

Influence of the cell wall on the organisation and
dynamics of intracellular components in
Arabidopsis thaliana

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Abstract

This study used live cell imaging of fluorescently labelled marker proteins to quantify the organisation and dynamics of the actin cytoskeleton and to determine if and how it was affected when the connection between the cell wall and plasma membrane was disturbed. Two image analysis techniques were developed to measure the dynamic remodelling of the actin cytoskeleton: *Pairwise image subtraction* and *Cumulative fluorescence intensity*.

Both these analysis techniques showed that the actin cytoskeleton had a decreased rate of bulk remodelling when the cell wall-plasma membrane-actin continuum was disrupted either by plasmolysis or with isoxaben, a drug that specifically inhibits cellulose deposition. Changes in the rate of actin remodelling also affected its functionality as observed by alteration in Golgi body motility. The possibility that the changes seen were part of a general abiotic stress response by the actin cytoskeleton was refuted by showing that temperature stress did not decrease the rate of bulk remodelling. The effect of complete cell wall removal on the actin cytoskeleton was also investigated, but there was little difference in bulk remodelling between protoplasts immediately post-cell wall removal and after 24 h of cell wall re-growth.

In addition to actin cytoskeleton remodelling, this study also aimed to discover if plasma membrane protein mobility was altered in cells with a perturbed cell wall. It is known that the mobility of plasma membrane proteins can be increased in plant cells that have had their cell wall removed or separated from the plasma membrane. In this study, cell wall perturbations were caused by mutations leading to a lack of detectable xyloglucan. Some of the plasma membrane proteins examined had altered mobility in cells with a disturbed cell wall, but others did not, suggesting different components in the cell wall affect specific plasma membrane associations. Together these results show that disturbing the cell wall-plasma membrane interface reduces the dynamic remodelling of the actin cytoskeleton and affects plasma membrane protein mobility.

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Abbreviations

aa	Amino acid
a.u.	Arbitrary units
ABD	Actin binding domain
ACT	Actin
ADF	Actin depolymerising factor
ADP	Adenosine diphosphate
AGP	Arabinogalactan protein
AIP1	Actin interacting protein 1
ANOVA	Analysis of variance
APAP1	Arabinoxylan pectin arabinogalactan protein 1
Arp 2/3	Actin related protein 2/3
AtFH1	<i>Arabidopsis thaliana</i> formin homology 1
ATP	Adenosine triphosphate
BDM	2,3-Butanedione monoxime
bp	Base pairs
BY-2	Bright yellow 2
CP	Capping protein
CESA	Cellulose synthase A
CI	Confidence interval
CLSM	Confocal laser scanning microscope
CSC	Cellulose synthase complex
CSI1	Cellulose synthase-interacting 1
CSL	Cellulose synthase-like
C-terminal	Carboxy terminal
DCB	2,6-Dichlorobenzonitrile
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ER	Endoplasmic reticulum
F-actin	Filamentous actin

FAPP1	Phosphatidylinositol-4-phosphate adaptor protein-1
FH	Formin homology
FIM	Fimbrin
FLS2	Flagellin sensing 2
FP	Fluorescent protein
FRAP	Fluorescence recovery after photobleaching
G-actin	Globular actin
GIPC	Glycosyl inositol phosphorylceramides
GFP	Green fluorescent protein
GFP-FABD2	GFP-Fimbrin actin binding domain 2
GPI	Glycosylphosphatidylinositol
GRP1	General receptor for phosphoinositides, isoform 1
HRGP	Hydroxyproline-rich <i>O</i> -glycoprotein
LB	Luria-Bertani broth
LCB	Long-chain base
LTi6b	Low temperature induced 6b
MAP4	Microtubule-associated protein 4
MAP-GFP	Myristoylated and palmitoylated-GFP
MFR	Maximum fluorescence recovery
ML-7	Myosin light chain kinase inhibitor
MS	Murashige and Skoog medium
MTOC	Microtubule organising centre
MYA2	<i>Arabidopsis thaliana</i> myosin 2
NET3C	Networked protein 3C
NPSN11	Novel plant SNARE 11
N-terminal	Amino terminal
RT	Room temperature
OCTOPUS	Optics clustered to output unique solutions
PA	Phosphatidic acid
paGFP	Photoactivatable-GFP
(GFP)-PAP	(GFP-)prenylated GFP
PCR	Polymerase chain reaction
PH	Pleckstrin homology

PIN1	Pin-formed 1
PM	Plasma membrane
PME	Pectin methylesterase
PLD	Phospholipase D
PRF	Profilin
PtdIns	Phosphatidylinositol
PtdIns4P	Phosphatidylinositol 4-phosphate
PtdIns(4,5)P ₂	Phosphatidylinositol 4,5-bisphosphate
mRFP	Monomeric red fluorescent protein
RNAi	Ribonucleic acid interference
ROI	Region of interest
SD	Standard deviation
SEM	Standard error of mean
SINE	SUN domain-interacting nuclear envelope
SNARE	<u>S</u> oluble <u>N</u> -ethylmaleimide-sensitive factor <u>A</u> ttachment protein <u>R</u> Eceptors
ST	Sialyl transferase
SUN	Sad1p, UNC-84
SYP121	Syntaxin of plants 121
TAIR	The Arabidopsis information resource
T-DNA	Transfer DNA
TGN	<i>trans</i> -Golgi network
TIRF	Total internal reflection fluorescence
TM	Transmembrane domain
VAEM	Variable-angle epi-fluorescence microscope
VAP27	Vesicle-associated protein 27
VLN	Villin
WAK	Cell wall-associated kinase
WT	Wild type
XXT	Xyloglucan xylosyltransferase
YFP	Yellow fluorescent protein

Chapter One

Introduction

The overall aim of this work was an investigation of the cell wall-plasma membrane-actin cytoskeleton continuum, specifically the influence that the cell wall has over the other two components. This work addressed the following questions: if the cell wall is perturbed, what are the effects on the organisation of the actin cytoskeleton and also, how does this affect the movement of plasma membrane proteins? Live cell imaging using fluorescent protein markers was primarily used during this study as a way to record dynamics and how they change in cells with a perturbed cell wall. This chapter gives a brief introduction to the many cellular structures and processes that are involved in, or pertinent to, the cell wall-plasma membrane-actin cytoskeleton continuum and finishes with a brief description of the microscopes used during the course of this study.

All cells are bounded by a plasma membrane. In addition plants, fungi and bacteria have an all-encompassing cell wall. The composition of the cell walls found in these three kingdoms is quite different, but they fulfil the same basic functions: they provide structural support, resist osmotic pressures from within the cell and give protection by acting as a barrier to the extracellular environment. In addition the cell wall can also act to provide a signalling platform with some proteins linking from cell wall through plasma membrane to cell interior. In *Arabidopsis thaliana* the cell wall-plasma membrane-actin cytoskeleton continuum can be very dynamic (Figure 1.1) (Martinière and Runions, 2013). It is proposed that the cell wall can act as a stable structure for dynamic intracellular components to remodel around (Baluska *et al.*, 2003, McKenna *et al.*, 2014). Filaments of actin and tubulin are found throughout the cytoplasm, forming cytoskeletal networks in constant flux that are hypothesised to be able to respond to signals anywhere in the cell at any time (Michelot *et al.*, 2006).

Until relatively recently, organisation of the actin cytoskeletal network was not even considered in light of the cell wall (Green, 1962, Baluska *et al.*, 2003, Telewski, 2006). However, several recent studies have successfully shown that the cell wall and constituents thereof can be and are involved in organising intracellular components (Paredez *et al.*, 2008, Martinière *et al.*, 2011a, Martinière *et al.*, 2012). This work aims to improve understanding of the influences of the cell wall by analysing both dynamics of the actin cytoskeleton and plasma membrane resident proteins in different states of cell wall disarray.

1.1 Plant cell wall - plasma membrane - cytoskeleton continuum

The cell wall-plasma membrane-cytoskeleton continuum is an important route for the bidirectional flow of information between extracellular and intracellular processes. Seifert and Blaukopf (2010) hypothesise that due to turgor pressure compressing the plasma membrane against the cell wall they are ‘mechanically connected’. They posit that this would allow the cell wall-plasma membrane to send signals about cell wall integrity based on changes in the mechanical force that is perceived (Seifert and Blaukopf, 2010).

So far the best described continuum between the cell wall, plasma membrane and cytoskeleton is that between the Cellulose Synthase Complex (CSC) and microtubules (see Sections 1.3.3 and 1.2.5). The microtubule network guides the CSC along the plasma membrane as it lays down cellulose fibrils; it is not a passive transporter of cellulose, but actually determines the position of the fibrils (Paredez *et al.*, 2006). If the microtubule cytoskeleton is depolymerised then the localisation pattern of the Cellulose Synthase proteins (CESA) is disrupted; however, their motility is not affected, suggesting that cellulose polymerisation itself is the driving force (Paredez *et al.*, 2006).

Lei *et al.* (2014) and references therein showed that the converse is also true; that if the CSC or CSC-associated proteins are mutated then the microtubules are affected. For example, if CESA6 contains a specific missense mutation (*prc1-20*) then the orientation and the organisation of the microtubule network are affected; it is not just due to signalling about the loss of cell wall integrity (Paredez *et al.*, 2008). This shows that the cell wall is

not a passive structure, but one with influence; the same effect has also been shown with drugs that disrupt the cell wall. These drugs are herbicides thought to inhibit cellulose synthesis. One of the most commonly used is isoxaben which specifically interacts with CESA3 and CESA6 as certain mutations in either of these genes confer resistance to isoxaben (Scheible *et al.*, 2001, Desprez *et al.*, 2002). However, it was also shown that when a plant cell is treated with isoxaben, the microtubule network becomes disordered, yet microtubules themselves were shown to be unaffected by isoxaben (Fisher and Cyr, 1998). This shows that while microtubules are vital in guiding the deposition of cellulose microfibrils, certain components of the cell wall are necessary for the correct organisation of the microtubule network.

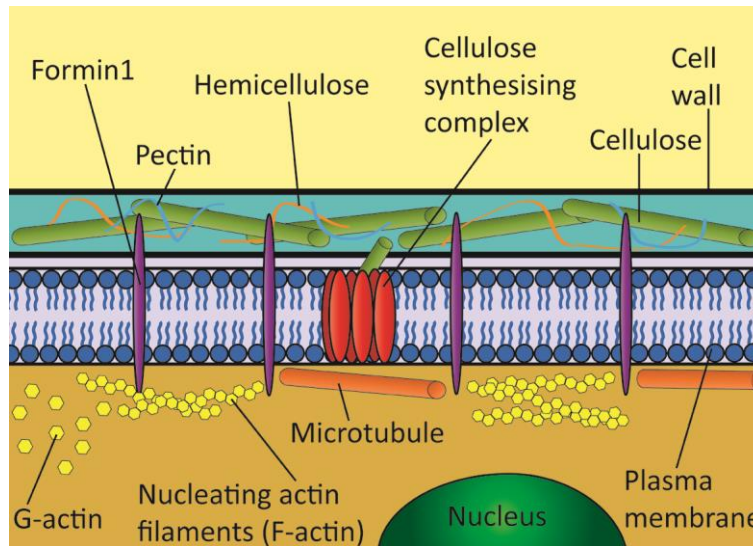


Figure 1.1 Cell wall-plasma membrane-cytoskeleton continuum.

Schematic showing some of the major components of the plant cell wall and an example of a protein (Formin 1) in the putative cell wall-plasma membrane-cytoskeleton continuum.

Above is a brief overview on the complex relationship between microtubules and the cell wall. However, while many of the interdependencies between the microtubule cytoskeleton and the cell wall are known, the interactions between the cell wall and the actin cytoskeleton are not well documented. It is known that the actin cytoskeleton is important for the organisation of the cell wall in its role as a transport network (Sampathkumar *et al.*, 2013). For example, the actin cytoskeleton is important for the motility of Golgi bodies (Boevink *et al.*, 1998, Nebenfuhr *et al.*, 1999, Akkerman *et al.*, 2011) and the Golgi bodies are necessary for the construction and maintenance of the cell

wall. In addition, it is also known that the actin cytoskeleton is important for plasma membrane organisation, for example, it is thought to be involved in the formation of transient clusters of plasma membrane proteins (Gowrishankar *et al.*, 2012). However, the influence of the cell wall on the actin cytoskeleton has not been well studied and this study hypothesises that the cell wall acts as an organising centre for actin cytoskeleton remodelling.

1.1.1.1 Extracellular matrix to cytoskeleton linkers in animal cells

The *A. thaliana* genome seems to lack homologues of the genes that in animals encode linkers of the extracellular matrix: genes such as integrins and cadherins, as well as many actin-associated proteins that are vital in animals (Meagher and Fechheimer, 2003, Baluska *et al.*, 2003). Baluska *et al.* (2003) and Kohorn (2000) suggest seven candidate families that could hypothetically link the plant cell wall to the cytoskeleton: arabinogalactan proteins (AGPs; see Section 1.3.1.4), cell wall-associated kinases (WAKs), pectins (see Section 1.3, pg. 19), cellulose synthases (CesAs; see Section 1.3.3), class VIII myosins (see Section 1.2.4.1), phospholipase Ds (PLDs) and formins (see Section 1.1.2).

Integrins are a family of widely-expressed bi-directional receptors (they signal 'outside-in' and 'inside-out') that mediate attachment of the cell to the extracellular matrix (ECM; Barczyk *et al.*, 2010). They can be turned 'on' or 'off' to initiate or halt the binding of 19 of the 90 proteins that are involved in large complexes known as cell-matrix adhesions which function to translate extracellular changes to intracellular signals (Rossier and Giannone, 2016). Integrins are connected to the actin cytoskeleton by talin, which binds their cytoplasmic tail, either to cause a conformational change in the integrin as part of the signalling 'inside-out' or as a direct link to the actin cytoskeleton if the integrin is altered by an extracellular change (Anthis and Campbell, 2011).

1.1.2 Cell wall-plasma membrane-actin cytoskeleton linker protein example: Formin

The cell wall is important for sensing changes; these can be changes in the extracellular environment or signals in the apoplast or alterations in the integrity of the cell or cell wall itself. Cell wall integrity is a crucial aspect of cell survival and so it is necessary that the

cell can sense these changes, or if the cell wall itself is impaired, in order that it can respond effectively and in as timely a manner as possible. It is thought that plants have a complex mechanism to monitor the integrity of the cell wall and any perceived weakness in it would lead to modifications of the wall and changes in metabolism to counteract the weakness (Hamann, 2015).

Therefore it is important that there are proteins with extracellular domains that can propagate signals from outside to inside of the cell. One example is the wall associated kinases (WAKs), a family of proteins thought to be able to sense pectins, both full-length and fragmented (Kohorn and Kohorn, 2012). Another is AtFH1 (*A. thaliana* Formin Homology 1), an actin nucleating protein: there is some excellent evidence to suggest that it acts as a linking molecule from the actin cytoskeleton through the plasma membrane to the cell wall (Martinière *et al.*, 2011a). For example, it has been shown that an intact extracellular domain is necessary for its actin nucleating function (Martinière *et al.*, 2011a).

Formins are a family of evolutionarily conserved actin nucleating and polymerising proteins, although the exact manner in which they nucleate actin filaments is yet to be precisely determined (Breitsprecher and Goode, 2013). Most plant formins have two Formin Homology (FH) domains: FH1 and FH2 (Deeks *et al.*, 2002). FH1 acts as a ‘docking site’ for globular actin (G-actin) monomers and FH2 works as a dimer and interacts with either the side of the actin filament or as a ‘leaky’ cap for the growing end (Yin *et al.*, 2014).

A. thaliana has 21 formins divided between two classes which are distinguished by the presence or absence of a highly specialised N-terminal sequence (Grunt *et al.*, 2008). AtFH1 to AtFH11 are in Group I (as shown by the position of formin in Figure 1.1) and AtFH12 to AtFH21 are in Group II; there is a Group III, but this is only found in plants that have flagellate sperm (see Section 1.2.4 on dyneins) (van Gisbergen and Bezanilla, 2013). In *Physcomitrella patens* all six Group I formins have been silenced using RNAi (RNA interference); the resulting moss were viable, but 46% smaller than control moss and contained fewer than half the number of cells (Vidali *et al.*, 2009). .

1.1.2.1 *A. thaliana* Formin Homology 1

A linker protein in *A. thaliana* is AtFH1 (*A. thaliana* Formin Homology 1), which contains two Formin Homology domains (FH1 and FH2), a signalling peptide, a transmembrane domain and an N-terminal region rich in serine and proline (Martinière *et al.*, 2011a). Profilin-actin complexes bind to the FH1 domain thus increasing the local concentration of actin monomers to allow polymerisation; and whilst the FH2 domain can cap the barbed end of the filament, it is also involved in elongation of the filament (van Gisbergen and Bezanilla, 2013). For AtFH1 the FH1 Domain is necessary for actin nucleation and it also regulates the FH2 domain by changing it from a strong actin filament capper to a 'leaky' cap which allows polymerisation to occur at the barbed end (Michelot *et al.*, 2005). AtFH1 is a non-processive formin so instead of binding to the barbed end of an actin filament and causing nucleation from there, it binds to the side of a filament to nucleate actin; in addition, it can also bundle actin filaments (see Figure 1.1) (Michelot *et al.*, 2006).

The N-terminal region is about 100 amino acids long and composed of four domains, including an extracellular domain (A, B, C and D); when the N-terminal region tagged with red fluorescent protein was expressed alone it localised to the cell wall in a transient assay (Martinière *et al.*, 2011a). Domain C is homologous to the glycosylated domain in extensin and it is probably involved in binding to the cell wall (Martinière *et al.*, 2011a). For example, it has been reported that extensins can bind pectins as well as proteins and so could be incorporated directly into the cell wall (Qi *et al.*, 1995) (Section 1.3.1, pg. 19).

If AtFH1 is overexpressed the actin cytoskeleton appears disordered, it becomes an extremely dense mesh of filaments (Martinière *et al.*, 2011a). However, if domain C, the domain that is homologous to the extensin motif, is deleted from AtFH1 and this deletion construct is overexpressed then the actin cytoskeleton appears normal (Martinière *et al.*, 2011a). This means that the cell wall interaction is necessary for AtFH1 to function correctly in the organisation of the actin cytoskeleton.

1.2 Cytoskeleton

1.2.1 Actin cytoskeleton

1.2.1.1 Evolution of the actin cytoskeleton

The actin cytoskeleton is an extremely dynamic network that runs throughout the cytoplasm and is highly conserved throughout eukaryotes. It is involved in the transportation of vesicles around the cell, it connects various organelles to each other and it is vital for cell division and for pathogen responses (McKinney and Meagher, 1998, Meagher and Fechheimer, 2003). In eukaryotes, the actin cytoskeleton became involved in such a diverse range of processes it limited the amount of change that could occur within the molecule that did not adversely affect any interactions or function of the protein (Gunning *et al.*, 2015). However, it appears that while the DNA sequences for actins and for actin-binding proteins may have changed between plants, animals and protists, the protein structure has not changed and *Homo sapiens* cytoplasmic actin gene β -actin ACTB can rescue *A. thaliana act8 act7* mutants (Kandasamy *et al.*, 2012).

1.2.1.2 The actin protein

Actin is a self-associating, ATP-binding protein of about 42 kDa that is a globular when monomeric (G-actin); when polymerised it forms double helical filaments about 7-9 nm in diameter with a right-handed twist (F-actin) (Staiger and Blanchoin, 2006, Alberts *et al.*, 2014). Actin filaments dissociate in a timed fashion via the hydrolytic state of ATP: the longer an actin protein has been part of a filament the higher the chance that it will hydrolyse its bound ATP molecule (Alberts *et al.*, 2014). This gives polarity to the filament: the plus end is defined by actin molecules containing ATP and is called the barbed end (due to its appearance when bound with myosins), whereas the minus end is termed the pointed end and contains actin molecules with ADP (Alberts *et al.*, 2014). .

Once the actin proteins have dissociated from a filament they can be recycled or sequestered within the cytoplasm allowing rapid polymerisation: plants are unusual in that the ratio of monomeric actin to actin filaments is high, thus allowing the network to be very dynamic (Staiger and Blanchoin, 2006). Coupled with a selection of different actin proteins and a large variety of actin binding proteins it creates the potential for a hugely complex, but well-regulated network that can be changed on an individual filament basis (Gunning *et al.*, 2015).

1.2.1.3 The actin cytoskeleton in *A. thaliana*

A. thaliana has ten actin genes grouped into two classes, those that are expressed in vegetative tissues and those that are only expressed in reproductive tissues (Kandasamy *et al.*, 2009). The vegetative class consists of two subclasses with actins act2 and act8 in one and act7 in the other (Blanchoin *et al.*, 2010). The reproductive class has three subclasses: act11, act1 and act3, act4 and act12; while act5 and act9 are considered pseudogenes (Slajcherová *et al.*, 2012). It is possible that the different proteins have different polymerisation rates or bundling affinities, thus creating a more flexible system while allowing greater control (Meagher *et al.*, 1999). Blanchoin *et al.* (2010) showed that when act2 expression levels were knocked-down the expression of act7 increased and the plants had the same above-ground morphology as wild-type, whereas the roots were found to be extremely stunted and branched. In addition, Kandasamy *et al.* (2010) showed that ACT7 is differently distributed in *A. thaliana* nuclei compared to ACT2 and ACT8.

The actin filaments create the physical forces in an otherwise semi-rigid and immobile plant cell that are necessary for various cellular processes, such as cytoplasmic streaming and organelle movement (Wolf *et al.*, 2014). The actin cytoskeleton resembles a meshwork spread throughout the cell (Figure 1.2).

The actin cytoskeleton is extremely flexible: it is constantly being dismantled and re-formed, events that are termed ‘catastrophes’ and ‘rescues’. In plants actin filaments have been found to have high assembly and elongation rates which can generate a filament long enough to span from sidewall to sidewall in approximately 10 seconds (hypocotyl epidermal cells) (Henty-Ridilla *et al.*, 2013). In *A. thaliana* hypocotyls it has been proposed that it is possible for approximately 630 actin monomers to be added to a growing filament per second (a growth rate of 1.7 $\mu\text{m/s}$): this allows the cell to react extremely quickly to any stimuli (Henty-Ridilla *et al.*, 2013). Actin filaments can also be grouped and twisted by actin-binding proteins and then the resulting bundles can be further grouped by other actin binding proteins to produce large structures containing hundreds of filaments (Claessens *et al.*, 2008).

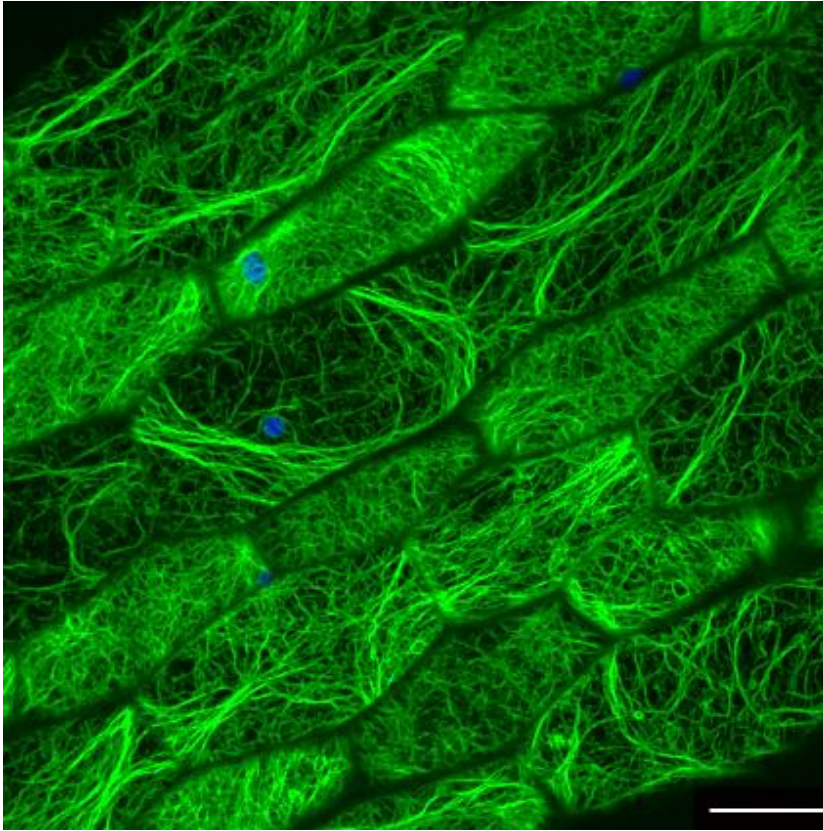


Figure 1.2 GFP-FABD2 labelled actin in *A. thaliana* seedlings.

GFP-FABD2 labelled actin in the hypocotyl of *A. thaliana* seedlings three days after germination. Scale bar = 20 μm .

For further pictures of the actin cytoskeleton in *A. thaliana* cells, see Chapters 4 and 5.

1.2.2 Actin binding proteins

The genome of *A. thaliana* putatively contains 150 actin interacting proteins that can interact with actin by bundling, capping, severing, anchoring, cross-linking, nucleating or moving along the filaments (Meagher and Fechtmeier, 2003). Actin interacting proteins, are also vital for different cellular processes such as pollen tube growth, cell polarity, cell expansion, cytokinesis and cell division. *A. thaliana* and other angiosperms possess a different set of actin binding proteins compared to animal cells: for example they lack direct homologues of many vital mammalian genes, such as α -actinin or zyxin (Meagher and Fechtmeier, 2003). However, the actin binding proteins that have been confirmed in *A. thaliana* have much larger families than the corresponding protein families in metazoans. For example, *A. thaliana* is thought to have 21 formins, while humans only

have 13 and *A. thaliana* has 12 Actin Depolymerising Factor (ADF) proteins, but humans have only three (Gunning *et al.*, 2015).

It is thought that the reason there are so many actin regulating proteins in general (and why many of the protein families listed in Table 1.1 are large) is to allow specificity in the system. Having many proteins allows the cell to specialise one protein for one spatio-temporal point. For example, in human cell culture it has been shown that cortical actin nucleation is specifically dependent on one formin protein (mDia1) despite the presence of at least eight other formins and the ARP2/3 complex (Bovelian *et al.*, 2014).

Table 1.1 Examples of actin binding proteins and protein families.

These examples were chosen because they are either directly referred to during this work or are important for those proteins or processes which are discussed.

Protein Families	Function	Publications
Myosin	Motor proteins	(Madison and Nebenfuhr, 2013)
Formin	Filament nucleation, mostly	(Cvrckova, 2013)
Phospholipase D (PLD)	Involved in signal transduction	(Eliás <i>et al.</i> , 2002)
Networked proteins (NET)	Membrane compartment connectors	(Hawkins <i>et al.</i> , 2014)
Actin-related proteins 2/3 (ARP2/3)	Filament nucleation, in a branching manner	(Yanagisawa <i>et al.</i> , 2013)
Villin	Severing, capping and bundling	(Thomas, 2012)
LIM (LIM-domain containing proteins)	Bundling	(Thomas, 2012)
Fimbrin	Bundling	(Thomas, 2012)
Profilin	Binds G-actin and aids filament elongation	(Sun <i>et al.</i> , 2013)
Capping protein/Actin interacting protein 1 (CP/AIP1)	Caps the barbed ends of filaments	(Li <i>et al.</i> , 2012a)
Actin depolymerising factor (ADF)/Cofilin	Binds G-actin and halts filament elongation	(Michelot <i>et al.</i> , 2007)
SUN domain-interacting nuclear envelope (SINE)	Nuclear positioning in guard cells	(Zhou <i>et al.</i> , 2014)
Cyclase-associated proteins (CAP)	Cytoskeleton organisation and cellular development	(Deeks <i>et al.</i> , 2007)
Networked (NET) proteins	Mediates actin-membrane interactions	(Deeks <i>et al.</i> , 2012)

1.2.2.1 Actin Related Protein 2/3

The Actin Related Protein 2/3 (ARP 2/3) complex is a key component in cell shape determination in *A. thaliana* (Yanagisawa *et al.*, 2013). ARP 2/3 is made up of seven subunits which together nucleate and polymerise actin; it is thought to be regulated in part

by microtubules, thus linking two major components of the cytoskeleton (Petrasek and Schwarzerova, 2009). It nucleates from the side of a filament and always nucleates a new filament at a 70° angle, creating a branched network (Yanagisawa *et al.*, 2013).

In *A. thaliana* pavement leaf cells ARP2/3 is mostly inactive, the subunits are found in the nucleus and on the surfaces of organelles (Zhang *et al.*, 2013). *A. thaliana* mutants in four of these subunits all have ‘distorted’ trichomes and defects in hypocotyl, pavement and root hair cells (Uhrig *et al.*, 2007).

1.2.2.2 Cofilin

ADF/Cofilin is a member of the Actin Depolymerising Factor (ADF)/cofilin family of proteins, it binds both globular and filamentous actin, but has a higher affinity for ADP-actin (Carrier *et al.*, 1997). ADF/Cofilin is involved in severing actin filaments as well as de-polymerisation, the latter is possibly because severing in conjunction with the activity of capping protein promotes de-polymerisation (Andrianantoandro and Pollard, 2006). Andrianantoandro and Pollard (2006) hypothesised that a lower concentration of ADF/Cofilin increases de-polymerisation and severing events, whilst high concentrations increase nucleation events.

ADF/Cofilin twists filaments: undecorated actin twists 2.15 – 2.17 units per turn, whereas an actin filament bound with cofilin twists 2.22 units per turn which causes a strain to develop between actin molecules (McGough *et al.*, 1997). Severing relieves the strain that the twist has imposed upon the filament, but only if the dissociation of the actin molecules needs less energy than the removal of cofilin from the filament (Andrianantoandro and Pollard, 2006).

In *A. thaliana* plants lacking ADF4 the amount of bundled filaments were increased, severing events were decreased 2.5-3 fold and both filament length and lifetime were significantly increased (Henty *et al.*, 2011).

1.2.2.3 Profilin

Profilin binds the barbed end of actin and promotes actin filament assembly, but only at the barbed end of the filament; it binds globular actin with high affinity and filamentous actin with lower affinity (Kang *et al.*, 1999, Blanchoin *et al.*, 2014, Pernier *et al.*, 2016).

Profilin inhibits globular actin binding spontaneously: actin molecules bound to profilin can only be added to a filament interacting with another actin binding protein, such as a formin or ARP 2/3 (Blanchoin *et al.*, 2014).

Profilin also competes with other proteins that bind to the barbed end of actin, such as capping protein: in this case when capping protein is bound to the barbed end, then profilin will sequester globular actin molecules (Pernier *et al.*, 2016). It was recently found to encourage large changes in the length of actin filaments thus causing a 'mild dynamic instability' (Pernier *et al.*, 2016).

However, in *A. thaliana* plants lacking PRF1 the nucleation frequency of actin filaments was decreased as well as the filament elongation rate and filament length, conversely filament lifetime was increased (Cao *et al.*, 2016).

1.2.2.4 Fimbrin

Fimbrins have two actin binding domains (ABDs) which are both comprised of a pair of calponin-homology domains arranged one in front of the other; this means that they can act as crosslinkers, causing actin to be tightly bundled (Kovar *et al.*, 2000). In *A. thaliana* fimbrin cross-links actin filaments and probably protects them from profilin-mediated depolymerisation (Kovar *et al.*, 2000).

In *A. thaliana* pollen tubes lacking FIM5, actin filaments appear to be more curved and do not form a dense network in the cortex, the filaments are also less dynamic and more disorganised (Zhang *et al.*, 2016a).

1.2.2.5 Capping protein

Capping protein binds to the barbed ends of actin filaments with high affinity, preventing de-polymerisation and polymerisation; it has also been shown to localise to Golgi bodies in *A. thaliana* (Huang *et al.*, 2003, Jimenez-Lopez *et al.*, 2014). It is thought to act partially redundantly with both villin and Actin Interacting Protein1 (AIP1) (Li *et al.*, 2012a). Capping protein is an efficient nucleator of actin filament assembly and it is regulated by both phosphatidic acid and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂), either directly or by controlling the localisation of scaffold proteins that provide an environment

favourable for specific protein interactions (Section 1.5.2, pg. 31) (Li *et al.*, 2012a, Huang *et al.*, 2003).

A. thaliana lines lacking capping protein displayed denser actin networks than wild type as reduced capping protein levels increased the amount of filaments with free ends (Li *et al.*, 2012a). These mutants were also insensitive to increases and decreases in phosphatidic acid levels (Li *et al.*, 2012a).

1.2.2.6 Villins

Villins were discovered in intestinal cells in mammals, they are involved in the complex structure of the actin cytoskeleton in microvilli and appear to have similar functions to the villins found in *A. thaliana* (Friederich *et al.*, 1999). There are five villin proteins in *A. thaliana*; they regulate actin by nucleating, capping or severing filaments and also bundling them, which may or may not be influenced by calcium (Thomas, 2012). Villins usually contain six gelsolin homology domains and a villin headpiece domain with actin binding properties (Huang *et al.*, 2015).

In *A. thaliana* plants lacking VLN2 and VLN3 the actin cytoskeleton displayed very few bundles; there also appeared to be a lack of co-ordination in cell elongation and the organs of the plant did not grow correctly (van der Honing *et al.*, 2012).

1.2.3 Cytoplasmic streaming

Cytoplasmic streaming is the movement of organelles around the cytoplasm in eukaryotic cells that ‘mechanically’ moves the cytosol (Geitmann and Nebenfuhr, 2015). It has been shown in BY-2 cells that this active transport of organelles in the cell produces ‘hydrodynamic flow’ and it is this which causes movement of the cytosol (Esseling-Ozdoba *et al.*, 2008). It is vital for cell homeostasis and cell expansion; it is caused by myosins (see Section 1.2.4.1) that move their associated cargoes moving along the actin network (Stefano *et al.*, 2014). In angiosperms the most commonly recorded velocities are slower than 10 $\mu\text{m}/\text{sec}$, whilst in some algae it can be as fast as 100 $\mu\text{m}/\text{sec}$ (Shimmen and Yokota, 2004). However, it is not easy to analyse as the speed is generally variable

between organelle types and also extremely sensitive to changes in temperature (Holweg, 2007).

1.2.4 Molecular motors

Motor proteins provide intracellular motility in conjunction with the cytoskeleton. There are three motor protein families found in eukaryotes: myosins, kinesins and dyneins. However, while fungi and animals have dynein, flowering plants do not (two subclasses within the Gymnosperms also lack dynein, while it is thought the other Gymnosperms do have it); it is thought that angiosperms lost dynein when they lost the flagellar apparatus (Lawrence *et al.*, 2001, Brandizzi and Wasteneys, 2013). Although *Physcomitrella patens* also lacks dynein (Tijs Ketelaar, personal communication).

Myosins and kinesins use the energy provided by ATP hydrolysis to transport vesicles or organelles along the cytoskeletal network; myosins travel along actin filaments while kinesins move along microtubules (Lee and Liu, 2004). They are found in all eukaryotic organisms and are essential to such processes as cell growth and cell division. In *A. thaliana*, there are 17 myosin genes divided between two subfamilies (class VIII and class XI) and 61 kinesin genes in 10 subfamilies (Zhu and Dixit, 2011a).

1.2.4.1 Myosins

Several groups have tried to determine if myosins are tissue or organelle specific, but as yet no myosin can be definitively said to be tied to a specific function (Madison and Nebenfuhr, 2013). However, it has been shown that an application of a myosin inhibiting chemical will alter the ‘rate of change of convolutedness’, that is, the amount of buckling and straightening that actin filaments undergo (Staiger *et al.*, 2009). It has also been shown that *A. thaliana* plants that lack multiple myosin genes have an altered actin morphology in certain cell types: thick bundling is reduced and the orientation of the filaments alters from longitudinal to transverse (Peremyslov *et al.*, 2010).

Cai *et al.* (2014) examined the actin cytoskeleton in growing and non-growing epidermal cells of *A. thaliana* cells lacking Myosins XI-1, XI-2 and XI-K. They found that there were fewer filaments in the cortex and these were more bundled and disorganised

compared to wild type (Cai *et al.*, 2014). Park and Nebenfuhr (2013) investigated a single mutant of *A. thaliana* myosin xi-k and found that although the organisation of the actin cytoskeleton appeared normal, it was less dynamic. The authors could not conclude whether this effect was direct or indirect, but suggested that myosins in general might be involved in ‘straightening’ actin bundles on the long axis of polarised cells, as they have been shown to alter the ‘convolutedness’ of actin filaments (Staiger *et al.*, 2009, Park and Nebenfuhr, 2013).

1.2.4.2 Kinesins

Of the motor proteins only the kinesin superfamily is common to all eukaryotes, but different combinations of the 17 families are found in different organisms (Wickstead *et al.*, 2010). In *A. thaliana* kinesins from ten of the families are found, although the families Kinesin-7 and Kinesin-14 have the most member proteins (Zhu and Dixit, 2011a). Some proteins in the Kinesin-14 family contain domains that are not found in organisms outside the Archaeplastidae (a group consisting of algae and land plants), which suggest that the expansion of that particular family is for plant specific functions (Wickstead *et al.*, 2010, Zhu and Dixit, 2011a).

In *A. thaliana*, kinesins are particularly important in cell division: as interactors with the microtubular cytoskeleton they are involved in the formation of the preprophase band, the spindle apparatus and the phragmoplast (see Section **Error! Reference source not found.**) (Lipka *et al.*, 2014). Kinesins are also involved in cell wall deposition; for example, FRA1 (a class IV kinesin) is somehow involved in microfibril organisation, as a loss-of-function mutant results in disordered microfibrils (Zhu and Dixit, 2011b).

1.2.5 Microtubule cytoskeleton

Like the actin cytoskeleton, microtubules make up a dynamic structure which is constantly growing and shrinking and which can generate enough force to move small organelles (Fujita and Wasteneys, 2014). Microtubules consist of tubulin, a ubiquitous globular protein, which arranges head-to-tail in heterodimers of α -tubulin and β -tubulin, tightly bound together in pairs by non-covalent bonds (Pelletier *et al.*, 2010). These heterodimers form longitudinal bonds to make proto-filaments of which 13 will form lateral bonds to

create long, hollow tubules (Howard and Hyman, 2007). There is a third type of tubulin, γ -tubulin, which is much less common and involved in the nucleation of new microtubules (Pastuglia and Bouchez, 2007).

Microtubules can spontaneously assemble if the concentration of tubulin is high enough; however, microtubule-organising centres (MTOCs) allow formation of microtubules at lower concentrations of tubulin while also acting as a regulator for nucleation (Brown and Lemmon, 2007). Although plants do not have all the structures that in other eukaryotes act as MTOCs, they do have γ -tubulin which allows nucleation from pre-existing microtubules at specific angles (Yang and Pon, 2002).

1.2.5.1 Microtubule binding proteins

Analysis of the genome of *A. thaliana* suggests that there are over 200 microtubule-interacting proteins that can interact with microtubules by bundling, capping, severing, cross-linking, nucleating or moving along the filaments (Table 1.2) (Meagher and Fechheimer, 2003). These proteins, while all interacting with microtubules in some way, are vital for different cellular processes such as cell wall construction, cytokinesis and cell division (Sedbrook, 2004).

Table 1.2 Examples of microtubule binding proteins and protein families.

These are a selection of microtubule-binding proteins to give a broad overview of the kinds of functions that have been observed.

Protein Families	Function	Publications
Kinesin	Motor proteins	(Wickstead <i>et al.</i> , 2010)
Formins	ATFH4/14	(Deeks <i>et al.</i> , 2010, Li <i>et al.</i> , 2010)
Phospholipase D (PLD)	Signalling	(Eliás <i>et al.</i> , 2002)
Katanin	ATP-dependent severing	(Sedbrook, 2004)
Microtubule plus-end tracking proteins (+TIP)/CLASP/EB1	Microtubules polymerisation and MAP recruitment	(Sedbrook, 2004)
Spiral 1/2/3	Modulating microtubule severing	(Wightman <i>et al.</i> , 2013)
Cellulose synthase-interacting 1 (CSI1)	Links microtubules to CSCs	(Li <i>et al.</i> , 2012b)
Microtubule associated protein (MAP65)	Crosslinking	(Sedbrook, 2004)
MOR1	Polymerisation	(Sedbrook, 2004)
Wave-dampened 2 (WVD2)	Bundling?	(Sedbrook, 2004)
XMAP215	Stabilising	(Howard and Hyman, 2007)

1.2.5.2 Microtubule and actin cytoskeleton interactions

The actin and microtubule cytoskeletons are found together in the cortex of plant cells and are both involved in processes such as cell division and cell growth, although with differing roles (Schneider and Persson, 2015). They can influence each other and can also be connected physically by proteins that can interact with both networks (Schneider and Persson, 2015).

For example, Smertenko *et al.* (2010) treated BY-2 protoplasts expressing GFP-Lifeact with taxol or APM (a microtubule de-polymerising agent) and found that when microtubules were depolymerised actin filaments elongated 30% faster and shortened quicker, but there were no statistical differences between control cells and those with

stabilised microtubules. In *A. thaliana* hypocotyl cells when the actin cytoskeleton was stabilised using jasplakinolide, microtubule orientation became disordered (Sampathkumar *et al.*, 2011). The actin cytoskeleton was completely de-polymerised by overnight incubation in latrunculin B (an actin depolymerising agent) and the drug washed out, then when the actin signal began to recover, it did so along microtubules (Sampathkumar *et al.*, 2011). However, in cells treated with both latrunculin B and oryzalin (a microtubule depolymerising agent) when latrunculin B was washed out, but oryzalin was re-applied, the actin cytoskeleton did not recover properly, it re-formed as puncta that moved erratically (Sampathkumar *et al.*, 2011). A similar situation was found with the microtubule cytoskeleton in cells treated with both drugs, but with oryzalin washed out and latrunculin B re-applied (Sampathkumar *et al.*, 2011). This shows that the actin and microtubule cytoskeleton interact and are dependent on each other and some of the proteins that link them have been identified.

Proteins or protein complexes have been shown to link the actin and microtubule cytoskeletons. For example, AtFH4 is a group I formin from *A. thaliana* that nucleates actin filaments, but interacts directly with microtubules; possibly the microtubule cytoskeleton acts as a scaffold for AtFH4 (Deeks *et al.*, 2010). A sub-group of kinesin-14 in *A. thaliana* all contain one calponin homology domain which can bind actin when the kinesins dimerise (Schneider and Persson, 2015). Their exact function in *A. thaliana* is not known, but they are thought to be involved in nuclear positioning and to have two roles: one during interphase and one during mitosis (Schneider and Persson, 2015). Schneider and Persson (2015) speculated that they could transport actin filaments along microtubules (they are minus-end directed) or that they could be functional homologues for dynein in angiosperms.

Lastly, NET3C (Table 1.1) is an actin binding protein that associates with the plasma membrane, while Vesicle-Associated Protein 27 (VAP27) is an endoplasmic reticulum protein that also associates with the plasma membrane and microtubules (Wang *et al.*, 2014). They form a complex with at least each other and possibly additional proteins, thus linking the plasma membrane, actin, endoplasmic reticulum and microtubules (Wang *et al.*, 2014).

1.3 Plant cell wall

The cell wall is a relatively large structure compared to many structures inside the cell and it is made up of many complex carbohydrates and proteins. This study is concerned with the influence of the cell wall on the actin cytoskeleton, so it is first necessary to briefly describe the principle components of the cell wall and how they are thought to interact.

Plants possess two types of cell wall. The primary cell wall surrounds every cell: it is relatively extensible and it is composed of carbohydrates and proteins. The secondary cell wall is a more rigid structure produced in specific circumstances: cell type, age or in response to certain stresses, after cell growth has ceased. It is composed of carbohydrates and polyphenolic compounds. Both types of cell wall provide vital structural support, mechanical strength and they are the first, physical barrier that pathogens must overcome to invade a cell (Pilling and Hofte, 2003).

1.3.1 Plant primary cell wall

The primary cell wall is considered as such because it surrounds a newly produced cell that remains capable of growing by cell expansion, whereas the secondary cell wall surrounds differentiated cells that require a great amount of structural reinforcement (Cosgrove, 2005). The majority of cell walls are roughly 5% of the cell volume and are squeezed between the plasma membranes of neighbouring cells (Szymanski and Cosgrove, 2009). The thickness of the cell wall of *N. tabacum* suspension cells was estimated from electron micrographs to be about 0.1 μm (Iraki *et al.*, 1989).

By dried weight, the primary cell wall is made up of anywhere between 15-40% cellulose, 20-30% xyloglucans (see Section 1.3.4) and 30-50% pectic polysaccharides, with the rest of the material a mixture of arabinoxylans and cell-wall proteins (Cosgrove and Jarvis, 2012). The chemical structure of cellulose is the same in the cell walls of different plant species, but the degree to which it crystallises and the length of the chain vary (Burton *et al.*, 2010).

1.3.1.1 Cellulose

Cellulose is the major load-bearing component of the cell wall, it acts as a scaffold due to its strength and because it is generally unaffected by enzyme activity (Figure 1.1; Table 1.3) (Cosgrove, 2005). It is made of 1-4-linked- β -D-glucose chains which almost instantly form hydrogen bonds and weaker Van der Waals forces and crystallise into microfibrils (Carpita, 2011, Harris *et al.*, 2010). These chains are laid down by cellulose synthase complexes moving along the microtubule network (see Section 1.2.5). In contrast, the other components of the cell wall are synthesised in the Golgi apparatus primarily through the action of glycosyltransferases, of which *A. thaliana* has approximately 400 (Scheible and Pauly, 2004). Unless further modification is necessary they are then transported to the plasma membrane in secretory vesicles (Fangel *et al.*, 2012).

1.3.1.2 Hemicelluloses

Hemicelluloses are a diverse group of polysaccharides that have large side chains (Table 1.3), which are often made from xylose, galactose and fucose and are extracted from plant cell walls with a strong alkaline solution (Gutjahr and Nick, 2006, Cosgrove, 2005). They bind tightly to cellulose via hydrogen bonds, but cannot form microfibrils themselves because of their branched and modified structure (Keegstra, 2010, Cosgrove, 2005).

1.3.1.3 Pectins

Pectins are extremely complex acidic polysaccharides, containing galacturonate and rhamnogalaturonans, which can be extracted using hot water or dilute acid (Cosgrove, 2005). These polysaccharides make up about 35% of the primary cell wall in dicotyledonous plants and they bind together and form a cross-linked matrix with cellulose microfibrils (Mohnen, 2008, Carpita, 2011). The structure of pectin can be changed by methyl-esterification or de-esterification, thus making it more or less loose; for example, de-esterification is followed by a halting of cell growth (Mohnen, 2008).

Pectins probably link to other pectins via their backbones and to hemicelluloses with a tight association, such as a covalent link (Cosgrove, 2005, Mohnen, 2008). It has also been reported that there is an arabinogalactan protein that can covalently bind both pectin and hemicellulosic polysaccharides; in the mutant of this protein, pectins and xylans were more

easily extracted compared to wild-type (Arabinoxylan Pectin Arabinogalactan Protein1 (APAP1)) (Tan *et al.*, 2013).

Table 1.3 The major carbohydrate groups found in the primary plant cell wall.

Carbohydrates	Function	Publications
Cellulose	Major load-bearing component	(Fry, 2004)
Callose	Developmental roles and stress response	(Chen and Kim, 2009)
Xyloglucan	Cross-links cellulose	(Fry, 2004)
Xylans	Cross-link cellulose	(Fry, 2004)
Mixed linked glycans (MLGs)	Cross-link cellulose, assembled at the plasma membrane	(van de Meene <i>et al.</i> , 2016)
Homogalacturonan, Rhamnogalaacturonan, Rhamnogalaacturonan II, Xylogalacturonan	Pectins – form a gel-like matrix	(Pelloux <i>et al.</i> , 2007)

1.3.1.4 Plant cell wall proteins

Embedded within the polysaccharide matrix are the cell wall proteins. About 17% of the genome of *A. thaliana* probably encodes cell wall proteins: including expansins, hydroxyproline-rich *O*-glycoproteins (HRGPs) and cell wall associated kinases (WAKs) (Table 1.4) (Jamet *et al.*, 2006). Expansins can cause cell wall extension within seconds, without any apparent enzymatic activity and without permanently altering the elasticity or strength of the wall (McQueen-Mason *et al.*, 1992, Cosgrove, 2016). They bind to the cellulose in primary cell walls at specific ‘bio-mechanical hot-spots’ causing a looser wall thus inducing ‘wall creep’ (Cosgrove, 2015).

One of the larger families of cell wall proteins is that of hydroxyproline-rich *O*-glycoproteins, the protein component of which are made up of complex macromolecules demonstrating a wide range of functions (Hijazi *et al.*, 2014). This family consists of three broad categories of protein: extensins, which are only moderately glycosylated; AGPs, which are very glycosylated; and the much less studied, hydroxyproline-rich proteins, which are variably glycosylated (Hijazi *et al.*, 2014). Extensins are a large family of

complex proteins that probably form scaffolds at the cell wall, although not much is known about their exact functions (Hijazi *et al.*, 2014).

AGPs always contain at least one arabinogalactan side chain, but other than that they are very diverse in structure: they are sometimes in tight association with pectin and are thought to be involved in various developmental processes, including tip growth and secondary wall deposition (Seifert and Roberts, 2007). However, glycoproteins are difficult to work with, as Seifert and Roberts (2007) emphasises: “sugars cannot be cloned!”.

Lastly, cell wall associated kinases are known to bind pectins: although the exact binding mechanism is unknown, it is thought that they have a higher affinity for de-esterified pectin (Kohorn and Kohorn, 2012). They are involved in cell expansion and defence responses, probably as pectin receptors due to their differing affinities for pectins in various states of esterification (Kohorn and Kohorn, 2012).

1.3.2 Plant secondary cell wall

The secondary cell wall is constructed between the primary cell wall and the plasma membrane, after the cell has finished expanding. The secondary cell wall contains lignin, a polyphenolic compound, and it has a different combination of carbohydrates (Cosgrove and Jarvis, 2012). It has more xylans and glucomannans than the primary cell wall, fewer xyloglucans and pectins and less water (Cosgrove and Jarvis, 2012, Mellerowicz and Sundberg, 2008). Lignin is a complicated mix of different phenolic polymers, the composition of which varies between species and even between individual cell wall layers (Vanholme *et al.*, 2010). Monolignols, which make up lignin, are transported to the apoplast, where they are polymerised by various enzymes in an oxidative manner (Weng and Chapple, 2010). If *A. thaliana* has a small reduction in lignin content then the plants develop normally; however, if the amount of lignin is severely reduced then plants grow stunted and are sterile (Vanholme *et al.*, 2010). While the secondary cell wall is important for a plant, it is not under investigation in this study.

Table 1.4 The major protein families found in the primary plant cell wall.

Protein Families	Function	Publications
Expansins	Wall loosening	(Cosgrove, 2005)
Arabinogalactans (AGPs)	Development, signalling and maybe mechanical properties	(Seifert and Blaukopf, 2010)
Extensins	Wall extension	(Lamport <i>et al.</i> , 2011)
Proline-rich proteins (PRPs)	Damage response and development	(Fowler <i>et al.</i> , 1999)
Wall associated kinases (WAKs)	Wall maintenance and signalling	(Kohorn, 2000)
Leucine-rich repeat-extensin proteins (LRX)	Cell morphogenesis of root hairs	(Baumberger <i>et al.</i> , 2003)
Peroxidases	Dehydrogenation using hydrogen peroxide	(Vanholme <i>et al.</i> , 2010)
Pectin methylesterase (PME)	Pectin methylesteration	(Pelloux <i>et al.</i> , 2007)

1.3.3 Cellulose synthase complexes

Cellulose synthase complexes (CSC) are made up of six rosette subunits (see Section 1.3.3; Figure 1.3), each subunit of which is hypothesised to be composed of three proteins (Vandavasi *et al.*, 2016). It is possible that the rosette subunits can each make up to six glucan chains at any one time, although it is now thought that they make only three chains, so that one cellulose microfibril is made up of 18 chains (Vandavasi *et al.*, 2016). These chains form cellulose microfibrils 3-5 nm wide and several micrometres long (Cosgrove, 2005). CSCs are made up of a combination of six cellulose synthase proteins (CESAs) of which there are ten in *A. thaliana* (McFarlane *et al.*, 2014). However, there is no data to confirm in what arrangement the different CESA proteins are located within the complex (Li *et al.*, 2014). Each CSC is hypothesised to be at least 4 mDa with other proteins directly or indirectly associated; however, to date, only a few associated proteins have been definitively demonstrated (Taylor, 2008).

It has been shown that the CSCs move, on average, $0.33 \mu\text{m}/\text{min}$ through the plasma membrane (Paredes *et al.*, 2006). They move along microtubule tracks through an association with CESA interactive protein 1 (CS1) (Li *et al.*, 2012b); although the force for movement is provided by the polymerisation of the microfibrils themselves (Diotallevi and Mulder, 2007). Cellulose microfibrils are laid down in an ordered pattern, initially in elongating cells they are extruded transversely in a helical manner until a certain developmental point is reached and they reorient (Benatti *et al.*, 2012). This polymerisation/depolymerisation can be eliminated by stopping the movement of the underlying microtubules, a subject which will be discussed in Section 1.2.5 (pg. 15) (Chan *et al.*, 2010).

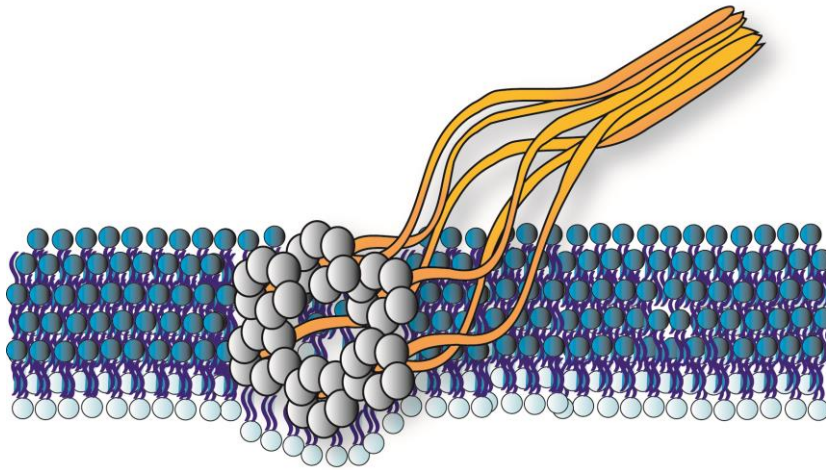


Figure 1.3 Schematic showing the CSC on the plasma membrane with a cellulose microfibril being extruded.

The six rosette subunits are shown extruding a random number of cellulose microfibrils which crystallise in the cell wall.

There has been much work carried out on the CESA proteins and the structures that they form. It has been demonstrated that the proteins CESA1, -3 and -6 are involved in the synthesis of primary cell wall cellulose, while CESA4, -7 and -8 make cellulose for the secondary cell wall (see Section 1.3.2) (Carpita, 2011). CESA1 and -7 can partially rescue defects in certain *cesa* mutants, but it appears there is selectivity in the system to make sure that the CESA proteins are either in primary or secondary complexes, not both (Carroll *et al.*, 2012). CESA2, -5 and -9 are thought to be partially redundant with CESA6, while CESA10 has not yet been characterised (Carroll *et al.*, 2012, McFarlane *et al.*, 2014). The CESA protein family also contains sub-families, such as the CELLULOSE SYNTHASE-

LIKE (CSL) proteins, which also function in the production of complex carbohydrates (Liepman and Cavalier, 2012).

1.3.3.1 Isoxaben: cellulose biosynthesis inhibitor

Isoxaben is a cellulose biosynthesis inhibitor that stops glucose incorporation into cellulose and causes removal of the majority of CSCs from the plasma membrane within 20 minutes (Desprez *et al.*, 2002, Paredez *et al.*, 2006). It has been hypothesised that isoxaben acts as a ‘steric inhibitor’ which stops CESAs changing conformation to switch ‘on’ (Tateno *et al.*, 2016). Isoxaben interferes specifically with the proteins CESA3 and CESA6, as certain point mutations within these proteins confer tolerance, possibly by altering the topology of the protein thus preventing isoxaben binding (Desprez *et al.*, 2002, Tateno *et al.*, 2016). One of the mutations in Cesa3 that confers tolerance (S377F) is very close to a conserved region that is involved in substrate or acceptor binding and also to the catalytic site itself (Sethaphong *et al.*, 2013). Interestingly, it does not affect the amount of cellulose in mutant *A. thaliana* plants, but it does affect the process of cellulose crystallisation (Sethaphong *et al.*, 2013).

1.3.4 Xyloglucans

The most abundant hemicellulose in the primary cell wall of dicotyledons is xyloglucan: it can make up around a quarter of the dry weight of the primary cell wall (Fry, 1989). Xyloglucan has the same chemical backbone as cellulose. The molecule can be anywhere from 0.15 to 1.5 μm in length and it can form hydrogen bonds with cellulose (Fry, 1989). Until recently it was thought that xyloglucan cross-linked cellulose microfibrils, while pectins in-filled around them creating a gel-like matrix (Cosgrove and Jarvis, 2012). However, Cavalier *et al.* (2008) found that a plant can lack any detectable xyloglucan with only small changes in phenotype relative to wild type (Section 1.3.4.1).

One study used an extensometer to measure ‘wall creep’ when cells were treated with three small endoglucanases (Park and Cosgrove, 2012). They showed that digesting specifically either enzyme-accessible cellulose or xyloglucan, alone and together, was insufficient to induce ‘wall creep’ and even digesting pectin before treatment gave the same result (Park and Cosgrove, 2012). However, one fungal enzyme (Cel12A) that had the ability to digest

cellulose and xyloglucan simultaneously did cause a high rate of ‘wall creep’ (Park and Cosgrove, 2012). The authors concluded that the areas acted upon by expansins and similar proteins are places where cellulose and xyloglucans are linked intimately such that there is only space for one enzyme to act upon both carbohydrates simultaneously (Park and Cosgrove, 2012).

Therefore it is hypothesised that xyloglucans bind cellulose microfibrils together at ‘biomechanical hotspots’ (Bashline *et al.*, 2014). This model has xyloglucan located in areas where the cellulose microfibrils are in very close contact, perhaps acting as an adhesive layer (Bashline *et al.*, 2014).

1.3.4.1 *xxt1-xxt2 mutant*

There are several genes involved in the biosynthesis of xyloglucans, including at least three xylosyltransferase proteins, which are involved in the transfer of xylosyl which is essential for the production of detectable xyloglucan (Zabotina *et al.*, 2008). It is thought that *A. thaliana* has only three xyloglucan xylosyltransferases genes and mutants have been characterised for all of them: *xxt1*, *xxt2* and *xxt5* (Zabotina *et al.*, 2012). Plants lacking XXT1 had 10% less xyloglucan, plants lacking XXT2 had a 20% reduction in xyloglucan content while plants lacking XXT5 showed a 50% decrease in xyloglucan content (Cavalier *et al.*, 2008, Zabotina *et al.*, 2008). Plants that no longer had functional XXT1 or XXT2 proteins had no detectable xyloglucan at all (Cavalier *et al.*, 2008, Park and Cosgrove, 2012).

Plants were generated that lacked various different combinations of each of these three genes and it was found that they all had slightly different alterations in their cells walls, suggesting that plants can compensate for the loss of a structural element (Zabotina *et al.*, 2012). *xxt1 xxt2* and *xxt1 xxt5* plants showed some increase in the amount of pectins produced when compared to wild type (Zabotina *et al.*, 2012). It was also shown that there was an increase in xylan content (see Table 1.3) in the *xxt1 xxt2* plants and that the cell walls were less able to extend, displaying a decreased sensitivity to expansins (Park and Cosgrove, 2012). Additionally, the cell walls of the *xxt1 xxt2* plants were more sensitive to treatments that made pectins and xylans ‘looser’; these results all point to xyloglucan being important for growth of the plant, although it also shows that the mutant can compensate using other carbohydrates (Cosgrove, 2014).

The organisation of cellulose microfibrils in the *xxt1 xxt2* mutant in hypocotyl epidermal cells was disturbed: most microfibrils were parallel rather than in differently oriented layers, each with a different microfibril orientation as exhibited by wild type plants (Xiao *et al.*, 2016). One possible interpretation is that xyloglucan could be involved in the bidirectional feedback between cellulose deposition and the microtubule cytoskeleton (Tijs Ketelaar, personal communication). The microtubule cytoskeleton was also disordered and microtubules were slower to grow and faster in shrinking as well as being more sensitive to oryzalin, whilst pressure caused the microtubule cytoskeleton to fragment (Xiao *et al.*, 2016). The authors suggest that as this mutant has weaker walls, xyloglucan is important for cell wall strength, suggesting that the ‘biomechanical hotspots’ where it is probably found are load-bearing (Cosgrove, 2014, Xiao *et al.*, 2016). Interestingly, in the *xxt1 xxt2* mutant the CSCs were found to be more clustered and to have a reduced density and velocity (Xiao *et al.*, 2016). In addition, mRNA levels were quantified in the *xxt1-xxt2* mutant and there were found to be increases and decreases in expression levels in microtubule-binding proteins, receptors in the cell wall and proteins that function in cellulose synthesis itself (Xiao *et al.*, 2016).

1.4 Formation of the cell wall *de novo*

1.4.1 Protoplasts

Protoplasts are walled cells that have had their wall removed, apart from generative cells in pollen which have no cell wall. Although the occasional example has been found in nature, such as certain cells in the endosperm in *Haemanthus* species (African blood lilies) (Zhou *et al.*, 1986). They can be considered a versatile and useful experimental tool (Yoo *et al.*, 2007). Protoplasts are usually produced by incubating leaf material with digestive enzymes (e.g. cellulase, pectinase, etc) that specifically degrade cell wall components. Davey *et al.* (2005) (and references within) outline the many, varied uses for protoplasts since the first isolation attempts in the early 1960s. They are often used to generate stable transgenic lines of plant cell suspension cultures. They have also been used to create transgenic plants; for example, using somatic hybridisation to produce commercial potato strains that are resistant to blight and pathogenic nematodes (Austin *et al.*, 1993).

Protoplasts are also used for studying certain processes, such as the secretory pathway, the re-establishment of polarity and cell wall regrowth (Denecke *et al.*, 2012, Zaban *et al.*, 2013).

1.4.1.1 Plasmolysis

Plasmolysis is a complicated process that is not fully understood: it occurs when a cell surrounded by a wall is exposed to hyper-osmotic or drought conditions causing the plasma membrane containing all the cell contents to pull away from the cell wall. The protoplast can survive plasmolysis, though the amount and duration depends on the species. For example, *Ramonda serbica*, the resurrection plant, can survive three weeks of drought by altering the types of lipid and the ratio of protein to lipid content in the plasma membrane; and after rehydration these alterations revert (Quartacci *et al.*, 2002).

The cellular contents of a plasmolysed cell are still connected to the cell wall via Hechtian strands on anticlinal (adjacent to other cells) and periclinal walls; these are just not desmotubules as periclinal walls lack plasmodesmata (Oparka, 1994). Hechtian strands can contain at least microtubules, actin and some endoplasmic reticulum (Oparka, 1994, Lang *et al.*, 2014). During plasmolysis the protoplast can shrink down to 15% of its original volume before the membrane is no longer semi-permeable (Palta and Lee-Stadelmann, 1983). It has also been shown that while cytoplasmic streaming is disrupted by plasmolysis, the number of vesicles around Golgi bodies are unaffected for 30 minutes and that organelle motility via cytoplasmic streaming continued during plasmolysis (Oparka, 1994, Lang *et al.*, 2014).

During plasmolysis CSCs are removed from the plasma membrane and are localised to specific vesicles that physically interact with Golgi bodies, a process that takes approximately six minutes (Crowell *et al.*, 2009). Endler *et al.* (2015) showed that in etiolated hypocotyl cells after 30 minutes of treatment with 200 mM NaCl, there are very few CSCs left in the plasma membrane and that CESA proteins can be detected in specific vesicles in the cytoplasm. Gutierrez *et al.* (2009) showed that the rate of CESA delivery to the plasma membrane was 4.8 ± 0.7 events $\mu\text{m}^{-2}\text{h}^{-1}$ for untreated etiolated *A.thaliana* epidermal hypocotyl cells and 0.04 ± 0.04 events $\mu\text{m}^{-2}\text{h}^{-1}$ for cells treated with 200 mM mannitol (a concentration that did not cause plasmolysis) for three hours. Gutierrez *et al.* (2009) showed that the same osmotic stress caused a reduction in the motility of

endosomes and Golgi bodies and suggested that osmotic stress affects membrane trafficking.

In *Nicotiana tabacum* cells adapted to osmotic stress, the cell walls contain approximately 50% fewer polysaccharides compared to un-stressed cells (Iraki *et al.*, 1989). Of these there was much less cellulose, a higher proportion of hemicelluloses and while the amount of pectin was roughly the same, it was a different kind of pectin that was much more loosely bound into the cell wall (Iraki *et al.*, 1989).

1.5 Plasma membrane

The plasma membrane is important as an interface between the extracellular cell wall and the intracellular cytoplasm. It is a phospholipid bilayer approximately 5 nm thick (see Section 1.5.2): this is thicker than the membranes of either the endoplasmic reticulum or the Golgi bodies (Nicolson, 2014, Hurst and Hemsley, 2015). During synthesis and trafficking, plasma membrane proteins are adapted, such as with transient lipid modifications like S-acylation in order to avoid inappropriate degradation (Hurst and Hemsley, 2015). S-acylation can cause the protein to ‘tilt’ in the membrane, which is hypothesised to have many interesting consequences, such as altering membrane tension, although to date this has only been shown in mammalian systems (Hurst and Hemsley, 2015).

The plasma membrane is a dynamic structure with many of its lipid and protein constituents joined together by non-covalent bonds and able to move around. For example, an unhindered lipid molecule can diffuse about $2 \mu\text{m}^2\text{s}^{-1}$ (covalent bonds are formed when atoms share electrons whereas non-covalent bonds are via electromagnetic interactions) (Alberts *et al.*, 2014). It is proposed that perhaps as much as a quarter of the plasma membrane volume is protein; these are inserted throughout the plasma membrane and are involved in many physiological processes (Malinsky *et al.*, 2013). Alberts *et al.* (2014) states that in the average membrane although 50% of the mass is protein, there are 50 lipid molecules for each single protein.

There are several different ways that proteins can associate with the plasma membrane. Integral membrane proteins have one or more transmembrane domains that are inserted into the lipid bilayer. These proteins are often transporters, pumps or channels, such as PIP2;1, an aquaporin that aids in water diffusion through the plasma membrane (Santoni *et al.*, 2003). They can also be part of signalling cascades that trigger responses to changes in the extracellular environment (Weichsel *et al.*, 2010). Transmembrane domains are important for protein insertion into the membrane, but can also be part of the mechanism to target proteins to the correct destination. Brandizzi (2002) showed that protein targeting to membranes is partially dependent on the length of their hydrophobic domain: when they gave a Golgi-targeted protein a longer hydrophobic domain it was instead trafficked to the plasma membrane.

Peripheral membrane proteins are not directly inserted into the plasma membrane, but are associated either via covalent bonds or by interactions with other membrane-bound proteins (Weichsel *et al.*, 2010). These include proteins like RPM1, which confers resistance to bacterial pathogens and is found on the cytoplasmic side of the plasma membrane (Boyes *et al.*, 1998). There are several different binding mechanisms by which a peripheral protein can associate with the membrane. One of these mechanisms is post-translational lipid modification, which is important, often essential, for correct localisation (Stael *et al.*, 2011).

In mammalian cells it is considered that the biggest influences on the lateral mobility of membrane-associated proteins are: protein-protein interactions, protein-lipid interactions, protein scaffolds, and cytoskeletal tethering or corralling (Barrantes, 2014). While it is known that properties of the plasma membrane (such as viscosity) vary between cell types and even between membrane domains, it is considered that they are less influential factors (Barrantes, 2014). In *A. thaliana* it has been shown that both lipid domains and both the microtubule and actin cytoskeletons have a small effect on the lateral diffusion of membrane proteins compared to the presence or absence of the cell wall (Martinière *et al.*, 2012).

1.5.1 Plasma membrane protein lipid modifications

There are four kinds of lipid anchors known in *A. thaliana*: prenylation, N-terminal myristoylation, palmitoylation/S-acylation and C-terminal glycolipids such as glycosylphosphatidylinositol-anchors (GPI) (Friso and van Wijk, 2015). These are all variants of post-translational modifications that add a lipid chain which can covalently attach to the inner leaflet of a membrane, thus associating a protein with a membrane (Alberts *et al.*, 2014). The GPI-anchor is a very strong association, in some biochemical ways like an integral membrane protein (Ytterberg and Jensen, 2010).

1.5.2 Plasma membrane lipids

Plants have a greater diversity of lipids than animals and the mix of lipids in the plasma membrane is particularly varied (Grosjean *et al.*, 2015). Lipids are synthesised in the endoplasmic reticulum, although some also require additional processing in the Golgi apparatus (Alberts *et al.*, 2014). Lipids are uncharged; they usually move in a membrane by lateral diffusion as they can change places with their neighbours, although they rarely move from one leaflet to another, unless acted upon by enzymes (Alberts *et al.*, 2014). The length and amount of saturation of the fatty acid chains specifies the strength of the van der Waals forces that connect lipids, which then defines the properties of that section of membrane (Alberts *et al.*, 2014). A membrane region can be fluid or more solid: for example, the longer, more saturated chains of sphingolipids make a more stable, less fluid membrane (Alberts *et al.*, 2014).

All eukaryotes possess the three main groups of lipids: sterols, sphingolipids and glycerolipids; although only glycerolipids are the same between plants, animals and fungi, sterols and sphingolipids are different for each kingdom (Cacas *et al.*, 2016). Additionally, the lipid composition of inner and outer plasma membrane leaflets is different and tightly regulated (Cacas *et al.*, 2016).

