that it may produce alterations in endomembrane organisation (Horstman et al., 2014; Tao et al., 2019). The latter has however been focussed on studies investigating interactions between permanently membrane-associated proteins (Tao et al., 2019). BiFC is therefore best used as a complement to other methods of investigating protein-protein interactions. Indeed, control experiments conducted here indicate a degree of interaction between n/cYFP: RAB-A5c and p35S:: n/cYFP. Whilst this is unlikely to fully account for the spatially-restricted YFP signal seen in the BiFC assays, additional controls to further strengthen the data presented here could include co-expression of secRFP: RLP4/-L trunc: n/cYFP with putatively-inactive n/cYFP: RAB-A5c[S26N] or of

n/cYFP: RAB-A5c with secRFP: RLP44 trunc. Future consideration of the possible direct interaction between split YFP-tagged RLP4/RLP4-L and RAB-A5c should also complement the approach taken here with an alternative method testing for a direct interaction. Confirmation of the nature of the interaction between RAB-A5c, RLP4 and RLP4-L is likely to be of high interest in future functional characterisation of RLP4 and RLP4-L, as a direct interaction between RAB-A5c and RLP4/RLP4-L would imply that trafficking of RLP4 and RLP4-L is directly dependent on and proportional to activity of RAB-A5c. Such a scenario would indicate that RAB-A5c is tightly-associated with the functional organisation and output of edge-directed trafficking, rather than simply being a supporting trafficking component in this pathway

Finally, data presented in this chapter further demonstrates that the trafficking dynamics and subcellular distribution of RLP4: RFP and RLP4-L: RFP are distinct from those of RLP44: RFP, which is believed to function in brassinosteroid-mediated sensing of cell wall integrity, and responses to integrity disruption (Wolf et al., 2014). Edge-directed trafficking has been proposed as a possible route for local modification of the cell wall (Kirchhelle et al., 2016, 2019) and the fluorescently-tagged RLP4/RLP4-L ECDs of are cell wall associated, indicating that RLP4 and RLP4-L may occupy a cell wall related function within edge-directed trafficking. The noticeable differences between trafficking and subcellular distribution of fluorescently-tagged RLP4/RLP4-L and RLP44 and their truncations demonstrate that the observations made here for trafficking of RLP4 and RLP4-L are not universal features of cell-wall associated RLPs, and may indeed be linked to specialised functions of RLP4 and RLP4-L.

Chapter 5: Perturbation of trafficking of YFP: RAB-A5c, RLP4: RFP and RLP4-L: RFP provides further insights into organisation and possible regulation of edge-directed trafficking

5.A: Introduction

The discovery that fluorescently-tagged RLP4 and RLP4-L label cell edge compartments and the PM and that this localisation is disrupted by expression of RAB-A5c[N125I] demonstrates the existence of an edge-directed pathway to the cell surface that is dependent on RAB-A5c function. As well as providing a foundation for greater functional understanding of edge-directed trafficking during morphogenesis, fluorescently-tagged RLP4 and RLP4-L also provide the first opportunity to visualise the dynamics of this trafficking pathway in the absence of YFP: RAB-A5c. Through use of Rab dominant-negative mutant variants and pharmacological agents, Kirchhelle and colleagues proposed that RAB-A5c defines a pathway from the TGN/EE to the cell surface via cell edge compartments, and that this pathway is dependent on both the actin and microtubule cytoskeletons (Kirchhelle et al., 2016). Localisation of RLP4: RFP/RLP4-L: RFP to the TGN/EE, cell edge compartments and to the PM in a polarised manner supports these previous predictions that edge-directed trafficking arises from the TGN/EE and is a polarised route to the cell surface. Moreover, RLP4 and RLP4-L are permanently membrane-associated, whereas RAB-A5c cycles between a cytosolic and membrane-associated state. RLP4 and RLP4-L may therefore allow for investigation of trafficking dynamics of edge-directed trafficking upstream and downstream of the points of RAB-A5c membrane-association and membrane-dissociation. RLP4 and RLP4-L presumably pass through the Golgi prior to the TGN/EE and entry into RAB-A5c-defined edge-directed trafficking, and so it is possible that they are dependent on other trafficking regulators prior to association with RAB-A5c. Other Rab-A GTPases are suspected to be regulators of trafficking to and from the TGN/EE, raising the possibility that

edge-directed trafficking may be dependent on the function of Rab-A GTPases other than RAB-A5c. Such dependence could conceivably arise from other Rab-A GTPases underpinning essential TGN/EE functions upstream of RAB-A5c-defined edge-directed trafficking. Alternatively, Rab-A GTPases may function as a part of a Rab cascade in which upstream Rab GTPases recruit interaction factors essential for activation of downstream Rab GTPases.

I therefore examined the effects of perturbations of the endomembrane and cytoskeletal systems on trafficking of RLP4: RFP and RLP4-L: RFP, in comparison with YFP: RAB-A5c, in order to gather new insights into the regulation of edge-directed trafficking. I also investigated the effects of disruption of other Rab GTPases on edge-directed trafficking of RLP4: RFP, RLP4-L: RFP and YFP: RAB-A5c in order to determine the possible dependence on edge-directed trafficking on function of other potentially upstream-acting Rab GTPases.

5.B: Edge-directed trafficking is sensitive to pharmacological disruption of the endomembrane and cytoskeletal systems

Kirchhelle and colleagues previously described the effect of pharmacological disruptors of the endomembrane and cytoskeletal systems on YFP: RAB-A5c (Kirchhelle et al., 2016). In order to assess the extent of the similarities of responses between previously-known and newly-identified components of edge-directed trafficking to such inhibitors, lines co-expressing YFP: RAB-A5c and pUB:: RLP4: RFP or pUB:: RLP4-L: RFP were used to investigate the effect of pharmacological disruptions of the endomembrane and cytoskeletal systems on edge-directed trafficking.

As already discussed, treatment with the mycotoxin Brefeldin-A (BFA) inhibits recycling traffic to the PM in *Arabidopsis*, and cause aggregations of the *Arabidopsis* TGN/EE into 'BFA-bodies'. BFA treatment abolishes localisation of YFP: RAB-A5c to cell edge compartments and re-localises it to BFA-bodies (Kirchhelle et al., 2016). Treatment with BFA also disrupts RLP4: RFP and RLP4-L: RFP localisation to cell edge compartments, re-localising them to BFA-bodies where they co-localise with YFP: RAB-A5c (Figure 5.1A & B). Some pUB:: RLP4: RFP and pUB:: RLP4-L: RFP however persisted at the PM after treatment with BFA, even when localisation to cell edge compartments was completely abolished (Figure 5.1A & B, white arrows). Concanamycin-A (ConcA) is also often employed as a pharmacological inhibitor of TGN/EE function in *Arabidopsis*, through its action as an inhibitor of V-ATPase activity (Dröse et al., 1993; Huss et al., 2002; Dettmer et al., 2006; Viotti et al., 2010; Luo et al., 2015). Treatment with ConcA abolished localisation of both YFP: RAB-A5c and pUB:: RLP4: RFP/RLP4-L: RFP to cell edge compartments, re-localising them to large intracellular aggregates (Figure 5.1C & D). Interestingly, the intracellular aggregates labelled by YFP: RAB-A5c and pUB:: RLP4: RFP/RLP4-L: RFP after treatment with ConcA did not appear to completely overlap.

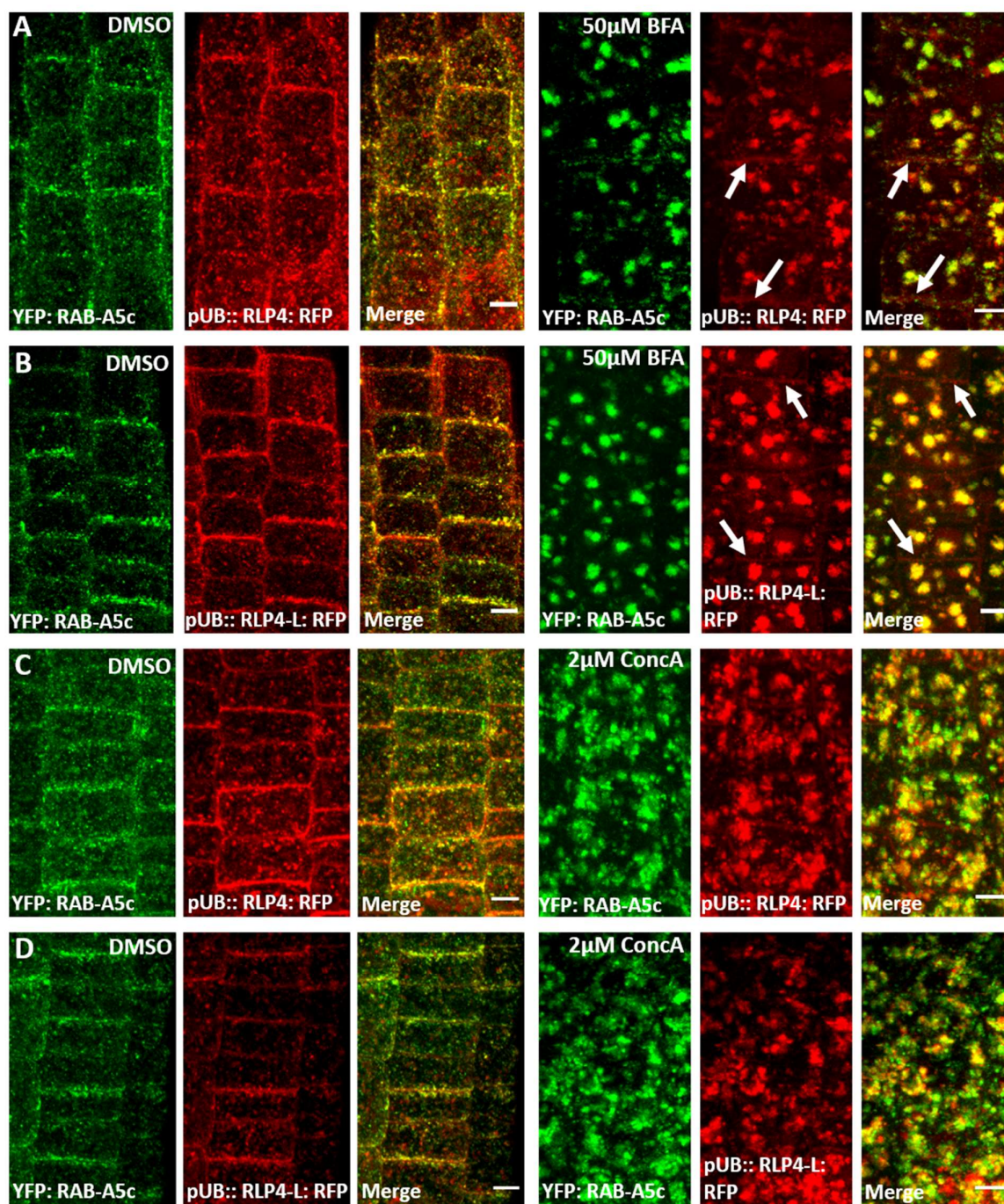


Figure 5.1: Treatment with Brefeldin-A and Concanamycin-A abolishes pUB:: RLP4: RFP and pUB: RLP4-L: RFP localisation to cell edge compartments. (A, B) 3D confocal stack projections of lateral roots meristematic cells co-expressing YFP: RAB-A5c (left) and pUB:: RLP4: RFP (A, middle) or pUB:: RLP4-L: RFP (B, middle). White arrows = PM. 50 μM BFA or equivalent volume, 30 minutes. (C,D) 2 μM ConcA or equivalent volume DMSO, 90 minutes. All from 10 day old T2 seedlings. Scale bars, 5 μm.

YFP: RAB-A5c localisation to cell edge compartments is also sensitive to pharmacological disruption of both actin and microtubules, through treatment with Latrunculin-B (LatB) and Oryzalin respectively (Kirchhelle et al., 2016). Oryzalin abolished both YFP: RAB-A5c and pUB:: RLP4: RFP/RLP4-L: RFP localisation to cell edge compartments, resulting in their intracellular accumulation. Some RLP4: RFP/RLP4-L: RFP remained at the PM following oryzalin treatment (Figure 5.2B). Strikingly, treatment with Oryzalin re-localised YFP: RAB-A5c to the PM in many affected cells, which had not been previously reported (Figure 5.2C, white arrows; Kirchhelle et al., 2016). Re-localisation of YFP: RAB-A5c to the PM in cells clearly penetrated by Oryzalin was not however universally seen. This may indicate that this re-localisation may be dependent on the degree of Oryzalin penetration or on other factors such as the developmental stage of the cell. LatB treatment abolished localisation to cell edge compartments and the PM of both pUB::RLP4:RFP and pUB::RLP4-L:RFP (Figure 5.2D). Whether complete abolition of pUB::RLP4:RFP and pUB::RLP4-L:RFP localisation to the PM by LatB is due to a distinct requirement for actin in RLP4 and RLP4-L localisation to the PM or due to greater penetration of LatB compared to Oryzalin is unclear. Interestingly however, treatment with LatB did not re-localise YFP: RAB-A5c to the PM, indicating that this re-localisation is specific to disruption of microtubules. pUB:: RLP4: RFP and pUB:: RLP4-L: RFP localisation is therefore sensitive to both disruption of secretory and recycling traffic, as well as to disruption of the cytoskeleton, in a manner broadly similar to YFP: RAB-A5c.

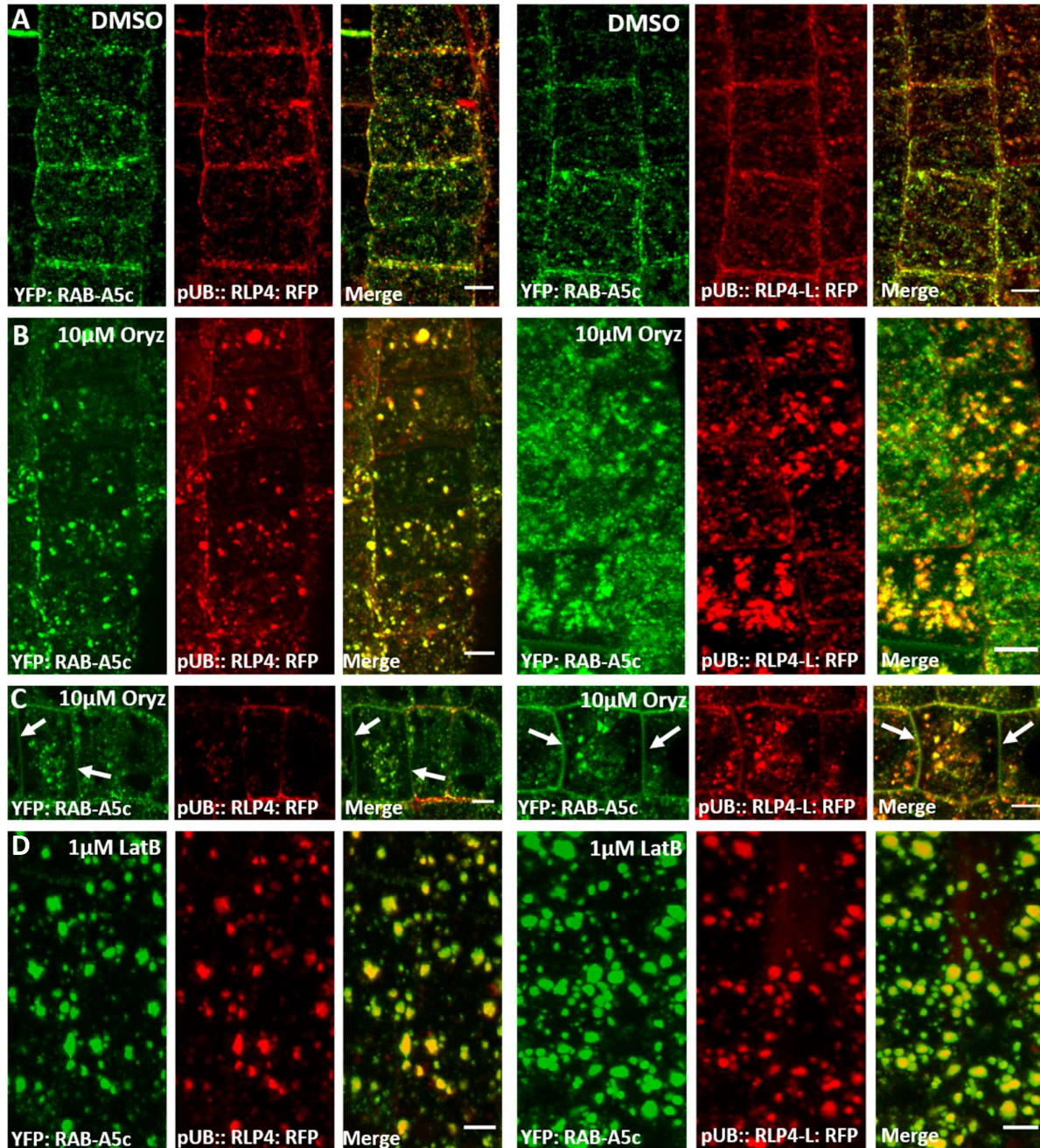


Figure 5.2: Disruption of the actin and microtubule cytoskeleton inhibits pUB:: RLP4: RFP and pUB:: RLP4-L: RFP localisation to cell edge compartments, but effects YFP: RAB-A5c localisation differentially. (A, B) 3D confocal stack projections of lateral root meristematic cells co-expressing YFP: RAB-A5c (left) and pUB:: RLP4: RFP or pUB:: RLP4-L: RFP (middle). (C) higher magnification confocal slices. (D) 3D confocal stack projections. DMSO, 180 minutes (A). 10µM Oryzalin, 180 minutes (B, C). 1µM Latrunculin-B (LatB), 180 minutes (D). All from 10 day old T2 seedlings. Scale bars, 5µm. White arrows = YFP: RAB-A5c re-localisation to the PM.

Investigating the specificity of RAB-A5c[N125I]-driven inhibition of edge-directed trafficking

Expression of RAB-A5c[N125I] re-localises fluorescently-tagged edge-directed trafficking components RAB-A5c, RLP4 and RLP4-L away from the cell surface to a predominantly intracellular localisation pattern, indicating that this trafficking pathway is dependent on RAB-A5c function. Whilst expression of RAB-A5c[N125I] does not affect bulk exocytic or endocytic trafficking (Kirchhelle et al., 2016), I further sought to test the specificity of inhibition edge-directed trafficking by RAB-A5c[N125I] expression by investigating whether expression of dominant negative mutant variants of other TGN/EE-localised Rab-A GTPases could disrupt the trafficking of RLP4 and RLP4-L. For this, I chose to use dominant negative mutant variants of two of the most widely-studied members of the Rab-A clade, RAB-A2a and RAB-A4b. Expression of dominant negative RAB-A2a results in cytokinesis defects as well as broadly stunted growth (Chow et al., 2008), and RAB-A4b and its effectors have been implicated in maintenance of polarised growth of *Arabidopsis* root hairs (Preuss et al., 2004; Preuss et al., 2006) however, specific defects in root anisotropic growth like those produced by AtRPS5a>DEX>A5c[N125I] expression have not been linked to inhibition of either RAB-A2a or RAB-A4b.

5.C: Edge-directed trafficking is sensitive to expression of dominant-negative mutant variants of the Rab-A GTPases RAB-A2a and RAB-A4b

I crossed plant lines expressing dominant-negative mutants of RAB-A2a (AtRPS5a>DEX>A2a[N125I]) or Rab-A4b (AtRPS5a>DEX>A4b[N125I]) with plants co-expressing YFP: RAB-A5c and pUB:: RLP4: RFP or pUB:: RLP4-L: RFP. 72 hours after induction of AtRPS5a>DEX>A2a[N125I] and AtRPS5a>DEX>A4b[N125I], YFP: RAB-A5c edge compartment localisation was completely lost in lateral roots, localising to the cytosol as well as intracellular structures (Figures 5.3 and 5.4). Localisation of pUB:: RLP4: RFP and pUB:: RLP4-L: RFP to cell edge compartments was also abolished in lateral roots after 72 hours of expression of both AtRPS5a>DEX>A2a[N125I] and

AtRPS5a>DEX>A4b[N125I] and their localisation to the PM was also noticeably much reduced (Figures 5.3 and 5.4), with localisation to intracellular structures predominating. After 72 hours of RAB-A2a[N125I] and RAB-A4b[N125I] expression, extensive cytokinesis defects including multinucleate cells, incomplete and floating cell walls were present in lateral roots. Cell death was also evident in many cases. In order to reduce the likelihood that disruption of YFP: RAB-A5c, RLP4: RFP and RLP4-L: RFP localisation by expression of AtRPS5a>DEX>A2a[N125I] and AtRPS5a>DEX>A4b[N125I] may be a secondary consequence of serious cytokinesis defects or cell death, I examined the localisations of YFP: RAB-A5c, pUB:: RLP4: RFP and pUB:: RLP4-L: RFP after the shorter expression induction period of 24 hours. Removal of YFP: RAB-A5c, pUB:: RLP4: RFP and pUB:: RLP4-L: RFP from cell edge compartments and the cell periphery also occurred after 24 hours of expression of AtRPS5a>DEX>A2a[N125I] and AtRPS5a>DEX>A4b[N125I] (Appendix 2, Figures 6 & 7).

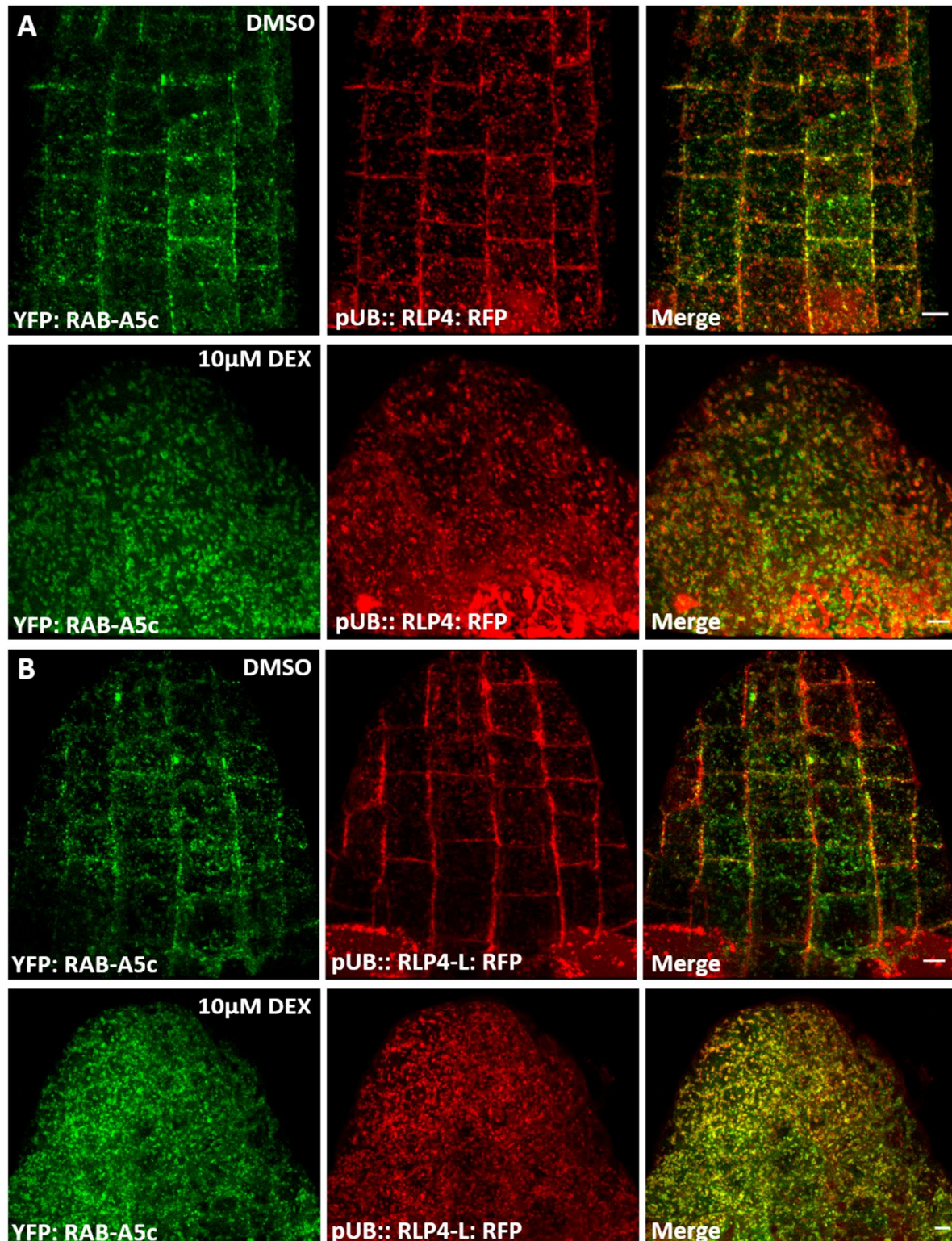


Figure 5.3: Expression of AtRPS5a>DEX>A2a[N125I] disrupts localisation of YFP: RAB-A5c, pUB:: RLP4: RFP and pUB:: RLP4-L: RFP in lateral roots. (A, B) 3D confocal stack projections of lateral roots co-expressing YFP: RAB-A5c (left) and pUB:: RLP4: RFP (A, middle) or pUB:: RLP4-L: RFP (B, middle) and AtRPS5a>DEX>A2a[N125I]. 10μM DEX or equivalent volume DMSO, 72 hours. All from 10 day old F3 seedlings. Scale bars, 5μm.