In the plant plasma membrane, sterols are represented by stigmasterol, campesterol and β -sitosterol; the proportions of these present in a membrane define its fluidity, permeability and structure and consequently influence its function (Grosjean *et al.*, 2015). The

sphingolipids found in plants are glycosyl inositol phosphorylceramides (GIPCs), glycosylceramides, ceramides and free long-chain bases (LCBs) (Pata *et al.*, 2010). Cacas *et al.* (2016) found that GIPCs made up approximately 60-80% of the plasma membrane outer leaflet, but only 30-40% of the total plasma membrane lipid content, implying that they are heavily enriched at the outer leaflet. The authors suggest that the long chain fatty acids found on GIPCs would ‘interdigitate’ from the outer leaflet into the inner, possibly to stabilise the outer leaflet (Cacas *et al.*, 2016). Glycerolipids in the plasma membrane of *N. tabacum* were found to possess the same head groups as those found on the glycerolipids of animal cells, but the fatty acyl chains were shorter (the longest in *N. tabacum* were 20 carbon molecules as opposed to 24 carbons) and were generally more saturated (Mongrand *et al.*, 2004).

Polyphosphoinositides are derivatives of phosphatidylinositol (PtdIns) and consist mostly of saturated fatty acids; they are important in animal, yeast and plant cells as primary and secondary signalling molecules (Furt *et al.*, 2010). They are involved in many cellular processes such as trafficking and signalling and are found not just at the plasma membrane, but also in the membranes of the endoplasmic reticulum, Golgi bodies and the nucleus (Saarikangas *et al.*, 2010). In most cells, both PtdIns and all polyphosphoinositides only comprise approximately 10% of the total lipid content, phosphatidylinositol 4-phosphate (PtdIns4P) is the most common polyphosphoinositide in plant membranes and it is the precursor to phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) (Vermeer *et al.*, 2009, Hammond and Balla, 2015). They are important because of their low abundance, they can cause interacting proteins to be selectively targeted to specific membrane domains (as long as they are present) as well as activating or inactivating said proteins (Saarikangas *et al.*, 2010).

1.5.3 Plasma membrane microdomains

Membrane microdomains are usually considered as lateral compartments in the plasma membrane that have a different composition and function to the surrounding membrane, regardless of their stability, size or how they formed (Malinsky *et al.*, 2013). They can be distinct from lipid rafts, which are defined as areas of membrane enriched in sterols and sphingolipids that are dynamic and transient (Malinsky *et al.*, 2013). Lastly, detergent-resistant membranes (DRMs) or detergent-insoluble membranes (DIMs) are just that these

are membranes extracted under those specific conditions; however, their existence *in vivo* as organised, meaningful structures has yet to be proved (Malinsky *et al.*, 2013).

It is thought that closely related proteins are targeted to similar microdomains in the plasma membrane, possibly to keep certain activities zoned (Malinsky *et al.*, 2013). Membrane microdomains have a specific lipid lateral composition that differs between the inner and outer leaflet; microdomains are also thicker than the surrounding membrane (Konrad and Ott, 2015).

Residence within a microdomain can be due to lipid modification; it has been shown that S-acylation, which is reversible, is necessary, but not sufficient, for many integral mammalian raft proteins (Levental *et al.*, 2010). In *A. thaliana* a mutant in S-acylation showed a different microdomain organisation to wild type (Konrad *et al.*, 2014), but the protein lifetime and the stability of the microdomain has not yet been examined (Hurst and Hemsley, 2015).

Although many of the theories and evidence for membrane microdomains have come from work on mammalian cells, their importance is being proven in plant cells. Demir *et al.* (2013) showed that interaction between anion channel proteins led to their activation and association with membrane microdomains was the preferred location for this activation. Jarsch *et al.* (2014) investigated the Remorin family in *A. thaliana* and discovered that the microdomains they labelled were immobile, but maintained by lateral protein diffusion. However, when they compared FRAP analyses on protein associated with an immobile domain and protein diffused within the membrane they found no differences in diffusion rate (Jarsch *et al.*, 2014). Suggesting that the mobility of the protein was due to the protein itself rather than its inclusion in a microdomain.

1.6 Endomembrane system

1.6.1 Vacuole

While the cell wall provides strength and support, it is only partially responsible for maintaining turgor pressure and thus cell shape and tissue integrity. Turgor pressure is created by the vacuole acting together with the cell wall. A full vacuole (whether lytic and/or protein storage vacuole depends on the cell type and developmental stage), which can make up to 90% of the cell volume, appresses the cytoplasm against the plasma membrane-cell wall continuum (Zhang *et al.*, 2014). The lytic vacuole can be the end-point for macromolecules synthesised in the endoplasmic reticulum or for proteins that need to be degraded, whilst some cells contain storage vacuoles (Zhang *et al.*, 2014).

1.6.2 Golgi apparatus

Cellulose is synthesised at the plasma membrane by CSCs (Section 1.3.3, pg. 23), but pectins and hemicelluloses (Section 1.3.1) are synthesised in Golgi bodies. These are motile stacks of polarised membrane-bound compartments that are physically close to the endoplasmic reticulum if not somehow connected (Kim and Brandizzi, 2014, Robinson *et al.*, 2015). Carbohydrate backbones are synthesised in specific compartments within the Golgi body; as they pass through different compartments in the Golgi carbohydrate residues are transferred onto them before being trafficked to the plasma membrane (Section 1.5, pg. 29) (Worden *et al.*, 2012).

In *A. thaliana* the Golgi apparatus is made up of hundreds of small Golgi bodies (approximately 1 μm diameter) which are extremely motile. The number of Golgi bodies per cell and the number of cisternae of which they are comprised is dependent on the cell type and the developmental stage (Driouich *et al.*, 2012). What polysaccharide is produced or modified in which cisternae also differs between cell type and development; and few proteins have been definitively localised (Driouich *et al.*, 2012).

1.6.2.1 Actin cytoskeleton – Golgi body connection

The motility of Golgi bodies was shown to be mainly due to the actin cytoskeleton (Boevink *et al.*, 1998, Nebenfuhr *et al.*, 1999, Akkerman *et al.*, 2011), although Kinesin-13A is known to interact with Golgi bodies (Lu *et al.*, 2005). Using dominant negative myosin mutants in a transient system, six myosins were found to reduce Golgi body motility (Avisar *et al.*, 2009). However, when the same group used *A. thaliana* they discovered that myosin XIK reduced motility much more than the other five (Avisar *et al.*, 2012).

1.6.3 Exocytosis

Proteins or carbohydrates leave the Golgi body through the *trans*-Golgi network (TGN; described as a ‘post-office’) to be trafficked to their destination in vesicles (van de Meene *et al.*, 2016). One destination is the plasma membrane. Exocytosis and endocytosis are two processes that control the composition of the plasma membrane and therefore its structure and proper functioning (Paez Valencia *et al.*, 2016).

Secretion of Golgi-derived vesicle contents into extracellular space via exocytosis is a process conserved across eukaryotes that is necessary for several vital cellular processes (Zhang *et al.*, 2010). It is also the process by which pectins and hemicelluloses are delivered to the cell wall in plant cells, although this rule is not always true: it also depends on the plant species and type of carbohydrate (Wilson *et al.*, 2015). In order for exocytosis to occur the vesicle is ‘tethered’ to the destination membrane through the interactions of specific SNARE proteins on both partners and this process is promoted by various protein complexes (Zhang *et al.*, 2010). SNARE proteins are SNAP receptors (Soluble N-ethylmaleimide-sensitive factor Attachment protein REceptors) necessary for the controlled regulation of the fusion of vesicles with the destination membrane (Hachez *et al.*, 2014).

Each compartment also has its own specific pH: from the endoplasmic reticulum to the vacuole, the compartments get gradually more acidic, although still more alkaline than their corresponding animal counterparts (Martinière *et al.*, 2013). For example, the early endosome is more acidic than the TGN, which causes the ligands of the mannosyl 6-

phosphate receptor (involved in sorting lysosomal enzymes) to disengage (Robinson, 2014).

1.6.4 Endocytosis

Endocytosis is the process by which plasma membrane proteins and extracellular substances are internalised and enter the endosomal pathway, this can be essential for the proper functioning of proteins such as receptors (Paez Valencia *et al.*, 2016). These proteins are contained within vesicles which can be trafficked through various compartments, such as the TGN to a destination such as the vacuole for degradation, or they could be recycled back to the plasma membrane (Robinson *et al.*, 2008). The section of membrane which will be removed is deformed to create a pit by a protein ‘coat’ that will be shed after the vesicle is separated from the donor membrane (Paez Valencia *et al.*, 2016).

A good example of the complexity and importance of endocytosis is the plasma membrane receptor FLAGELLIN SENSING2 (FLS2). FLS2 is endocytosed when activated by its ligand (flg22) and it is targeted to late endosomes and from there to the vacuole (Beck *et al.*, 2012). However, it is also constitutively recycled in its inactive form via a different pathway common to other plasma membrane proteins such as PIN-FORMED (PINs); it cycles from the plasma membrane to early endosomes and this is independent of its co-receptor (Beck *et al.*, 2012).

1.7 Confocal, TIRF and fluorescent proteins

Several of the studies discussed in this thesis and many of the proteins, structures and processes were investigated using microscopy, in particular, live-cell microscopy. The two types of microscopes used in this study were confocal laser scanning and Total Internal Reflection Fluorescence (TIRF).

1.7.1.1 *Confocal laser scanning microscopy*

Confocal microscopes have a pinhole in the plane of the image that is placed ‘confocally’ (having the same foci) to the area illuminated on the specimen which stops any signal from out-of-focus objects reaching the detector (Pawley, 2006). The size of the pinhole is controlled by the user; a smaller aperture means that more photons (particles of light rather than electromagnetic waves) are excluded so the resolution is increased (Pawley, 2006). In particular, photons that originate far from the focal plane in the ‘z’ dimension are removed (Pawley, 2006). However, a reduction in the number of photons reaching the detector also means a reduction in signal generally (Pawley, 2006). To counteract this the exposure time can be increased and in addition a sensitive detector can be used. In the confocal microscopes used in this study, the signal was detected by photomultiplier tubes (PMTs). When a photon strikes a pixel ‘wall’ in the PMT an electronic signal is created that is amplified (this is the ‘multiplication’ step in a photomultiplier tube and it is controlled by the gain settings selected by the user) finally the electrons exit the detector as digital signal (Pawley, 2006).

Confocal microscopes have excellent ‘xy’ resolution and good axial (‘z’-axis) resolution and can scan in four dimensions (‘x’, ‘y’, ‘z’ and ‘t’) (Pawley, 2006). They can image relatively quickly in time, so that it is possible to capture relatively rapid cellular changes. In this study all signal was produced by fluorescent proteins that were imaged using lasers (light amplification by stimulated emission of radiation) which excite fluorophores at specific wavelengths in a very defined space. In this study confocal microscopy was used to image the dynamics of the actin cytoskeleton and Golgi bodies in mature epidermal leaf pavement cells of *A. thaliana*.

1.7.1.2 *Total internal reflection fluorescence microscopy*

TIRF microscopes work on a very different principle to confocal microscopes. The laser is emitted through and accepted by the objective lens; when it reaches a boundary between two objects with different refractive indices it can be reflected off the object with the higher refractive index if the angle of incidence is correct (Martin-Fernandez *et al.*, 2013). The ‘critical angle of incidence’ is the angle of incidence from when the beam of light is first totally internally reflected (Konopka and Bednarek, 2008). If the angle of incidence is greater than the ‘critical angle of incidence’ it is reflected back into the object with the higher refractive index (usually the coverslip); this is known as total internal reflection

(Martin-Fernandez *et al.*, 2013). Total internal reflection produces an evanescent wave (electromagnetic field) which decays exponentially so that only fluorophores within 400 nm of the boundary are excited (see Section 1.7.1.3 for VAEM) (Konopka and Bednarek, 2008).

It was hypothesised that the heterogeneity and thickness of the plant cell wall would prevent accurate TIRF; however, that has been shown not to be the case. Vizcay-Barrena *et al.* (2011) showed that it is possible to image fluorescent proteins in plants cells, not just at the plasma membrane, but also in the cytoplasm and even in the tonoplast.

1.7.1.3 Variable-angle epifluorescence microscopy

Variable-angle epi-fluorescence microscopes (VAEM) work on a similar principle to TIRF microscopes. However, during TIRF the laser is above the ‘critical angle of incidence’ and is totally reflected internally, causing only a very limited layer of the sample to be illuminated (Konopka and Bednarek, 2008). During VAEM, the angle of the laser is sub-critical, causing a variable depth of sample to be illuminated because the laser is slanted through the sample and no evanescent field is produced (Vizcay-Barrena *et al.*, 2011). When the laser is angled obliquely the field of illumination is narrow; this can give excellent contrast in parts of the field of view, as is seen in TIRF (Konopka and Bednarek, 2008). Many studies have taken advantage of this technology to investigate the movements of single actin filaments (Staiger *et al.*, 2009, Smertenko *et al.*, 2010, Henty-Ridilla *et al.*, 2014, Cao *et al.*, 2016).

1.7.1.4 Single molecule imaging

TIRF microscopes can be used to image and track single molecules tagged with fluorescent proteins in living plant cells, because of their high signal-to-noise ratio (Vizcay-Barrena *et al.*, 2011). This is done using several controls, such as the size of the fluorescing dot, whether they ‘blink’ as fluorescent proteins are expected to and whether they bleach, as autofluorescence does not bleach (Vizcay-Barrena *et al.*, 2011).

1.8 Aims

The aim of the work described in this thesis was to determine whether the cell wall has an influence over the remodelling of the actin cytoskeleton and mobility of plasma membrane-resident proteins in *A. thaliana*. To establish this, the following were carried out:

1. Develop image analysis methods to measure the dynamics of the actin cytoskeleton in a manner that is appropriate for the kind of data collected during the study period, i.e. to quantify the bulk remodelling of the actin cytoskeleton.
2. Use the tools developed in the first aim to measure changes in the organisation and dynamics of the actin cytoskeleton when the cell wall-plasma membrane interface is disturbed via plasmolysis, using isoxaben treatment or when the cell wall is removed entirely. The tools were also used to explore the possibility that any changes seen in the actin cytoskeleton during the conditions mentioned above are part of a generalised abiotic stress response.
3. Investigate the dynamics of plasma membrane-resident proteins in wild type and an *A. thaliana* line lacking detectable xyloglucan. To use single molecule tracking of plasma membrane proteins to investigate changes in mobility when the cell wall is perturbed by enzymes that digest specific carbohydrates within the cell wall.

Chapter Two

Methods

2.1 Genotyping *A. thaliana*

2.1.1 Polymerase Chain Reaction

Genotyping PCRs were carried out using the primers listed in Table 2.1, the reagents detailed in Table 2.2 and the conditions in Table 2.3. A negative control containing a commensurate amount of nuclease-free water instead of the template DNA was included in every reaction. The reactions were carried out using a BioRad Thermal Cycler [BioRad, Hemel Hempstead, UK]. The gels from these PCR reactions are shown in Appendix Figure D.1, pg. 287.

Table 2.1 Primers for PCR reactions.

Genes/primers	Forward	Reverse
xxt1	GAAATCTCGAGACCGGACT AATAAACCT	ATCCCCCAATAACCGTGCAA GTAATAAC
xxt2	CCAAAGAGCTTTACGCCAA T	CGCTTGTTAGGTCCGATGAA
LB SALK	ATTTTGCCGATTTCGGAAC	-
AtFH1	GTCTCCGTCACGTGCGTTA GC	TTGTTGTTTAACGACTTCGC C

* AtFH1 are from Rosero *et al.* (2013).

**All primers from Invitrogen [Thermo Fisher Scientific Inc., Massachusetts, US].

Table 2.2 PCR reagents.

Reagents	25 µl reaction
Template DNA	*1 to 5 µl
10 mM Forward primer	0.5 µl
10 mM Reverse primer	0.5 µl
10 mM dNTPs (NEB)	0.5 µl
5x Buffer (NEB)	5 µl
Taq polymerase (NEB)	0.125 µl
Nuclease-free water	Enough to make 25 µl in total

*NEB [NEB, Massachusetts, US]

Table 2.3 Genotyping PCR conditions.

Process	Temperature	Duration	Additional information
1) Initial denaturation	95 °C	30 s	
2) Denaturation	95 °C	20 s	
3) Annealing	46 °C	30 s	
4) Extension	68 °C	30 s	Go back to 2) and repeat steps 2) to 4) 30 times
5) Final extension	68 °C	5 min	
6) Hold	16 °C	Indefinitely	

2.1.2 Agarose gel electrophoresis

A 1% gel was made using molecular grade agarose [Melrose Laboratories Ltd., Ipswich, UK] and 1x TAE buffer (TAE: 40 mM Tris, 20 mM acetic acid and 1 mM EDTA; [AppliChem GmbH, Darmstadt, Germany]). The agarose was melted then cooled to 50 °C to allow the addition of 0.625 µg/ml of ethidium bromide [Thermo Fisher Scientific Inc.] and poured into gel running moulds with the correct sized combs for the amount of PCR reaction to be run. When the gel was set, the combs were removed and the gel covered in 1% TAE. All PCR reactions were done either using a coloured buffer or 6x DNA loading dye (15% Ficoll [Sigma-Aldrich Company Ltd., Missouri, US] and 0.25% w/v Bromophenol blue [Sigma-Aldrich Company Ltd.]). In addition a 1 kbp DNA ladder was always used [NEB]. The voltage was set to 95V and the gel was run for about 30 min. The gels were visualised using a UV transilluminator [Ultra-Violet Products Ltd.,

Cambridge, UK] and a picture taken using a Uvisave gel documentation camera [UVIttec Ltd., Cambridge, UK].

2.2 Bacteria

Bacteria were grown as described in Section 2.3.2.

2.2.1 LB media

LB media was made using 2.5 g LB with agar included [Melfords Laboratories Ltd., Suffolk, UK] made up to 100 ml with dH₂O, then autoclaved.

2.2.2 Antibiotics

Antibiotics were only added to media that had a temperature cooler than 50°C.

Table 2.4 Antibiotics used during this study.

Antibiotic			Stock (µg/ml)	Company
BASTA	(Glufosinate ammonium)	-	10	Sigma-Aldrich Company Ltd.
Hygromycin B			50	Invitrogen [Thermo Fisher Scientific Inc.]
Kanamycin			100	Sigma-Aldrich Company Ltd.
Spectinomycin			50	Melfords Laboratories Ltd.

2.3 Plants

2.3.1 Growth of *A. thaliana*

2.3.1.1 *Seeds grown on agar*

Murashige and Skoog media (MS; 400 ml) was made using 0.88 g Murashige and Skoog [Duchefa Biochemie; Haarlem, The Netherlands]; 4 ml 0.05% MES pH 5.7 (4-Morpholine-ethanesulfonic acid sodium salt; [Sigma-Aldrich Company Ltd.]); 2.4 g Phytigel [Duchefa]; made up to 400 ml with de-ionised dH₂O and autoclaved.

The liquid MS media was poured into sterile Petri dishes of different sizes depending on the experiment. If antibiotics were necessary they were added when the media had cooled to below 50°C (Table 2.4).

To lightly sterilise seeds of *A. thaliana* a dilute bleach solution was used and seeds were incubated for approximately 3 min (10% NaClO [Acros Organics, Geel, Belgium] and 100 µl 10% Tween 20 [AppliChem GmbH] per 50 ml) and then washed three times in sterile dH₂O and kept in water or once in 70% EtOH for 3 min [Sigma-Aldrich Company Ltd.] and dried on sterile filter paper. If immersed in liquid, they were sown onto MS-agar containing plates using a P10 pipette and if stored dry they were sown using a sterile toothpick. The seeds were then stratified in the fridge for at least two and no more than six days before being placed in a plant growth chamber [Panasonic, Osaka, Japan] with the conditions set to: 18h light - 6h dark at 22 °C.

2.3.1.2 *Seeds grown on soil*

See Section above for sterilisation and vernalisation protocol. A 24-module tray was filled with Levingtons F2 Seed and Modular Compost [Scotts Miracle-Gro, Marysville, Ohio, USA] that had been pre-treated with 0.2 g/L Intercept 70WG [Crop Care, Murarrie, Australia] according to the instructions of the manufacturers. The seeds were sown directly onto the surface of the compost and allowed to germinate in light on the top shelf in the greenhouse set to: 18h light - 6h dark at 22°C; [CambridgeHOK, East Yorkshire, UK]). The plants were then grown to the age required.

2.3.2 Stable transformation of *A. thaliana*

The day before dipping, 100 ml cultures of the appropriate *A. tumefaciens* cultures were inoculated and left shaking at 28°C with the correct antibiotics in LB (Table 2.4 and Section 2.2.1). After approximately 24 h of growth the cultures were spun down at 4000 rpm [Eppendorf, Hamburg, Germany] for 20 min in 50 ml Falcon tubes. The pellets were re-suspended in a 5% sucrose [Sigma-Aldrich Company Ltd.] solution containing 50 µl Silwett L-77 [Lehle Seeds, Texas, US] per 100 ml and the bacterial mix made up to 50 ml with the sucrose solution in a 50 ml Falcon tube.

The plants to be dipped were organised and labelled and the stems carefully but firmly twined together. The inflorescences were then dipped into the correct bacteria culture in the tube for approximately 20 s with agitation. The plants were then laid on their side in a tray and covered and sealed with cling film. The next day the cling film was removed and the day after that the plants were stood upright. See Section below for seed collection and selecting from floral dips.

2.3.3 Selection of stable lines of *A. thaliana*

Seeds were collected from floral-dipped parent plants (Section above) and then sterilised if they were to be sown on agar, but not if they were to be sown on silicon dioxide sand (Section 2.3.1.1) for sterilisation and vernalisation protocol. If on agar (Section 2.3.1.1) the appropriate antibiotics were added at the correct temperature and when the plate was set, the seeds were either pipetted or sprinkled onto the surface. 20 ml silicon dioxide [Sigma-Aldrich Company Ltd.] was poured into a 9 cm Petri dish and saturated with 10 ml of solution containing ¼ MS and the appropriate antibiotics. If necessary, the silicon dioxide was repeatedly saturated with the same solution until all the seedlings had died or been removed. When a seedling was obviously resistant to the selecting antibiotic it was carefully removed from its position and placed gently into soil (Section 2.3.1.2). All seedlings were grown as normal except that after two or three weeks they were checked for fluorescence under the confocal microscope (Section 2.5). The seeds were collected and stored at room temperature in paper bags.

2.4 Protoplasts from *A. thaliana*

The protocol used in this study is based on Yoo *et al.* (2007) with adaptations suggested by Dr Mike Deeks, a Lecturer in Plant Biology at the Department of Biosciences in the University of Exeter (Exeter, UK).

2.4.1 Growth of *A. thaliana*

Seeds of fluorescently tagged *A. thaliana* were sown in a 24-module tray (Section 2.3.1.2) and grown in Sanyo plant incubators for one week (settings: 16h light - 8h dark, 22 °C), then moved to the top shelf of greenhouse and used when 3-4 weeks old.

2.4.2 Reagents for protoplasting

Reagents for protoplasting were made as follows: 0.2 M MES pH5.7 (4-Morpholineethanesulfonic acid sodium salt [Sigma-Aldrich Company Ltd.]; 0.8 M Mannitol [Sigma-Aldrich Company Ltd.]; 1 M CaCl₂ [Thermo Fisher Scientific Inc]; 2 M KCl [Sigma-Aldrich Company Ltd.]; 10% BSA [Sigma-Aldrich Company Ltd.]). These solutions were filter sterilised using 0.45 µm filters and 10 ml syringes.

2.4.3 Protoplasting procedure

Before use, the MES was heated to 70°C for 3 min and allowed to cool. Then MES, mannitol and 1.5% Cellulase ‘Onozuka’ R-10 [from *Trichoderma viride*; Yakult Pharmaceutical Industry Co., Ltd., Tokyo, Japan] and 0.4% Macerozyme R-10 [from *Rhizopus* sp.; Yakult Pharmaceutical Industry Co., Ltd.] were added to a 20 ml Sterilin tube. The enzyme solution was incubated at 55 °C for 10 min then cooled to room temperature and CaCl₂ and BSA were added before being made up to volume with sterile deionised water (the final volume used was 10 ml per condition). The media was then filter sterilised with 0.45 µm filters and 10 ml syringes. If necessary, isoxaben was added prior to filtration and 10 ml of the enzyme solution poured into 9 cm Petri dishes.

The rosette leaves 5, 6, 7 and 8 from 7 – 8 mature *A. thaliana* plants were used for protoplasting. Each leaf was quickly washed in a dilute bleach solution (1:5; NaClO) and then rinsed in sterile deionised water and allowed to dry. A sharp razor blade was used (and changed frequently) to cut 0.5 – 1 mm strips either side of the midrib. Each side of the leaf was quickly submerged in the enzyme solution and left abaxial side down. The leaves were then vacuum infiltrated twice and left at room temperature in the dark for approximately 3 hs.

The W5 wash solution and W1 overnight solution were made as follows:

W5 was made using: 2 mM MES (pH5.7); 154 mM NaCl [Thermo Fisher Scientific Inc.]; 125 mM CaCl₂; and 5 mM KCl. W1 was made using: 4 mM MES (pH5.7); 0.5 M Mannitol; and 20 mM KCl.

The protoplast mix was diluted with an equal volume of W5 and the mesh to be used was also wetted with W5. The protoplast mix was carefully filtered through the mesh into a 9 cm Petri dish and then pipetted into a 50 ml Falcon tubes – either with a Pasteur pipette or a sterile 1 ml pipette tip with the end removed to avoid bursting the protoplasts.

The protoplast mix was allowed to settle and then approximately 750 µl of the protoplasts were removed (again, used Pasteur pipette or a sterile 1 ml pipette tip with the end removed) into an Eppendorf tube and 750 µl W1 solution was added to this tube. The protoplasts were allowed to settle again and then either used for imaging (one drop on a glass bottomed dish [Ibidi, Planegg, Germany]) or 1 ml was left overnight at room temperature in the light in a 6 well culture plate for imaging the next day [CellStar; Greiner, Kremsmünster, Germany].

2.5 Confocal microscopy

An inverted Zeiss LSM 510, Zeiss LSM 510 Meta and a Zeiss LSM 880 [Zeiss, Jena, Germany] (Zeiss microscopes: Axio-Vert, Axio Imager Z.1 and Axio Observer Z.1, respectively) were used to image green fluorescent protein (GFP) and yellow fluorescent protein (YFP) labelling of various proteins with the 63x oil immersion objective (all

Numerical Apertures 1.4). In all cases the oil added to the coverslip was Immersol 518 F [Zeiss]. The coverslip was attached to the glass slide using double-sided sticky tape [Hobbycraft, Dorset, UK] to create a rectangular chamber in which the leaf section or seedling could be placed.

When leaf cells were imaged, a 0.5 cm² section was excised (for treatments see Sections 2.6) and placed on a 0.8 – 1 mm glass slide abaxial side up. A water based solution was applied to the section or to a 22 x 50 mm coverslip and the coverslip placed on the leaf section. When imaging hypocotyls, whole seedlings were gently removed from the solution and incubated in ½ MS and placed on a glass slide. Then 80 µl of the solution that they were incubated in was added to the chamber on the slide and the coverslip very gently placed on top to create a seal.

Table 2.5 Settings used to image fluorescent proteins on three confocal microscopes used during this study.

The LP 650 channel is to remove autofluorescence.

Fluorescent protein	Excitation	Emission	Beam splitter	Microscope
GFP	488 nm	BP 505-550 LP 650	HFT 488/543; NFT 635 VIS	Inverted Zeiss 510
GFP	488 nm	BP 505-550	HFT 405/488/543/633; NFT 635 VIS; NFT 490	Zeiss 510 Meta
YFP	514 nm	BP 530 - 600	HFT 458/514; NFT 515	Zeiss 510 Meta
YFP	514 nm	530 - 600	488/561	Zeiss 880
GFP	488 nm	490 - 550	488/561	Zeiss 880

2.6 Image Analysis

2.6.1 Dynamics assay

Individual cells were imaged using a zoom of two and then a rectangular ROI of 200 by 150 pixels. A Z-stack of 4 μm was taken with 1 μm intervals between slices, thus giving five slices in total. This was repeated twenty times with no delay between stacks so that the time series lasted approximately 105 s (approximately 5.5 s between each time-frame). Z-stacks were taken so that a greater amount of network was in the field of view. Cells that stably-expressed a fluorescent Golgi body marker were imaged differently. The same ROI was used, but no Z-stack and 200 time-frames with no delay; the time series lasted approximately 90 s (approximately 0.46 s between each time-frame). The gain was decided on a sample by sample basis and the zoom level was always two.

2.6.2 Plasmolysis assay

The same type of leaves as were used for protoplasts (Section 2.4) were used for plasmolysis experiments: rosette leaves 5, 6, 7 and 8. Pieces of leaf were cut with a clean razor into sections of approximately 0.5 cm^2 and incubated in 0.6 M mannitol [Sigma-Aldrich Company Ltd.] for 30 min. The abaxial side of the leaves were imaged and only epidermal pavement leaf cells used. Control sections were viewed directly after excision from leaves and were mounted in dH_2O . After incubation, half the pieces submerged in 0.6 M mannitol were mounted in 0.6 M mannitol and imaged and the other half were rinsed in dH_2O and then incubated in dH_2O for another 30 min before they too were mounted in dH_2O and imaged. See Table 2.5 for imaging settings.

2.6.3 Temperature assay

The same type of leaves were used as for the plasmolysis experiments, that is the rosette leaves 5, 6, 7 and 8. Pieces of leaf were cut with a clean razor into sections of approximately 0.5 cm^2 and incubated in $\frac{1}{2}$ MS plus MES for 30 min or 1 h. They were

incubated at room temperature (approximately 22°C) or in a water bath at 37°C or in a fridge at 4°C.

2.6.4 Isoxaben assay

Leaves were used as described above (Section 2.6.3). Pieces of leaf were cut with a clean razor into sections of approximately 0.5 cm² and incubated in ½ MS plus MES for 1 h or 4 hs with the addition of either 20 µM isoxaben in 0.01% DMSO [Fluka, Sigma-Aldrich Company Ltd.] or 0.01% dimethyl sulfoxide [DMSO; Sigma-Aldrich Company Ltd.].

2.6.5 Fluorescence Recovery After Photobleaching

Fluorescence Recovery After Photobleaching (FRAP) is a way to quantify protein mobility. In this study two microscopes were used for FRAP (Table 2.1) with two different protocols. The Zeiss 510 Meta was used with a protocol from Martinière *et al.* (2011a) which specified the 63x objective, a zoom level of 5 and two regions of interest (ROI) with a radius of 2.4 µm. The scanning laser was set at very low intensities (either 488 nm or 514 nm set between 3 – 7% depending on the fluorescence level) and one ROI was bleached with the 405 nm laser set at 100% for 10 – 20 iterations. The fluorescence intensity was then measured every 1.5 s for a total of 60 s: the bleaching was between scan five and six. However, in many time-series the sample started to drift in the last 20 s, so in the subsequent analysis the last 20 s were excluded and only the first 40 s considered.

The Zeiss 880 microscope was used with the 63x objective, a zoom level of 2 and three regions of interest (ROI). The first ROI was a rectangle of 200 by 150 to limit scanning time, the other two ROIs were circles with a radius of 25 pixels. The second ROI was where the fluorescence was bleached and the recovery monitored, while the third was a control to check for unintended bleaching and drift. The scanning laser was set very low (488 nm set between 1 – 2% depending on the fluorescence level) and the bleach ROI was bleached with the 488 nm laser set at 100% for 10 – 20 iterations. The bleach was between scan 30 and 31. The fluorescence intensity was then measured every 0.06 s for approximately 17 s.

The fluorescence intensity (from the second ROI) was normalised for each cell using Microsoft Excel (Microsoft, Redmond, Washington, US) using the average of the pre-bleach scans and setting the first post-bleach scan as 0%. The formula used was:

$$= \text{Sum of } ((\text{time frame} - \text{first post} - \text{bleach scan}) \div (\text{pre} - \text{bleach average} - \text{first post} - \text{bleach scan}) \times 100)$$

These values were pasted into GraphPad Prism 5 [GraphPad Software Inc.] and nonlinear regression with a one-phase association was used to fit a global curve to the dataset. This method of analysis also produces an R^2 value which states how appropriate the global curve is in describing the dataset: the higher the value, the better the fit. It also provides plateau and half-time values for every cell imaged. These values were used for statistical analysis. An F-test was performed to establish whether the variances were significantly different or not. If they were, then an unpaired t-test with Welch's correction was used, if they were not, then an unpaired t-test was used.

2.6.6 Actin spatio-temporal dynamics analysis

Experimental set-up and imaging conditions were described in Sections 2.6.1 and Table 2.5 for. The 3D movies were analysed using ImageJ [NIH; (Rasband, 1997-2016)] and the values computed using Excel [Microsoft, Washington, US]. The methods are detailed in Chapter 3 (Section 3.4.3.1 pg. 60 and Section 3.4.3.3). The plug-in for the *Cumulative fluorescence intensity* analysis will be published separately in a peer-reviewed journal in collaboration with Dr Axel Poulet (Emory University, Atlanta, US) who led in the development of the plug-in.

2.6.7 Volocity analysis

Volocity [PerkinElmer; Massachusetts, US] was used to track the movements of Golgi bodies over time and measure the amount and type of movement. The protocol used had to be altered for each movie as each cell had different levels of fluorescence and Golgi body distribution. The commands used were: 'Find Objects' with fluorescence identified using the percentage function, as bleaching may occur with levels from 100% to 13-35%;

‘Separate Objects’ with objects usually defined as $1\ \mu\text{m}^3$; ‘Exclude Objects’ set much lower, often as $0.1\ \mu\text{m}^3$; and lastly, ‘Track Objects’ with a limiting movement distance set at $1\ \mu\text{m}$. The measurements collected were organised in Excel and analysed using GraphPad [GraphPad Software Inc., California, US]. Statistical analysis was carried out using either Kruskal-Wallis or unpaired t-tests with Welch’s correction.

2.7 Statistics and Graphs

2.7.1 Box plots

Box plots were used to show the distribution of the data for many of the experiments in this study. In all cases, they represent as follows:

The top of the box is the upper quartile, beneath this line lie three quarters of the data points, the line within the box represents the median value (the data point that is situated exactly in the middle of the ranked data points) and the bottom of the box is the lower quartile, below which lies one quarter of the data points in the sample. The distance between the median and the upper quartile is the upper quartile range containing one quarter of the data points and the distance between the median and the lower quartile is the lower quartile range, also containing one quarter of the data points. The Tukey whiskers represent, 1.5 times the lower interquartile range (lower whisker) and 1.5 times the upper interquartile range (upper whisker). Any points outside the whiskers are represented as dots and can be considered outliers.

2.7.2 Statistical tests

Several statistical tests were employed in this study, all were carried out in GraphPad Prism 5 [GraphPad Software Inc.], although the data was organised prior to analysis in Microsoft Excel [Microsoft]. For datasets which appeared to have very different variances, the t-test with Welch’s correction was used. For datasets which appeared to have similar variances, but did not appear to follow a Gaussian distribution (according to the histogram), the Kruskal-Wallis test was used as it is a non-parametric test. For all other

datasets a one-way ANOVA was used if there were three or more groups to compare, or a t-test if there were just two groups.

2.8 Total Internal Reflection Fluorescence (TIRF) single molecule imaging at Harwell

Five day old *A. thaliana* seedlings were used for single molecule Total Internal Reflection Fluorescence (TIRF) imaging at the Central Laser Facility within the Rutherford Appleton Laboratory in Harwell. The TIRF microscope was a Zeiss Observer Z1 [Zeiss] that was custom-adapted and equipped with a 100x objective (100x AlphaPInApo lens NA=1.46). Only GFP-tagged plants were used, so the fluorophores were excited with an Omicron 488 nm laser, a HQ 525/550 nm filter [Chroma Technology Corporation, Vermont, US], and an electron-multiplication CCD [iXon; Andor, Belfast, NI]. The stage was temperature controlled and kept at 22°C. All samples used had low expression for accurate molecule tracking.

First, control data was collected using fluorescent beads followed by biased and dark images. Image collection was done with the laser at 10% power and an image taken every 0.05 s with the gain at 300 or less. Three proteins were imaged, one of them (paGFP-LTI6b) required bleaching with the laser at 100% power for 10 s prior to image collection. Each cell was imaged for 90 s, thus collecting 1200 images. Three seedlings were imaged for each condition, with four cells imaged per seedling. The seedlings were incubated for 1 h before imaging in either ½ MS, or ½ MS containing 1.5% cellulase (Section 2.4.2), or 1.5% pectinase [Sigma-Aldrich Company Ltd.].

2.8.1 TIRF single molecule image analysis

The data collected was prepared by Dr Dan Rolfe, a team leader within OCTOPUS at CLF at the Rutherford Appleton Laboratory in Harwell. The data was analysed using a programme that Dr Dan Rolfe created and accessed through a Virtual Private Network (VPN) that he manages. The dataset to be analysed was selected and then opened by the

program. All the tracks of the single molecules were made visible and then the outline of the cell was traced, but well within its boundaries so that only tracks on the side of the cell facing the coverslip were included. Once this had been done for all the datasets the analysis was completed by Dr Rolfe and Mean Square Displacement curves generated (daniel.rolfe@stfc.ac.uk). The custom-written program takes into account both photobleaching of fluorescent molecules and their blinking profiles in order to ensure that single fluorescent proteins are tracked individually and so that the tracks of two proteins are not conflated (Rolfe *et al.*, 2011).

Chapter Three

Image analysis tools for the quantification of the spatio-temporal dynamics of the actin cytoskeleton

3.1 Introduction

This study aimed to measure bulk remodelling of the cortical actin cytoskeleton, so that rather than tracking the movement of individual filaments, the alterations within a section of the cell were recorded and analysed. Bulk remodelling is a very broad term that includes network changes such as polymerisation, depolymerisation, severing, annealing and the forming and breaking of junctions. This focus on bulk remodelling is so that the amount of change in a relatively large piece of network can be quantified rather than single filament dynamics and without discriminating between different kinds of change. It describes alterations within the network: it is not a description of whether a strand has moved back and forth in a limited area. The swaying of a strand is movement, but not a change in the network, whereas bulk remodelling is a measure of wholesale change within the actin network (Figure 3.1, pg. 65). Rather than separately analysing network changing events, this study makes no distinction between them and instead focusses on bulk remodelling to provide a global understanding of actin dynamics.

No previously developed and published methods were found to be appropriate for this study to measure bulk remodelling. In general, they required excellent spatial and

temporal resolution (as is achieved using Total Internal Reflection Fluorescence microscopy (TIRF), which was not available for the majority of this study (Staiger *et al.*, 2009, Smertenko *et al.*, 2010). Some were developed to analyse the much less dynamic microtubule cytoskeleton (Komis *et al.*, 2014), while many required complex mathematical, computational or biophysical techniques (Breuer *et al.*, 2014, Kimori *et al.*, 2015). In addition, several of the experiments undertaken during this study used specimens with low levels of fluorescence intensity (mature tissue often seemed to have lower levels of fluorescence), which precluded the use of many published methods. Lastly, some of the published studies were developed to track single actin filaments whilst one of the aims of this study was to quantify bulk actin dynamics on a cellular scale.

The following sections introduce, with more detail, the studies that influenced the development of the image analysis tools described in this chapter:

Lindeboom *et al.* (2013) developed several image analysis tools, one of which they termed “time-phased walking subtraction” which is similar to the initial step of the first analysis method described in this chapter (Section 3.4.3.1, pg. 60). They subtracted time-frames that were three frames apart (the phase shift) and selected the positive and negative differences for growth and shrinkage, respectively (Lindeboom *et al.*, 2013). They used it to track the growing and shrinking ends of microtubules as part of an investigation into microtubule array reorganisation (Lindeboom *et al.*, 2013).

van der Honing *et al.* (2011) used a macro for ImageJ developed by Van Bruaene *et al.* (2004), (Rasband, 1997-2016) that merges two time-frames (one red and green) and the filaments that are static are coloured yellow. Van Bruaene *et al.* (2004) developed it to measure microtubules, but van der Honing *et al.* (2011) adapted it for the actin cytoskeleton in a way that is very similar to the first analysis method described in this chapter (Section 3.4.3.1, pg. 60). van der Honing *et al.* (2011) used it to determine the percentage of actin bundle relocation within 5 s to compare the organisation of the actin cytoskeleton when using different markers. Nick *et al.* (2009) measured fluorescence differences between two time-frames: specifically, the fluorescence present in time-frame one but absent from that same location in time-frame two and the fluorescence present in time-frame two that is absent from that same location in time-frame one. This is similar to Van Bruaene *et al.* (2004) and van der Honing *et al.* (2011) but was not chosen in this

study because it is possible to measure the same filament twice, once in time-frame one in its first position and again in time-frame two if it has moved into an area devoid of fluorescent signal in time-frame one (although it is possible to extract positional information).

Higaki *et al.* (2010) developed several image analysis tools to evaluate the structure of the actin cytoskeleton in stomatal cells in *A. thaliana*. However, their workflow begins by skeletonising the actin network, in which case it is necessary to have well defined filaments, otherwise the skeletonisation step does not produce an accurate representation of the network (Higaki *et al.*, 2010). Staiger *et al.* (2009) used Variable Angle Epi-fluorescence Microscopy (VAEM; see Section 1.7.1.3, pg. 38) to collect a series of metrics, including elongation rate, severing frequency and the filament origin point. They also developed a measurement of ‘convolutedness’ to describe the shape made by the filament: the ratio of filament length to the longest length of bounding rectangle that is defined by said filament (Staiger *et al.*, 2009).

Many studies have also used Fluorescence Recovery After Photobleaching (FRAP; see Section 2.6.5, pg. 49) to good effect in studying the actin cytoskeleton, in both plant and animal cells: for example, FRAP has been used to study the binding kinetics of different cytoskeleton markers. This was informative in those studies as they were investigating the characteristics of two actin marker proteins (van der Honing *et al.*, 2011). This study was not measuring binding kinetics, but rather the amount of change in the network, so FRAP was not used in this chapter or Chapters 4 and 5 (Figure 3.1, pg. 65).

The methods developed in this chapter to measure the bulk remodelling of the actin cytoskeleton in this study were: *Pairwise image subtraction* and *Cumulative fluorescence intensity*. These are described in detail in Sections 3.4.3.1 (pg. 60) and 3.4.3.3 (pg. 63).

3.2 Collaborators

The *Cumulative fluorescence intensity* method was developed in collaboration with, but written by, Dr Axel Poulet at Oxford Brookes University (Oxford, UK), specifically for this study.

3.3 Aims

This chapter details the development of methods to quantify bulk remodelling of the actin cytoskeleton on a cellular scale. The aim was to develop methodologies to quantify the spatio-temporal dynamics of the actin cytoskeleton in *A. thaliana* by:

- a) Development of a method for measuring bulk remodelling of the actin cytoskeleton using the ‘subtract’ function in the ‘Image Calculator’ of ImageJ (Rasband, 1997-2016).
- b) Collaborate with a bioinformatician to create a new method of measuring bulk remodelling of the actin cytoskeleton using ImageJ.

3.4 Methods

3.4.1 Markers to visualise the actin cytoskeleton and its dynamics

Two of the most commonly used fluorescent protein markers for the actin cytoskeleton in *A. thaliana* are the actin binding domains in fimbrin and Lifeact. There are two binding domains in *A. thaliana* the fimbrin AtFIM1 (in all fimbrins), each approximately 27 amino acids in length (Fimbrin Acting Binding Domain (FABD) and Fimbrin Acting Binding Domain 2 (FABD2)) (Kovar *et al.*, 2000, Sheahan *et al.*, 2004, Ketelaar *et al.*, 2004). The second binding domain seems to be more conserved than the first (Kovar *et al.*, 2000). There is the possibility that fluorescently tagged AtFIM1 stabilises the network (Kovar *et al.*, 2000). If cytoplasmic streaming is measured, the organelles in GFP-FABD2-tagged lines move 20% slower than those of wild-type plants, although different expression levels were not compared in that study (Holweg, 2007).

Lifeact is the first 17 amino acids from ABP140, an actin-binding protein from *Saccharomyces cerevisiae*, which is only found in this yeast and close relatives (Riedl *et al.*, 2008). van der Honing *et al.* (2011) used three different *A. thaliana* containing Lifeact-Venus lines with varying amounts of fluorescent protein expression. They found that Lifeact labelled the fine, very dynamic filamentous actin in the root hair subapical region (van der Honing *et al.*, 2011). They also found that reorganisation was reduced in cells that expressed Lifeact-Venus, compared to those labelled with GFP-FABD2 (van der Honing *et al.*, 2011). Rocchetti *et al.* (2014) showed that transiently expressing Lifeact-GFP (in *N. tabacum* leaves) in conjunction with a Golgi body marker (ST-GFP) significantly reduced the velocity of the Golgi bodies (also by approximately 20%) and caused them to move in a markedly more directional manner than if the Golgi body marker was expressed alone.

Smertenko *et al.* (2010) used FRAP to show that GFP-Lifeact has a four-fold faster exchange rate of binding filamentous actin compared to GFP-FABD (2.56 ± 0.70 s compared to 13.2 ± 1.3 s as recorded by Sheahan *et al.* (2004)). van der Honing *et al.* (2011) also performed FRAP on GFP-FABD2 and Lifeact-Venus in *A. thaliana* root epidermal cells and found a similar situation: Lifeact-Venus had a half-time of 2.7 ± 1.4 s,

whilst GFP-FABD2 had a half-time of 6.9 ± 2.7 s which was a significant difference. The authors suggest this is due to the lower affinity for actin filaments exhibited by Lifeact (van der Honing *et al.*, 2011).

For this study both GFP-FABD2 and GFP-Lifeact were used: constructs and lines were made by (Ketelaar *et al.*, 2004, Sheahan *et al.*, 2004) and Smertenko *et al.* (2010) respectively (Figure 3.2, pg. 66). Either of these markers may cause a change in actin cytoskeleton morphology or possibly label slightly different populations of filaments (Dyachok *et al.*, 2014). Therefore it was interesting to discover if the actin cytoskeleton behaved in the same way in response to various stimuli when labelled with each marker.

3.4.2 Qualitative description of the spatio-temporal dynamics of the cytoskeleton in *A. thaliana*

The easiest visual method to judge movement is to see several time-frames at once in a way that differentiates filaments that have changed. To this end, three time-points from a time-series were selected and recoloured so that the earliest time-point was red, the middle one green and the last one blue. These can then be overlaid using software such as Adobe Photoshop or ImageJ (Rasband, 1997-2016) and where there has been change between time-points the colours of the filaments will be distinct and visible. Where there has been no change the filaments will appear white because of the superimposition of red, green and blue and intermediate colours (such as purple) if only two frames align (Figure 3.3, pg. 67).

This method, while effective and illustrative is qualitative, not quantitative. Therefore several different methods were developed or co-opted in an effort to assess bulk remodelling of the actin cytoskeleton in a quantifiable manner.

3.4.3 Quantitative description of the spatio-temporal dynamics of the actin cytoskeleton in *A. thaliana*

3.4.3.1 Pairwise image subtraction of GFP-FABD2 marked actin cytoskeleton

Several studies influenced the development of this method, chiefly: Higaki *et al.* (2010), van der Honing *et al.* (2011) and Lindeboom *et al.* (2013). *Pairwise image subtraction* is designed to measure any alterations in the network; Figure 3.4 (pg. 65) shows the difference between a static network and a network that has undergone many alterations.

Image processing was done using ImageJ (Rasband, 1997-2016). All time-series showing the actin cytoskeleton were collected in 3D for this study. The first step was to use 'Maximum intensity projection' to project each 3D time-frame of a 20 frame time-series, resulting in 20 2D images (Step 1; each time-series approximately 105 s long; Figure 3.5, pg. 69). Then the dataset was thresholded to reduce background fluorescence to zero. Due to the wide range of fluorescence intensities between mature plants all thresholding was done manually so that as many background pixels had a value of '0' as possible while minimising the loss of filaments with a fluorescence intensity near zero (Step 2; Figure 3.5, pg. 69). The dataset was cropped spatially so that it was the size of the ROI (Region Of Interest) used when making the images (in this study the ROI was always 200 x 150 pixels so that every image had exactly the same number of pixels: 30,000; Step 3; Figure 3.5).

Then the image was 'filtered' using a median filter of 0.2 pixels in order to further reduce the value of any left-over noise and to enhance any fainter filaments (Step 4; Figure 3.5, pg. 69). A median filter was chosen because the fluorescence intensity values produced are actual values in the image (see Appendix Figure A.1, pg. 249). Median filtration is considered to be quite good at preserving edges which is important when dealing with a filamentous network as it contains many edges (<http://homepages.inf.ed.ac.uk/rbf/HIPR2/median.htm>). Median filters are also effective at getting rid of 'salt and pepper noise' (Rasband, 1997-2016) which would result in erroneously measuring remodelling as present when it is absent. In addition, the filter used was small enough that it should not falsely remove actual fluorescent signal. The amount of change in the network can be easily assessed in a qualitative manner in order to check that this method of analysis is producing meaningful results (Figure 3.6).

An ImageJ macro was written to do the filtering and the subtraction of the resultant images; there are 20 time-frames in each dataset, so 19 comparisons must be made for each time-series (Steps 5 and 6 Figure 3.5; Figure 3.7; Table 3.1). The macro starts with the first two time-frames ('1' and '2') and proceeds sequentially: '2' and '3', '3' and '4' and so on. In each case the first time-point is subtracted from the subsequent one, leaving only the pixels that are in a different location in the second time-point compared to the first (Step 5; Figure 3.5; and Figure 3.7). These pixels are considered to be the part of the network that has remodelled.

If a filament is present and stationary in time-frames one, two and three but disappears before time-frame four, it will be measured as not remodelled between time-frames one and two, and time-frames two and three. As the filament was present in time-frame three, not in time-frame four, this will mean a smaller value of bulk remodelling. However, if the difference is used rather than subtraction then some filaments will be counted twice (see Appendix Figure A.2). The remodelling seen during this study has usually involved the changes to filaments rather than their disappearance. Therefore, for this analysis it is a necessary compromise, however, *Cumulative fluorescence intensity* (Section 3.4.3.3, pg. 63) would deal well with this situation. Lastly, it was not possible to discriminate between the different types of alteration in the network, particularly not polymerisation and depolymerisation as the entire bundle was rarely in view. This means that it is impossible to distinguish between growth/shrinkage or with the movement of the filament either in out of view.

The last step uses the plugin 'Color Pixel Counter' (Rasband, 1997-2016) (http://imagejdocu.tudor.lu/doku.php?id=plugin:color:color_pixel_counter:start): this counts the number of pixels containing fluorescence intensity above a set value. The macro permitted the automation of repetitive steps that do not require any user input and it results in a log window that can be copied straight into a programme such as Excel [Microsoft]. The data is normalised in Excel so the numbers produced can be averaged to produce a mean value for the whole dataset and compared statistically between treatments.

The counting is done for every time-frame as well as the subtracted images so that the subtracted image can be compared to the first time-frame of the pair in order to express the difference (the number of fluorescent pixels left in the subtracted image) as a proportion

for every pair of time-frames (Step 6; Figure 3.5; and Table 3.1, pg. 72). The data is normalised using the equation:

$$\textit{Proportion of bulk remodelling} = \textit{subtracted image} \div \textit{first image}$$

This is the proportion that is used to express the bulk remodelling index. Small output values indicate little network remodelling has taken place between time-points, because it means that the difference between time-frames was a small proportion of the total fluorescence. Larger values imply that a lot of remodelling occurred because the difference will be a greater proportion of the total fluorescence, so more of the filaments were in a different place in the second time-frame of the pair compared to the first.

In this study the value set for pixels to be counted by the plugin ('Color Pixel Counter') (Rasband, 1997-2016) as fluorescent was '1'; as every dataset had been thresholded manually and then filtered it was considered that almost all the visible fluorescence should be information rather than noise. However, to test this assumption one dataset was analysed using a set value of '30' to see if this altered the results. While this procedure did not change the relationships between the different treatments, it did slightly change the statistical results (see Appendix Figure A.3 and Table A.1). This was due to the loss of information resulting from the incidental removal of thin bundles¹ with very low fluorescence intensity.

To test the effect of the median filter, the same dataset was analysed in the same manner, but filtered with a different filter (Gaussian blur of 0.5 pixels in all three dimensions) on the same dataset to see if that altered the results and this time there was no difference in relationship or statistics (see Appendix Figure A.4 and Table 1.1). The Gaussian blur filter is similar to the median filter, but smooths using a Gaussian distribution: the degree of smoothing is based on the standard deviation chosen (sigma; <http://homepages.inf.ed.ac.uk/rbf/HIPR2/median.htm>).

Pairwise image subtraction (using a median filter and counting every pixel with a value of '1' and above) has been used as reported in Chapters 4 and 5 to measure the bulk dynamics

¹ Due to technical reasons, it is not possible to be certain whether filaments are single actin filaments or thin bundles of actin filaments. Therefore, throughout this work, they will be referred to as thin bundles, as this is the most likely scenario.

of GFP-FABD2 marked actin cytoskeleton as these were considered to be the most appropriate parameters for this study.

3.4.3.2 Mean fluorescence intensity using Pairwise image subtraction of GFP-FABD2 marked actin cytoskeleton

To analyse the organisation of the actin cytoskeleton (via changes in intensity between thick and thin fluorescent bundles), the same subtraction method was used as described in the previous section (Steps 1 through 5; Figure 3.5) except there was no filtering step (Step 4; Figure 3.5, pg. 69). The same datasets were used throughout for both *Pairwise image subtraction* and Mean fluorescence intensity from *Pairwise image subtraction*. The plugin used in this analysis ('Calculate Mean' in ImageJ; (Rasband, 1997-2016) (<https://imagej.nih.gov/ij/plugins/calculate-mean.html>)) does not count the total number of pixels at each fluorescence intensity level, but rather calculates the mean of the fluorescent intensities within a given ROI. The data is normalised using the equation:

$$\begin{aligned} & \text{Mean fluorescence intensity of remodelled filaments \%} \\ &= \text{fluorescence intensity (subtracted image} \div \text{first image)} \times 100 \end{aligned}$$

Mean fluorescence intensity percentage was calculated (Step 5; Figure 3.5) in order that different treatments could be compared.

Mean fluorescence intensity from *Pairwise image subtraction* was used as reported in Chapters 4 and 5 to measure the organisation of GFP-FABD2 marked actin cytoskeleton.

3.4.3.3 Cumulative fluorescence intensity of GFP-Lifeact marked actin cytoskeleton

This method was developed in collaboration with Dr Axel Poulet (Oxford Brookes University). The program was written as an ImageJ plugin (Rasband, 1997-2016). Steps 1 through to 4 (Figure 3.5) from Section 3.4.3.1 (pg. 60) were followed for time-series data, then the datasets were converted to 8-bit and processed using the 'batch process' function in the plugin. If a pixel has an intensity value of '1' or more (where '0' is black and '255' is the highest intensity level), then it was assigned a value of '1' while every pixel with an intensity value of '0' keeps that value (Figure 3.8, pg. 73).

At each pixel position the plugin sums the fluorescence for all time-frames within the time-series. This produces a number from ‘0’ to ‘19’ for each pixel location. These numbers are called ‘fluorescence intensity values’: they indicate how many times during the time-series each individual pixel was occupied with fluorescence. The first time-frame with a fluorescence intensity >0 carries no weight since an initial comparison is necessary for the algorithm; however, this also helps in reducing any residual noise which would appear in only a single time-point. Where there has been no change in the network throughout the entire time-series the pixel will carry a value of ‘19’. However, where the network has changed constantly the cumulative value will be lower (Figure 3.9, pg. 74).

For every dataset each pixel location was assigned a value from ‘0’ – ‘19’, from the pixels where no fluorescence was present (‘0’) through to the pixels where fluorescence was present in every time-frame (‘19’; Figure 3.10 and Table 3.2, pg. 76). The total fluorescence was calculated for each time-series and the distribution of fluorescence intensity values was determined. For example, in a network that did not remodel, fluorescence intensity values from the *Cumulative fluorescence intensity* procedure would be either ‘19’ (actin filaments present) or ‘0’ (actin filaments absent). In a network that did remodel, fluorescence intensity values would be distributed throughout the range from ‘0’ to ‘19’. Each fluorescence intensity value was then presented as a proportion of the total fluorescence value to give a representation of the number of pixels for each amount of change. This normalises the data and allows statistical comparisons between different treatments.

One-way Analysis Of Variance (ANOVA) was chosen to compare each fluorescence intensity value for each experiment: this detects differences between the different treatments. For example, the proportion of the total fluorescence that has an intensity value of ‘1’ can be compared between mock and the different treatments. Although the variance may differ between datasets and not all datasets nicely fit a Gaussian curve, it was decided that ANOVA is a robust enough test to cope with these discrepancies. The *Cumulative fluorescence intensity* technique (using a median filter) was used as reported in Chapters 4 and 5 to measure the bulk dynamics of GFP-Lifeact marked actin cytoskeleton.

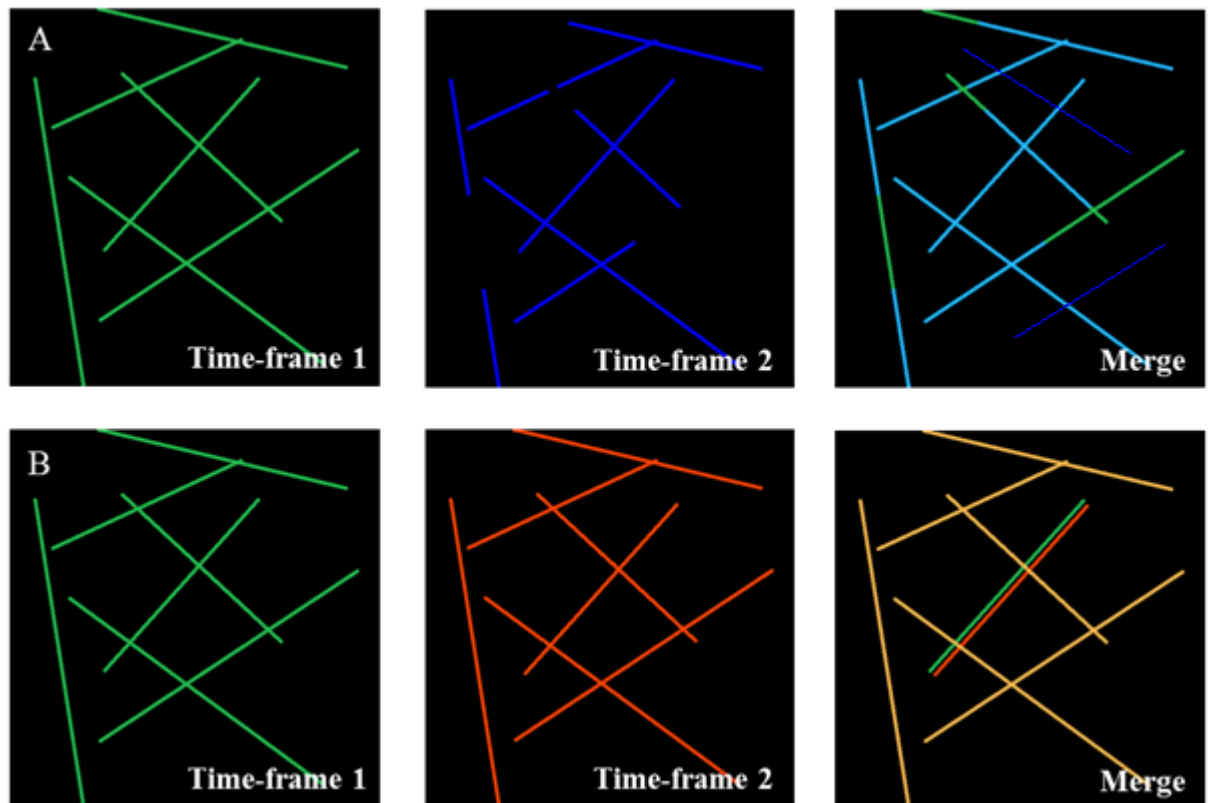


Figure 3.1 Diagram to define the difference between bulk remodelling and simple movement.

Row A: In the merged image when the filaments are light blue the network has remained the same, but where only green is visible the network has altered. In this instance it is clear that severing events, depolymerisation and polymerisation have taken place.

Row B: In the merged image when the filaments are yellow the network has remained the same. In the middle of the image two filaments are not yellow, this is the same filament in both images and it has just shifted slightly in the second time-frame compared to the first. It is clear that the organisation of the network remains the same; therefore this is simple movement, rather than remodelling.

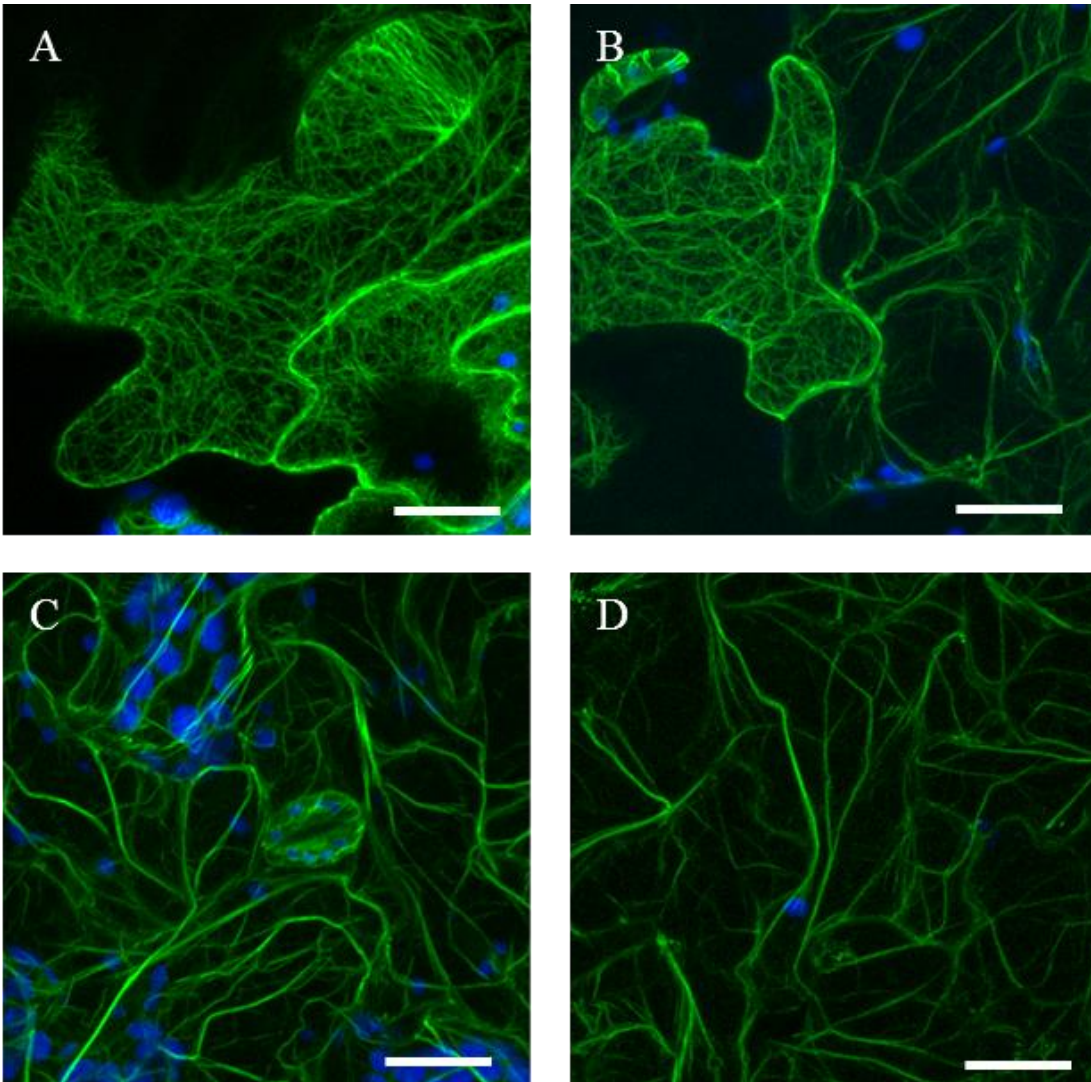


Figure 3.2 Differences in actin cytoskeleton morphologies compared between two live cell markers: GFP-FABD2 (A and B), which usually decorates a dense meshwork and GFP-Lifeact (C and D), which displays many thick bundles.

Abaxial surface of epidermal pavement cells from mature *A. thaliana* leaves that stably-expressed either GFP-FABD2 (A and B) or GFP-Lifeact (C and D). Projected images from confocal z-stacks.

Scale bar = 20 μm .

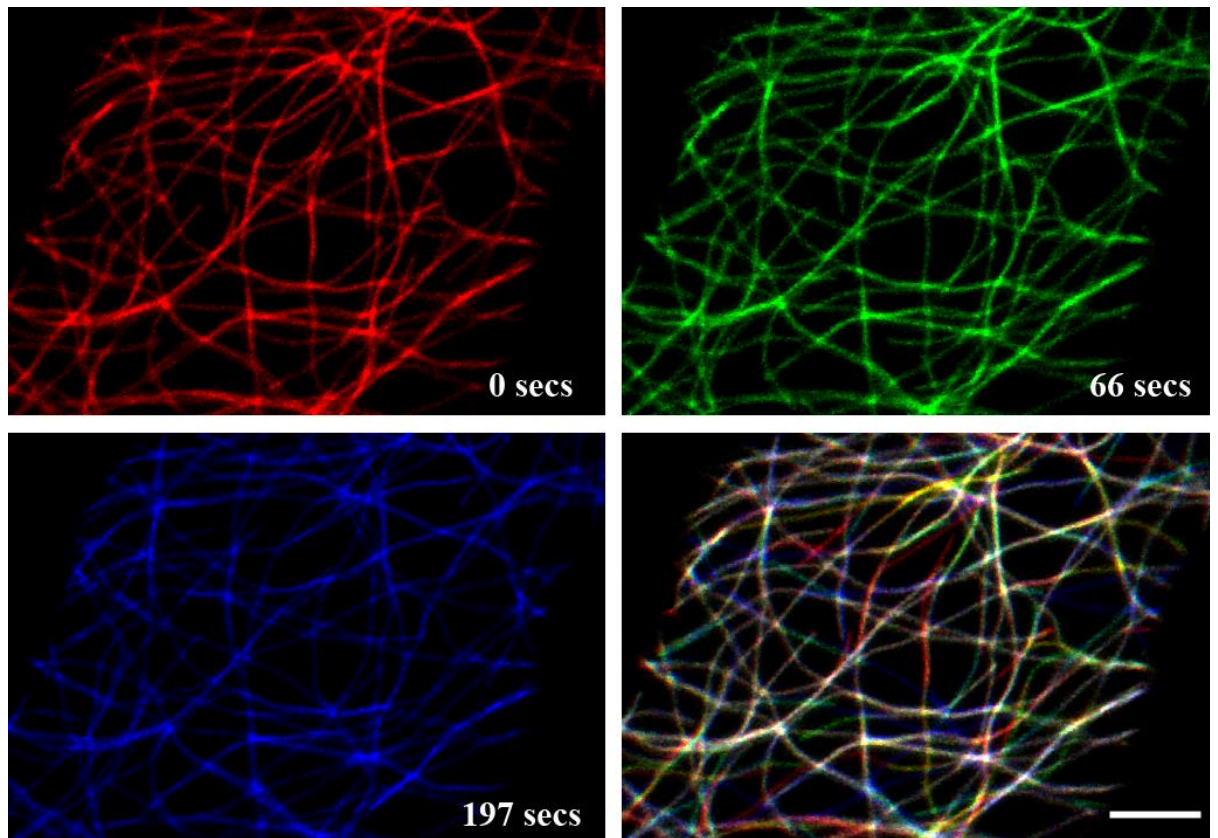


Figure 3.3 Time-lapse three dimensional (xyz) confocal micrographs of GFP-MAP4 at three separate time-points. All three time-points merged in final frame.

Leaf epidermal pavement cells from a transgenic line of *A. thaliana* that stably-expressed GFP-MAP4. On the bottom right is the cell with the three time-points overlaid: the first time-point is red, the middle time-point is green and the last one is blue. In the overlay when the filaments have moved they are in colour, but when they have not moved they appear white as the colours are superimposed.

Scale bar = 5 μm .

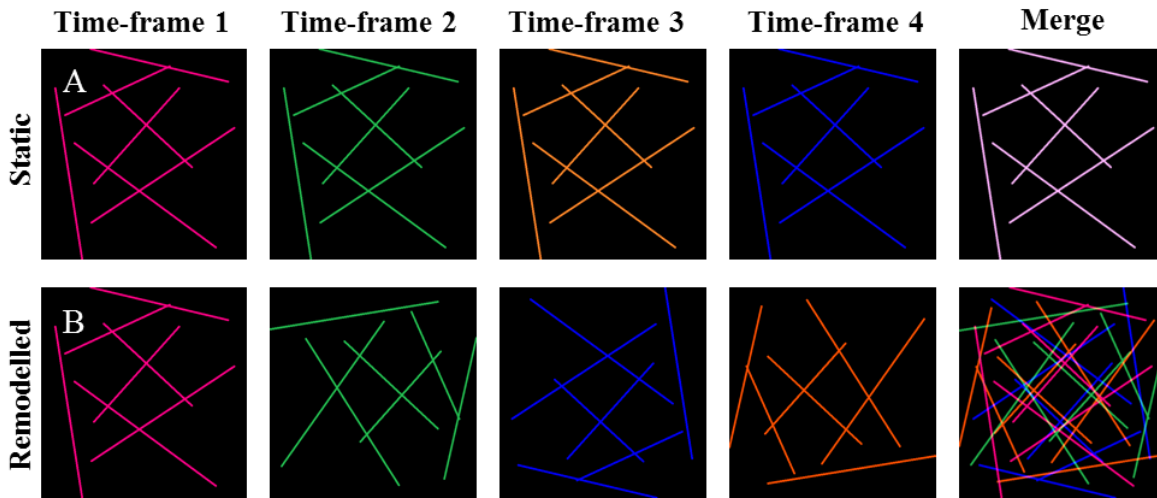


Figure 3.4 Schematic representation of two networks, with and without remodelling, depicted at four time-points, the fifth image in each row is a merge of all the time-points.