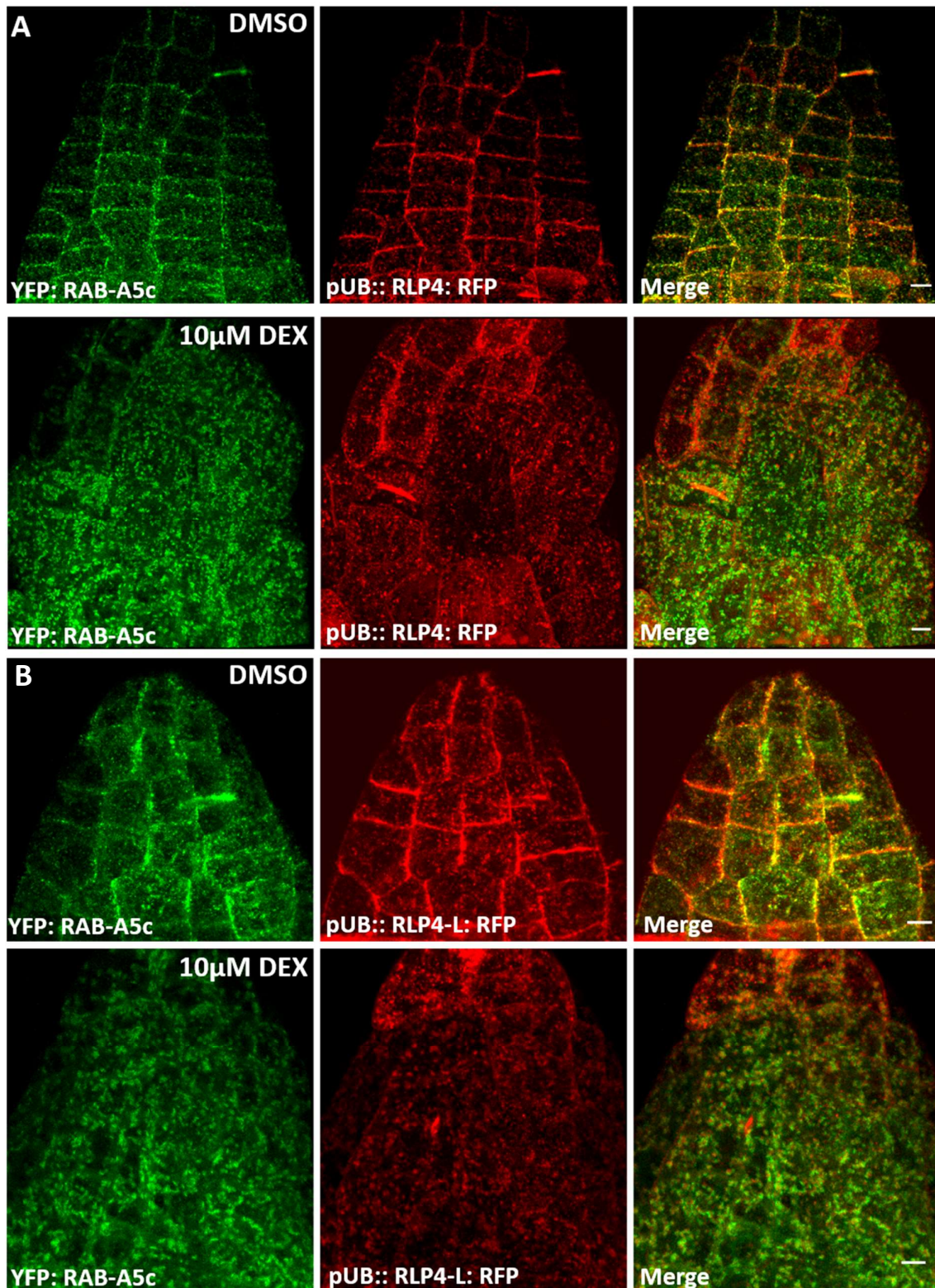


Figure 5.4: Expression of *AtRPS5a*>*DEX*>*A4b*[N125I] disrupts localisation of YFP::RAB-A5c, pUB::RLP4:RFP and pUB::RLP4-L:RFP in lateral roots. (A, B) 3D confocal stack projections of lateral roots co-expressing YFP::RAB-A5c (left) and pUB::RLP4:RFP (A, middle) or pUB::RLP4-L:RFP (B, middle) and *AtRPS5a*>*DEX*>*A4b*[N125I]. 10µM DEX or equivalent volume DMSO, 72 hours. All from 10 day old T2 seedlings. Scale bars, 5µm.

5.D: Edge-directed trafficking in lateral roots is not sensitive to expression of a Rab-E GTPase dominant-negative mutant

Edge-directed trafficking is therefore sensitive to disruption of the function of other Rab-A GTPases by expression of dominant negative Rab-A mutant variants. In order to examine whether disruption of edge-directed trafficking can be achieved by expression of dominant negative mutants of Rab GTPases from other clades, or whether this is specific to inhibition of Rab-A clade members, I examined the response of YFP: RAB-A5c, pUB:: RLP4: RFP and pUB:: RLP4-L: RFP to expression of a dominant-negative mutant variant of the Rab-E clade member RAB-E1d. Members of the Rab-E clade are believed to define a secretory pathway between the Golgi and the PM (Zheng et al., 2005; Camacho et al., 2009; Speth et al., 2009; Mayers et al., 2017), in contrast to the TGN/EE-to-PM pathways believed to be defined by Rab-A GTPases. Expression of RAB-E1d[N125I] does not produce a robustly-selectable phenotype in *Arabidopsis* (Niloufer Irani et al., unpublished data) and so, expression of the alternative dominant negative mutant RAB-E1d[S26N] (AtRPS5a>DEX>E1d[S26N]), which features a Serine (S) to Asparagine (N) substitution, was chosen instead. S→N mutations of Rab GTPases are predicted to stabilise their interactions with GDP, titrating out upstream interactors of the endogenous Rab GTPase and inhibiting membrane recruitment or activation (Olkkonen and Stenmark, 1997; Sohn et al., 2003; Kotzer et al., 2004; Lee et al., 2004; Chow et al., 2008). Expression of AtRPS5a>DEX>E1d[S26N] in *Arabidopsis* results particularly in stunted growth of aerial tissues, as well as stunted growth of primary roots and reduced lateral root emergence. RAB-E1d[S26N] does not however cause cytokinesis defects in roots or lateral roots (Niloufer Irani et al., unpublished data). I therefore crossed plants expressing AtRPS5a>DEX>E1d[S26N] with plants co-expressing YFP: RAB-A5c and pUB:: RLP4: RFP or pUB:: RLP4-L: RFP. Expression of AtRPS5a>DEX>E1d[S26N] for 72 hours had no effect on the localisations of YFP: RAB-A5c, pUB:: RLP4: RFP or pUB:: RLP4-L: RFP in lateral roots (Figure 5.5) indicating that expression of RAB-E1d[SN] does not affect trafficking of fluorescently-tagged RLP4 and RLP4-L in lateral roots, and that the ability to disrupt edge-directed trafficking may be confined to expression of dominant-negative mutants of Rab-A clade members.

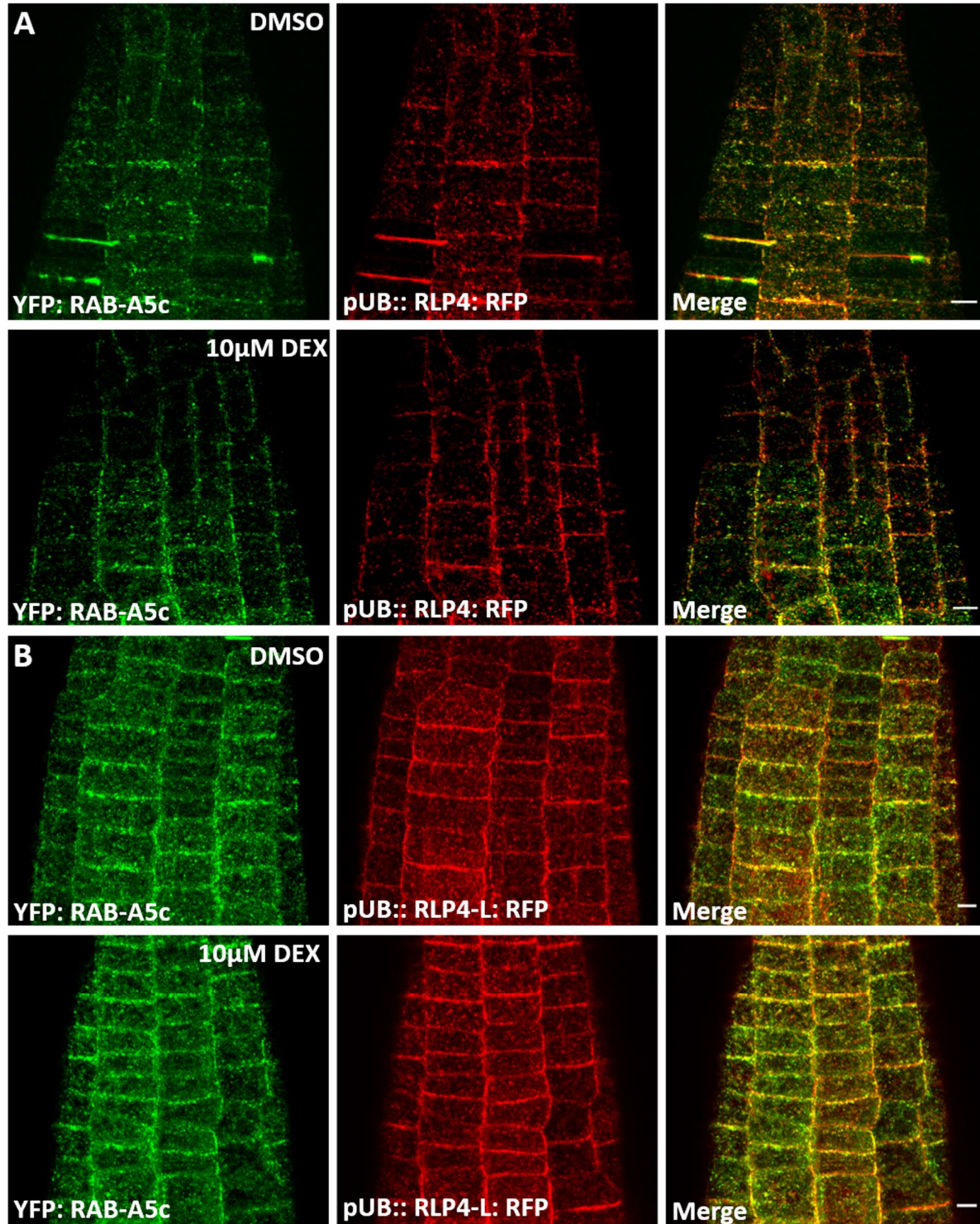


Figure 5.5: Expression of AtRPS5a>DEX>E1d[S26N] does not disrupt localisation of YFP: RAB-A5c, pUB:: RLP4: RFP and pUB:: RLP4-L: RFP in lateral roots. (A, B) 3D confocal stack projections of lateral roots co-expressing YFP: RAB-A5c (left) and pUB:: RLP4: RFP (A, middle) or pUB:: RLP4-L: RFP (B, middle) and AtRPS5a>DEX>E1d[S26N]. 10µM DEX or equivalent volume DMSO, 72 hours. All from 10 day old F3 seedlings. Scale bars, 5µm.

5.E: Dominant-negative Rab-A mutant variants disrupt the localisation of YFP: RAB-A5c during treatment with BFA

Dominant-negative mutants of Rab-A GTPases, but not a dominant-negative mutant of a Rab-E GTPase, are therefore capable of mis-localising YFP: RAB-A5c, RLP4: RFP and RLP4-L: RFP away from cell edge compartments and the cell surface. YFP: RAB-A5c, RLP4: RFP and RLP4-L: RFP all predominantly localise to intracellular compartments after expression of Rab-A dominant-negative mutant variants. The intracellular compartments labelled by YFP: RAB-A5c and RLP4: RFP/RLP4-L: RFP upon expression of Rab-A dominant-negative mutants did not however appear to overlap completely and were morphologically different, indicating that these components of edge-directed trafficking may respond differently to expression of dominant-negative mutants of Rab-A GTPases. In order to begin to investigate this further, I treated lines co-expressing Rab-A dominant-negative mutant variants, YFP: RAB-A5c and RLP4/RLP4-L: RFP with BFA. BFA, as already discussed, causes aggregation of the TGN/EE into BFA bodies, with TGN/EE-components labelling the core of the BFA body. Golgi-localised proteins are excluded from this core, instead clustering in a characteristic pattern around the periphery of the BFA bodies (Grebe et al., 2003; Robinson et al., 2008; Chow et al., 2008; Uemura et al., 2014; Naramoto et al., 2014). After 24 hours of AtRPS5a>DEX>A5c[N125I].2, AtRPS5a>DEX>A2a[N125I] or AtRPS5a>DEX>A4b[N125I] expression, RLP4: RFP/RLP4-L: RFP labelled the centre of BFA bodies, indicating TGN/EE localisation of RLP4: RFP/RLP4-L: RFP in the presence of Rab-A dominant-negative mutant variants (Figure 5.6B & C). YFP: RAB-A5c-labelled BFA bodies however differed noticeably in morphology from those in DMSO-treated controls, and labelled structures that clustered around the periphery of the BFA bodies (Figure 5.6B & C, white arrows). This was in contrast to the DMSO control lines in which both RLP4-L: RFP and YFP: RAB-A5c uniformly labelled the core of BFA bodies (Figure 5.6B & C). This indicates that expression of Rab-A dominant-negative mutant variants mis-localise different components of edge-directed trafficking distinctly, with YFP: RAB-A5c appeared to be mis-localised to the Golgi compared to RLP4-L: RFP at the TGN/EE.

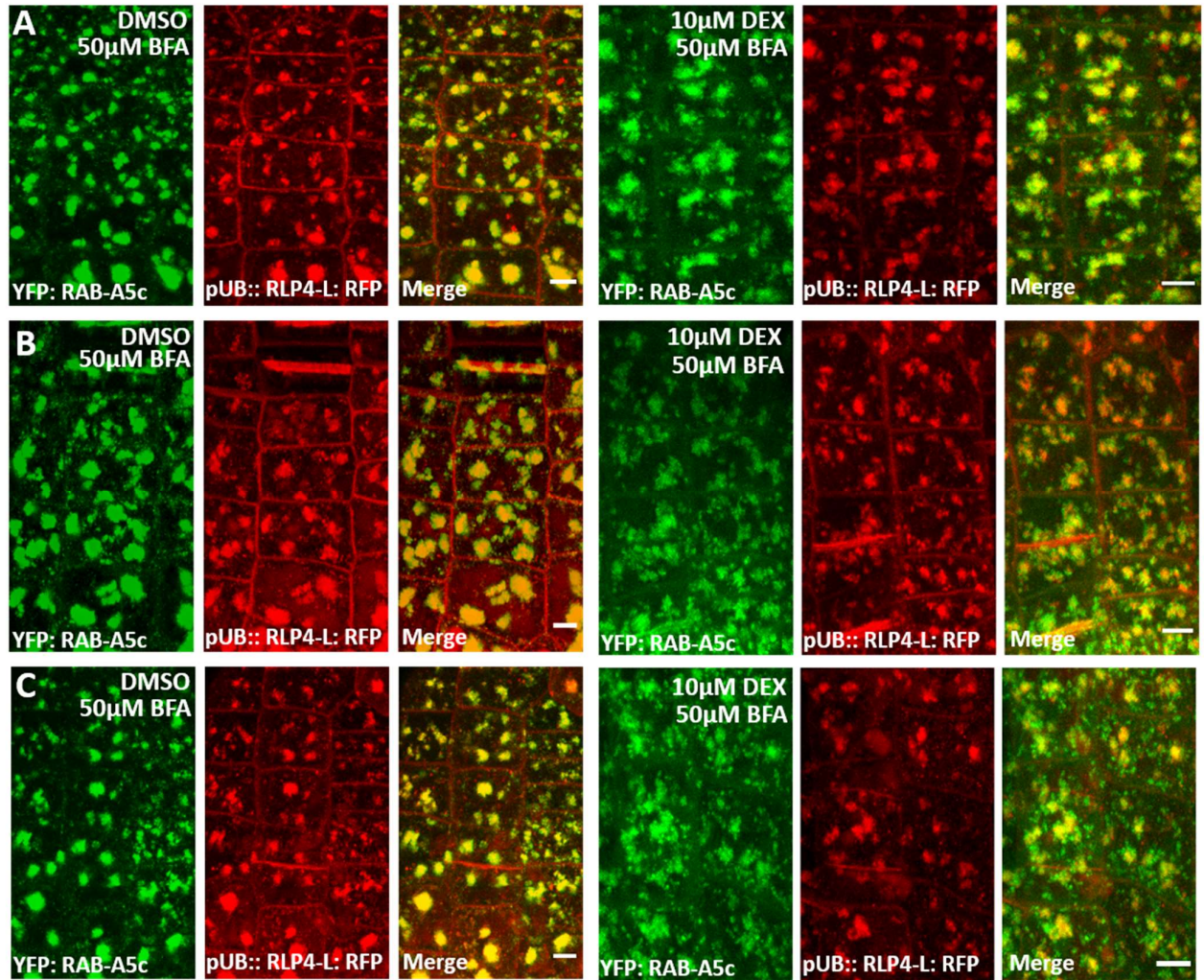


Figure 5.6: Expression of Rab-A GTPase dominant-negative mutant variants alters the localisation of YFP: RAB-A5c after treatment with BFA. (A) 3D confocal stack projections of lateral roots co-expressing YFP: RAB-A5c (left) and pUB:: RLP4-L: RFP (middle) and AtRPS5a>DEX>A5c[N125I].2 (B) AtRPS5a>DEX>A2a[N125I] (C) AtRPS5a>DEX>A4b[N125I]. 10μM DEX or equivalent volume DMSO for 24 hours, then 50μM BFA, 30 minutes. All from 10 day old F3 seedlings. Scale bars, 5μm.

5.F: RAB-A5c[N125I] mis-localises YFP: RAB-A5c to the Golgi, which may be the first point of RAB-A5c association with the endomembrane system

Kirchhelle and colleagues proposed that RAB-A5c defines a pathway between the TGN/EE and cell surface based on the observations that YFP: RAB-A5c[N125I] localises to the TGN/EE and that YFP: RAB-A5c itself partially labels the TGN/EE (Kirchhelle et al., 2016). N→I substitution mutants of Rab GTPases are generally believed to form a stable complex with Rab GEFs, sequestering these away from the endogenous Rab GTPase and preventing its activation (Schmitt et al., 1996; Jones et al., 1995; Olkkonen & Stenmark, 1997; Batoko et al., 2000; Goh et al., 2007). If this is true, YFP: RAB-A5c dynamics in the presence of RAB-A5c[N125I] could therefore represent the organisation of RAB-A5c upstream of activation by its cognate GEF. It is interesting therefore that when the pool of RAB-A5c's GEF is presumably sequestered by RAB-A5c[N125I], YFP: RAB-A5c retains association with the endomembrane system. Retention of membrane association of Rab GTPases has however been reported in the absence of their GEF or putative GEF (Cabrera & Ungermann, 2013; Qi et al., 2011; Kalde et al., 2019), indicating that Rab GEFs are not essential for at least partial membrane association of some Rab GTPases, contrary to traditional models of Rab GTPase cycling. Expression of RAB-A5c[N125I] (as well as RAB-A2a[N125I] and RAB-A4b[N125I]) alters the localisation of YFP: RAB-A5c during treatment with BFA, shifting it to a peripheral distribution pattern that is indicative of Golgi-localisation. To confirm that YFP: RAB-A5c does indeed re-localise to the Golgi in the presence of RAB-A5c[N125I], I crossed plants expressing the Golgi-marker MEMB12: mCh (Geldner et al., 2009) into plants co-expressing YFP: RAB-A5c and AtRPS5a>DEX>A5c[N125I].2. After 24 hours of RAB-A5c[N125I] expression, the structures labelled by YFP: RAB-A5c visibly co-localised well with MEMB12: mCh (Figure 5.7A & B), indicating that RAB-A5c[N125I] expression re-localises YFP: RAB-A5c from the cell edge and TGN/EE to the Golgi. This observation indicates that that YFP: RAB-A5c may first associate with the endomembrane system independently of its GEF at the Golgi, and that this may be the first point of association of YFP: RAB-A5c with the secretory pathway rather than the previously-assumed TGN/EE.

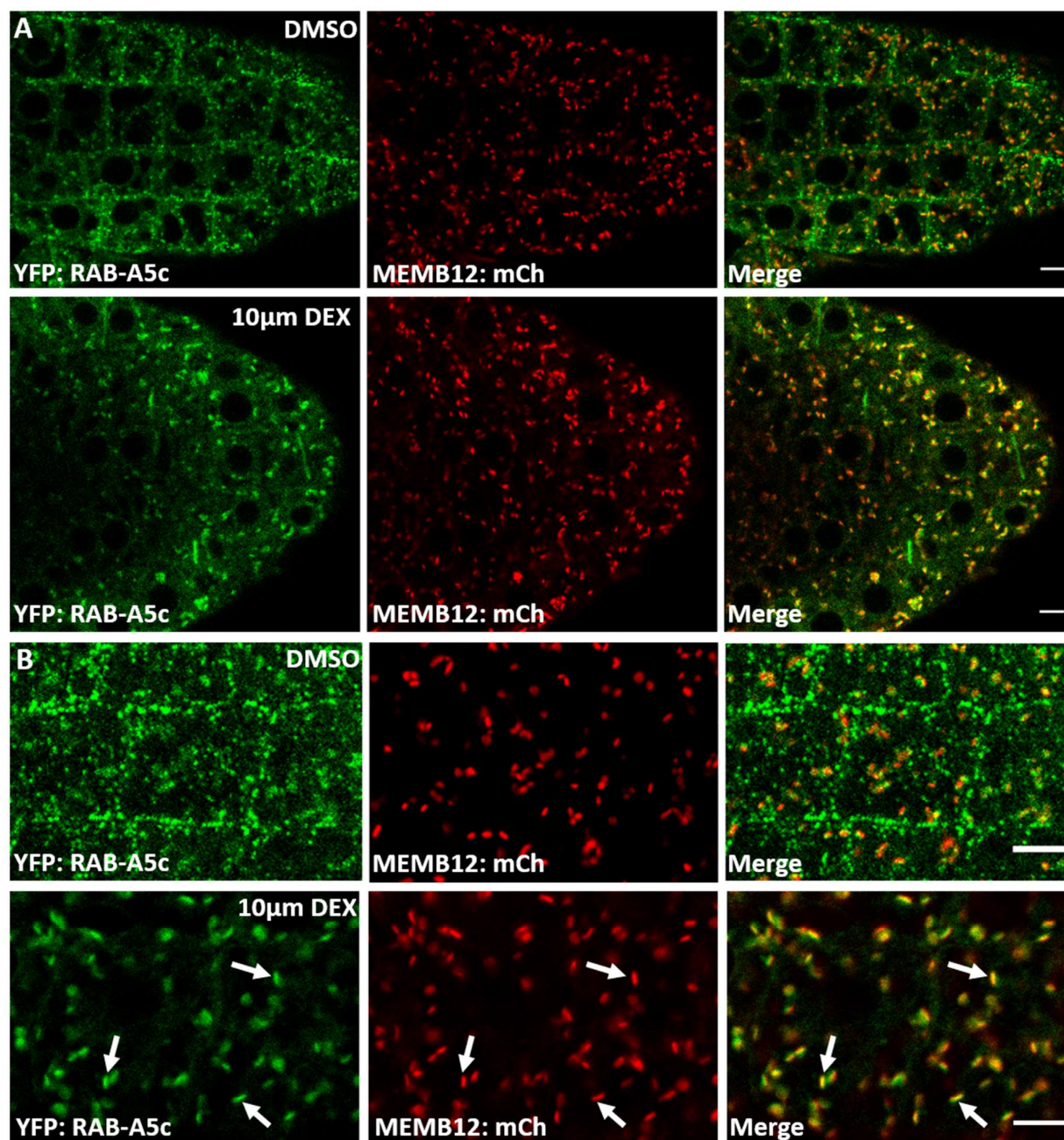


Figure 5.7: Expression of *AtRPS5a*>DEX>*A5c*[N125I] re-localises YFP: RAB-A5c to compartments that co-localise with the Golgi marker MEMB12: mCh (white arrows). (A,B) confocal slices of lateral roots co-expressing YFP: RAB-A5c (left), MEMB12: mCh (middle) and *AtRPS5a*>DEX>[N125I].2. (C,D) higher resolution confocal slices. 10µM DEX or equivalent volume DMSO, 24 hours. All from 10 day old F2 seedlings. Scale bars, 5µm.

5.G: Expression of dominant-negative RAB-A5c[N125I] can disrupt the localisation of other fluorescently-tagged Rab-A GTPases

The localisation disruption of YFP: RAB-A5c, pUB:: RLP4: RFP and pUB:: RLP4-L: RFP by expression of other dominant-negative Rab-A mutant variants was surprising and raises several questions. The most fundamental of these is whether the disruption of edge-directed trafficking by expression of dominant-negative RAB-A2a and RAB-A4b mutant variants is due to genuine dependence of edge-directed trafficking on the functionality of these TGN/EE-localised GTPases or, whether expression of dominant-negative RAB-A2a and RAB-A4b mutant variants can directly inhibit the functionality of Rab-A5 GTPases through a non-specific mode of action. The latter of these scenarios raises the possibility that the effects of RAB-A5c[N125I] expression on control of growth anisotropy and cell geometry in lateral roots may be partly due to disruption of other Rab-A GTPases, in addition to inhibition of RAB-A5c and edge-directed trafficking. This possibility of non-specific inhibition by Rab dominant negative mutants has been raised before, and work has shown that effects of some Rab N→I mutants can be at least partially suppressed by co-expression of the wild-type Rab GTPase. For example, transient expression of dominant negative AtRAB1b[N121I] (Rab1 = Rab-D; Rutherford & Moore, 2002) in *N.tabacum* leaf epidermal cells inhibits secretion of secGFP however, co-expression of wild-type AtRAB1b restores secGFP secretion (Batoko et al., 2000). Moreover, Kirchhelle and colleagues show that co-expression of wild-type RAB-A5c (AtRPS5a>DEX>A5c) and YFP: RAB-A5c with AtRPS5a>DEX>A5c[N125I] can suppress primary root length inhibition in a dose-dependent manner, and also suppresses lateral root morphogenesis defects (Kirchhelle et al., 2016). In this experiment, it is assumed that if RAB-A5c[N125I] specifically inhibits a RAB-A5c-dependent process, co-expression with wild-type RAB-A5c should be able to quantitatively suppress the resulting phenotypes. Conversely, if RAB-A5c[N125I] also acts to inhibit a distinct pathway through interaction with a common interactor shared with other Rab-A GTPases, co-expression of wild-type RAB-A5c may be expected to enhance observed phenotypes, through introduction of an additional competitive interaction factor (Kirchhelle et al., 2016). The result of this experiment is therefore

taken to indicate that phenotypic defects caused by RAB-A5c[N125I] expression are likely due to specific inhibition of a RAB-A5c-dependent function. It is therefore expected that while expression of dominant-negative RAB-A2a[N125I] and RAB-A4b[N125I] can disrupt YFP: RAB-A5c localisation, RAB-A5c[N125I] expression should not be able to reciprocally disrupt the localisations of other Rab-A GTPases. I therefore tested whether expression of the RAB-A5c[N125I] was also capable of reciprocally mis-localising fluorescently-tagged RAB-A2a and RAB-A4b, and crossed plants expressing AtRPS5a>DEX>A5c[N125I].2 with plants expressing either YFP: RAB-A2a or GFP: RAB-A4b.

In interphase cells, YFP: RAB-A2a localises to a subdomain of the TGN/EE and also partially to the PM (Chow et al., 2008; Figure 5.8A & C). After 24 hours of AtRPS5a>DEX>A5c[N125I] expression, YFP: RAB-A2a localisation differed noticeably compared to the controls. YFP: RAB-A2a in the presence of AtRPS5a>DEX>A5c[N125I].2 localised more uniformly to discrete intracellular compartments, as well as labelling the cytosol. YFP: RAB-A2a persisted also at the PM somewhat after 24 hours of AtRPS5a>DEX>A5c[N125I].2 expression, but this appeared to be reduced (Figure 5.8B & D). RAB-A4b has also been shown to localise to the TGN/EE (Preuss et al., 2004; Kang et al., 2011), and GFP: RAB-A4b was found here to localise to discrete intracellular compartments (Figure 5.9A & B). After 24 hours of AtRPS5a>DEX>A5c[N125I].2 expression however, GFP: RAB-A4b localisation was disrupted, localising to compartments of reduced intensity and less uniform shape (Figure 5.9A & B). Expression of AtRPS5a>DEX>A5c[N125I].2 is therefore capable of disrupting the localisation of other fluorescently-tagged Rab-A clade members, just as dominant-negative mutant variants of other Rab-A GTPases are capable of disrupting YFP: RAB-A5c localisation.

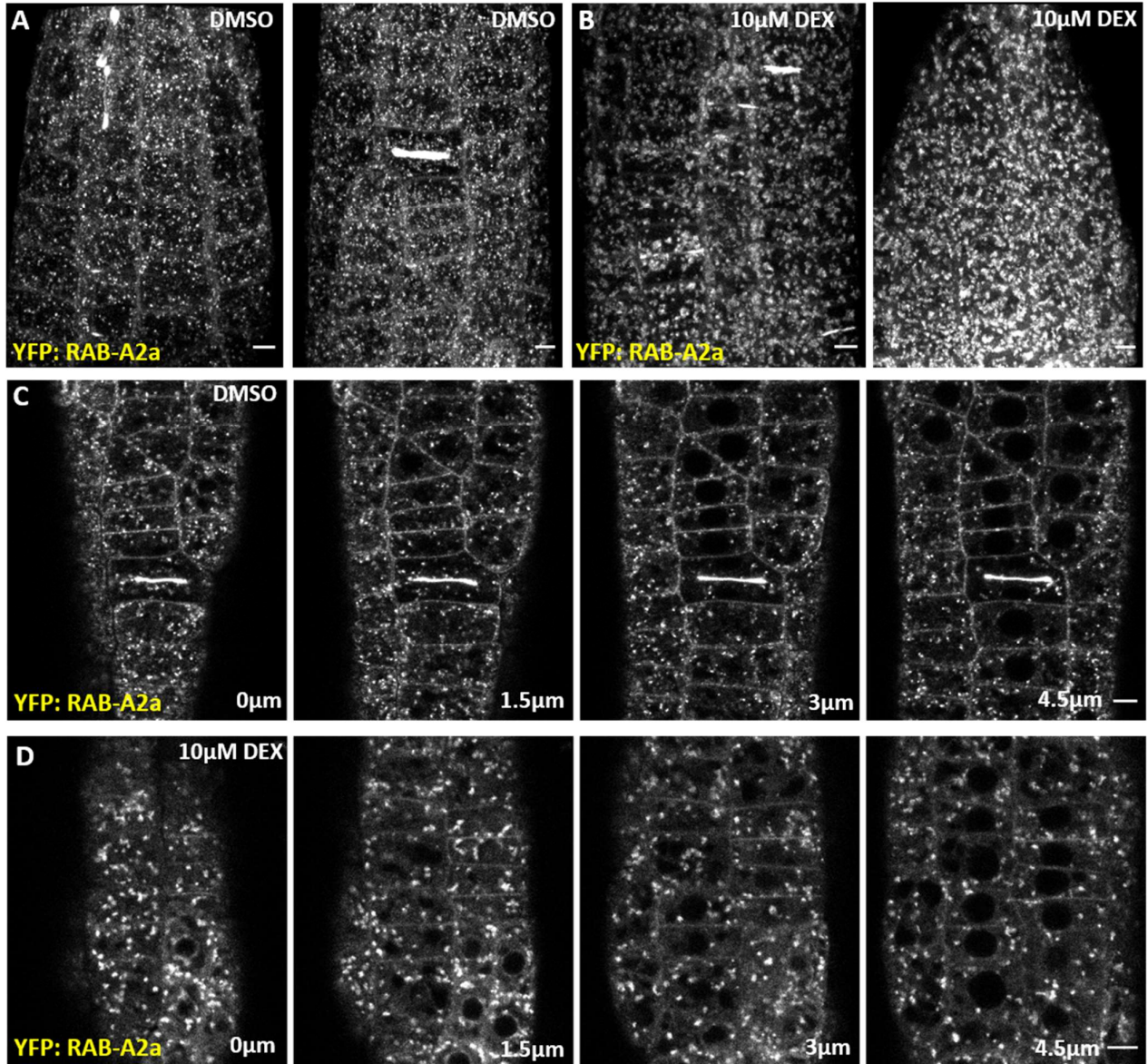


Figure 5.8: Expression of *AtRPS5a*>*DEX*>*A5c*[N125I] mis-localises YFP: RAB-A2a. (A, B) 3D confocal stack projections of lateral roots co-expressing YFP: RAB-A2a and *AtRPS5a*>*DEX*>*A5c*[N125I].2 (C,D) sequential confocal slices of lateral roots, 1.5μm intervals. 10μM DEX (B, D) or equivalent volume DMSO (A,C), 24 hours. All from 10 day old F1 seedlings. Scale bars, 5μm.

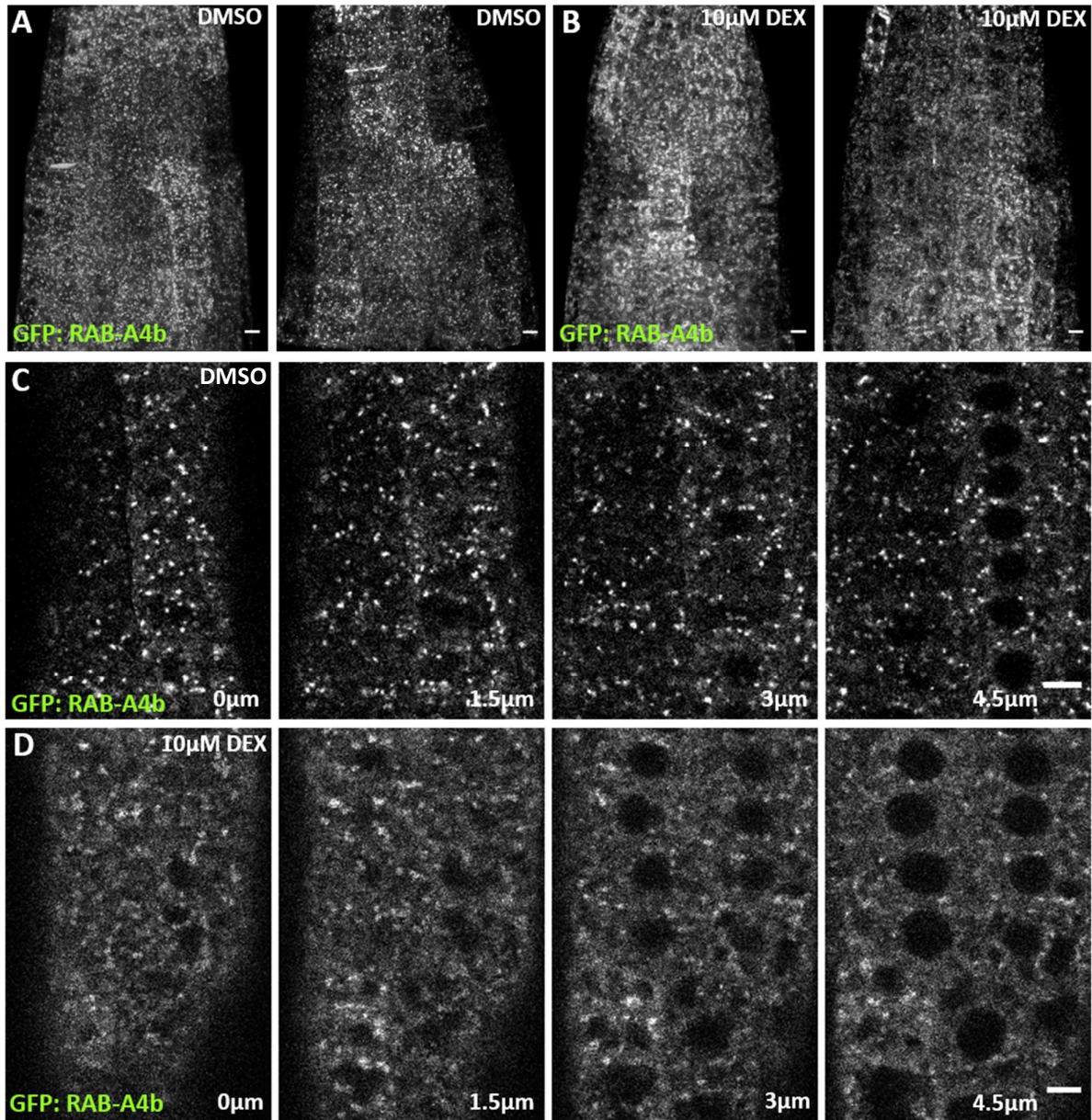


Figure 5.9: Expression of AtRPS5a>DEX>A5c[N125I] mis-localises GFP: RAB-A4b. (A,B) 3D confocal stack projections of lateral roots co-expressing GFP: RAB-A4b and AtRPS5a>DEX>A5c[N125I]. (C,D) sequential confocal slices of lateral roots, 1.5μm intervals. 10μM DEX (B,D) or equivalent volume DMSO (A,C), 24 hours. All from 10 day old F1 seedlings. Scale bars, 5μm.

5.H: Summary & Discussion

In this chapter, I have shown that expression of dominant-negative mutant variants of the Rab-A GTPases RAB-A2a and RAB-A4b can disrupt edge-directed trafficking and that expression of dominant-negative RAB-A5c[N125I] can also disrupt the localisations of both YFP: RAB-A2a and GFP: RAB-A4b. One possible explanation for this is that these dominant-negative mutants act specifically, but perturb aspects TGN/EE function that prevent the proper trafficking of other TGN/EE-localised Rab GTPases. However, RAB-A5c[N125I] expression does not disrupt bulk exocytic or endocytic trafficking (i.e. secretion of secGFP, PM localisation of PIN2 or uptake of FM4-64 into BFA bodies; Kirchhelle et al., 2016), nor does expression of RAB-A2a[N125I] disrupt bulk secretory or late-endosomal traffic (i.e. secretion of secGFP or localisation of late-endosome markers; Astrid Woollard & Ian Moore, unpublished data) indicating that TGN/EE function is not grossly perturbed by expression of Rab-A dominant-negative mutants. It remains possible however that more subtle TGN/EE defects caused by expression of Rab-A dominant-negative mutant variants are sufficient to mislocalise other Rab-A GTPases.

Dominant-negative mutants of Rab GTPases are believed to perturb function of endogenous Rab GTPases through sequestering upstream interactors of Rab GTPases, preventing activation of the endogenous Rab GTPase. Commonly-used Rab N→I mutant variants are believed to function through sequestering Rab GEFs away from endogenous Rab GTPases by forming a stable complex with Rab GEFs (Schmitt et al., 1996; Jones et al., 1995; Olkkonen & Stenmark, 1997; Batoko et al., 2000; Goh et al., 2007). The fact that expression of RAB-A2a[N125I] and RAB-A4b[N125I] can disrupt edge-directed trafficking, and that RAB-A5c[N125I] can mis-localise YFP: RAB-A2a and GFP: RAB-A4b therefore may indicate that these Rab GTPases share common upstream interaction factors, possibly a shared Rab GEF. An alternative possibility is that endogenous Rab-A GTPases interact preferentially with different upstream factors, but that these are sufficiently promiscuous to also be sequestered by different Rab-A dominant-negative mutant variants. One possible shared upstream interactor

factor of Rab GTPases is the TRAPP II complex, which has been shown to function upstream of the Rab-A GTPases RAB-A1c and RAB-A2a, and is a possible *Arabidopsis* Rab GEF (Qi et al., 2011; Qi & Zheng, 2011; Kalde et al., 2019). TRAPP II preferentially co-immunoprecipitated with dominant-negative YFP: RAB-A2a[S26N] (which is predicted to be arrested upstream of Rab activation) compared to YFP: RAB-A2a and putatively constitutively-active YFP: RAB-A2a[Q71L] (Kalde et al., 2019). Whether TRAPP II also interacts with dominant-negative variants of other Rab-A GTPases remains unclear however, the core TRAPP component AtBET5 was identified as a putative RAB-A5c interactor in our comparative proteomics approach. Furthermore, I have also shown that the localisation of the RFP-tagged TRAPP component pUB:: BET5: RFP localisation is possibly altered by expression of dominant-negative RAB-A5c[N125I], indicating that TRAPP components may be a candidate for common disruption by Rab-A dominant-negative mutants. Possibly GEF-associated YFP: RAB-A5c[N125I] localises to the TGN/EE but whether the compartments to which pUB:: BET5: RFP re-localises to upon RAB-A5c[N125I] expression are also TGN/EEs remains unknown. Whether TRAPP II functions upstream of Rab-A5 or other Rab-A GTPases in addition to RAB-A2a and RAB-A1c is also unknown. If TRAPP II or TRAPP components are indeed sequestered by Rab-A dominant negative N→I mutant variants as possible common interaction factors of Rab-A GTPases, then it could be predicted that mutants in TRAPP components, like expression of Rab-A dominant-negative mutant variants, may mis-localise YFP: RAB-A5c and possibly other Rab-A GTPases to the Golgi. Experiments have now been initiated to test this. It is also possible that shared interactors sequestered by Rab-A dominant negative mutant variants do not function equivalently against different Rab-A GTPases. Such a scenario could arise from a 'Rab cascade' within Rab-A GTPases. In yeast and metazoan Rab cascades, upstream Rab GTPases recruit effectors (such as Rab GEFs) essential for the organisation and function of downstream Rab GTPases, with downstream Rab GTPases subsequently recruiting effectors (such as Rab GAPs) promoting the removal of upstream Rab GTPases from membranes (reviewed by Novick, 2016). No Rab cascades have so far been identified in plants however, *Arabidopsis* Rab-F members are known to recruit the MON1-CCZ

complex to late endosomes, which functions as a GEF for downstream late endosome/tonoplast-localised Rab-G GTPases (Cui et al., 2014).

Regardless of the mechanism by which Rab-A dominant-negative mutant variants may function, the disruption of edge-directed trafficking by RAB-A2a[N125I] and RAB-A4b[N125I] expression in addition to by RAB-A5c[N125I] expression raises important questions as to whether loss of control of growth anisotropy and cell geometry in lateral roots is due specifically to disruption of RAB-A5c function and edge-directed trafficking by RAB-A5c[N125I]. Co-expression of YFP: RAB-A5c has already been shown to suppress the effects of RAB-A5c[N125I] expression (Kirchhelle et al., 2016). Experiments have now been initiated to test whether conditional expression of other Rab-A GTPases can also suppress the phenotypic effects of RAB-A5c[N125I] expression, in order to further examine the specificity of the developmental disruption caused by RAB-A5c[N125I].

Use of dominant-negative RAB-A5c[N125I] and pharmacological agents has identified interesting features of association and dissociation of RAB-A5c with the secretory pathway. Golgi-localisation of YFP: RAB-A5c in the presence of RAB-A5c[N125I] indicates that RAB-A5c may first associate with the endomembrane system at the Golgi upstream of its as-yet unidentified GEF. This would predict the existence of a Rab GDI-DF at the Golgi capable of promoting at least partial membrane-association of RAB-A5c in a targeted manner. Rab GDI-DFs are virtually uncharacterised in *Arabidopsis*, and none has so far been identified for RAB-A5c. Interestingly however, work has localised some *Arabidopsis* homologues of the yeast GDI-DF PRA1 to the Golgi, and over-expression of the PRA1 member AtPRA1.F4 reduces export of certain proteins from the Golgi, indicating the possible presence of active Rab GDI-DFs at the Golgi (Kamei et al., 2008; Lee et al., 2017). Recent work has however identified PLANT-UNIQUE RAB5 EFFECTOR 2 (PUF2) as a possible 'platformer' of two Rab-F GTPases, recruiting them to membranes in a spatially-controlled manner before activation by the Rab-F GEF VPS9a (Ito et al., 2018). PUF2 has however not been classified as a Rab GDI-DF and it is possible that a similarly novel factor Rab interactor may promote RAB-A5c membrane association. In addition to

the possibility that RAB-A5c may associate with the Golgi upstream of its cognate GEF, dominant-negative YFP: RAB-A2a[S26N] also localises to the Golgi (Chow et al., 2008) indicating that other Rab-A GTPases may also begin their membrane association at the Golgi upstream of interaction with their cognate GEF. YFP: RAB-A5c[N125I] localises to the TGN/EE and YFP: RAB-A5c[S26N] partially labels unidentified intracellular compartments (Kirchhelle et al., 2016; Carol Walton & Ian Moore, unpublished data). Whether the compartments to which YFP: RAB-A5c[S26N] localises, and to which YFP: RAB-A2a and YFP: RAB-A4b localise in the presence of RAB-A5c[N125I] are also Golgi remains unknown and this will be the subject of future investigation.

At the opposite end of the secretory pathway, prolonged treatment with Oryzalin re-localises YFP: RAB-A5c to the PM. Putatively constitutively active YFP: RAB-A5c[Q71L] partially labels the PM (Kirchhelle et al., 2016), and edge-directed trafficking has been shown through investigation of trafficking of fluorescently-tagged RLP4 and RLP4-L to define a pathway between the TGN/EE and PM, supporting a scenario in which RAB-A5c loses membrane association shortly before or after reaching the PM. Persistence of YFP: RAB-A5c association with the PM after treatment with Oryzalin, but not actin-disrupting LatB, indicates that removal of RAB-A5c from membranes may be specifically microtubule-dependent. Re-localisation of YFP: RAB-A5c to the PM by treatment with Oryzalin had not been previously described (Kirchhelle et al., 2016). Here, Oryzalin was applied in solid ½ Murashige & Skoog (MS) medium, allowing for a prolonged treatment of 180 minutes compared to the shorter liquid application done by Kirchhelle and colleagues (Kirchhelle et al., 2016). Re-localisation of YFP: RAB-A5c to the PM by treatment with Oryzalin may therefore be promoted by prolonged incubation and a high degree of penetration. This provides evidence that actin and microtubules function distinctly in support of edge-directed trafficking, and that an intact microtubule cytoskeleton may be necessary for removal of RAB-A5c from the PM, or to prevent RAB-A5c from reaching the PM. Dissociation of Rab GTPases from membranes is generally believed to be facilitated by activity of Rab GAPs, which promote GTP hydrolysis and reversion to the GDP-bound cytosolic Rab GTPase form. Re-localisation of YFP: RAB-A5c to the PM after treatment with Oryzalin

raises the interesting possibility that RAB-A5c's Rab GAP may be microtubule-associated or microtubule-dependent, and facilitate dissociation of RAB-A5c from membranes at the cell periphery.

Chapter 6: Discussion & Outlook

6.A: Hypotheses for the function of RLP4 and RLP4-L

This thesis has identified the first known components of edge-directed trafficking beyond Rab-A5 GTPases, the Receptor-Like Proteins RLP4 and RLP4-L. Fluorescently-tagged RLP4 and RLP4-L are likely the first known cargoes of RAB-A5c as their localisation is disrupted by expression of RAB-A5c[N125I], and transient BiFC assays indicate possible direct interactions between split YFP-tagged RAB-A5c and RLP4/RLP4-L in the secretory pathway. The identification of RLP4 and RLP4-L has demonstrated that edge-directed trafficking is a post-Golgi route to the cell surface and that cell edges are also defined as a discrete polar domain within the PM of *Arabidopsis* cells. These observations and investigation of the trafficking behaviour of fluorescently-tagged RLP4 and RLP4-L truncated variants have provided the first insights into possible functions of RLP4 and RLP4-L. This work has also raised questions about the organisation of edge-directed trafficking, the extent to which common factors may underpin edge-directed trafficking as well as other Rab-A GTPase trafficking pathways, and the validity of techniques that have previously been used to investigate this. Continuation of the work begun in this thesis will therefore focus both on functional characterisation of RLP4 and RLP4-L, as well as further investigation of trafficking factors supporting edge-directed trafficking and their arrangement in the *Arabidopsis* endomembrane system.

Fluorescently-tagged RLP4 and RLP4-L are the first identified non-GTPase proteins capable of localising to cell edge compartments in *Arabidopsis* lateral roots. Fluorescent protein-fusions of RLP4 and RLP4-L also localise to the TGN/EE and the PM, and expression of RAB-A5c[N125I] abolishes their localisation to cell edge compartments, shifting them from the cell periphery to a predominantly intracellular localisation pattern. RLP4: RFP and RLP4-L: RFP are polarised within the PM compared to an evenly-distributed PM marker and attenuate with increased distance from adjacent cell edges at the outer lateral surface. These observations confirm that RAB-A5c defines an

edge-directed trafficking pathway to the cell surface, and identify RLP4 and RLP4-L as the first known components of this trafficking pathway. Fluorescently-tagged truncated variants of RLP4 and RLP4-L lacking intracellular domains and TMDs show that the ECDs of RLP4 and RLP4-L associate with the cell wall in *Arabidopsis* lateral roots, possibly in a polarised manner. Moreover, fluorescently-tagged truncated variants of RLP4 and RLP4-L lacking ECDs do not localise to cell edge compartments or the PM. Treatment with pharmacological disruptors of post-Golgi trafficking and endocytosis partially re-localises these truncations to the PM, indicating that the ECDs of RLP4 and RLP4-L may be required to stabilise their localisation at the cell surface. Finally, BiFC indicates that RLP4 and RLP4-L may be direct interactors of RAB-A5c and that this interaction may specifically occur at the TGN/EE. Bringing together these data, I now propose two hypotheses for the function of RLP4 and RLP4-L in edge-directed trafficking, incorporating previous predictions that edge-directed trafficking may be a route for local modification of the cell wall at edges (Kirchhelle et al., 2016, 2019).

Hypothesis 1: RLP4/RLP4-L act as cargo receptors.

The first hypothesis proposes that RLP4 and RLP4-L may be cargo receptors, and associate with carbohydrate cargoes or cell wall-modifying proteins via their N-terminal sequences/ECDs and organise their transport to the cell wall via cell edge compartments, resulting in local modification of the cell wall at edges (Figure 6.1). The N-terminal sequences/ECDs of RLP4 and RLP4-L are cell wall binding and are predicted to be lumen-facing within the Golgi, TGN/EE and cell edge compartments before fusion with the PM. It is possible that RLP4 and RLP4-L bind a carbohydrate cargo in the Golgi or TGN/EE and transport this to the cell wall, via cell edges, in edge-directed trafficking (Figure 6.1). As fluorescently-tagged truncated variants of RLP4 and RLP4-L lacking their ECDs label the Golgi and TGN/EE, this model assumes that RLP4 and RLP4-L require their N-terminal sequences/ECDs for proper export from the Golgi and TGN/EE to the plasma membrane, and possibly that cargo-binding via their N-terminal sequences/ECDs is a required step for their export from the Golgi & TGN/EE.

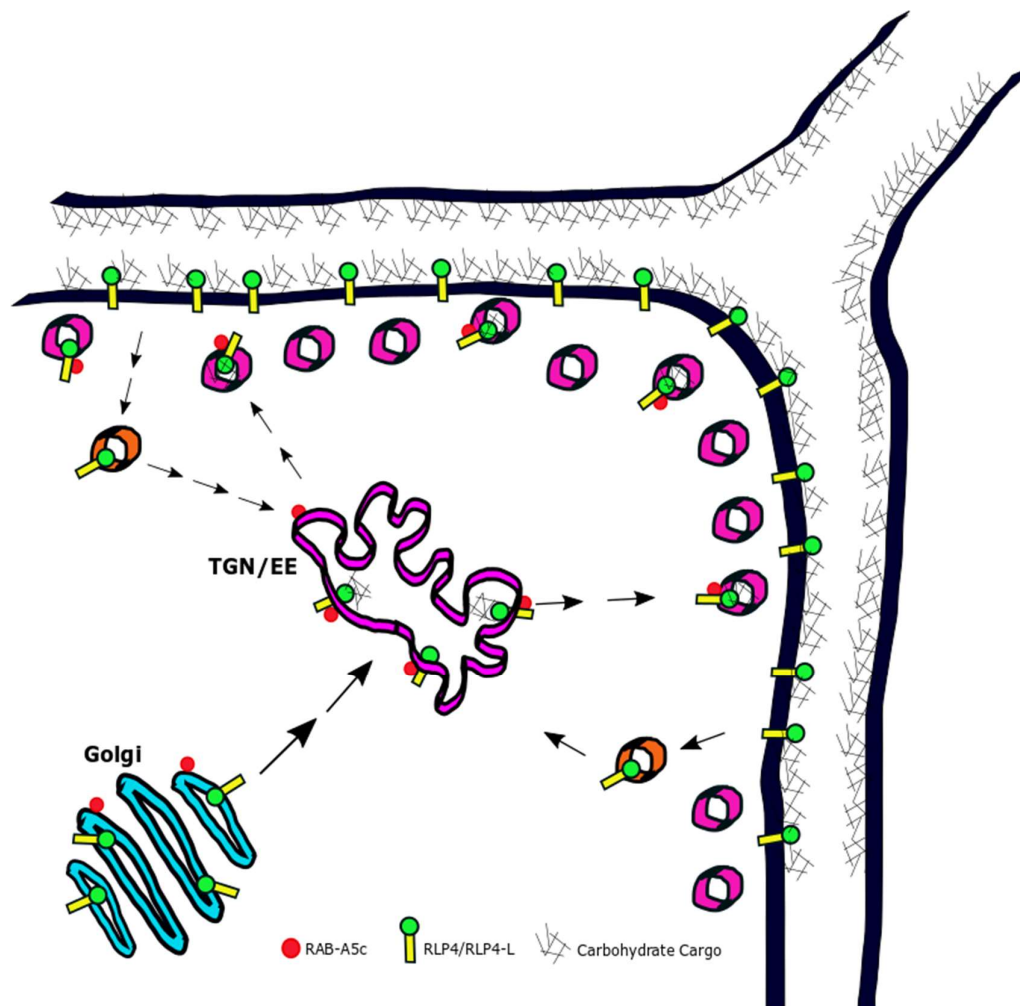


Figure 6.1: RLP4 and RLP4-L are cargo receptors transporting a carbohydrate or cell wall modifying cargo via edge-directed trafficking in *Arabidopsis* lateral root epidermal cells. RLP4 and RLP4-L traffic from the Golgi to TGN/EE, probably independently of RAB-A5c. A direct interaction between RAB-A5c and RLP4/RLP4-L may arise at the TGN/EE. RLP4 and RLP4-L bind carbohydrate cargoes or cell-wall modifying agents through their N-terminal sequences and organise their transport to the cell wall via cell-edge compartments, resulting in selective modification of cell wall composition at cell edges.

This hypothesis suggests that RLP4 and RLP4-L support previously predicted local modification of cell walls around cell edges (Kirchhelle et al., 2016, 2019) via an edge-directed trafficking pathway, through either transport of specific carbohydrates or cell-wall modifying factors to cell edges. Polarised distribution of cell wall components around cell edges and corners has been reported in a wide variety of plant tissues and species, including for pectins and structurally-supportive lignins

(reviewed by Elliott & Kirchhelle, 2019). Enrichment of de-methylesterified pectins, which has been linked to local cell wall strengthening, at cell edges has been reported in parenchymous tissues of diverse plant species (Bush et al., 2001; Parker et al., 2001; Jarvis et al., 2003; Guillemin et al., 2005; Szymańska-Chargot et al., 2016; Wolf & Greiner, 2016). Cell edges are also known to be enriched in phenolic compounds in some plant species (Parr et al., 1996; Waldron et al., 1997; Parker et al., 2003; Suslov et al., 2009). In the case of Chinese water chestnut, enrichment of ferulic acid in the cell wall at cell edges has been proposed to locally strengthen the cell wall by inducing cross-linking of esterified pectins (Parr et al., 1996; Waldron et al., 1997; Parker et al., 2003). Edge-enrichment of structurally-supportive lignins has also been described in several cases (Tirumalai et al., 1996; Agarwal, 2006; Zeng et al., 2017; Yi Chou et al., 2018). The identity of a possible edge-polarised/enriched cell wall component in growing lateral roots of *Arabidopsis* remains unknown however, work is now ongoing to investigate the relative *in vivo* distributions of major cell wall components at the outer surface of *Arabidopsis* lateral root epidermal cells using available antibody probes. The possible existence of an edge-polarised cell wall component(s) deposited by edge-directed trafficking raises questions as to why this may be necessary during lateral root growth. Kirchhelle and colleagues used *in silico* finite element models to demonstrate that local disruption of mechanical properties at cell edges can disrupt cell morphology *in vitro* in a manner similar to that produced by expression of RAB-A5c[N125I] *in vivo* (Kirchhelle et al., 2016). Moreover, more recent work has identified cell geometric edges as significant foci for mechanical strain in idealised *in silico* finite element models of growing plant tissues (Charlotte Kirchhelle, unpublished data), indicating that polarised deposition of cell wall components at cell geometric edges during growth may be a compensatory response to locally mechanically modify the cell wall at cell edges and alleviate such mechanical strain. *In vivo* assessment of cell edges as mechanically distinct regions of the cell surface remains limited however, work using onion peel epidermal cells has identified cell edges as softer than the adjacent cell faces during indentation experiments, and also as having a lower cellulose content than adjacent cell faces (Suslov et al., 2009; Routier-Kierzkowska et al., 2012). Tensile

loading experiments indicated that cell edges of onion peel epidermal cells were however stronger than the adjacent cell faces (Zamil et al., 2013; Zamil et al., 2014). Whether such differences in local cell wall strength are directly linked to the intrinsic mechanical properties of a polarised cell wall material in all cases remains unknown. Interestingly, in the abscission zones of *Arabidopsis* floral organs, edge-polarised lignification has been proposed to restrict the diffusion of cell wall digesting enzymes, concentrating them around the abscission site and promoting local cell wall loosening (Lee et al., 2018). Whether a similar mechanism could also function around meristematic cells of growing tissues remains unclear however, staining with Phloroglucinol-HCl indicates that little, if any, lignin is present in young lateral roots of *Arabidopsis* (Kirchhelle, 2016. DPhil Thesis). The identity of a possible edge-polarised cell wall component in *Arabidopsis* young lateral roots therefore remains unknown.