Row A is a static network that has not undergone remodelling. Each time-frame is the same as the previous one and when they are all merged (image on the far right), all the filaments are superimposed and appear pink. If the *Pairwise image subtraction* method is used on this network, the output is '0', because nothing has changed.

Row B is a network that has remodelled: each time-frame is different to the previous. When all four time-frames are merged, every colour is visible and there is no light pink, which would indicate stasis. If the *Pairwise image subtraction* method is used on this network, the output is '0.95', because there is so much movement, almost none of the network remains in the same location.

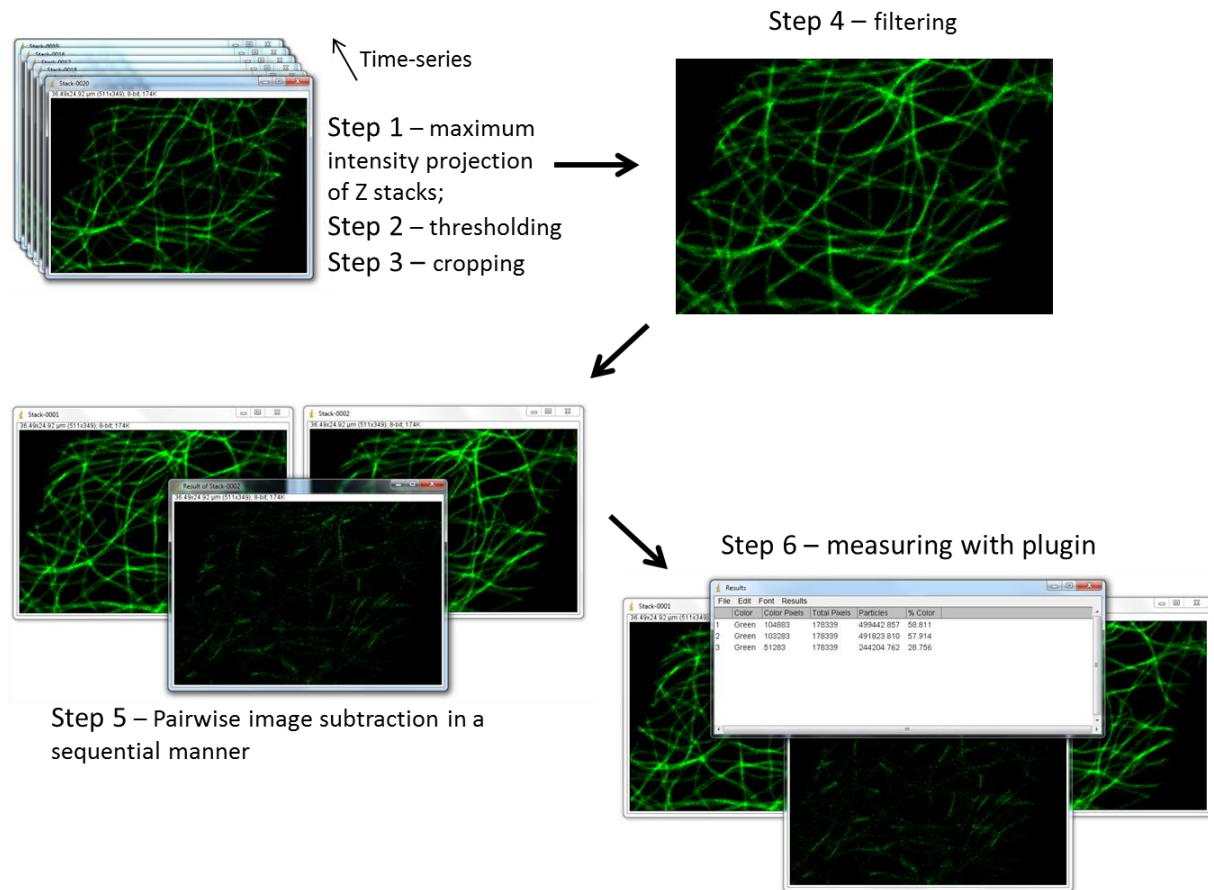


Figure 3.5 Workflow for Pairwise image subtraction.

This example uses the time-series of GFP-MAP4 from Figure 3.3. Steps 1 - 3 detail production of maximum intensity projections of all the Z-stacks within the 3D time-series followed by thresholding so that the background has a value of '0' and finally cropping so all images are 200 by 150 pixels (30,000 pixels in total). In step 4 the time-series are filtered using a median filter of size 0.2 (Figure 3.6 for details). Step 5 is sequential subtraction using the 'Image calculator' in ImageJ (Figure 3.7 for details).

Lastly Step 6 is the use of the plugin 'Color Pixel Counter' (http://imagejdocu.tudor.lu/doku.php?id=plugin:color:color_pixel_counter:start) that counts the number of pixels containing a fluorescent intensity above a set value and produces a log window with the results. A small value indicates little remodelling occurred, while a larger value indicates much remodelling. Figure inspired by Higaki *et al.* (2010).

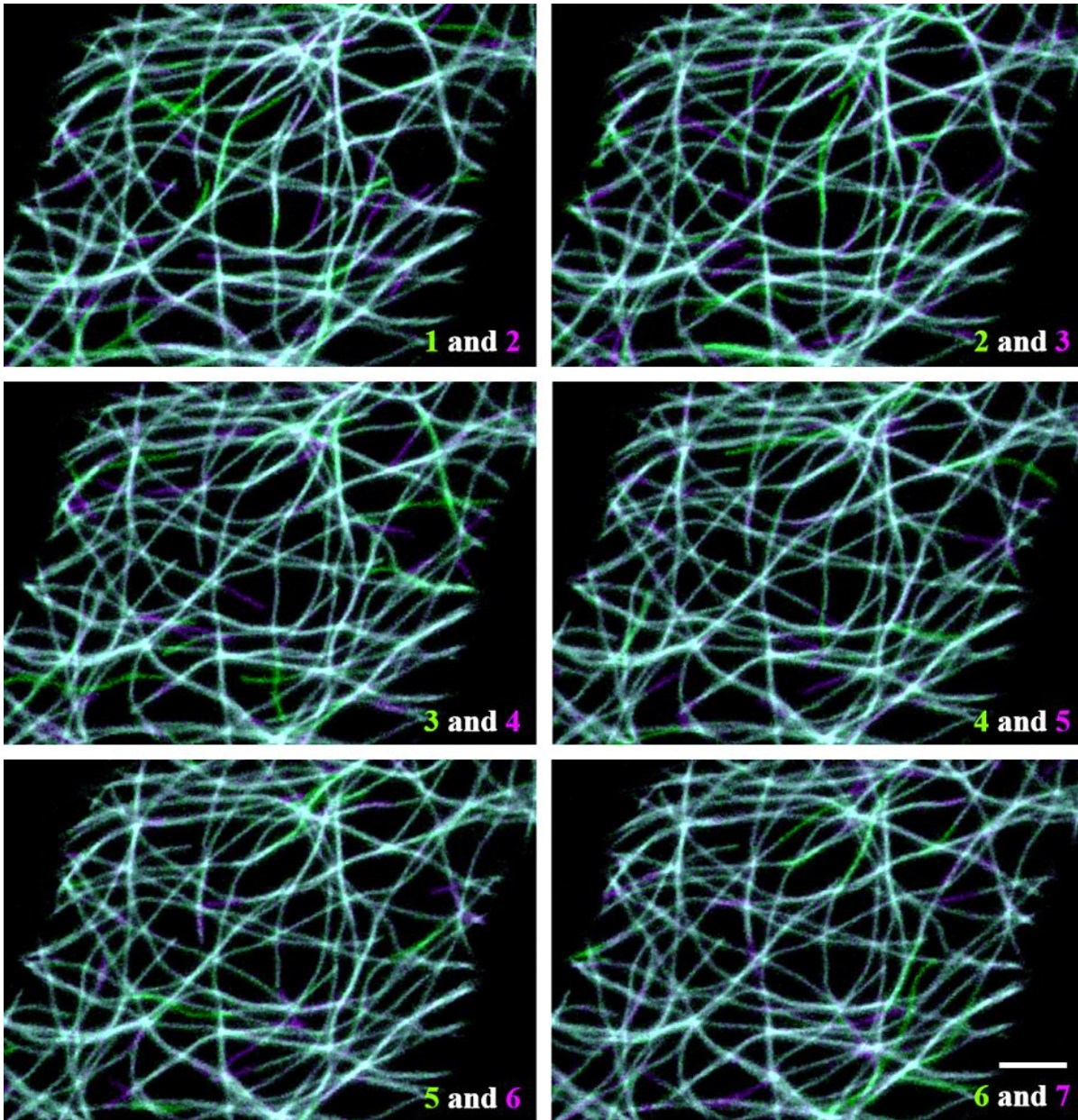


Figure 3.6 Step 4 of the *Pairwise image subtraction* method.

This example uses the GFP-MAP4 time-series from Figure 3.3; this is not part of the method, it is merely illustrative. Pairs of time-frames were alternately coloured so that green represents pixel locations in the first frame from the pair and magenta represents pixels from the second. Superimposition of the green and magenta results in a cyan colour that indicates no network remodelling between frames, while visible green and magenta indicate regions where remodelling has occurred within the network. The first seven time-frames are shown here in pairs (33 s between each time-frame). The amount of change between time-frames is shown in a qualitative manner, so time-frame one is merged with time-frame two, time-frame two is merged with time-frame three and so on.

Scale bar = 5 μm .

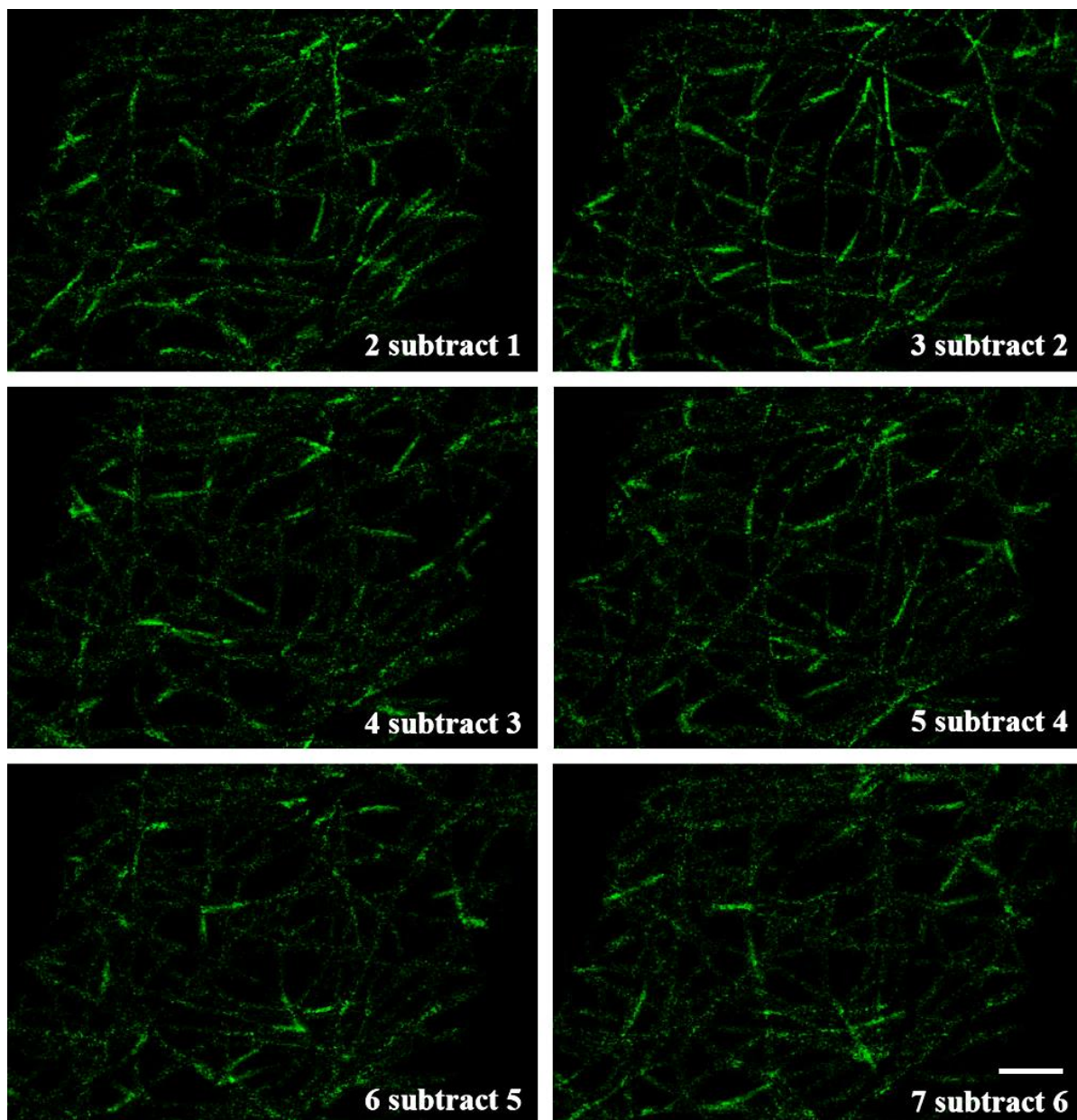


Figure 3.7 Step 5 of the *Pairwise image subtraction* method.

This example uses the GFP-MAP4 time-series from Figure 3.3; workflow is outlined in Figure 3.5. In this case the first seven time-frames were used for the subtraction (33 s between each time-frame). Sequential subtraction was carried out using 'Image calculator' in ImageJ (Rasband, 1997-2016): it was done using pairs of sequential images, so that time-frame one was subtracted from time-frame two, time-frame two was subtracted from time-frame three and so on. Green in each image represents the network change between the first and second time-frame in each pair. The numbers on each image state which time-frames are in view and which was subtracted from which. Scale bar = 5 μm .

Table 3.1 Step 6 of the *Pairwise image subtraction* method.

This example uses the GFP-MAP4 time-series from Figure 3.3; workflow is outlined in Figure 3.5. The ImageJ ‘Color Pixel Counter’ plugin (Rasband, 1997-2016) (http://imagejdocu.tudor.lu/doku.php?id=plugin:color:color_pixel_counter:start) produces a percentage value representing the number of pixels that contain fluorescence, i.e. fluorescence intensity >0. Here the images in Figure 3.6 (pg. 70) and Figure 3.7 were analysed and the right-most column (subtracted image divided by first image) indicates the relative rate of remodelling. Low values (approaching 0.0) represent no remodelling and higher values (approaching 1.0) represent rapid remodelling.

Time-frame	Fluorescence pixels	Total # pixels	% Fluorescence pixels	Subtracted image divided by first image
1	104883	178339	58.811	
2	103283	178339	57.914	
2 minus 1	51283	178339	28.756	0.489
2	103283	178339	57.914	
3	103082	178339	57.801	
3 minus 2	51863	178339	29.081	0.502
4	102145	178339	57.276	
3	103082	178339	57.801	
4 minus 3	50722	178339	28.441	0.492
5	101480	178339	56.903	
4	102145	178339	57.276	
5 minus 4	50559	178339	28.35	0.495
6	101507	178339	56.918	
5	101480	178339	56.903	
6 minus 5	49299	178339	27.643	0.486
7	101979	178339	57.183	
6	101507	178339	56.918	
7 minus 6	53936	178339	30.244	0.531
8	99006	178339	55.516	
7	101979	178339	57.183	

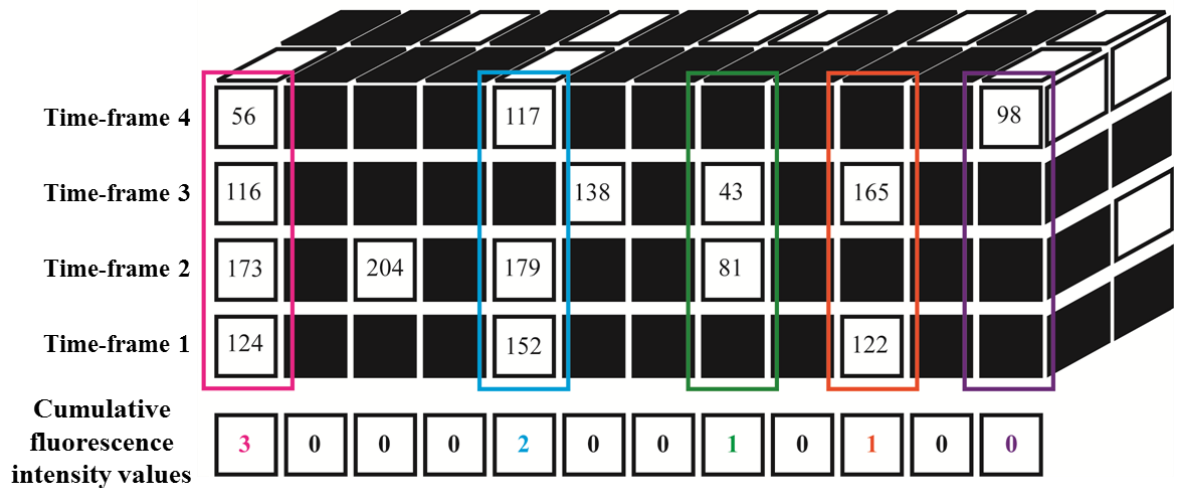


Figure 3.8 Schematic of the *Cumulative fluorescence intensity* analysis.

This schematic presents a 3D array of pixels (xyt). A z-stack is collected at each time-point and maximally projected, although this is not essential, as only x and y information is necessary per time-frame. Each pixel location is then tested for fluorescence. The numbers in the white boxes indicate some level of fluorescence between 1–255 (e.g. 56 in the top left pixel, time-frame 4) although the amount of fluorescence is irrelevant. All pixels with fluorescence intensity >0 are then assigned a value of ‘1’ and pixels with no fluorescence are assigned a value of ‘0’. Assigned values are then summed down columns to arrive at the cumulative fluorescence intensity value for that pixel location across the entire time-series. The first value of ‘1’ in each column is not counted in this calculation since an initial comparison is necessary.

The *Cumulative fluorescence intensity* for each pixel in this scheme is displayed in a box below the pixel location. The pink box on the left outlines a pixel that contains signal at every time-frame in the time-series; using the *Cumulative fluorescence intensity* method this pixel will have a value of ‘3’. The amount of signal in each pixel is not considered, merely whether it is present or absent. The blue box on the left outlines a pixel that contains signal in three time-frames in the time-series, so this pixel will have a value of ‘2’. The green and orange boxes in the middle outline pixels that contain signal in only two time-frames in the time-series, so these pixels will each have a value of ‘1’. The purple box on the right outlines a pixel that contains signal in only one time-frame, so this pixel will have a value of ‘0’. All other pixel locations in this schematic have a *Cumulative fluorescence intensity* of ‘0’.

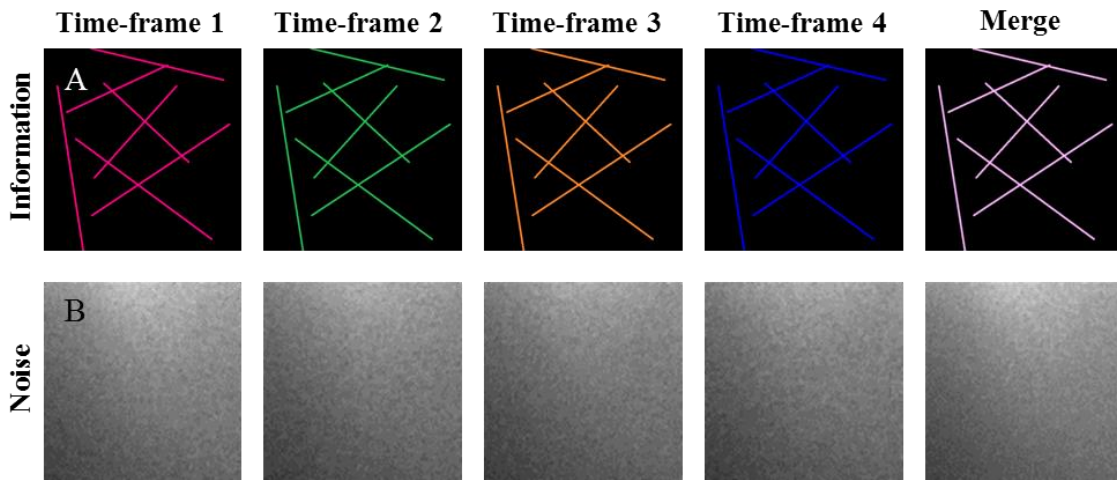


Figure 3.9 Diagram to illustrate the difference between a static network and noise.

This figure shows two sets of four time-frames with a merge of each the four on the far right.

Row A is the static network with no alterations from Figure 3.4 (pg. 68). If the *Cumulative fluorescence intensity* method is used on this network, the proportion of the fluorescence intensity value at '3' will be 1.0: because the filaments are stationary, no other fluorescence intensity values are present.

Row B is noise from a time-series, not informative signal. If the *Cumulative fluorescence intensity* method is used on this noise, the proportion of the fluorescence intensity value at '0' will approach 1.0; everything has changed and so no pixels have any cumulative fluorescence intensity.

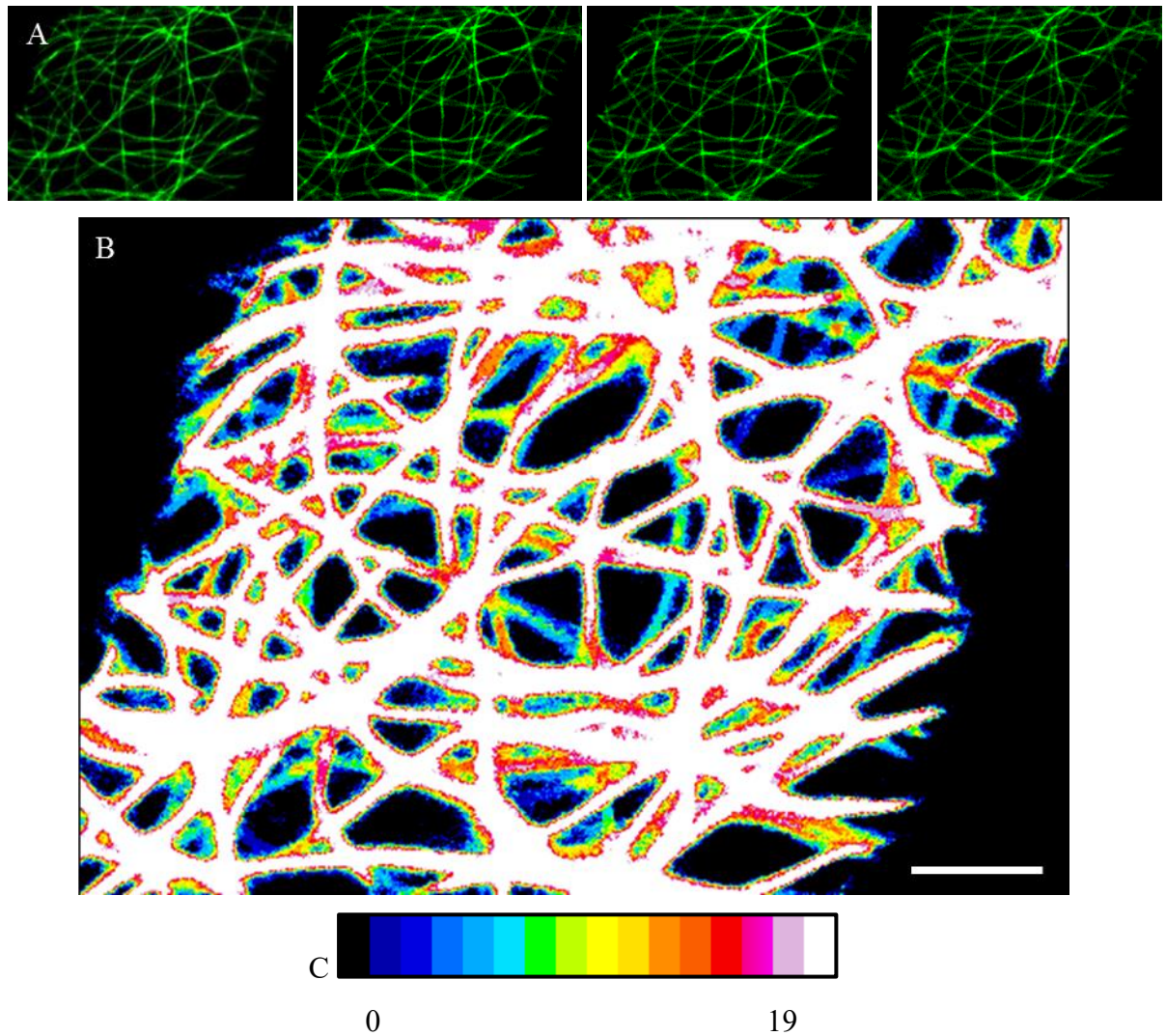


Figure 3.10 Consecutive time-points of three dimensional confocal micrographs and the final summing of all 20 time-points with a heat map.

GFP-MAP4 tagged microtubule cytoskeleton from Figure 3.3 (pg. 67).

Row A is the first four time-frames from this time-series containing 20 time-frames in total (time-lapse = 33 s per frame).

Row B is all 20 time-points of the time-series (mentioned above) which were analysed using the *Cumulative fluorescence intensity* method (627 s in total). The colours correspond to fluorescence intensity values for each pixel from '0' to '19': blue shades are smaller values and red and white are larger values. See Section 3.4.3.3 (pg. 63) for the method and Table 3.2 (below) for the values themselves. Scale bar = 5 μ m.

Row C is the heat map for (B) with an indication of which values correspond to which colours (Rasband, 1997-2016).

Table 3.2 Cumulative fluorescence intensity distribution in the GFP-MAP4 tagged microtubule cytoskeleton in Figure 3.10.

See Section 3.4.3.3 (pg. 63) for the method. The image-wide sum of the number of pixels with cumulative fluorescence intensities from 1 to 19 is 142657 (total pixels minus the '0' values), thus giving the total number of fluorescent pixels.

Fluorescence intensity values	Number of pixels	Calculations # pixels/total # fluorescent pixels	Normalised values as a ratio
0	35682	-	-
1	9914	9914/142657	0.069495
2	6127	6127/142657	0.042949
3	4941	4941/142657	0.034636
4	4466	4466/142657	0.031306
5	4652	4652/142657	0.03261
6	4526	4526/142657	0.031726
7	4414	4414/142657	0.030941
8	4083	4083/142657	0.028621
9	4032	4032/142657	0.028264
10	4005	4005/142657	0.028074
11	4071	4071/142657	0.028537
12	4207	4207/142657	0.02949
13	4259	4259/142657	0.029855
14	4408	4408/142657	0.030899
15	4928	4928/142657	0.034544
16	5461	5461/142657	0.038281
17	6570	6570/142657	0.046055
18	10158	10158/142657	0.071206
19	47435	47435/142657	0.332511
Total pixels	178339 (total >0 = 142657)		

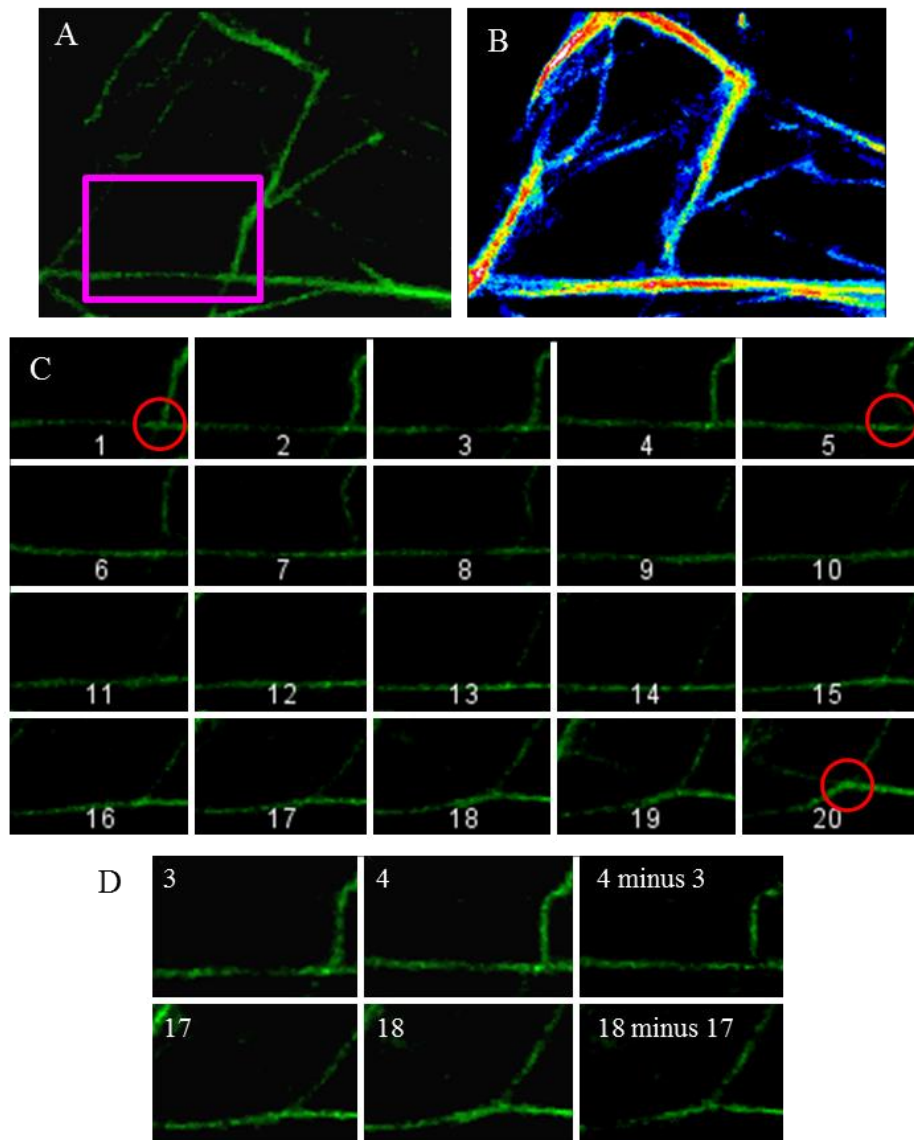


Figure 3.11 Diagram to show why *Pairwise image subtraction* analysis is not effective in measuring remodelling in GFP-Lifeact marked networks.

(A) The first time-frame from the time-series in (C); this is from a leaf epidermal pavement cell from a transgenic line of *A. thaliana* that stably-expressed GFP-FABD2. The area examined in (C) and (D) is outlined by the pink rectangle.

(B) Visual result from *Cumulative fluorescence intensity* analysis of the cell in (A)

(C) The region outlined in (A) at 20 numbered time-frames. Remodelling occurred within the regions marked by the three red circles, outside these a good deal of simple movement has occurred, but not remodelling.

(D) The same region as (C), but showing two pairs of time-frames and their subsequent ineffective subtractions (as in Figure 3.7). Due to simple movement, the result would indicate that remodelling had occurred when this is not the case as verified by visual inspection of the time-series.

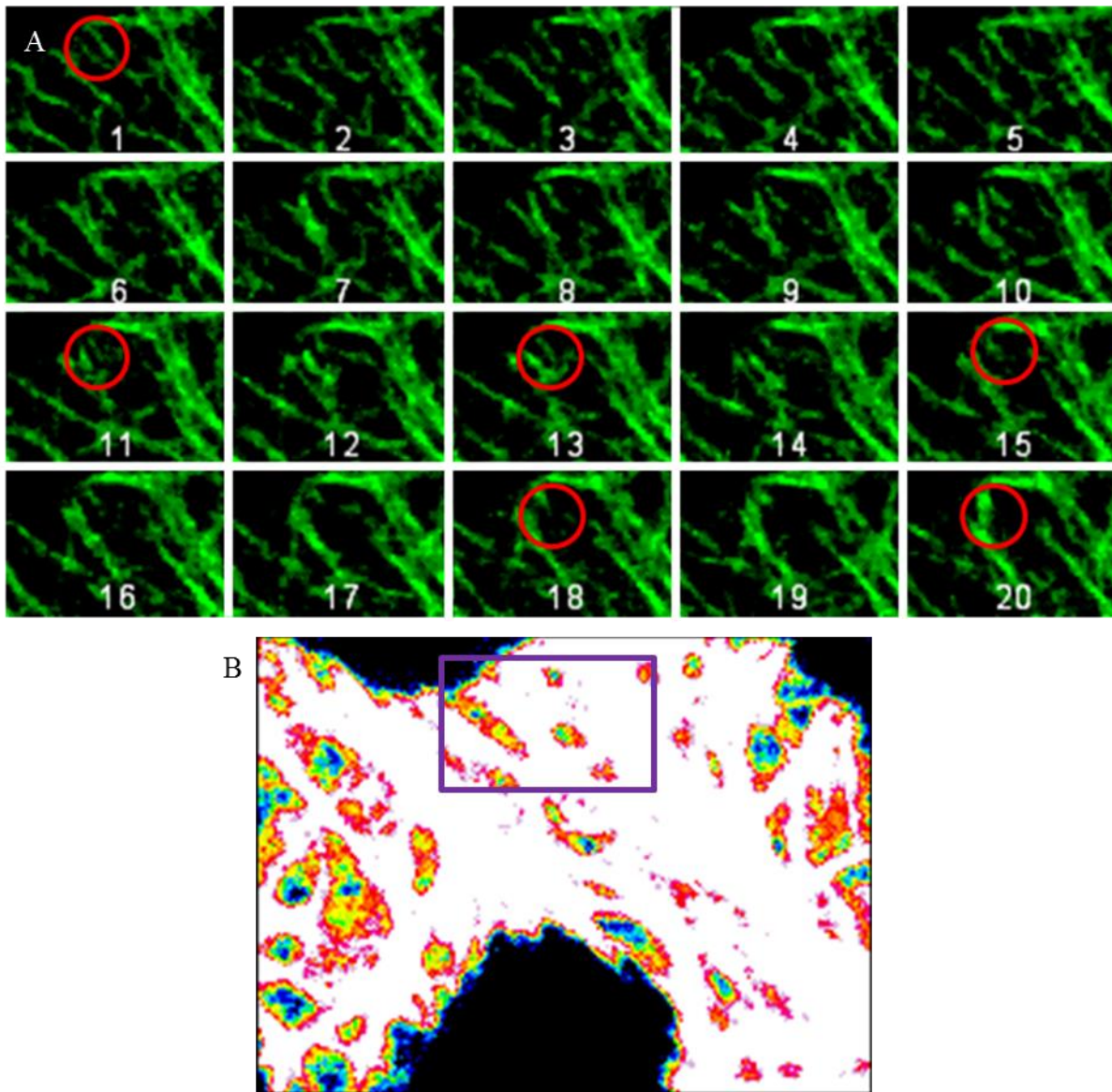


Figure 3.12 Diagram to show why *Cumulative fluorescence intensity* analysis is not effective in measuring remodelling in GFP-FABD2 marked networks.

(A) Region from a time-lapse 3D confocal micrograph at 20 numbered time-frames taken from a leaf epidermal pavement cell from a transgenic line of *A. thaliana* that stably-expressed GFP-FABD2. Remodelling visibly occurs within the red circles, but at the same location, so using *Cumulative fluorescence intensity* analysis it would be counted as not remodelled

(B) Visual result from *Cumulative fluorescence intensity* analysis of the same cell. The area examined in (A) is outlined by the purple rectangle; it can be seen that all of the remodelling has been designated as static network.

3.5 Discussion

Visualising the dynamics of the cytoskeleton can be done simply in a qualitative manner by overlaying different time-points in different colours, thus showing the change in the network over time. For example, in Figure 3.3 (pg. 67) the different colours show that between each time-point the network has undergone remodelling. However, while this is an accurate representation of the amount of change within the actin cytoskeleton it is not quantitative and does not allow statistical comparisons between different conditions.

3.5.1 Methods to quantitatively measure the bulk remodelling of the actin cytoskeleton

The methods developed in this study to quantify changes in the actin cytoskeleton are *Pairwise image subtraction* with GFP-FABD2 and *Cumulative fluorescence intensity* with GFP-Lifeact (Sections 3.4.3.1, pg. 60 and 3.4.3.3). These methods are used in the subsequent two chapters to analyse the bulk remodelling of the actin cytoskeleton.

GFP-FABD2 tagged networks are often dense so filaments and strands generally do not move (i.e. simple movement is minimal); therefore any changes in the position of the filaments are usually due to remodelling of the cytoskeleton. *Pairwise image subtraction* gives a binary answer to whether there has been change (e.g. yes or no). This means that movement causes inaccurate measurements about the amount of remodelling in the network (Figure 3.1, pg. 65). However, erroneous calculation of remodelling due to filament remodelling back into the space previously occupied by a filament is minimised as these measurements were taken between sequential pairs of time-frames and this kind of event is unlikely to have occurred during the short time between frames.

Using *Cumulative fluorescence intensity* to measure the dynamics of a network decorated with GFP-Lifeact was effective. The filaments are sparsely distributed and there is a higher proportion of empty field of view and thus a reduced chance of remodelling over a previously occupied area. Although there is simple movement of filaments (Figure 3.1, pg. 65), this approach can quantify the amount in that the cumulative fluorescence

intensity values are on a scale: lots of movement ('cfi' values = '1', '2', '3', '4' or '5') through to very little or no movement ('cfi' values = '16', '17', '18' or '19'). Movement itself does not invalidate the results of this method as it does with *Pairwise image subtraction* (Figure 3.10, pg. 75). In addition, the threshold for the *Cumulative fluorescence intensity* method was chosen as '0' because all the time-series were manually thresholded to retain information. In another scenario it would be possible to change the threshold of the plugin to '10', for example, and say that all values of '9' or below are just background. This could increase the amount of automation with consistent datasets.

3.5.1.1 Caveats

Using *Pairwise image subtraction* to measure the dynamics of an actin network marked with GFP-Lifeact was not accurate. This is because its sparse and bundled nature means there are often thick strands of actin running through the cortex which do not remodel but which can move (Figure 3.11, pg. 77). This movement produces spurious results suggesting that the actin network is remodelling at a high rate when, in fact, it maintains a static shape that changes position. Therefore, *Pairwise image subtraction* is not a robust analysis technique when lateral drift features as part of the dynamic nature of a network.

For exactly the opposite reason, *Cumulative fluorescence intensity* is inaccurate in describing remodelling of the actin cytoskeleton labelled with GFP-FABD2. The network is often so dense that if a filament remodels it does so into a space previously occupied by another filament and therefore change is measured as no change (Figure 3.12).

3.6 Conclusion

Over the last few years many ways to measure cytoskeletal dynamics have been published; however, none of these methods were found to be completely appropriate for this study. A method of measuring the bulk remodelling of the actin cytoskeleton on a cellular scale was developed. Termed '*Pairwise image subtraction*' it utilises the 'subtract' function in the '*Image Calculator*' of ImageJ (NIH) (Rasband, 1997-2016). However, it appears to work better with the more dense networks of GFP-FABD2 rather than the sparser networks of GFP-Lifeact. This method will be used in the next two chapters as part of the investigation into the influence of the cell wall on the organisation and dynamics of the actin cytoskeleton.

In collaboration with a bioinformatician, a novel method of measuring actin cytoskeleton dynamics was developed. Called '*Cumulative fluorescence intensity*', it was written as a plug-in for ImageJ (NIH) (Rasband, 1997-2016). However, it does not work well with the dense networks of GFP-FABD2, but is more informative when analysing the more sparse networks of GFP-Lifeact. This method will also be used in the next two chapters as part of the investigation into the influence of the cell wall on the organisation and dynamics of the actin cytoskeleton.

Using large datasets in different experimental contexts in the next chapters will help validate the effectiveness of these two different approaches to quantitatively measuring the organisation and dynamics of the actin cytoskeleton. The next two chapters investigate the dynamics of the actin cytoskeleton in cells in which the cell wall-plasma membrane interface was perturbed.

Chapter Four

The effects of cell wall disruption on actin cytoskeleton dynamics

4.1 Introduction

As detailed previously there is well documented evidence for bidirectional feedback between the cell wall and the microtubule cytoskeleton (see Section 1.2.5.2, pg. 17). It is also well known that the actin cytoskeleton is important for transporting Golgi bodies and vesicle to the cell wall (see Section 1.6.2, pg. 34). However, little work has been done to determine whether the cell wall can influence the actin cytoskeleton, perhaps acting in an organisational capacity. This chapter addresses this question by investigating how disruption of the cell wall and its links to the cytoplasm affect the organisation and mobility of the actin cytoskeleton. The analysis of the experiments outlined below was developed and described in Chapter 3 (see Section 3.4.3.1, pg. 60 and Section 3.4.3.3). Figure 4.1 (pg. 99) details the workflow for these analysis methods and Figure 4.2 details how these methods were used in the experimental work (i.e. during the application of various types of stress to the cell, to investigate the influence of the cell wall on actin cytoskeleton morphology and remodelling).

Plasmolysis was used to disrupt the cell wall-cell connections and isoxaben to specifically disrupt the cell wall in a targeted manner. They were used in combination with fluorescent actin markers to analyse the actin cytoskeleton by confocal microscopy (Figure 4.1). Plasmolysis is a well-known phenomenon in which the protoplasm, that is the plasma membrane and all the cell contents, pull away from the cell wall (Figure 4.3; see Section 1.4.1.1, pg. 28). It is a naturally occurring process which preserves cell integrity during

situations such as drought or salt stress (Oparka, 1994). During this process there are still connections to the cell wall via structures termed 'Hechtian strands' (Oparka, 1994). In this study plasmolysis was used as a method to investigate the dynamics of the actin cytoskeleton when the protoplasm was physically separated from the cell wall.

To complement the global effects of plasmolysis, this study also used isoxaben to cause targeted and specific alteration to the cell wall (see Section 1.3.3.1, pg. 25; Figure 4.2). Isoxaben is a pre-emergent herbicide: it kills seedlings by interfering specifically with one of the major proteins involved in cellulose synthesis (Tateno *et al.*, 2016). This study is concerned with the influence of the cell wall; therefore it was necessary to exclude the possibility of a general stress response that the actin cytoskeleton always displays in the same manner regardless of the abiotic stress inflicted on the cell. To this end plant cells were temperature stressed to determine if the cytoskeleton reacted in the same manner as when the cell was plasmolysed (Figure 4.2).

In addition, this study sought to establish whether there were any differences between two different fluorescent protein markers for the actin cytoskeleton (GFP-FABD2 and GFP-Lifeact; see Section 3.4.1, pg. 58). In case any dynamic alteration was due to the actin cytoskeleton incorporating fluorescent proteins, a marker for Golgi bodies was also used as this should not directly affect the dynamics of the actin cytoskeleton (ST-GFP). The movement of Golgi bodies can be used as a proxy for network structure and dynamics, as the primary means of their movement is along actin filaments (Sparkes *et al.*, 2008, Akkerman *et al.*, 2011, Martinière *et al.*, 2011a, Rocchetti *et al.*, 2014).

4.2 Collaborators

The *Cumulative fluorescence intensity* plugin was developed in collaboration with, but written by, Dr Axel Poulet, at Oxford Brookes University (Oxford, UK), specifically for this study.

4.3 Aims

The aim of this chapter was to study the influence of the cell wall on the spatio-temporal organisation and dynamics of the actin cytoskeleton using techniques that perturb the cell wall-plasma membrane interface. These included:

- a) Using plasmolysis to analyse the organisation and dynamics of the actin cytoskeleton under conditions that cause separation of the protoplasm from the cell wall. These experiments were carried out using two different fluorescent protein markers (GFP-FABD2 and GFP-Lifeact) for the actin cytoskeleton in the event the marker has an effect on the organisation and dynamics of the network. This was repeated with a marker for Golgi bodies (ST-GFP) in the event that any marker bound to the actin cytoskeleton changes the ability of the network to respond to alterations.
- b) Analysis of the GFP-FABD2 and GFP-Lifeact labelled actin cytoskeleton under heat stress and cold stress to ascertain if there is a general stress response regardless of how the cell has been abiotically stressed.
- c) Comparing cells treated with isoxaben against a mock treatment to analyse the organisation and dynamics of the GFP-FABD2 labelled actin cytoskeleton when a specific component of the cell wall has been compromised.

4.4 Results

All analysis in this chapter was carried out on mature stably-transformed *A. thaliana* plants and only abaxial epidermal leaf pavement cells were imaged; in addition, all time-series analysed in this chapter last approximately 100 s. The GFP-FABD2 expressing line was made by Ketelaar *et al.* (2004) and Sheahan *et al.* (2004), the GFP-Lifeact expressing line was made by Smertenko *et al.* (2010) and the ST-GFP expressing line was made by Saint-Jore (2001).

4.4.1 Does the physical separation of the cell wall from the protoplasm affect the actin network?

Mature Fimbrin Actin Binding Domain 2-GFP (GFP-FABD2) stably-expressing *A. thaliana* plants were used and abaxial leaf epidermal pavement cells were imaged using confocal microscopy under different conditions to determine whether the cell wall has an organisational influence over the spatio-temporal dynamics of the actin cytoskeleton. The control cells were viewed directly after excision from the leaf, whilst the plasmolysed cells were incubated for 30 min in a solution containing 0.6 M mannitol (Figure 4.2, pg. 100; see Section 2.6.2, pg. 48). In addition, some of the plasmolysed samples were subsequently re-hydrated in water and imaged, as were cells that had only been incubated in water with no prior incubation in mannitol. These last cells were considered to have been treated hypo-osmotically (Figure 4.2).

4.4.1.1 Qualitative assessment of GFP-FABD2 during plasmolysis

Mature GFP-FABD2 stably-expressing *A. thaliana* plants were used and abaxial leaf epidermal pavement cells were imaged using confocal microscopy during two minute time-series. Three time-points (at 0 s, 50 s and 105 s, respectively) were selected and re-coloured red, green and blue in Adobe Photoshop [Adobe Systems Incorporated; San Jose, CA; USA]. These were then overlaid to show how much remodelling occurred under different conditions. Where colour is visible in the overlay remodelling has occurred, but where the filaments are white the network has remained stationary (Figure 4.4, pg. 102).

Control cells (no incubation) that stably-expressed GFP-FABD2 displayed a range of morphologies in their actin cytoskeleton. However, the most predominant was a close-knit meshwork of filaments with few bundles and very few visible thin bundles (Figure 4.4-A). When incubated in mannitol, cells plasmolysed and the actin morphology changed: large bundles of filaments crossing the region of interest (ROI) were far more prominent and less of the ‘meshwork’ seen in the control cells was in evidence (Figure 4.4-B). In addition, the fluorescence was brighter in these cells, possibly due to the concentration of GFP into the bundles. In contrast, re-hydrated cells (incubated in mannitol and then incubated in dH₂O) almost always had much lower fluorescence levels than the control cells (Figure 4.4-C). In these cells, the network was more similar to the control cells than the plasmolysed cells, except they also seemed to have more bundles. However, this could be a consequence of the lower fluorescence levels, so finer filaments were not visualised. In the hypo-osmotic cells (incubated in dH₂O), the network appeared similar to the networks seen in the re-hydrated cells (Figure 4.4-D). See Appendix Figure B.1, pg. 256 and Figure B.2 for all cells.

4.4.1.2 Pairwise image subtraction of GFP-FABD2 marked actin cytoskeleton during plasmolysis

Pairwise image subtraction was introduced in the previous chapter as a way to quantify the amount of bulk remodelling of the actin cytoskeleton (see Section 3.4.3.1). Briefly, using pairs of time-frames, the first time-frame is subtracted from the second and the remaining fluorescence is calculated as a proportion of the total initial fluorescence; therefore a static network would have a low value and a dynamic network would have a higher value. This proportion represents the total amount of network change during the time interval and is reported as the ‘bulk remodelling index’ in arbitrary units (a.u.) which is equivalent to area change per unit time.

Approximately 30 datasets per condition were compared using an unpaired t-test with Welch’s correction (Figure 4.5, pg. 103; see Section 2.7.2, pg. 51). The bulk remodelling index of the GFP-FABD2 marked actin cytoskeleton was calculated as the mean and standard deviation (arbitrary units, a.u.): control was 0.5558 ± 0.0549 , plasmolysed was 0.5273 ± 0.0441 , re-hydrated was 0.6013 ± 0.0885 and hypo-osmotic was 0.5558 ± 0.0487 (see Appendix Table B.1). The bulk remodelling index was significantly lower in the plasmolysed sample compared to the control ($p < 0.0001$) and the variances were

significantly different ($p < 0.0001$). On the other hand, the bulk remodelling index of the actin cytoskeleton was significantly higher in the re-hydrated sample compared to the control ($p < 0.0001$) and the variances were significantly different ($p < 0.0001$). There was no difference between the control and the hypo-osmotic sample ($p = 0.9951$), although the variances were significantly different ($p = 0.0036$).

4.4.1.3 Mean fluorescence intensity using Pairwise image subtraction of GFP-FABD2 marked actin cytoskeleton during plasmolysis

Mean fluorescence intensity measured using *Pairwise image subtraction* was introduced in the previous chapter as an indicator of the organisation of the actin cytoskeleton (see Section 3.4.3.2). The same initial method as used in *Pairwise image subtraction* was employed, except that then the mean fluorescence intensity was measured rather than the total amount of fluorescence.

The same dataset as used in the previous analysis was compared using an unpaired t-test with Welch's correction (Figure 4.6, pg. 104). The mean fluorescence intensity of the GFP-FABD2 marked actin cytoskeleton was calculated as the mean and standard error of mean (no units as these are proportions): the control was 0.3132 ± 0.0046 , plasmolysed was 0.2432 ± 0.0039 , re-hydrated was 0.3491 ± 0.0053 and hypo-osmotic was 0.3558 ± 0.0034 (see Appendix Table B.2). The mean fluorescence intensity of the actin cytoskeleton was significantly lower in the plasmolysed sample compared to the control ($p < 0.0001$) and the variances were significantly different ($p < 0.0001$). The mean fluorescence intensity of the actin cytoskeleton was significantly higher in both the re-hydrated and hypo-osmotic samples compared to the control ($p < 0.0001$) and the variances were significantly different ($p < 0.0001$).

4.4.1.4 Qualitative assessment of GFP-Lifeact during plasmolysis

Mature GFP-Lifeact stably-expressing *A. thaliana* plants were used and leaf abaxial epidermal pavement cells were imaged using confocal microscopy over two min. The experiment was carried out as described in Figure 4.2 (pg. 100).

Control cells (no incubation) expressing GFP-Lifeact appeared to be quite similar in their actin cytoskeleton morphology with most cells possessing a sparse and bundled network (Figure 4.7-A, pg. 105). When plasmolysed (incubated in mannitol) the cells seemed to

possess the same numbers of bundles as were visible in the control cells, but they were no longer as straight, they appeared slightly ‘wavy’ or ‘kinked’ (Figure 4.7-B). In cells that had been re-hydrated (incubated in mannitol and then incubated in dH₂O) the network appeared similar to the networks observed in control cells (Figure 4.7-C). Hypo-osmotic cells (incubated in dH₂O) displayed a network that shared features with both control cells and re-hydrated cells; certainly it was still bundled (Figure 4.7-D).

4.4.1.5 Cumulative fluorescence intensity of GFP-Lifeact marked actin cytoskeleton during plasmolysis

Cumulative fluorescence intensity was introduced in the previous chapter as a way to measure any alterations in the actin cytoskeleton tagged with GFP-Lifeact (see Section 3.4.3.3). Briefly, each pixel was recorded as having fluorescence or no fluorescence for all time-points in a time-series and then these were summed to produce a set of values that describe the amount of alteration in the actin cytoskeleton.

Figure 4.8 (pg. 106) and Figure 4.9 show the *Cumulative fluorescence intensity* for each treatment: control, plasmolysed, re-hydrated and hypo-osmotic (approximately 25 datasets per condition). See Appendix Figure B.3, pg. 259 and Figure B.4 for all cells. The cells were chosen at random, the first fluorescing pavement cell seen in a new area of the leaf was imaged in order to avoid bias. Short intervals were used in the time-series because the actin cytoskeleton both remodels and moves quickly. The conclusion from the individual images is of a general trend either towards more or less remodelling depending on the condition, but also that there is variation within each condition.

Figure 4.10 (pg. 108) shows the average of each condition at every fluorescence intensity for the cells shown in Figure 4.8 and Figure 4.9. They have all been normalised against the total fluorescence of each dataset, so there should be no bias for size of cell or the amount of cytoskeleton in view. The fluorescence intensity of the different treatments has been graphed against the control which has a value of one for every *Cumulative fluorescence intensity* point. To compare the treatments, one-way ANOVA was used with a Tukey post-test for each fluorescence intensity value, so that there are 19 tests. The p-value for every ANOVA can be seen in Table 4.1 (pg. 109). Those in bold with one or more asterisks denote the fluorescence intensities at which significant differences were observed; the

datasets between which the differences lie are indicated in the table and described in the text below.

The proportion of each condition at the 1st fluorescence intensity value was calculated as the mean and standard deviation (no units as these are proportions): control was 0.2695 ± 0.09911 , plasmolysed was 0.2063 ± 0.07478 , re-hydrated was 0.2007 ± 0.11160 and hypo-osmotic was 0.2717 ± 0.14060 (see Appendix Table B.3). One-way ANOVA indicated experiment-wide differences between datasets ($p = 0.0213$), but using the Tukey post-test no significant differences were found between individual treatments (all $p > 0.05$) when correcting for multiple statistical tests (Table 4.1). Then for the next 14 fluorescence intensity values one-way ANOVA found no significant differences between treatments ($p > 0.05$; Table 4.1).

The proportion of each condition at the 16th fluorescence intensity value was calculated as the mean and standard deviation: control was 0.0058 ± 0.00719 , plasmolysed was 0.0157 ± 0.01365 , re-hydrated was 0.0156 ± 0.02079 and hypo-osmotic was 0.0111 ± 0.01505 . One-way ANOVA found a small, significant difference between treatments ($p = 0.0461$), but using the Tukey post-test no significant differences were found between individual treatments (all $p > 0.05$; Table 4.1).

The 17th, 18th and 19th fluorescence intensity values gave the same statistical results: using the Tukey post-test the proportion of fluorescence intensity at these values was significantly higher in the plasmolysed sample compared to the control (17th and 18th $p < 0.05$, 19th $p < 0.01$). One-way ANOVA found significant differences between treatments ($p = 0.0192$, $p = 0.0209$ and $p = 0.0093$, respectively; Table 4.1). The proportion of each condition at the 17th fluorescence intensity value was calculated as the mean and standard deviation: control was 0.0044 ± 0.00674 , plasmolysed was 0.0151 ± 0.01403 , re-hydrated was 0.0135 ± 0.01718 and hypo-osmotic was 0.0096 ± 0.01404 . The proportion of each condition at the 18th fluorescence intensity value was calculated as the mean and standard deviation: control was 0.0042 ± 0.00750 , plasmolysed was 0.0150 ± 0.01367 , re-hydrated was 0.0120 ± 0.01500 and hypo-osmotic was 0.0092 ± 0.01472 . The proportion of each condition at the 19th fluorescence intensity value was calculated as the mean and standard deviation: control was 0.0059 ± 0.01500 , plasmolysed was 0.0298 ± 0.03793 , re-hydrated was 0.0138 ± 0.01871 and hypo-osmotic was 0.0140 ± 0.02665 .

The first and the last three fluorescence intensity values had significant differences between treatments (Table 4.1) which means that there was an increased or decreased amount of fluorescence in the intensity values that indicate either change or rigidity in the network.

4.4.1.6 Dynamics of ST-GFP marked Golgi bodies during plasmolysis

All the above analysis was done using stably-transformed *A. thaliana* lines with the actin cytoskeleton ubiquitously and constitutively bound with artificial proteins themselves tagged with fluorescent proteins. In the event that these actin binding proteins themselves affect the working of the network, these experiments were repeated using a stably-transformed *A. thaliana* line containing a rat sialyl transferase transmembrane domain tagged with GFP (ST-GFP) which localises to the Golgi bodies (Saint-Jore, 2001). It is known that the movement of Golgi bodies in plant cells is mostly actin cytoskeleton dependent (see Section 1.6.2.1, pg. 35) and previous studies have used Golgi body motility as a way to evaluate the morphology of the actin cytoskeleton (Sparkes *et al.*, 2008, Martinière *et al.*, 2011a, Rocchetti *et al.*, 2014). In this study Golgi body movement was measured using Volocity software, which is one of the more accurate tracking softwares available (Bros *et al.*, 2015) [PerkinElmer, Massachusetts, USA].

The protocol was optimised to each time-series to obtain the most accurate description of the amount and kind of movement present (see Section 2.6.7, pg. 50). Mature ST-GFP stably-expressing *A. thaliana* plants were used and abaxial epidermal pavement leaf cells were imaged using confocal microscopy for approximately 90 s. Using Volocity it was possible to show a cell overlaid with all the tracks of the Golgi bodies that move through the field of view during the time-series and these tracks can be annotated with the velocity of the Golgi body at that particular time-point (Figure 4.11, pg. 110). The measurements listed below are all from the same experiment comparing treatments of approximately 30 cells each (the number of Golgi bodies per condition is on each graph).

Figure 4.12 (pg. 111) shows the average velocities of ST-GFP marked Golgi body populations analysed using the Kruskal-Wallis test with Dunn's Multiple Comparison test (a non-parametric ANOVA with post-test; see Section 2.7.2). The average velocity of ST-GFP was calculated as the mean and standard error of mean ($\mu\text{m}/\text{sec}$): control was 0.6856 ± 0.0149 , plasmolysed was 0.5204 ± 0.0151 , re-hydrated was 0.7651 ± 0.0195 and hypo-

osmotic was 0.7427 ± 0.0181 (see Appendix Table B.4, pg. 262). When the cell was plasmolysed the velocity was significantly lower compared to the control ($p < 0.001$). The re-hydrated velocity was higher compared to the control, but there was no significant difference ($p > 0.05$). Under hypo-osmotic conditions the velocity was significantly higher compared to the control ($p < 0.05$). The velocity was significantly lower in the plasmolysed sample compared to both the re-hydrated and hypo-osmotic samples ($p < 0.001$), but no significant difference between re-hydrated and hypo-osmotic samples ($p > 0.05$).

The displacement rate is a description of how much time an object has taken to move between two specified points as measured in a straight line [Volocity User Guide]. A low displacement rate means an object has taken a long time to move a short distance, whilst a high value means the object took a short time to move a long distance.

Figure 4.13 (pg. 112) shows the displacement rate of ST-GFP marked Golgi body populations analysed as above. The displacement rate of ST-GFP was calculated as the mean and standard error of mean ($\mu\text{m}/\text{sec}$): control was 0.2839 ± 0.0130 , plasmolysed was 0.2087 ± 0.0137 , re-hydrated was 0.3431 ± 0.0177 and hypo-osmotic was 0.3130 ± 0.0171 (see Appendix Table B.5). When cells were plasmolysed the displacement rate was significantly lower compared to control ($p < 0.001$). When re-hydrated and under hypo-osmotic conditions there was no significant difference between these conditions or between either of them compared to the control ($p > 0.05$). The displacement rate of the plasmolysed sample was significantly lower compared to both the re-hydration and hypo-osmotic ($p < 0.001$).

The meandering index is a ratio describing how complex the movement of an object is between two points. It is the displacement rate divided by average velocity (see above) and calculates a value between 0 and 1. If an object has a meandering index close to 1 then it has moved in a straight line between two points and if it has a value close to 0 then it has a complicated saltatory motion, i.e. ‘wiggling’ (Rocchetti *et al.*, 2014).

Figure 4.14 (pg. 113) shows the meandering index of ST-GFP marked Golgi body populations analysed as above. The meandering index of ST-GFP was calculated as the mean and standard deviation (no units as this is an index): control was 0.3305 ± 0.2529 ,

plasmolysed was 0.2762 ± 0.2608 , re-hydrated was 0.3517 ± 0.2651 and hypo-osmotic was 0.3357 ± 0.2587 (see Appendix Table B.6). When cells were plasmolysed the meandering index was significantly lower compared to control ($p < 0.001$). When re-hydrated and under hypo-osmotic conditions there was no significant difference between these conditions or between either of them compared to the control ($p > 0.05$). The plasmolysed sample was significantly lower compared to both the re-hydration and hypo-osmotic ($p < 0.001$).

Figure 4.15 (pg. 114) shows the number of time-frames that the ST-GFP marked Golgi body population (all Golgi bodies identified within the field of view during the experiment) were present in the field of view, analysed using an unpaired t-test with Welch's correction. The time span of ST-GFP was calculated as the mean and standard deviation (units are time-frames): control was 45.52 ± 44.14 , plasmolysed was 75.54 ± 58.18 , re-hydrated was 52.59 ± 47.17 and hypo-osmotic was 53.73 ± 47.14 (see Appendix Table B.7). In plasmolysed cells there were significantly more time-frames that the Golgi bodies were in view compared to control cells, re-hydrated cells and the cells under hypo-osmotic conditions ($p < 0.0001$, $p < 0.0001$ and $p < 0.0001$ respectively) and the variances were all significantly different at $p < 0.0001$. In re-hydrated and hypo-osmotically treated cells the number of time-frames was significantly higher compared to control cells ($p = 0.0016$ and $p = 0.0006$, respectively), but the variances were not significantly different at $p = 0.0540$ and $p = 0.0713$, respectively. There was no significant difference between the re-hydration and hypo-osmotic samples ($p = 0.6525$) and the variances were not significantly different ($p = 0.9885$).

4.4.2 Are these general abiotic stress responses?

To establish whether the morphology of the actin cytoskeleton observed when the cell has been plasmolysed is a general response of the network to any abiotic stress both GFP-FABD2 and GFP-Lifeact expressing *A. thaliana* mature abaxial epidermal pavement leaf cells were stressed using incubations at 37°C and 4°C (mock treatments were at room temperature; all conditions were incubated in ½ MS; see Section 2.6.3, pg. 48). It was hypothesised that if there is a general abiotic stress response then the network would be

similar to those in plasmolysed cells (Section 4.4.1, pg. 85). However, it should be noted that temperature stress, while abiotic, is not a physical stress like plasmolysis.

4.4.2.1 Qualitative assessment of GFP-FABD2 when confronted with an abiotic stress

Mature GFP-FABD2 stably-expressing *A. thaliana* plants were imaged using confocal microscopy over approximately two min. The experiment was carried out as described in Figure 4.2 (pg. 100).

Cells incubated for 30 min at each temperature (Figure 4.16-A, pg. 115) displayed a range of morphologies. Mock treated cells incubated at room temperature for one h appeared very similar to mock 30 min (at room temperature), although perhaps there were more bundles visible in more of the networks (Figure 4.17-A, pg. 116). A smaller proportion of cells incubated at 37°C for 30 min appeared to contain fewer bundles of filaments than the mock 30 minute cells (but with the proviso of a small ‘n’ number for this sample; Figure 4.16-B). Some of these cells also contained punctate structures around the network.

Cells incubated at 37°C for one h appeared similar to those incubated for 30 min, except that somehow they look disorganised: there did not appear to be higher order structures, as though the network was not connected as it should be (but again with the proviso of a small ‘n’ number for this sample Figure 4.17-B). These observations were the same when cells incubated at 37°C for one h were compared to mock 1 h cells.

Cells incubated at 4°C for 30 min appeared to contain more bundles than either mock 30 min or 37°C 30 min and they also seemed to have a lower level of fluorescence overall (Figure 4.16-C). Cells incubated at 4°C for one h looked more similar to mock one h than 4°C 30 min, although perhaps there were a higher proportion of cells that contained a lot of bundles (Figure 4.17-C). They certainly looked different to the cells incubated at 37°C for one h: there did not appear to be the same loss of organisation.

4.4.2.2 Pairwise image subtraction of GFP-FABD2 marked actin cytoskeleton during stress

Roughly 20 datasets per condition were compared using the Kruskal-Wallis test with Dunn’s Multiple Comparison test (a non-parametric ANOVA with post-test). Figure 4.18

(pg. 117) shows the mean bulk remodelling index of the GFP-FABD2 marked actin cytoskeleton; the mean $\pm$ standard deviation (arbitrary units, a.u.) were: mock 30 min 0.6295 ± 0.07563 , mock 1 h 0.6548 ± 0.08943 , 37°C 30 min 0.6382 ± 0.07369 , 37°C 1 h 0.6595 ± 0.09044 , 4°C 30 min 0.6498 ± 0.07553 and 4°C 1 h 0.6509 ± 0.09647 (see Appendix Table B.8).

The bulk remodelling index was significantly higher in the mock 1 h sample compared to the mock 30 min ($p < 0.001$), but there was no significant difference when the 37°C 30 min was compared to the 37°C 1 h, or when the 4°C 30 min was compared to the 4°C 1 h ($p > 0.05$). The bulk remodelling index of the 37°C 30 min compared to mock 30 min at room temperature showed no significant differences ($p > 0.05$), but 4°C 30 min was significantly higher compared to mock 30 min ($p < 0.001$). However, there were no significant differences when either the 37°C 1 h or the 4°C 1 h were compared to the mock 1 h ($p > 0.05$). There were also no significant differences in the bulk remodelling index when the 37°C 30 min was compared to the 4°C 30 min or the 37°C 1 h compared to the 4°C 1 h ($p > 0.05$).

4.4.2.3 Mean fluorescence intensity using Pairwise image subtraction of GFP-FABD2 marked actin cytoskeleton during stress

The same experimental datasets were used for this analysis as were used in the previous analysis; however, as detailed in Chapter 3 this analysis measures mean fluorescence intensity instead of whether fluorescence is present or absent. Figure 4.19 (pg. 118) shows the mean fluorescence intensity of the GFP-FABD2 marked actin cytoskeleton calculated as the mean and standard error of mean (no units as these are proportions): mock 30 min 0.4728 ± 0.0051 , mock 1 h 0.5409 ± 0.0062 , 37°C 30 min 0.5313 ± 0.0079 , 37°C 1 h 0.5455 ± 0.0091 , 4°C 30 min 0.5069 ± 0.0061 and 4°C 1 h 0.5278 ± 0.0065 (see Appendix Table B.9).

The mean fluorescence intensity was significantly higher in the mock 1 h at room temperature compared to mock 30 min ($p < 0.001$), but there was no significant difference when the 37°C 30 min was compared to the 37°C 1 h, or when the 4°C 30 min was compared to the 4°C 1 h ($p > 0.05$). The mean fluorescence intensity of both the 37°C 30 min and the 4°C 30 min samples were significantly higher compared to the mock 30 min ($p < 0.001$). However, there were no significant differences when either 37°C 1 h or the

4°C 1 h samples were compared to the mock 1 h ($p > 0.05$). There were also no significant differences when the 37°C 30 min was compared to the 4°C 30 min or the 37°C 1 h compared to the 4°C 1 h ($p > 0.05$).

4.4.2.4 Qualitative assessment of GFP-Lifeact when confronted with an abiotic stress

Mature GFP-Lifeact stably-expressing *A. thaliana* plants were used and abaxial leaf epidermal pavement cells were imaged using confocal microscopy over two min. The experiment was carried out as described in Figure 4.2 (pg. 100).

Mock treated cells that have been incubated for 30 min at room temperature generally showed a sparse and bundled network (Figure 4.20-A, pg. 119). Cells that have been mock treated for one h displayed morphologies similar to that of mock 30 minute cells (Figure 4.21-A, pg. 120).

Cells incubated at 37°C for 30 min appeared to have fewer bundles than mock 30 min at room temperature cells (Figure 4.20-B). A few punctate structures and acquosomes were visible (Smertenko *et al.*, 2010) (Figure 4.16-B, pg. 115). In addition it looked as though some of the networks were made up of ‘wavy’ or ‘kinky’ filaments (Figure 4.7-B, pg. 105). In cells incubated at 37°C for one h the filaments and bundles did not appear straight, they looked similar to the network seen in the cells at 37 °C for 30 min (Figure 4.21-B; Figure 4.17-B, pg. 116). Punctate structures were seen, but no acquosomes were noted, although perhaps that was due to the reduced levels of fluorescence.

Cells incubated at 4°C for 30 min appear extremely similar to mock 30 min at room temperature (Figure 4.20-C). Cells incubated at 4°C for one h appeared more bundled than those seen at 4°C for 30 min (Figure 4.21-C).

4.4.2.5 Cumulative fluorescence intensity of GFP-Lifeact marked actin cytoskeleton during abiotic stress

Figure 4.22 (pg. 121) and Figure 4.23 show the *Cumulative fluorescence intensity* for 20 time-frames for six treatments: mock 30 min at room temperature, mock 1 h at room temperature, 37°C for 30 min, 37°C for 1 h, 4°C for 30 min and 4°C for 1 h (approximately 25 datasets per condition).

Figure 4.24 (pg. 123) shows the average of each condition at every fluorescence intensity for the cells treated for 30 min and shown in Figure 4.22; Figure 4.25 shows the average of each condition at every fluorescence intensity for the cells treated for one h and shown in Figure 4.23 (see Appendix Table B.10 for values). The fluorescence intensity of the different treatments was graphed against the room temperature mock treatments (30 min and one h, respectively), having a value of one for every *Cumulative fluorescence intensity* point. To compare the treatments, one-way ANOVA was used with a Tukey post-test for each fluorescence intensity value, so that there were 19 tests. The p-value for every ANOVA can be seen in Table 4.2 (pg. 125), those in bold with one or more asterisks are significant. For the first and from the fourth to the ninth fluorescence intensity value, one-way ANOVA found no significant differences between treatments ($p > 0.05$).

At the second, third, tenth, 11th, 12th, 15th, 16th and 17th fluorescence intensity values the p-values given by the one-way ANOVA were significant, but using the Tukey post-test differences were found between conditions that cannot be properly compared, as they are not controlled for time of incubation (see Section 2.6.3). Using the Tukey post-test significant differences in comparable conditions were found at the 13th, 14th and 18th fluorescence intensity values between either the 1 h 4°C sample compared to the 1 h 37°C or the 30 min 4°C sample compared to the 30 min 37°C.

The proportion of each condition at the 19th fluorescence intensity value was calculated as the mean and standard deviation (no units as these are proportions): mock 30 min 0.0070 ± 0.0174 , mock 1 h 0.0056 ± 0.0118 , 37°C 30 min 0.0257 ± 0.0429 , 37°C 1 h 0.0166 ± 0.0497 , 4°C 30 minute 0.0047 ± 0.0089 and 4°C 1 h 0.0038 ± 0.0088 . There was a significant difference between treatments ($p = 0.0329$, one-way ANOVA), but the Tukey post-test revealed no significant differences between individuals treatments ($p > 0.05$).