Hypothesis 2: RLP4/RLP4-L define an edge-based cell wall signalling module.

The second hypothesis proposes that RLP4 and RLP4-L may be components of an edge-localised signalling module. In this hypothesis, RLP4 and RLP4-L are transported in edge-directed trafficking to the PM via cell edges, where they associate with an edge-enriched cell wall component through their ECDs. In common with other RLPs, RLP4 and RLP4-L may then associate with one or more RLKs in an edge-localised signalling module (Figure 6.2). Such an edge-localised signalling module containing cell-wall binding RLP4 and RLP4-L may signal the abundance of a particular cell wall component at cell edges, or possibly the mechanical status of the cell wall at cell edges. If edge-directed trafficking is indeed a route for local cell wall modification at cell edges, the output of such an edge-localised signalling module is assumed in this model to be feedback against edge-directed trafficking itself, possibly modulating the delivery of specific cell wall components, or cell wall modifying factors, to the cell edge, whose transport is orchestrated by other as-yet unidentified components and supporting factors of edge-directed trafficking. It should also be noted that the two hypotheses presented here are not necessarily mutually exclusive and that RLP4 and RLP4-L may

functional dually to organise cargoes intracellularly and perceive a signalling stimulus at the cell surface.

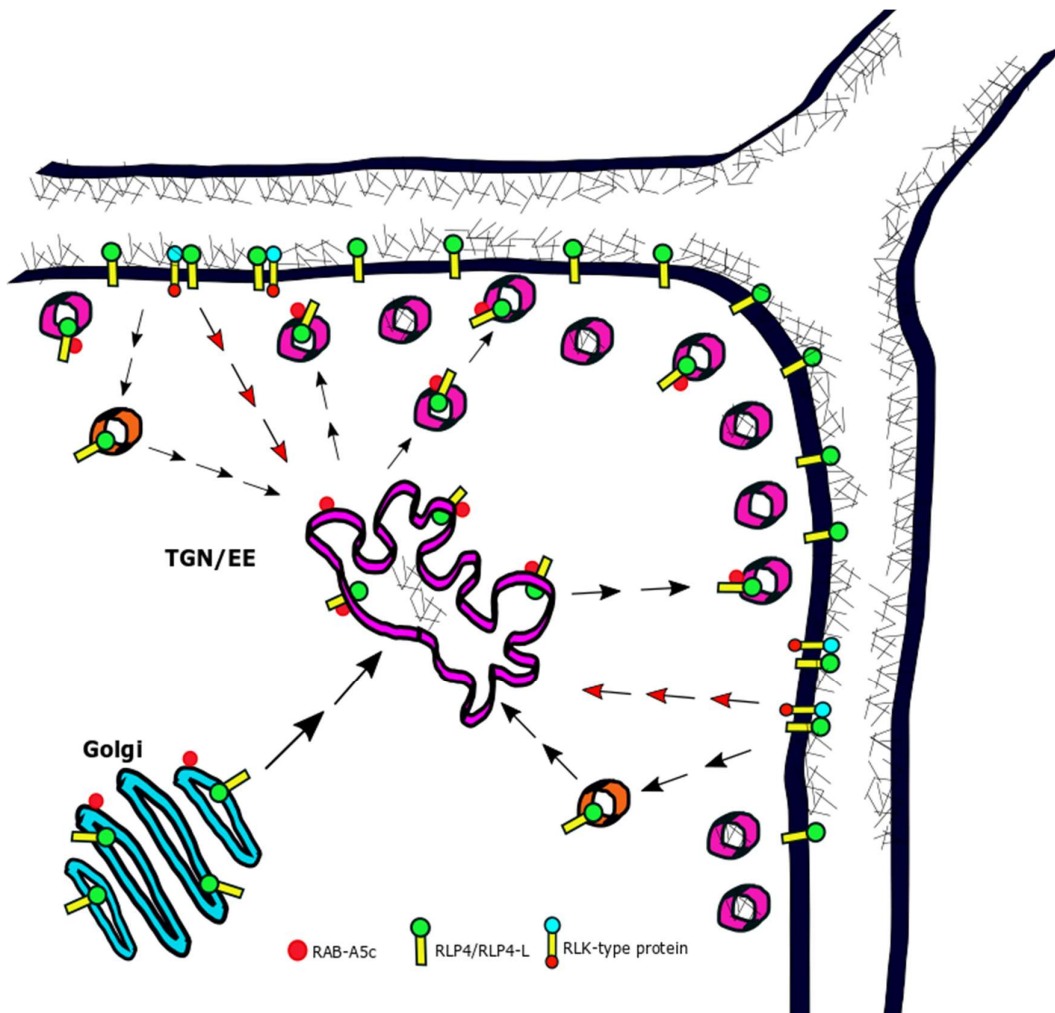


Figure 6.2: RLP4 and RLP4-L are components of an edge-localised signalling module. RLP4 and RLP4-L traffic from the Golgi to TGN/EE, probably independently of RAB-A5c. A direct interaction between RAB-A5c and RLP4/RLP4-L may arise at the TGN/EE. In the PM, RLP4 and RLP4-L associate with an RLK-type protein, forming an edge-localised signalling module possibly sensing cell wall composition or mechanical status at cell edges. The output of this signalling module may be against edge-directed trafficking itself.

If RLP4 and RLP4-L are indeed found to interact with an RLK-type protein as part of a possible edge-localised signalling module, it remains an open question as to what the signalling stimulus for this may be. One possibility is that RLP4 and RLP4-L may associate specifically with an edge-enriched cell wall component via their ECDs, and that this maintains a population of RLP4 and RLP4-L at the cell

surface in a dose-dependent manner for formation of signalling modules. The signalling output of such a complex could, in turn, influence the deposition of edge-enriched cell wall component(s) or modifying components, which are presumably deposited via edge-directed trafficking itself (Figure 6.3a). It is interesting to consider whether dose-dependent maintenance of an RLP4/RLP4-L signalling module at cell edges by a polarised cell wall component is itself sufficient to generate a signalling output, or whether a separate stimulus may be required (Figure 6.3a vs. b). It is also possible that RLP4 and RLP4-L associate with the cell wall indiscriminately and form a signalling module which perceives a separate stimulus (Figure 6.3c). This latter scenario would indicate that polarised deposition of RLP4 and RLP4-L at the cell surface via edge-directed trafficking is the main driving force in maintenance of cell-edge polarised PM distribution of RLP4: RFP and RLP4-L: RFP. However, fluorescently-tagged membrane-associated ECDs of RLP4 and RLP4-L, which do not label cell-edge compartments remain polarised within the PM, indicating that RLP4 and RLP4-L may be capable of stable polarisation at the cell surface independently of edge-directed trafficking. The nature of a possible separate stimulus remains purely speculative at this stage however, other work has identified cell geometric edges as significant foci for mechanical strain within *in silico* finite element models of growing plant tissues (Charlotte Kirchhelle., unpublished data). It is therefore possible that a RLP4/RLP4-L signalling module is responsive to mechanical strain at cell edges, either directly or indirectly such as through perception of a cell wall shearing component. The MLDs of FERONIA have been shown to bind to the secreted RALF1 peptide to negatively regulate cell growth in roots (Haruta et al., 2014; Du et al., 2016), raising the possibility that such an entirely separate ligand may be perceived by a possible RLP4/RLP4-L signalling module. Why such a scenario would specifically require an edge-localised signalling module is however unclear.

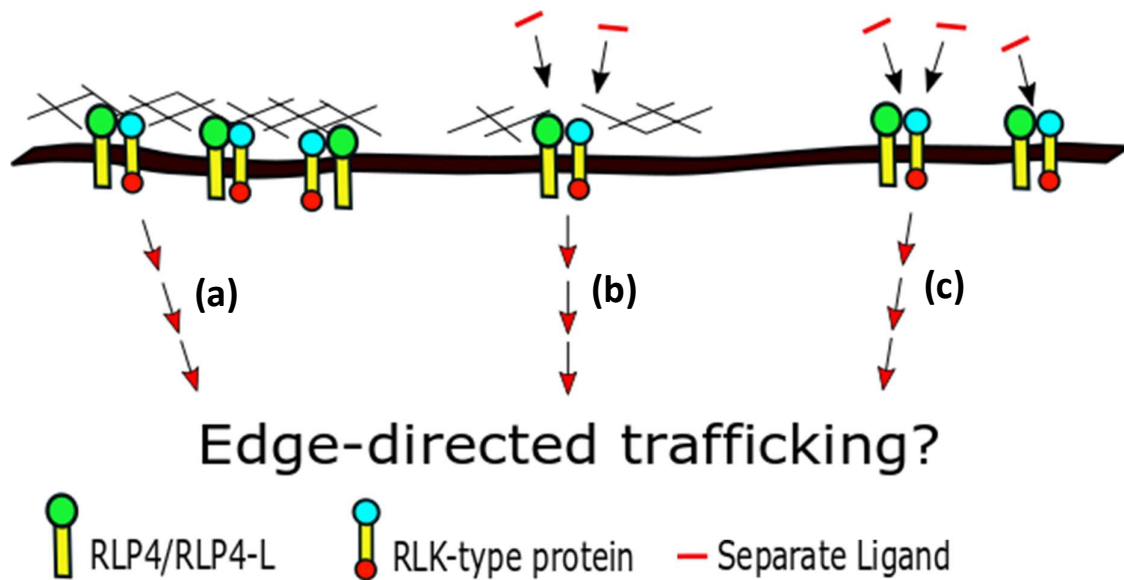


Figure 6.3: Possible scenarios for an RLP4/RLP4-L edge-localised signalling module.

(a) RLP4/RLP4-L associate with an edge-restricted cell wall component that is co-deposited by edge-directed trafficking, stabilising their presence at the cell surface in a dose-dependent manner. Formation of a constitutively-active signalling module follows. (b) The edge-localised dose-dependent signalling module formed by RLP4/RLP4-L is responsive to a specific stimulus, possibly a cell wall shearing product, perceived either by RLP4/RLP4-L or an associated RLK. (c) RLP4/RLP4-L associate with the cell wall indiscriminately or specifically with an evenly-distributed component and form a signalling module. Polar deposition by edge-directed trafficking is therefore the main driver of RLP4/RLP4-L edge-restriction.

6.B: Models for trafficking of Rab-A GTPases and supporting factors of edge-directed trafficking

Work in this thesis has questioned previously-held assumptions regarding the organisation of edge-directed trafficking and given initial indications of some of the regulatory factors that may support it. Expression of RAB-A5c[N125I] re-localises YFP: RAB-A5c to the Golgi, indicating that this may be the first point of RAB-A5c association with the endomembrane system, rather than the TGN/EE as previously assumed (Kirchhelle et al., 2016). N→I substitution mutant variants are believed to function through titrating Rab GEFs away from wild-type Rab GTPases, preventing their activation (Schmitt et al., 1996; Jones et al., 1995; Olkkonen & Stenmark, 1997; Batoko et al., 2000). Re-localisation of YFP: RAB-A5c to the Golgi by expression of RAB-A5c[N125I] therefore may indicate that initial association of RAB-A5c with the Golgi is independent of its GEF, but that its GEF or other

factors sequestered by RAB-A5c[N125I] are required for subsequent localisation to the TGN/EE and cell edge compartments. Whilst no GEF for RAB-A5c has yet been identified, TRAPP^{II} has been suggested as a putative GEF for some Rab-A GTPases (Qi et al., 2011; Qi & Zheng, 2011; Kalde et al., 2019). Interestingly, expression of RAB-A5c[N125I] putatively alters the localisation of YFP::BET5, an RFP-tagged TRAPP component, lending support to the possibility that RAB-A5c[N125I] may perturb the function of wild-type RAB-A5c by sequestering TRAPP^{II}, a possible GEF. Surprisingly, expression of dominant-negative mutant variants of TGN/EE-localised RAB-A2a and RAB-A4b also disrupts edge-directed trafficking. Expression of RAB-A5c[N125I] can also disrupt localisation of YFP::RAB-A2a and YFP::RAB-A4b, questioning whether the phenotypes produced by expression of RAB-A5c[N125I] and dominant-negative mutants of other Rab-A GTPases are due to specific inhibition of the target GTPase, or broader disruption of Rab-A function. This also raises the possibility that, despite their unique localisation pattern, Rab-A5 GTPases may share common interaction factors with other Rab-A GTPases. Finally, prolonged disruption of microtubules through application of Oryzalin re-localises YFP::RAB-A5c to the PM, which had not been described in previous microtubule-disruption of YFP::RAB-A5c localisation (Kirchhelle et al., 2016), and indicates that microtubules may support removal of RAB-A5c from the PM at the end of edge-directed trafficking. These observations support a new model for trafficking of RAB-A5c and other Rab-A GTPases in the post-Golgi secretory pathway of *Arabidopsis* (Figure 6.4).

In this model, RAB-A5c (and possibly other Rab-A GTPases) first associate with the Golgi through the action of an unidentified Golgi-localised factor such as a Rab GDI-DF, but independently of their GEF (possibly TRAPP^{II}; Figure 6.4.1a). RAB-A5c traffics from the Golgi to the TGN/EE where it associates with RLP4 and RLP4-L and defines an edge-directed trafficking pathway (Figure 6.4.1b). TRAPP^{II} components are TGN/EE localised (Qi et al., 2011; Naramoto et al., 2014; Ravikumar et al., 2018), indicating that TRAPP^{II} may act as a Rab GEF at the TGN/EE. At the PM, RAB-A5c removal from, or prevention of association with, the PM is promoted by microtubules, possibly through action of a microtubule-dependent Rab GAP (Figure 6.4.1c). During expression of RAB-A5c[N125I], and possibly

dominant-negative mutants of other Rab-A GTPases, TRAPP^{II} is sequestered away from wild-type RAB-A5c. RAB-A5c therefore associates with the Golgi but cannot progress further (Figure 6.4.2a). RLP4 and RLP4-L likely predominantly localise to the TGN/EE in the presence of RAB-A5c[N125I], indicating that their Golgi-to-TGN/EE trafficking is independent of RAB-A5c function (Figure 6.4.2b).

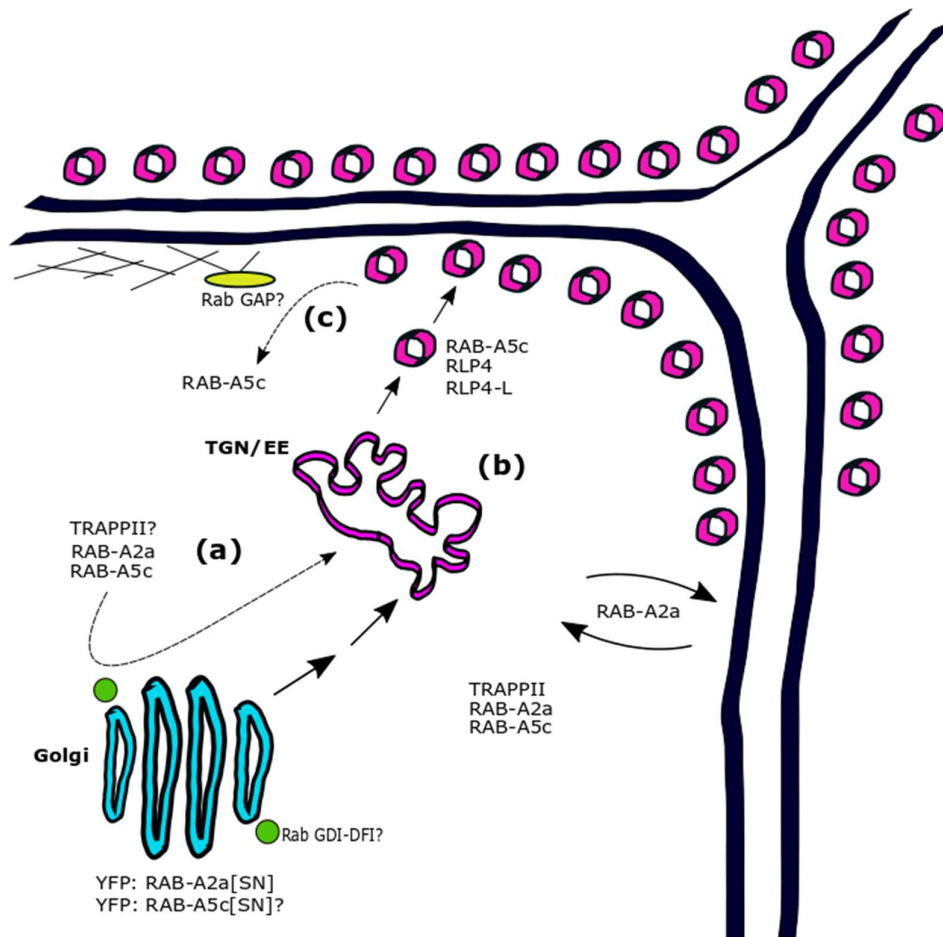
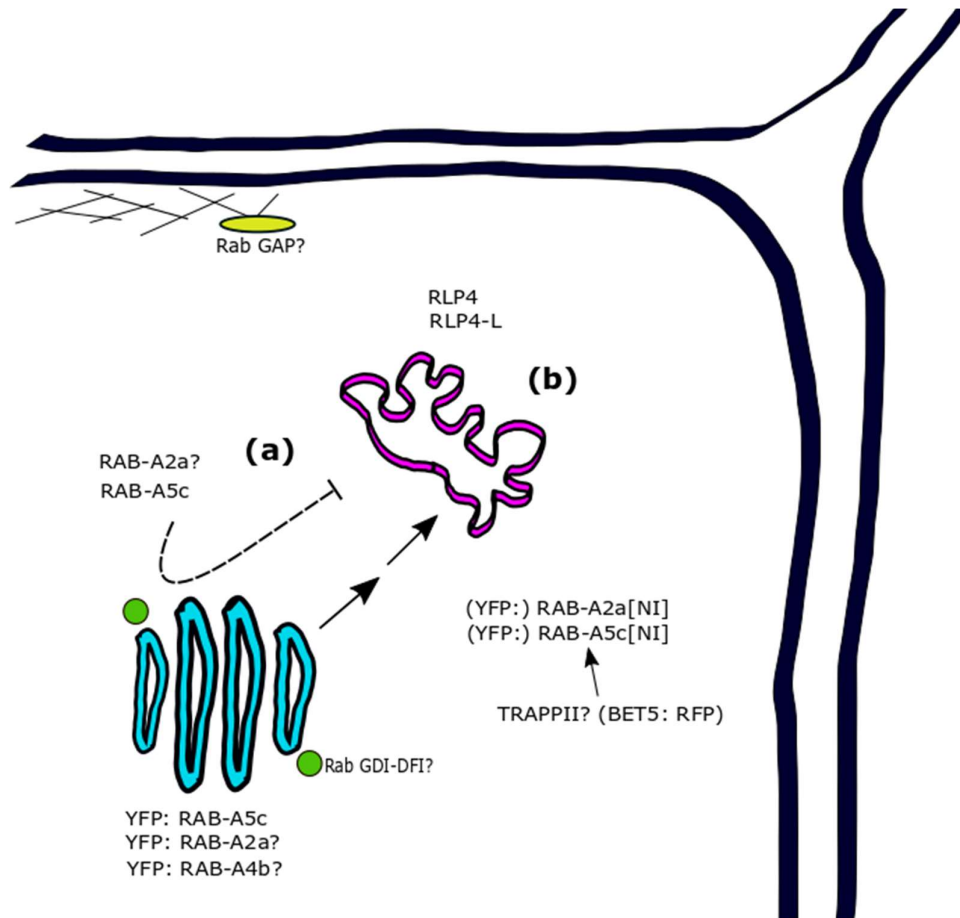


Figure 6.4: Trafficking of Rab-A5c and other Rab-A GTPases in the post-Golgi secretory pathway.

6.4.1: Rab-A5c and possible interacting factors in *Arabidopsis* lateral root epidermal cells. (a) RAB-A5c, and likely also RAB-A2a, are recruited to the Golgi by either a common or separate mechanism. TRAPP^{II} (which associates with Golgi-localised YFP: RAB-A2a[SN]) may also be first recruited to the Golgi. RAB-A5c, RAB-A2a and TRAPP^{II} traffic to the TGN/EE with maturation from the Golgi. (b) RAB-A5c defines specialised edge-directed trafficking from the TGN/EE whilst RAB-A2a may define a polarised recycling route. TRAPP^{II} subunits localise to the TGN/EE as do putatively GEF-bound YFP: indicating that TRAPP^{II} GEF function may occur at the TGN/EE. (c) Edge-compartments fuse with the PM. RAB-A5c removal from the PM is microtubule-dependent, possibly involving a microtubule-associated or microtubule-regulated Rab GAP.



6.4.2: : Rab-A5c and possible interacting factors in *Arabidopsis* lateral root epidermal cells in the presence of RAB-A5c[N125I]. (a) YFP: RAB-A5c steady-state localises to the Golgi in the presence of RAB-A5c[N125I], indicating that RAB-A5c may be recruited to the Golgi independently of its GEF. RAB-A5c[N125I] blocks transport of YFP: RAB-A5c from the Golgi to the TGN/EE. (b) RLP4 and RLP4-L possibly arrest at the TGN/EE in the presence of RAB-A5c[N125I] indicating that Golgi-to-TGN/EE transport of RLP4 and RLP4-L is independent of RAB-A5c. The TRAPP component BET5: RFP may be re-localised to the TGN/EE by RAB-A5c[N125I], indicating that Rab-A dominant negatives may sequester TRAPP II to the TGN/EE. Recruitment of YFP: RAB-A5c to the Golgi, but not subsequent localisation to the TGN/EE and edge compartments, may therefore be independent of TRAPP II.

Outlook & Future Work

6.C: Trafficking and function of RLP4 and RLP4-L

Both of the hypotheses presented for the function of RLP4 and RLP4-L predict that these RLPs may support the deposition of one or more polarised cell wall components or cell wall modifying factors at cell geometric edges in growing tissues of *Arabidopsis*. Whilst polarisation of cell wall components has been described in diverse plant species and tissues (reviewed by Elliott & Kirchhelle, 2019), none have yet been identified in *Arabidopsis* lateral roots. Work is now ongoing to investigate the relative *in vivo* distributions of major cell wall components at the outer surface of *Arabidopsis* lateral root epidermal cells using available antibody probes. Moreover, fluorescently-tagged ECDs of RLP4 and RLP4-L are capable of association with the *Arabidopsis* cell wall however, it remains unclear whether this is due to direct binding to carbohydrates or to an indirect interaction through association with cell-wall binding proteins, where this association first occurs, and whether this association is with a polarised cell wall component. The presence of MLDs in the N-terminal/ECD sequences of RLP4 and RLP4-L indicates a candidate protein domain through which such a direct or indirect interaction with the cell wall may occur. Work will therefore be undertaken to identify carbohydrate epitopes with which RLP4 and RLP4-L may associate with through their N-terminal/ECD sequences. Full-length N-terminal regions of RLP4 and RLP4-L, and truncations of these lacking MLDs, will be expressed heterologously and purified for use in *in vitro* carbohydrate binding assays (Ruprecht et al., 2017). The results of this may also inform *in vivo* analysis of cell wall component distribution in lateral root epidermal cells. Fluorescently-tagged truncated variants of RLP4 and RLP4-L lacking their N-terminal MLDs (but retaining other regions of the ECD) have already been constructed in order to also investigate the extent to which this domain contributes to trafficking of RLP4 and RLP4-L, and their polarisation within the PM. In addition, the recent description of a protocol for glycomic analyses of isolated subcellular compartments may provide a method for glycomic analysis of cell edge compartments (Wilkop et al., 2019). This would be particularly interesting for investigating whether

cell edge compartments may transport additional carbohydrate cargoes to those possibly organised by/associated with RLP4 and RLP4-L.

The possibility that RLP4 and RLP4-L may form an edge-localised signalling module within the PM of *Arabidopsis* lateral root epidermal cells predicts a possible interaction between RLP4 and RLP4-L and an as-yet unidentified RLK-type protein, as other RLPs are known to do. In order to identify possible RLK partners of RLP4 and RLP4-L, as well as other interactors of these proteins and possible additional components of edge-directed trafficking, proteomic analysis of RLP4 and RLP4-L will be undertaken through co-immunoprecipitation of these RLPs followed by mass spectrometry, using the protocol developed by Kalde and colleagues (Kalde et al., 2019). Pull downs against RLP4: GFP and RLP4-L: GFP expressed from their native promoters will be undertaken alongside repeat pull-downs against YFP: RAB-A5c in order to highlight possible additional components of edge-directed trafficking through identification of shared interaction candidates. Pull-downs against intracellular-shifted (secRFP:: RLP4 trunc and secRFP:: RLP4-L trunc) and cell-surface shifted (RLP4 ECD MA: RFP and RLP4-L ECD MA: RFP) truncated variants of RLP4 and RLP4-L may also be used to further refine possible candidate interactors of RLP4 and RLP4-L likely to be of particular interest for future investigation. If RLP4 and RLP4-L organise the transport of proteinaceous cell-wall modifying factors to the cell wall via cell edges, the identity of these may also be revealed through co-immunoprecipitation analyses against RLP4: GFP, RLP4-L: GFP and fluorescently-tagged truncated variants. It has been suggested that cell wall LRR-extensins may associate with the extracellular MLDs of FERONIA (Dünser et al., 2019) however, it remains to be seen whether such proteins linked to cell wall modification also associate with RLP4 and RLP4-L.