The first and the last fluorescence intensity values showed no significant difference between the mock conditions and the temperature stressed conditions, which is where change is expected if there was a difference in the dynamics of the network (see Section 3.4.3.3, pg. 63). This means that there was not an increased or decreased amount of fluorescence in the intensity values that indicate change or rigidity in the network.

4.4.3 Does the same response occur with specific alteration of the cell wall?

To establish whether the morphology of the actin cytoskeleton observed when the cell has been plasmolysed is specific to plasmolysis or is a general response to the disruption of the cell wall-plasma membrane, cells were treated with isoxaben. Isoxaben is a pre-emergent herbicide that causes the cellulose synthase complexes (CSCs; see Section 1.3.3, pg. 23) to be removed from the plasma membrane, therefore no cellulose can be extruded into the cell wall (Paredes *et al.*, 2006).

Isoxaben was chosen as this specifically targets two of the cellulose synthase proteins within the cellulose-synthesising complex (see Section 1.3.3.1). GFP-FABD2 *A. thaliana* mature leaf abaxial epidermal pavement cells were treated with 20 μ M isoxaben or 0.01% DMSO as a mock treatment in $\frac{1}{2}$ MS (see Section 2.6.4, pg. 49).

4.4.3.1 Qualitative assessment of GFP-FABD2 marked actin cytoskeleton during isoxaben treatment

Mature GFP-FABD2 stably-expressing *A. thaliana* plants were imaged using confocal microscopy over approximately two min. The experiment was carried out as described in Figure 4.2 (pg. 100).

Mock treated cells (dimethyl sulfoxide (DMSO) treatment) at one h displayed a range of morphologies (Figure 4.26-A, pg. 126), but fewer cells had actin bundles and more cells displayed a meshwork (Figure 4.17, pg. 116). The cells that had been treated with isoxaben for one h appeared very similar to the cell mock treated with DMSO for one h, except that there were fewer cells displaying filamentous mesh-works than the mock one h (Figure 4.26-B).

Mock treated cells (DMSO) at four hs appeared similar to mock one h (DMSO) except that they seemed to have more bundles (Figure 4.26-C). Isoxaben-treated cells at four hs appeared similar to isoxaben-treated at one h and mock treated four hs, except that there appeared to be more cells exhibiting bundles (Figure 4.26-D).

4.4.3.2 Pairwise image subtraction of GFP-FABD2 marked actin cytoskeleton during isoxaben treatment

Approximately 30 datasets per condition were compared using the Kruskal-Wallis test with Dunn's Multiple Comparison test (Figure 4.27, pg. 127; a non-parametric ANOVA with post-test). The bulk remodelling index of the GFP-FABD2 marked actin cytoskeleton was calculated as the mean and standard deviation (arbitrary units, a.u.): mock 1 h was 0.6222 ± 0.05656 , 20 μM isoxaben-treated 1 h was 0.6103 ± 0.05670 , mock 4 hs was 0.6087 ± 0.05847 and 20 μM isoxaben-treated 4 hs was 0.5967 ± 0.04860 (see Appendix Table B.11). The bulk remodelling index of the actin cytoskeleton was significantly lower in the 20 μM isoxaben-treated 1 h sample and the mock 4 h compared to the mock 1 h ($p < 0.01$ and $p < 0.001$, respectively). The bulk remodelling index of the actin cytoskeleton was also significantly lower in the 20 μM isoxaben-treated 4 h sample compared to the mock 4 h and the 20 μM isoxaben-treated 1 h sample ($p < 0.001$ and $p < 0.01$, respectively).

4.4.3.3 Mean fluorescence intensity using Pairwise image subtraction of GFP-FABD2 marked actin cytoskeleton during isoxaben treatment

The same experimental datasets were used for this analysis as were used in the previous analysis; however, as detailed in Chapter 3 (Section 3.4.3.2, pg. 63) this analysis measures mean fluorescence intensity instead of whether fluorescence is present or absent. (Figure 4.28, pg. 128) shows the mean fluorescence intensity of the GFP-FABD2 marked actin cytoskeleton was calculated as the mean and standard error of mean (no units as these are proportions): mock 1 h was 0.4717 ± 0.0042 , 20 μM isoxaben-treated 1 h was 0.4542 ± 0.0055 , mock 4 hs was 0.4438 ± 0.0050 and 20 μM isoxaben-treated 4 hs was 0.4004 ± 0.0031 (see Appendix Table B.12). The mean fluorescence intensity of the actin cytoskeleton was significantly lower in the 20 μM isoxaben-treated 1 h sample and the mock 4 h compared to mock 1 h ($p < 0.01$ and $p < 0.001$, respectively). The mean fluorescence intensity of the actin cytoskeleton was significantly lower in the 20 μM isoxaben-treated 4 h sample compared to the mock 4 h and the 20 μM isoxaben-treated 1 h sample (both $p < 0.001$).

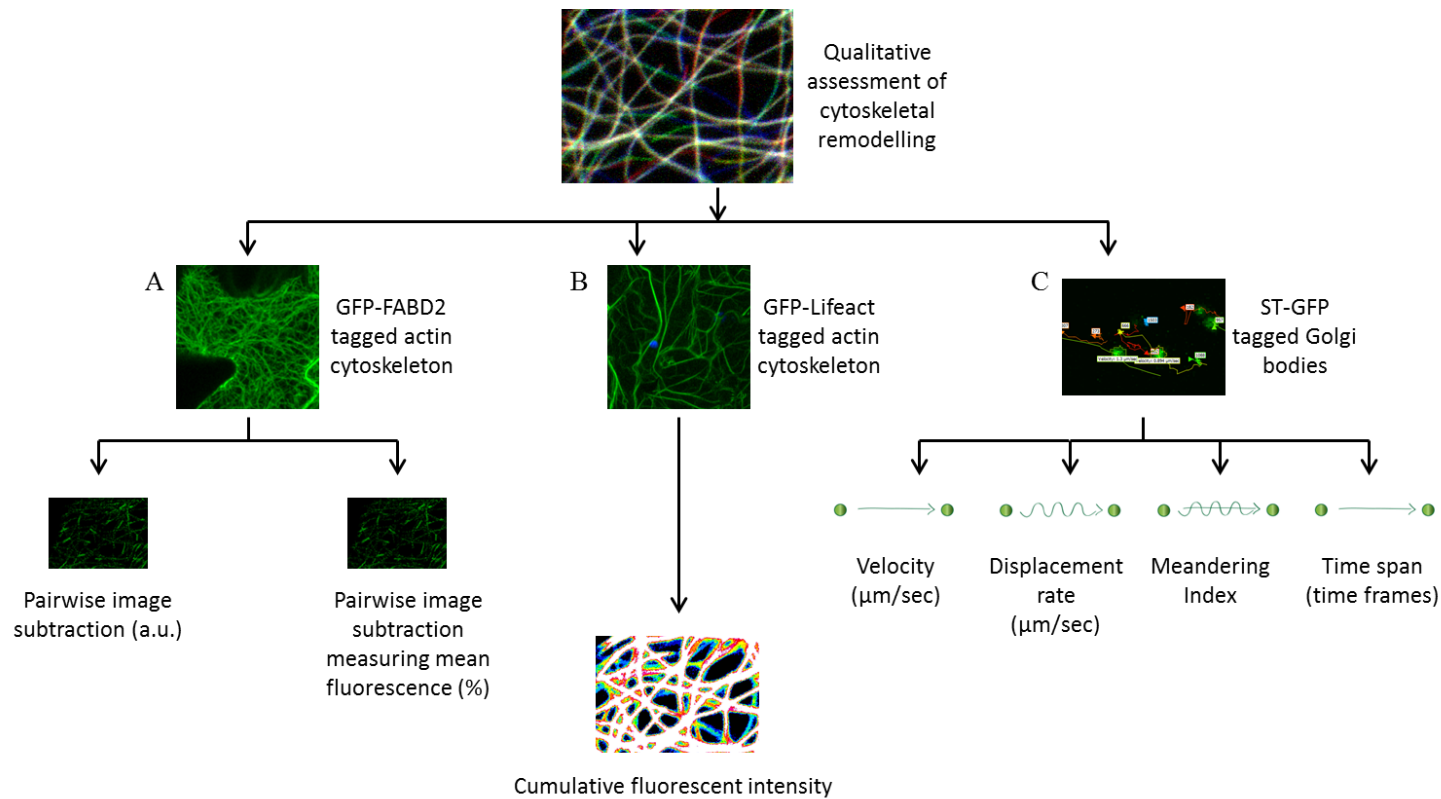


Figure 4.1 Workflow for the analysis employed to investigate the influence of the cell wall on the organisation and dynamics of the actin cytoskeleton.

(A) *Pairwise image subtraction* and *Pairwise image subtraction* measuring mean fluorescence were used to analyse the GFP-FABD2 tagged cytoskeleton in the experiments outlined in Figure 4.2. (B) *Cumulative fluorescent intensity* was used to analyse the GFP-Lifeact tagged cytoskeleton in the experiments outlined in Figure 4.2. (C) Volocity software was used to quantify the motility of ST-GFP tagged Golgi bodies by measuring their velocity, displacement rate, meandering index and the number of frames they remained in view in the experiments outlined in Figure 4.2.

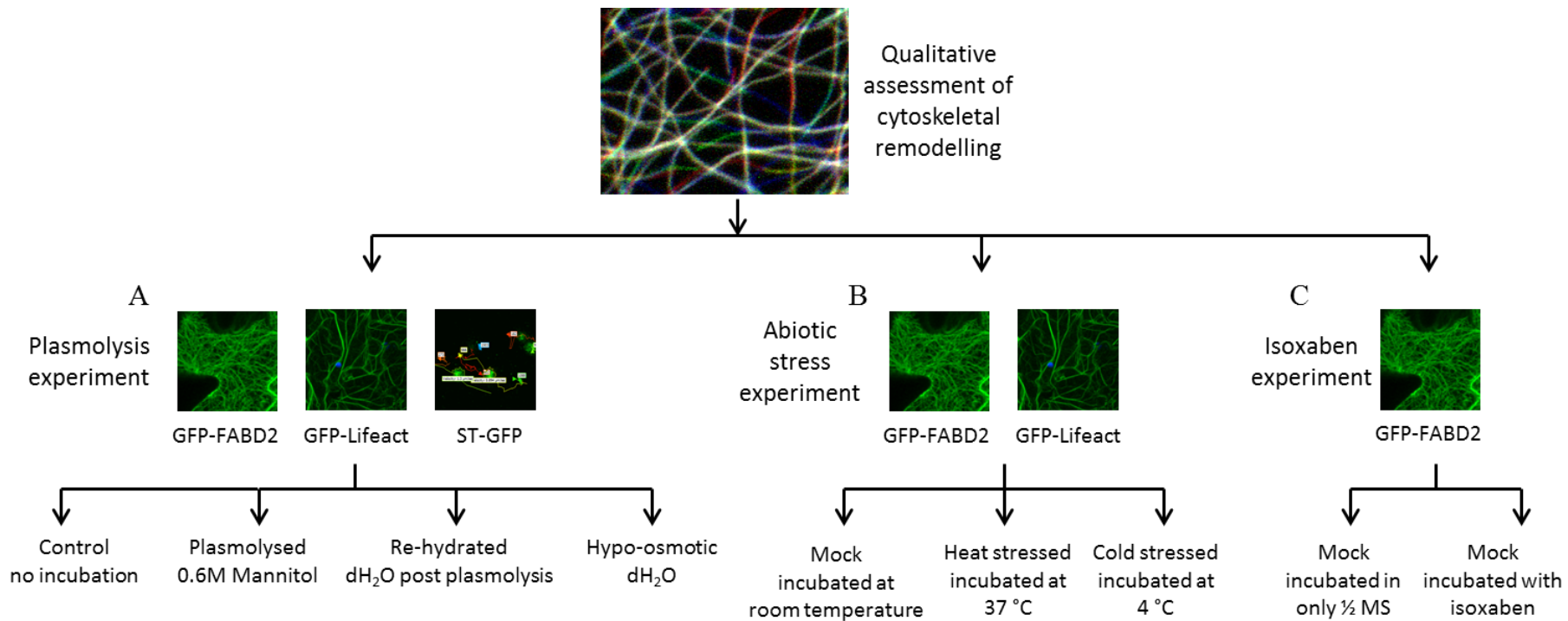


Figure 4.2 Workflow for investigating the influence of the cell wall on the organisation and dynamics of the actin cytoskeleton.

(A) The plasmolysis experiment was carried out using three different markers and four different conditions to image the actin cytoskeleton (or Golgi bodies) when the cytoplasm is physically separated from the cell wall via plasmolysis and when it was appressed to it (Section 4.4.1, pg. 85). (B) The abiotic stress experiment was carried out using two different markers and three different conditions to image the actin cytoskeleton when it was abiotically stressed by high or low temperatures (Section 4.4.2, pg. 92).

(C) The isoxaben experiment was carried out using one marker and two different conditions to image the actin cytoskeleton when treated with isoxaben to affect cell-wall structure (Section 4.4.3, pg. 97).

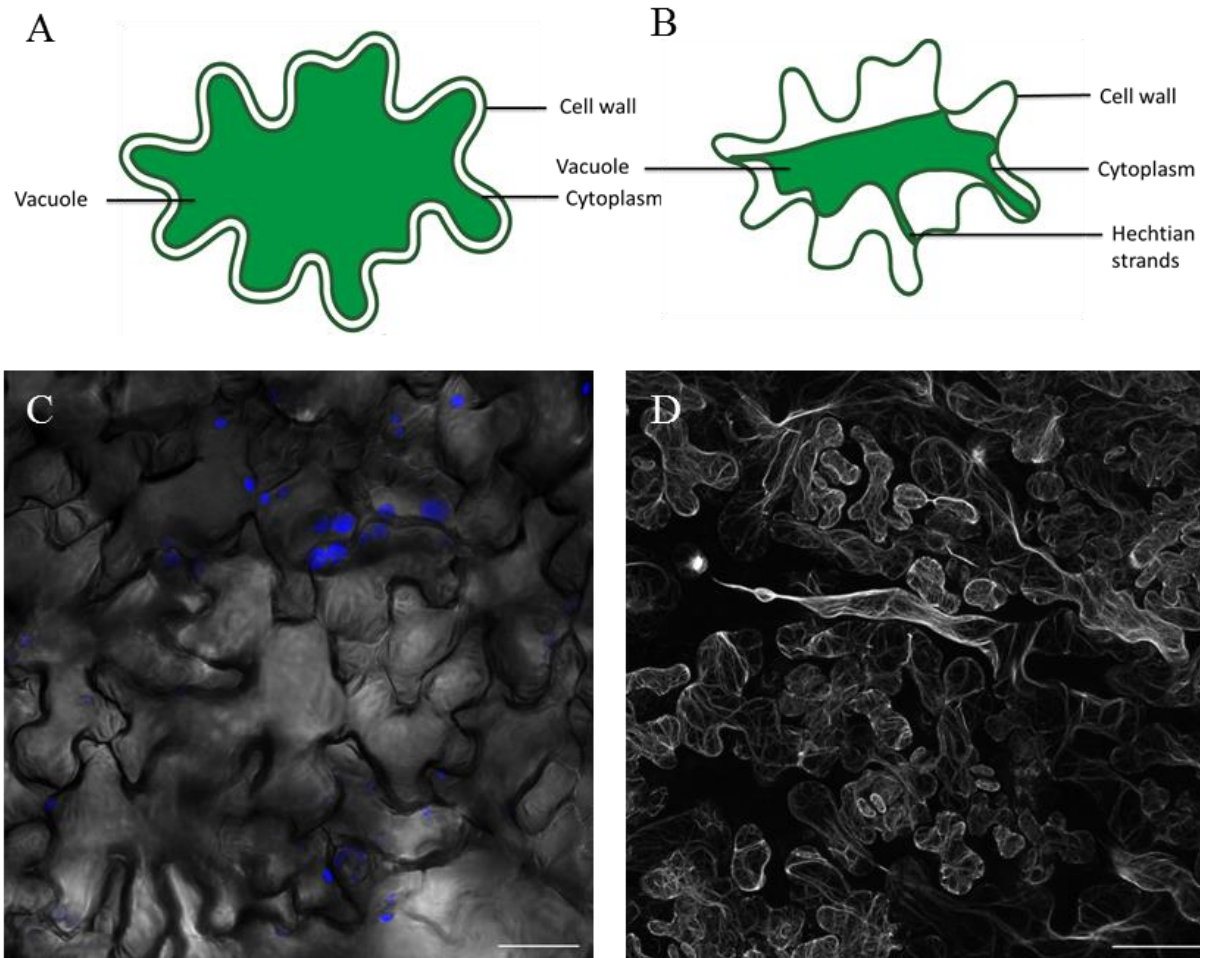


Figure 4.3 Plasmolysis schematic and plasmolysis in mature *A. thaliana* epidermal pavement leaf cells.

(A) An epidermal pavement leaf cell under normal hydration conditions. The protoplasm, i.e. the plasma membrane and cellular contents (green), is tightly appressed to the cell wall.

(B) An epidermal pavement leaf cell is altered during plasmolysis. The protoplasm (green) pulls away from the cell wall as the turgor pressure in the vacuole decreases. The separation is not complete as connections (Hechtian strands) (Oparka, 1994) remain attached to the cell wall.

(C) Epidermal pavement leaf cells undergoing the effects of plasmolysis. The 'crinkled' appearance of the cell is caused by cell wall buckling inward due to the loss of turgor pressure in the vacuole. Scale bar = 20 μm .

(D) Epidermal pavement leaf cells from a transgenic line of *A. thaliana* stably expressing GFP-FABD2. The actin cytoskeleton is present in the cortical cytoplasm just inside the plasma membrane, so it acts as a marker of the protoplasm shrinkage. Scale bar = 50 μm .

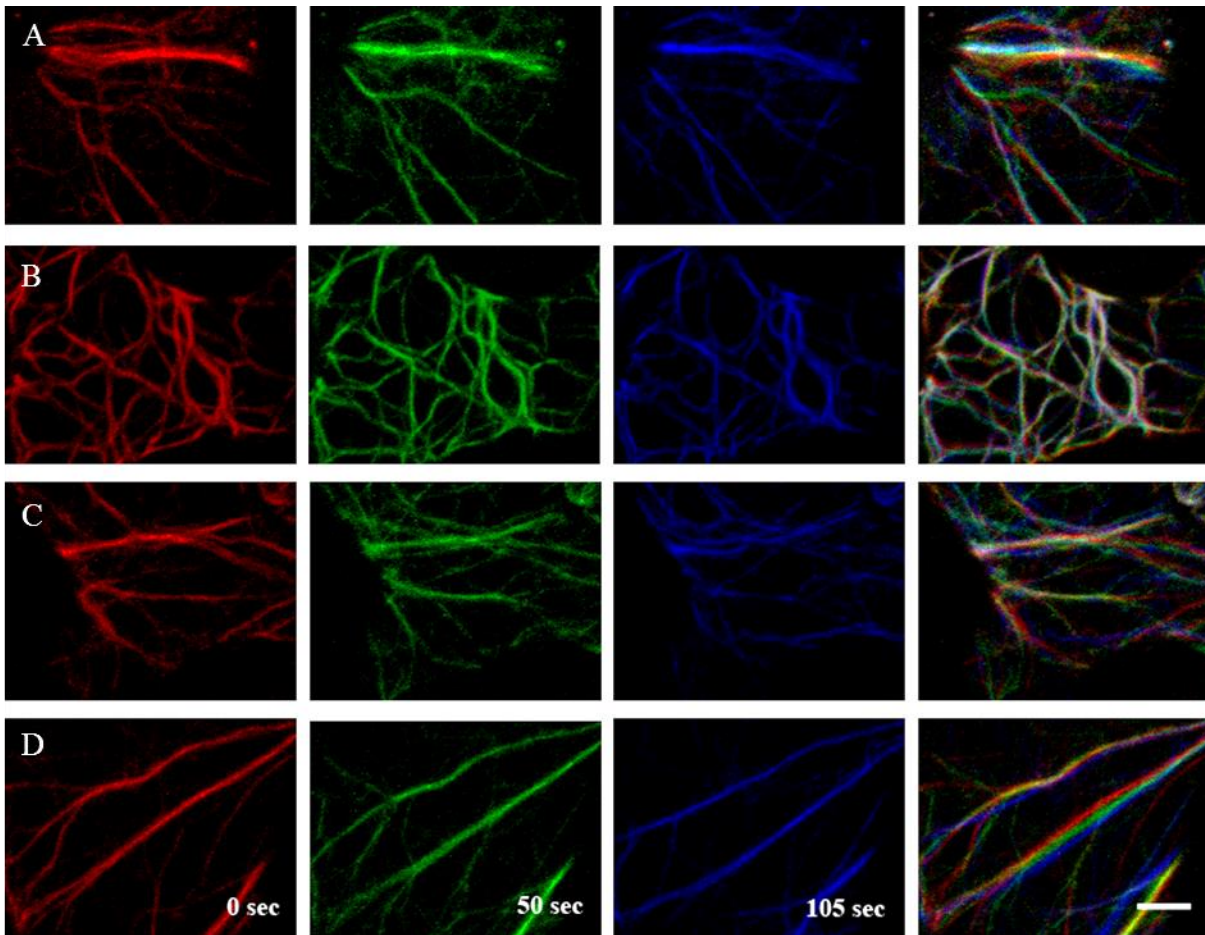


Figure 4.4 Time-lapse three dimensional CLSM images of GFP-FABD2.

Mature epidermal pavement leaf cells from a transgenic line of *A. thaliana* stably expressing GFP-FABD2. The red, green and blue images are 3D projections of a single time-point from a time-series. On the far right is each cell with its corresponding three time-points overlaid: in each row the first time-point is red (0 s), the middle time-point is green (50 s) and the last time-point is blue (105 s). In the overlay when the filaments have moved they appear colourful, but when they do not move they appear white as the colours are superimposed.

(A) Non-treated control. (B) Plasmolysed sample, incubated in 0.6 M mannitol for 30 min. (C) Re-hydrated sample: incubated in 0.6 M mannitol for 30 min and then removed, washed in water and then incubated in water for a further 30 min to increase turgor pressure again. (D) Hypo-osmotic sample: incubated in water for 30 min.

Scale bar = 5 μ m.

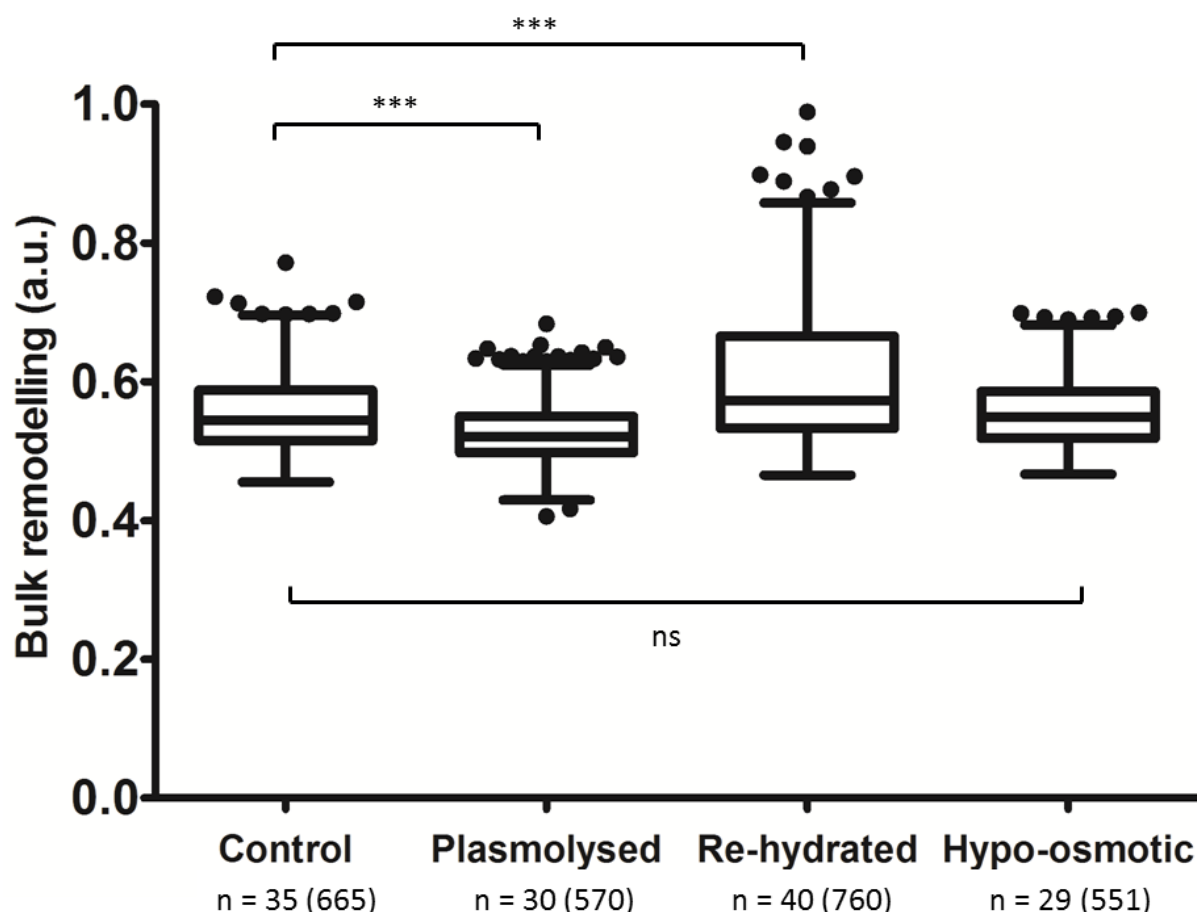


Figure 4.5 Results from *Pairwise image subtraction* analysis of cells expressing GFP-FABD2 with different treatments.

The boxplots show the data for epidermal pavement leaf cells from a transgenic line of *A. thaliana* stably expressing GFP-FABD2 under the four experimental conditions shown in Figure 4.4 and analysed using the *Pairwise image subtraction* method (see Section 3.4.3.1, pg. 60). The mean and standard deviation (arbitrary units, a.u.): control was 0.5558 ± 0.0549 , plasmolysed was 0.5273 ± 0.0441 , re-hydrated was 0.6013 ± 0.0885 and hypo-osmotic was 0.5558 ± 0.0487 . The value 'n' is the number of cells imaged and the number in parentheses the total of the 19 comparisons made per cell. The results are detailed in Appendix Table B.1. The asterisks refer to the p-value: ***p < 0.001 and 'ns' indicates no significant difference; only p-values relating to the control are shown on the figure.

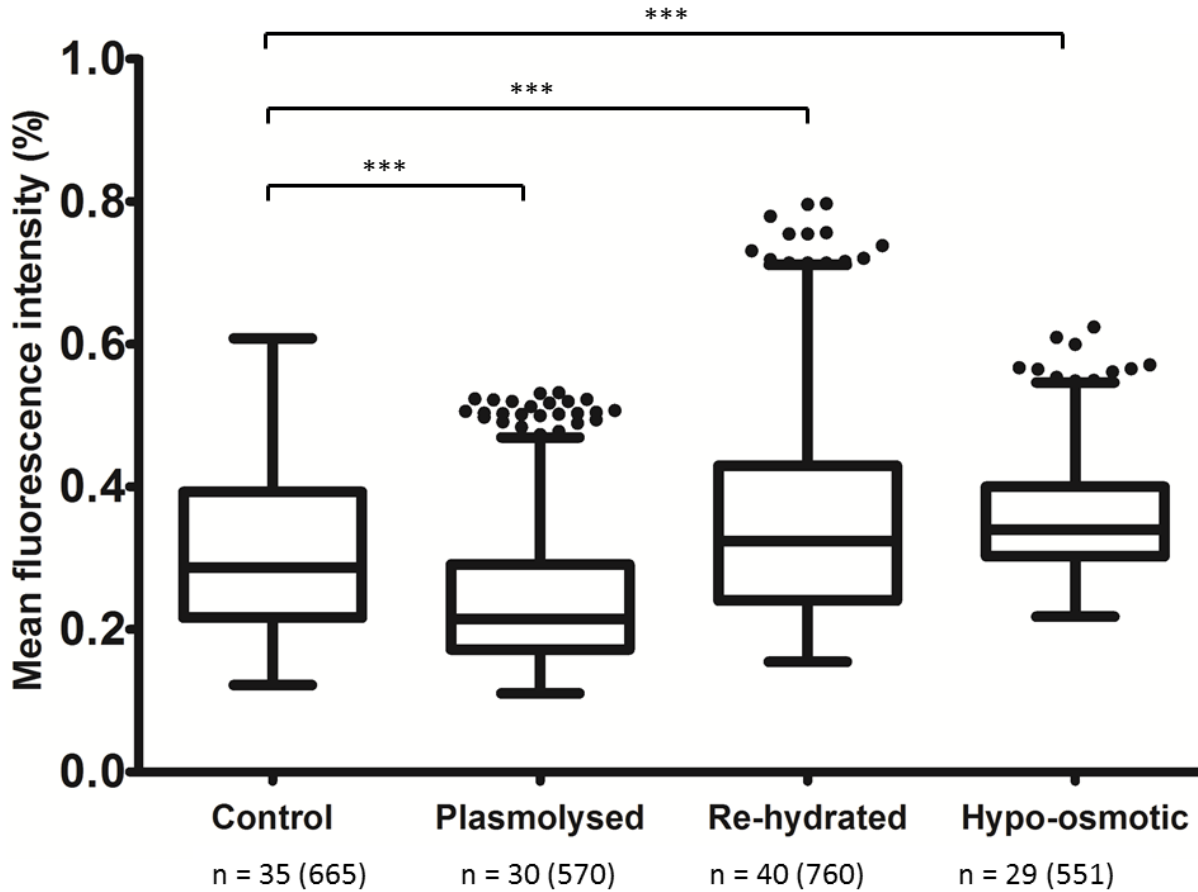


Figure 4.6 Results from *Pairwise image subtraction* measuring mean fluorescence analysis of cells expressing GFP-FABD2 with different treatments.

The boxplots show the data for epidermal pavement leaf cells from a transgenic line of *A. thaliana* stably expressing GFP-FABD2 under the four experimental conditions shown in Figure 4.4 and analysed using the *Pairwise image subtraction* method to measure mean fluorescence intensity (see Section 3.4.3.2). The mean and standard error of mean (no units as these are proportions): the control was 0.3132 ± 0.0046 , plasmolysed was 0.2432 ± 0.0039 , re-hydrated was 0.3491 ± 0.0053 and hypo-osmotic was 0.3558 ± 0.0034 . The value 'n' is the number of cells imaged and the number in parentheses the total of the 19 comparisons made per cell. The results are detailed in Appendix Table B.2. The asterisks refer to the p-value: ***p < 0.001; only p-values relating to the control are shown on the figure.

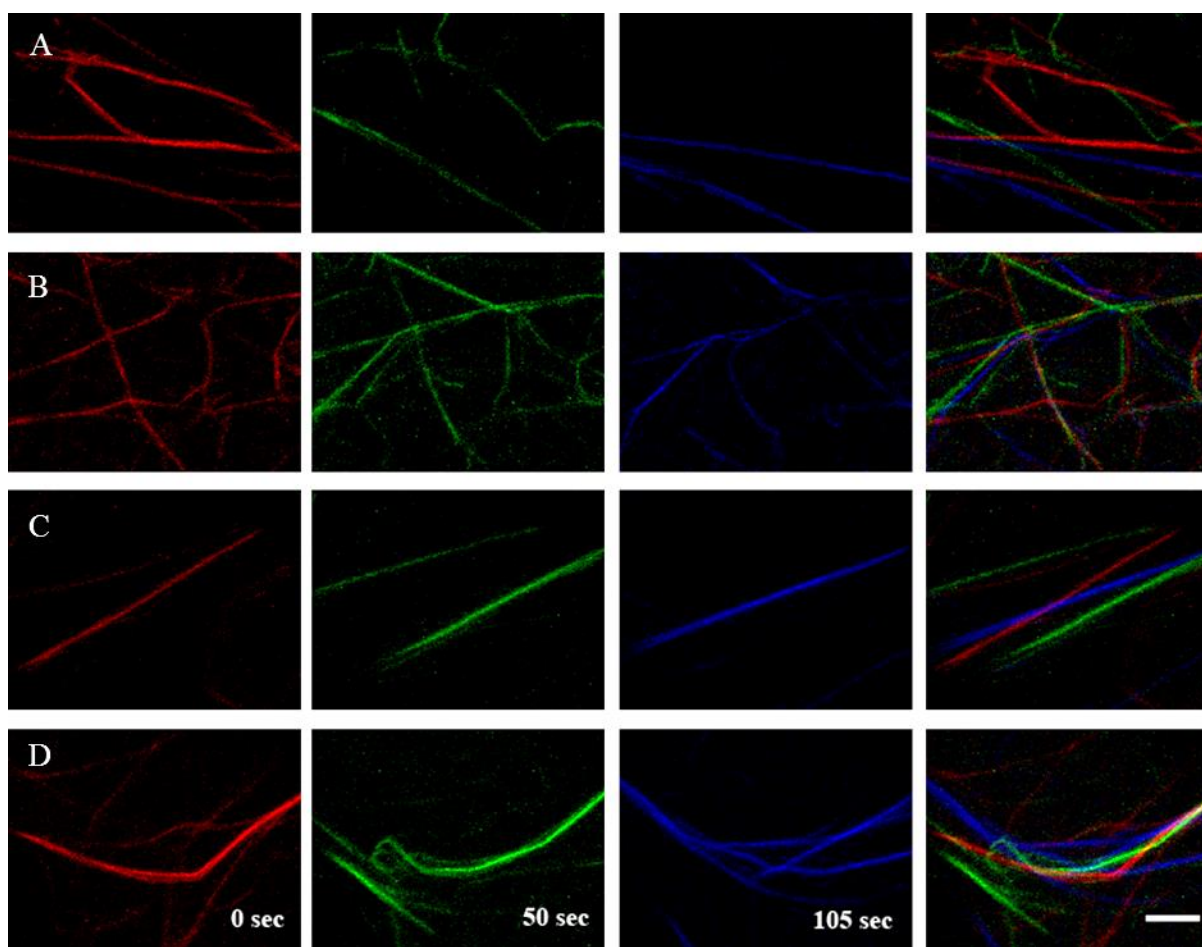


Figure 4.7 Time-lapse three dimensional CLSM images of GFP-Lifeact in cells with different treatments.

Mature epidermal pavement leaf cells from a transgenic line of *A. thaliana* stably expressing GFP-Lifeact. The red, green and blue images are 3D projections of a single time-point from a time-series. On the far right is each cell with its corresponding three time-points overlaid: in each row the first time-point is red (0 s), the middle time-point is green (50 s) and the last time-point is blue (105 s). In the overlay when the filaments have moved they appear colourful, but when they do not move they appear white as the colours are superimposed.

(A) Non-treated control. (B) Plasmolysed sample, incubated in 0.6 M mannitol for 30 min. (C) Re-hydrated sample: incubated in 0.6 M mannitol for 30 min and then removed, washed in water and then incubated in water for a further 30 min to increase turgor pressure again. (D) Hypo-osmotic sample: incubated in water for 30 min.

Scale bar = 5 μ m.

In Figure 4.8 and Figure 4.9 the cells shown here are denoted with an asterisk: control cell 10; plasmolysed cell 10; re-hydrated cell 11; and hypo-osmotic cell 17.

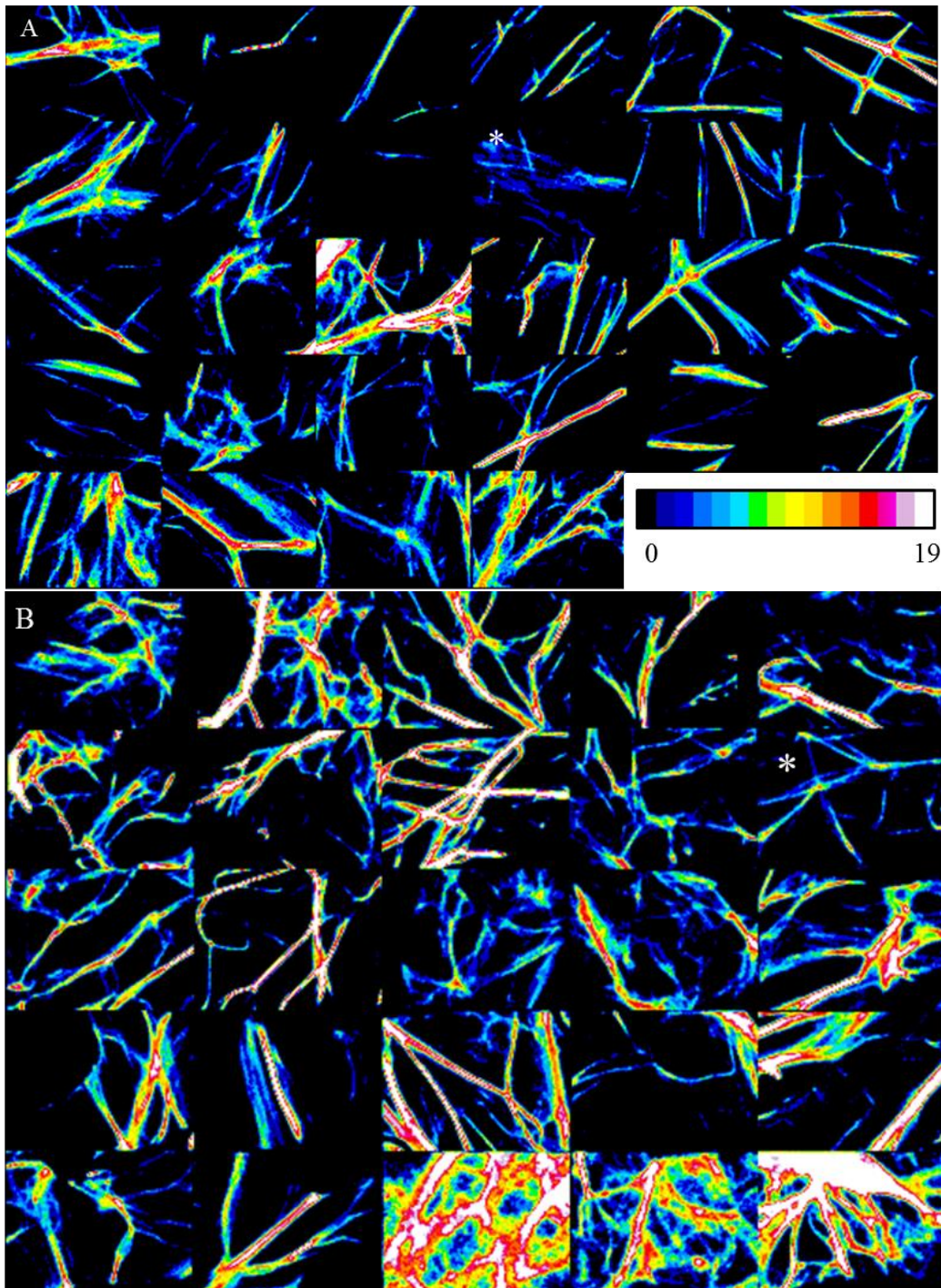


Figure 4.8 Visual results from the *Cumulative fluorescence intensity* analysis on cells with different treatments expressing GFP-Lifeact.

Heat map shows the fluorescence intensity value per pixel, where white represents stationary filaments and darker colours represent lots of movement.

(A) 28 different control cells that had no treatment. (B) 25 cells plasmolysed in 0.6 M mannitol for 30 min. These heat maps were constructed as described in Section 3.4.3.3, pg. 63. Asterisks denote cells shown in Figure 4.7.

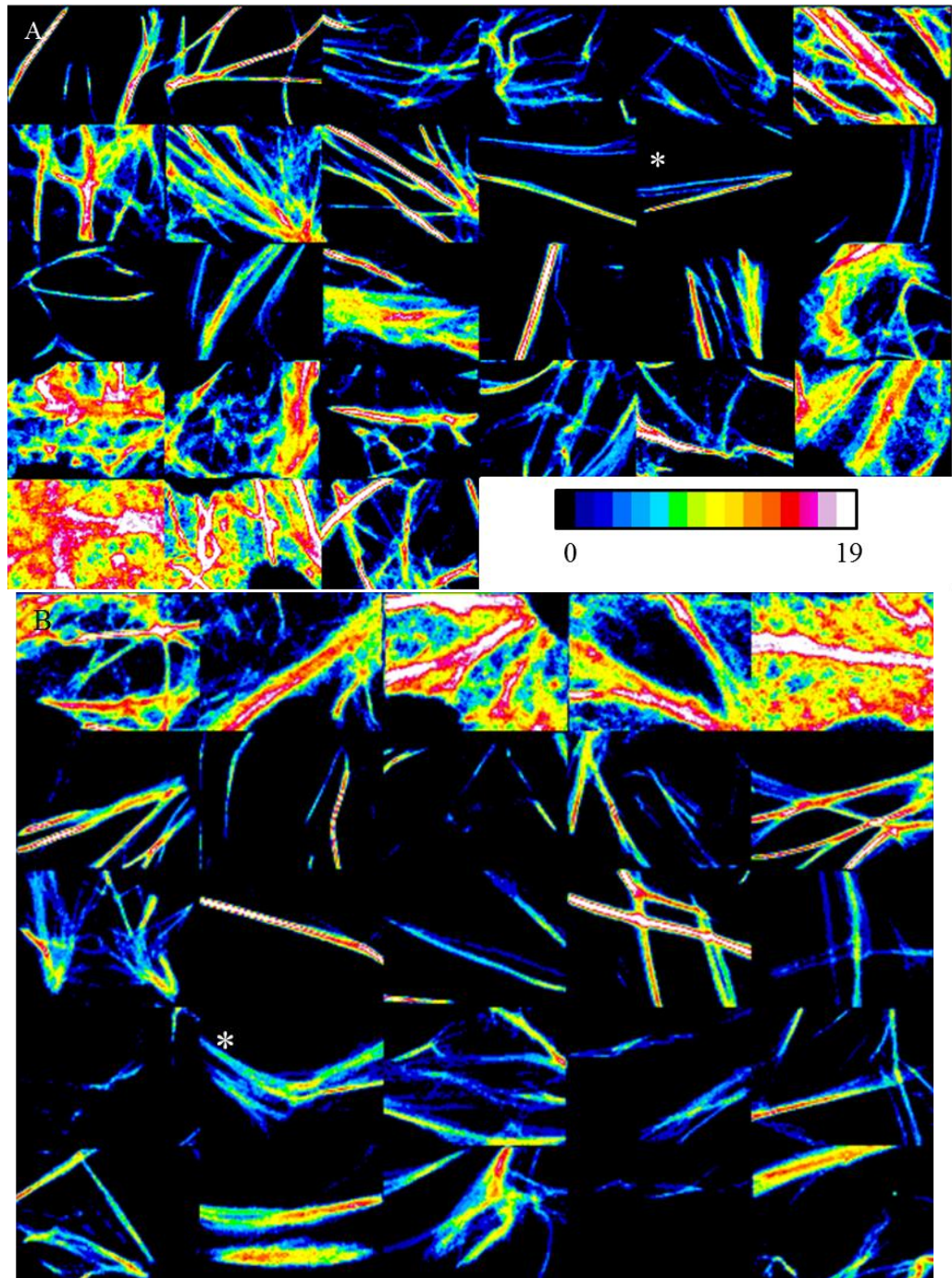


Figure 4.9 Visual results from the *Cumulative fluorescence intensity* analysis on cells with different treatments expressing GFP-Lifeact.

Heat map shows the fluorescence intensity value per pixel, where white represents stationary filaments and darker colours represent lots of movement.

(A) 27 cells plasmolysed in 0.6 M mannitol for 30 min and then incubated for a further 30 min in dH₂O. (B) 25 cells incubated for 30 min in dH₂O. These heat maps were constructed as described in Section 3.4.3.3. Asterisks denote cells shown in Figure 4.7.

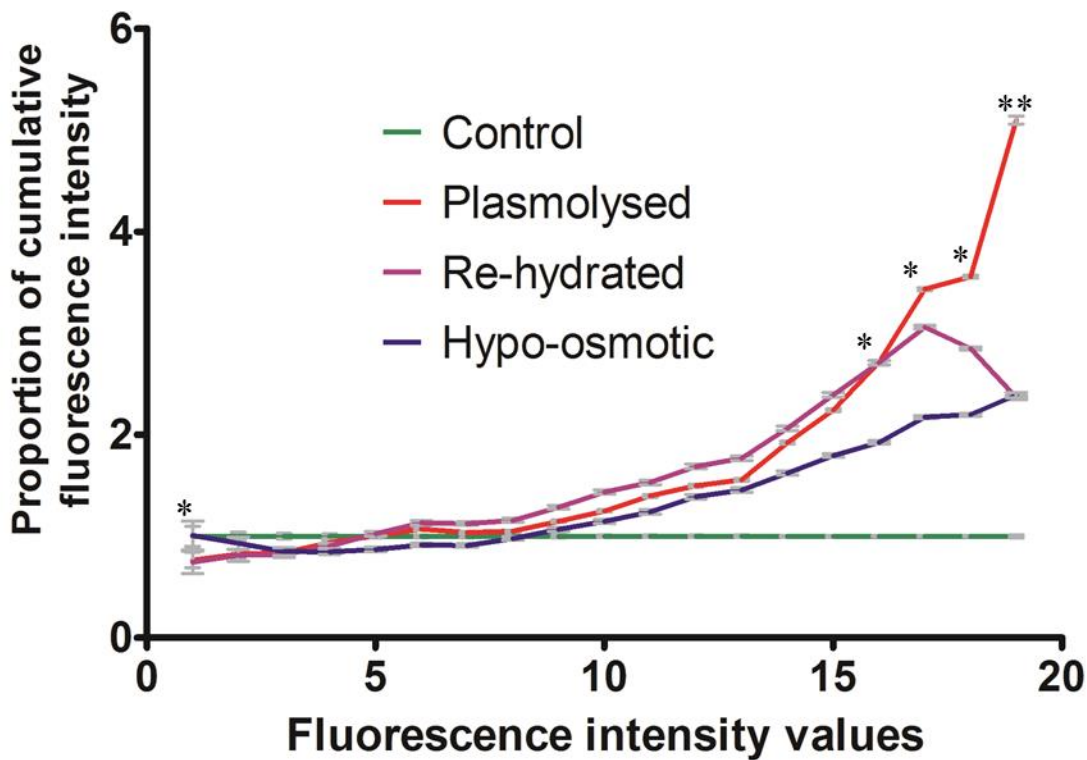


Figure 4.10 Results from *Cumulative fluorescence intensity* analysis of cells expressing GFP-Lifeact with different treatments.

Bulk remodelling of the actin cytoskeleton marked with GFP-Lifeact under the four experimental conditions: control in green with values normalised to '1' (n = 28 cells), plasmolysed in red (n = 25 cells), re-hydrated in purple (n = 27 cells) and hypo-osmotic in blue (n = 25 cells). This graph was constructed using the analysis from the *Cumulative fluorescence intensity* method, the error bars are standard deviation. The results are detailed in Appendix Table B.3. The asterisks refer to the differences between control and plasmolysed samples: *p < 0.05 and **p < 0.01, see Table 4.1 (below). All conditions are expressed as a ratio compared to the control, hence the control itself is always one.

Table 4.1 The p-value for every cumulative fluorescence intensity value analysed using one-way ANOVA.

Fluorescence intensity values of cells with different treatments expressing GFP-Lifeact that have been analysed using the *Cumulative fluorescence intensity* method (see Appendix Table B.3). The asterisks refer to the p-values: *p < 0.05 and **p < 0.01.

Fluorescence intensity value	p value from one-way ANOVA	p value from Tukey post-test
1	0.0213 *	
2	0.0715	
3	0.0567	
4	0.1993	
5	0.1592	
6	0.0578	
7	0.1229	
8	0.3639	
9	0.2021	
10	0.1053	
11	0.1264	
12	0.1777	
13	0.1646	
14	0.0967	
15	0.0724	
16	0.0461 *	
17	0.0192 *	Control vs plasmolysed *
18	0.0209 *	Control vs plasmolysed *
19	0.0093 **	Control vs plasmolysed **

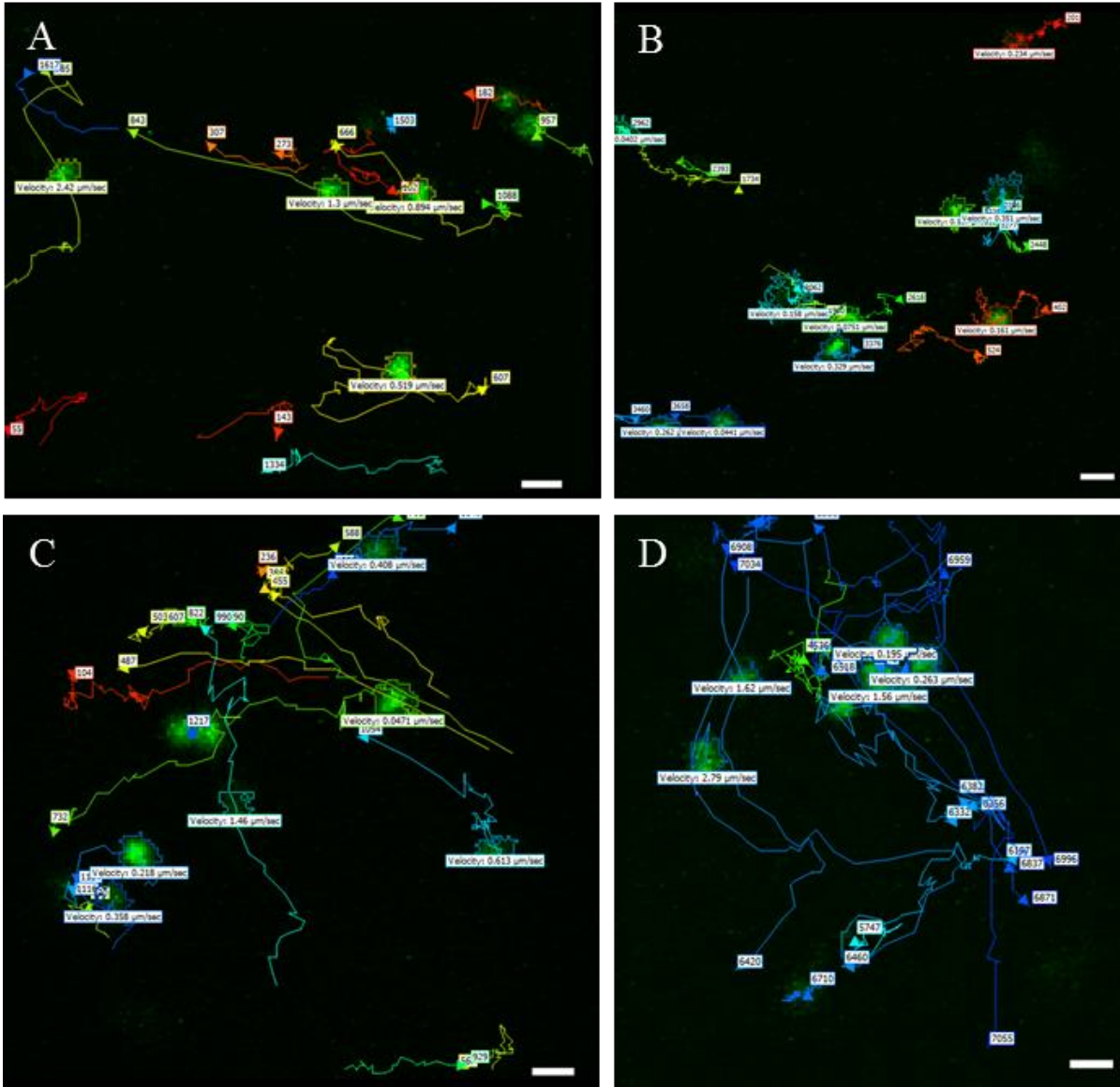


Figure 4.11 Full length tracks of ST-GFP tagged Golgi bodies in cells annotated with their velocity in the pictured time-point with different treatments.

Epidermal pavement leaf cells from a transgenic line of *A. thaliana* stably expressing ST-GFP.

(A) Control cells that had no incubation; (B) cells plasmolysed in 0.6 M mannitol for 30 min; (C) cells plasmolysed in 0.6 M mannitol for 30 min and then incubated for a further 30 min in dH₂O; (D) cells incubated for 30 min in dH₂O.

The tracks are labelled with numbers and the velocity of each Golgi body currently in view is shown.

Scale bar = 1.5 μm and the total elapsed time was 92 s.

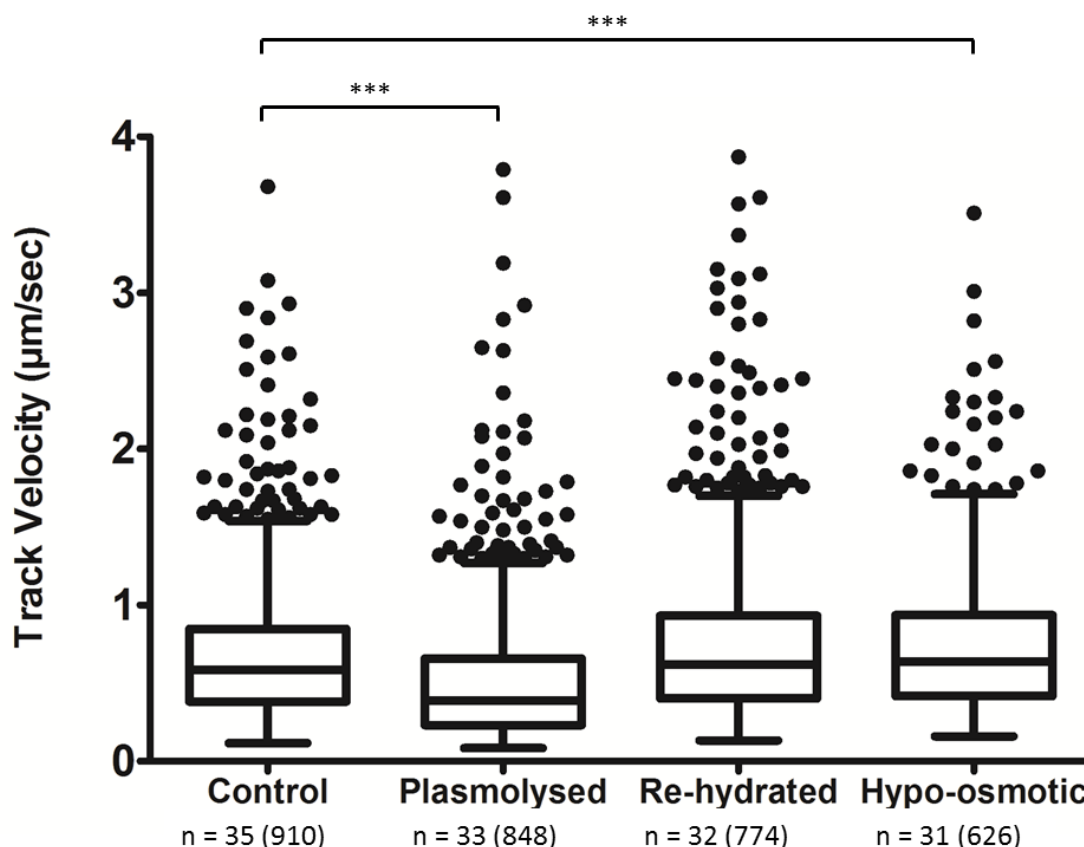


Figure 4.12 Average velocities of Golgi bodies in cells expressing ST-GFP with different treatments.

Track velocity of Golgi bodies in mature epidermal pavement leaf cells from a transgenic line of *A. thaliana* stably expressing ST-GFP under the four experimental conditions. The track velocities have been analysed using Volocity and the results are detailed in Appendix Table 5.4. The average velocity of Golgi bodies tagged with ST-GFP was calculated as mean and standard error of mean (µm/s): control was 0.6856 ± 0.0149 , plasmolysed was 0.5204 ± 0.0151 , re-hydrated was 0.7651 ± 0.0195 and hypo-osmotic was 0.7427 ± 0.0181 . The number of cells imaged is indicated on the plot and the total number of Golgi bodies is indicated in the following parentheses. The asterisks refer to the p-value: *** $p < 0.001$; only p-values relating to the control are shown on the figure.

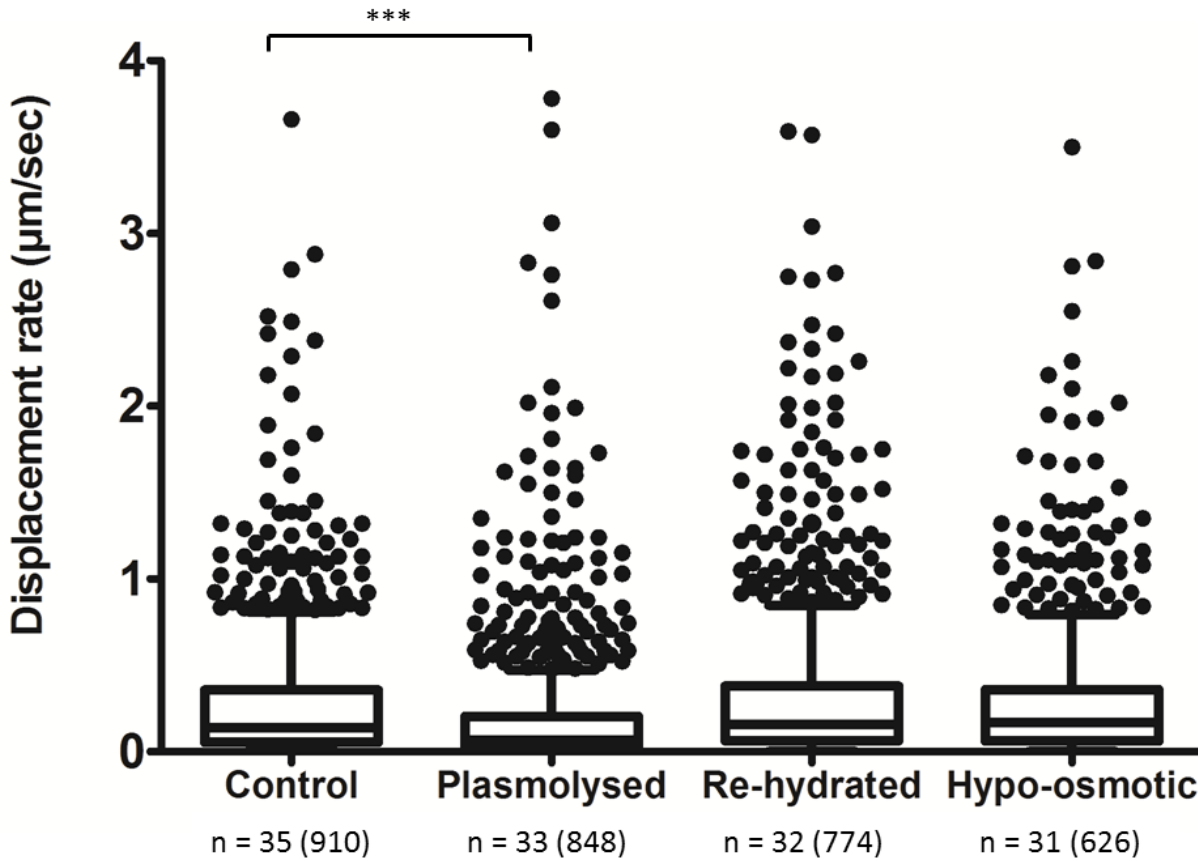


Figure 4.13 Average displacement rate of Golgi bodies in cells expressing ST-GFP with different treatments.

Displacement rate of Golgi bodies in mature epidermal pavement leaf cells from a transgenic line of *A. thaliana* stably expressing ST-GFP under the four experimental conditions. The displacement rates have been analysed using Volocity and the results are detailed in Appendix Table B.5. The displacement rate of ST-GFP calculated as the mean and standard error of mean (µm/s): control was 0.2839 ± 0.0130 , plasmolysed was 0.2087 ± 0.0137 , re-hydrated was 0.3431 ± 0.0177 and hypo-osmotic was 0.3130 ± 0.0171 . The number of cells imaged is detailed on the graph and in the following parentheses is the total number of Golgi bodies. The asterisks refer to the p-value: *** $p < 0.001$; only p-values relating to the control are shown on the figure.

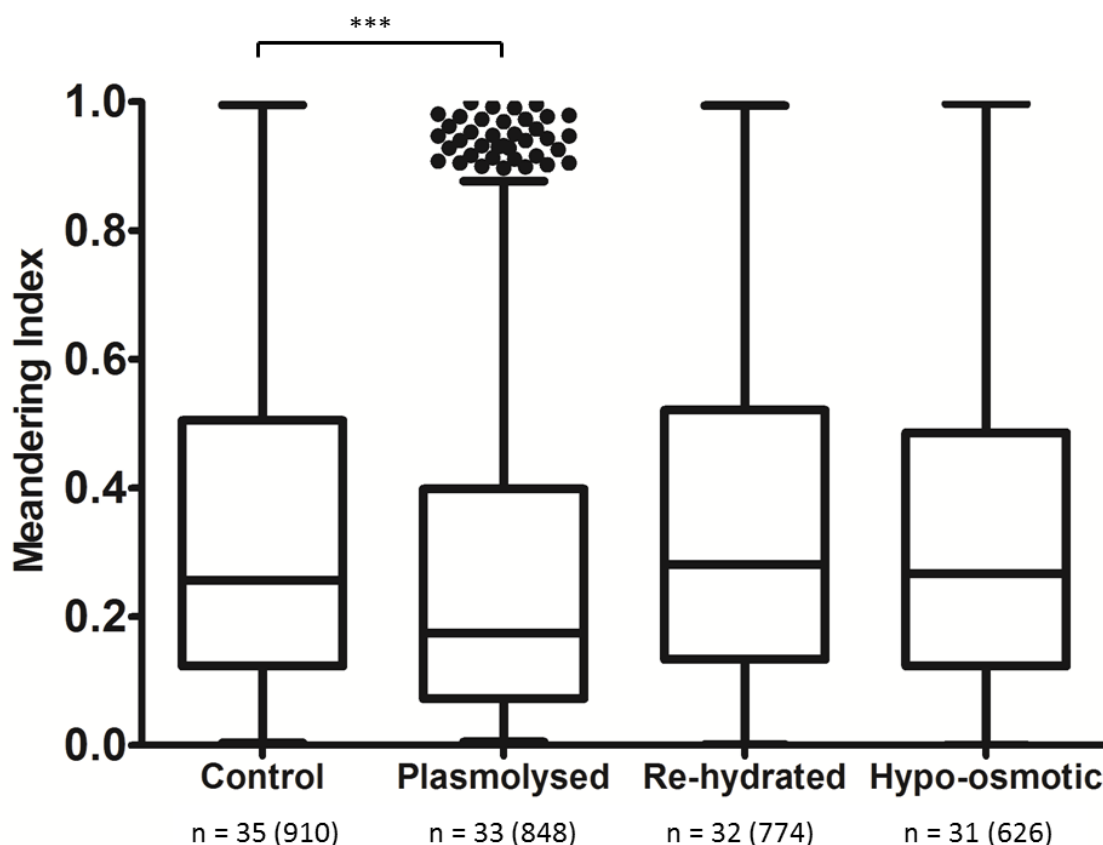


Figure 4.14 Meandering index of Golgi bodies in cells expressing ST-GFP with different treatments.

Meandering index of Golgi bodies in mature epidermal pavement leaf cells from a transgenic line of *A. thaliana* stably expressing ST-GFP under the four experimental conditions. The meandering index of ST-GFP calculated as the mean and standard deviation (no units as this is an index): control was 0.3305 ± 0.2529 , plasmolysed was 0.2762 ± 0.2608 , re-hydrated was 0.3517 ± 0.2651 and hypo-osmotic was 0.3357 ± 0.2587 . The meandering indices have been analysed using Volocity and the results are detailed in Appendix Table B.6. The number of cells imaged is detailed on the graph and in the following parentheses is the total number of Golgi bodies. The asterisks refer to the p-value: *** $p < 0.001$; only p-values relating to the control are shown on the figure.

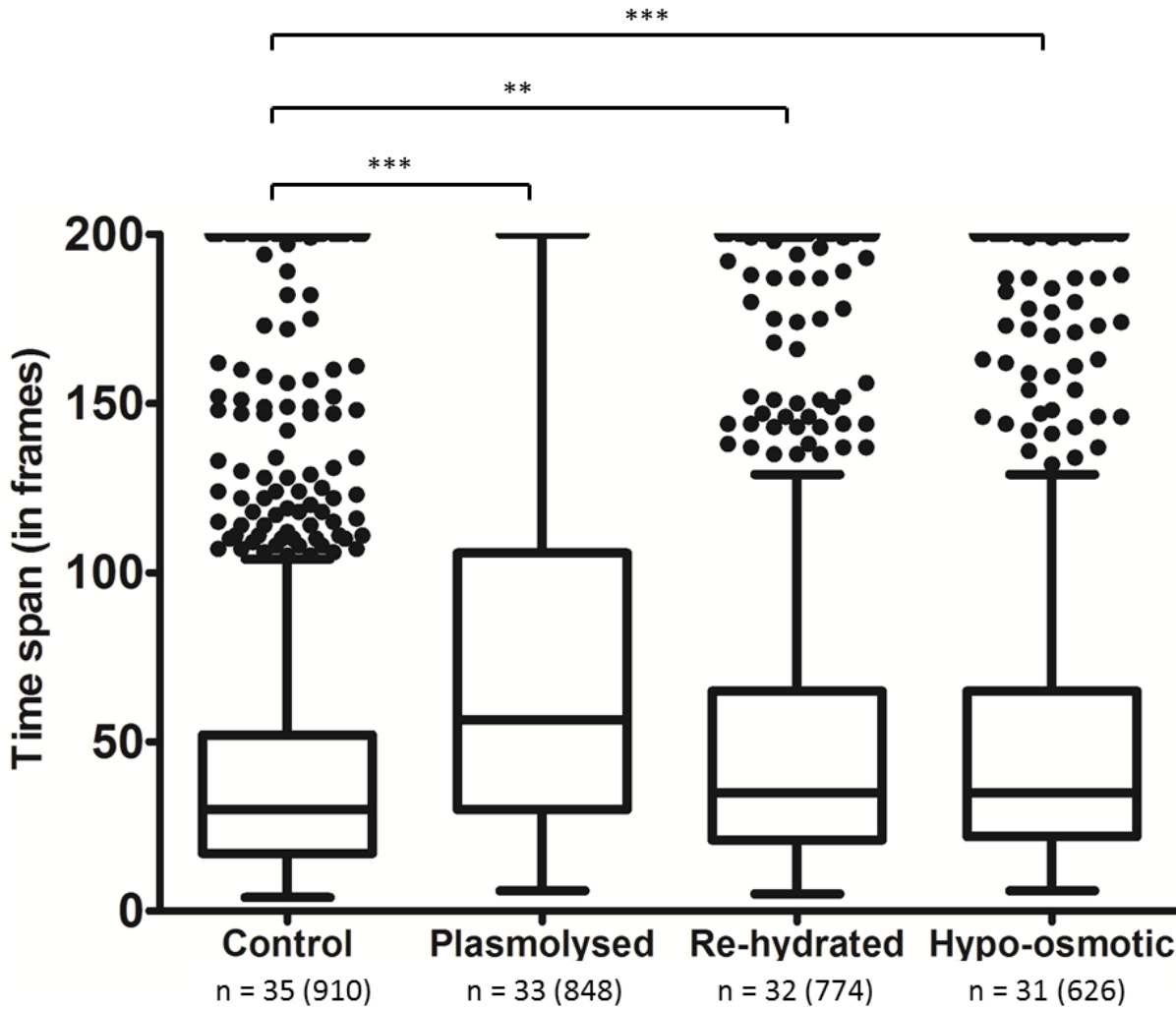


Figure 4.15 Time span that Golgi bodies remain in the field of view in cells expressing ST-GFP with different treatments.

The length of time that Golgi bodies are present in view in mature epidermal pavement leaf cells from a transgenic line of *A. thaliana* stably expressing ST-GFP under the four experimental conditions. The time span of ST-GFP calculated as the mean and standard deviation (units are time-frames): control was 45.52 ± 44.14 , plasmolysed was 75.54 ± 58.18 , re-hydrated was 52.59 ± 47.17 and hypo-osmotic was 53.73 ± 47.14 . The time spans have been analysed using Volocity and the results are detailed in Appendix Table B.7. The number of cells imaged is detailed on the graph and in the following parentheses is the total number of Golgi bodies. The asterisks refer to the p-value: ** $p < 0.01$ and *** $p < 0.001$; only p-values relating to the control are shown on the figure.

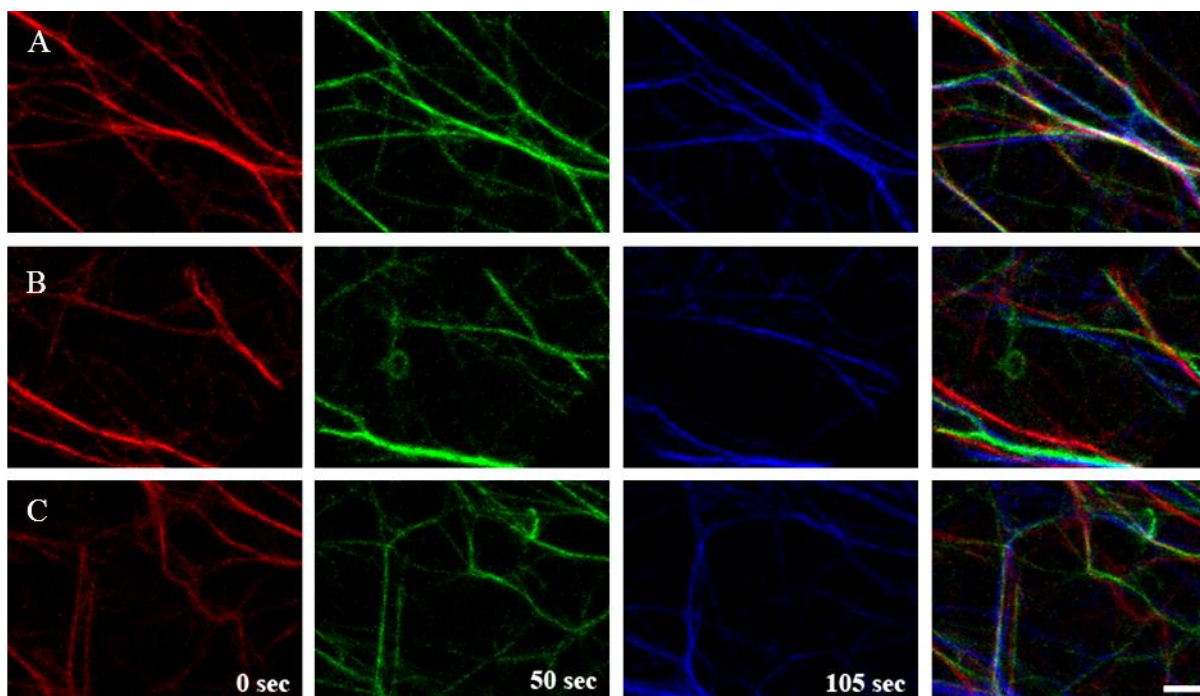


Figure 4.16 Time-lapse three dimensional CLSM images of GFP-FABD2 in cells that have been temperature stressed for 30 min.

Mature epidermal pavement leaf cells from a transgenic line of *A. thaliana* stably expressing GFP-FABD2 shown in confocal micrographs. The red, green and blue images are 3D projections of a single time-point from a time-series. On the far right is each cell with its corresponding three time-points overlaid: in each row the first time-point is red (0 s), the middle time-point is green (50 s) and the last time-point is blue (105 s). In the overlay when the filaments have moved they appear colourful, but when they do not move they appear white as the colours are superimposed.

(A) Mock sample incubated in $\frac{1}{2}$ MS for 30 min at room temperature.

(B) Sample incubated in $\frac{1}{2}$ MS at 37°C for 30 min.

(C) Sample incubated in $\frac{1}{2}$ MS at 4°C for 30 min.

Scale bar = 5 μ m.

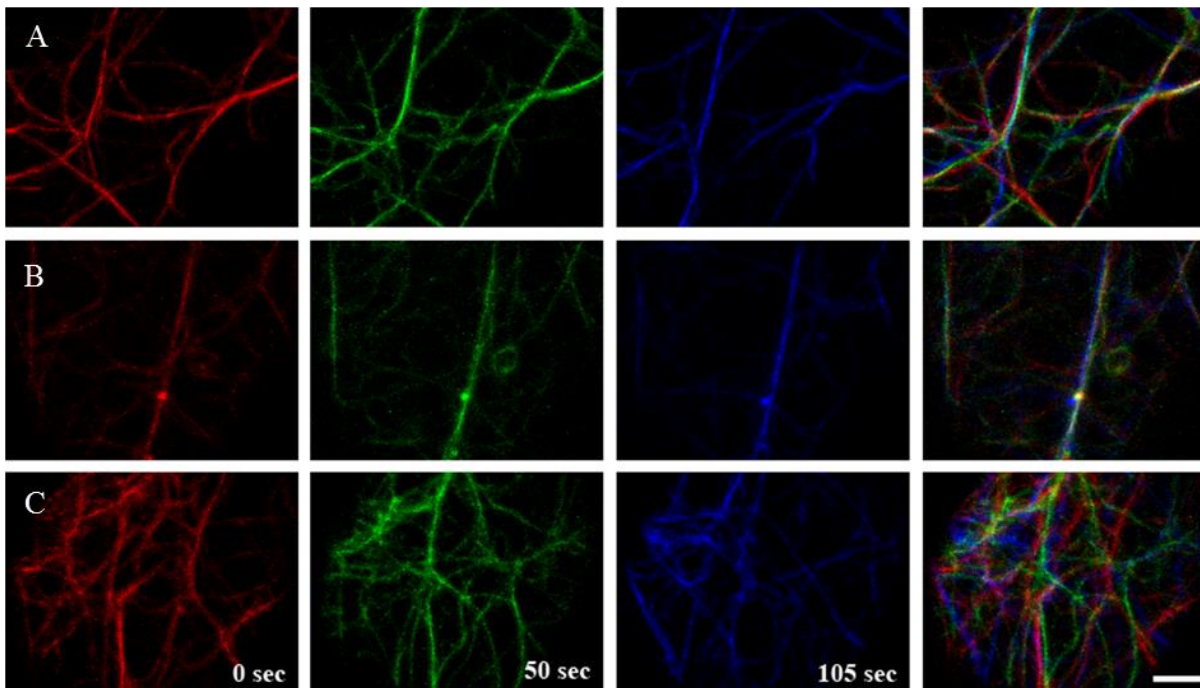


Figure 4.17 Time-lapse three dimensional CLSM images of GFP-FABD2 in cells that have been temperature stressed for one h.

Mature epidermal pavement leaf cells from a transgenic line of *A. thaliana* stably expressing GFP-FABD2 shown in confocal micrographs. The red, green and blue images are 3D projections of a single time-point from a time-series. On the far right is each cell with its corresponding three time-points overlaid: in each row the first time-point is red (0 s), the middle time-point is green (50 s) and the last time-point is blue (105 s). In the overlay when the filaments have moved they appear colourful, but when they do not move they appear white as the colours are superimposed.

(A) Mock sample incubated in $\frac{1}{2}$ MS for 1 h at room temperature.

(B) Sample incubated in $\frac{1}{2}$ MS at 37°C for 1 h.

(C) Sample incubated in $\frac{1}{2}$ MS at 4°C for 1 h.

Scale bar = 5 μ m.

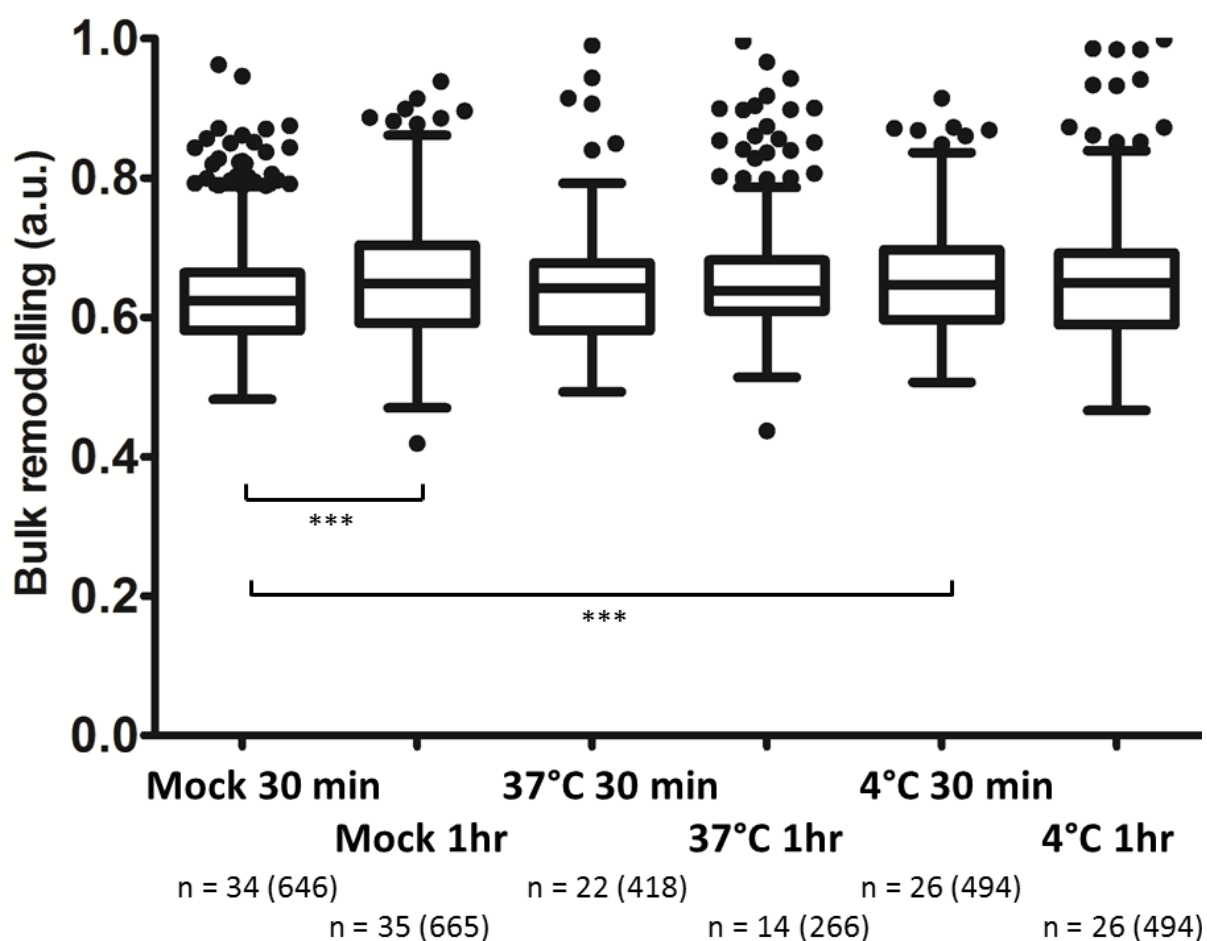


Figure 4.18 Results from *Pairwise image subtraction* analysis on cells expressing GFP-FABD2 that have been temperature stressed.

The boxplots show the data for mature epidermal pavement leaf cells from a transgenic line of *A. thaliana* stably expressing GFP-FABD2 under six experimental temperature conditions shown in Figure 4.16 and Figure 4.17 and analysed using the *Pairwise image subtraction* method (see Section 3.4.3.1). The mean and standard deviation (arbitrary units, a.u.) were: mock 30 min 0.6295 ± 0.076 , mock 1 h 0.6548 ± 0.089 , 37°C 30 min 0.6382 ± 0.074 , 37°C 1 h 0.6695 ± 0.090 , 4°C 30 min 0.6498 ± 0.076 and 4°C 1 h 0.6509 ± 0.096 . The number in parentheses is the total of the 19 comparisons made per cell and the results are detailed in Appendix Table B.8. The asterisks refer to the p-value: ***p < 0.001; only p-values relating to the control are shown on the figure.

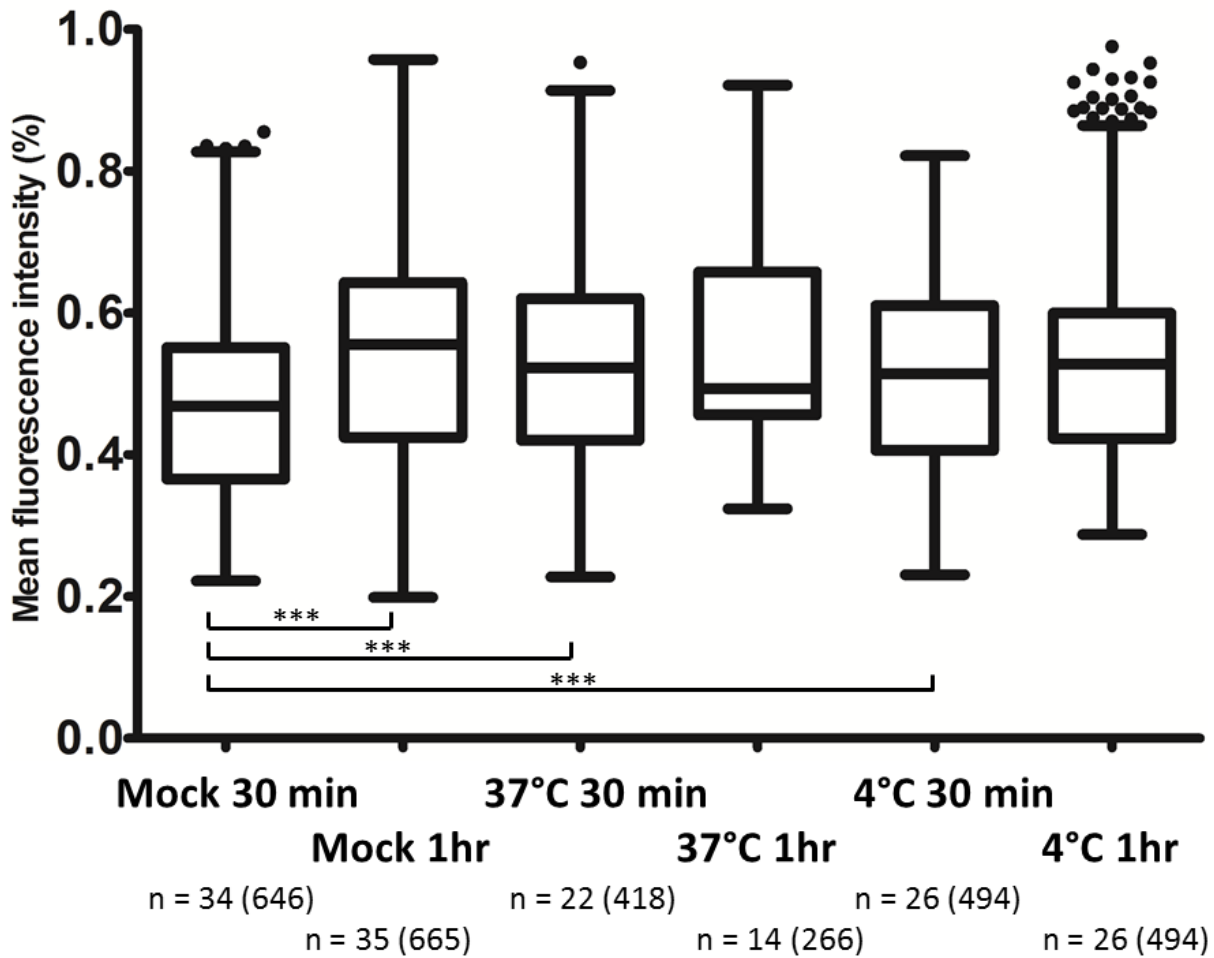


Figure 4.19 Results from *Pairwise image subtraction* measuring mean fluorescence intensity from cells expressing GFP-FABD2 that have been temperature stressed.

The boxplots show the data for mature epidermal pavement leaf cells from a transgenic line of *A. thaliana* stably expressing GFP-FABD2 under six experimental conditions shown in Figure 4.16 and Figure 4.17. They were analysed using the *Pairwise image subtraction* measuring mean fluorescence intensity method (see Section 3.4.3.2). The mean and standard error of mean (no units as these are proportions): mock 30 min 0.4728 ± 0.0051 , mock 1 h 0.5409 ± 0.0062 , 37°C 30 min 0.5313 ± 0.0079 , 37°C 1 h 0.5455 ± 0.0091 , 4°C 30 min 0.5069 ± 0.0061 and 4°C 1 h 0.5278 ± 0.0065 . The number in parentheses is the total of the 19 comparisons made per cell and the results are detailed in Appendix Table B.9. The asterisks refer to the p-value: ***p < 0.001; only p-values relating to the control are shown on the figure.

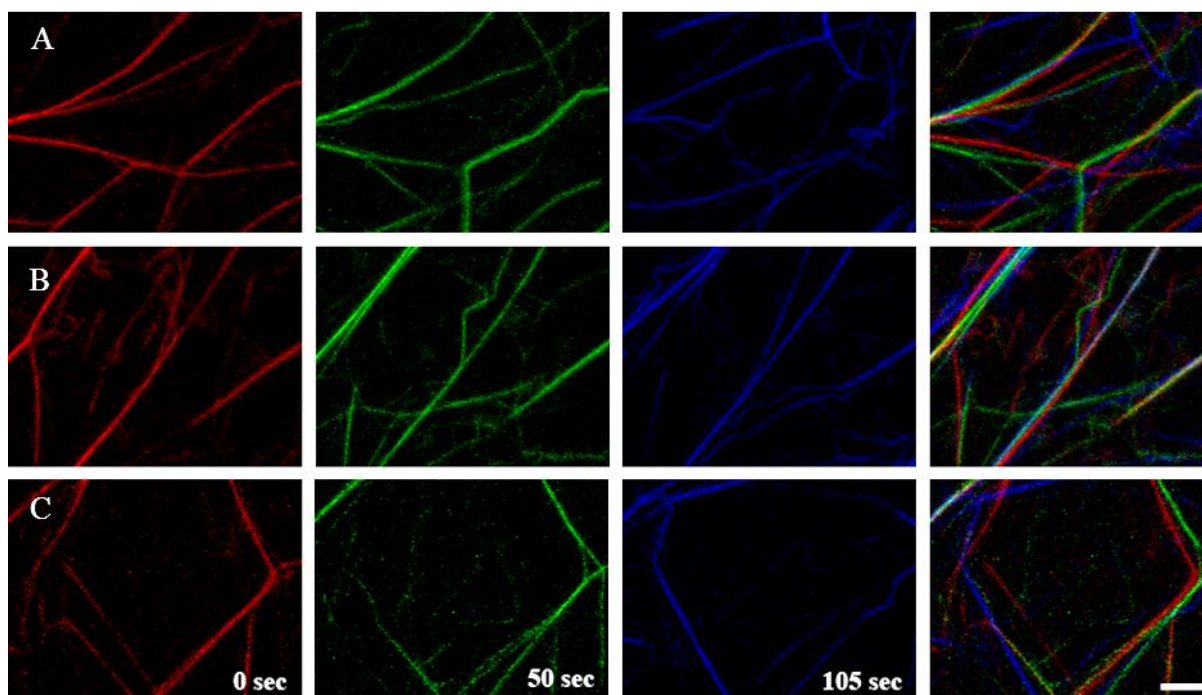


Figure 4.20 Time-lapse three dimensional CLSM images of GFP-Lifeact in cells that have been temperature stressed for 30 min.

Mature epidermal pavement leaf cells from a transgenic line of *A. thaliana* stably expressing GFP-Lifeact shown in confocal micrographs. The red, green and blue images are 3D projections of a single time-point from a time-series. On the far right is each cell with its corresponding three time-points overlaid: in each row the first time-point is red (0 s), the middle time-point is green (50 s) and the last time-point is blue (105 s). In the overlay when the filaments have moved they appear colourful, but when they do not move they appear white as the colours are superimposed.

(A) Mock sample incubated in $\frac{1}{2}$ MS for 30 min at room temperature.

(B) Sample incubated in $\frac{1}{2}$ MS at 37°C for 30 min.

(C) Sample incubated in $\frac{1}{2}$ MS at 4°C for 30 min.

Scale bar = 5 μ m.

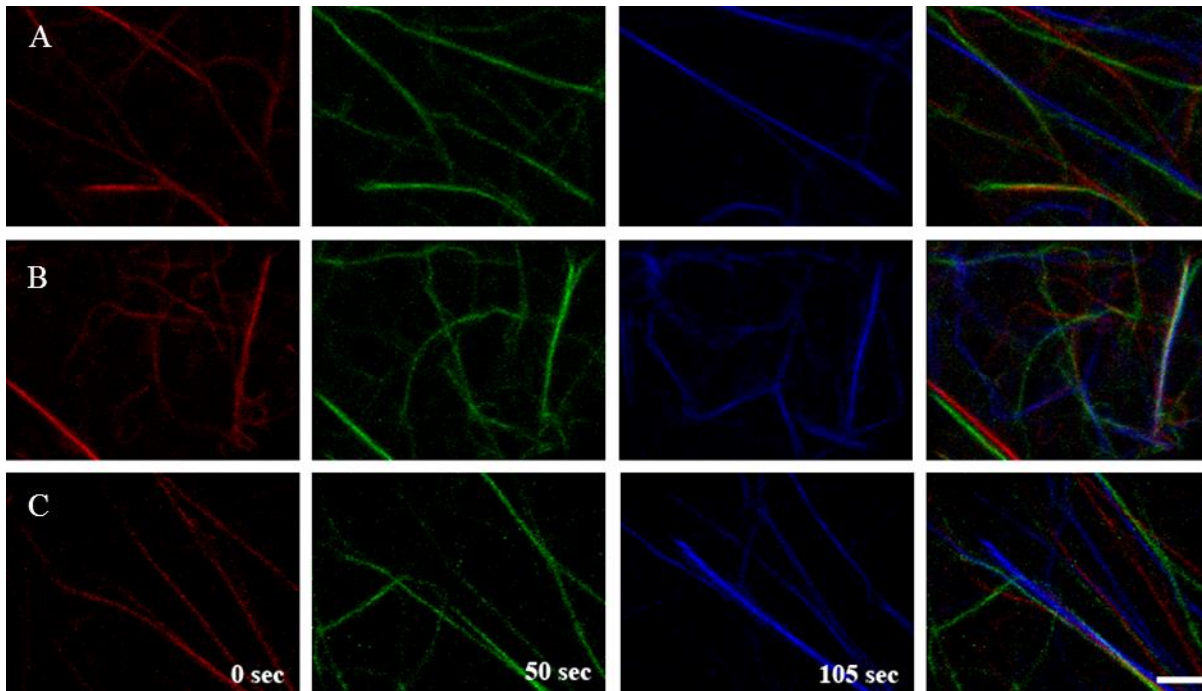


Figure 4.21 Time-lapse three dimensional CLSM images of GFP-Lifeact in cells that have been temperature stressed for one h.

Mature epidermal pavement leaf cells from a transgenic line of *A. thaliana* stably expressing GFP-Lifeact shown in confocal micrographs. The red, green and blue images are 3D projections of a single time-point from a time-series. On the far right is each cell with its corresponding three time-points overlaid: in each row the first time-point is red (0 s), the middle time-point is green (50 s) and the last time-point is blue (105 s). In the overlay when the filaments have moved they appear colourful, but when they do not move they appear white as the colours are superimposed.

(A) Mock sample incubated in $\frac{1}{2}$ MS for 1 h at room temperature.

(B) Sample incubated in $\frac{1}{2}$ MS at 37°C for 1 h.

(C) Sample incubated in $\frac{1}{2}$ MS at 4°C for 1 h.

Scale bar = 5 μ m.

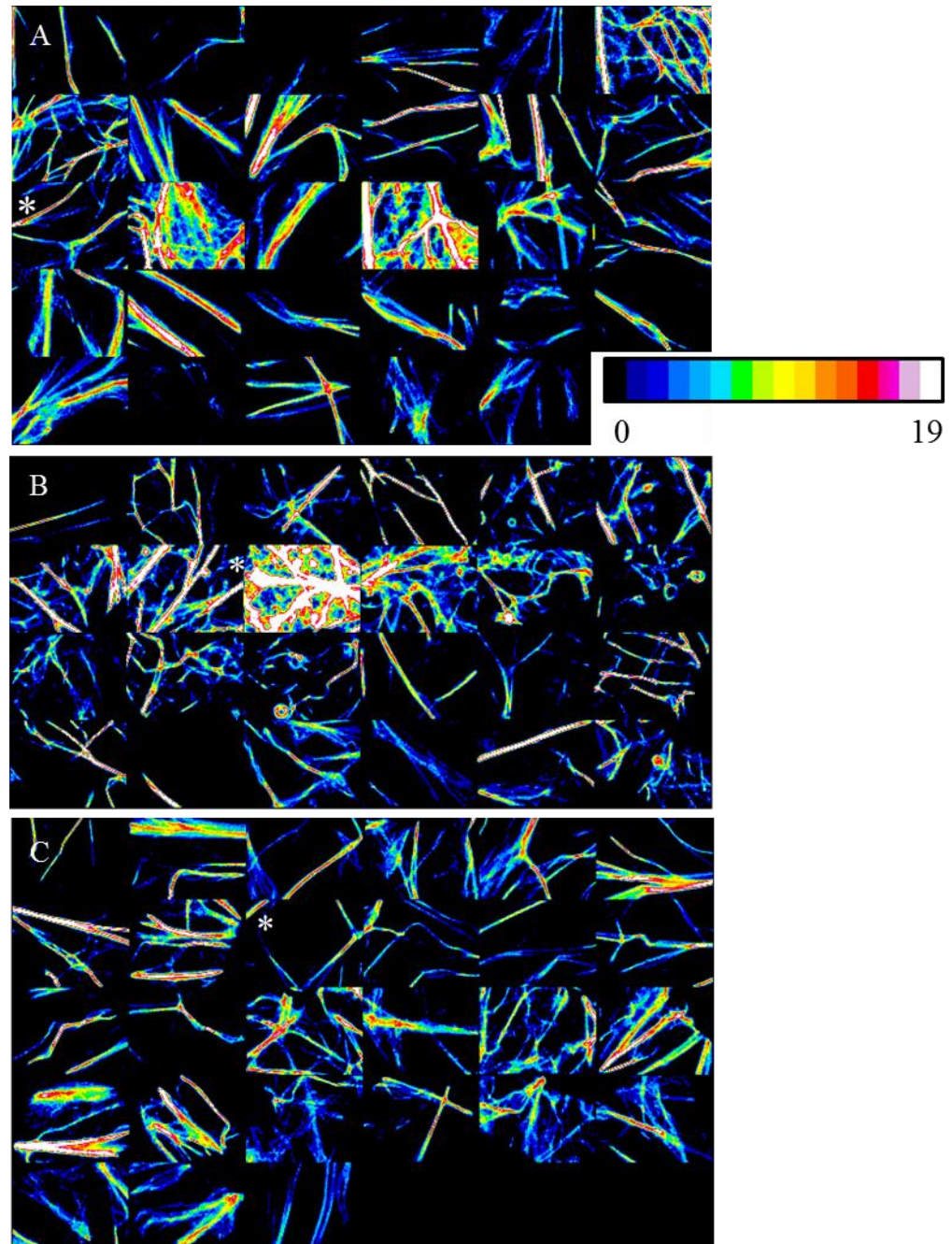


Figure 4.22 Visual results from the *Cumulative fluorescence intensity* analysis on cells expressing GFP-Lifeact that have been temperature stressed for 30 min.

Heat map shows the fluorescence intensity value per pixel, where white represents stationary filaments and darker colours represent lots of movement.

(A) 29 mock cells incubated at room temperature; (B) 24 cells incubated at 37°C; and (C) 27 cells incubated at 4°C. These heat maps were constructed as described in Section 3.4.3.3, pg. 63. Asterisks denote cells shown in Figure 4.20.

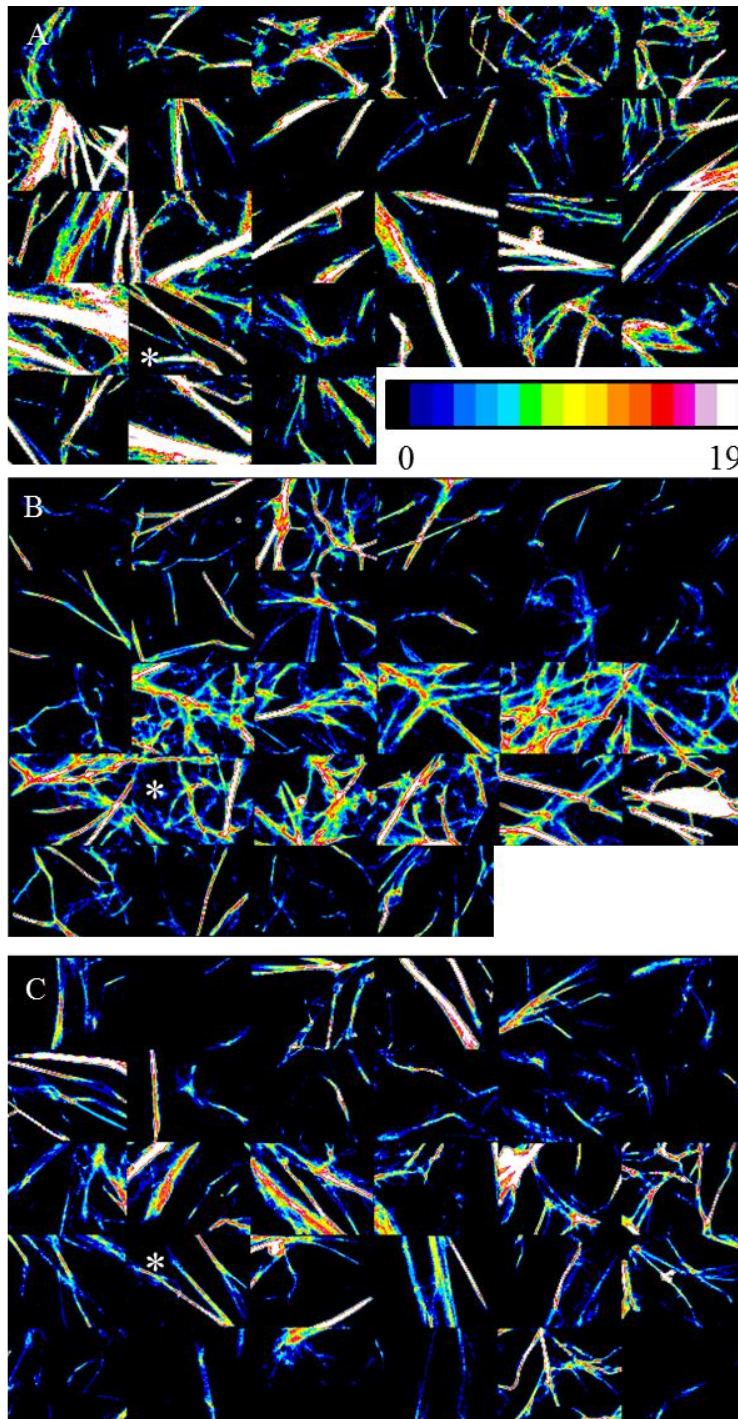


Figure 4.23 Visual results from the *Cumulative fluorescence intensity* analysis on cells expressing GFP-Lifeact that have been temperature stressed for one h.

Heat map shows the fluorescence intensity value per pixel, where white represents stationary filaments and darker colours represent lots of movement.

(A) 27 mock cells incubated at room temperature; (B) 28 cells incubated at 37°C; and (C) 30 cells incubated at 4°C. These heat maps were constructed as described in Section 3.4.3.3. Asterisks denote cells shown in Figure 4.21.

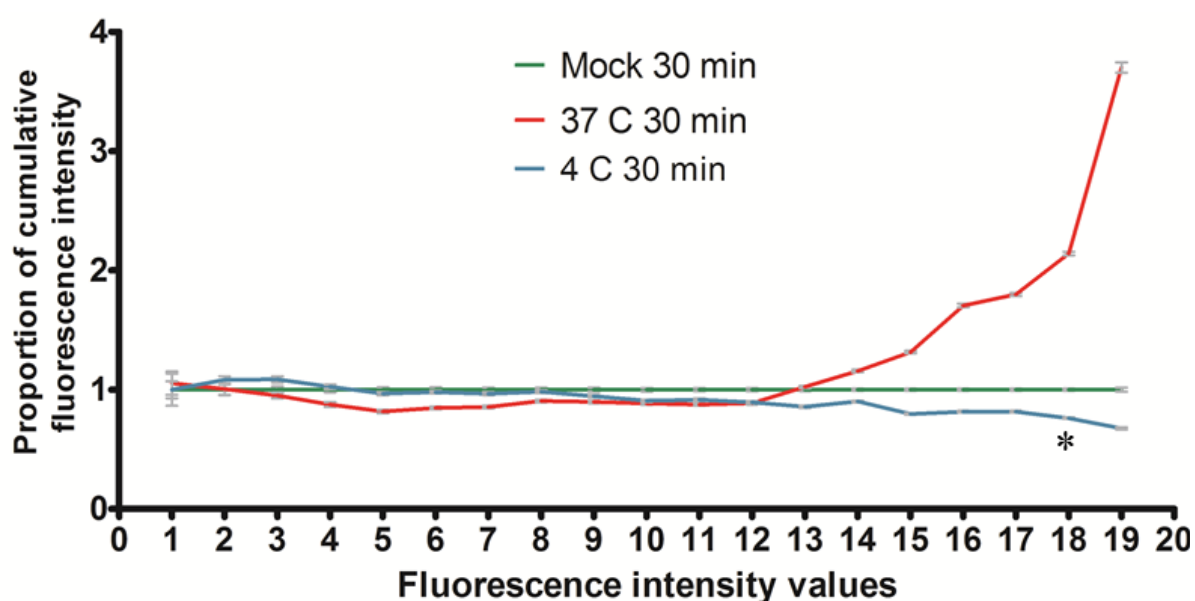


Figure 4.24 Results from *Cumulative fluorescence intensity* analysis of cells expressing GFP-Lifeact that have been temperature stressed for 30 min.

Mature epidermal pavement leaf cells from a transgenic line of *A. thaliana* stably expressing GFP-Lifeact under three experimental conditions (mock 30 min at RT with values normalised to '1' (n = 29 cells), 37°C 30 min (n = 24 cells) and 4°C 30 min (n = 27 cells)) shown in Figure 4.22, the error bars are standard deviation. The results are detailed in Appendix Table B.10 and the p-values are in Table 4.2 (below); here all conditions are expressed as a ratio compared to the mock. The asterisks refer to the difference found between 37°C 30 min and 4°C 30 min: *p < 0.05.

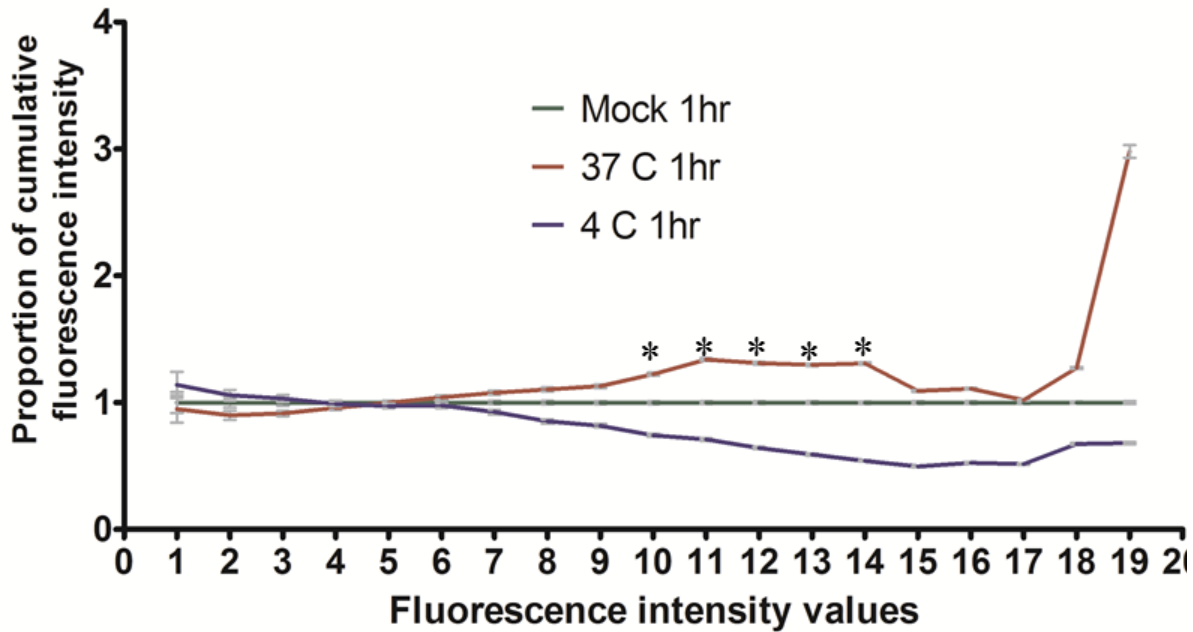


Figure 4.25 Results from *Cumulative fluorescence intensity* analysis of cells expressing GFP-Lifeact that have been temperature stressed for one h.

Mature epidermal pavement leaf cells from a transgenic line of *A. thaliana* stably expressing GFP-Lifeact under three experimental conditions (mock 1 h at RT with values normalised to '1' (n = 27 cells), 37°C 1 h (n = 28 cells) and 4°C 1 h (n = 30 cells)) shown in Figure 4.23, the error bars are standard deviation. The results are detailed in Appendix Table B.10 and the p-values are in Table 4.2 (below); here all conditions are expressed as a ratio compared to the mock. The asterisks refer to the differences found between 37°C 1 h and 4°C 1 h: *p < 0.05.

Table 4.2 The p-value of every cumulative fluorescence intensity value analysed in temperature stress experiments using one-way ANOVA.

Fluorescence intensity values of cells expressing GFP-Lifeact that have been temperature stressed have been analysed using the *Cumulative fluorescence intensity* method (see Appendix Table B.10). The asterisks refer to the p-value: *p<0.05 and **p < 0.01.

Fluorescence intensity value	p-value from one-way ANOVA	p-value from Tukey post-test
1	0.3770	
2	0.0169 *	
3	0.0161 *	
4	0.1800	
5	0.1540	
6	0.4874	
7	0.4726	
8	0.3104	
9	0.1936	
10	0.0262 *	1h 37 °C vs 1h 4 °C *
11	0.0082 **	1h 37 °C vs 1h 4 °C *
12	0.0138 *	1h 37 °C vs 1h 4 °C *
13	0.0168 *	1h 37 °C vs 1h 4 °C *
14	0.0175 *	1h 37 °C vs 1h 4 °C *
15	0.0308 *	
16	0.0379 *	
17	0.0410 *	
18	0.0181 *	30 min 37 °C vs 30 min 4 °C *
19	0.0329 *	

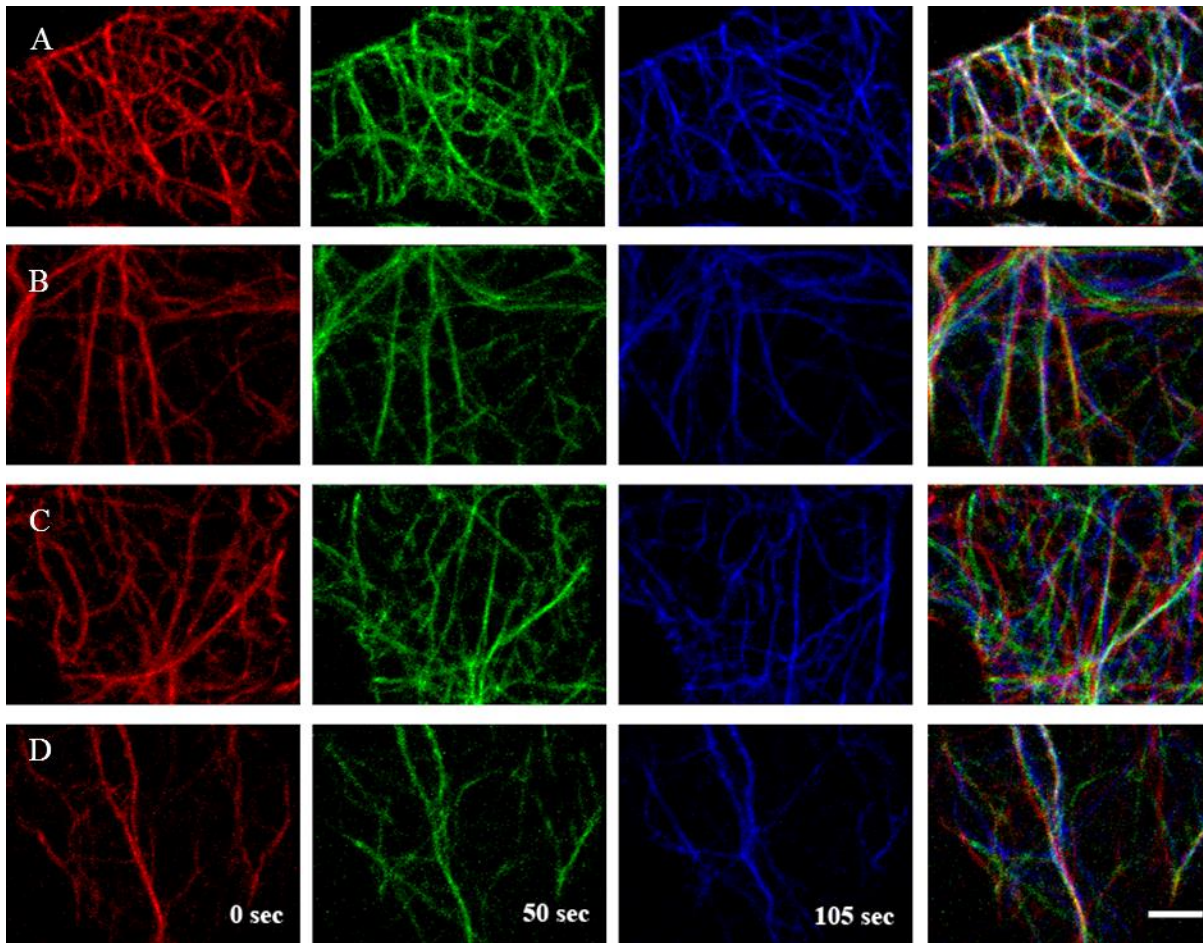


Figure 4.26 Time-lapse three dimensional CLSM images of GFP-FABD2 that have been treated with isoxaben.

Mature epidermal pavement leaf cells from a transgenic line of *A. thaliana* stably expressing GFP-FABD2 shown in confocal micrographs. The red, green and blue images are 3D projections of a single time-point from a time-series. On the far right is each cell with its corresponding three time-points overlaid: in each row the first time-point is red (0 s), the middle time-point is green (50 s) and the last time-point is blue (105 s). In the overlay when the filaments have moved they appear colourful, but when they do not move they appear white as the colours are superimposed.

(A) Mock sample that has been incubated in $\frac{1}{2}$ MS for 1 h without isoxaben.

(B) Sample that has been incubated in $\frac{1}{2}$ MS with 20 μ M isoxaben for 1 h.

(C) Mock sample that has been incubated in $\frac{1}{2}$ MS for 4 hs without isoxaben.

(D) Sample that has been incubated in $\frac{1}{2}$ MS with 20 μ M isoxaben for 4 hs.

Scale bar = 5 μ m.

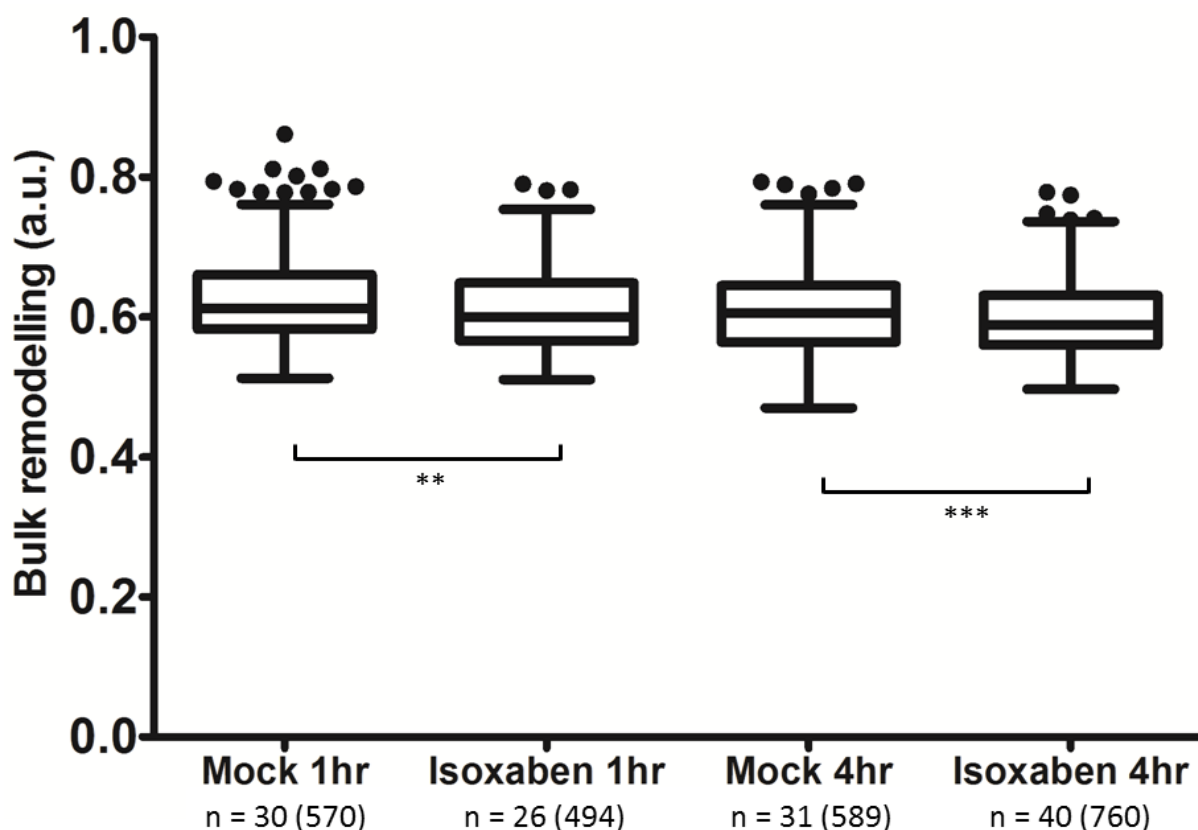


Figure 4.27 Results from *Pairwise image subtraction* analysis on cells with different treatments expressing GFP-FABD2.

The boxplots show the data for mature epidermal pavement leaf cells from a transgenic line of *A. thaliana* stably expressing GFP-FABD2 under four experimental conditions: mock 1 h without isoxaben (n = 30 cells), 20 μ M isoxaben for 1 h (n = 31 cells), mock 4 h without isoxaben (n = 26 cells), and 20 μ M isoxaben for 4 h (n = 40 cells). These are shown in Figure 4.26 and analysed using the *Pairwise image subtraction* method (see Section 3.4.3.1, pg. 60). The mean and standard deviation (arbitrary units, a.u.): mock 1 h was 0.6222 ± 0.057 , 20 μ M isoxaben-treated 1 h was 0.6103 ± 0.057 , mock 4 h was 0.6087 ± 0.058 and 20 μ M isoxaben-treated 4 h was 0.5967 ± 0.049 . The number in parentheses is the total of the 19 comparisons made per cell and the results are detailed in Appendix Table B.11. The asterisks refer to the p-value: **p < 0.01 and ***p < 0.001; only p-values relating to the control are shown on the figure.

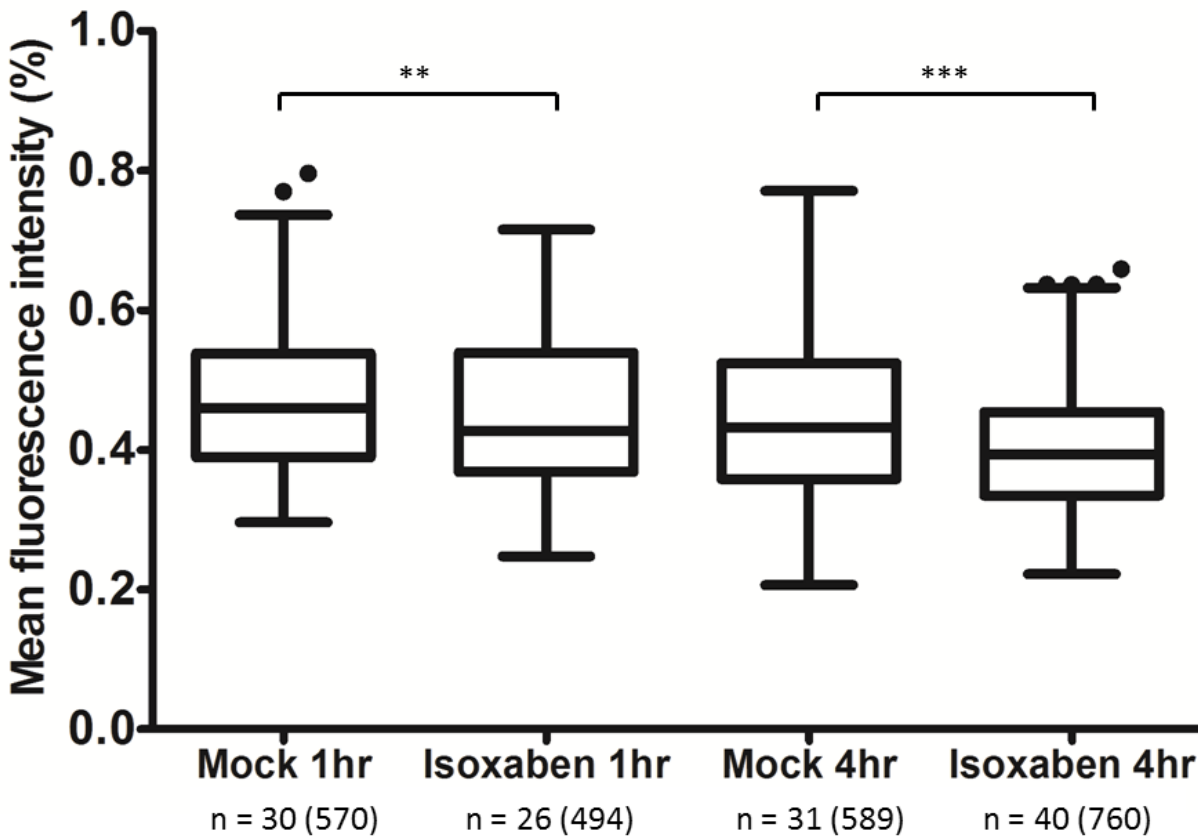


Figure 4.28 Results from *Pairwise image subtraction* measuring mean fluorescence intensity on cells expressing GFP-FABD2 that have been treated with isoxaben.

The boxplots show the data for mature epidermal pavement leaf cells from a transgenic line of *A. thaliana* stably expressing GFP-FABD2 under four experimental conditions. These are shown in Figure 4.26 and analysed using the mean fluorescence intensity from the *Pairwise image subtraction* method (see Section 3.4.3.2). The mean and standard error of mean (no units as these are proportions): mock 1 h was 0.4717 ± 0.0042 , 20 μ M isoxaben-treated 1 h was 0.4542 ± 0.0055 , mock 4 h was 0.4438 ± 0.0050 and 20 μ M isoxaben-treated 4 h was 0.4004 ± 0.0031 . The number in parentheses is the total of the 19 comparisons made per cell and the results are detailed in Appendix Table B.12. The asterisks refer to the p-value: **p < 0.01 and ***p < 0.001; only p-values relating to the control are shown on the figure.

4.5 Discussion

This chapter began the investigation into the influence of the cell wall on the organisation and dynamics of the actin cytoskeleton by using plasmolysis to separate the protoplasm from the cell wall. It also explored whether the differences seen in plasmolysed cells were in fact due to a general abiotic stress response initiated in the cells. In addition, cells were treated with isoxaben, a chemical which perturbs cell-wall structure by interfering with cellulose production in a specific manner. In this chapter, use of markers and methods of analysis, introduced and outlined in the previous chapter, to quantify the dynamics of the actin cytoskeleton in these cells are described. Lastly, ST-GFP, a marker for Golgi bodies, was also imaged in case the actin cytoskeleton markers interfered with the dynamics of the network. The behaviour of the Golgi bodies was measured with the software Volocity (PerkinElmer).

Sheahan *et al.* (2004) showed using FRAP (Fluorescence Recovery After Photobleaching) on *A. thaliana* seedlings that stably-expressed GFP-FABD2 had a faster fluorescence recovery time in leaf epidermal cells than in root hair cells. The actin cytoskeleton has been reported as being arranged in a more random fashion in isotropic cells (cells uniform in all direction) rather than arranged longitudinally, as can be found in anisotropic cells (cells with directional dependency); this possibly corresponds with factors such as fluorescence recovery time (Kotzer and Wasteney, 2006). This suggests that there are tissue specific differences in the behaviour of the network. Therefore in this study, all of the analyses of the actin cytoskeleton were done on the abaxial side of mature epidermal leaf cells from *A. thaliana*. The morphology of the actin cytoskeleton was variable, but rarely anisotropic (see Appendix Figure , pg. 256).

Due to these many and unknown variables, mannitol was chosen as the plasmolysing agent because it is a non-metabolisable sugar, so the cells should not react to the mannitol itself. Lang *et al.* (2014) and references therein state that plasmolysis is complete after 30 min and that plasmolysed cells can survive for longer than 24 hs, depending on the experiment and type of cell.

4.5.1 Qualitative description of the actin cytoskeleton in cells with a perturbed cell wall

The actin cytoskeleton visualised in cells that stably-expressed GFP-FABD2 and GFP-Lifeact appeared very different and the GFP-FABD2 actin cytoskeleton displayed a greater morphological variability than the actin cytoskeleton marked with GFP-Lifeact (see Appendix Figure B.1 and Figure B.3). However, plasmolysed GFP-FABD2 cells tended to exhibit a greater degree of bundling of actin filaments compared to control cells (Section 4.4.1.1, pg. 85). Lang *et al.* (2014) imaged *A. thaliana* hypocotyl cells that stably-expressed GFP-ABD (actin binding domain from AtFIM1; (Kovar *et al.*, 2000) and were plasmolysed using 0.8 M mannitol (this study used 0.6 M mannitol); they stated that actin filaments reorganised rapidly as the protoplasm shrank, but did not become ‘wavy’. This study did not image as the protoplast shrank, but that could be an interesting next step, to establish when actin bundles form during plasmolysis. However, the marker used by Lang *et al.* (2014) was different to both markers used in this study, although bundles were also apparent in their images.

GFP-Lifeact control cells were mostly bundled; when plasmolysed, the amount of bundling appeared to remain the same, but the bundles seemed to have more ‘kinks’ or to be a bit more ‘wavy’ (Section 4.4.1.4, pg. 87). Lang *et al.* (2014) imaged cells that stably-expressed a microtubule marker and they stated that the microtubules bundled more and became ‘wavy’, although not in the same way as GFP-Lifeact in this study. It should also be noted that when a microtubule appears to be ‘wavy’ it is actually buckling and that this requires a lot of pressure (Tijds Ketelaar, personal communication). This suggests that the microtubule cytoskeleton reacts in a different way to plasmolysis than the actin cytoskeleton.

Lang *et al.* (2014) compared the actin bundles observed in plasmolysed cells to the stress fibres² that are found in animal cells. They, and others, suggest that these structures are important for shape and volume regulation during plasmolysis (Wojtaszek *et al.*, 2007, Lang *et al.*, 2014). Wojtaszek *et al.* (2007) showed that actin filaments associated with the plasma membrane reorganise to stabilise the protoplast during plasmolysis in maize roots.

² Stress fibres are well known in animal cells, but do not exist in plant cells. Any mention of stress fibres in plant cells is as a descriptive indicator or possible suggestion of their mechanical role.

Komis *et al.* (2002) showed that in plasmolysed *Chlorophyton comosum* leaf cells, fine actin filaments disappeared and were replaced with thick actin bundles in the cortex. They also treated cells with Cytochalasin-B before plasmolysis which caused plasmolysis to last for longer and for the protoplast to culminate in a smaller volume compared to mock cells (Komis *et al.*, 2002). The authors concluded that the actin cytoskeleton is important for regulation of the shape and volume of the protoplast during plasmolysis (Komis *et al.*, 2002). This is an interesting hypothesis and it would be the next step for this study to determine the biological function of the actin bundles observed in plasmolysed cells.

When GFP-FABD2 cells were rehydrated (after plasmolysis) and treated hypo-osmotically there appeared to be more bundles compared to the control cells. Lang *et al.* (2014) ‘de-plasmolysed’ cells with a 15 minute incubation of 0.4 M mannitol for 15 min and then with distilled water for a further 15 minute incubation. They found that the ‘cyto-architecture’ of the actin cytoskeleton remained the same throughout ‘de-plasmolysis’ and that after the end of this process the network was organised the same as control cells (Lang *et al.*, 2014). When GFP-Lifeact cells were rehydrated (after plasmolysis) the amount of bundling remained the same as the control cells, although there were ‘kinks’ in evidence. When GFP-Lifeact cells were treated hypo-osmotically they appeared very similar to the control cells. This study concluded that there did appear to be some differences in the GFP-FABD2 tagged actin cytoskeleton between the control cells and the re-hydrated cells, although the data was very variable. However, there was no significant difference when the GFP-Lifeact tagged actin cytoskeleton in the re-hydrated cells was compared to the control cells. Perhaps this is due to variability in the data, or to subtle differences in the markers.

Lastly, in GFP-FABD2 cells treated with isoxaben, the network appeared more bundled compared to the mock treated cells (Section 4.4.3.1, pg. 97; see Appendix Figure B.10 and Figure B.11). However, the bundles apparent in those cells did not look the same as the bundles visualised in the plasmolysed cells (Figure 4.4, pg. 102).

To explore the possibility that there is a general response of the actin cytoskeleton to abiotic stresses, epidermal leaf cells that stably-expressed GFP-FABD2 and GFP-Lifeact were temperature stressed (Section 4.4.2, pg. 92). The cells were mock incubated with ½ MS; however, the mock treated cells appeared very similar to the re-hydrated and hypo-osmotically-treated cells from Section 4.4.1 (pg. 85). When GFP-FABD2 cells were heat

stressed at 37°C, the structures of the networks appeared similar to the mock-treated cells, except that there were punctae around the network. When GFP-FABD2 cells were cold stressed at 4°C, the network appeared very similar to the mock-treated cells, although there was a reduction in fluorescence.

GFP-Lifeact mock-treated epidermal leaf cells appeared very similar to the re-hydrated cells from Section 4.4.1.4 (pg. 87). When GFP-Lifeact cells were heat stressed at 37°C, the network appeared disorganised with some acuquosomes present (Smertenko *et al.*, 2010). Whereas when GFP-Lifeact cells were cold stressed at 4°C, the network appeared very similar to the control cells from Section 4.4.1.4 (pg. 87).

4.5.2 Plasmolysis affects the organisation and dynamics of the actin cytoskeleton when marked with GFP-FABD2

Using the marker GFP-FABD2 and the *Pairwise image subtraction* method (see Section 3.4.3.1) the bulk remodelling index of the actin cytoskeleton was found to be significantly lower in the plasmolysed sample compared to the control (Figure 4.5, pg. 103). The mean of the control was 0.56 and the plasmolysed sample mean was 0.53. This implies that the networks in the plasmolysed sample were more static and unchanging than the networks in control cells. The hypothesis of this study was that the cell wall somehow influenced the organisation and dynamics of the actin cytoskeleton and these results show that separation of the protoplasm from cell wall did cause a change.

Komis *et al.* (2002) hypothesise that the actin bundles they observed in plasmolysed *C. comosum* leaf cells might help mitigate the physical stresses caused by gross deformation of the plasma membrane or alternately that they strengthen the plasma membrane and the tonoplast. Mathur (2005) theorised that there is a zonation in diffuse-growing cells, similar to that seen in tip-growing cells, that underlying the plasma membrane is a 'fine cortical mesh' and below this actin bundles and microtubules. Mathur (2005) goes on to compare this situation with that found in mobile animal cells that possess lamellipodia (leading edge of the cell) with an 'actin-rich zone' at the leading edge.

In animal cells it appears that two of the major functions of the actin cytoskeleton are cell movement and cell shape, whilst the microtubule cytoskeleton is the primary long distance transport network (Alberts *et al.*, 2014). Turgor pressure in plant cells capable of changing the shape of the cell wall must be approximately 0.6 MPa, while at best an actin network is hypothetically able to generate 0.01 MPa (Szymanski and Cosgrove, 2009). Perhaps in a plasmolysed cell without the support of a cell wall, the actin cytoskeleton is needed for support (van der Honing *et al.*, 2007)?

4.5.2.1 Stress fibres

Thick bundles were often visualised in plasmolysed cells in this study and stress fibres in animal cells are used to provide mechanical support to maintain cellular integrity (Claessens *et al.*, 2008). In animal cells the extracellular matrix is connected to the cytoskeleton at focal adhesions by stress fibres which are antiparallel bundles of actin filaments held together with myosin II and α -actinin (a long cross-linking protein) (Burridge and Wittchen, 2013, Blanchoin *et al.*, 2014). Stress fibres can generate tension but are also load-bearing and 'mechanosensitive': their presence indicates the relationship of the cell with its environment, both chemical and physical (Burridge and Wittchen, 2013). They are commonly seen in animal cells grown in tissue culture, but not often observed in cells from intact tissue; the tissues that possess these structures in their cells are generally undergoing 'high mechanical forces/shear stresses', such as endothelial cells in the blood vessels (Burridge and Wittchen, 2013).

Interestingly, when stress fibres disassemble it is often due to a loss of tension; indeed, it has been hypothesised that the tension is sensed by the filaments themselves which then become more susceptible to severing by cofilin (Hayakawa *et al.*, 2011). In an elegant experiment where an actin filament was tensed via a myosin-coated bead that was controlled using optical trapping, the same group showed that it took more than twice as long for a tensed filament to be severed by cofilin, than a relaxed filament (43.2 ± 5.1 s and 15.1 ± 3.7 s, respectively) (Hayakawa *et al.*, 2011). If the same situation is found in plasmolysed plant cells then this could be a biochemical mechanism that results in more stable networks.

4.5.2.2 Future directions

An interesting avenue of research would be to determine if there are similarities between the actin bundles in plasmolysed plant cells and the stress fibres seen in animal cells. Komis *et al.* (2003) showed that if *C. comosum* leaf cells were pre-treated with either of two myosin inhibitors 2,3-Butanedione monoxime (BDM) or ML-7 (myosin light chain kinase inhibitor) and then plasmolysed, the protoplast became an abnormal shape with a much reduced volume compared to untreated plasmolysed cells. In addition, when treated with BDM (but not in the case of ML-7 treatment) these cells did not possess thick bundles of actin underlying the plasma membrane as seen in untreated plasmolysed cells.

This does not prove that the bundles are like stress fibres; in animal cells stress fibres are formed from actin, α -actinin and myosin II (Blanchoin *et al.*, 2014). BDM has been shown to affect myosins, but not actin or kinesins (Cramer and Mitchison, 1995), and microtubules are known to 'buckle' during plasmolysis (this is a response to force), suggesting they are not the major mechanical support during plasmolysis (Tijs Ketelaar, personal communication) (Lang *et al.*, 2014). Ueda *et al.* (2010) showed that two myosins in plant cells (XI-K and MYA2/XI-2) were necessary for thick actin bundles and streaming of the endoplasmic reticulum. It is also thought that myosins in plant cells are not only able to move on actin bundles, but also accept them as cargo (Szymanski and Cosgrove, 2009). It is hypothesised that myosins bound to actin bundles can generate enough force (approximately 100 pN per filament) to overcome tonoplast membrane tension to create transvacuolar strands for trafficking organelles (Szymanski and Cosgrove, 2009). All these studies (coupled with the findings from this study that the actin bundles formed during plasmolysis are more stable) invite the hypothesis that these are structures capable of providing mechanical support during plasmolysis.

4.5.2.3 Re-hydration and hypo-osmotic treatments

The bulk remodelling index of the actin cytoskeleton was significantly higher in the re-hydrated sample compared to the control, but there was no difference between the control and hypo-osmotic. The mean of the control was 0.56 ± 0.06 , the re-hydrated was 0.60 ± 0.09 and the hypo-osmotic was 0.56 ± 0.05 (Figure 4.5, pg. 103). This implies that in re-hydrated cells the networks remodel more than the networks in control cells, whereas the hypo-osmotic cells seem to have the same remodelling index as the control. Both samples have been treated with water so that the turgor pressure of the vacuole is increased and it presses the cytoplasm against the cell wall-plasma membrane interface. If a lack of cell

wall reduces remodelling, perhaps it is possible that the opposite situation causes an increase in remodelling this is seen in the re-hydrated sample, but not in the hypo-osmotic. This suggests that the increase in the bulk remodelling index seen in the re-hydrated cells is linked to recovery processes post-plasmolysis. This also demonstrates that the plasmolysis carried out in this study was not fatal or irreversible, because the cells re-hydrated and the actin cytoskeleton remained dynamic.

4.5.2.4 Mean fluorescence intensity of the Pairwise image subtraction method

In this case, the marker GFP-FABD2 was analysed using the *Pairwise image subtraction* method, but the mean fluorescence intensity was measured rather than whether the fluorescence was present or not (see Section 3.4.3.2). The mean fluorescence of the actin cytoskeleton was significantly lower in the plasmolysed sample compared to the control (Figure 4.6, pg. 104). In this case only the fluorescence intensity of the filaments that have moved was scored, thus allowing an estimation of the kind of filaments that have moved.

The plasmolysed sample had a reduction in mean fluorescence intensity which suggests that the stationary filaments have higher fluorescent intensities than the filaments that are not stationary. This suggests that the thick bundles that are formed during plasmolysis do not remodel and the filaments that are remodelling are thinner and have correspondingly lower fluorescence intensities (Figure 4.6, pg. 104). It could also be due to more or less of the marker binding to the actin bundles or the marker protein masking the signal. The mean fluorescence intensity of the actin cytoskeleton was increased in both the re-hydrated and hypo-osmotic samples compared to the control. Again, this suggests that there are some thicker filaments in the control that do not remodel, whereas in the re-hydrated and hypo-osmotic samples much more remodelling is occurring. This means that a range of filaments are mobile giving a higher mean fluorescence intensity. This is interesting as the previous results suggested there was no difference in the dynamics between control and hypo-osmotic-treated cells, instead perhaps there is a difference in the organisation of the network.

4.5.3 Plasmolysis also affects the organisation and dynamics of the actin cytoskeleton when marked with GFP-Lifeact

A different marker was used to test whether the results obtained with GFP-FABD2 were due to the action of the fluorescent marker rather than being representative of what happens to the actin cytoskeleton during plasmolysis. Investigating plasmolysis using GFP-Lifeact analysed with *Cumulative fluorescence intensity* at the first fluorescence intensity value, the ANOVA found a significant difference, although the Tukey post-test found no differences between columns (Table 4.1, pg. 109). At the 16th fluorescence intensity value the ANOVA found a significant difference, although the Tukey post-test found no differences between columns.

At the 17th, 18th and 19th fluorescent intensities the proportion of fluorescence intensity in the plasmolysed sample compared to the control was found to be significantly higher. This means that there is comparatively more fluorescence at the three values which correspond to the filaments that have moved the least or not at all (see Section 3.4.3.3). This reinforces the results from the GFP-FABD2 marked actin cytoskeleton experiments analysed with the *Pairwise image subtraction* method (also with measuring the mean fluorescence intensity; Section 4.4.1.2, pg. 86).

Smertenko *et al.* (2010) imaged GFP-Lifeact and described two different kinds of network rearrangements: the first consisted of elongation, severing and shortening events and the other was bundling, unbundling, stretching and retraction. Interestingly, they observed that the latter group of dynamics was more prevalent in transiently transformed *N. bethamiana* than stably-expressing *A. thaliana* or *N. tabacum* BY-2 cells (Smertenko *et al.*, 2010). They also discovered that (in root epidermal cells) the brighter or thicker filaments underwent the same dynamic processes as fine filaments, just at a much slower rate (Smertenko *et al.*, 2010). The next steps for this study would be to carry out a similar survey to categorise the kinds of remodelling undergone by the actin cytoskeleton under different conditions, comparing both fine filaments and thick bundles.

There were no differences found between the control and the re-hydrated or the hypo-osmotic samples at any fluorescence intensity value. Although both had a higher proportion of fluorescence intensity at the higher fluorescence intensity values, there were

no significant differences, probably due to large standard deviations. This is different to the situation found with GFP-FABD2, perhaps reflecting differences in the markers or maybe differences in analysis; however, it was noted that in re-hydrated and hypo-osmotic-treated cells there were more bundles when visualised with GFP-FABD2. In this situation categorising different kinds of remodelling would be very interesting.

4.5.3.1 *Comparison of GFP-FABD2 and GFP-Lifeact*

van der Honing *et al.* (2011) compared the dynamic reorganisation of actin filaments imaged with GFP-FABD2 and Lifeact-Venus (in *A. thaliana* epidermal root cells) and found that actin bundles relocate at a much lower rate in Lifeact-Venus compared to GFP-FABD2. van der Honing *et al.* (2011) suggested that Lifeact labels a slightly different population of filaments because it decorates very fine actin filaments in the subapical region in root hairs whereas GFP-FABD2 does not. They also showed that Lifeact-Venus has a higher fluorescence recovery rate after photobleaching than GFP-FABD2, although this is probably due to the lower affinity of Lifeact for actin filaments (van der Honing *et al.*, 2011).

It is interesting that during this study both the GFP-FABD2 labelled networks and the GFP-Lifeact labelled networks had a reduced bulk remodelling index when plasmolysed (Sections 4.4.1.2, pg. 86 and 4.4.1.4, pg. 87). This suggests that they label a very similar network in epidermal leaf cells and that the reduction in dynamics in plasmolysed cells is a standard response in that cell type.

4.5.4 Plasmolysis affects the motility of Golgi bodies when marked with ST-GFP

It is known that there is variation in Golgi body movements as one region of a cell can simultaneously contain unmoving and moving Golgi bodies along with Golgi bodies within a cytoplasmic stream that move extremely fast (Runions *et al.*, 2006, Sparkes *et al.*, 2008, Akkerman *et al.*, 2011, Martinière *et al.*, 2011a). It has also been shown that using different markers for the actin cytoskeleton alters the structure of the network enough that Golgi body motility is altered (Rocchetti *et al.*, 2014).

Golgi body velocity is the average speed over the track length and it is reduced in the plasmolysed sample compared to control and increased in the hypo-osmotic samples compared to the control, but remains unchanged in the re-hydrated sample compared to the control (Figure 4.12, pg. 111). Golgi bodies in the re-hydrated cells have a higher mean track velocity than in control cells, but the standard deviation is particularly large, precluding any definitive statement of difference. The results in the previous sections showed a reduction in actin cytoskeleton remodelling in plasmolysed cells (Figure 4.5, pg. 103 and Table 4.1109). However, the network is not a dense mesh in plasmolysed cells; therefore the reduction in Golgi body motility must be due to factors other than those observed by Martinière *et al.* (2011a).

The displacement rate is the straight-line distance from the starting position of the track to the last position of the track divided by the time taken for the Golgi body to move that distance [Volocity User Guide; PerkinElmer]. Golgi bodies in plasmolysed cells had a reduced displacement rate compared to control cells (Figure 4.13, pg. 112). This means it took a longer time for the Golgi bodies to move the same distance or they moved a shorter distance in the same amount of time. However, there was no difference in the displacement rate between the control and the re-hydrated and hypo-osmotic cells.