Mutant analyses of RLP4 and RLP4-L have so far failed to give significant insights into their possible functions in edge-directed trafficking. CRISPR-Cas9 *rlp4* and *rlp4l* mutants as well as a double *rlp4*, *rlp4l* mutant background plants are indistinguishable from wild-type plants under normal growth conditions (Sebastian Wolf et al., unpublished data). *rlp4*, *rlp4l* plants are however hyper-sensitive to

salinity and possibly also to disruption of cellulose synthase activity through treatment with isoxaben, indicating that they may be mildly developmentally compromised and that this only becomes phenotypically visible upon challenging conditions (Sebastian Wolf et al., unpublished data). Genetic or pharmacological perturbation of microtubules during expression of RAB-A5c[N125I] synergistically enhances lateral root phenotypes (Kirchhelle et al., 2019). Disruption of microtubule organisation in the *rlp4*, *rlp4l* background could therefore also be tested for possible synergy. Work has shown that knockout mutants of RLPs often retain a wild-type-like phenotype, indicating that RLPs may be able to act redundantly (Wang et al., 2008). Over-expression phenotypic analyses have however been successfully used in functional characterisation of some RLPs (Wolf et al., 2014; Yan et al., 2014). No obvious phenotypic defects were observed in plants expressing fluorescent protein fusions of RLP4 or RLP4-L from the UBQ10 promoter however, it is not known if fluorescently-tagged RLP4 or RLP4-L remain functional. Moreover, it is possible that any detrimental phenotypes caused by constitutive over-expression of fluorescently-tagged RLP4 and RLP4-L from the UBQ10 promoter result in negative selection against high-expressing individuals. Therefore, in order to investigate any possible phenotypic effects of over-expression of RLP4 and RLP4-L, constructs allowing for conditional over-expression of RLP4 and RLP4-L could be generated using the pOp/LhGr system (Craft et al., 2005). In addition, some of the fluorescently-tagged truncated variants of RLP4 and RLP4-L exhibited noticeable variation in brightness (e.g. secRFP: RLP4-L trunc was consistently brighter than secRFP: RLP4 trunc), indicating that some of these truncated variants may exert a dominant-negative effect. Conditional over-expression constructs for truncated variants of RLP4 and RLP4-L could therefore also be generated to investigate the possibility that these may exert dominant-negative effects. Proteomic analyses of RLP4 and RLP4-L may also provide additional targets for mutant analyses and further investigation of RLP4 and RLP4-L function – particularly any possible RLK partner for RLP4 and RLP4-L.

6.D: Trafficking of Rab-A GTPases and supporting factors of edge-directed trafficking

Mis-localisation of YFP: RAB-A5c to the Golgi in the presence of RAB-A5c[N125I] suggests that RAB-A5c may first associate with the endomembrane system at the Golgi, possibly independently of its cognate GEF. A particularly interesting question is whether other Rab-A GTPases also begin their membrane association at the Golgi, and whether this may be unpinned by a common recruitment mechanism. YFP: RAB-A2a[S26N] is known to localise to the Golgi, reinforcing the possibility that other Rab-A GTPases may also begin their membrane association at the Golgi (Chow et al., 2008). To investigate this further, Golgi and TGN/EE markers will be introduced into lines co-expressing AtRPS5a>DEX>RAB-A5c[N125I].2 and YFP: RAB-A2a/GFP: RAB-A4b.

If Rab-A dominant negative N→I mutants disrupt the localisation of Rab-A GTPases by sequestering a possibly shared GEF that is not required for their initial membrane localisation, TRAPP II is a strong candidate for this. Expression of RAB-A5c[N125I] putatively alters the localisation of the TRAPP component pUB:: BET5: RFP, possibly shifting it towards the TGN/EE. Whether expression of RAB-A5c[N125I] or other Rab-A dominant negative mutant variants are also capable of altering the localisation of pUB:: BET5: RFP and fluorescently-tagged TRAPP II-specific components will be the subject of future investigation. In such a scenario, it would be predicted that mutants in TRAPP II components should mis-localise Rab-A GTPases in a similar manner to that caused by expression of Rab-A N→I mutants. Kalde and colleagues show that *trappii* mutants mis-localise YFP: RAB-A2a at the growing cell plate, but did not investigate the nature of the intracellular compartments labelled by YFP: RAB-A2a in *trappii* mutant backgrounds in interphase cells (Kalde et al., 2019). YFP: RAB-A5c and YFP: RAB-A2a could therefore be introduced into TRAPP II component mutant *trs120* or *trs130* plants (Thellman et al., 2010), along with markers of the Golgi and TGN/EE, in order to investigate this further. However, definitive assessment of whether TRAPP II acts a Rab GEF for one or more Rab-A GTPases in *Arabidopsis* will require investigation using an *in vitro* nucleotide-exchange assay,

alongside a wider range of comparative co-immunoprecipitation analyses against fluorescently-tagged mutant variants of Rab-A GTPases (Kalde et al., 2019).

The identity of a Rab GDI-DF or other factor capable of platforming RAB-A5c and possibly other Rab-A GTPases at the Golgi also remains unknown. Co-immunoprecipitation and proteomic analyses against YFP-tagged dominant-negative Rab-A S→N mutants could be used to identify candidates for this, possibly in combination with co-immunoprecipitation targeting YFP-tagged wild-type Rab-A GTPases co-expressed with dominant-negative Rab-A mutant variants (e.g. YFP: RAB-A5c in an induced vs. uninduced AtRPS5a>DEX>A5c[N125I] background). Interestingly, comparative proteomic analyses against YFP: RAB-A2a and dominant-negative YFP: RAB-A2a[S26N] identified two PRA1 proteins, which are possible Rab GDI-DFs in *Arabidopsis*, as candidate interactors of RAB-A2a (Kalde et al., 2019; Kalde & Moore, unpublished data). One of these PRA1 proteins, AtPRA1.B4, localises to compartments in *Arabidopsis* that were often associated with ER-strands, indicating that it may localise to the Golgi (Kamei et al., 2008). Whether these candidates act to promote membrane association of RAB-A2a *in planta*, and are possibly shared by other Rab-A GTPases, remains unknown.

Pharmacological disruption of microtubules through prolonged treatment with oryzalin partially re-localised YFP: RAB-A5c to the PM. Putatively constitutively-active YFP: RAB-A5c[QL] partially localises to the PM in *Arabidopsis* lateral roots however YFP: RAB-A5c does not, indicating that RAB-A5c is removed from membranes either shortly before or shortly after reaching the PM in edge-directed trafficking. Partial re-localisation of YFP: RAB-A5c upon disruption of microtubules indicates that removal of RAB-A5c from membranes, which is presumably promoted by the activity of a Rab GAP, may be microtubule-dependent. Interestingly, previous work has identified a putative Rab GAP for RAB-A5c through a yeast two-hybrid screen using wild-type and dominant-negative Rab mutant variants as bait. The putative Rab GAP At2g37290 interacted specifically with RAB-A5c[Q71L] in yeast two-hybrid assays whilst a different putative Rab GAP, At3g55020, interacted specifically with RAB-

A2a[Q71L] (Michal Hála & Ian Moore, unpublished data). This indicates that RAB-A5c and RAB-A2a removal from membranes may be supported by activity of different Rab GAPs. Whether either these Rab GAPs acts against RAB-A5c or RAB-A2a *in planta* and whether possible activity of At2g37290 is microtubule-dependent, remains unknown. Treatment with endocytosis inhibitors Wortmannin and Tyrphostin A23 also mis-localised YFP: RAB-A5c to the PM in some cases. Whether this is due to a direct role for endocytosis in removal of YFP: RAB-A5c from the PM, or due to an indirect disruption of an as-yet unknown RAB-A5c trafficking regulator by these drugs remains unknown.

The work presented in this thesis has highlighted the need for proteomic analysis against newly-identified components of edge-directed trafficking, and mutant variants of RAB-A5c, in order to further molecular and functional understanding of edge-directed trafficking. However, the proteomically-obtained list of candidate interactors of YFP: RAB-A5c used as a starting point for this thesis contained several candidates interactors that either could not be easily cloned or gave no fluorescence when expressed as fluorescent protein fusions in *Arabidopsis*. These include the two highest-ranking candidate interactors of YFP: RAB-A5c according to the comparative analysis, At3g15356 and At1g52000 (Appendix 1, Figure 1), both of these are putatively annotated as lectin-type proteins. Future work should therefore also include further efforts to express and characterise these remaining candidate interactors *in planta*, the significance of which may be highlighted further by additional proteomic characterisation of newly identified components of edge-directed trafficking.

Chapter 7: Materials & Methods

Plant materials and growth conditions

7.A: Material sources and growth

All stable transgenic expression was performed in the *Arabidopsis thaliana* Columbia ecotype. BiFC experiments were performed using *Nicotiana tabacum* plants at around 5 weeks old. *Arabidopsis* lines expressing AtRPS5a>DEX>RAB-A2a[N125I], AtRPS5a>DEX>RAB-A4b[N125I] and AtRPS5a>DEX>RAB-E1d[S26N] are unpublished and were generated by Niloufer Irani. Lines expressing GFP: RAB-A4b from *pUB:: GFP: RAB-A4b* were also generated by Niloufer Irani. SAIL T-DNA insertion lines SAIL_119_B07 (*rab-a5c*) and SAIL_273_B12 (*rab-a5b*) were characterised and crossed by Charlotte Kirchhelle to generate *rab-a5b rab-a5c* T-DNA lines (Kirchhelle et al., 2016 and unpublished data; see Figure 7.2). The sources of all published plant materials are listed below.

<u>Arabidopsis line</u>	<u>Publication Source</u>
pRAB-A5c::YFP:RAB-A5c	Kirchhelle et al., 2016
AtRPS5a>DEX>RAB-A5c[N125I]	Kirchhelle et al., 2016
pVHAa1::VHA-a1:GFP	Dettmer et al., 2006
pUB::YFP:NPSN12	WAVE collection, Geldner et al., 2009
pUB::RFP:NPSN12	WAVE collection, Geldner et al., 2009
pUB::SYP32:mCh	WAVE collection, Geldner et al., 2009
pUB::MEMB12:mCh	WAVE collection, Geldner et al., 2009
p35S::N-ST:YFP	Batoko et al., 2000; Grebe et al., 2003
pRAB-A2a:: YFP: RAB-A2a	Chow et al., 2008

Arabidopsis lateral roots were imaged after 10-12 days after germination unless stated otherwise on full or half-concentration Murashige and Skoog (MS) plates (Sigma-Aldrich, Poole, UK) with 0.8% (w/v) Bacto-agar (Appleton Woods, Birmingham, UK) and 1% (w/v) sucrose. Seeds grown on MS or ½ MS plates were stored at 4°C for 2 days prior to germination. For conditional dominant-negative mutant expression, plants were either germinated on or transferred to ½ MS plates (as described above) containing 10µM DEX (dissolved in DMSO). For non-induction controls, the plates contained an equivalent volume of DMSO. All plants were grown at 20°C in a 16 hour: 8 hour day-night cycle.

A

B

C

D

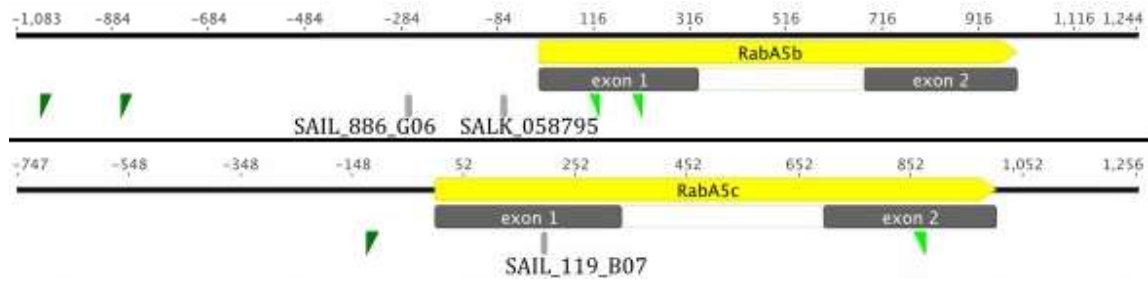
Figure 7.1: *rlp4* and *rlp4l* mutant lines generated by CRISPR-Cas9 (generated, characterised and provided by Ann-Kathrin Schuerholz and Sebastian Wolf, University of Heidelberg).

Gg = Restriction Site **■** = Insertion **■** = Stop codon

(A) cDNA sequence of RLP4 from 1 to 279bp. (B) A G insertion at position 185 disrupts a HypCH4V restriction site and creates an in-frame downstream stop codon. Lines were genotyped using primers GGATTAGTTGTGGAGCTAG and TTGACTACTCCAACCAGATT and the resultant product tested for sensitivity to HypCH4V.

(C) cDNA sequence of RLP4-L from 1 to 351bp. (D) A T insertion at position 286 disrupts a BstNI restriction site and creates an in-frame downstream stop codon. Lines were genotyped using primers AAAGTGAATTCTTCTGTGTT and ATCTCAAGAGAAAACAAGAG and the resultant product tested for sensitivity to BstNI.

A

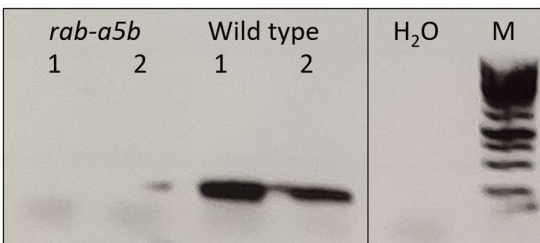


B

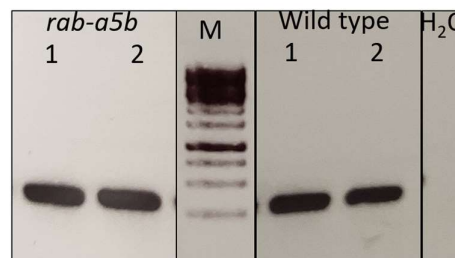


C

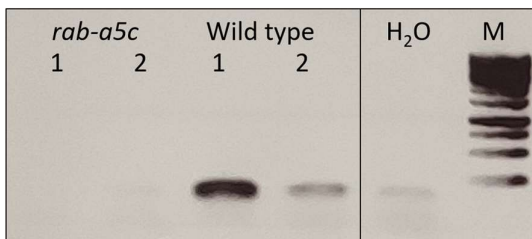
RAB-A5b RT1
CACTGCTGGTCAAGAGAGGT + GCCGTGTCACAATGAGTGT



GAPDH (positive control)
AGAGGAGCAAGGCAGTTAGTG + CACTTGAAGGGTGGTGCTAAA



RAB-A5c RT1
CGATGAGCTCAACACTCATTCTG + CAACGCAGAGGTCTCCATGA



GAPDH (positive control)
AGAGGAGCAAGGCAGTTAGTG + CACTTGAAGGGTGGTGCTAAA

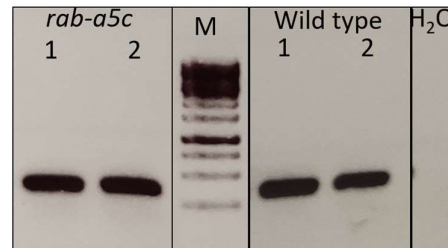


Figure 7.2: Characterisation of *rab a5b* and *rab a5c* insertional mutant lines (characterised and provided by Charlotte Kirchhelle).

(A) Available insertion mutants in RAB-A5b and RAB-A5c. Putative insertion site indicated in grey, forward primer position used for genotyping in dark green, reverse primer in light green. RAB-A5b contains two putative insertion lines upstream of exon 1. RAB-A5c contains a putative insertion line in exon 1. (B) Genotyping of RAB-A5b and RAB-A5c insertion mutants. (C) RT-PCR of *rab-a5b* (SAIL_886_G06) and *rab-a5c* (SAIL_119_B07) homozygous insertion mutant lines.

7.C: Floral Dipping and Manual Crossing of *Arabidopsis*

Transformation of *Arabidopsis* by floral dipping with *Agrobacterium tumefaciens* was done according to Clough and Bent., 1998. T1 transgenic seed were washed in 70% ethanol for 5 minutes and then for 10 minutes in a solution of 20% thin bleach in ethanol. Seed were then suspended in sterile 0.1% agarose and spread onto selection plates. At least two independent transformation events were performed for each construct and subsequent generations derived from these two independent lines. For manual crossing of *Arabidopsis* transgenic lines, maternal line flowers on approximately 5 week old plants were emasculated using forceps and gently enclosed in cling film to prevent dehydration. Paternal line pollen was applied 24 hours after emasculation.

7.D: Transient Expression in *Nicotiana tabacum*

For Bimolecular Fluorescent Complementation (BiFC) assays, constructs were transiently expressed in *Nicotiana tabacum* leaves. Overnight *Agrobacterium* cultures were centrifuged at 3,000rpm for 10 minutes and cells resuspended in 10mM MgCl₂ with 10μM acetosyringone. The cell suspension was introduced into the basal side of mature leaves via syringe and leaf samples imaged 48 hours later.

PCR amplification and Cloning

7.E: Bacterial Strains

Escherichia Coli strains DH5 α and DB3.1 were used for Gateway Cloning, with *Escherichia coli* (*E.coli*) DH5 α also used for restriction-based cloning of YFP:RAB-A5b and split-YFP-tagged Rab GTPases. For transformation of *Arabidopsis*, *Agrobacterium tumefaciens* GV3101::pMP90 was used.

7.F: Bacterial Growth and Transformation

LB medium was used for all bacterial growth, with LB + Agar (LBA) plates used for growth of colonies. LB medium contained 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) sodium chloride, with X% (w/v) agar added to give LBA medium. SOC medium was used for incubation of bacteria after transformation – 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10mM sodium chloride, 2.5mM potassium chloride, 10mM magnesium chloride and 20mM glucose.

For *E.coli* transformation, no more than 10% volume DNA was added to the cell suspension and incubated on ice for 30 minutes. Cells were then heat-shocked for 2 minutes at 42°C and subsequently suspended in 600 μ l SOC. This was then incubated at 37°C for 1 hour with gentle shaking. 75 to 100 μ l was spread onto an LBA selection plate and incubated overnight at 37°C. For transformation of *Agrobacterium tumefaciens*, no more than 10% volume of a 1:100 dilution of DNA was added to electrocompetent *Agrobacterium* cells and subsequently shocked using a Gene Pulser Electroporation Cuvette (Bio-Rad Laboratories, Watford, UK), before resuspension in 1mL SOC. This was incubated at 28°C for 2-4 hours with gentle shaking, and 75-100 μ l spread onto an LBA selection plate. Plates were incubated at 28°C for 2 days.

7.G: Plasmid Sources

For Gateway cloning, pDONR207 and pENTR™ were used as DONOR vectors (Invitrogen, Paisley, UK). DESTINATION pUB-DEST vectors used for fluorescent protein fusions and BiFC were obtained from published sources (Grefen et al., 2010). Hygromycin-resistant variants of pUB: YFP-DEST (Grefen et

al., 2010) were generated by Bernadette Gehl and used for transformation of *rab a5b rab a5c* lines with pRLP4: RLP4: YFP and pRLP4L: RLP4-L: YFP. For cloning of YFP:RAB-A5b and generation of split-YFP-tagged RAB-A5c, the vector pBINPLUS was used (van Engelen et al., 1995). For blunt-ended cloning, pJET1.2/blunt (Thermo Fisher Scientific, Hemel Hempstead, UK) was used as an intermediate vector.

7.H: PCR

Promoter and gene amplification for cloning was done using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Hemel Hempstead, UK) according to the manufacturer's specification. The basic PCR reaction began with an initial temperature of 98°C for 1 minute, followed by a cycle of 10 seconds at 98°C, 20 seconds at the annealing temperature and a variable time period at 72°C for amplification. Reactions ended with 10 minutes at 72°C. The number of cycles, the annealing temperature and the amplification time varied according to the requirements of the individual PCR amplification.

7.I: PCR Product Purification and Gel Extraction

PCR products were purified for cloning using a QIAquick PCR Purification Kit (Qiagen, Manchester, UK) and extracted from 1% agarose gels using a QIAquick Gel Extraction Kit (Qiagen, Manchester, UK). Both protocols followed those of the manufacturer and the DNA was eluted into 30µl of the provided elution buffer heated to 50°C.

7.J: Plasmid DNA isolation from bacteria

For isolation of plasmid DNA from overnight *E.coli* cultures, cultures were spun at 3000rpm for 10 minutes and DNA isolated from pelleted cells using QIAprep Spin Miniprep Kit (Qiagen, Manchester, UK), according to the manufacturer's instructions. Plasmid DNA was eluted into 30µl of the provided Elution Buffer.

For isolation of DNA from *Agrobacterium* cultures, cultures were pelleted by centrifugation at 3000rpm for 10 minutes and pellets re-suspended in a resuspension buffer (50mM glucose, 25mM Tris-HCl pH 7.5, 10mM EDTA) with 10µl lysozyme (10mg/ml). After 5 minutes on ice, 200µl of lysis buffer (0.2M NaOH, 1% SDS) was added, the mixture inverted several times and left on ice for 10 minutes. 300µl neutralisation buffer (3M potassium acetate pH 4.8, 46% acetic acid) was then added, the mixture inverted several times and spun at 13,000rpm (4°C) for 20 minutes. 800µl supernatant was then mixed with 560µl isopropanol and spun at 13,000rpm (4°C) for 30 minutes. The resultant pellet was washed with 70% ethanol before air drying and re-suspended in 50µl sterile dH₂O. For verification of *Agrobacterium* plasmid identity, isolated *Agrobacterium* DNA was transformed in *E.coli* as described above and subsequently isolated for restriction digests.

7.K: Genomic DNA isolation from *Arabidopsis*

Arabidopsis seedlings or leaf samples were used for the isolation of genomic DNA for PCR. Tissue samples were macerated using small sterile pestles and 400µl DNA extraction buffer added immediately afterwards (200mM Tris-HCl pH 7.5, 250mM NaCl, 25mM EDTA and 0.5% SDS). Samples were briefly vortexed and incubated at room temperature for 10 to 30 minutes with light shaking. Samples were then centrifuged at 13,000rpm for 1 minute and 300µl supernatant mixed with an equal volume of isopropanol. After 2 minutes at room temperature, this was spun at 13,000rpm for 10 minutes and the supernatant discarded. DNA pellets were washed with 70% ethanol and subsequently air-dried. Dry pellets were re-suspended in 50µl sterile dH₂O.

7.L: Cloning strategies

Gateway cloning

With the exception of RAB-A5b, pUB-DEST vectors (Grefen et al., 2010) were used for expression of fluorescent-protein fusions with candidate interactors. N- or C-terminal fusions were selected based on basic protein prediction software (TAIR; www.arabidopsis.org & Uniprot; www.uniprot.org) and

existing literature. If no reason to favour either a N- or C-terminal fusion was found, a C-terminal fusion was used. The presence of signal peptides and extracellular domains favoured C-terminal fusions for most of the candidate interactors however, two notable exceptions were identified. The first of these was HIP26 (At4g38580), a member of the Heavy metal-associated Isoprenylated Plant Protein (HIP) family. HIP proteins contain C-terminal isoprenylation domains and thus have previously been expressed as N-terminal fusions with fluorescent proteins (Barth et al., 2004; Barth et al., 2009; Cowan et al., 2018) to avoid disruption of this modification. The second exception was the remorin-like protein At4g00670. Remorins are plant-specific membrane-associated proteins that have been implicated in the formation of membrane microdomains and a variety of associated physiological processes. Remorin C-terminal regions contain conserved coiled-coil domains and are involved in remorin oligomerisation (Bariola et al., 2004; Raffaele et al., 2007; Marin et al., 2012; Toth et al., 2012) and has been shown in at least one case to be necessary for peripheral-association of a remorin with the plasma membrane (Perraki et al., 2012). It was therefore decided to use N-terminal fusions of both HIP26 and the remorin-like protein At4g00670. Each candidate interactor was initially introduced into the DONR vector pDON207 and upon sequencing confirmation, pUB-DEST vectors pUBC-RFP-DEST or pUBN-RFP-DEST (Grefen et al., 2010). pUB:: RLP4/RLP4-L ECD: RFP, pUB:: RLP4/RLP4-L ECD MA: RFP and pUB:: RLP44: RFP were all also expressed using pUBC-RFP-DEST.

N-terminally tagged RLP4 and RLP4-L

To generate N-terminally tagged RLP4 and RLP4-L, coding sequences were amplified with a repeated Gly Gly Ala linker sequence in place of their endogenous signal peptides. secRFP was then amplified with a 3' Gly Gly Ala linker sequence and the two sequences joined by overlap PCR to give secRFP: RLP4 and secRFP: RLP4-L. These sequences were then inserted into the vector pUB-DEST (Grefen et al., 2010) using Gateway recombination to give pUB:: secRFP: RLP4 and pUB:: secRFP: RLP4-L. Truncated pUB:: secRFP: RLP4 trunc, pUB:: secRFP: RLP4-L trunc and pUB:: secRFP: RLP44 trunc were all also generated by the same method, with a Gly Gly Ala linker placed immediately ahead of the

endogenous TMDs and intracellular sequences, giving secRFP: RLP4 trunc, secRFP: RLP4-L trunc and secRFP: RLP44 trunc. For use in BiFC, these sequences lacking a stop codon were inserted into pUB-cYFP-DEST and pUB-nYFP-DEST (Grefen et al., 2010) using Gateway recombination.

Native-promoter driven RLP4 and RLP4-L

In order to express RLP4 and RLP4-L from their native promoters, the UBQ10 promoter was removed from pUB-DEST (-RFP, -YFP and -eGFP) vectors by digestion with PmeI and PspXI and the vector closed by ligation, giving pX-xFP-DEST. The promoter sequences, 5'-UTRs and coding regions of RLP4 and RLP4-L were then amplified by PCR together and inserted into pX-DEST variants using Gateway Recombination, giving pRLP4: RLP4: xFP and pRLP4L: RLP4-L: xFP

YFP: RAB-A5b and constructs for BiFC

YFP: RAB-A5b was cloned downstream of its endogenous promoter (defined as 4.9kb upstream of the start codon) into the vector pBINPLUS (van Engelen et al., 1995) using the cloning strategy described by Kirchhelle and colleagues for RAB-A5c (Kirchhelle et al., 2016). To generate split-YFP-tagged RAB-A5c constructs for use in BiFC, YFP was removed from p35S:: YFP: RAB-A5c in pBINPLUS (Moore et al., unpublished; van Engelen et al., 1995) through digestion with KpnI & BamHI and replaced with nYFP/cYFP sequences to generate p35S:: nYFP: RAB-A5c and p35S:: cYFP: RAB-A5c respectively. YFP: RAB-A5c was removed from p35S:: YFP: RAB-A5c by digestion with KpnI & Ascl and replaced with nYFP/cYFP sequences to generate p35S:: nYFP and p35S:: cYFP for use in BiFC controls.

7.M: Oligonucleotide Primers

All primers were synthesised by Sigma-Aldrich (Poole, UK). Primer sequences are given below in

Table 1.