The meandering index is the ratio of the displacement rate to the velocity and it describes the deviation the Golgi body made from a straight line: a value of '1' means a perfect straight line, the closer the value to '0', the more the Golgi body deviated from a straight line or 'wiggled' [Volocity User Guide; PerkinElmer]. Golgi bodies in plasmolysed cells had a lower meandering index compared to control cells (Figure 4.14, pg. 113). This means the Golgi bodies moved in a less direct and more saltatory manner. There was no difference in the meandering index between the control and the re-hydrated and hypo-osmotic cells.

The number of time-frames that the Golgi bodies are present in view is increased in the plasmolysed, re-hydrated and hypo-osmotic cells compared to the control (Figure 4.15, pg. 114). For the plasmolysed cells this makes sense with regards to the previous data, but the only other definitive evidence is that the hypo-osmotic cells have an increased track velocity compared to control cells (Figure 4.12, pg. 111). Therefore the Golgi bodies are moving faster, but remain within view for a longer period without moving in a more 'wiggly' manner. There is no significant difference in displacement rates, possibly

because the variation in the data is large. However, the displacement rate is increased in the re-hydrated and hypo-osmotic cells, so perhaps they move quicker and more directly over greater distances. The Golgi body track length was increased in plasmolysed, re-hydrated and hypo-osmotic cells compared to the control (data not shown).

In plasmolysed cells the actin cytoskeleton has a different morphology and bulk remodelling is reduced, this is expected to impact on Golgi body motility and all the measures detailed here record reduced motility (Section 4.4.1.6, pg. 90). It is either a physical cause from the alteration in the actin cytoskeleton from mostly thin bundless to a greater proportion of thick strands and bundles, or a reduction in overall cytoplasmic streaming, although this does not stop during plasmolysis (Figure 4.4, pg. 102) (Lang *et al.*, 2014). Possibly Golgi bodies cannot easily move along bundled actin filaments, perhaps myosins do not interact efficiently with thicker bundles. Or it may be that the loss of organisation within the network adversely impacts on the ability of Golgi bodies to be trafficked in an efficient fashion. However, there could be non-actin related reasons, such as less myosin available for binding?

However, it is generally considered that organelles move faster on actin filaments that are bundled together, possibly because more myosins can bind to a bundle compared to a single filament thus providing a greater force for motion (Geitmann and Nebenfuhr, 2015) and references therein. For example, Martinière *et al.* (2011a) showed that when AtFH1 is over-expressed, thus causing a dense actin network, Golgi body motility is reduced. Akkerman *et al.* (2011) stated that Golgi bodies moved faster and more directionally on bundled actin filaments compared to fine filaments. However, in plasmolysed cells, that contain many actin bundles, the Golgi bodies have reduced velocity (Figure 4.12, pg. 111). Lastly, CSCs are removed from the plasma membrane during plasmolysis and synthesis of cell wall carbohydrates is also reduced (Iraki *et al.*, 1989, Gutierrez *et al.*, 2009).

4.5.5 The actin cytoskeleton does not appear to have a general dynamic response to abiotic stresses

Cold acclimation is the process by which temperate plants (such as *A. thaliana*) become able to withstand freezing temperatures and it is initiated by a period of low, but not

freezing temperatures: it can be carried out in the laboratory by using temperatures between 2 – 6°C (Xin and Browse, 2000). However, the short duration of the temperature stresses used in this study would not allow plants the time to become acclimated: they were sudden changes for plants that were used to nearly constant temperatures of 22°C. Likewise, *A. thaliana* responds to an increase in temperature, but it has been shown that exposure to temperatures of 37°C caused different epigenetic changes (Liu *et al.*, 2015). However, as mentioned previously, temperature stress is not a physical stress. These experiments could be repeated using centrifugation or bending of the tissue as physical stresses.

4.5.5.1 Temperature-stressed GFP-FABD2

Using the marker GFP-FABD2 and the *Pairwise image subtraction* method, the effects of cold and heat stress on the bulk remodelling of the actin cytoskeleton were measured in mature epidermal pavement leaf cells. Of all the conditions examined the only significant differences were found when the mock 30 min (room temperature) treatment, which had the lower bulk remodelling index, was compared to mock 1 h and 4°C 30 minute treatments (Figure 4.18, pg. 117). In the case of the former, this increase in bulk remodelling must be due to the incubation itself. When examining the hypo-osmotic samples in previous experiments sometimes the dynamics of the actin cytoskeleton have been different to the control, which suggests that just incubating cells in liquid can alter the bulk remodelling index (Figure 4.5, pg. 103). Therefore, the increase in the bulk remodelling index may have been due to the doubling of the incubation time, an interesting next step would be to use perfluorodecalin to determine if this causes a similar change in the actin cytoskeleton (Littlejohn *et al.*, 2010).

Using the marker GFP-FABD2 and the mean fluorescence intensity from the *Pairwise image subtraction* gave greater standard deviations. The mean fluorescence intensity of mock 1 h, 37°C 30 min and 4°C 30 min were significantly higher compared to mock 30 min (Figure 4.19, pg. 118). These results indicate the same effect as the previous analysis method except for the additional significant difference found between 37°C 30 min and mock 30 min.

Why should the 4°C 30 min sample have an increased rate of bulk remodelling compared to mock 30 min? If there were any change at all with a cold treatment it would be expected

to reduce the rate of bulk remodelling, rather than increase it: slower velocities of organelles were found in wild-type plants when the temperature was lowered by 3-4°C (Holweg, 2007). It is not unsurprising if a heat treatment causes greater movement in a system, in this case the increase in the 37°C 30 min compared to mock 30 min (Figure 4.18, pg. 117). However, neither relationship is repeated with the 1 h conditions; perhaps the difference is due to early responses to the stress as a short term adaptation which is not sustained as the stress continues. It should also be noted that 37°C 1 h has a very small sample size (see Appendix Table B.8, pg. 267).

It appears that the reduction in the bulk remodelling index of the actin cytoskeleton seen in plasmolysed cells is not part of a generalised stress response as no reduction at all was seen with either cold or heat treatments. Kreps *et al.* (2002) examined the change in transcription of *A. thaliana* seedlings that had been stressed with cold, salt and mannitol (hyper-osmotic) and found that they had less than 5% of changed genes in common. Interestingly, even salt and mannitol stressed seedlings only shared 174 changed genes (out of 2,409), while cold-stressed seedlings had twice as many changed genes as either the salt or mannitol stressed seedlings (Kreps *et al.*, 2002). However, the authors also state the longer the period of the stress the more specific the response, which means fewer shared transcriptome changes (Kreps *et al.*, 2002). The actin cytoskeleton during this study did not become more dissimilar from the mock treatments the longer the duration of the stress, however, this study did not look at gene expression, although that is an interesting next step. In addition, perhaps the actin cytoskeleton is part of the immediate reaction, whilst the process of gene regulation takes longer to occur or perhaps the actin changes are a necessary pre-cursor to changes in gene expression.

When *A. thaliana* plants that expressed GFP-Fimbrin were treated with cadmium there was no apparent change in the organisation of the network, however, the authors did not examine its dynamics (Rodriguez-Serrano *et al.*, 2009). They investigated the effect of cadmium on peroxisomal dynamics (they move on the actin cytoskeleton via myosins) in epidermal cells and found that the velocity of peroxisomes increased when the cells were treated with cadmium (Rodriguez-Serrano *et al.*, 2009). This is the opposite of what was found with Golgi bodies in plasmolysed cells in this study, again arguing against a general stress response by the actin cytoskeleton.

4.5.5.2 Temperature-stressed GFP-Lifeact

Investigating the effect of heat and cold stress on actin remodelling using GFP-Lifeact analysed with *Cumulative fluorescence intensity* found few significant differences (Section 4.4.2.5, pg. 95). The 37°C 1 h sample was significantly different to the 4°C 1 h sample at fluorescence intensity values: 10th, 11th, 12th and 13th (Table 4.2, pg. 125). These are not values that indicate an increase or a decrease in the amount of change in the network, which suggests that these two conditions cause a difference in the amount of filaments which are neither continuously remodelling nor stationary (Figure 4.23, pg. 122). However, there was no difference between both treatments and the mock one h sample at any fluorescence intensity value.

The 37°C 30 min sample was significantly different to the 4°C 30 min sample only at the 18th fluorescence intensity value. This indicates that the 37°C 30 min sample has significantly more stationary filaments than the 4°C 30 min sample (Figure 4.22, pg. 121). However, there was no difference between both treatments and the mock 30 min sample at any fluorescence intensity value.

4.5.5.3 Temperature stresses in general

There was no difference in remodelling between the mock treatments and the respective 37°C treatments even though the networks appeared different. However, Malerba *et al.* (2010) examined the effect of heat stress on the actin cytoskeleton (in BY-2 cells) and found that five min at 50°C caused fragmentation of the actin network and also a decrease in the amount of actin protein, but no differences were seen with five min at 35°C or at 45°C. Wang *et al.* (2012) used *A. thaliana* seedlings and found that while some longitudinal bundles remained intact after 40 min, treatment at 45°C also caused fragments of actin filaments to be seen in the cytoplasm.

In this study a mild heat stress changed the organisation of the actin cytoskeleton, but not its dynamics. Muller *et al.* (2007) exposed *A. thaliana* seedlings to heat treatments of 35-38°C for several hours and found that the network did not fragment, but did form thick bundles. Muller *et al.* (2007) found that heat treatments of 40-42°C for 7-25 min caused fragmentation and disappearance of the actin cytoskeleton in *A. thaliana* seedlings, but if the seedlings were returned to 20°C, then the network re-forms. Interestingly, although no thick bundles formed during the stress, after two hours of recovery certain cell types did possess thick bundles that were then mostly disassembled as recovery continued (Muller *et*

al., 2007). A conclusion could be that heat stress is more likely to cause fragmentation of the network rather than an alteration in the dynamics.

4.5.6 Targeted cell wall damage affects the organisation and dynamics of the actin cytoskeleton when marked with GFP-FABD2

Using the marker GFP-FABD2 and the *Pairwise image subtraction* method, the effects of isoxaben treatment were measured in mature leaf epidermal pavement cells. The bulk remodelling index of the actin cytoskeleton was significantly lower in the isoxaben-treated 1 h sample compared to mock 1 h (Figure 4.27, pg. 127). It was also significantly lower in the isoxaben-treated 4 h sample compared to mock 4 h. This reinforces previous results suggesting that the cell wall is part of the mechanism that regulates the actin cytoskeleton in time and space.

Gutierrez *et al.* (2009) showed that two h of isoxaben treatment caused a reduction in motility of Golgi bodies and an accumulation of CSC-containing vesicles in the cell cortex which also had reduced motility. This is interesting as it appears to corroborate the results described above; however, it is possible that as both Golgi bodies and CSC-containing vesicles are unable to reach the cell wall, they somehow ‘block’ the actin cytoskeleton from remodelling. The next step in this experiment would be to investigate another cellulose biosynthesis inhibitor, such as DCB (2,6-dichlorobenitrile), which causes more CESA proteins to accumulate in the membrane whilst almost completely halting CSC motility (Tateno *et al.*, 2016). If the actin cytoskeleton is not so ‘busy’, is there still a reduction in the bulk remodelling index? In addition, instead of tracking Golgi bodies, various fluorescent markers for vesicles and post-Golgi compartments could be tracked to see if there is also a decrease in their velocity during isoxaben and DCB treatments. Wojtaszek *et al.* (2007) state that disrupting specific cell wall components changed the organisation of the actin cytoskeleton; for example, they found pectins were particularly important for anchoring actin.

The bulk remodelling index was lower in the mock 1 h treatment in this experiment compared to the mock 1 h treatment in the temperature stress experiment, however, in this

case the mock was treated with DMSO, perhaps causing a slight difference. Using the marker GFP-FABD2 and the mean fluorescence intensity from the *Pairwise image subtraction* method, the effects of isoxaben treatment were measured in mature epidermal pavement leaf cells. Again, the mean fluorescence intensity of the actin cytoskeleton was significantly lower in isoxaben-treated 1 h compared to mock 1 h (Figure 4.28, pg. 128). It was also significantly lower in the isoxaben-treated 4 h sample compared to mock 4 h. This was unexpected as the isoxaben-treated cells did not appear to have a greater proportion of thick bundled actin strands to thin actin bundles (probably sub-cortical bundles).

Remodelling was altered when cells were treated with isoxaben and the organisation of the network was also disturbed as was found with plasmolysis. Although, in general, all four conditions (mock 1 h, isoxaben-treated 1 h, mock 4 h and isoxaben-treated 4 h) were considered not only to have very similar morphologies, but also to contain more bundles than the control cells in the first experiment (see Appendix Figure B.10 and Figure B.11). Perhaps disruption of different cell wall components in a specific manner causes different reactions from the actin cytoskeleton, possibly because of the way linking elements associate with the cell wall-plasma membrane interface. It is also possible that disruption of different cell wall components affects other structures or processes within the cell, such as the microtubule cytoskeleton or a stress-response signalling cascade, and it is these indirect influences that cause alterations in the dynamics of the actin cytoskeleton.

4.6 Conclusion

The data presented in this chapter suggest that the cell wall has specific organisational control over the spatio-temporal dynamics of the actin cytoskeleton. It was shown that bulk remodelling of the actin cytoskeleton is reduced when a cell is plasmolysed, whereas it is often increased when the cell is re-hydrated or incubated in a hypo-osmotic solution. This finding was partly reproduced with a different method of analysis using GFP-Lifeact and using Golgi body motility as a proxy for actin cytoskeletal dynamics. It was also shown that the change in the actin network when the cell wall-plasma membrane-cytoskeleton continuum was perturbed is not part of a general stress response.

Lastly, the bulk remodelling of the actin cytoskeleton was shown to be reduced when mature epidermal pavement leaf cells had been treated with isoxaben which disrupts specific proteins within the cellulose synthesising complex. This chapter is the start to an investigation into whether the cell wall has any influence over the dynamics of the actin cytoskeleton using plasmolysis and isoxaben, a targeted cell wall disrupting agent. The next chapter explores what the consequences for the actin cytoskeleton if the cell wall is removed entirely.

Chapter Five

The effects of cell wall removal on actin cytoskeleton dynamics

5.1 Introduction

The previous chapter discussed the effects on actin cytoskeleton morphology and dynamics of perturbing the cell wall-plasma membrane interface and showed that these are not part of a general abiotic stress response. The next step was to remove the cell wall and examine the consequences to the dynamics of the actin cytoskeleton.

In this chapter, data is presented from experiments in which the cell wall was removed using digestive enzymes and then allowed to regrow for 24 h (thus cells with minimal cell wall can be compared to cells that have had 24 h to regrow their cell wall). This was carried out using cells that expressed cytoskeletal markers, so that the dynamics and organisation of the actin cytoskeleton could be quantified and compared between cells with minimal cell wall and those with substantial regrowth of cell wall.

As in the previous chapter, the experiment was repeated with two markers for the actin cytoskeleton. Protoplasts derived from mature *A. thaliana* leaf epidermal pavement cells stably expressing GFP-FABD2 had the dynamics of their actin cytoskeletons analysed using *Pairwise image subtraction* with and without measuring the mean fluorescence intensity. The dynamics of the actin cytoskeleton of the same cell types stably expressing GFP-Lifeact were analysed using *Cumulative fluorescence intensity*. In addition, protoplasts derived from mature *A. thaliana* leaf epidermal pavement cells stably expressing ST-GFP were imaged and the Golgi bodies were tracked using the Volocity software, so that their movement and behaviour could be analysed and compared between

conditions. All this can be seen in Figure 4.1, which details the image analysis methods for the different markers.

5.2 Collaborators

The *Cumulative fluorescence intensity* method was developed by Dr Axel Poulet, at Oxford Brookes University (Oxford, UK), specifically for this study. Dr Mike Deeks, a Lecturer in Plant Biology at the Department of Biosciences in the University of Exeter (Exeter, UK), advised on the protoplast work.

5.3 Aims

The aim of this chapter was to study the influence of the cell wall on the dynamics and organisation of the actin cytoskeleton using protoplasts. This included:

- a) Using confocal microscopy to analyse and compare the dynamics and organisation of the actin cytoskeleton under two different conditions – immediately after cell wall removal (0 h) and after 24 h of cell wall regrowth. The experiments above were analysed with two different fluorescent protein markers for the actin cytoskeleton, in the event the marker has an effect on the dynamics and organisation of the network; and then again with a different fluorescent protein marker for Golgi bodies, in the event that any marker bound to the actin cytoskeleton alters the network, as was done in the previous chapter.
- b) Using confocal microscopy to compare protoplasts treated with isoxaben against a mock treatment to analyse the dynamics and organisation of the actin cytoskeleton when the cell wall is growing back in a compromised manner.

5.4 Results

All protoplasts were made from mature stably-expressing *A. thaliana* epidermal leaf cells. All confocal microscope derived time-series analysed in this chapter lasted approximately 100 s. The GFP-FABD2 expressing line was made by Ketelaar *et al.* (2004) and Sheahan *et al.* (2004), the GFP-Lifeact expressing line was made by Smertenko *et al.* (2010) and the ST-GFP expressing line was made by Saint-Jore (2001).

5.4.1 Analysis of bulk actin cytoskeleton remodelling visualised with GFP-FABD2 under conditions of cell wall removal and isoxaben treatment using confocal microscopy

Protoplasts were made from mature *A. thaliana* plants stably-expressing GFP-FABD2, but only the leaf epidermal pavement cells were imaged. Mesophyll cells were excluded because of their abundant chloroplasts which alter actin cytoskeleton spatial organisation (Figure 5.1, pg.149). The cells were imaged under a mock treatment (0.01% DMSO) and a treatment of 20 μ M isoxaben (see Section 2.6.4, pg. 49). Both samples were imaged at 0 h, which here is defined as when the cell wall has been digested and the cells have been washed (see Section 2.4.3). They were also imaged 24 h later after the cells had been left without enzymes for the cell wall to grow back.

5.4.1.1 Qualitative assessment of GFP-FABD2 at 0 h and 24 h after cell wall removal

Protoplasts made from the leaf epidermal pavement cells of mature *A. thaliana* plants stably-expressing GFP-FABD2 were imaged using confocal microscopy for approximately 2 min. As in the previous chapter, remodelling can be described in a qualitative fashion by overlaying time points (Figure 5.2, pg.150). Three time points (0 s, 50 s and 105 s) were selected and re-coloured red, green and blue in Adobe Photoshop for four protoplast samples: one immediately post-cell wall removal and one after 24 h of cell wall regrowth, both with and without isoxaben [Adobe Systems Incorporated; San Jose, CA; USA].

Mock 0 h cells usually displayed a dense meshwork of actin filaments, but could have large bundled structures running around the cell with large areas of the cortex seemingly empty of filaments (Row A in Figure 5.2). Isoxaben-treated 0 h cells appeared very similar to the mock 0 h cells (Row B in Figure 5.2). Mock 24 h cells were very similar in appearance to mock 0 h cells, but seemed to have more bundles and strands around the cell cortex (Row C in Figure 5.2). Isoxaben-treated 24 h cells appeared very similar to the isoxaben-treated 0 h cells (Row D in Figure 5.2). See Appendix Figure C.1, pg. 274 and Figure C.2 for images of all cells.

5.4.1.2 Pairwise image subtraction of GFP-FABD2 marked actin cytoskeleton in protoplasts 0 h and 24 h after cell wall removal with and without the addition of isoxaben

Pairwise image subtraction was introduced in Chapter 3 and used in the previous chapter (see Section 3.4.3.1, pg. 60). In brief, using pairs of time-frames, the first time-frame is subtracted from the second and the remaining fluorescence is calculated as a proportion of the total initial fluorescence. Therefore a static actin cytoskeleton would have a lower value and a constantly changing actin cytoskeleton would have a higher value.

Approximately 30 cells per condition of protoplasts stably-expressing GFP-FABD2 were compared using the Kruskal-Wallis test and Dunn's Multiple Comparison test (a non-parametric ANOVA with post-test; Figure 5.3, pg.151; see Section 2.7.2, pg. 51). The bulk remodelling index of the GFP-FABD2 marked actin cytoskeleton was calculated as the mean and standard deviation (arbitrary units, a.u.): mock 0 h was 0.5299 ± 0.03884 , 20 μM isoxaben-treated 0 h was 0.5241 ± 0.03994 , mock 24 h was 0.5233 ± 0.04463 and 20 μM isoxaben-treated 24 h was 0.5185 ± 0.04198 (Figure 5.3 and Appendix Table C.1).

In mock-treated protoplasts that have had 24 h to regrow their cell wall, significantly less bulk remodelling occurred than in protoplasts imaged directly post-cell wall removal ($p < 0.001$; Figure 5.3). The bulk remodelling index for isoxaben-treated protoplasts at 0 h is significantly smaller than that of mock-treated protoplasts at the same time point ($p < 0.01$; Figure 5.3). There are no significant differences between isoxaben-treated 24 h and the isoxaben-treated 0 h ($p > 0.05$; Figure 5.3) or between the mock 24 h and the isoxaben-treated 24 h sample ($p > 0.05$; Figure 5.3). More bulk remodelling occurred at the 0 h time-points than the 24 h time-points and the bulk remodelling of the 0 h protoplasts was more sensitive to isoxaben treatment.

5.4.1.3 *Mean fluorescence intensity using Pairwise image subtraction of GFP-FABD2 marked actin cytoskeleton in protoplasts 0 h and 24 h after cell wall removal with and without the addition of isoxaben*

Mean fluorescence intensity measured using *Pairwise image subtraction* was introduced in Chapter 3 and used in the previous chapter (see Section 3.4.3.2). In brief, the same method is used as pairwise image subtraction, except that the mean fluorescence intensity is measured rather than the total amount of fluorescence.

The same dataset as used in the previous analysis of protoplasts stably-expressing GFP-FABD2 was compared as above (Figure 5.4, pg.152). The mean fluorescence intensity of the GFP-FABD2 marked actin cytoskeleton was calculated as the mean and standard deviation: mock 0 h was 0.2671 ± 0.0034 , 20 μ M isoxaben treated 0 h was 0.2706 ± 0.0033 , mock 24 h was 0.2745 ± 0.0037 and 20 μ M isoxaben treated 24 h was 0.2712 ± 0.0038 (Figure 5.4 and Appendix Table C.2). There were no significant differences between any of the conditions ($p = 0.3621$; Figure 5.4) indicating that whatever remodelling may or may not have occurred at each time-point and condition, it is the same kind of filaments that move and remain stationary. This suggests that there is not a different proportion of bundles to thin bundless in any of the conditions.

5.4.2 Analysis of bulk actin cytoskeleton remodelling visualised with GFP-Lifeact under conditions of cell wall removal and isoxaben treatment using confocal microscopy

5.4.2.1 *Qualitative assessment of GFP-Lifeact at 0 h and 24 h after cell wall removal*

Protoplasts made from the leaf epidermal pavement cells of mature *A. thaliana* plants stably-expressing GFP-Lifeact were imaged using confocal microscopy for approximately 100 s (Figure 5.5, pg.153).

Protoplasts expressing GFP-Lifeact displayed a large range of morphologies. Mock 0 h cells usually displayed thick bundles or strands (Row A in Figure 5.5). Isoxaben-treated 0 h cells, mock 24 h cells and isoxaben-treated 24 h cells all appeared to have quite similar morphologies (Rows B, C and D, respectively in Figure 5.5).

5.4.2.2 Cumulative fluorescence intensity of GFP-Lifeact marked actin cytoskeleton in protoplasts 0 h and 24 h after cell wall removal with and without the addition of isoxaben

Cumulative fluorescence intensity was introduced in Chapter 3 as a way to measure any alterations in the actin cytoskeleton tagged with GFP-Lifeact; it was also used in the previous chapter (see Section 3.4.3.3). In brief, each pixel was recorded as having fluorescence, or no fluorescence, for all time points in a time-series and then these were summed to produce a set of values that describe the amount of change.

Figure 5.6 (pg.154) shows the cumulative fluorescence intensities of mock 0 h and isoxaben-treated 0 h, whilst Figure 5.7 (pg. 155) shows mock 24 h and isoxaben-treated 24 h (approximately 30 cells per condition). These have all been normalised against the total fluorescence of each time-series, so there should be no bias for size of cell or the amount of cytoskeleton in view. The fluorescence intensity of the different treatments has been graphed against mock 0 h having a value of ‘one’ for every *Cumulative fluorescence intensity* point (Figure 5.8, pg.156). To compare the treatments, one-way ANOVA was used with a Tukey post-test for each fluorescence intensity value, resulting in 19 tests. The p-value for every ANOVA can be seen in Table 5.1 (pg. 157); those in bold are significantly different.

One-way ANOVA found significant differences between treatments at the third and fourth fluorescence intensity values ($p = 0.0465$ and $p = 0.0300$ respectively; Table 5.1). The proportion of each condition at the third fluorescence intensity value was calculated as the mean and standard deviation (no units as these are proportions): mock 0 h was 0.0779 ± 0.0313 , isoxaben-treated 0 h was 0.0779 ± 0.0331 , mock 24 h was 0.0927 ± 0.0362 and isoxaben-treated 24 h was 0.0957 ± 0.0388 (Table 5.1). The proportion of each condition at the fourth fluorescence intensity value was calculated as the mean and standard deviation: mock 0 h is 0.0660 ± 0.0224 , isoxaben-treated 0 h was 0.0652 ± 0.0240 , mock 24 h was 0.0770 ± 0.0241 and isoxaben-treated 24 h was 0.0792 ± 0.0300 (Table 5.1). Using the Tukey post-test all column comparisons were found to have no significant differences ($p > 0.05$ in both cases; Table 5.1).

The first two and the last 15 fluorescence intensity values have a p-value of $p > 0.05$, so there were no differences in the proportions of the fluorescence intensities at these values

(Table 5.1). This also means that there was not an increased or decreased amount of fluorescence in the intensity values that can indicate movement or stability in the network.

5.4.3 Analysis of bulk actin cytoskeleton remodelling visualised with ST-GFP under conditions of cell wall removal and isoxaben treatment using confocal microscopy

As a proxy for the movement of the actin cytoskeletal network (Sparkes *et al.*, 2008, Martinière *et al.*, 2011a, Rocchetti *et al.*, 2014), the movement of Golgi bodies was analysed using Volocity software ([PerkinElmer]; see Section 2.6.7, pg. 50). The measurements listed below are all from the same dataset of approximately 30 cells per condition (the number of Golgi per condition is on each graph). Protoplasts made from the leaf epidermal pavement cells of mature ST-GFP stably-expressing *A. thaliana* plants were imaged using confocal microscopy for approximately 90 s. Using Volocity it was possible to show a cell overlaid with all the tracks of the Golgi bodies that moved through the field of view during the time-series and these tracks can be annotated with the velocity of the Golgi body at that particular time point (Figure 5.9, pg. 158). Golgi bodies in protoplasts were much easier to track than Golgi bodies in whole cells (see Section 4.4.1.6, pg. 90): this suggests that their movements were much slower and less complicated.

5.4.3.1 Dynamics of ST-GFP marked Golgi bodies at 0 h and 24 h after cell wall removal with and without the addition of isoxaben

Figure 5.10 (pg.159) shows the velocity of ST-GFP marked Golgi body populations analysed using the Kruskal-Wallis test with Dunn's Multiple Comparison test (a non-parametric ANOVA with post-test). The average velocity of ST-GFP tagged Golgi bodies was calculated as mean and standard error of mean ($\mu\text{m/s}$): mock 0 h was 0.3338 ± 0.0098 , isoxaben-treated 0 h was 0.3436 ± 0.0064 , mock 24 h was 0.4089 ± 0.0059 and isoxaben-treated 24 h was 0.4093 ± 0.0055 (see Appendix Table C.3, pg. 277). After 24 h the velocity was significantly higher in both the mock and the isoxaben-treated compared to their respective 0 h ($p < 0.001$; Figure 5.10). However, there were no significant differences between 0 h sample or between the 24 h samples ($p > 0.05$; Figure 5.10).

The displacement rate is a description of how much time an object has taken to move between two specified points as measured in a straight line. A low displacement rate means an object has taken a long time to move a short straight-line distance, whilst a high value means the object took a short time to move a long distance. Figure 5.11 (pg. 160) shows the displacement rate of ST-GFP marked Golgi body populations analysed using an unpaired t-test with Welch's correction. The average displacement rate of ST-GFP tagged Golgi bodies was calculated as mean and standard error of mean ($\mu\text{m/s}$): mock 0 h was 0.07583 ± 0.0071 , isoxaben-treated 0 h was 0.06138 ± 0.0034 , mock 24 h was 0.1095 ± 0.0044 and isoxaben-treated 24 h was 0.09382 ± 0.0034 (see Appendix Table C.4).

After 24 h, the displacement rate in both the mock and the isoxaben-treated compared to their respective 0 h was significantly higher ($p < 0.0001$; Figure 5.11) and the variances were significantly different ($p < 0.001$) between the two isoxaben-treated samples, but not between the two mock samples ($p = 0.3383$; Figure 5.11). There were no significant differences between the two 0 h conditions ($p = 0.0678$) but the variances were significantly different ($p < 0.001$; Figure 5.11). However, the displacement rate of Golgi bodies in the isoxaben-treated 24 h sample compared to the mock 24 h sample was significantly lower ($p = 0.0048$) and the variances were significantly different ($p < 0.001$; Figure 5.11).

The meandering index is a ratio describing how complex the movement of an object is between two points. It is calculated using the track velocity and displacement rate (see above) and calculates a value between 0 and 1. If an object has a meandering index close to '1', then it has moved in a straight line between two points and if it has a value close to '0' then it has made a very complicated motion, such as 'wiggling' (Rocchetti *et al.*, 2014). Figure 5.12 (pg. 161) shows the meandering index of ST-GFP marked Golgi body populations analysed using the Kruskal-Wallis test with Dunn's Multiple Comparison test. The meandering index of ST-GFP tagged Golgi bodies was calculated as mean and standard deviation (no units as this is an index): mock 0 h was 0.1925 ± 0.1794 , isoxaben-treated 0 h was 0.1741 ± 0.1556 , mock 24 h was 0.2441 ± 0.1847 and isoxaben-treated 24 h was 0.2220 ± 0.1798 (see Appendix Table C.5). After 24 h the meandering index in both the mock and the isoxaben-treated compared to their respective 0 h was significantly lower ($p < 0.001$; Figure 5.12). There was no significant difference between the two 0 h conditions ($p > 0.05$; Figure 5.12). However, the meandering index of the mock 24 h

sample compared to the isoxaben-treated 24 h was significantly higher ($p < 0.05$; Figure 5.12).

Figure 5.13 (pg. 162) shows the number of time-frames that the ST-GFP marked Golgi body populations were present in the field of view, analysed using an unpaired t-test with Welch's correction. The time-frames were calculated as mean $\pm$ standard deviation (units are time-frames): mock 0 h was 87 ± 63.68 , isoxaben-treated 0 h was 81.81 ± 60.04 , mock 24 h was 59.52 ± 46.27 and isoxaben-treated 24 h was 59.08 ± 50.42 (see Appendix Table C.6). After 24 h the number of time frames that Golgi bodies were present in the field of view was significantly lower for both the mock and the isoxaben-treated compared to their respective 0 h ($p < 0.0001$; Figure 5.13) and the variances were significantly different ($p < 0.0001$). There was no significant difference between the two 0 h conditions ($p = 0.2618$; Figure 5.13) and the variances were not significantly different ($p = 0.2570$). There was no significant difference between the 24 h samples ($p = 0.8625$), but the variances were significantly different ($p = 0.02$; Figure 5.13).

Figure 5.14 (pg. 163) shows the length of the tracks made by the ST-GFP marked Golgi body population, analysed using an unpaired t-test with Welch's correction. The track lengths were calculated as mean $\pm$ standard deviation (μm): mock 0 h was 10.69 ± 6.2 , isoxaben-treated 0 h was 11.39 ± 7.9 , mock 24 h was 9.74 ± 6.5 and isoxaben-treated 24 h was 9.58 ± 6.9 (see Appendix Table C.7). The track length in mock 24 h sample compared to mock 0 h was significantly lower ($p = 0.0305$; Figure 5.14) but the variances were not significantly different ($p = 0.3699$). The track length in isoxaben-treated 24 h compared to isoxaben-treated 0 h was also significant lower ($p < 0.0001$; Figure 5.14) and the variances were significantly different ($p = 0.0022$). There were no significant differences between mock and isoxaben-treated at 0 h ($p = 0.1707$) although the variances were significantly different ($p < 0.0001$; Figure 5.14). There were no significant differences between mock and isoxaben-treated at 24 h ($p = 0.6453$) and the variances were not significantly different either ($p = 0.0732$; Figure 5.14).

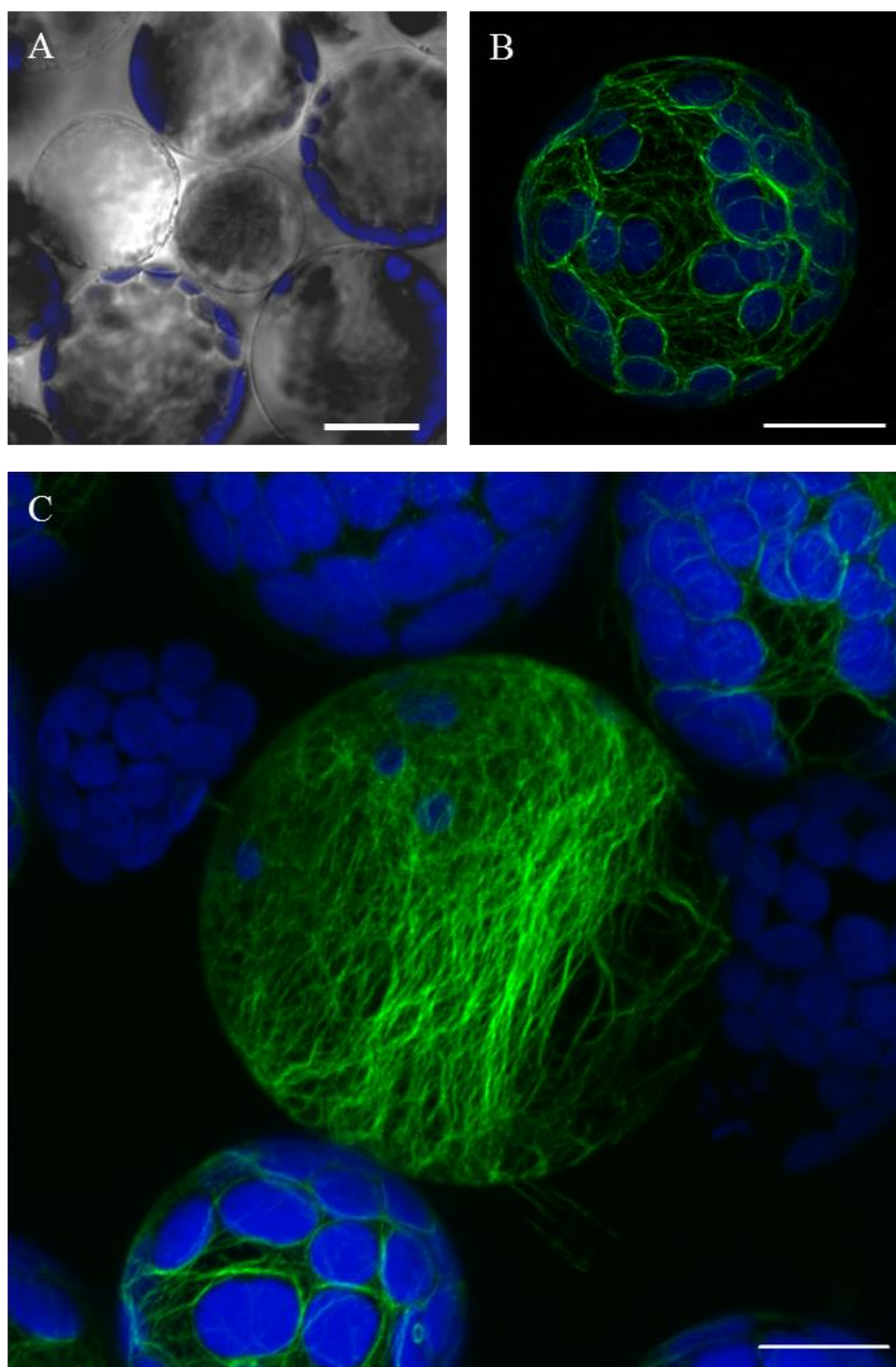


Figure 5.1 Confocal micrographs of protoplasts from mature *A. thaliana* leaf cells, both epidermal and mesophyll-derived.

A) Protoplasts derived from mesophyll cells contain many chloroplasts, unlike those of epidermal cells. B) Mesophyll-derived protoplast in which the actin cytoskeleton can be seen as a ‘cage’ surrounding the chloroplasts. C) The central protoplast is derived from an epidermal cell. There are very few chloroplasts and they are small; this is the cell type that was chosen for analysis.

In all images blue is auto-fluorescence from chloroplasts and green is GFP-FABD2.

Scale bar: A) and B) = 20 μm and C) = 10 μm .

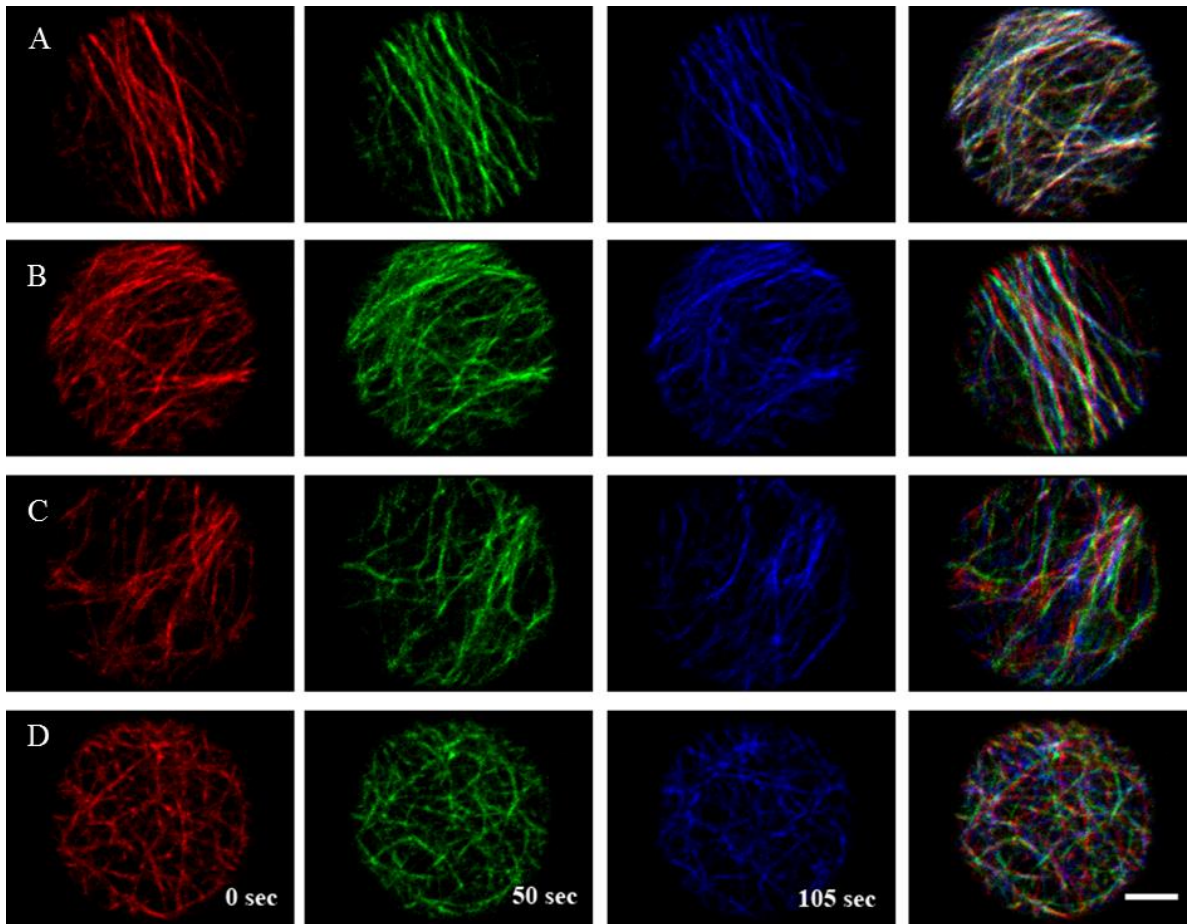


Figure 5.2 Time-lapse three dimensional CLSM images of protoplasts expressing GFP-FABD2.

Confocal micrographs of protoplasts derived from mature *A. thaliana* leaf epidermal pavement cells made from lines stably expressing GFP-FABD2. They were imaged at 0 h and 24 h after cell wall removal, with and without isoxaben treatment. The red, green and blue images are 3D projections of a single time-point from a time-series. On the far right is each cell with its corresponding three time-points overlaid: in each row the first time-point is red (0 s), the middle time-point is green (50 s) and the last time-point is blue (105 s). In the overlay when the filaments have moved they appear colourful, but when they do not move they appear white as the colours are superimposed.

(A) Mock treated 0 h time-point.

(B) 20 μ M isoxaben-treated 0 h time-point.

(C) Mock treated 24 h time-point.

(D) 20 μ M isoxaben-treated 24 h time-point.

Scale bar = 5 μ m.

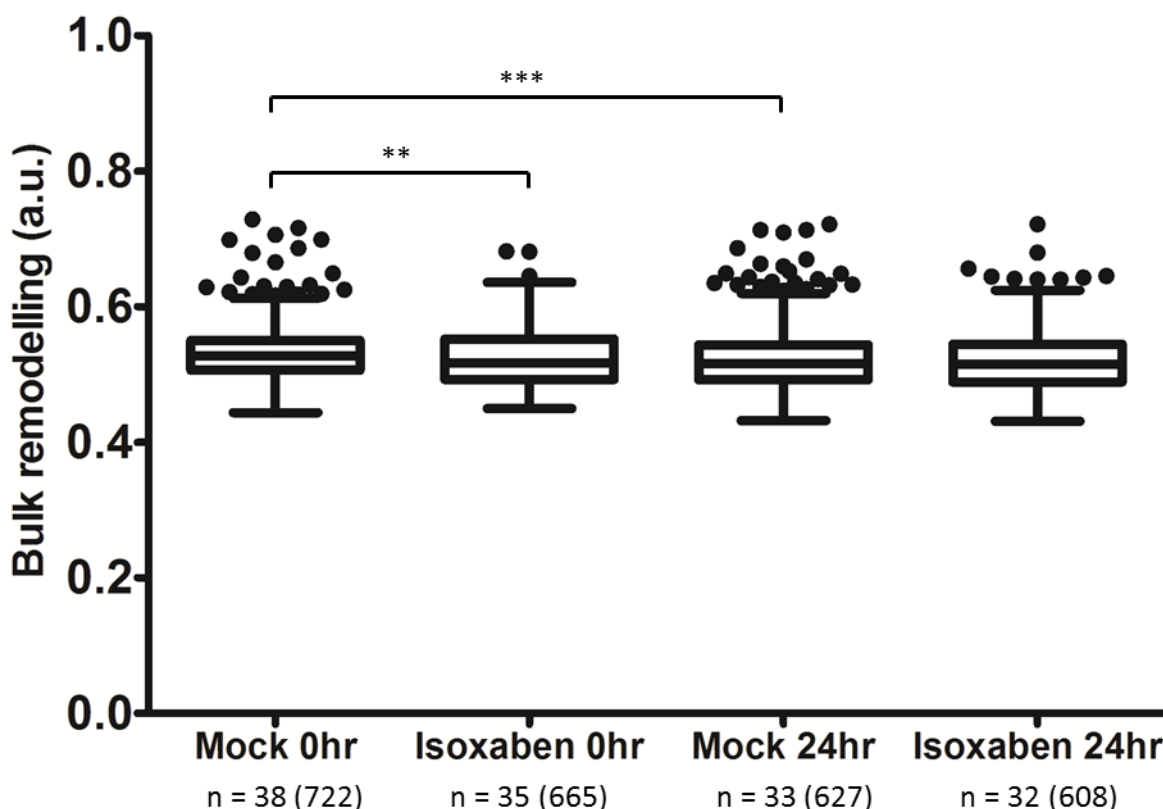


Figure 5.3 Results from *Pairwise image subtraction* analysis on protoplasts expressing GFP-FABD2.

The boxplots show the data for protoplasts originating from leaf epidermal pavement cells from a transgenic line of *A. thaliana* stably expressing GFP-FABD2 at different conditions and time points. These have been analysed using the *Pairwise image subtraction* method (see Section 3.4.3.1); for detailed results see Appendix Table C.1. The mean and standard deviation (arbitrary units, a.u.): mock 0 h was 0.5299 ± 0.039 , 20 μ M isoxaben-treated 0 h was 0.5241 ± 0.040 , mock 24 h was 0.5233 ± 0.045 and 20 μ M isoxaben-treated 24 h was 0.5185 ± 0.042 . The value 'n' is the number of cells imaged and the number in parentheses the total of the 19 comparisons made per cell. The asterisks refer to the p-value: **p < 0.01 and ***p < 0.001.

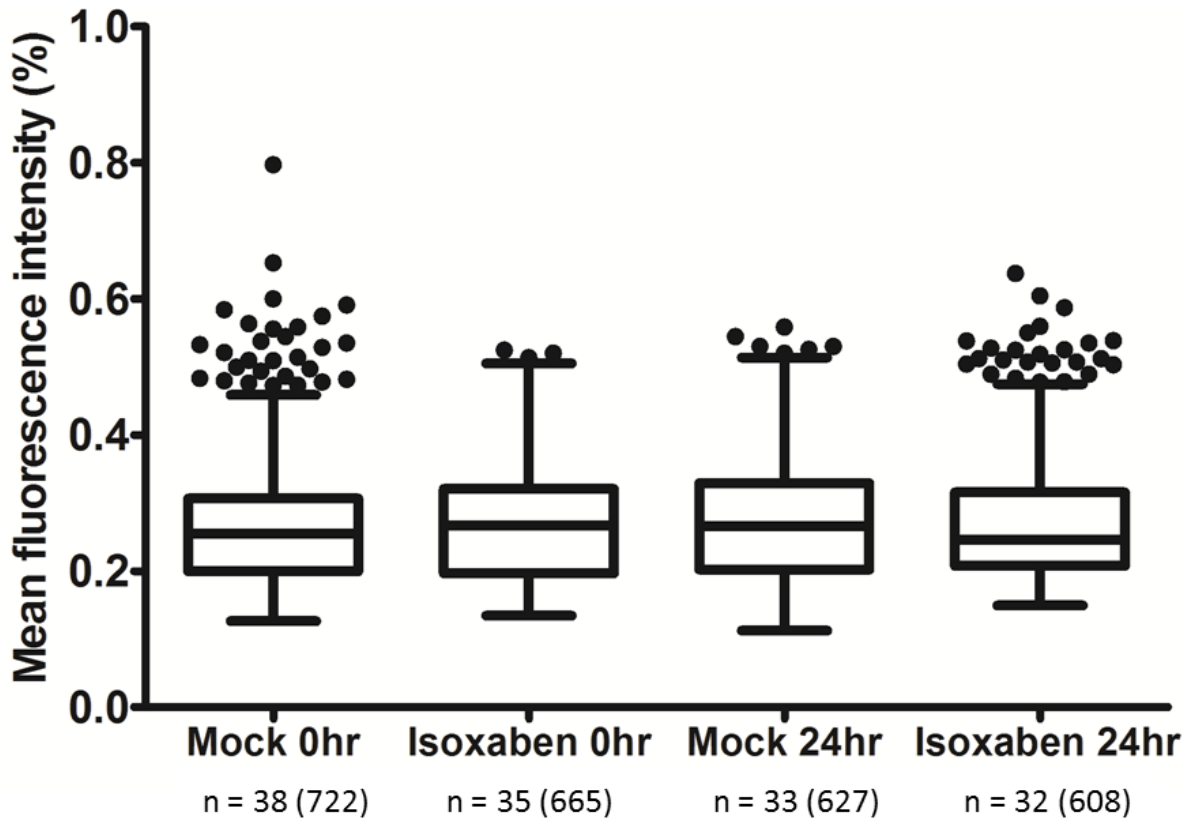


Figure 5.4 Results from *Pairwise image subtraction* measuring mean fluorescence analysis on protoplasts expressing GFP-FABD2.

The boxplots show the data for protoplasts originating from leaf epidermal pavement cells from a transgenic line of *A. thaliana* stably expressing GFP-FABD2 at different conditions and time points. These have been analysed using the *Pairwise image subtraction* measuring mean fluorescence intensity method (see Section 3.4.3.2); for detailed results see Appendix Table C.2. The mean and standard deviation: mock 0 h was 0.2671 ± 0.0034 , 20 μ M isoxaben treated 0 h was 0.2706 ± 0.0033 , mock 24 h was 0.2745 ± 0.0037 and 20 μ M isoxaben treated 24 h was 0.2712 ± 0.0038 . The value ‘n’ is the number of cells imaged and the number in parentheses the total of the 19 comparisons made per cell.

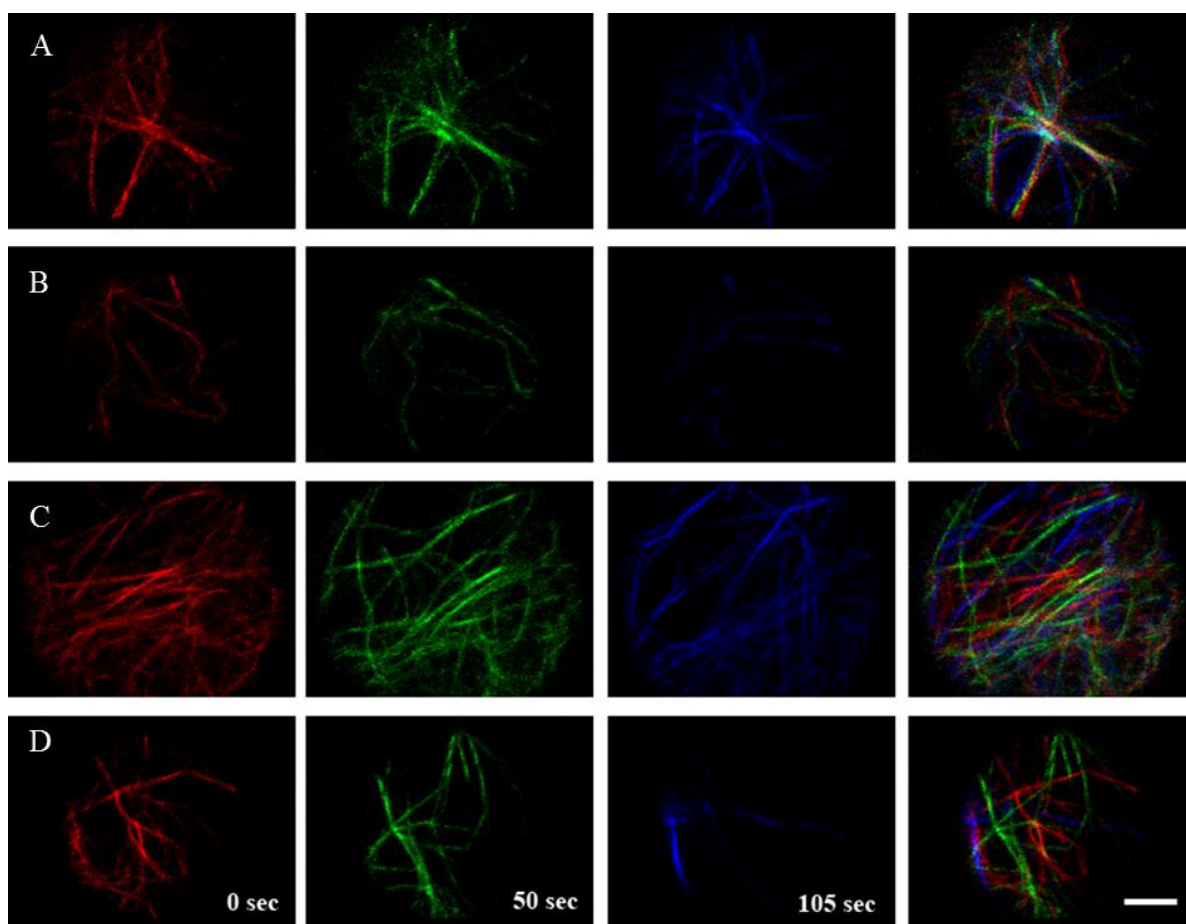


Figure 5.5 Time-lapse three dimensional CLSM images of protoplasts expressing GFP-Lifeact.

Confocal micrographs of protoplasts derived from mature *A. thaliana* leaf epidermal pavement cells made from lines stably expressing GFP-Lifeact. They were imaged at 0 h and 24 h after cell wall removal, with and without isoxaben treatment. The red, green and blue images are 3D projections of a single time-point from a time-series. On the far right is each cell with its corresponding three time-points overlaid: in each row the first time-point is red (0 s), the middle time-point is green (50 s) and the last time-point is blue (105 s). In the overlay when the filaments have moved they appear colourful, but when they do not move they appear white as the colours are superimposed.

(A) Mock treated 0 h time-point. (B) 20 μ M isoxaben-treated 0 h time-point.

(C) Mock treated 24 h time-point. (D) 20 μ M isoxaben-treated 24 h time-point.

Scale bar = 5 μ m.

In Figure 5.6 and Figure 5.7 the cells shown here are denoted with an asterisk: mock 0 h cell 29; mock 24 h cell 30; isoxaben-treated 0 h cell 23; and isoxaben-treated 24 h cell 20.

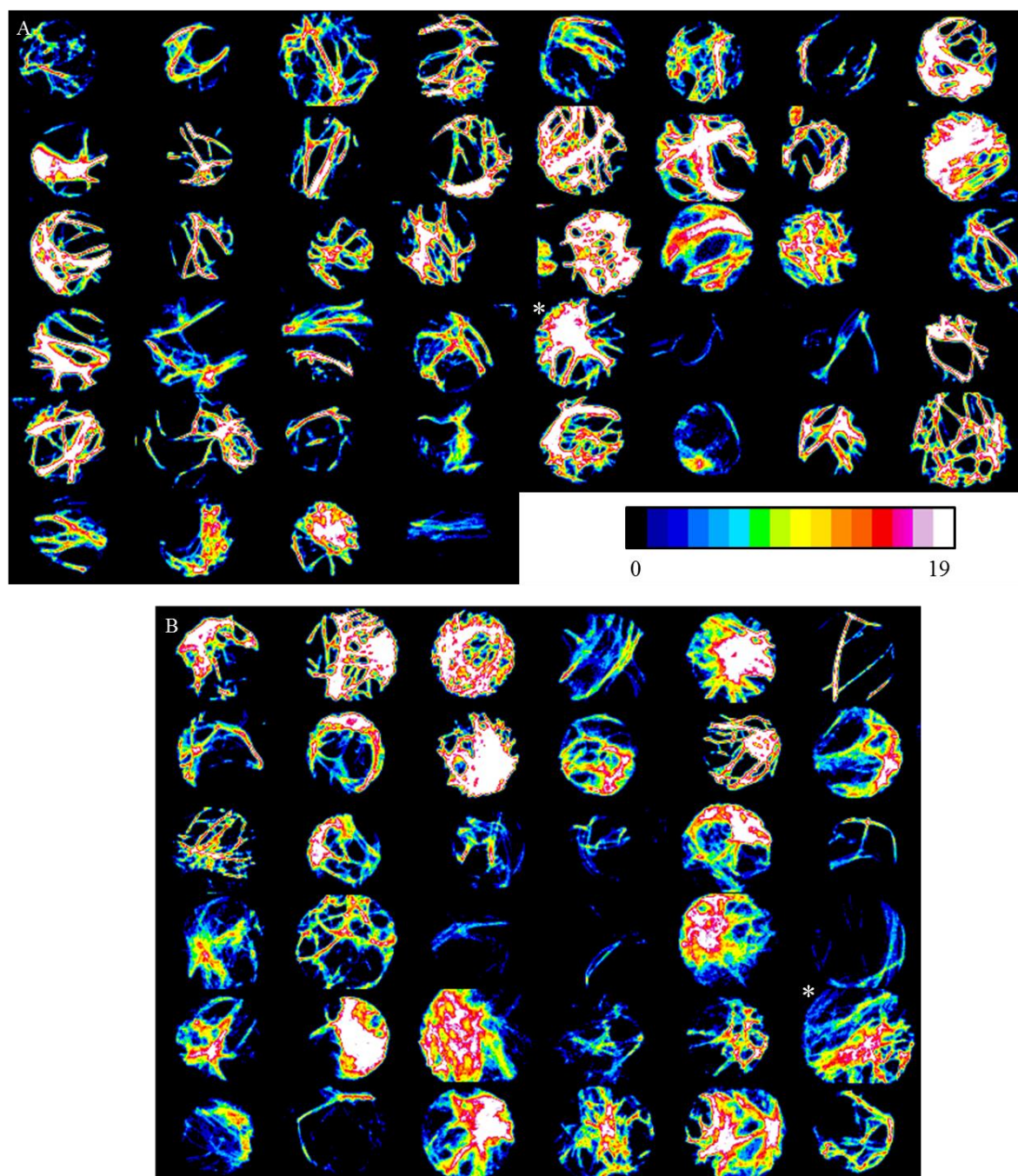


Figure 5.6 Visual results from the *Cumulative fluorescence intensity* analysis on protoplasts expressing GFP-Lifeact that have been mock treated.

Heat map shows the fluorescence intensity value per pixel, where white represents stationary filaments and darker colours represent lots of movement.

(A) 44 mock-treated protoplasts imaged at 0 h; (B) 36 mock-treated protoplasts imaged at 24 h. These heat maps were constructed as described in Section 3.4.3.3. Asterisks denote cells shown in Figure 5.5.

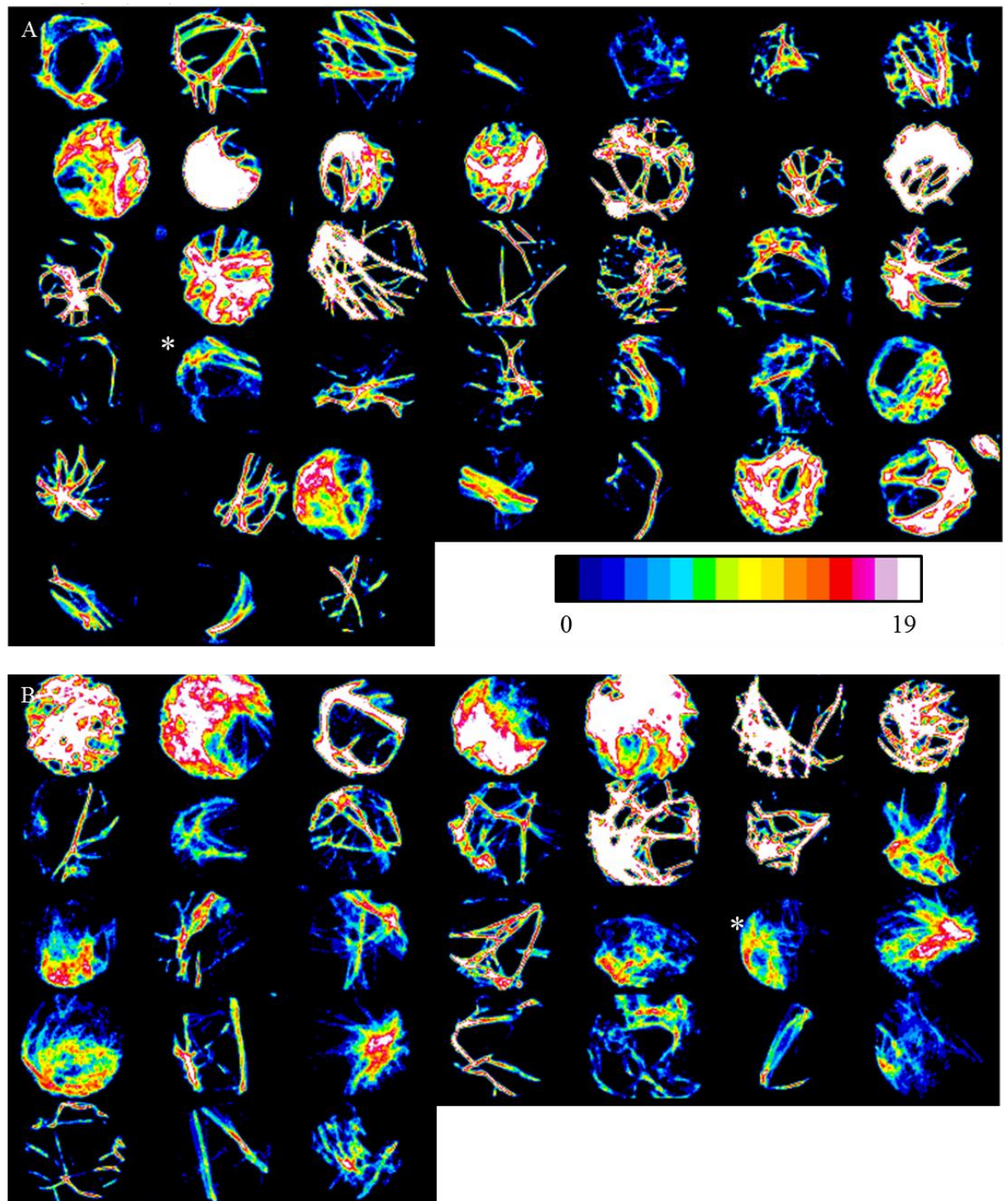


Figure 5.7 Visual results from the *Cumulative fluorescence intensity* analysis on protoplasts expressing GFP-Lifeact that have been treated with isoxaben.

Heat map shows the fluorescence intensity value per pixel, where white represents stationary filaments and darker colours represent lots of movement.

(A) 38 isoxaben-treated protoplasts imaged at 0 h; (B) 31 isoxaben-treated protoplasts imaged at 24 h. These heat maps were constructed as described in Section 3.4.3.3. Asterisks denote cells shown in Figure 5.5.

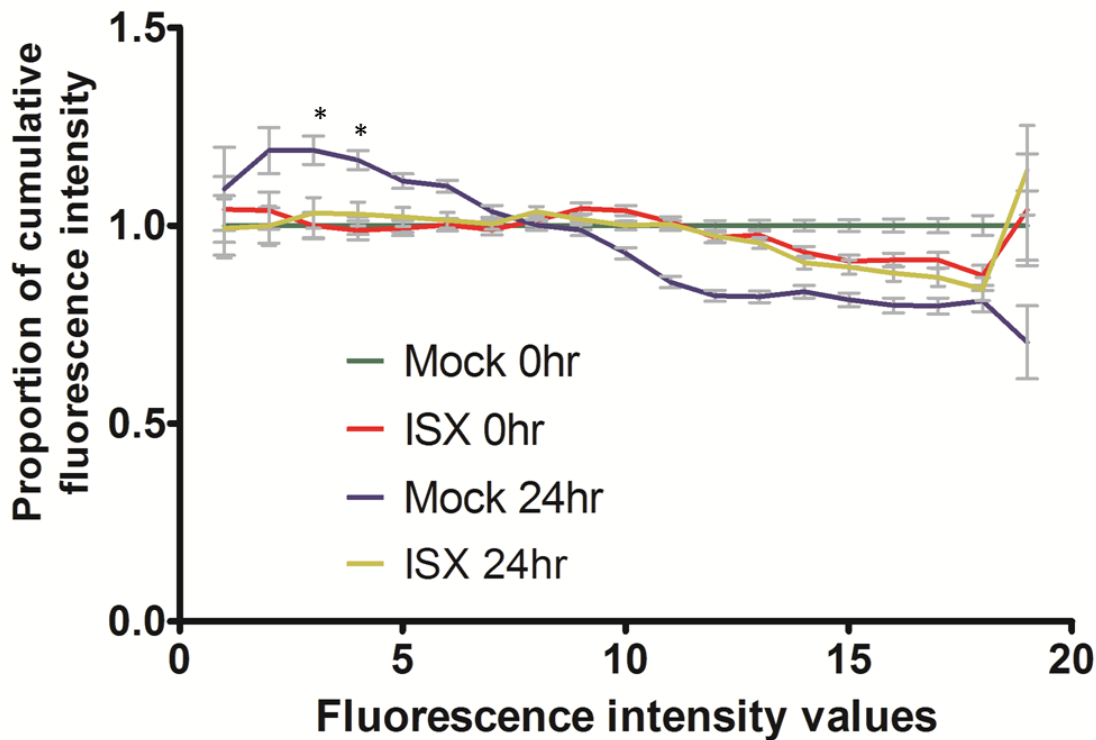


Figure 5.8 Results from *Cumulative fluorescence intensity* analysis of protoplasts expressing GFP-Lifeact.

Line graphs representing the bulk remodelling of the actin cytoskeleton marked with GFP-Lifeact. Mock 0 h (n = 44 cells) values were normalised to '1' in order to compare with experimental treatments: 20 μ M isoxaben-treated 0 h (n = 38 cells), mock 24 h (n = 36 cells) and 20 μ M isoxaben-treated 24 h (n = 31 cells). This graph was made using the analysis from the *Cumulative fluorescence intensity* method, for an additional graph see Appendix Figure C.3. The asterisks refer to the p-value from the one-way ANOVA: *p < 0.05. The Tukey post-test found all column comparisons to have no significant differences (p > 0.05).

Table 5.1 The p-value of every fluorescence intensity value analysed using one-way ANOVA.

Fluorescence intensity values from protoplasts expressing GFP-Lifeact analysed using the *Cumulative fluorescence intensity* method. The asterisks refer to p-values returned by the one-way ANOVA: *p < 0.05.

Fluorescence intensity value	p-value
1	0.8779
2	0.2250
3	0.0465 *
4	0.0300 *
5	0.1517
6	0.0578
7	0.1229
8	0.3639
9	0.2021
10	0.1053
11	0.1204
12	0.0653
13	0.0899
14	0.1718
15	0.1768
16	0.2054
17	0.2694
18	0.3854
19	0.6630

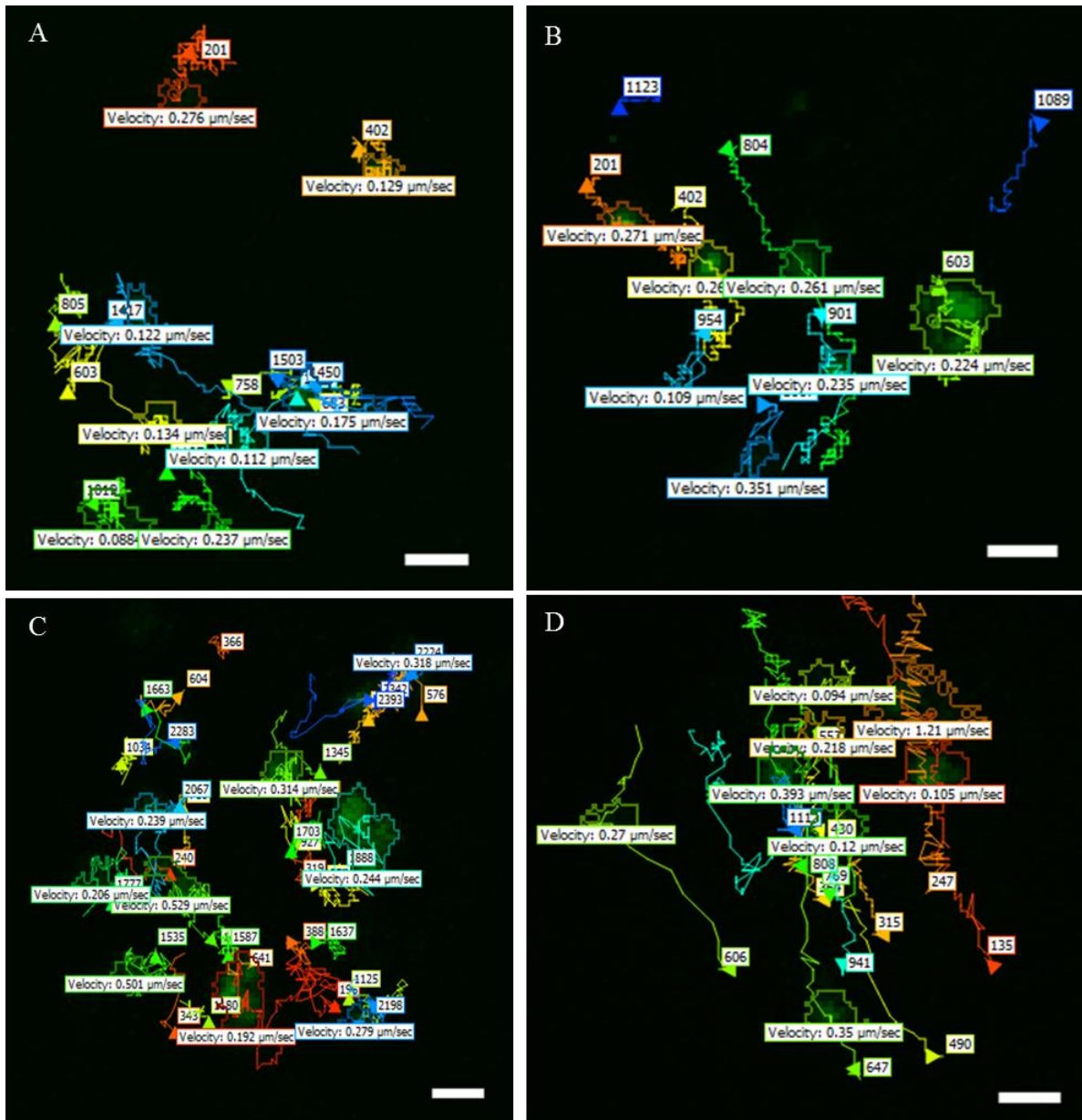


Figure 5.9 Confocal micrograph of protoplasts derived from leaf epidermal pavement cells from a transgenic line of *A. thaliana* stably expressing ST-GFP annotated with the current velocity of the Golgi bodies present.

(A) Golgi bodies in one mock-treated cell at 0 h after cell wall removal.

(B) Golgi bodies in one isoxaben-treated cell at 0 h after cell wall removal.

(C) Golgi bodies in one mock-treated cell at 24 h after cell wall removal.

(D) Golgi bodies in one isoxaben-treated cell at 24 h after cell wall removal.

The tracks are labelled by number and the velocity of each Golgi body currently in view is shown as measured in Velocity (see Section 2.6.7, pg. 50). Scale bar = $1.5 \mu\text{m}$ and the total elapsed time of each time-series was 92 s.

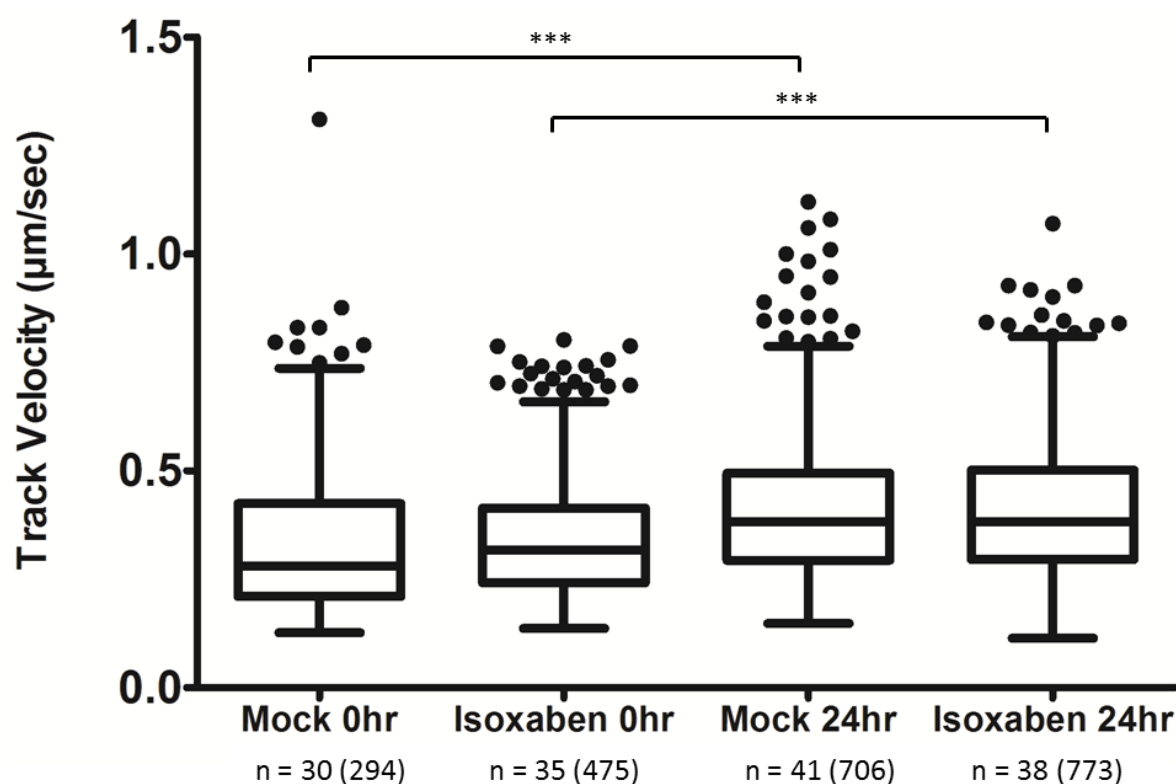


Figure 5.10 Average velocities of Golgi bodies in protoplasts expressing ST-GFP.

Track velocity of Golgi bodies in leaf epidermal pavement cells from a transgenic line of *A. thaliana* stably expressing ST-GFP at mock 0 h. The track velocities have been analysed using Velocity; for detailed results see Appendix Table C.3, pg. 277. The average velocity of ST-GFP tagged Golgi bodies was calculated as mean and standard error of mean (µm/s): mock 0 h was 0.3338 ± 0.0098 , isoxaben-treated 0 h was 0.3436 ± 0.0064 , mock 24 h was 0.4089 ± 0.0059 and isoxaben-treated 24 h was 0.4093 ± 0.0055 . The number in parentheses is the total number of Golgi tracked per cell. The asterisks refer to the p-value: *** $p < 0.001$.

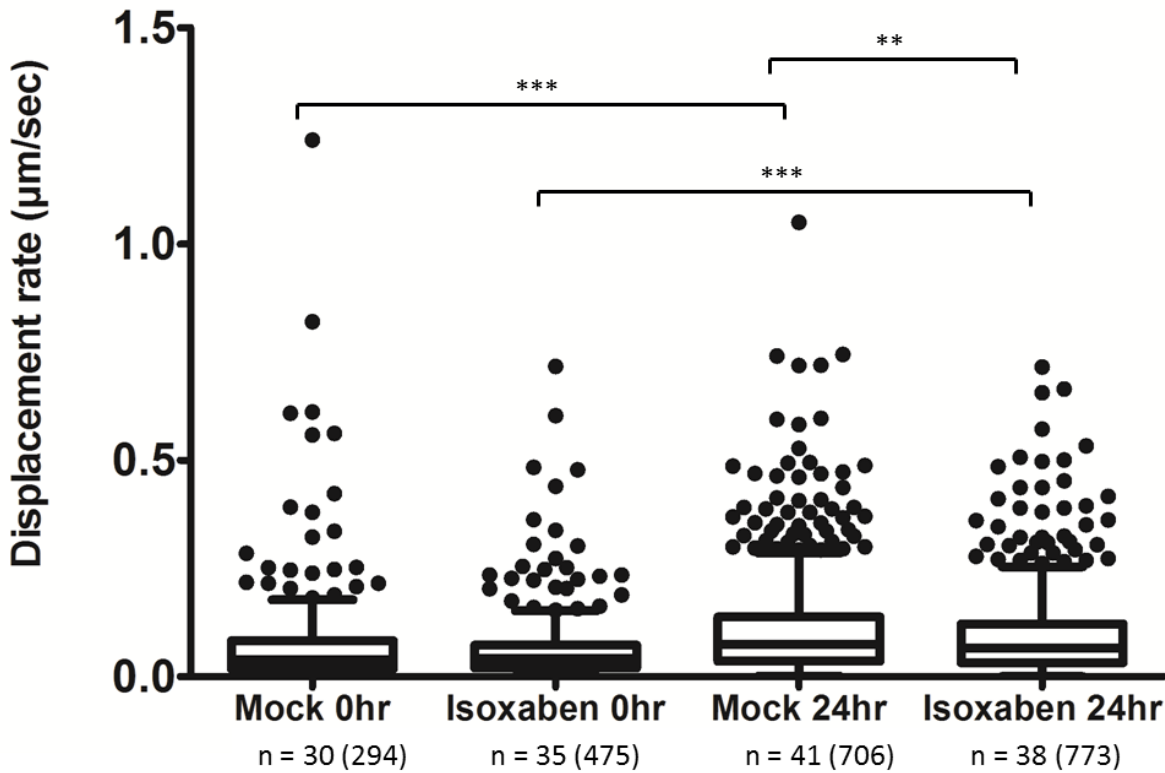


Figure 5.11 Average displacement rate of Golgi bodies in protoplasts expressing ST-GFP.

Displacement rate of Golgi bodies in leaf epidermal pavement cells from a transgenic line of *A. thaliana* stably expressing ST-GFP. The displacement rates have been analysed using Volocity; for detailed results see Appendix Table C.4. The average displacement rate of ST-GFP tagged Golgi bodies was calculated as mean and standard error of mean (μm/s): mock 0 h was 0.07583 ± 0.0071 , isoxaben-treated 0 h was 0.06138 ± 0.0034 , mock 24 h was 0.1095 ± 0.0044 and isoxaben-treated 24 h was 0.09382 ± 0.0034 . The number in parentheses is the total number of Golgi tracked per cell. The asterisks refer to the p-value: ** $p < 0.01$ and *** $p < 0.001$.

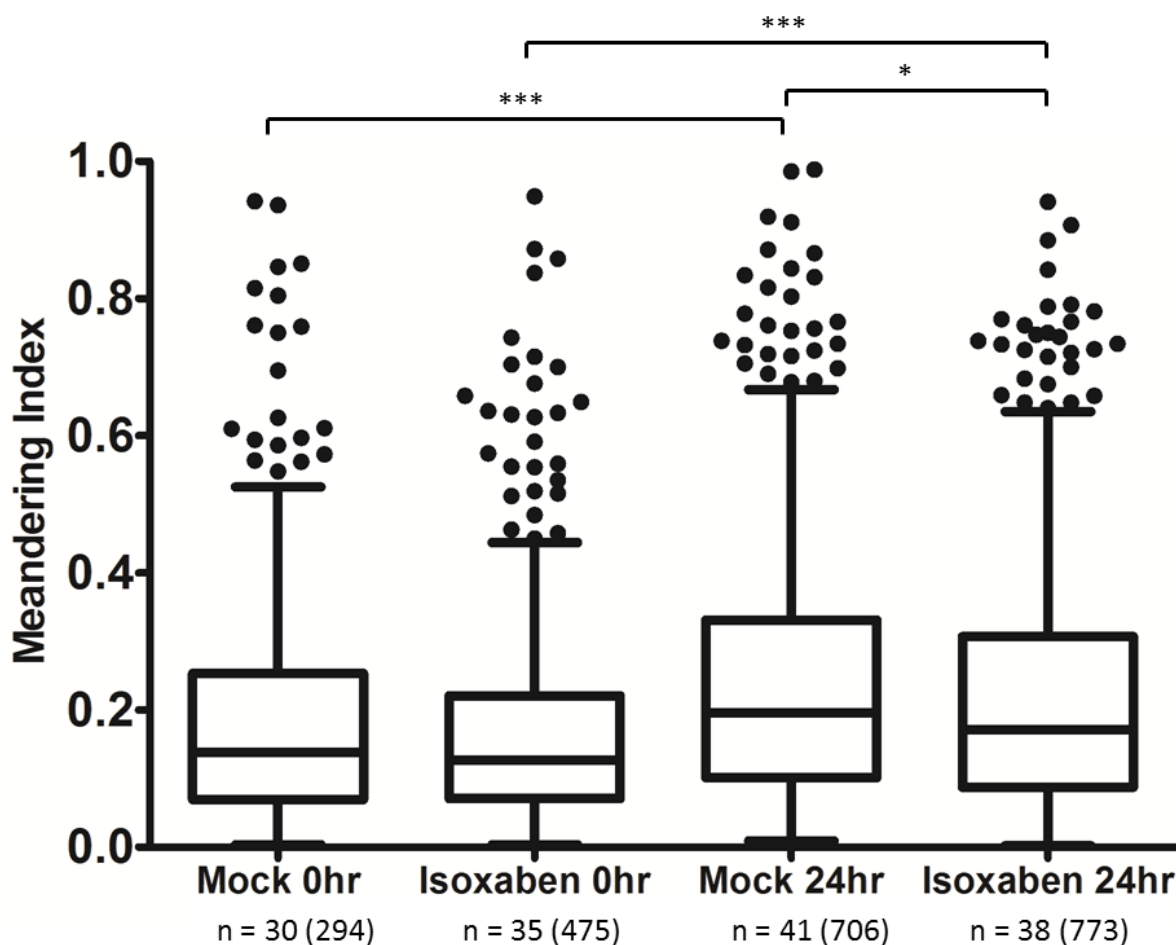


Figure 5.12 Meandering index of Golgi bodies in protoplasts expressing ST-GFP.

Meandering index of Golgi bodies in leaf epidermal pavement cells from a transgenic line of *A. thaliana* stably expressing ST-GFP. The meandering index has been calculated in Volocity; for detailed results see Appendix Table C.5. The meandering index of ST-GFP tagged Golgi bodies was calculated as mean and standard deviation (no units as this is an index): mock 0 h was 0.1925 ± 0.18 , isoxaben-treated 0 h was 0.1741 ± 0.16 , mock 24 h was 0.2441 ± 0.19 and isoxaben-treated 24 h was 0.2220 ± 0.18 . The number in parentheses is the total number of Golgi tracked per cell. The asterisks refer to the p-value: * $p < 0.05$ and *** $p < 0.001$.

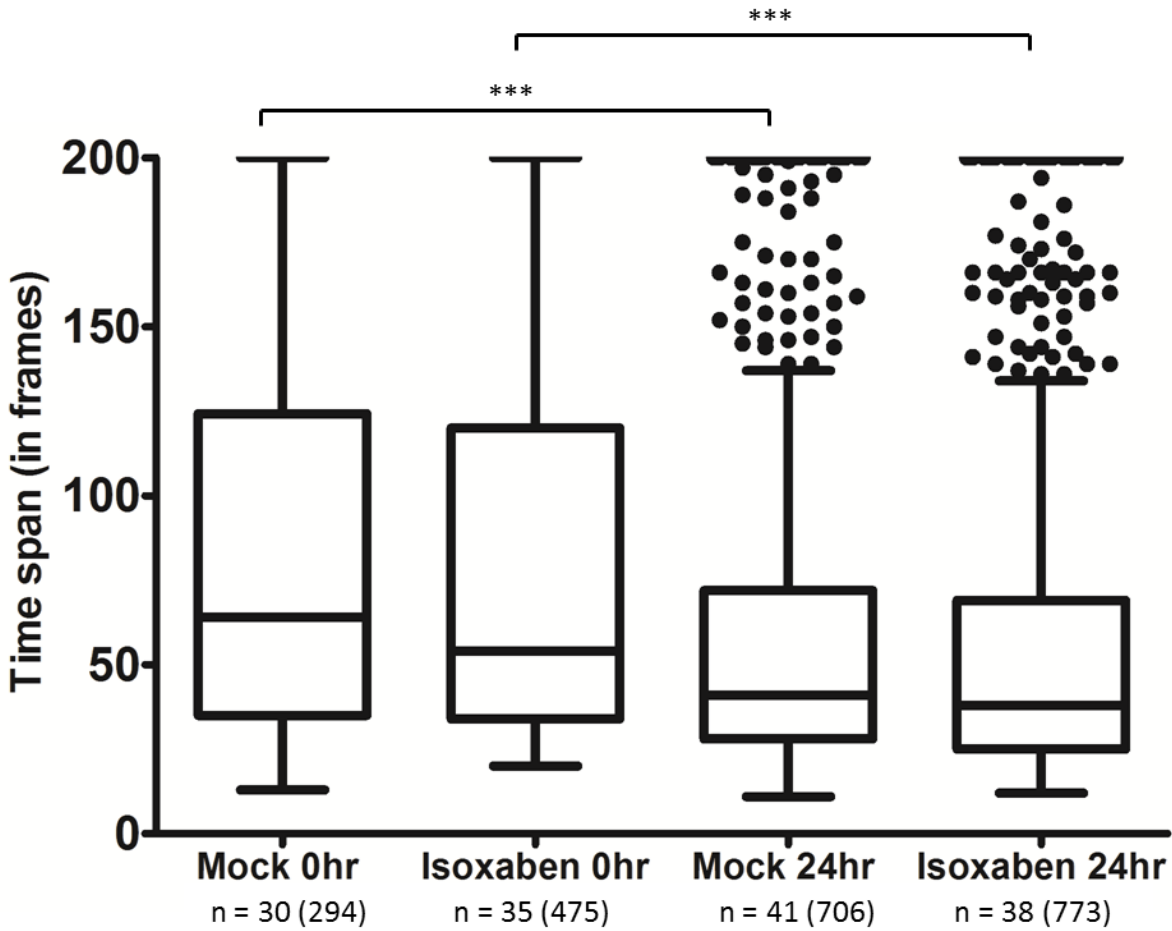


Figure 5.13 Time span that Golgi bodies remain in the field of view in protoplasts expressing ST-GFP.

The length of time that Golgi bodies are present in view in leaf epidermal pavement cells from a transgenic line of *A. thaliana* stably expressing ST-GFP. The time spans have been analysed using Volocity; for detailed results see Appendix Table C.6. The time-frames were calculated as mean $\pm$ standard deviation (units are time-frames): mock 0 h was 87 ± 63.68 , isoxaben-treated 0 h was 81.81 ± 60.04 , mock 24 h was 59.52 ± 46.27 and isoxaben-treated 24 h was 59.08 ± 50.42 . The number in parentheses is the total number of Golgi tracked per cell. The asterisks refer to the p-value: ***p < 0.001.

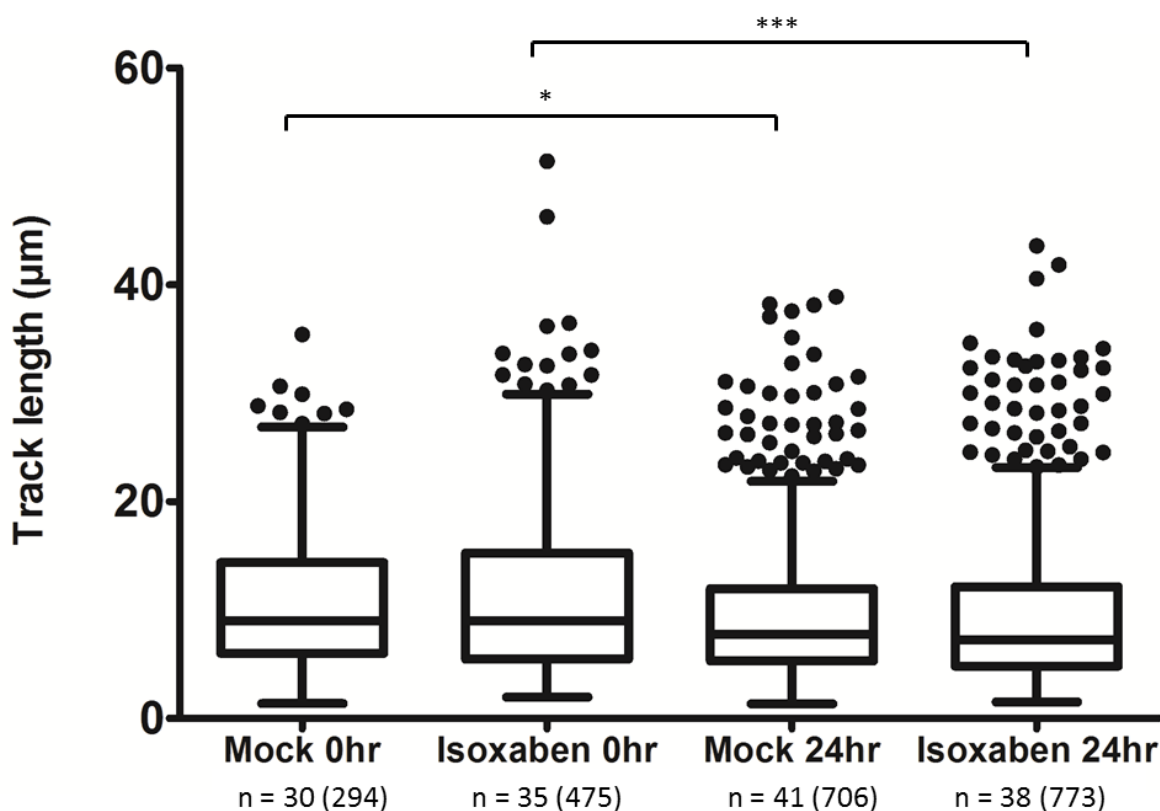


Figure 5.14 Track lengths of Golgi bodies in protoplasts expressing ST-GFP.

The track length of Golgi bodies in leaf epidermal pavement cells from a transgenic line of *A. thaliana* stably expressing ST-GFP. The track lengths have been analysed using Volocity; for detailed results see Appendix Table C.7. The track lengths were calculated as mean $\pm$ standard deviation (μm): mock 0 h was 10.69 ± 6.2 , isoxaben-treated 0 h was 11.39 ± 7.9 , mock 24 h was 9.74 ± 6.5 and isoxaben-treated 24 h was 9.58 ± 6.9 . The number in parentheses is the total number of Golgi tracked per cell. The asterisks refer to the p-value: * $p < 0.05$ and *** $p < 0.001$.

5.5 Discussion

This chapter continued the investigation into the influence of the cell wall on the organisation and dynamics of the actin cytoskeleton, with the use of protoplasts. The same stably-expressing *A. thaliana* lines and methods of analysis as were used as in the previous chapter were used in this chapter. Again, isoxaben was used (see Section 1.3.3.1, pg. 25).

5.5.1 Qualitative description of actin cytoskeleton morphology in protoplasts

The GFP-FABD2 tagged actin cytoskeleton in protoplasts often appears as a dense meshwork of filaments (Figure 5.1, pg. 149 and Figure 5.2, pg. 150). Zaban *et al.* (2013) categorised the morphology of both the microtubule and actin cytoskeletons in protoplasts as the cell regenerated. From 0 24 h post cell wall removal was called ‘Stage 0’ and described the network visualised via GFP-FABD2 in BY-2 cells, which was comprised of randomly orientated filaments (Zaban *et al.*, 2013).

This study found a large variation in the morphology of the actin cytoskeleton tagged with GFP-FABD2 within each condition examined (0 h and 24 h after cell wall removal, with and without isoxaben-treatment). Qualitative description is limited in being able to define changes between conditions, but Zaban *et al.* (2013) define the criteria for a cell to enter their ‘Stage 1’ as the presence of a cell wall and they describe the actin cytoskeleton as still lacking any orientation, whilst some filaments appeared thicker than others. In this study the protoplasts of pavement cells imaged were all isotropic as were most of their actin networks, although sometimes it would appear that several actin bundles were orientated in the same direction. The entirety of a cell was never visualised, i.e. Z-stacks were made only through the upper 4 µm of the cell, so this cannot be confirmed. Certainly some filaments appeared thicker than others, but this also seemed to be the case for cells imaged at what Zaban *et al.* (2013) would term ‘Stage 0’.

The GFP-Lifeact tagged actin cytoskeleton in protoplasts at both times-points usually appeared as thick bundles (Figure 5.5, pg.153) and it also displayed large variation in its morphology within each experimental treatment.

5.5.2 Quantitative description of actin cytoskeleton morphology and dynamics in protoplasts

Pairwise image subtraction was used to analyse bulk remodelling of GFP-FABD2 marked actin filaments in protoplasts at 0 h after cell wall removal and protoplasts that were allowed to regrow their cell walls for 24 h, with and without isoxaben (Section 5.4.1.2, pg.149). Compared to the results from the plasmolysis experiments, which used this analysis technique in the previous chapter (see Section 4.4.1.2, pg. 86), there were no obvious differences (Figure 5.3, pg.151). However, there were significant differences between conditions in the protoplast experiments.

The rate of bulk remodelling of both 24 h samples, i.e. 24 h mock-treated and isoxaben-treated, was significantly lower compared to their respective 0 h samples (Section 5.4.1.2, pg.149). This is the opposite of what was expected, as the actin cytoskeleton in plasmolyed cells and those treated with isoxaben showed a reduction in bulk remodelling. It is possible that what was observed was actually the process of cell wall regeneration, which required a greater degree of actin cytoskeleton remodelling than expected at 0 h. This is partially confirmed because the 0 h isoxaben-treated sample was significantly lower compared to the 0 h mock (Section 5.4.1.2). This is the expected result as it agrees with the results from the previous chapter, when cells treated with isoxaben showed a reduction in bulk remodelling.

The rates of bulk remodelling in protoplasts are very similar to that found in plasmolysed cells in Chapter 4. This is partly to be expected as part of the protoplasting procedure involves plasmolysis of the cells (see Section 2.4.2, pg. 45). However, the actin cytoskeleton looks different in plasmolysed cells compared to protoplasts, so the reduction of bulk remodelling in protoplasts is possibly for a different reason (see Figure 4.4, Figure 5.2, pg. 150 and Figure 5.5).

Pairwise image subtraction measuring mean fluorescence intensity was used to analyse the organisation of GFP-FABD2 marked actin filaments in protoplasts that had been allowed to regrow their cell walls for 0 h and 24 h, with and without isoxaben (see Section 5.4.1.3, pg. 150). There were no significant changes between any of the samples using this method. This is very interesting as taken with the results above it implies that either there

is a change in the proportion of actin filaments to bundles in protoplasts, thus arguing a loss of organisation; or both filaments and thick bundles are remodelling or not remodelling the same amount.

Compared to the control values in Section 4.4.1.3 (pg. 87), the mean fluorescence intensity of the actin cytoskeleton is much reduced in protoplasts at both 0 h and 24 h, with the exception of plasmolysed cells, when it is lower than in all protoplast conditions (see Figure 4.6 and Figure 5.4, pg. 152).

Cumulative fluorescence intensity was used to analyse the organisation of GFP-Lifeact marked actin filaments in protoplasts that had been allowed to regrow their cell walls for 0 h and 24 h, with and without isoxaben (see Section 5.4.2.2, pg. 151). Only at the third and fourth fluorescence intensity values were significant differences found, suggesting that using this method there are no differences between conditions (Table 5.1, pg. 157). The mock 24 h cells have slightly more movement than mock 0 h, but they are not statistically different (Figure 5.8, pg. 156). The two 0 h conditions are almost identical and the two 24 h conditions are extremely similar. There are some differences between the 0 h conditions and their respective 24 h conditions.

Smertenko *et al.* (2010) imaged GFP-Lifeact in BY-2 protoplasts using TIRF microscopy and found that actin filament dynamics were similar to those found in cotyledon pavement and root epidermal cells, except filaments stopped elongating earlier, grew slower and shrank slower. This is very similar to the situation found both with GFP-FABD2 analysed with *Pairwise image subtraction*, and GFP-Lifeact analysed with *Cumulative fluorescence intensity*.

The results in the plasmolysis experiment in Chapter 4 using *Cumulative fluorescence intensity* show that in the plasmolysed sample compared to control, the proportion of the fluorescent intensities at the three largest values ('17', '18' and '19') was significantly higher, which corresponds to the filaments that have moved the least or not at all (Figure 4.10). This result was not seen when comparing the different protoplast conditions against each other. It is possible that there are differences which would not be evident in a two minute time-series at this resolution. Together, this suggests that there are no changes in the dynamics of the actin cytoskeleton between 0 h and 24 h when imaged and analysed in this manner (Stage 0 according to Zaban *et al.* (2013)).

5.5.3 Golgi body motility in protoplasts

The speed of Golgi bodies is decreased in both 0 h samples compared to the respective 24 h samples (Figure 5.10, pg. 159). This implies that the actin cytoskeleton is in a different state in the 0 h sample compared to the 24 h, as motility of Golgi bodies and cytoplasmic streaming is ultimately heavily reliant on the actin network (Boevink *et al.*, 1998, Nebenfuhr *et al.*, 1999). Zaban *et al.* (2013) states that between 12 to 24 h after the cell wall is removed, as a new wall is being generated there is ‘vivid cytoplasmic streaming’ within the protoplasts. This contrasts with the lack of movement found during this study, either from the actin cytoskeleton (Section 5.4.1, pg. 148 and Section 5.4.2, pg. 150) or from the Golgi bodies (Section 5.4.3, pg. 152).

As the Golgi bodies are involved in cell wall rebuilding in both samples, the biggest difference is that there is minimal cell wall at 0 h after protoplasting. No huge changes were detected in the remodelling of the actin cytoskeleton, therefore it is possible that the changes observed in Golgi body motility reflect regeneration of the cell wall. Also, for both the actin cytoskeleton and Golgi bodies, the process of cell wall rebuilding may be so huge that it masks any organisational consequences of lacking a cell wall. Perhaps if the samples had been imaged again at 48 h when the cell wall should be fully regenerated (Alex Martinière and John Runions, personal communication), then a more consistent difference (one seen with both markers) might have been detected in the actin cytoskeleton, and Golgi body motility might be increased further.

Akkerman *et al.* (2011) showed that Golgi bodies exhibit slow and undirected movement in the early elongation zone of *A. thaliana* roots, but in the late elongation zone the Golgi bodies showed two types of movement depending on the kind of actin network which they were travelling over. Where the Golgi wiggle the actin network has a fine filamentous morphology and where they are fast and directional it is bundled (Akkerman *et al.*, 2011). In this study the actin cytoskeleton often appeared bundled (Section Figure 4.4 and Figure 4.26). This does correlate with the large standard deviations seen with all Golgi body data and suggests the presence of two or more sub-populations of Golgi bodies within the protoplasts exhibiting a range of behaviours that may represent different aspects of Golgi body function (Figure 5.9, pg. 158).

The motility of the Golgi bodies in protoplasts was very different than observed in whole cells. Only in the two 24 h samples (Figure 5.10) do any Golgi exceed 1 $\mu\text{m/s}$, compared to that recorded in Section 4.4.1.6 or that in the late elongation zone of the root where they reached 7 $\mu\text{m/s}$ (Akkerman *et al.*, 2011). That same study also showed that the interval between time-frames is important when analysing Golgi body motility, the interval must be least 0.4 s or shorter (Akkerman *et al.*, 2011). This study always used the same interval between time-frames: 0.4 s. This suggests that although the actin network appears to have a large proportion of bundles, the Golgi continue to move as though they were travelling along fine filaments.

There was no difference in track velocity between control samples and those treated with 20 μM isoxaben. This suggests that in the case of velocity, the most important factor is time, i.e. to what degree the cell wall has regenerated. When the average track velocities of ST-GFP marked Golgi bodies from the whole cells are compared to protoplasts, there is a difference in maximum rates. All whole cell conditions have maximum average velocities over 3.5 $\mu\text{m/s}$ whereas in protoplasts the largest maximum average velocity is only 1.3 $\mu\text{m/s}$ (for mock 0 h). The mean rates for protoplasts range from 0.33 to 0.41 $\mu\text{m/s}$ whereas in whole cells the rates are from 0.52 to 0.77 $\mu\text{m/s}$; the mean average track velocity of Golgi bodies in protoplasted cells is 0.52 $\mu\text{m/s}$, which is still much higher than protoplasts after 24 h of cell wall regeneration (0.41 $\mu\text{m/s}$).

The average displacement rate of Golgi bodies is decreased in both 0 h samples compared to their respective 24 h samples (Figure 5.11, pg. 160). This means that Golgi bodies, on average, took a longer time to move a shorter straight-line distance, suggesting a general reduction in movement as well as speed. This is in contrast to a reduction in the bulk remodelling index of the actin cytoskeleton in the 24 h sample compared to the 0 h. There was also a decrease in displacement rate in the 24 h sample treated with 20 μM isoxaben compared to the control sample. This is very interesting as it suggests that not just the absence of the cell wall, but a perturbed cell wall also affects the type of movement of Golgi bodies. No difference is seen between the two 0 h samples; however, very little cell wall would be present at this time point to become perturbed.

The mean of the average displacement rate of ST-GFP marked Golgi bodies is much lower in protoplasts compared to whole cells: protoplast values range from 0.06 to 0.11 $\mu\text{m/s}$, whereas whole cell values go from 0.21 to 0.34 $\mu\text{m/s}$. The Golgi bodies of plasmolysed

cells have an average displacement rate of 0.21 $\mu\text{m/s}$, higher than all the averages from the different protoplast conditions.

The last measurement used to analyse Golgi body movement is the meandering index. Briefly, the meandering index is the ratio between the displacement rate and the track velocity, to express the complexity of the movement (Rocchetti *et al.*, 2014); the lower the meandering index the more complicated the movement i.e. it is saltatory. The meandering index of the Golgi bodies is decreased in both 0 h samples compared to their respective 24 h samples (Figure 5.12, pg. 161). Again, this is in contrast to the actin cytoskeleton in the 24 h sample compared to the 0 h, as Golgi bodies have a more complicated movement when the bulk remodelling index is higher.

In addition, the meandering index of the mock 24 h sample compared to the isoxaben-treated 24 h was significantly higher. This means that the Golgi bodies in the isoxaben treated sample tended to have a more complicated, wiggling type of movement compared to the control sample. Protoplast values range from 0.17 to 0.24 whereas whole cell values are from 0.27 to 0.35. This means that Golgi bodies in protoplasts have more convoluted movement patterns compared to Golgi bodies in whole cells.

The time that Golgi bodies remain in the field of view was significantly decreased in both 24 h samples compared to the respective 0 h samples (Figure 5.13, pg. 162). There were no significant differences between the two 0 h conditions or between the two 24 h conditions. In whole cells the data for control, re-hydrated and hypo-osmotic conditions had similar ranges compared to protoplasts after 24 h of cell wall regrowth (see Section 4.4.1.6). In plasmolysed cells Golgi bodies were found to remain in the field of view for an average of 76 time-frames, in protoplasts immediately after cell wall removal they remained in view for an average of 87 or 82 time-frames (mock and isoxaben, respectively). In protoplasts 24 h after cell wall removal, Golgi bodies remained in view for an average of 60 or 59 time-frames (mock and isoxaben, respectively), whereas in whole cells Golgi bodies remained in view for an average of 46, 53 or 54 time-frames (control, re-hydrated and hypo-osmotic, respectively).

Lastly, the track length of the Golgi bodies is significantly decreased in 24 h conditions compared to their respective 0 h treatment (Figure 5.14, pg. 163). The longer track lengths suggest that Golgi bodies are more likely to remain roughly in the same area which means

they can be tracked for a longer period. In conjunction with the information on the length of time that a Golgi body stayed in the field of view, this suggests that Golgi bodies move more after 24 h of cell wall regeneration.

Therefore, the speed of the Golgi bodies increased over time, but did not change when the cell wall was altered by isoxaben treatment, whereas a cell wall altered by isoxaben also caused a change in the way that the Golgi bodies moved. Isoxaben causes the protein complexes which extrude cellulose into the cell wall to be removed from the plasma membrane and internalised (Tateno *et al.*, 2016). This means that no, or extremely little, cellulose synthesis can take place. Although Golgi bodies are not directly involved in cellulose production, they contain a lot of the enzymes necessary for the manufacture of pectins and hemicelluloses. In a cell that cannot properly synthesise cellulose, it might be expected that there would be an increased production of pectin or hemicelluloses to compensate for the lack of cellulose. An interesting next step would be to quantify the amount of different types of carbohydrate produced at different time-points during protoplast cell wall regeneration.

Golgi body dynamics were analysed in conjunction with microtubule markers and it was found that multiple Golgi bodies pause at the same position on microtubules, anywhere from 8 to 73 s (Crowell *et al.*, 2009). The authors hypothesise that this behaviour is part of the mechanism that determines where CSCs are inserted into the plasma membrane (Crowell *et al.*, 2009). It is known that the organisation of the microtubule cytoskeleton is disrupted by isoxaben treatment and that plant lines confirmed to be resistant to isoxaben treatment have mutations in *CesA6* and *CesA3* (Scheible *et al.*, 2001, Desprez *et al.*, 2002). The analysis presented in this chapter is probably not sensitive enough to detect Golgi bodies pausing on microtubules for as little as 8 s. However, treatment with isoxaben caused a change in the way Golgi bodies moved, so perhaps this is an indication that Golgi body pausing events have been reduced.

There are several proteins hypothesised to link the cell wall and Golgi bodies. Katamari/Murus3 (*A. thaliana*) is a xyloglucan galactosyltransferase localised to Golgi bodies that was shown to possess an actin cytoskeleton organising function in addition to its enzymatic role in cell wall biosynthesis (Tamura *et al.*, 2005). This protein combines three processes or structural features which create a biochemical link between actin organisation and cell wall synthesis, possibly even a physical link. Mutations in *Fragile-*

fiber 1 kinesin in *A. thaliana* do not have altered cellulose deposition, but do have a decreased secretion rate of pectin and deformed Golgi bodies (Zhu and Dixit, 2011b).

Another kinesin, AtKinesin-13A, was shown to localise to Golgi bodies and to be involved in evenly distributing them along microtubules for controlled cell wall deposition, although it does not seem to affect their motility (Lu *et al.*, 2005). Recently, Zhang *et al.* (2016b) described two proteins, STELLO1 and 2, the double mutants of which were hypersensitive to isoxaben and their hypocotyl cellulose content was reduced by approximately 50%. These proteins localise to Golgi bodies, CesA3 and CesA6 and also are involved in the distribution of CesA proteins within Golgi bodies (Zhang *et al.*, 2016b). The rate of CSC delivery to the plasma membrane was more than halved in the double mutant and the motility of the CSCs in the membrane was also reduced (Zhang *et al.*, 2016b). This same kinesin is also implicated in the movement of Golgi-derived vesicles on the microtubule cytoskeleton (Zhu *et al.*, 2015).

The results from these studies suggest indirect connections between cell wall deposition and Golgi body motility, which is interesting when considering the increase in motility from Golgi bodies in protoplasts at 24 h compared to those at 0 h. However, there are few differences in Golgi body motility between mock treated cells and isoxaben-treated cells. This was unexpected; it was expected that Golgi body motility would be affected if they play a key role in cellulose deposition, however, they might compensate by increasing production of pectins and hemicelluloses and they may be dwelling longer to deposit cargo. It would be interesting to analyse the proportions of cell wall carbohydrates in isoxaben-treated and untreated protoplasts.

5.6 Conclusion

The data presented in this chapter showed that the actin cytoskeleton behaved differently in protoplasts compared to whole cells, but that there were few differences in bulk remodelling between 0 h and 24 h after cell wall removal.

The actin cytoskeleton marked with GFP-FABD2 had a decreased rate of bulk remodelling at 24 h after cell wall removal compared to cells at 0 h and isoxaben treatment had no effect on bulk remodelling. There did not seem to be any changes in network organisation between time-points, or between treated and non-treated. No differences at all were found using the marker GFP-Lifeact for any condition.

This study has shown that track velocity, the time Golgi bodies are visible in the field of view and the length of their tracks are all dependent on the time since the cell wall was removed, whereas treating with isoxaben caused no change in their movement. The displacement rate and the meandering index of the Golgi bodies in protoplasts is also dependent on the time since the cell wall was removed, but there was also a significant difference between the mock and isoxaben treated samples 24 h after cell wall removal.

The final chapter investigates the cell wall-plasma membrane interface in closer detail through a combined approach of a cell wall mutant, the herbicide isoxaben and the cell wall digesting enzymes used in this chapter.

Chapter Six

The effects of an altered cell wall on plasma membrane protein mobility and behaviour

6.1 Introduction

The previous two chapters focussed on the relationship between the primary cell wall and the actin cytoskeleton, a broad study investigating the influence of the cell wall on the network as a whole. This chapter instead looks at the influence of the cell wall on the plasma membrane and how disrupting the interactions between the two affects the plasma membrane, specifically the mobility of resident proteins.

Previous studies have shown that either disrupting the cell wall globally or altering specific components within causes a change in the bulk movement of plasma membrane proteins and their behaviour at the single molecule level (Martinière *et al.*, 2012). These studies used wild type plants with a normal, intact cell wall, which was then perturbed or removed. In the study presented here, this approach was also used. However, in addition, T-DNA knockout plants with a perturbed cell wall were also studied. The mutations occurred in the genes for xyloglucanase one and two (*xxt1-xxt2*), which leads to a complete silencing of both genes (Cavalier *et al.*, 2008). As stated in Section 1.3.4 (pg. 25), although xyloglucan makes up a large part of the dicotyledonous cell wall, a plant without any detectable xyloglucan is still viable. The xyloglucan-deficient line was chosen because it is well characterised and many investigations have centred on the analysis of its altered cell wall state (Cavalier *et al.*, 2008, Zabortina *et al.*, 2012, Park and Cosgrove, 2012). It was

also chosen for this study because while growth is affected by the absence of detectable xyloglucan, seed production is not affected.

Fluorescence Recovery After Photobleaching (FRAP; see Section 2.6.5, pg. 49) is a commonly used technique that facilitates the study of bulk protein movement (Martinière *et al.*, 2012). In this study *txt1-txt2* mutants were stably-transformed with fluorescent-tagged plasma membrane proteins to examine whether the absence of xyloglucan affects the bulk movement of individual proteins. Two of these lines were also treated with isoxaben to investigate the consequences of impairing two components of the cell wall (xyloglucan and cellulose) simultaneously (see Section 1.3.3.1, pg. 25 for isoxaben). The plasma membrane-resident proteins used in this study were as follows.

LTI6b is a protein (Low Temperature Induced 6b; Genbank Accession: AF104221; At3g05890): it is a very small low temperature inducible protein (only 54 amino acids) with an unknown function (Table 6.1, pg. 186 and Figure 6.2, pg. 187) that has two predicted helical transmembrane domains (Figure 6.4, pg. 188; (Cutler *et al.*, 2000). LTI6b has four amino acids in a loop separating the two transmembrane domains within the apoplast; which in conjunction with the transmembrane domains makes it different from the other proteins used in this study (Figure 6.1, pg. 175).

All three of the constructs listed below have the fluorescent protein in the symplastic space and were termed 'minimal' proteins by Martinière *et al.* (2012) as they only consist of transmembrane domains and a fluorescent protein tag. MAP-GFP is the first 36 amino acids from AtGPA1 attached to GFP; it is myristoylated at position 2 and palmitoylated/S-acylated at position 5 and both modifications are necessary for AtGPA1 to associate with the plasma membrane (AT2G26300; Table 6.1, pg. 186 and Figure 6.1, pg. 175) (Ma *et al.*, 1990, Adjobo-Hermans *et al.*, 2006, Martinière *et al.*, 2012). GFP-PAP is a prenylated domain attached to GFP, it includes nucleotides 243-297 from AtAGG1 (AT3G63420; Table 6.1, pg. 186 and Figure 6.1, pg. 175) (Mason and Botella, 2000, Martinière *et al.*, 2012). Both these proteins are subunits of the heterotrimeric G-protein in *A. thaliana*. Lastly YFP-PI is a biosensor: the YFP is a fluorescent tag for the lipid-binding domain, specifically the pleckstrin homology domain of the human phosphatidylinositol-4-phosphate adaptor protein-1 (FAPP1) that binds phosphatidylinositol 4-phosphate (PtdIns4P; Table 6.1, pg. 186 and Figure 6.1, pg. 175) (Levine and Munro, 2002, Vermeer *et al.*, 2009).

FRAP enables measurement of protein diffusion rates, but it does not allow characterisation of the movement trajectories of individual protein molecules. In the last part of this chapter, single molecule tracking using Total Internal Reflection Fluorescence (TIRF) microscopy was employed to examine the behaviour of single proteins. It has been used previously to show that isoxaben treatment alters the size of the area in which paGFP-LTI6b moves (Martinière *et al.*, 2012). This chapter uses single molecule tracking to investigate the effects of digesting cellulose and pectin (see Section 1.7.1.4, pg. 38) on the movement and behaviour of individual molecules of three different plasma membrane resident proteins (paGFP-LTI6b, pFLS2::FLS2-GFP and pPIN1::PIN1-GFP). This allows accurate quantification of the movement of proteins, including their directionality, linearity and how constrained the molecule is within its environment, in this case the plasma membrane.

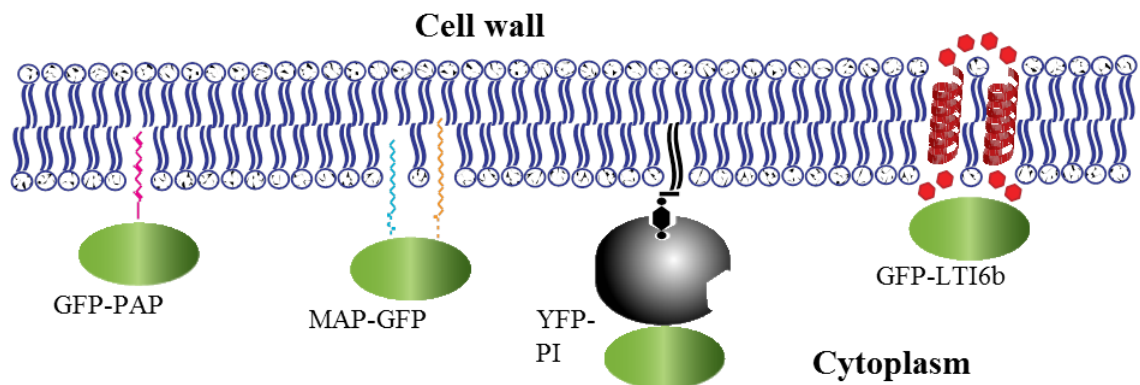


Figure 6.1 Schematic showing membrane binding domains.

This schematic shows the plasma membrane to illustrate the type of association of each plasma membrane-resident protein. The green oval attached to each membrane association type represents green fluorescent protein (GFP); the presence of GFP in the cytoplasm was determined by Martinière *et al.* (2012). The schematic is not to scale.

GFP-PAP: the pink membrane binding domain represents a 15-carbon farnesyl chain, it is attached to the protein by a cysteine link (Hemsley, 2015). MAP-GFP: the blue domain represents a 14-carbon myristoyl group attached to a glycine residue; the yellow is an 18-carbon stearate or palmitate acid (Hemsley, 2015). YFP-PI: the black lipid in the membrane represents PtdIns4P and the grey shape represents the pleckstrin homology domain that binds PtdIns4P (Heilmann and Heilmann, 2015, Hammond and Balla, 2015). GFP-LTI6b: two helical transmembrane domains that are predicted to span the lipid bilayer, making a horseshoe shape with four amino acids in the apoplast and four in the cytoplasm (Figure 6.4, below). Schematic adapted from: Hammond and Balla (2015), Heilmann and Heilmann (2015) and Hemsley (2015).

6.2 Collaborators

The single molecule TIRF microscope is in the Central Laser Facility at the Rutherford Appleton Laboratory, Science and Technology Facilities Council, Research Complex at Harwell (Didcot, UK). This microscope was used with the support and assistance of Dr Stephen Webb, a team leader within OCTOPUS (Clarke *et al.*, 2011). The single molecule tracking data was analysed by Dr Dan Rolfe, also a team leader within OCTOPUS, using software he developed. Access to the facility, equipment and personnel time was facilitated by a Plant Cell Initiative Programme Access Grant to Professor Chris Hawes (Oxford Brookes University, UK).

6.3 Aims

The aim of this chapter was to study the influence of the cell wall on plasma membrane resident proteins using a cell wall mutant line. This included:

- a) Obtaining a characterised cell wall mutant line (*xxt1-xxt2*), confirming the mutation and transforming the cell wall mutant line with various plasma membrane resident proteins to generate stably fluorescing lines (GFP-LTI6b, GFP-PAP, MAP-GFP and YFP-PI).
- b) Using FRAP to quantify and compare the mobility of the various plasma membrane resident proteins in the cell wall mutant line, in this case *xxt1-xxt2*, to a wild type plant line.
- c) Using FRAP to analyse the effect of isoxaben treatment on the mobility of the various plasma membrane resident proteins in the *xxt1-xxt2* line and a wild type plant line.
- d) Using TIRF to analyse the movement and behaviour of individual proteins (paGFP-LTI6b, pFLS2::FLS2-GFP and pPIN1::PIN1-GFP) in seedlings treated with cellulase and pectinase.

6.4 Results

6.4.1 *A. thaliana* stable transformation and selection of lines expressing tagged plasma membrane proteins in *xxt1-xxt2*

A semi-reverse genetics approach was taken using an *A. thaliana* line known to lack detectable xyloglucan (Cavalier *et al.*, 2008). This *xxt1-xxt2* mutant was obtained from the Nottingham Arabidopsis Stock Centre (NASC; Stock number: N67828) and has T-DNA insertions (see Section 2.1, pg. 40) in two genes: xylosyltransferase 1 and 2 (*xxt1*, At3g62720 and *xxt2*, At4g02500 respectively) (Cavalier *et al.*, 2008, Park and Cosgrove, 2012). Cavalier *et al.* (2008) made this homozygous double mutant and confirmed the lack of *xxt1* and *xxt2* using RT-PCR in both single mutants and again in the double. Cavalier *et al.* (2008) also used oligosaccharide mass profiling (which involved MALDI-TOF-MS) and a suite of antibodies against xyloglucans to show that *xxt1-xxt2* lacked detectable xyloglucan. For this study a gentyotyping PCR was used to confirm that the correct seed stock was obtained from NASC; the primers used were the ones from Cavalier *et al.* (2008) (see Section 2.1, pg. 40; see Appendix Figure D.1, pg. 287 for gel images).

This mutant *xxt1-xxt2* line was floral dipped with a selection of plasma membrane markers that were chosen because they have a wide range of sizes and membrane associations, as described by Martinière *et al.* (2012) (Table 6.1, pg. 186). The plasma membrane markers were all constructed by Martinière *et al.* (2012) with the exception of GFP-LTlb6, YFP-SYP121 and YFP-PI, constructed by Kurup *et al.* (2005), Foresti *et al.* (2006) and Vermeer *et al.* (2009) respectively.

A leaf section of selected F₁ plants was taken to confirm fluorescence by confocal microscopy (see Section 2.5, pg. 46). In this manner, seven plasma membrane resident proteins were confirmed in the *xxt1-xxt2* background (GFP-LTlb6, MAP-GFP, GFP-PAP, YFP-PI, GFP-AGP4, GFP-NPSN11 and YFP-SYP121; Figure 6.2, pg. 187). However, in three lines (GFP-AGP4, GFP-NPSN11 and YFP-SYP121) fluorescence was lost once the plants were grown to the second generation and hence these lines were not further used in this study (Figure 6.3, pg. 188).

For the four fluorescent markers which retained fluorescence (GFP-LTI6b, MAP-GFP, GFP-PAP and YFP-PI; Figure 6.2, pg. 187) at least two independent mutant lines were generated from different F₁ plants; that is, from separate transformation events. This should control for the possibility that the markers were inserted into genes that would affect plasma-membrane resident protein mobility or that over-expression of the markers due to multiple insertion events would affect the phenotype. Unfortunately, in the case of YFP-PI the second line lost fluorescence at the second generation and no other F₁ plants were recovered.

6.4.2 Fluorescence Recovery After Photobleaching of plasma membrane resident proteins in WT vs *xxt1-xxt2*

Fluorescence recovery after photobleaching was introduced in Section 2.6.5, but briefly: repeated iterations of the scanning laser were used to bleach a region of interest (ROI) within the cell membrane at the cell upper surface. The ROI was a circle with a radius of 2.4 μm . Fluorescence intensity was then measured at 1.5 s intervals for 40 s. These FRAP parameters were identical to the ones used in a previous study to make the results obtained in this study comparable (Martinière *et al.*, 2012). Both the maximum fluorescence recovery (MFR) and half-time for each fluorescent protein marker were quantified. The MFR after 40 s recovery time indicates how much of the protein population is mobile, while the half-time is a measurement of how long it takes for the fluorescence to recover to half of the maximum recovery and therefore indicates speed of protein movement. Together the MFR and half-time describe the mobility of a protein population.

xxt1-xxt2 stably-expressing *A. thaliana* lines were transformed with plasma membrane markers during this study, but all the wild type lines used were made by Martinière *et al.* (2012), with the exception of YFP-PI which was made by Vermeer *et al.* (2009).

6.4.2.1 Fluorescence Recovery After Photobleaching of GFP-LTI6b

Figure 6.5 (pg. 189) shows the MFR and fluorescence recovery half-time for GFP-LTI6b in WT and in two *xxt1-xxt2* lines: line 1 and line 8. The MFR was calculated as the mean and standard deviation: GFP-LTI6b WT was $74 \pm 7.6320\%$, GFP-LTI6b in line 1 of *xxt1-xxt2* was $68.69 \pm 8.5510\%$ and GFP-LTI6b in line 8 was $72.64 \pm 10.33\%$ (Figure 6.5; pg.

189). The WT MFR was significantly higher compared to line 1 ($p = 0.0026$), but not compared to line 8 ($p = 0.3707$). There was also no significant difference between the MFR of the two mutant lines ($p = 0.0882$). This indicates that in line 1 of the *xxt1-xxt2* mutant a lower proportion of the GFP-LTI6b protein population was mobile than in the WT.

Half-times of fluorescence recovery in these lines were calculated as mean and standard deviation: GFP-LTI6b WT was 4.586 ± 0.8532 s, GFP-LTI6b in line 1 of *xxt1-xxt2* was 4.335 ± 1.432 s and GFP-LTI6b in line 8 was 4.070 ± 1.205 s (Figure 6.5; pg. 189). There was no significant difference between the half-times of the WT and line 1 ($p = 0.3696$). The WT half-time was significantly higher compared to line 8 ($p = 0.0409$). There was also no significant difference between the half-times of the two mutant lines ($p = 0.4088$). GFP-LTI6b, therefore, moves faster in the *xxt1-xxt2* mutant line 8 than in the WT.

Lastly, the results from the two *xxt1-xxt2* mutant lines were pooled and compared to the WT. The WT MFR was significantly larger than the pooled *xxt1-xxt2* mutant lines ($p = 0.02$; mean and standard deviation of pooled data was $70.64 \pm 9.61\%$). The WT half-time was significantly higher than the pooled *xxt1-xxt2* mutant lines ($p = 0.07$; mean and standard deviation of pooled data was 4.205 ± 1.322 s). This suggests that the GFP-LTI6b proteins in the *xxt1-xxt2* mutant lines in general move faster than in the WT and a lower proportion of the GFP-LTI6b protein population was mobile.

6.4.2.2 Fluorescence Recovery After Photobleaching of GFP-PAP

Figure 6.6 (pg. 190) shows the MFR and fluorescence recovery half-time for GFP-PAP in WT and in two *xxt1-xxt2* lines: line 1 and line 2. MFR was calculated as the mean and standard deviation: GFP-PAP WT was $67.83 \pm 12.23\%$, GFP-PAP in line 1 of *xxt1-xxt2* was $86.32 \pm 8.197\%$ and GFP-PAP in line 2 was $82.79 \pm 9.678\%$ (Figure 6.6, pg. 190). The WT MFR was significantly lower compared to both *xxt1-xxt2* line 1 and line 2 ($p < 0.0001$), but there was no significant difference between the two mutant lines ($p = 0.1125$). This indicates that in the *xxt1-xxt2* mutant a higher proportion of the GFP-PAP protein population was mobile than in the WT.

Half-times of fluorescence recovery were calculated as mean and standard deviation: GFP-PAP WT was 1.577 ± 0.9603 s, GFP-PAP in line 1 of *xxt1-xxt2* was 1.655 ± 0.5685 s and GFP-PAP in line 2 was 1.440 ± 0.4193 s (Figure 6.6, pg. 190). There was no significant

difference in the half-times between the WT and line 1 or line 2 ($p = 0.7105$ and $p = 0.5094$, respectively). There was also no significant difference in the half-times between the two mutant lines ($p = 0.1007$). This suggests that the GFP-PAP proteins in the *xxt1-xxt2* mutant move at the same speed as those in the WT.

6.4.2.3 Fluorescence Recovery After Photobleaching of MAP-GFP

Figure 6.7 (pg. 191) shows the MFR and fluorescence recovery half-time for MAP-GFP in WT and in two *xxt1-xxt2* lines: line 3 and line 4. The MFR was calculated as the mean and standard deviation: MAP-GFP WT was $86.54 \pm 10.74\%$, MAP-GFP in line 3 of *xxt1-xxt2* was $84.51 \pm 7.876\%$ and MAP-GFP in line 4 of *xxt1-xxt2* was $80.87 \pm 7.379\%$ (Figure 6.7, pg. 191). There were no significant difference between WT and line 3 ($p = 0.3986$), but the MFR of the WT compared to line 4 was significantly higher ($p = 0.0051$). There was also no significant difference in the MFR between the two mutant lines ($p = 0.0932$). This indicates that in the *xxt1-xxt2* mutant line 4, a lower proportion of the GFP-MAP protein population was mobile than in the WT.

Half-times of fluorescence recovery were calculated as mean and standard deviation: MAP-GFP WT was 1.822 ± 0.5524 s, MAP-GFP in line 3 of *xxt1-xxt2* was 1.593 ± 0.4616 s and MAP-GFP in line 4 was 1.425 ± 0.4478 s (Figure 6.7, pg. 191). There was no significant difference between the WT and line 3 ($p = 0.1154$), but the WT half-time was significantly higher compared to line 4 ($p = 0.0006$). There was also no significant difference in the half-times between the two mutant lines ($p = 0.1940$). This suggests that the GFP-MAP proteins in the *xxt1-xxt2* mutant move faster than those in the WT.

Lastly, the results from the two *xxt1-xxt2* mutant lines were pooled and compared to the WT. The WT MFR was significantly larger than the pooled *xxt1-xxt2* mutant lines ($p = 0.02$; mean and standard deviation of pooled data was $82.10 \pm 7.68\%$). The WT half-time was significantly higher than the pooled *xxt1-xxt2* mutant lines ($p = 0.001$; mean and standard deviation of pooled data was 1.4820 ± 0.4554 s). This suggests that the MAP-GFP proteins in the *xxt1-xxt2* mutant lines in general move faster than in the WT and a lower proportion of the MAP-GFP protein population was mobile.

6.4.2.4 Fluorescence Recovery After Photobleaching of YFP-PI

Figure 6.8 (pg. 192) shows the MFR and fluorescence recovery half-time for YFP-PI in WT and in one *xxt1-xxt2* line. The MFR was calculated as the mean and standard

deviation: $75.66 \pm 7.362\%$ and $60.38 \pm 12.83\%$ for YFP-PI in the WT and in *xxt1-xxt2* respectively (Figure 6.8, pg. 192). The MFR of the WT was significantly higher compared to the mutant line ($p < 0.0001$). This indicates that in the *xxt1-xxt2* mutant a lower proportion of the YFP-PI protein population was mobile than in the WT.

Half-times of fluorescence recovery were calculated as means and standard deviations: 1.771 ± 0.7580 s and 1.319 ± 0.9091 s for YFP-PI in the WT and in *xxt1-xxt2* respectively (Figure 6.8, pg. 192). There was no significant difference in half-times between the WT and mutant line ($p = 0.0724$). This suggests that the YFP-PI proteins in the *xxt1-xxt2* mutant move at the same speed as those in the WT.

6.4.3 Fluorescence Recovery After Photobleaching of plasma membrane resident proteins in *xxt1-xxt2* when treated with isoxaben

The markers GFP-PAP and YFP-PI show an obvious difference in MFR in the wild-type cell wall compared to the *xxt1-xxt2* mutant: GFP-PAP showed a higher proportion of mobile protein in both the *xxt1-xxt2* lines, whilst YFP-PI showed a higher proportion of mobile protein in the WT (see Figure 6.6, pg. 190 and Figure 6.8, pg. 192). While there were changes in MFR for both GFP-LTI6b and MAP-GFP, the differences were not as pronounced. Therefore, these two markers were used for a second mobility experiment, in which the cell wall was disrupted further through isoxaben treatment.

FRAP was used again, but with some minor parameter changes. As all the proteins investigated in the previous section (Section 6.4.2, pg. 178) have relatively high MFR values and short half-times, the FRAP protocol was altered so that more time points were imaged, but over a very short period. This allowed for a more detailed observation of the early recovery phase. In this experiment the bleaching ROI circle had a radius of 25 pixels ($9.87 \mu\text{m}^2$) and the fluorescence recovery was measured every 0.06 s for approximately 17 s. The seedlings were treated with $20 \mu\text{M}$ isoxaben as this had been shown previously to affect the mobility of paGFP-LTI6b at the single molecule level (Martinière *et al.*, 2012) (see Section 2.6.4, pg. 49). Therefore the seedlings were being imaged under conditions of

altered cellulose-deposition (isoxaben-treatment) and with no detectable xyloglucan (*xxt1-xxt2* lines) to detect any changes in bulk plasma membrane protein movement.

6.4.3.1 Fluorescence Recovery After Photobleaching of GFP-LTI6b

Two lines were used from Section 6.4.2 (pg. 178): GFP-LTI6b WT and GFP-LTI6b in line 8 of *xxt1-xxt2*. GFP-LTI6b in line 8 of *xxt1-xxt2* was used as the fluorescence intensity most closely matched that of the control line. Figure 6.9 and Figure 6.10 (pg.s 193 and 194) show the MFR and fluorescence recovery half-time for GFP-LTI6b in WT and in one mutant line, both with a mock treatment and a treatment with 20 μ M isoxaben. The MFR was calculated as the mean and standard deviation: GFP-LTI6b WT mock was $50.70 \pm 9.373\%$, GFP-LTI6b in line 8 of *xxt1-xxt2* mock was $54.35 \pm 7.064\%$, GFP-LTI6b WT isoxaben treated was $43.82 \pm 7.011\%$ and GFP-LTI6b in *xxt1-xxt2* line 8 isoxaben treated was $53.46 \pm 10.96\%$ (Figure 6.9, pg. 193). The WT mock MFR was significantly higher compared to the WT isoxaben treated ($p = 0.0089$), but not compared to *xxt1-xxt2* line 8 mock ($p = 0.0601$). There was also no significant difference between the MFR of *xxt1-xxt2* line 8 mock and *xxt1-xxt2* line 8 isoxaben treated ($p = 0.6845$).

This indicates that in the isoxaben-treated WT a lower proportion of the GFP-LTI6b protein population was mobile compared to mock-treated WT, but that GFP-LTI6b moved at the same speed in mock-treated line 8 of the *xxt1-xxt2* mutant compared to both mock-treated WT and isoxaben-treated line 8 of the *xxt1-xxt2* mutant.

Half-times of fluorescence recovery were calculated as mean and standard deviation: GFP-LTI6b WT mock was 5.911 ± 1.105 s, GFP-LTI6b in line 8 of *xxt1-xxt2* mock was 6.732 ± 1.990 s, GFP-LTI6b WT isoxaben treated was 4.144 ± 1.088 s and GFP-LTI6b in line 8 isoxaben treated was 5.911 ± 1.416 s (Figure 6.9, pg. 193). The WT mock half-time was significantly higher compared to WT isoxaben treated ($p < 0.0001$). The WT mock half-time was significantly lower compared to *xxt1-xxt2* line 8 mock ($p = 0.0283$). The *xxt1-xxt2* line 8 mock half-time was significantly higher compared to the *xxt1-xxt2* line 8 isoxaben treated ($p = 0.0473$).

This suggests that the GFP-LTI6b proteins in the mock -treated WT moved slower than in the isoxaben-treated WT, but faster than in the mock-treated line 8 of the *xxt1-xxt2* mutant. Also, that the GFP-LTI6b proteins in the mock-treated line 8 of the *xxt1-xxt2* mutant moved slower than in the isoxaben-treated line 8 of the *xxt1-xxt2* mutant.

6.4.3.2 Fluorescence Recovery After Photobleaching of MAP-GFP

Two lines were used from Section 6.4.2 (pg. 178): GFP-MAP WT and GFP-MAP in line 4 of *txt1-txt2*. GFP-MAP in line 4 of *txt1-txt2* was used as the fluorescence intensity most closely matched that of the control line.

Figure 6.11 and Figure 6.12 (pg.s 195 and 196) show the MFR and fluorescence recovery half-time for MAP-GFP in WT and in one mutant line, both with a mock treatment and a treatment with 20 μ M isoxaben. The MFR was calculated as the mean and standard deviation: MAP-GFP WT mock was $69.88 \pm 7.679\%$, MAP-GFP in line 4 of *txt1-txt2* mock was $71.01 \pm 7.393\%$, MAP-GFP WT isoxaben treated was $68.61 \pm 8.793\%$ and MAP-GFP in line 4 isoxaben treated was $69.30 \pm 5.649\%$ (Figure 6.11, pg. 195). There was no significant difference between WT mock MFR compared to the WT isoxaben treated ($p = 0.4798$), nor compared to *txt1-txt2* line 4 mock ($p = 0.5230$). There was also no significant difference between the MFR of *txt1-txt2* line 4 mock and *txt1-txt2* line 4 isoxaben treated ($p = 0.2947$). This indicates that in all lines and in all conditions the same proportion of the MAP-GFP protein population was mobile.

Half-times of fluorescence recovery were calculated as mean and standard deviation: MAP-GFP WT mock was 2.087 ± 0.5506 s, MAP-GFP in *txt1-txt2* line 4 mock was 2.283 ± 0.5246 s, MAP-GFP WT isoxaben treated was 2.268 ± 0.4707 s and MAP-GFP in *txt1-txt2* line 4 isoxaben treated was 2.371 ± 0.4549 s (Figure 6.11, pg. 195). There was no significant difference between WT mock half-time compared to the WT isoxaben treated ($p = 0.1055$), nor compared to *txt1-txt2* line 4 mock ($p = 0.1202$). There was also no significant difference between the half-time of *txt1-txt2* line 4 mock and *txt1-txt2* line 4 isoxaben treated ($p = 0.4664$). This suggests that the GFP-MAP proteins moved at the same speed under all conditions.

6.4.4 Single molecule tracking of plasma membrane proteins under conditions of cell wall disruption

Total Internal Reflection Fluorescence microscopy was introduced previously (see Section 1.7.1.2, pg. 37). Briefly: the microscope is put in TIRF mode and one cell is imaged for 1200 frames over 1 min, 0.05 s between frames (Martinière *et al.*, 2012). Afterwards, an

ROI is selected within the cell (see Section 2.8, pg. 52) and the regions were analysed by Dr Dan Rolfe at Central Laser Facility (Harwell, Didcot, UK) using specialist software and the results returned as Mean Square Displacement curves (MSD). These curves give a mean for the diffusion rate of a molecule ($\mu\text{m}^2/\text{s}$) for a given time and the area in which it diffused (μm^2), along with an error rate. Together the constrained diffusion and area of diffusion can describe the mobility of individual proteins. However, this data is preliminary and so no statistical analyses were carried out.

Three proteins were selected for single molecule imaging. They are a different set of plasma-membrane resident proteins to those used in the previous sections, as for accurate single molecule tracking a lower number of fluorescent molecules is required in the plasma membrane. This is so that individual molecular tracks do not cross and become confused by crowding. LTI6b is the same protein as described previously, except that in this case it is fused to a photoactivatable version of GFP (paGFP-LTI6b) which becomes fluorescent immediately upon activation with a 405 nm laser (Section 6.4.1, pg. 177) (Martinière *et al.*, 2011b, Martinière *et al.*, 2012). During these experiments, paGFP was not photoactivated as there is a naturally occurring small population of molecules that are fluorescent without any stimulus (Martinière *et al.*, 2012) and these are sufficient to provide the low density fluorescence environment needed. The other two markers used were Flagellin Sensing 2 (FLS2) and Pin Formed 1 (PIN1) which were expressed under their native promoters in FLS2 and PIN1 knock-out plants, respectively. pFLS2::FLS2-GFP was made by (Robatzek *et al.*, 2006, Chinchilla *et al.*, 2007) and pPIN1::PIN1-GFP was made by (Benkova *et al.*, 2003).

Experimentally, plants containing the proteins listed above were treated with cellulase, which digests cellulose, the main load-bearing component of the cell wall (see Section 1.3, pg. 19), and also with pectinase, which digests pectin, a group of complex carbohydrates that form a gel-like matrix in the cell wall.

6.4.4.1 Single molecule tracking of pFLS2::FLS2-GFP

pFLS2::FLS2-GFP had a decreased diffusion speed in the cellulase treated sample ($0.0049 \pm 0.0001 \mu\text{m}^2/\text{s}$) compared to the mock ($0.0062 \pm 0.0005 \mu\text{m}^2/\text{s}$) and an increased diffusion speed in the pectinase treated sample ($0.0150 \pm 0.0030 \mu\text{m}^2/\text{s}$) compared to both mock and cellulase treated (Figure 6.13, pg. 197). The area in which pFLS2::FLS2-GFP diffused was decreased in the cellulase treatment ($0.1024 \pm 0.0016 \mu\text{m}^2$) compared to mock ($0.25 \pm$

$0.09 \mu\text{m}^2$) and increased in the pectinase treated sample ($0.36 \pm 0.64 \mu\text{m}^2$). Treatment with both cellulase and pectinase resulted in an increase in variance relative to measurement of untreated samples (Figure 6.13, pg. 197).

This indicates that in the cellulase-treated sample the pFLS2::FLS2-GFP protein population moved slower and within a smaller area compared to mock-treated. However, in the pectinase-treated sample the pFLS2::FLS2-GFP protein population moved faster and within a larger area compared to mock-treated cells.

6.4.4.2 *Single molecule tracking of paGFP-LTI6b*

Conversely paGFP-LTI6b had an increased diffusion speed in both the cellulase ($0.074 \pm 0.003 \mu\text{m}^2/\text{s}$) and pectinase treated samples ($0.074 \pm 0.003 \mu\text{m}^2/\text{s}$) compared to the mock ($0.0063 \pm 0.002 \mu\text{m}^2/\text{s}$; Figure 6.14, pg. 198). The area in which paGFP-LTI6b diffused was increased in the cellulase treatment ($1.2544 \pm 0.0064 \mu\text{m}^2$) and decreased in the pectinase treatment ($0.81 \pm 0.01 \mu\text{m}^2$) compared to mock ($1.1449 \pm 0.0025 \mu\text{m}^2$). This finding is the opposite of the results obtained for pFLS2::FLS2-GFP (Figure 6.14, pg. 198).

This indicates that the paGFP-LTI6b protein population moved faster in both the cellulase-treated sample and the pectinase-treated sample, but in a larger area in the former condition and a smaller area in the latter condition, compared to mock-treated cells.

6.4.4.3 *Single molecule tracking of pPIN1::PIN1-GFP*

pPIN1::PIN1-GFP had a decreased diffusion speed in both the cellulase ($0.0048 \pm 0.0006 \mu\text{m}^2/\text{s}$) and pectinase treated samples ($0.0039 \pm 0.0004 \mu\text{m}^2/\text{s}$) compared to the mock ($0.0070 \pm 0.0020 \mu\text{m}^2/\text{s}$; Figure 6.15, pg. 199). The area in which pPIN1::PIN1-GFP diffused was decreased in both the cellulase ($0.0441 \pm 0.0016 \mu\text{m}^2$) and pectinase treated samples ($0.0256 \pm 0.0004 \mu\text{m}^2$) compared to mock ($0.0625 \pm 0.0009 \mu\text{m}^2$), the same situation as was found with the diffusion rate (Figure 6.15, pg. 199).

This indicates that the pPIN1::PIN1-GFP protein population moved slower and in a smaller area in both the cellulase-treated sample and the pectinase-treated sample compared to mock-treated.

Table 6.1 Fluorescently-tagged plasma-membrane resident proteins successfully transformed into the xylosyltransferase 1 and 2 (*xxt1-xxt2*) cell wall mutant line.