Table 1: Primer sequences used in this study

Primer Name	Nucleotide Sequence
RLP4 Gateway Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGATGCTTCGATTTATCCTAGCTTCTCTTCTC
RLP4 Gateway Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCAGACAACAAGCTCGGTCCATTTTCCAC
RLP4-L Gateway Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGCCCTTCTCTCCTTCTCTTC
RLP4-L Gateway Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTGCGAATTCAGTGGAAGAGTGGGC
RLP4 Promoter Gateway Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCAATTTAAACACCTAAGGAGTGACATAC GGTCGAGCTAGAGAAGAGTAGAG
RLP4-L Promoter Gateway Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCCTAAACAAAACCTACCACGAGCTTAAGAC TGAATGGAGAGGATAAGGAGAGGTG
RLP4 ECD Gateway Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTTGGCTCCAGAAGAAAGGTGAGGC
RLP4-L ECD Gateway Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTTACCCCTTTGGATAAG
RLP4 N-terminal Fusion Forward with linker	GGAGGAGCTGGAGGAGCTCGACCAGGTTTCGTTTTCTAGATTTAG
RLP4-L N-terminal Fusion Forward with linker	GGAGGAGCTGGAGGAGCTTCTCCATTTCAGTACGTCTCCTTCTC
secRFP Forward	CACCATGAAGACTAATCTTTTTCTCTTCTCATCTTTCACTTCTC

secRFP Reverse with linker	AGCTCCTCCAGCTCCTCCGGCGCCGGTGGAGTGGCG
RLP4 TMD with linker Forward	GGAGGAGCTGGAGGAGCTATTGGCATTGCATTCCGG
RLP4-L TMD with linker Forward	GGAGGAGCTGGAGGAGCTATAGCCATAGCCATATC
RLP4 Reverse with Stop	TCACTGTCGTCGTTTCCACCATATC
RLP4-L Reverse with Stop	CTATCTTCCTCTCCAGATGCAACACAAG
RLP4 TMD Gateway Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTC CTGTCGTCGTTTCCACCATATC
RLP4-L TMD Gateway Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCTTCCTCTCCAGATGCAACACAAG
RLP4 Reverse with Stop	TCAAGACAACAAGCTCGGTC
RLP4-L Reverse with Stop	CTATTGCGAATTCAGTGGAAGAGTG
RLP44 Gateway Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGACAAGGAGTCACCGGTTAC
RLP44 Gateway Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCGTAATCAGGCATAGATTGACTAATCTTACCTTC
RLP44 TMD with linker Forward	GGAGGAGCTGGAGGAGCTGGTTTGTCTGTGATGGC
RLP44 reverse with stop	TTAGTAATCAGGCATAGATTGACTAATCTTACC
RAB-A5b CDS Forward	GGCGCGCCTGGAGCAGGAATGGGTAAGGAAGATGACC
RAB-A5b CDS Reverse	GGTGGCGCGCCTTGTACTTGATCAACCATTCCATCTGTTA
RAB-A5b Promoter Forward	GATGCCTGCAGGAGCAGCCTCAAGCTATTTATGTACAAAC

RAB-A5b Promoter Reverse	TGCTGGTACCCTTCTTCTACTTCTTCGAGACTGTGGAG
At4g00670 Gateway Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGAGCCAAATATTCCGATCCAAAGAGG
At4g00670 Gateway Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGAAGCAGCTCAAAGATGACTTCTTGGG
At4g18070 Gateway Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGCCTTCAGGTGCTAAGAA
At4g18070 Gateway Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGTCACAGGATCCAGTAGATAA
NIP1-1 Gateway Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGCGGATATCTCGGGAAACGGC
NIP1-1 Gateway Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTC AGTGCTACCGATTCTCACGGTCTTTAGAAATGAAC
ATS3a Gateway Forward	GGGGACAAGT TTGTACAAAAAAGCAGGCTTCACCATGTGCAACTACTGCGAGTTCTCCA
ATS3a Gateway Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGACATTTAGATGCTCATGCAAACCATGAAATCCG
HIPP26 Gateway Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGGTGTTCTTGATCATGTCTCTGAAAT
HIPP26 Gateway Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCCATGACCACACAAGCCGCCGGG
At5g35735 Gateway Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGCTGCACTCTACTTGAGCTGTGATTCTGGG
At5g35735 Gateway Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGGCTCTTTTCTCCACTGGATATACAACG
CASPL1D1 Gateway Forward	GGGGACAAGT TTGTACAAAAAAGCAGGCTTCACC ATGGGTTATGAAACCAAATCGACTTTGGACAC
CASPL1D1 Gateway Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGGATTTTCTTGAGAGGGAGAGG
AtBET5 Gateway Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACC ATGCAGTTCTTCGGTGGATCTGAGATTAG
AtBET5 Gateway Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGAGATGCTCCTTACGTACTGGTCA

7.N: DNA Sequencing

All DNA sequencing of PCR-amplified promoters and genes was performed by Source Bioscience (Nottingham, UK) with samples prepared according to their specifications (www.sourcebioscience.com/sequencing).

7.O: Restriction Digests

Restriction digests of DNA were performed in a 25µl format: 2.5µl Reaction Buffer, 5µl DNA, 0.5µl enzyme (for double digests, 0.25µl of each enzyme was used) and 17µl dH₂O. Reactions were incubated for 1 hour at 37°C. For analytical digests, 7µl of a completed reaction was used for gel electrophoresis. All restriction enzymes were provided by NEB Ltd (Hitchin, UK).

7.P: Gateway and Non-Gateway Cloning

Gateway-primed PCR products were recombined into the donor vector pDONR207 (Life Technologies, UK) using Gateway BP Clonase II Enzyme Mix (Thermo Fisher Scientific, Hemel Hempstead, UK) according to the manufacturer's instructions. Cloned DNA sequences were verified by sequencing in the pDONR207 vector. Non-Gateway-primed PCR products were introduced into the donor vector pENTR/D-TOPO using a pENTR™/D-TOPO™ Cloning Kit (Thermo Fisher Scientific, Hemel Hempstead, UK). Recombination from the donor vector into the pUB-DEST vectors was done using Gateway LR Clonase II Enzyme Mix (Thermo Fisher Scientific, Hemel Hempstead, UK) also according to manufacturer's instructions.

For non-Gateway cloning, the vector pJET1.2/blunt (Thermo Fisher Scientific, Hemel Hempstead, UK) was used for amplification and sequencing of cloning fragments. Ligation reactions were set up according to the manufacturer's instructions and left at room temperature for 1 to 2 hours.

7.Q: DNA ligation

Ligation of sticky-end digests was done using T4 DNA ligase (Thermo Fisher Scientific, Hemel Hempstead, UK) with the mixture matching the provided instructions. Reactions were left overnight at room temperature before transformation into *E.coli*.

7.R: Dephosphorylation using Alkaline Phosphatase

Dephosphorylation of digested DNA ends was performed when required with Shrimp Alkaline Phosphatase (Thermo Fisher Scientific, Hemel Hempstead, UK). Reactions were incubated at 37°C for 15 minutes, followed by 45°C for 10 minutes and 65°C for 5 minutes. Dephosphorylated samples were purified using a QIAquick PCR Purification Kit (Qiagen, Manchester, UK) before use.

7.S: Antibiotics and Herbicides

Antibiotics and herbicides used for bacterial transformation and selection of transgenic plants were used at the following concentrations.

<u>Antibiotic/Herbicide</u>	<u>Selection Concentration</u>
Ampicillin	50µg/mL
Gentamycin	10µg/mL
Spectinomycin	100µg/mL
Kanamycin	50µg/mL
Carbenicillin	100mg/mL
Timentin	2mg/L
Gluofsinat-ammonium (BASTA)	10mg/L
Hygromycin	15µg/mL
Chloramphenicol	35µg/mL

7.T: Drug treatments

All drugs were dissolved in DMSO and diluted for use from stock solutions. Oryzalin, Latrunculin-B and DEX were applied in ½ MS + agar plates and all other drugs in ½ MS liquid medium. Brefeldin-A was applied at 50µM from a 50mM stock, Concanamycin-A at 2µM from a 2mM stock, Oryzalin at 10µM from a 10mM stock, Latrunculin-B at 1µM from a 1mM stock, Wortmannin at 50µM from a 50mM stock, Tyrphostin A23/A51 at 50µM from a 50mM stock and DEX at 10µM from a 10mM

stock. All were applied for the time period specified in the main text and obtained from Sigma Aldrich (Poole, UK). Responses recorded of fluorescent-protein fusions to drug treatments were based on observation of the response in at least 5 individual seedlings.

7.U: Confocal Microscopy

Confocal images were acquired on a Zeiss 880 CLSM (www.zeiss.com/microscopy). YFP was excited with a 488nm laser and collected between 503-581nm, mRFP1 excited with 561nm and collected between 581-754nm and eGFP excited with a 458nm laser and collected between 460-581nm. When YFP and eGFP were collected together, eGFP was excited using a 458nm laser and collected between 460-511nm and YFP excited with 488nm and collected between 520-581nm. Confocal microscopy was used to assess expression levels of fluorescent protein fusions in transgenic lines. For work involving RLP4: xFP and RLP4-L: xFP lines, those selected and propagated for use in this thesis were of lower expression level based on fluorescence intensity.

7.V: Image Analysis in Fiji

Maximum intensity projections, intensity adjustments cropping and figure assembly of confocal images for figures was done using Fiji (ImageJ-win64; Schindelin et al., 2012). For calculation of Mander's Correlation Coefficients, cytosolic background in confocal images was removed using a hysteresis filter in Fiji. To do this, average image background intensity was calculated based on 10 pixel samples for each fluorophore channel and the mean background intensity and maximum background intensity + 2SD (standard deviations) used as hysteresis thresholds. Hysteresis thresholding generates a binary mask of a given image, based on removing all signal below a set threshold (in this case, the mean background intensity) and retaining all signal between this and the upper threshold (maximum background intensity + 2SD) if it is connected to signal above the upper threshold. This generated a binary mask of each image channel, which was then inverted and subtracted from the raw image channel. Mander's values were then calculated in Fiji using the JACoP

plugin using the resulting image channel subtractions (Bolte and Cordelières, 2006). Mander's calculations were based on over 50 cells each from at least 7 individual seedlings for each line.

7.W: Image Analyses in MorphographX

2D segmentation for identifying lateral root surface and signal quantification

For 2D segmentation in MorphographX (Barbier de Reuille et al., 2015), YFP: NPSN12 image stacks were filtered using a Gaussian Blur filter with a radius of 0.2 μ m in all directions. The Edge Detect function was used to outline the organ surface with a threshold of between 5000 and 11000 depending on the degree of surface curvature. The Marching Cubes Surface tool was then used to generate a mesh based on this surface with a cube spacing of 3 μ m and threshold of 5000 and the Smooth and Subdivide tools used to refine this mesh further. The mesh was adjusted to better fit the YFP: NPSN12 image if necessary using the Shrink tool and the surface then projected onto the raw YFP: NPSN12 image. The Automated Watershed algorithm tool was used to automatically segment and numerically identify individual cells based on YFP: NPSN12 cell outlines and these manually merged in case of over-segmentation. Onto this final surface, YFP: NPSN12 and processed RLP4: RFP and RLP4-L: RFPs images were projected, and absolute signal intensity acquired within a border area up to 0.5 μ m , 1 μ m, 1.5 μ m and 2 μ m away from cell edges. Further surfaces projections were generated at a distance of 2-4 μ m and 4-6 μ m from the outer surface using YFP: NPSN12 and processed RLP4: RFP and RLP4-L: RFPs images to acquire absolute signal intensity at these depths. Each RLP4/4-L: RFP per unit area value was normalised against the YFP: NPSN12 value per unit area. The resultant value for each edge was then normalised against the corresponding value in the 0-0.5 μ m border area or 0-2 μ m depth respectively. Two-way ANOVA's and Tukey Post-Hoc tests were performed in R. Statistics were based on over 75 cells each from at least 4 individual seedlings for each line.

3D segmentation for removal of intracellular signal in RLP4: RFP and RLP4-L: RFP images

For 3D segmentation in MorphographX, YFP: NPSN12 image stacks were filtered using a Gaussian Blur filter with a radius of $0.3\mu\text{m}$ in all directions. Cells were segmented in 3D using a 3D auto-seeded watershed algorithm and subsequently manually curated to merge over-segmented cells and remove all but the epidermal cell layer. 3D cell meshes were identified from the segmented voxel data using the Marching Cube Surface 3D tool at cube size $0.8\mu\text{m}$. The Shrink Mesh function was used to shrink cell meshes by $0.25\mu\text{m}$ and $0.5\mu\text{m}$ respectively in all directions. Resulting meshes were used as 3D masks to trim to remove all intracellular signal from raw RLP4: RFP and RLP4-L: RFP images more than $0.25\mu\text{m}$ or $0.5\mu\text{m}$ away from the cell surface, respectively.

Appendix 1: Proteomic analysis of YFP: RAB-A5c and candidate interactor ranking

Co-immunoprecipitation against YFP-tagged Rab GTPases was performed by Dr Monika Kalde and mass spectrometry by Oxford Advanced Proteomics Facility. Data analysis and candidate interactor ranking was performed by Dr Ian Moore.

Co-immunoprecipitation protocol:

Plants expressing YFP: RAB-A5c (Kirchhelle et al., 2016), YFP: RAB-A2a (Chow et al., 2008) or YFP: RAB-G3f (Geldner et al., 2009) as well as control Col-0 plants were grown in liquid ½ MS medium for 14 days before application of 5µM NAA to induce lateral root proliferation. After a further 3 days of growth, root tissue was harvested and ground into double volume of extraction buffer (50mM HEPES pH7.5, 10% glycerol, 50mM NaCl, 5mM MgCl₂, 0.5mM PMSF) in the presence of cOmplete™ EDTA-free Protease Inhibitor (Sigma, Poole, UK). Samples were spun for 10 minutes at 13,000rpm to remove debris and incubated at 4°C with 1mM DTSSP crosslinker for 30 minutes (Sigma, Poole, UK). Samples were quenched for 15 minutes with 50mM Tris-HCl, which was also added to all buffers subsequently used. Microsomes were subsequently pelleted onto 50µl 2M sucrose through ultracentrifugation at 100,000g for 30 minutes. Microsomes were then solubilised through re-suspension in protein extraction buffer with 2% CHAPs added. Total microsome samples were removed at this point for analysis. The remaining samples were then spun again at 100,000g for 30 minutes and supernatant removed. α-GFP isolation was performed using µMACS GFP Isolation Kits (Miltenyi Biotec, Cologne, Germany). 200µl of α-GFP microbeads were added to 1mL protein

supernatant and incubated on ice for 60 minutes. Protein supernatant was added to isolation columns, the columns washed four times with protein extraction buffer with 2% CHAPs, and α -GFP microbeads with protein eluted with 50 μ l hot elution buffer. Protein extraction through tryptic on-gel digest and LC-MS/MS mass spectrometry were subsequently performed by Oxford Advanced Proteomics Facility. 20 samples were submitted for mass spectrometry with approximately 5g of plant tissue used per sample.

Samples submitted:

- α -GFP beads against YFP: RAB-A5c (3 samples – A5cBeads)
- α -GFP beads against YFP: RAB-A2a (5 samples – A2aBeads)
- α -GFP beads against YFP: RAB-G3f (3 samples – G3fBeads)
- α -GFP beads against Col-0 (3 samples- ColBeads)
- YFP: RAB-A5c total microsomes (2 samples- A5cTM)
- YFP: RAB-A2a total microsomes (2 samples – A2aTM)
- YFP: RAB-G3f total microsomes (2 samples – G3fTM)

Label-free quantitation of LC-MS/MS was performed using spectral index quantitation software (SINQ; Trudigan et al., 2011) by Oxford Advanced Proteomics Facility

Candidate Interactor Ranking

To begin, all proteins not present in each of the 3 YFP: RAB-A5c α -GFP beads were discounted leaving 315 proteins present in each YFP: RAB-A5c α -GFP beads sample. Average quantitative spectral index values of each category were calculated for each remaining hit, giving AvA5cBeads, AvA2aBeads, AvG3fBeads, AvColBeads and AvTM (Average of A5cTM, A2aTM and G3fTM values – Figure 1, columns A-E).

Three rank values were then calculated for each hit:

- Total Enrichment: $\frac{AvA5cBeads}{AvTM}$ (indicative of candidate enrichment in YFP: RAB-A5c pull downs compared to total microsomes. Figure 1, column F)
- A2a, Col Enrichment: $\frac{AvA5cBeads}{\frac{AvA2aBeads+AvColBeads}{2}}$ (indicative of candidate enrichment in YFP: RAB-A5c pull downs compared to pull downs against YFP: RAB-A2a and Col-0 samples. Figure 1, column G)
- G3f, Col Enrichment: $\frac{AvA5cBeads}{\frac{AvG3fBeads+AvColBeads}{2}}$ (indicative of candidate enrichment in YFP: RAB-A5c pull downs compared to pull downs against YFP: RAB-G3f and Col-0 samples. Figure 1, column H)

Each of these rank value categories was then ranked largest-to-smallest, with the largest hit value assigned 1 and the smallest hit value assigned 315. The ranked value for each hit was then summed across these three categories, giving a final SumRank value (Appendix 1, Figure 1; column I) that was itself ranked smallest-to-largest. Several high-ranking candidate interactors were however annotated as ribosomal or mitochondrial proteins (Appendix1, Figure 1; highlighted in red) and so these were removed from the list of initially considered candidate interactors as likely contaminants.

Appendix 1, Figure 1: Top-ranking candidate interactors of YFP: RAB-A5c ranked against abundance in bead pull-downs of plants expressing YFP: RAB-A2a and YFP: RAB-G3f and total microsome samples of plants expressing YFP: RAB-A5c, YFP: RAB-A2a and YFP: RAB-G3f. Candidates highlighted in red are those identified as likely contaminants from ribosomes, mitochondria or chloroplasts.

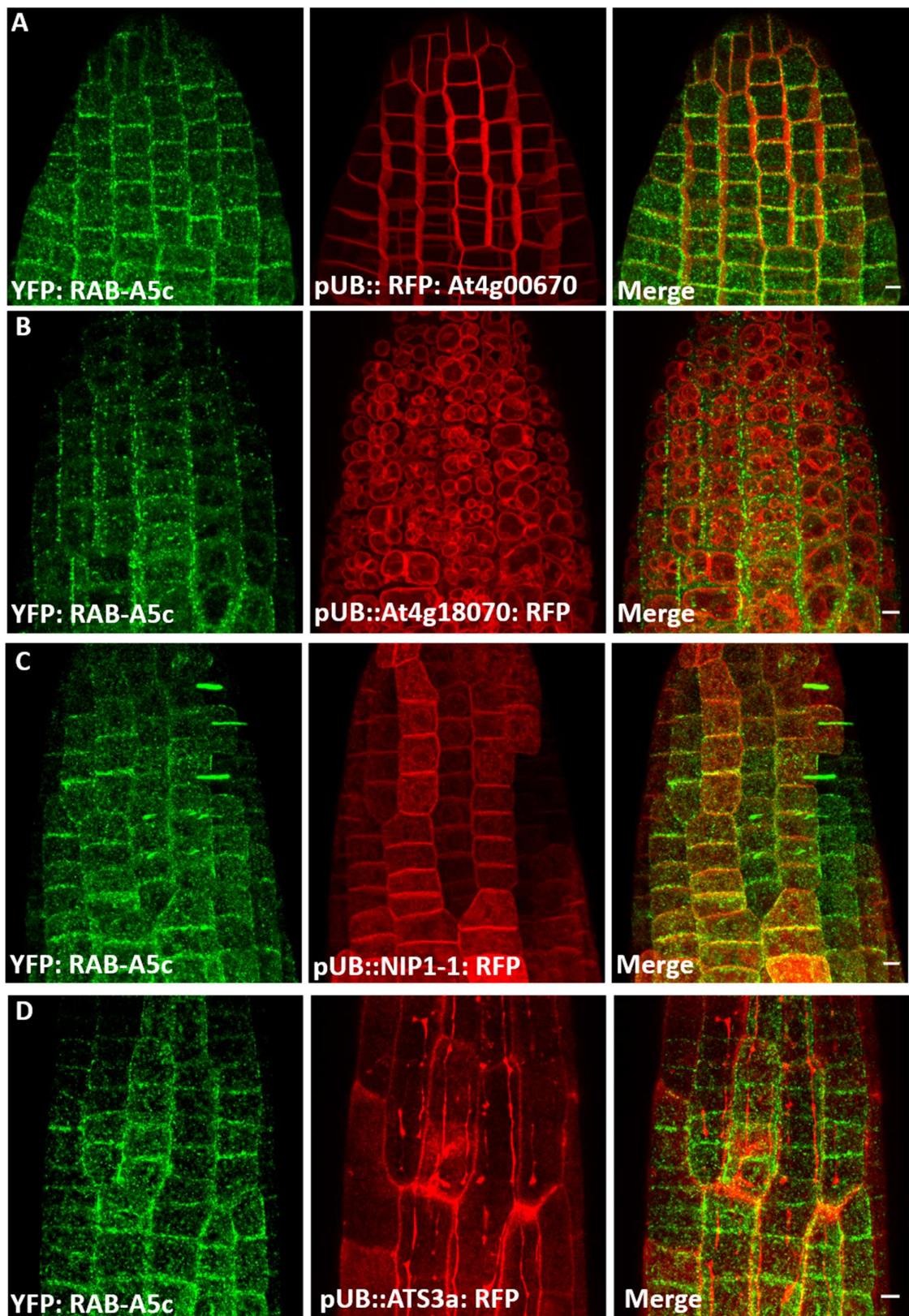
A: Average candidate intensity in A5c beads. B: Average candidate intensity in A2a beads. C: Average candidate intensity in G3f beads. D: Average candidate intensity in Col-0 beads. E: Average candidate intensity in Total Microsomes sample. F: Average intensity in A5c beads normalised against intensity in Total Microsomes. G: Average intensity in A5c beads normalised against intensity in A2a beads and Col-0 beads. H: Average intensity in A5c beads normalised against intensity in G3f beads and Col-0 beads. I: Final summed rank value of ranks for columns F, G & H.

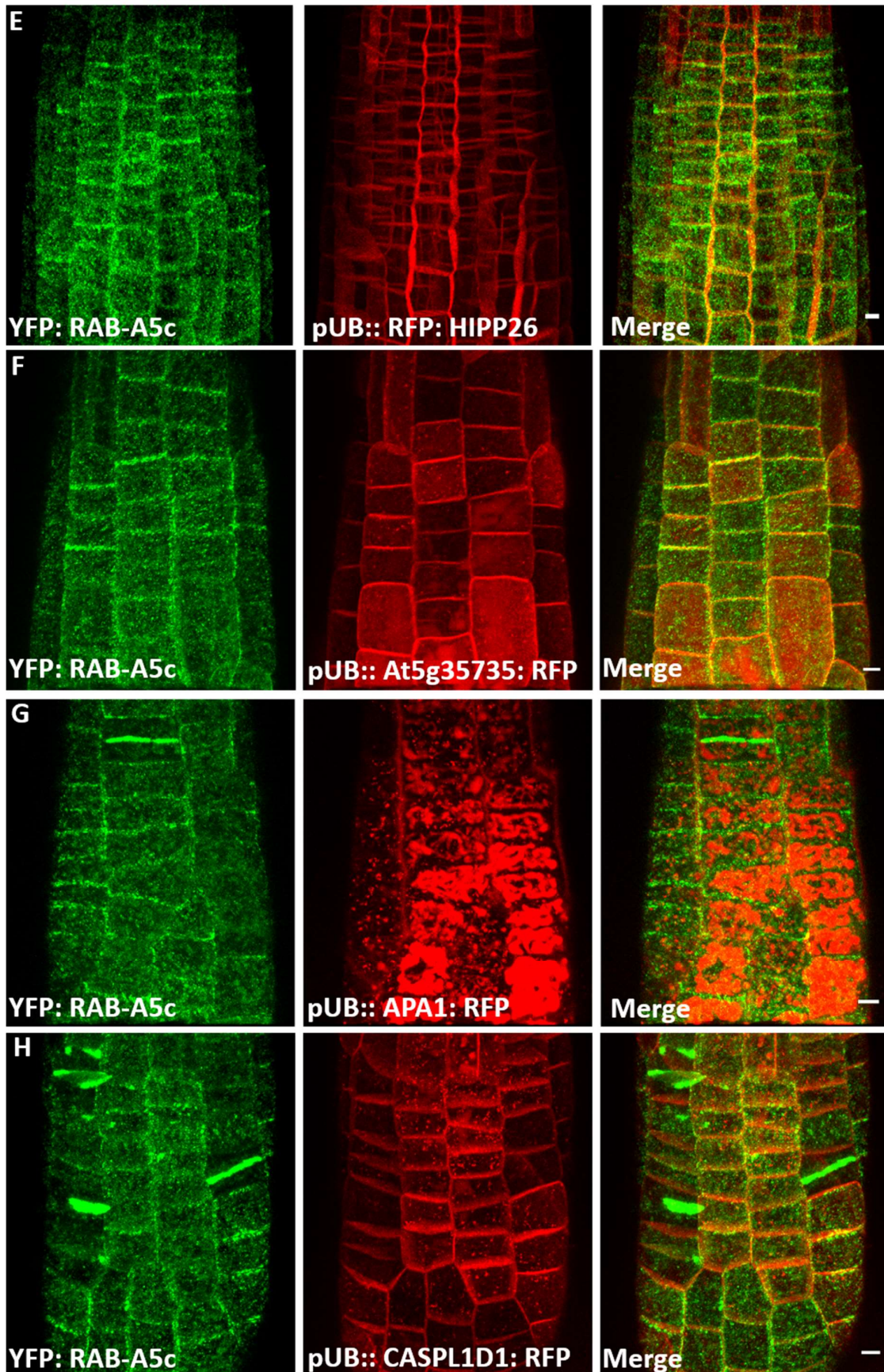
A B C D E F G H I

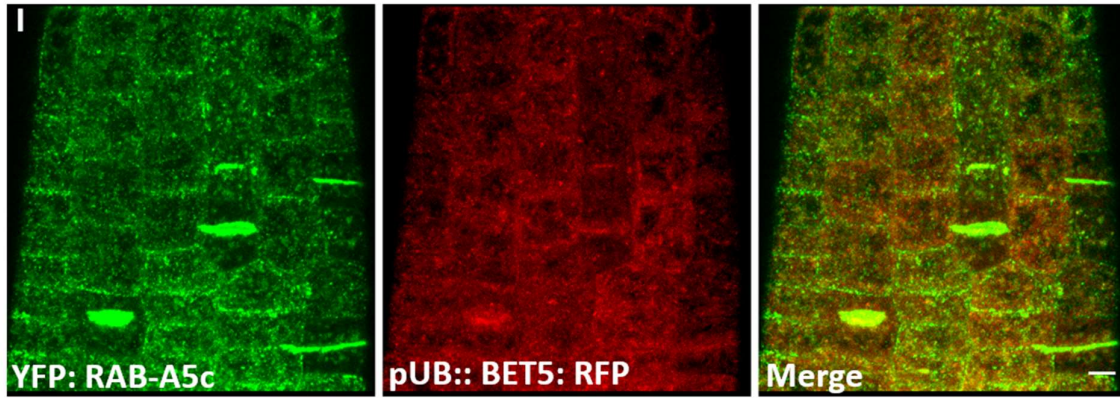
Protein	General Name	AvA5Beads	AvA2aBeads	AvG3fBeads	AvColBeads	AvTm	Total Enrichment	Rank	A2a, Col Enrichment	Rank	G3f, Col Enrichment	Rank	SumRank	Rank	Filtered Rank
P28187	RAB-A5c	0.0011295	2.8324E-07	2.394E-07	0	7.0042E-07	1611.196831	17	7941.083418	1	9387.856876	2	20	1	
O9LUR2	At3g15356	0.000002636	6.482E-08	0	0	0	4286.178862	6	79.81831945	8	4286.178862	6	20	2	1
O9ZU23	At1g52000	2.07553E-06	3.7004E-08	5.80533E-08	0	1.2592E-09	1107.443308	22	108.5700337	5	70.02080405	13	40	3	2
O9C5C1	At4g31330	4.69967E-06	4.7878E-07	2.31133E-07	0	0	7641.734417	1	19.5815365	26	40.4510178	16	43	4	3
F4IGD2	At4g04460	0.000006839	1.3856E-08	0	0	1.0628E-07	63.9806578	49	906.6684343	3	11120.3252	1	53	5	4
O6IDB4	At4g00670	5.38607E-06	4.526E-07	6.01E-08	0	1.5072E-08	343.3914152	30	23.73869804	20	175.6617207	10	60	6	5
O9SR55	RAB-A5b	4.71333E-06	0	0	0	0	21.1882801	79	7663.95664	2	7663.95664	4	85	7	6
O8VZ57	At4g18070	4.87933E-06	1.39358E-07	8.54947E-07	0	1.9562E-08	241.8304973	33	69.41322635	9	11.39795914	45	87	8	7
CIP1		6.6329E-06	1.21268E-07	1.66997E-06	0	6.3693E-09	949.682623	25	108.294013	6	7.937904775	63	94	9	8
SUC1		9.996E-07	1.00746E-07	0.000000223	0	0	1625.365854	16	19.60461285	25	8.915845337	59	100	10	9
O8W4H8	At1g54010	5.06967E-06	8.88848E-07	3.54257E-07	0	2.5545E-08	193.7945973	35	11.39151101	49	28.52240122	20	104	11	10
O8VZW1	NIP1-1	0.000004524	7.841E-07	5.212E-07	0	9.3827E-09	452.5055846	27	11.52127131	47	17.31906667	32	106	12	11
O681K2	AT53a	4.36433E-06	4.5486E-07	4.30033E-07	0	7.4167E-08	58.36100649	51	19.13803562	28	20.2397607	28	107	13	12
O9SZN7	HIPP26	0.000003152	2.9746E-07	2.967E-07	0	8.3167E-08	37.62159881	63	21.10549399	22	21.15933273	26	111	14	13
O84W97	MSL9	0.000002711	1.89136E-07	5.995E-07	0	2.03E-08	129.61989	40	28.48197682	15	9.025685416	58	113	15	14
O65390	APA1	0.00006486	0.000002437	0.000004561	0	4.1118E-06	15.77162672	89	53.20252806	11	28.43346346	21	121	16	15
O9FE58	At5g27770	3.07507E-06	9.75E-08	0	0	2.5667E-07	11.95447272	106	62.30460178	10	5001.084011	5	121	17	
At1g25570	F4ICJ5	9.87533E-07	2.3501E-07	4.43567E-08	0	3.6167E-09	233.3674675	34	8.360424427	74	43.32553378	15	123	18	16
At1g54000	Q1H583	0.000010395	1.62008E-06	1.42453E-06	0	1.6723E-07	61.93090985	50	12.82296415	44	14.58166269	36	130	19	17
F4HWL3	RLP4	1.41223E-06	3.45944E-07	2.80867E-07	0	0	2296.314363	11	8.135593871	77	10.01240709	49	137	20	18
At1g22210	O39080	3.87233E-06	7.0346E-07	0.000001648	0	0	6296.476965	3	10.99017535	54	4.695928807	102	159	21	
O9FE29	CASP1D1	2.12407E-05	3.72814E-06	4.61567E-06	0	6.9632E-07	30.4860113	67	11.39423906	48	9.20873598	56	171	22	19
At5g35735	O9FKH6	6.41333E-07	2.0194E-07	1.33307E-07	0	0	1042.818428	24	6.313268035	103	9.53956047	52	179	23	20
AtMg00285	O05000	1.3267E-06	3.2252E-07	5.11233E-07	0	0	2157.235772	12	8.195830116	76	5.177736293	92	180	24	
At1g73220	O9CAT6	1.00997E-06	2.13514E-07	0.000000451	0	0	1642.22222	15	9.406238746	67	4.466606225	104	186	25	21
O27G63	At5g62890	1.01503E-06	4.4448E-07	2.02567E-07	0	0	1650.467005	14	4.554680547	137	9.961235872	50	201	26	
O8RV04	At2g55795	0.000001499	4.01682E-07	2.10267E-07	0	7.9532E-08	18.70321078	85	7.440830752	85	14.17516431	38	208	27	
O9MAU6	At1g04980	6.432E-07	4.946E-08	0	0	2.495E-07	2.571617056	181	25.37778655	18	1045.853659	9	208	28	
O9M9W1	At3g05560	5.11733E-06	1.2358E-07	0	0	3.083E-06	1.659524076	200	82.00197634	7	8320.867209	3	210	29	
AtMg00580	P93313	9.551E-07	2.36848E-07	4.507E-07	0	0	1553.00813	18	8.023420896	80	4.226760782	113	211	30	
At5g67500	O9FXK3	1.22623E-05	0.0000003134	0.000003686	0	2.6828E-07	45.6030783	58	7.822286297	83	6.651244068	74	215	31	
O9SY88	BET5	1.29887E-05	5.0972E-06	2.25467E-06	0	2.6838E-07	48.2850453	57	5.095163282	122	11.51530286	43	222	32	22

Appendix 2: Expanded and additional figures

- **Appendix 2, Figure 1: Other candidate interactors of YFP: RAB-A5c do not localise to YFP: RAB-A5c-labelled cell edge compartments.**
- **Appendix 2, Figure 2: Domain comparisons and pairwise alignments of RLP4 and RLP4-L.**
- **Appendix 2, Figure 3: Expression of AtRPS5a>DEX>A5c[N125].1 does not substantially alter the localisation patterns of other initially considered candidate interactors of YFP: RAB-A5c, with the possible exception of pUB:: BET5: RFP.**
- **Appendix 2, Figure 4: Expression of AtRPS5a>DEX>A5c[N125].1 possibly shifts localisation of pUB:: BET5: RFP towards intracellular compartments.**
- **Appendix 2, Figure 5: Expression of AtRPS5a>DEX>[N125I].2 disrupts localisation of pRLP4:: RLP4: eGFP and pRLP4L: RLP4-L: RFP in lateral roots.**
- **Appendix 2, Figure 6: 24 hours of AtRPS5a>DEX>A2a[N125I] expression disrupts localisation of YFP: RAB-A5c, pUB:: RLP4: RFP and pUB:: RLP4-L: RFP in lateral roots.**
- **Appendix 2, Figure 7: 24 hours of AtRPS5a>DEX>A4b[N125I] expression disrupts localisation of YFP: RAB-A5c, pUB:: RLP4: RFP and pUB:: RLP4-L: RFP in lateral roots.**
- **Appendix 2, Figure 8: pUB: secRFP: RLP4 and pUB: secRFP: RLP4-L co-localise intracellularly with TGN/EE-localised VHA-a1: GFP but not with Golgi-localised ST: YFP in lateral roots.**







<u>Candidate Interactor</u>	<u>Figure Panel</u>	<u>Localisation in lateral roots</u>
At4g00670	A	Plasma membrane
At4g18070	B	Tonoplast
NIP1-1 (At4g19030)	C	Plasma membrane and endoplasmic reticulum (?)
ATS3a (At2g41475)	D	Extracellular
HIPP26 (At4g38580)	E	Plasma membrane
At5g35735	F	Plasma membrane and intracellular compartments
APA1 (At1g11910)	G	Vacuolar and some extracellular (?)
CASPL1D1 (AT4G15610)	H	Plasma membrane and intracellular compartments
AtBET5 (AT1G51160)	I	Intracellular compartments and cytosol

Appendix 2, Figure 1: Other candidate interactors of YFP: RAB-A5c do not localise to YFP: RAB-A5c-labelled cell edge compartments. (A, B, C, D, E, F, G, H, I) 3D confocal stack projections of lateral roots co-expressing YFP: RAB-A5c and RFP-tagged candidate interactors. All from 10 day old T2 seedlings. Scale bars 5µm.

A

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RLP4      1 mml--rfilasllssf--slysslarppayalriscgarknvrtpptyalwfkdiaytggvpanattp-tyitppiktiryfpise 82
RLP4-L    1 MPFSPSPF---LFLSSFFLLHLSSLSPFNTSFFIDCGS-PETSTDVFNRTWLPDQFYSGGSTAVVSEPLRFHLIAEKTIRYFPLSF 83

RLP4      83 gpnncynlrvvpkghysvriifglvdqpsfdkeplfdisiegtqisslksqw--ssqddqvfaealiflfggtaticfhstghgdpa 167
RLP4-L    84 GKKNCY-VVPLPPGRYYLRTFTVYDNYDGKSHSPSFDVSEGLVFSWRSPWPESLLRDGSYSDLFAFIGDGEELDCFYSIATDPP 169

RLP4      168 ilsiellqvddkaysfgegwgqgvilrtatrltcgtgksrfdedyrgdhwgdrfwnrmrsgksadsp-----ste 240
RLP4-L    170 VGSLEVLRQVDPSSYD-ADGTGQNVLLVNYGRSLSCGS-----DQW-GPGFTNHTDNFGRSQSDEDFRSEDSRSVARSLSTL 243

RLP4      241 etikkasvspnfypeglyqsavstddqpdltysldvepnrnysvwlhfaeidntitaegkrvfdvvingdtffedvdiikmsggry 327
RLP4-L    244 EKIKGVDAQPNYFPMKLYQTA-VTVSGGSLVYELEVDALDYLLWFHFSEIDSTVKKAGQRFVFLVFN-DNNVSRVDFVHEVGG-F 327

RLP4      328 aalvinatvtvsgrtltvvlqpkagghalinaievfeilitaefktlrdevsaiqkmkkaiglpisrfgwngdpcvppq-hpwsqancq 413
RLP4-L    328 AAYSLNYTVKNLSSTIVTVKLSSVSGAPIISGLENYAIVPADMATVPEQVTAMKALKDSLVRPDRMGWNGDPCAPTSDAWEGVSCR 414

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RLP4-L    415 PNSQGSALVIFQIDLSQGLKGFISEQISLLTNLSNLSSNTLSGQLPLGLGH-KSLVSLDLSNNQLTGPVPESL-TLSCLKLVLL 499

RLP4      501 ngnsisgkvpaavggriihrasfnftdnaglcgipglpac----gphlssgakigiafg---vsiafllivacamiwkrqnii 578
RLP4-L    500 NGNELQGVPEEVYSGVGHGIIIDLSGNKGLCGVPSLPTCPLWENGHLSKGGKIAIAISCVVFLILVFLVLYLCCI--WRGRHDYD 584

RLP4      579 ra----qiaargapyakkrthvshdiqmsrhghnnhgqartavengpslis 626
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B

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RLP4      1 mml--rfilasllssf--slysslarppayalriscgarknvrtpptyalwfkdiaytggvpanattp-tyitppiktiryfpise 82
RLP4-L    1 MPFSPSPF---LFLSSFFLLHLSSLSPFNTSFFIDCGS-PETSTDVFNRTWLPDQFYSGGSTAVVSEPLRFHLIAEKTIRYFPLSF 83

RLP4      83 gpnncynlrvvpkghysvriifglvdqpsfdkeplfdisiegtqisslksqw--ssqddqvfaealiflfggtaticfhstghgdpa 167
RLP4-L    84 GKKNCY-VVPLPPGRYYLRTFTVYDNYDGKSHSPSFDVSEGLVFSWRSPWPESLLRDGSYSDLFAFIGDGEELDCFYSIATDPP 169

RLP4      168 ilsiellqvddkaysfgegwgqgvilrtatrltcgtgksrfdedyrgdhwgdrfwnrmrsgksadsp-----ste 240
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RLP4      241 etikkasvspnfypeglyqsavstddqpdltysldvepnrnysvwlhfaeidntitaegkrvfdvvingdtffedvdiikmsggry 327
RLP4-L    244 EKIKGVDAQPNYFPMKLYQTA-VTVSGGSLVYELEVDALDYLLWFHFSEIDSTVKKAGQRFVFLVFN-DNNVSRVDFVHEVGG-F 327

RLP4      328 aalvinatvtvsgrtltvvlqpkagghalinaievfeilitaefktlrdevsaiqkmkkaiglpisrfgwngdpcvppq-hpwsqancq 413
RLP4-L    328 AAYSLNYTVKNLSSTIVTVKLSSVSGAPIISGLENYAIVPADMATVPEQVTAMKALKDSLVRPDRMGWNGDPCAPTSDAWEGVSCR 414

RLP4      414 idkntsrwfidgldidnqgikgflpndiskikhlqsnlennirggipaslgsvtslevidlsynsfngsipetigeltsirini 500
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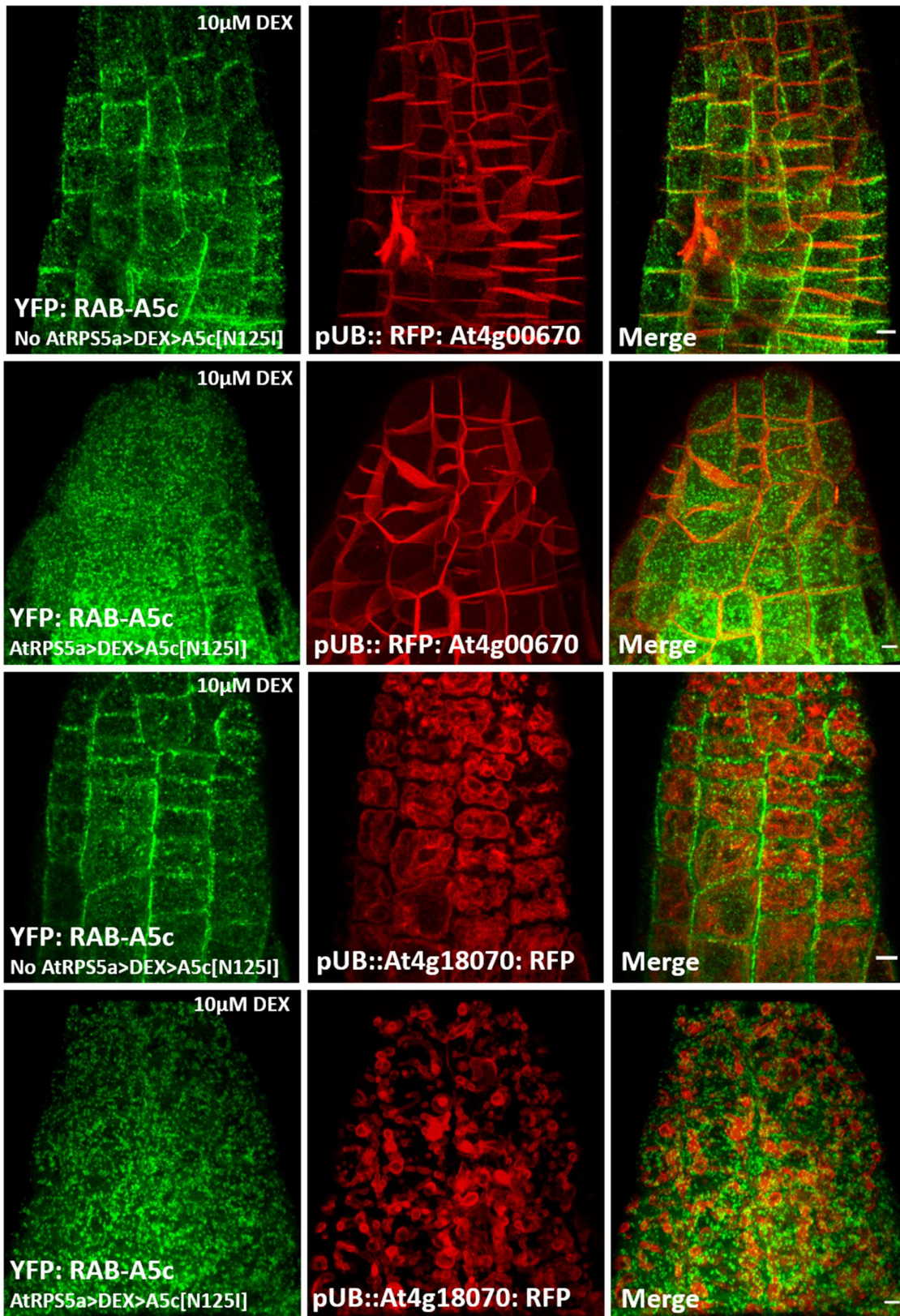
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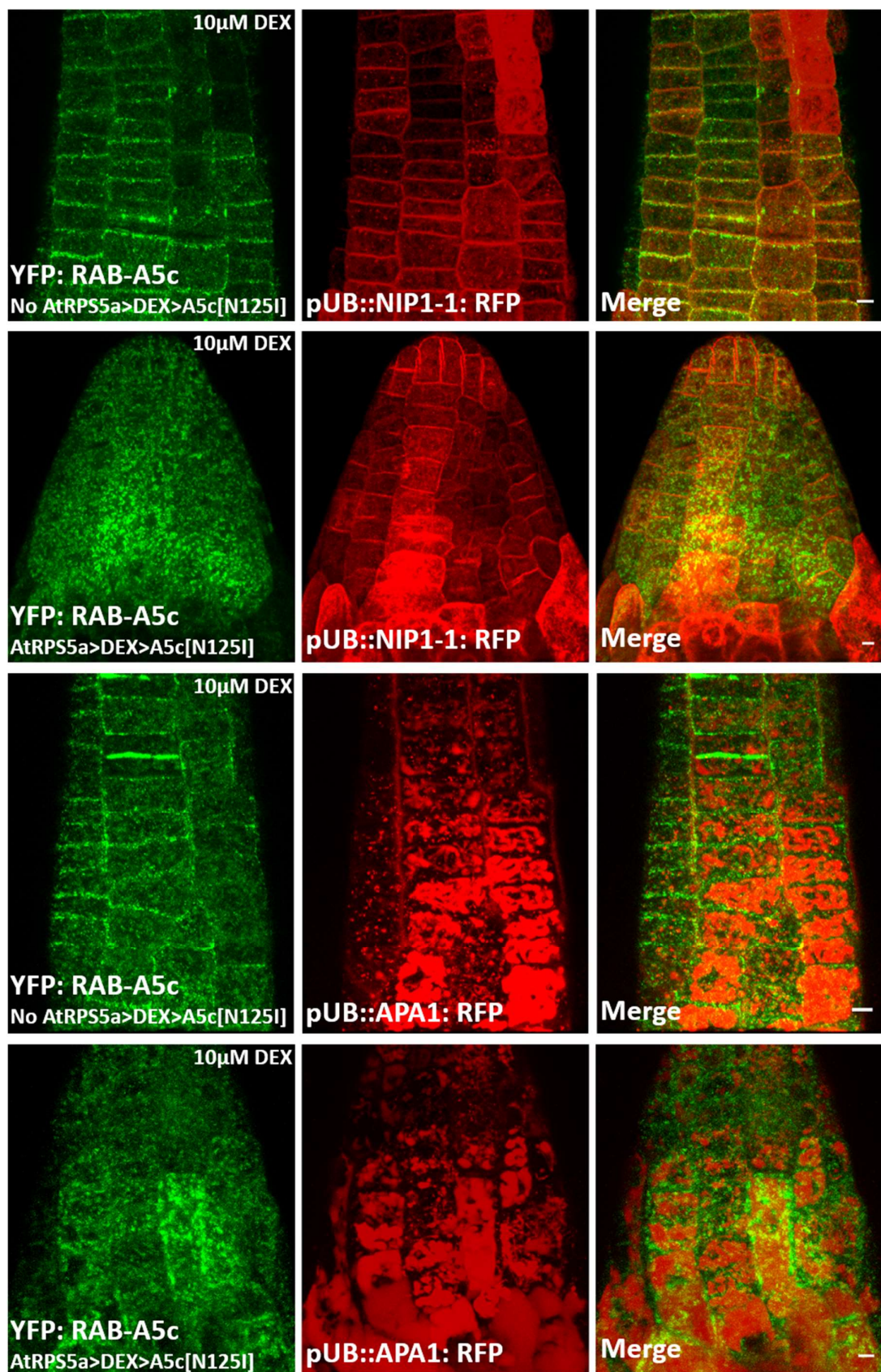
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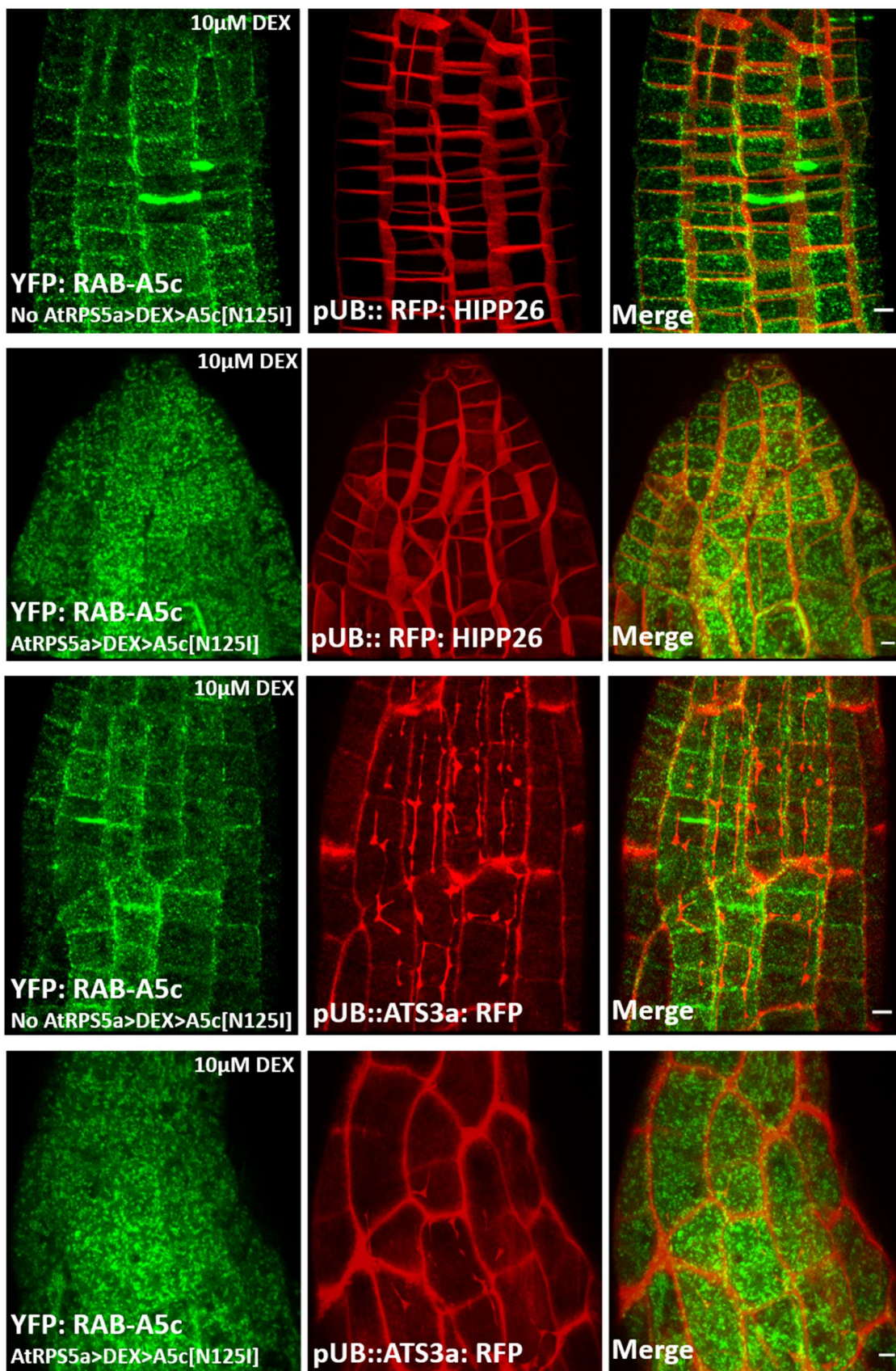
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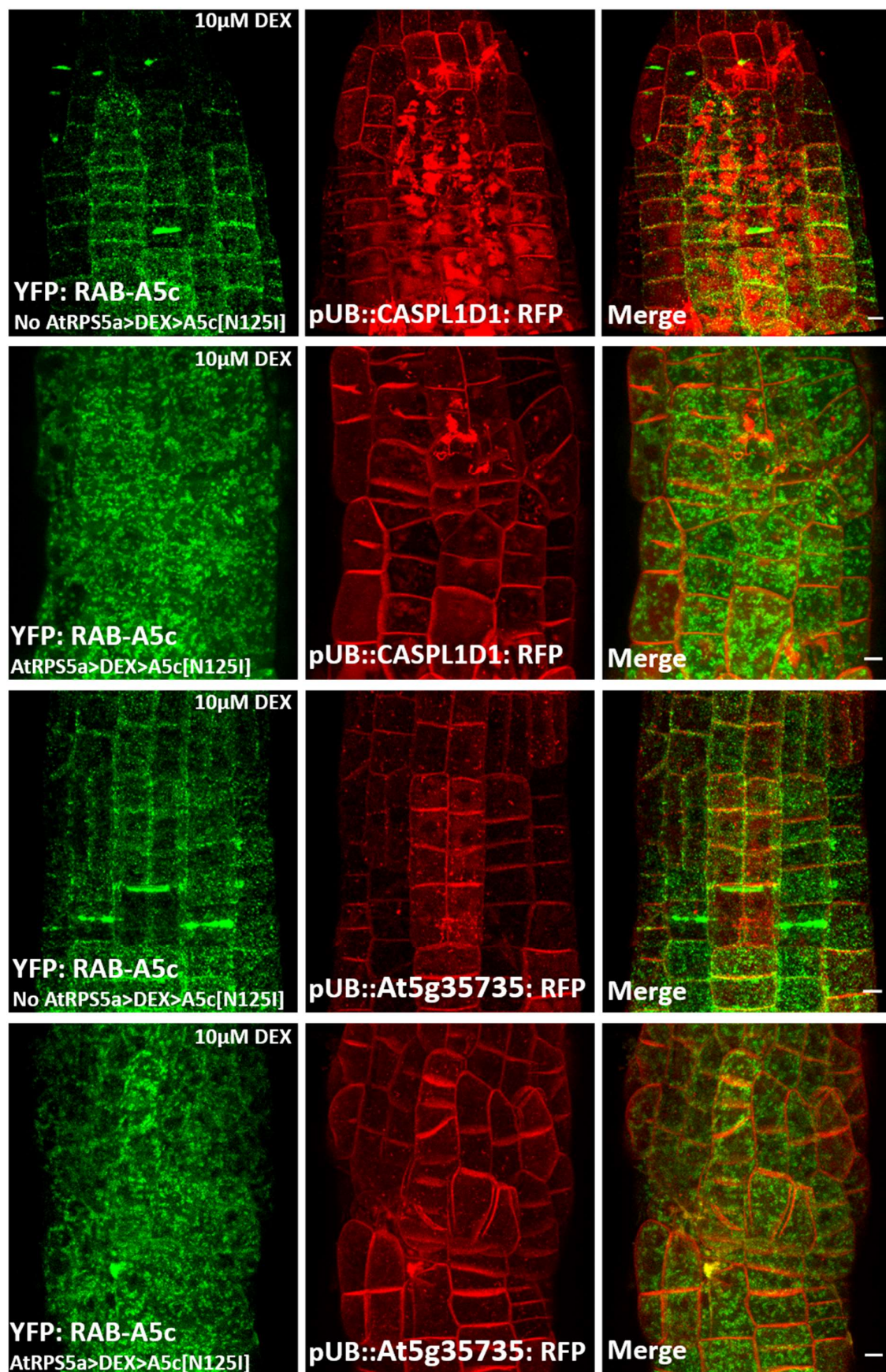
Appendix 2, Figure 2: Domain comparisons and pairwise alignments of RLP4 and RLP4-L.

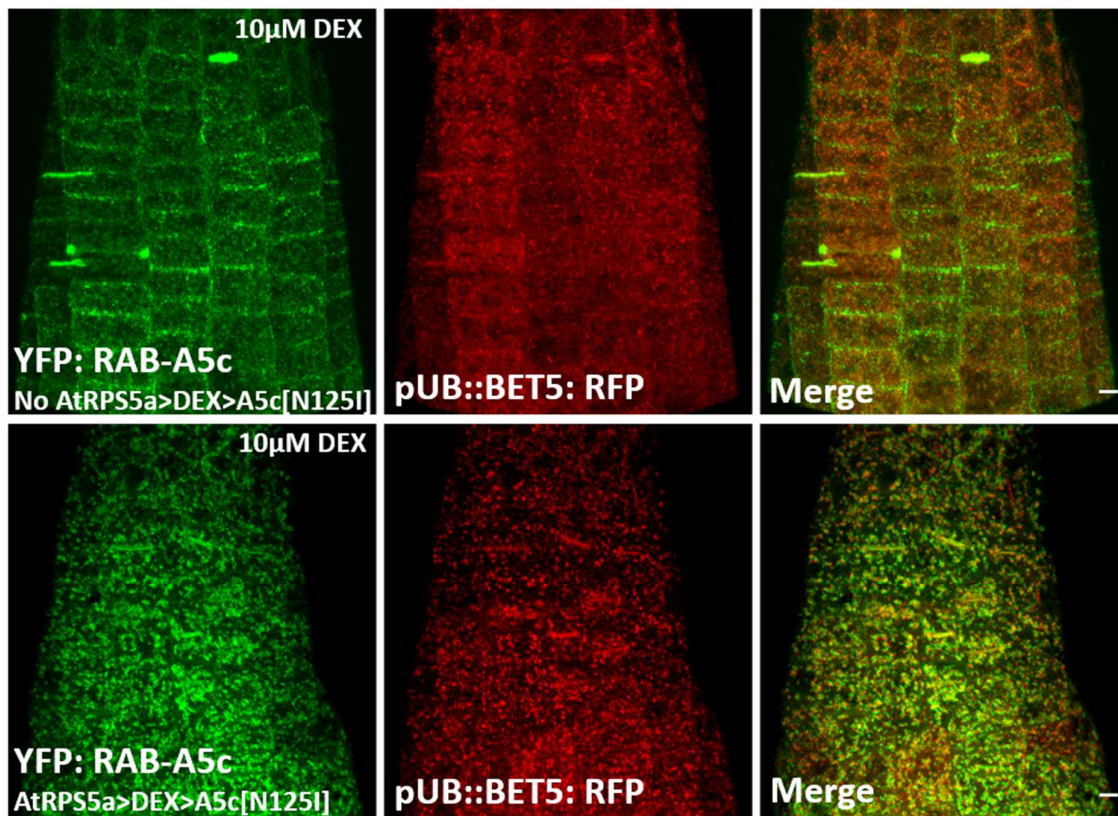
(A) pairwise domain comparison of RLP4 and RLP4-L. ■ = Malectin-Like Domain, ■ = Leucine Rich Repeat-containing region, ■ = Transmembrane Domain. (B) pairwise alignment of RLP4 and RLP4-L protein sequences. Text in red indicates conserved amino acid residues. Pairwise Identity = 34.7%, Pairwise Similarity = 50.2%. Alignment using Needleman-Wunsch algorithm (Needleman & Wunsch, 1970).



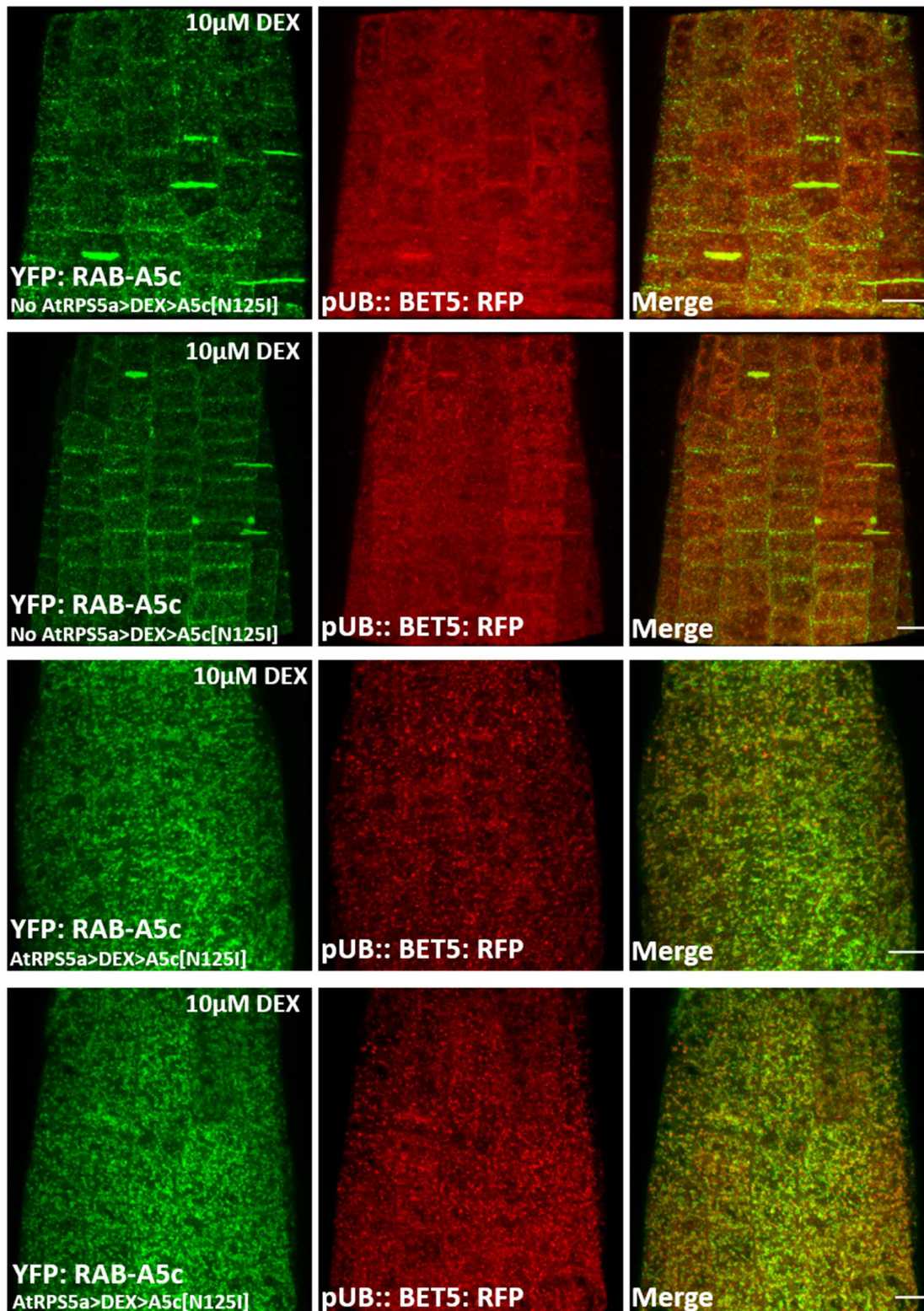




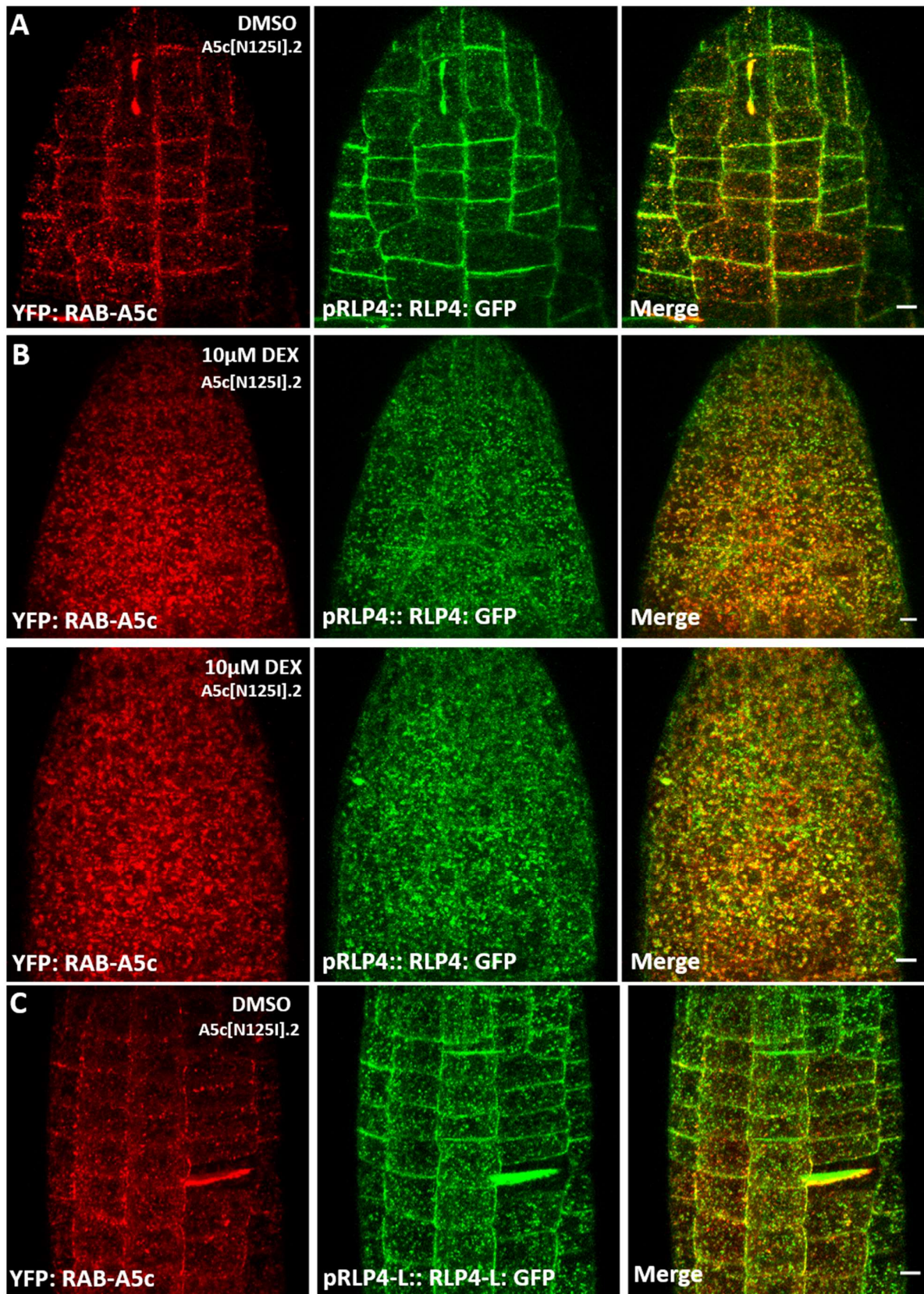


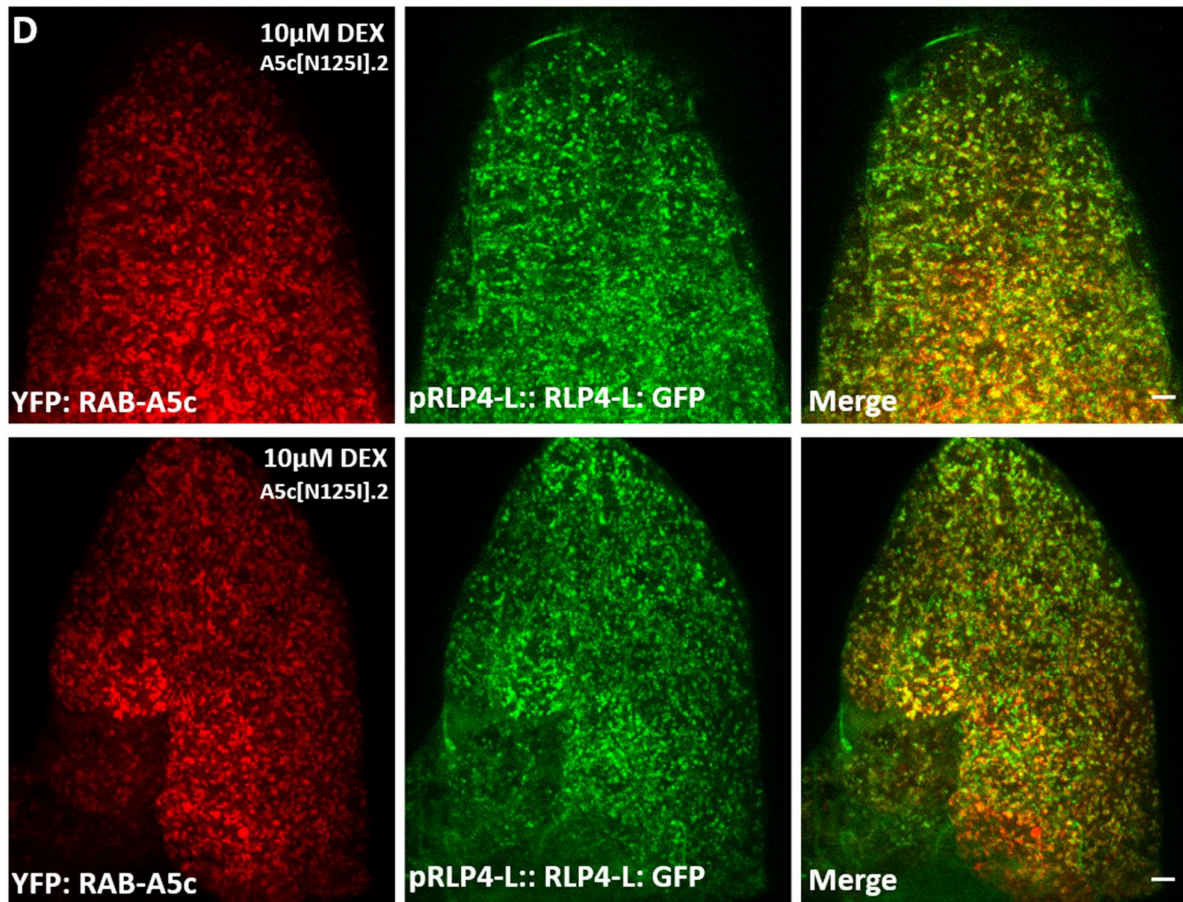


Appendix 2, Figure 3: Expression of AtRPS5a>DEX>A5c[N125].1 does not substantially alter the localisation patterns of other initially considered candidate interactors of YFP: RAB-A5c, with the possible exception of pUB:: BET5: RFP. 3D confocal stack projections of lateral roots co-expressing YFP: RAB-A5c and candidate interactors. Also co-expressing or lacking AtRPS5a>DEX>A5c[N125I]. 10μM DEX, 72 hours. All from 10 day old F2 seedlings. Scale bars 5μm.

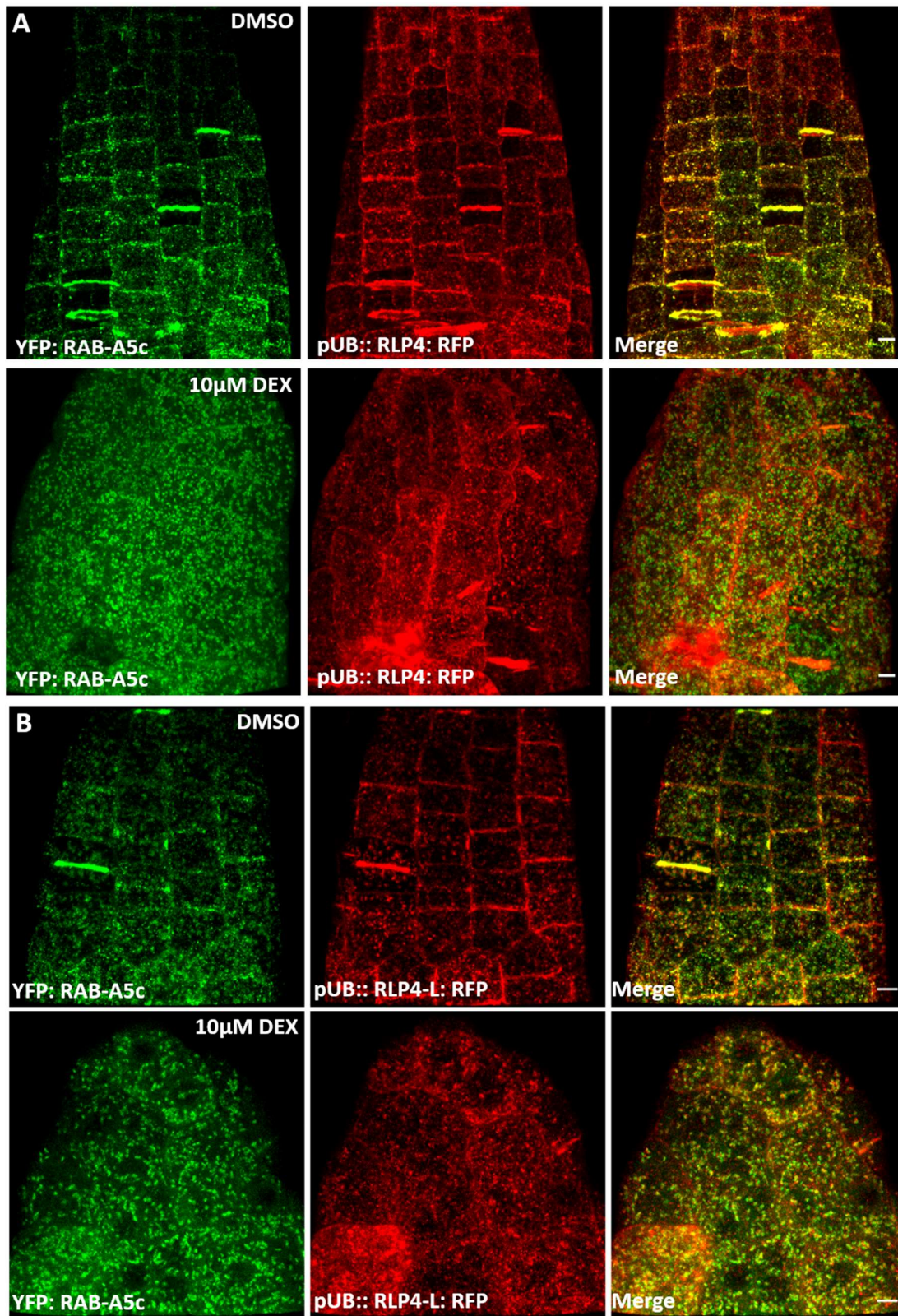


Appendix 2, Figure 4: Expression of AtRPS5a>DEX>A5c[N125].1 possibly shifts localisation of pUB:: BET5: RFP towards intracellular compartments. 3D confocal stack projections of lateral roots co-expressing YFP: RAB-A5c and pUB:: BET5: RFP, with or without AtRPS5a>DEX>A5c[N125].1. 10μM DEX, 72 hours. All from 10 day old F2 seedlings. Scale bars 10μm.

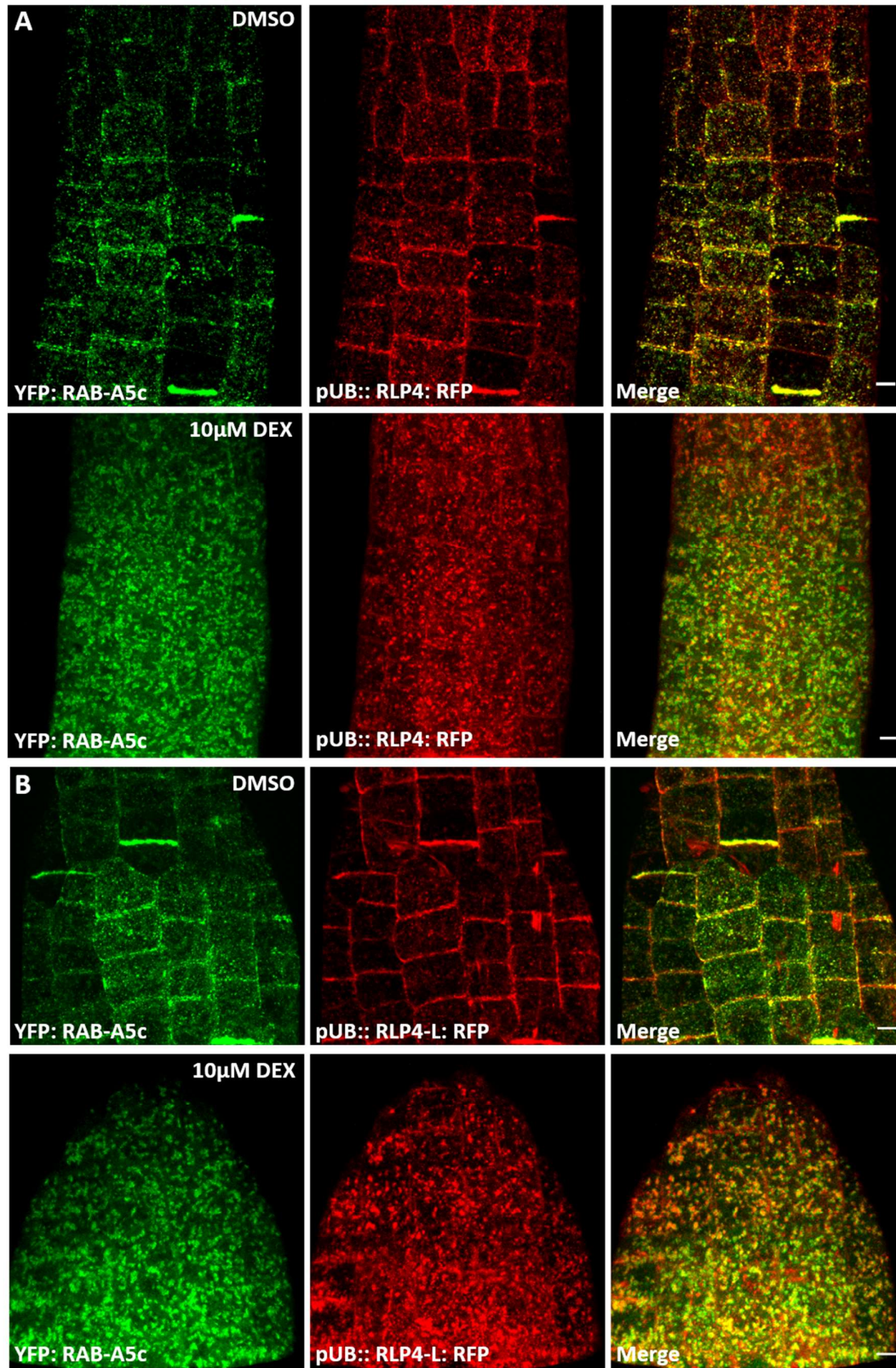




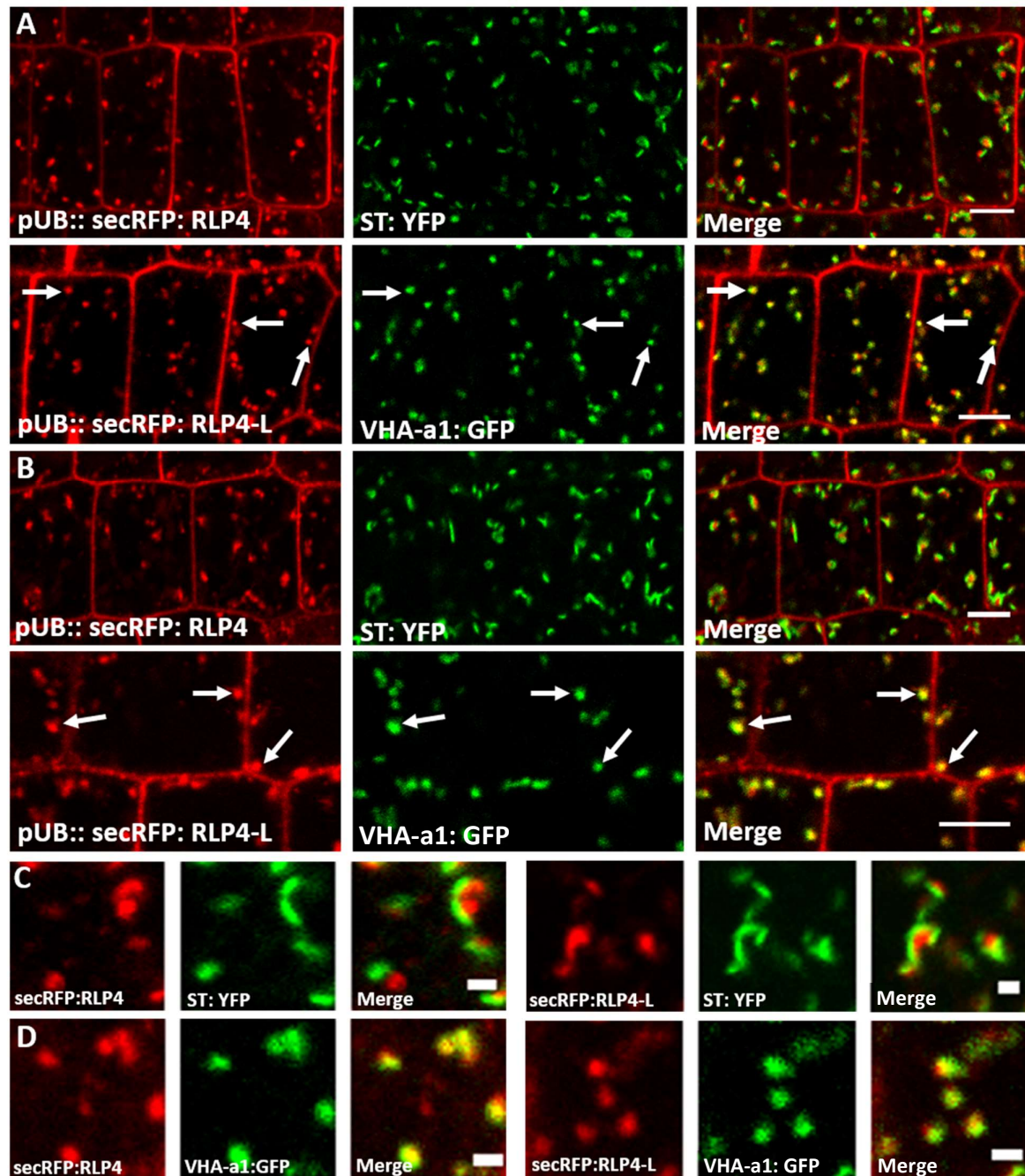
Appendix 2, Figure 5: Expression of *AtRPS5a>DEX>[N125I].2* disrupts localisation of pRLP4:: RLP4: eGFP and pRLP4L: RLP4-L: RFP in lateral roots. (A, B, C, D) 3D confocal stack projections of lateral roots co-expressing YFP: RAB-A5c (left) and pRLP4:: RLP4: eGFP (A, C, middle) or pRLP4L:: RLP4-L: eGFP (B, D, middle) and *AtRPS5a>DEX>[N125I].2*. 10μM DEX or equivalent volume DMSO, 72 hours. All from 10 day old F3 seedlings. Scale bars 5μm.



Appendix 2, Figure 6: 24 hours of *AtRPS5a*>*DEX*>*A2a*[N125I] expression disrupts localisation of YFP: RAB-A5c, pUB:: RLP4: RFP and pUB:: RLP4-L: RFP in lateral roots. (A, B) 3D confocal stack projections of lateral roots co-expressing YFP: RAB-A5c (left) and pUB:: RLP4: RFP (A, middle) or pUB:: RLP4-L: RFP (B, middle) and *AtRPS5a*>*DEX*>*A2a*[N125I]. 10 μ M DEX or equivalent volume DMSO, 24 hours. All from 10 day old F3 seedlings. Scale bars 5 μ m.



Appendix 2, Figure 7: 24 hours of AtRPS5a>DEX>A4b[N125I] expression disrupts localisation of YFP: RAB-A5c, pUB:: RLP4: RFP and pUB:: RLP4-L: RFP in lateral roots. (A, B) 3D confocal stack projections of lateral roots co-expressing YFP: RAB-A5c (left) and pUB:: RLP4: RFP (A, middle) or pUB:: RLP4-L: RFP (B, middle) and AtRPS5a>DEX>A4b[N125I]. 10µM DEX or equivalent volume DMSO, 24 hours. All from 10 day old F3 seedlings. Scale bars 5µm.



Appendix 2, Figure 8: pUB: secRFP: RLP4 and pUB: secRFP: RLP4-L co-localise intracellularly with TGN/EE-localised VHA-a1: GFP (white arrows) but not with Golgi-localised ST: YFP in lateral roots. (A, B) confocal slices of lateral root meristematic cells co-expressing pUB: secRFP: RLP4 (left) or pUB: secRFP: RLP4-L (left) and ST: YFP (A, middle) or VHA-a1: GFP (B, middle). Scale bars 5µm. (C,D) close up confocal slices. Scale bars 1µm. All from 10 day old T2 seedlings.

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