Gene	Location and association	Function	Publications
LTI6b	PM via transmembrane domains	Function as yet unknown	(Cutler <i>et al.</i> , 2000)
AGP4	PM via transmembrane domains	Function as yet unknown	(Martinière <i>et al.</i> , 2012)
NPSN11	PM via transmembrane domains	Membrane-bound SNARE	(Zheng <i>et al.</i> , 2002)
PI^a	PM via binding to PtdIns4P	- ^c	(Vermeer <i>et al.</i> , 2009)
PAP^a	PM via prenylation	- ^c	(Martinière <i>et al.</i> , 2012)
MAP^a	PM via myristoylation and palmitoylation/S-acylation	- ^c	(Martinière <i>et al.</i> , 2012)
SYP121	PM via transmembrane domains	Plasma membrane-resident syntaxin	(Sutter <i>et al.</i> , 2006)

^a These are not full-length genes, but modified so that the protein contains only the membrane anchoring domain; see Section 1.5.1, pg. 31 for full details.

^b These should have no ability to interact with anything other than the PM (Martinière *et al.*, 2012).

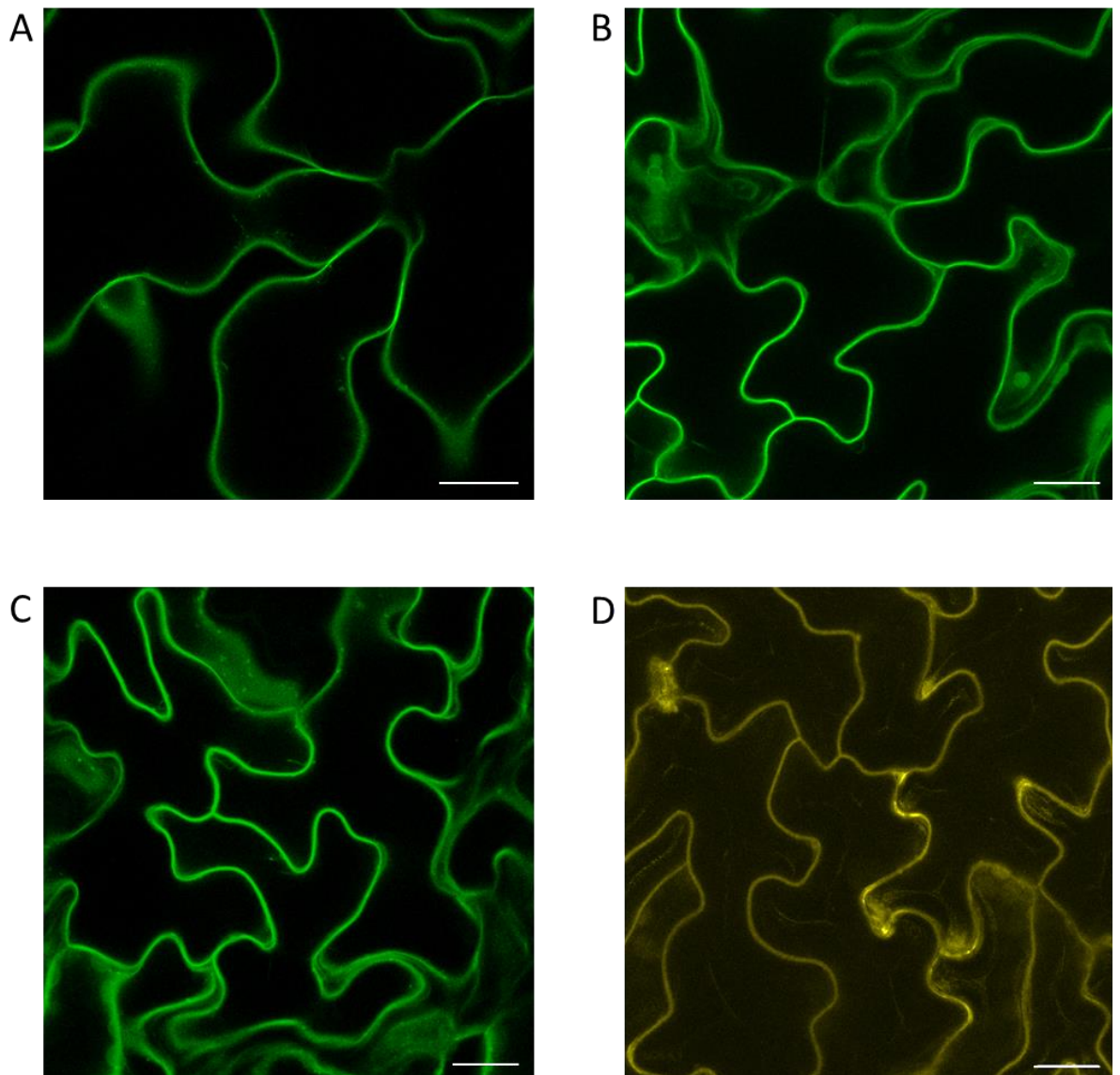


Figure 6.2 Fluorescent protein expression in *xxt1-xxt2* background plants. (A) GFP-LTI6b in green; (B) MAP-GFP in green; (C) GFP-PAP in green; and (D) YFP-PI in yellow. These proteins all have an even distribution in the plasma membrane, although some of them also label the cytoplasm or Golgi body membranes. Scale bar = 20 μm .

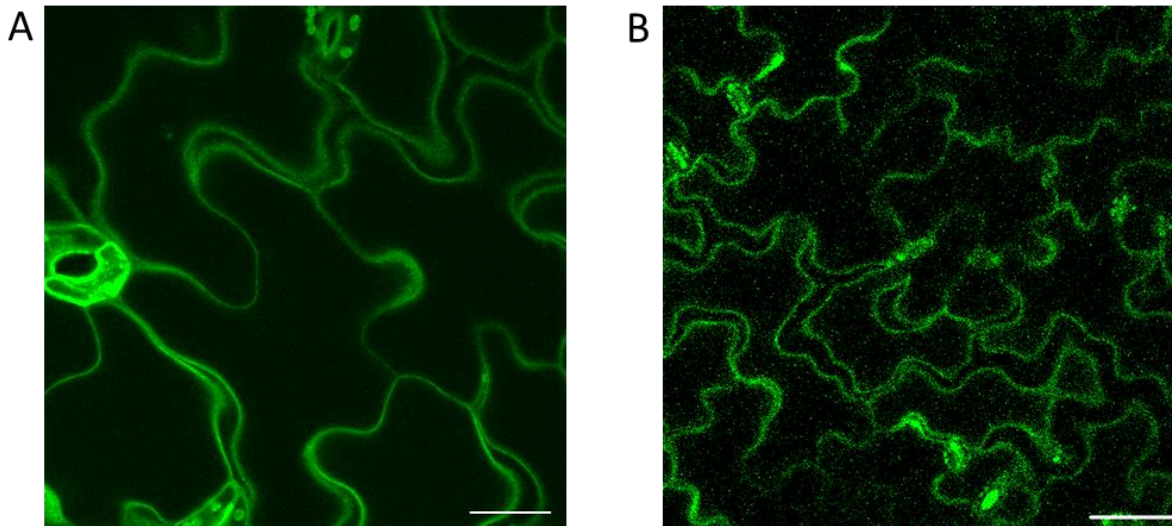


Figure 6.3 Fluorescent protein expression in *xxt1-xxt2* background plants. These are T_1 plants and fluorescence was lost in subsequent generations.

(A) GFP-AGP4 in green. Scale bar = 20 μm .

(B) GFP-NPSN11 in green. Scale bar = 50 μm .

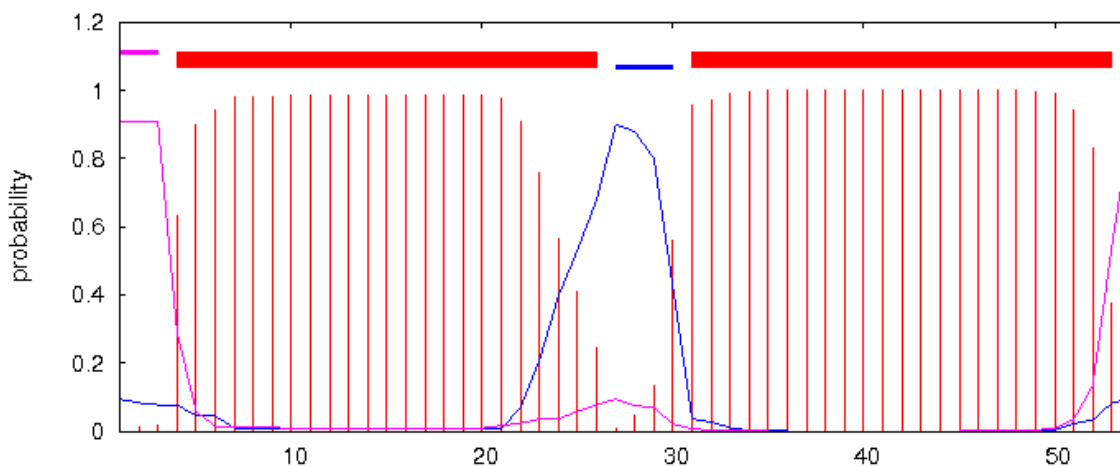


Figure 6.4 Hydrophobicity plot for LTI6b (At3g05890).

Hydrophobicity plot from TMHMM (<http://www.cbs.dtu.dk/services/TMHMM/>) for LTI6b showing the predicted transmembrane domains in red. In purple and in blue are the eight amino acids that are not within the transmembrane domain (AF104221 in Genbank).

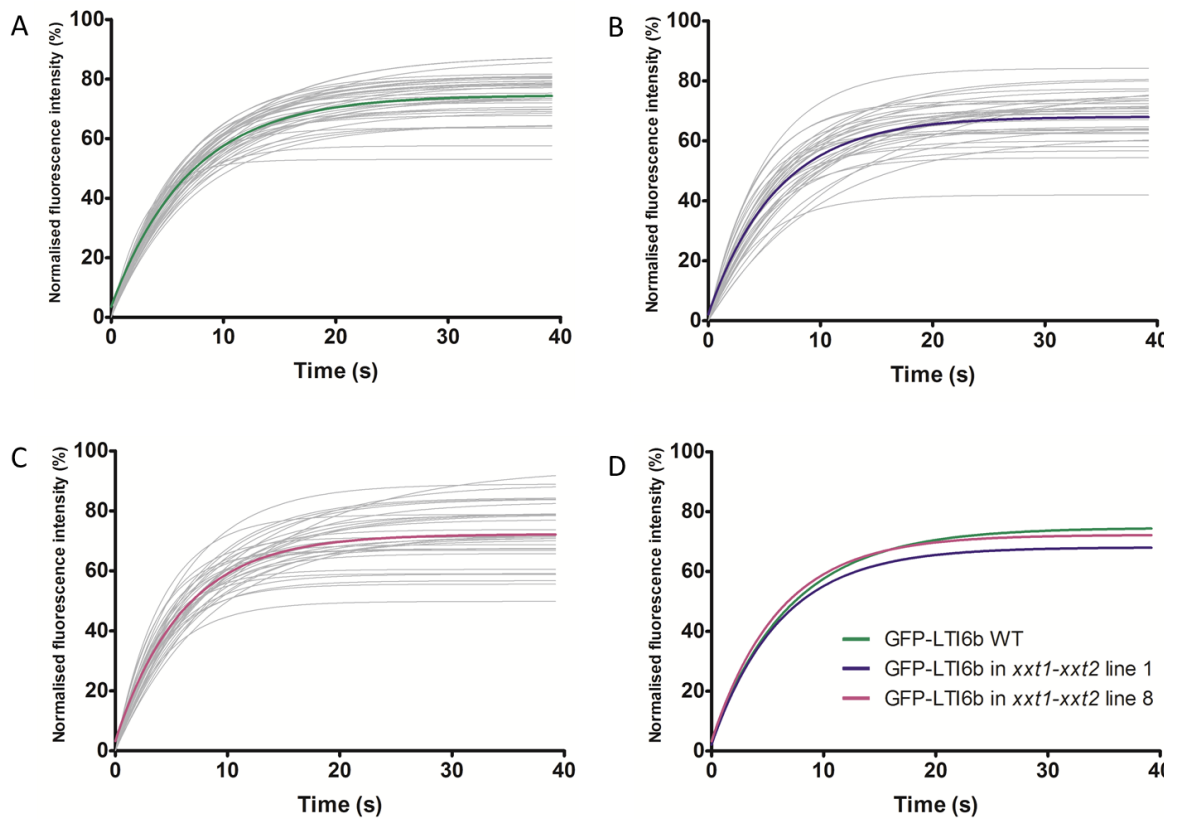


Figure 6.5 Fluorescence recovery curves of GFP-LTI6b in WT and *xxt1-xxt2* seedlings.

Plots of normalised fluorescence intensity vs time during fluorescence recovery within bleached regions. (A) GFP-LTI6b WT (global curve in green; 40 cells in 8 seedlings). (B) GFP-LTI6b in *xxt1-xxt2* line 1 (global curve in blue; 35 cells in 7 seedlings). (C) GFP-LTI6b in *xxt1-xxt2* line 8 (global curve in pink; 34 cells in 7 seedlings). (D) Fluorescence recovery curves of GFP-LTI6b WT (wild type; green), GFP-LTI6b in *xxt1-xxt2* line 1 (blue), and GFP-LTI6b in *xxt1-xxt2* line 8 (pink). Time zero on the graphs denotes the first measurement post-bleach.

The maximum fluorescence recovery (MFR) was calculated as the mean and standard deviation: GFP-LTI6b WT was $74 \pm 7.6320\%$, GFP-LTI6b in line 1 of *xxt1-xxt2* was $68.69 \pm 8.5510\%$ and GFP-LTI6b in *xxt1-xxt2* line 8 was $72.64 \pm 10.33\%$.

The half-times of fluorescence recovery were calculated as mean and standard deviation: GFP-LTI6b WT was 4.586 ± 0.8532 s, GFP-LTI6b in line 1 of *xxt1-xxt2* was 4.335 ± 1.432 s and GFP-LTI6b in *xxt1-xxt2* line 8 was 4.070 ± 1.205 s

Recovery data was normalised to percentage as described in Section 2.6.5.

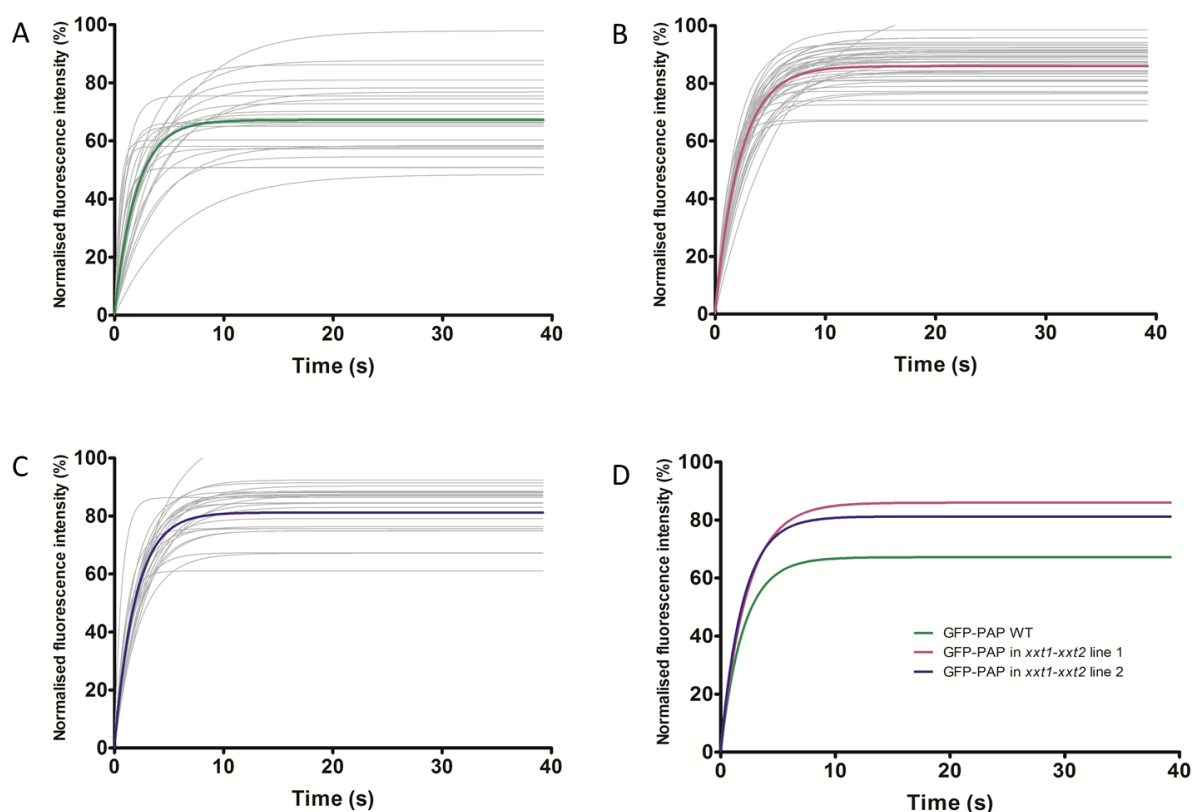


Figure 6.6 Fluorescence recovery curves of GFP-PAP in WT and *xxt1-xxt2* seedlings.

Plots of normalised fluorescence intensity vs time during fluorescence recovery within bleached regions. (A) GFP-PAP WT (global curve in green; 26 cells in 9 seedlings). (B) GFP-PAP in *xxt1-xxt2* line 2 (global curve in blue; 42 cells in 6 seedlings). (C) GFP-PAP in *xxt1-xxt2* line 1 (global curve in pink; 26 cells in 8 seedlings). (D) Fluorescence recovery curves of GFP-PAP WT (wild type; green), GFP-PAP in *xxt1-xxt2* line 1 (pink) and GFP-PAP in *xxt1-xxt2* line 2 (blue). Time zero on the graph denotes the first measurement post-bleach.

The MFR was calculated as the mean and standard deviation: GFP-PAP WT was $67.83 \pm 12.23\%$, GFP-PAP in line 1 of *xxt1-xxt2* was $86.32 \pm 8.197\%$ and GFP-PAP in *xxt1-xxt2* line 2 was $82.79 \pm 9.678\%$.

The half-times of fluorescence recovery were calculated as means and standard deviations: GFP-PAP WT was 1.577 ± 0.9603 s, GFP-PAP in line 1 of *xxt1-xxt2* was 1.655 ± 0.5685 s and GFP-PAP in *xxt1-xxt2* line 2 was 1.440 ± 0.4193 s.

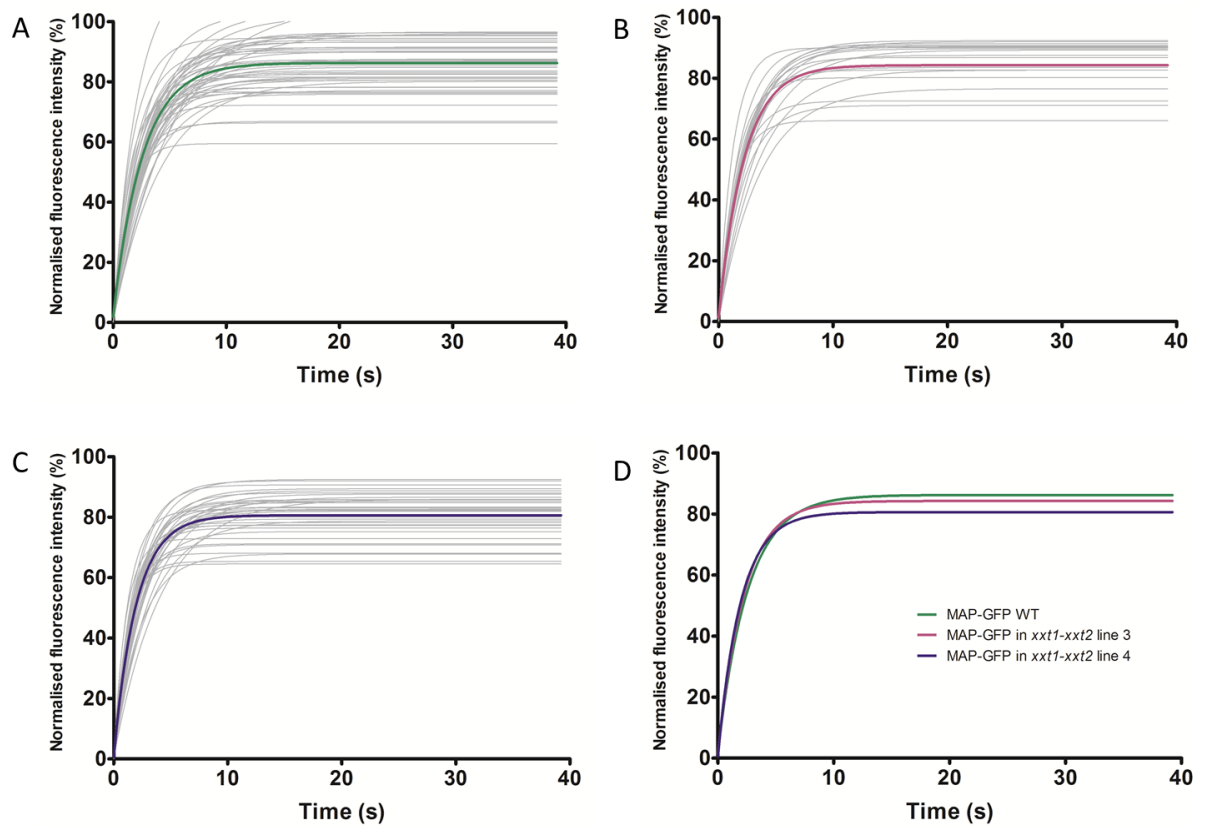


Figure 6.7 Fluorescence recovery curves of MAP-GFP in WT and *xxt1-xxt2* seedlings.

Plots of normalised fluorescence intensity vs time during fluorescence recovery within bleached regions. (A) MAP-GFP WT (global curve in green; 48 cells in 9 seedlings). (B) MAP-GFP in *xxt1-xxt2* line 4 (global curve in blue; 37 cells in 8 seedlings). (C) MAP-GFP in *xxt1-xxt2* line 3 (global curve in pink; 19 cells in 4 seedlings). (D) Fluorescence recovery curves of MAP-GFP WT (wild type; green), MAP-GFP in *xxt1-xxt2* line 4 (blue), and MAP-GFP in *xxt1-xxt2* line 3 (pink). Time zero on the graph denotes the first measurement post-bleach.

The MFR was calculated as the mean and standard deviation: MAP-GFP WT was $86.54 \pm 10.74\%$, MAP-GFP in line 3 of *xxt1-xxt2* was $84.51 \pm 7.876\%$ and MAP-GFP in line 4 of *xxt1-xxt2* was $80.87 \pm 7.379\%$.

The half-time means and standard deviations were calculated as: MAP-GFP WT was 1.822 ± 0.5524 s, MAP-GFP in line 3 of *xxt1-xxt2* was 1.593 ± 0.4616 s and MAP-GFP in *xxt1-xxt2* line 4 was 1.425 ± 0.4478 s.

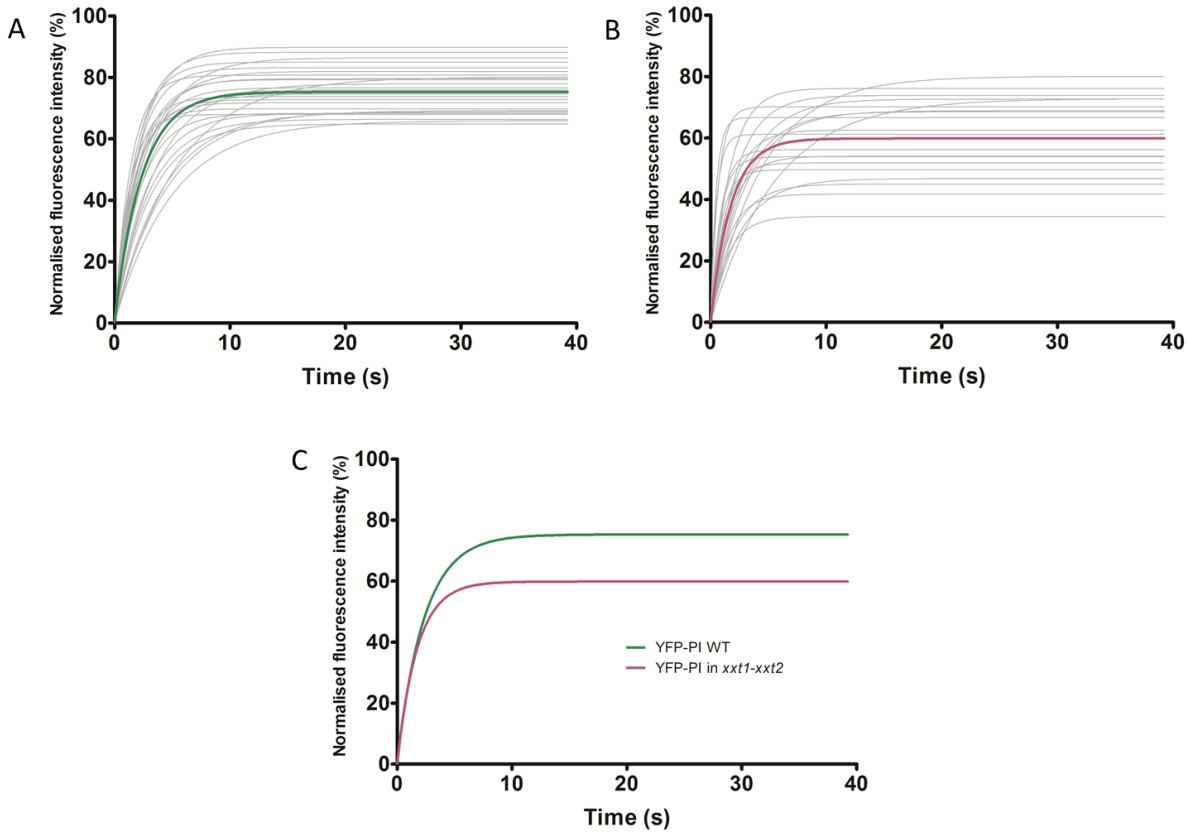


Figure 6.8 Fluorescence recovery curves of YFP-PI in WT and *xxt1-xxt2* seedlings.

Plots of normalised fluorescence intensity vs time during fluorescence recovery within bleached regions. (A) YFP-PI WT (global curve in green; 26 cells in 6 seedlings). (B) YFP-PI in *xxt1-xxt2* line (global curve in pink; 20 cells in 4 seedlings). (C) Fluorescence recovery curves of YFP-PI WT (wild type; green) and YFP-PI in *xxt1-xxt2* (pink). Time zero on the graph denotes the first measurement post-bleach.

The MFR was calculated as the mean and standard deviation: $75.66 \pm 7.362\%$ and $60.38 \pm 12.83\%$ for YFP-PI and YFP-PI in *xxt1-xxt2* respectively.

The half-time means and standard deviations were calculated as: 1.771 ± 0.7580 s and 1.319 ± 0.9091 s for YFP-PI and YFP-PI in *xxt1-xxt2* respectively.

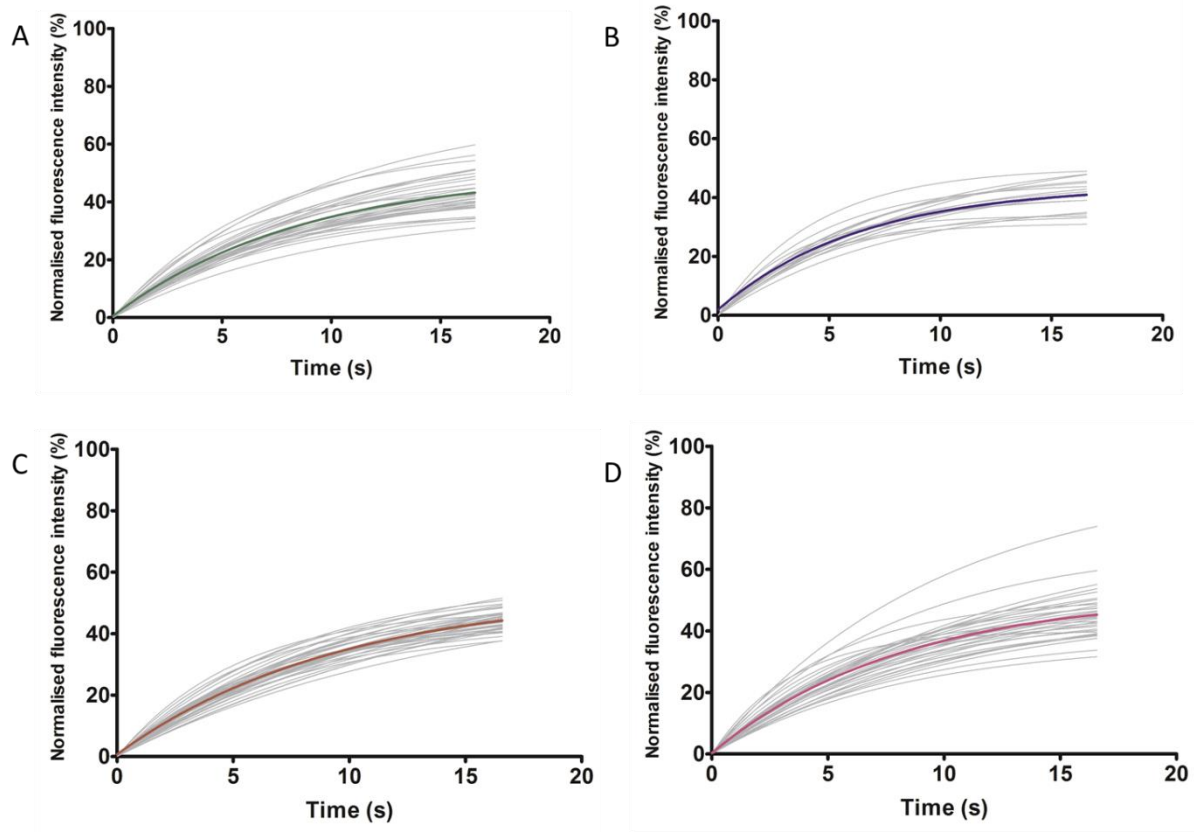


Figure 6.9 Fluorescence recovery curves of GFP-LTI6b in WT and *xxt1-xxt2* seedlings with and without isoxaben treatment.

Plots of normalised fluorescence intensity vs time during fluorescence recovery within bleached regions.

(A) GFP-LTI6b WT mock (global curve in green; 33 cells in 7 seedlings). (B) GFP-LTI6b WT isoxaben treated (global curve in blue; 18 cells in 4 seedlings). (C) GFP-LTI6b in *xxt1-xxt2* line 8 mock (global curve in red; 41 cells in 7 seedlings). (D) GFP-LTI6b in *xxt1-xxt2* line 8 isoxaben treated (global curve in pink; 34 cells in 8 seedlings). Time zero on the graphs denotes the first measurement post-bleach.

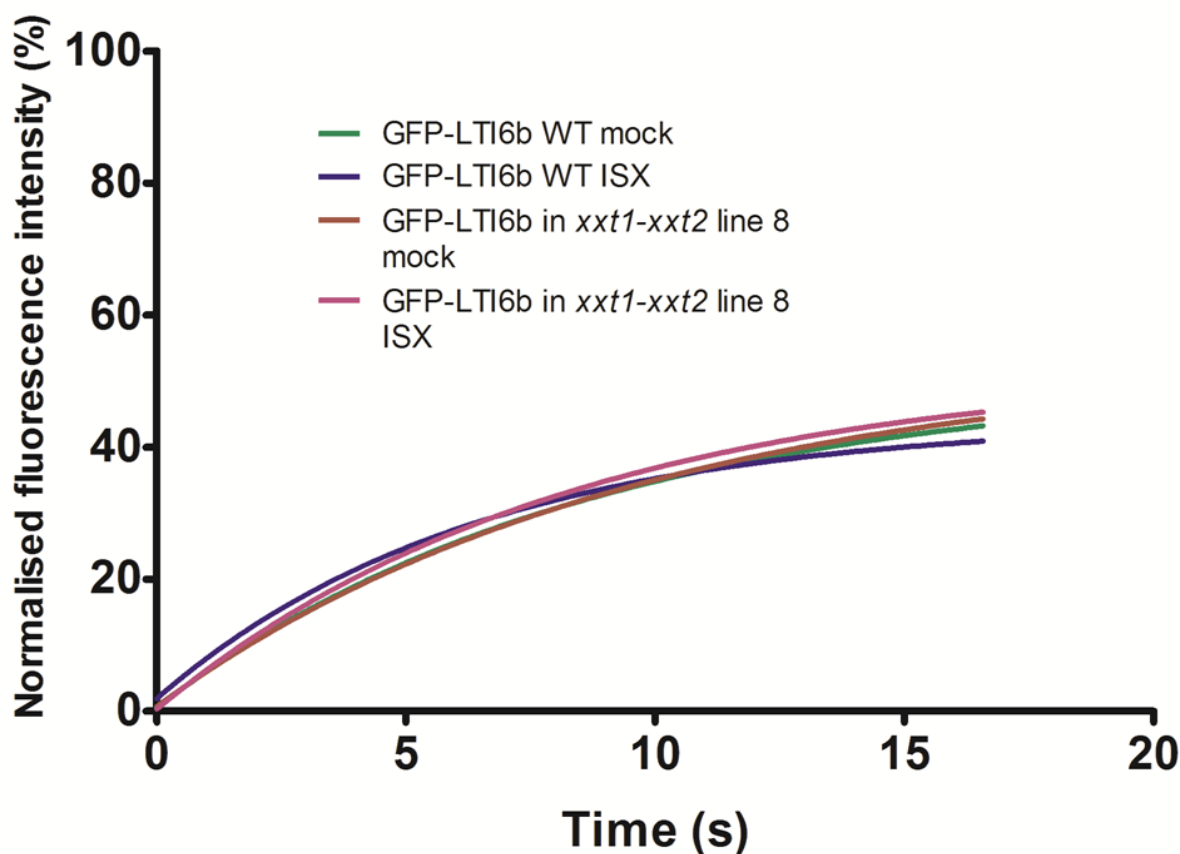


Figure 6.10 Fluorescence recovery global fit curves of GFP-LTI6b from Figure 6.9.

GFP-LTI6b WT mock (wild type; green), GFP-LTI6b WT isoxaben treated (blue), GFP-LTI6b in *xxt1-xxt2* line 8 mock (red) and GFP-LTI6b in *xxt1-xxt2* line 8 isoxaben treated (pink). Time zero on the graphs denotes the first measurement post-bleach.

The MFR was calculated as the mean and standard deviation: GFP-LTI6b WT mock was $50.70 \pm 9.373\%$, GFP-LTI6b in line 8 of *xxt1-xxt2* mock was $54.35 \pm 7.064\%$, GFP-LTI6b WT isoxaben treated was $43.82 \pm 7.011\%$ and GFP-LTI6b in line 8 isoxaben treated was $53.46 \pm 10.96\%$

The half-time means and standard deviations were calculated as: GFP-LTI6b WT mock was 5.911 ± 1.105 s, GFP-LTI6b in line 8 of *xxt1-xxt2* mock was 6.732 ± 1.990 s, GFP-LTI6b WT isoxaben treated was 4.144 ± 1.088 s and GFP-LTI6b in line 8 isoxaben treated was 5.911 ± 1.416 s.

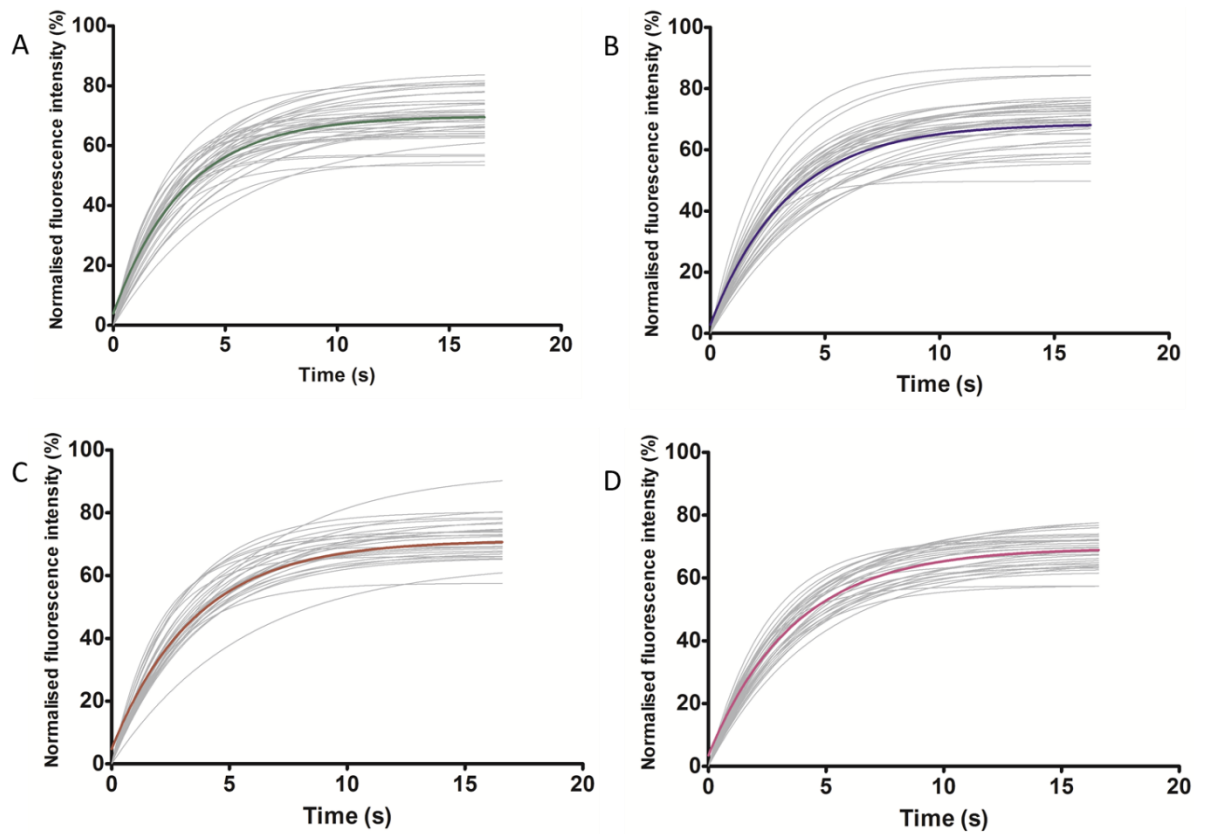


Figure 6.11 Fluorescence recovery curves of MAP-GFP in WT and *xxt1-xxt2* seedlings with and without isoxaben treatment.

Plots of normalised fluorescence intensity vs time during fluorescence recovery within bleached regions.

(A) MAP-GFP WT mock (global curve in green; 40 cells in 7 seedlings). (B) MAP-GFP WT isoxaben treated (global curve in blue; 45 cells in 8 seedlings). (C) MAP-GFP in *xxt1-xxt2* line 4 mock (global curve in red; 35 cells in 7 seedlings). (D) MAP-GFP in *xxt1-xxt2* line 4 isoxaben treated (global curve in pink; 32 cells in 6 seedlings). Time zero on the graphs denotes the first measurement post-bleach.

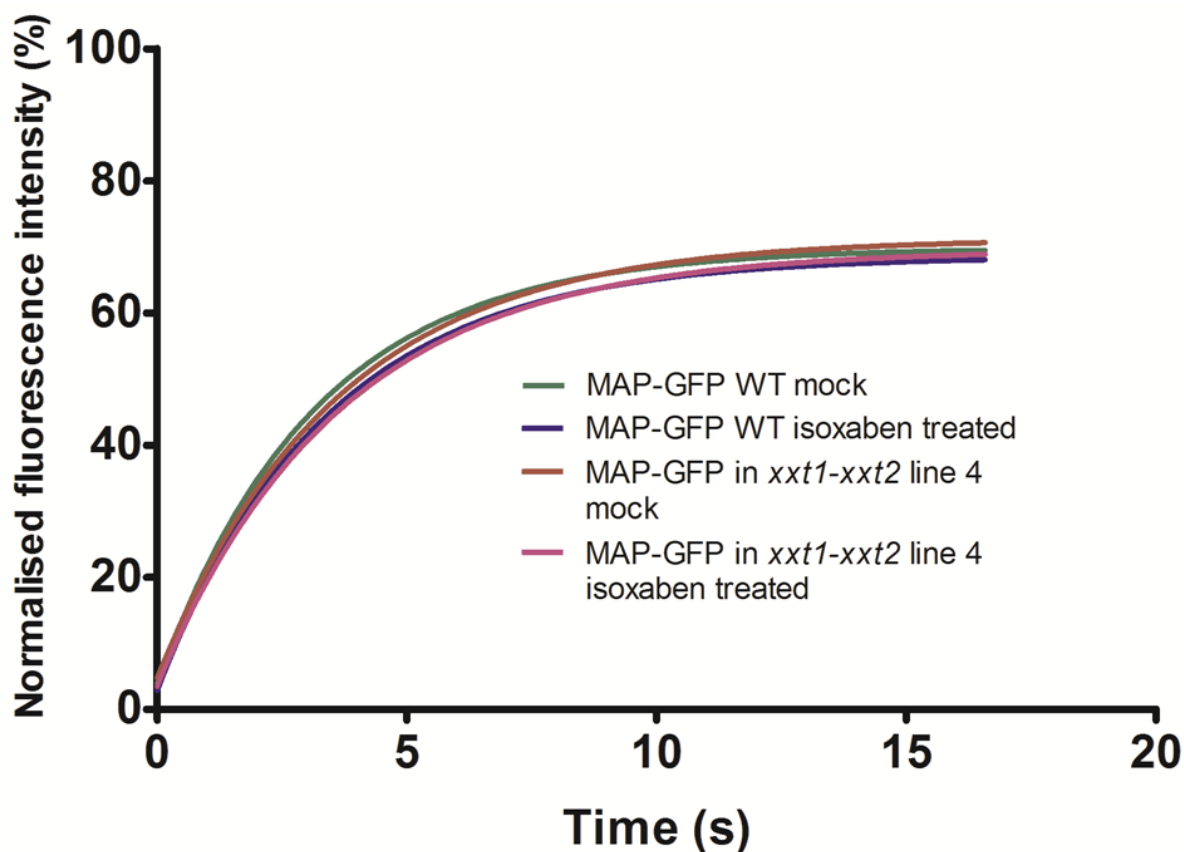


Figure 6.12 Fluorescence recovery global fit curves of MAP-GFP from Figure 6.11 (above).

MAP-GFP WT mock (wild type; green), MAP-GFP in *xxt1-xxt2* line 4 mock (blue), MAP-GFP WT isoxaben treated (red) and MAP-GFP in *xxt1-xxt2* line 4 isoxaben treated (pink). Time zero on the graphs denotes the first measurement post-bleach.

The MFR was calculated as the mean and standard deviation: MAP-GFP WT mock was $69.88 \pm 7.679\%$, MAP-GFP in line 4 of *xxt1-xxt2* mock was $71.01 \pm 7.393\%$, MAP-GFP WT isoxaben treated was $68.61 \pm 8.793\%$ and MAP-GFP in line 4 isoxaben treated was $69.30 \pm 5.649\%$.

The half-time means and standard deviations were calculated: MAP-GFP WT mock was 2.087 ± 0.5506 s, MAP-GFP in line 4 of *xxt1-xxt2* mock was 2.283 ± 0.5246 s, MAP-GFP WT isoxaben treated was 2.268 ± 0.4707 s and MAP-GFP in line 4 isoxaben treated was 2.371 ± 0.4549 s.

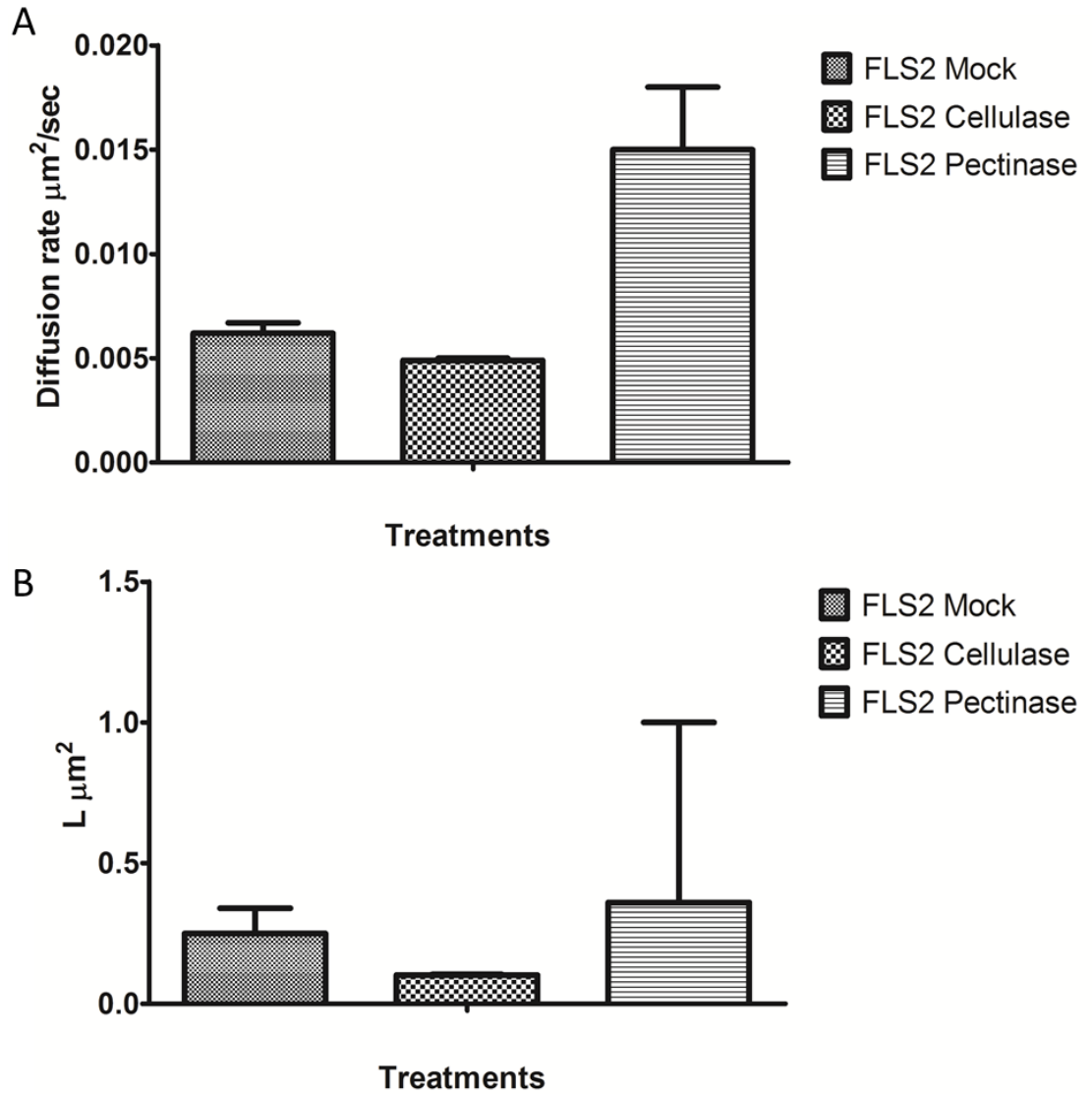


Figure 6.13 Diffusion of single pFLS2::FLS2-GFP molecules.

Single molecule tracking of pFLS2::FLS2-GFP within the plasma membrane. Both the rate of diffusion and the area that molecules are free to diffuse within were measured. (A) In cells treated with 1.5% cellulase pFLS2::FLS2-GFP molecules moved more slowly ($0.0049 \pm 0.0001 \mu\text{m}^2/\text{s}$) than in control cells ($0.0062 \pm 0.0005 \mu\text{m}^2/\text{s}$), while in cells treated with 1.5% pectinase pFLS2::FLS2-GFP molecules moved faster, although with a greater variance ($0.0150 \pm 0.0030 \mu\text{m}^2/\text{s}$). (B) In cells treated with 1.5% cellulase pFLS2::FLS2-GFP molecules moved within a smaller area ($0.1024 \pm 0.0016 \mu\text{m}^2$) than in control cells ($0.25 \pm 0.09 \mu\text{m}^2$), while in cells treated with 1.5% pectinase pFLS2::FLS2-GFP molecules moved in an apparently greater area, but note that variance has increased in the dataset ($0.36 \pm 0.64 \mu\text{m}^2$).

$n = 12$ cells per condition (4 cells from 3 different seedlings).

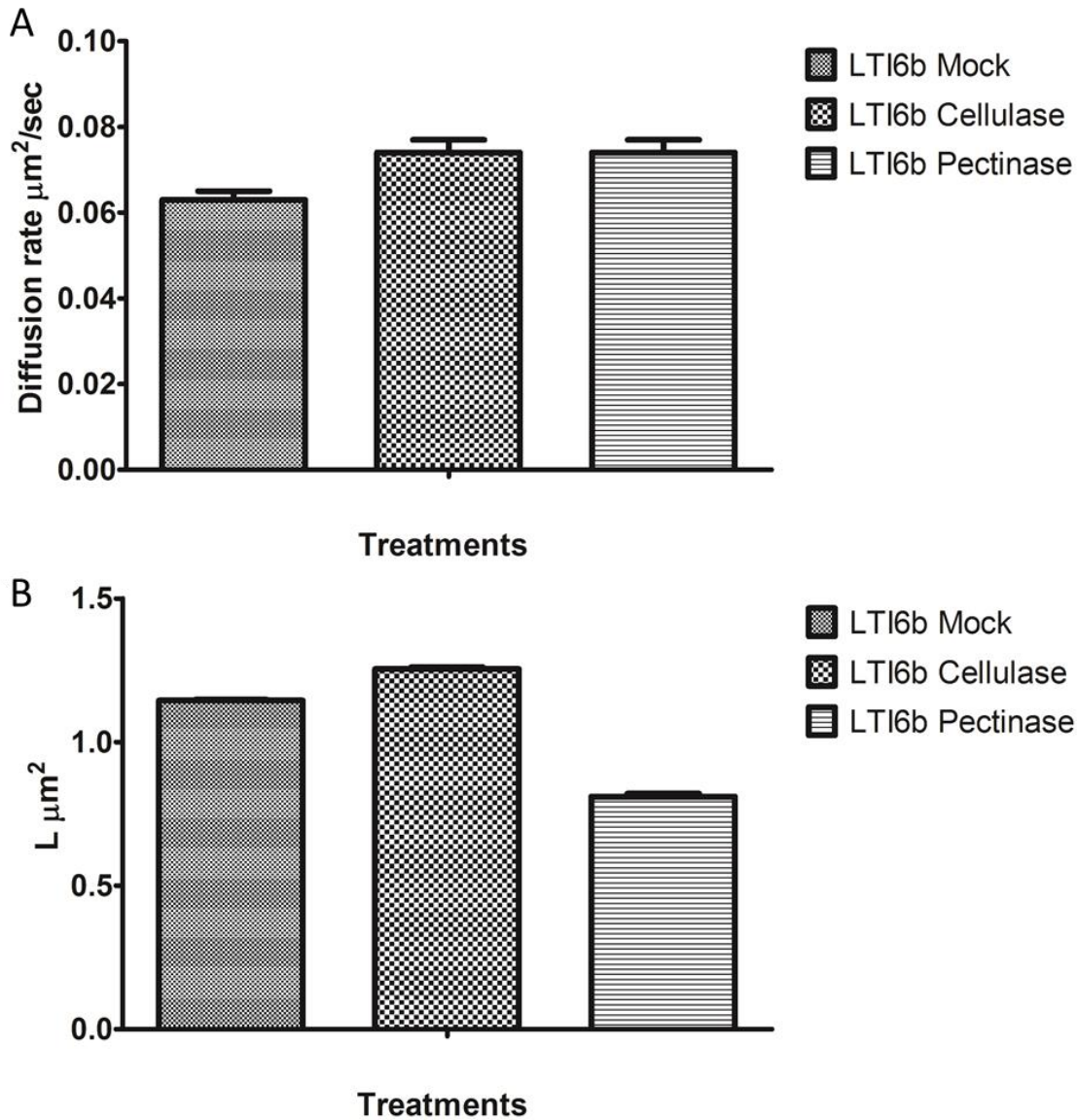


Figure 6.14 Diffusion of single paGFP-LTI6b molecules.

Single molecule tracking of paGFP-LTI6b within the plasma membrane. Both the rate of diffusion and the area that molecules are free to diffuse within were measured. (A) In cells treated with 1.5% cellulase ($0.074 \pm 0.003 \mu\text{m}^2/\text{s}$) and those treated with 1.5% pectinase ($0.074 \pm 0.003 \mu\text{m}^2/\text{s}$) paGFP-LTI6b molecules moved faster than in control cells ($0.063 \pm 0.002 \mu\text{m}^2/\text{s}$). (B) In cells treated with 1.5% cellulase ($1.2544 \pm 0.0064 \mu\text{m}^2$) paGFP-LTI6b molecules moved in an apparently greater area than in control cells ($1.1449 \pm 0.0025 \mu\text{m}^2$), while in cells treated with 1.5% pectinase paGFP-LTI6b molecules moved in an apparently reduced area, but note that variance has increased in the dataset ($0.81 \pm 0.01 \mu\text{m}^2$).

$n = 12$ cells per condition (4 cells from 3 different seedlings).

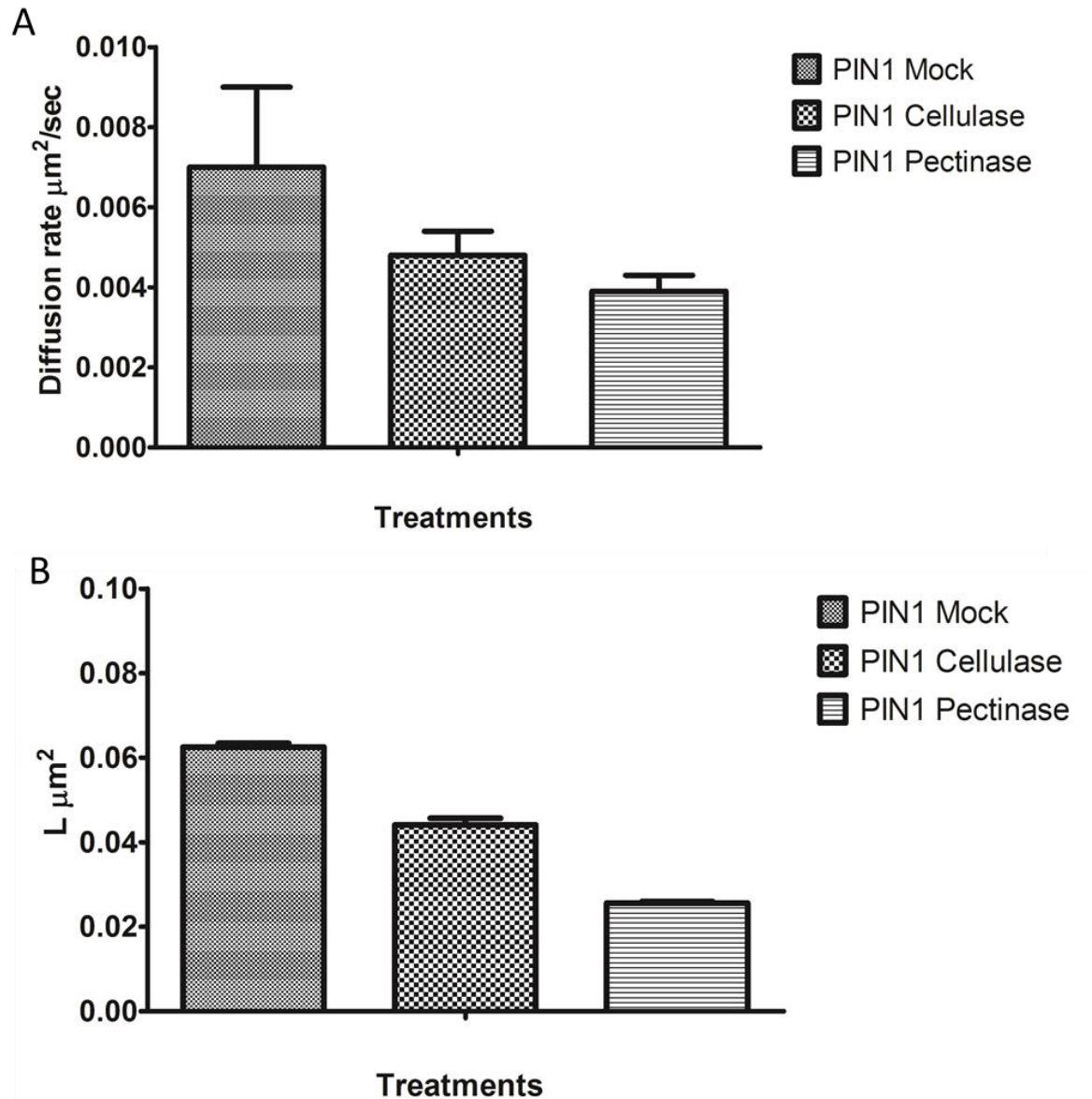


Figure 6.15 Diffusion of single pPIN1::PIN1-GFP molecules.

Single molecule tracking of paGFP-LTI6b within the plasma membrane. Both the rate of diffusion and the area that molecules are free to diffuse within were measured. (A) In cells treated with 1.5% cellulase ($0.0048 \pm 0.0006 \mu\text{m}^2/\text{s}$) and those treated with 1.5% pectinase ($0.0039 \pm 0.0004 \mu\text{m}^2/\text{sec}$) pPIN1::PIN1-GFP molecules moved more slowly than in control cells ($0.0070 \pm 0.0020 \mu\text{m}^2/\text{sec}$). (B) In cells treated with 1.5% cellulase ($0.0441 \pm 0.0016 \mu\text{m}^2$) pPIN1::PIN1-GFP molecules moved in an apparently reduced area than in control cells ($0.0625 \pm 0.0009 \mu\text{m}^2$), while in cells treated with 1.5% pectinase pPIN1::PIN1-GFP molecules moved in a much reduced area ($0.0256 \pm 0.0004 \mu\text{m}^2$).

$n = 12$ cells per condition (4 cells from 3 different seedlings).

6.5 Discussion

6.5.1 Selection screening of plasma membrane markers in the *xxt1-xxt2* mutant background

As described in Section 2.1, genotyping PCR was used to check the absence of xylosyltransferase 1 and 2 in an *A. thaliana* line sent from the Nottingham Arabidopsis Stock Centre (NASC; *xxt1-xxt2*); it confirmed what NASC and a previous study stated is a homozygous knock-out for those two genes (see Appendix Figure D.1, pg. 287) (Cavalier *et al.*, 2008). Fluorescence was confirmed for four plasma membrane-resident protein markers: GFP-LTI6b, MAP-GFP, GFP-PAP and YFP-PI (Section 6.4.1, pg. 177 and Figure 6.2, pg. 187; Table 6.1, pg. 186). However, whilst positive plants were found for the proteins AGP4, NPSN11 and SYP121 and fluorescence was visible in the first generation, by the second generation the fluorescence was no longer visible. These three proteins were the biggest (135 to 346 amino acids in length) and all have a known or hypothesised function (Table 6.1, pg. 186).

Xiao *et al.* (2016) tried to transform the same line with mCherry-TUA5 and found that the recovered transformants showed gross growth defects and sterility. Perhaps this line is sensitive somehow, so that certain functionally important over-expressed proteins were lethal and any transformants that survived had done so by silencing the proteins in question.

The morphological changes observed in *xxt1-xxt2* plants were the same as those reported by Cavalier *et al.* (2008); that is they grew slower, were smaller, had shorter roots and appeared to lack trichomes. In addition, the *xxt1-xxt2* plant lines that were successfully transformed and over-expressed fluorescent protein-tagged markers for the plasma membrane exhibited no additional morphological changes compared to the un-transformed *xxt1-xxt2* plants.

6.5.2 Fluorescence Recovery After Photobleaching of plasma membrane resident proteins in the *xxt1-xxt2* background

In this study three proteins were used that have been termed ‘minimal’: MAP-GFP is a small domain that is myristoylated and palmitoylated (36 amino acids plus GFP), while PAP is a small domain that is prenylated (55 amino acids plus GFP). All three of these lipid modifications occur on proteins that are integral to, or associated with, the inner leaflet of the plasma membrane (Hurst and Hemsley, 2015). The third ‘minimal’ protein is YFP-PI. This is a biosensor that binds PtdIns4P so that the mobility measured in this case is related to the mobility of the polyphosphoinositide (PtdIns; see Section 1.5.2, pg. 31) (Vermeer *et al.*, 2009). The PI in this study refers to a pleckstrin homology (PH) domain that is 98 amino acids (plus YFP) (Levine and Munro, 2002, Vermeer *et al.*, 2009). PH domains bind polyphosphoinositides with hydrogen bonds (Stahelin *et al.*, 2014).

Finally, LTI6b (At3g05890) is the only full length protein out of the four constructs used for FRAP in this study, but nothing is known of its function and no protein-protein interactions are predicted (Cutler *et al.*, 2000, Kurup *et al.*, 2005). It consists of 54 amino acids and has two helical transmembrane domains arranged in a Type IV topology with four amino acids forming a loop in the apoplast between them (Figure 6.4, pg. 188) (Martinière *et al.*, 2012).

Martinière *et al.* (2012) showed that protein crowding had little impact on lateral protein diffusion, so differences in the amount of protein in the membrane should not affect mobility. There were differences in protein mobility between wild-type and *xxt1-xxt2* plants and then additionally between the *xxt1-xxt2* lines expressing the same fluorescent construct. If it is true that the number of insertion events and therefore total amount of tagged protein does not affect mobility, perhaps different insertion events throughout the genome might cause a minor phenotype which affects protein mobility within the plasma membrane. For example, if an insertion occurred within a gene involved in lipid synthesis, this would probably affect membrane fluidity (Section 6.5.2.4, pg. 204). Martinière *et al.* (2012) also showed in *A. thaliana* seedlings expressing the ‘minimal’ proteins there was no increase in the MFR when they were treated with either oryzalin or cytochalasin D. Therefore in this study, the influence of the microtubule and actin cytoskeletons in terms of

‘corralling’ plasma membrane proteins was not investigated, although their impact on general protein mobility would be an interesting area of future research.

For three of the plasma membrane markers analysed (GFP-LTI6b, MAP-GFP and YFP-PI) the loss of detectable xyloglucan caused some decrease in the mean fluorescence recovery (MFR), which means that these proteins were relatively more immobile due to the alterations in cell wall structure. However, the notable exception to the above is GFP-PAP. This had an increase in the amount of mobile protein in the mutant lines compared to wild type, although there was no change in the rate (half-time) at which the protein diffused (Figure 6.6, pg. 190).

6.5.2.1 GFP-PAP and MAP-GFP in *xxt1-xxt2*

GFP-PAP associates with the inner leaflet of the plasma membrane via a prenyl group, which can be either a farnesyl or geranylgeranyl group (Zeng *et al.*, 2007). Both farnesylation and myristoylation are weak membrane associations, so can often be found in conjunction with another lipid modification if a tighter membrane association is necessary (Hemsley, 2015). This may mean that GFP-PAP has a looser attachment to the plasma membrane than MAP-GFP, which is held in place by S-acylation (also termed palmitoylation), a more stable lipid anchor in addition to the weaker myristoylation (Hemsley, 2015). The disruption in the cell wall-plasma membrane interface by the absence of detectable xyloglucan may have had a greater impact on the weakly-associated prenylated GFP-PAP, causing more of the protein to become mobile within the membrane (Figure 6.6, pg. 190; Section 6.5.2.4). Although, when the results from MAP-GFP in the two *xxt1-xxt2* mutant lines were pooled and compared to the wild type, the MAP-GFP proteins were found to move faster in the two *xxt1-xxt2* mutant lines, but a lower proportion of the population was mobile. However, because these results were not entirely consistent, MAP-GFP was used in a second set of FRAP experiments.

6.5.2.2 YFP-PI in *xxt1-xxt2*

YFP-PI showed a decrease in MFR in the mutant compared to wild type, meaning that a larger proportion of protein had become constrained in cells with walls lacking detectable xyloglucan (Figure 6.8, pg. 192). YFP-PI is derived from FAPP1 (Section 6.4.1, pg. 177) and it was designed to monitor PtdIns4P dynamics; it has been localised to the plasma membrane and the Golgi membrane in plant cells (Levine and Munro, 2002, Vermeer *et*

al., 2009). In this study, small organelles the same size and shape as Golgi bodies were observed in cells expressing YFP-PI in the wild type and in the mutant (data not shown). PtdIns lipids are much bigger than non-PtdIns lipids, so they project outwards, i.e. into the cytoplasm, from the inner leaflet of the plasma membrane and they are ‘engulfed’ by PH domains, so these domains do not insert very far into the membrane (Figure 6.2, pg. 187) (Knight and Falke, 2009). This suggests that PI should not be affected by changes in the cell wall-plasma membrane interface, but that the lipid it is binding is affected; somehow its mobility was decreased by the lack of detectable xyloglucan (Section 6.5.2.4).

In the *xxt1-xxt2* mutant it has been shown that cellulose microfibrils are more aligned than in wild-type (Xiao *et al.*, 2016). As the plasma membrane is appressed against the cell wall by turgor pressure, perhaps this physical change is enough to alter the mobility of both or either lipids and proteins; for example, they might get caught in ‘troughs’ between microfibrils.

Knight and Falke (2009) used single molecule tracking to investigate the PH domain from the General Receptor for Phosphoinositidies, isoform 1 (GRP1), which binds the rarer PtdIns(3,4,5) P_3 . They showed that it diffuses at the same rate as a single lipid molecule, but that it also has secondary binding domains that when occupied reduced its lateral diffusion (Knight and Falke, 2009). Ziemba and Falke (2013) used the same construct to track single molecules, which they recorded as $89.8 \pm 7.5\%$ of the protein population having a diffusion constant of $2.5 \pm 0.2 \mu\text{m}^2/\text{s}$. While it is not biologically or technically possible to compare these values with the ones obtained in this study, they both showed that a single PH domain is extremely mobile, which is also the conclusion of this study.

In addition, Ziemba and Falke (2013) showed that lateral diffusion of peripheral membrane proteins (in supported lipid bilayers) is determined by how many lipids the protein has bound and how far the protein domains insert into the membrane. If lateral diffusion is affected by these factors under ‘normal’ circumstances and the cell wall has an effect on plasma membrane mobility, then perhaps in the absence of detectable xyloglucan the mobility of differently associated membrane proteins is differently altered.

6.5.2.3 GFP-LTI6b in *xxt1-xxt2*

GFP-LTI6b, has a Type IV membrane anchor comprising two helical transmembrane domains separated by four amino acids, which form a loop into the apoplast (Figure 6.4, pg. 188). The MFR of GFP-LTI6b was decreased in *xxt1-xxt2* line 1 compared to wild type, but not in *xxt1-xxt2* line 8 compared to wild type (Section 6.4.2.1, pg. 178; Table 6.1, pg. 186). However, the exact opposite situation was found when half-times were examined: the half-time of *xxt1-xxt2* line 8 was decreased compared to wild type, but there was no difference between *xxt1-xxt2* line 1 and wild type (Section 6.4.2.1, pg. 178; Table 6.1, pg. 186). *xxt1-xxt2* line 1 had a reduced mobile protein fraction that took the same amount of time to recover as the wild type, whereas in *xxt1-xxt2* line 8 the same amount of protein recovered as wild type, but it recovered faster than wild type. When the results from the two *xxt1-xxt2* mutant lines were pooled and compared to the wild type, GFP-LTI6b proteins in general were found to move faster, but a lower proportion of the population was mobile. This suggests that protein interactions were affected by the lack of detectable xyloglucan in the cell wall, but the inconclusive results were the reason why this protein was used in the second set of FRAP experiments (Section 6.4.3, pg. 181).

6.5.2.4 Changes in *xxt1-xxt2* and their possible consequences

It has been reported that in the *xxt1-xxt2* mutant there are changes in the cellulosic and pectin components (Cavalier *et al.*, 2008, Park and Cosgrove, 2012). Antibodies against pectins showed a difference in distribution patterns and staining intensities (this could be due to accessibility), while there was a decrease in the amount of particular sugars (namely, rhamnose, fucose, arabinose, xylose and galactose) (Cavalier *et al.*, 2008). Park and Cosgrove (2012) found that it was a combination of xylans and pectins that became bio-mechanically more important in the absence of detectable xyloglucan. Therefore, an interesting next step would be to treat seedlings with a pectin biosynthesis inhibitor. They also showed that the decreased size of *xxt1-xxt2* plants was probably due to expansins losing their natural target in cell walls (Park and Cosgrove, 2012).

In the *xxt1-xxt2* mutant perhaps this loss of the natural target for association or interaction affects the cell wall-plasma membrane interface and alters protein mobility. This natural target may not have any relationship with the strength of an attachment to the plasma membrane, but may affect the dynamics of the lipids within the membranes. It is known that *A. thaliana* plants that lack functional genes involved in sterol biosynthesis have cell wall defects, such as cell wall gaps, and even dead cells (Schrack *et al.*, 2004). These

mutants also contain 50 to 70% less cellulose than wild type, but have normal levels of pectins and sugars and exhibit ectopic callose and lignin deposits (Schrick *et al.*, 2004). Another group suggested these phenotypes were due to PIN mis-localisation, although these are also plasma membrane proteins (Pullen *et al.*, 2010).

If sterols themselves are removed from the membrane there is a change in protein mobility. It has been shown that if cholesterol is removed from mammalian cells then the membrane viscosity is altered and protein diffusion is reduced (Shvartsman *et al.*, 2006, Barrantes, 2014). *A. thaliana* mutations in sterol biosynthesis had fewer sterol-dependent proteins, suggesting that disruption in the structure of the plasma membrane had a global effect on protein and lipid populations (Zauber *et al.*, 2014). If cell wall components affect lipid mobility (which can depend on the type of attachment), it is expected that the movement of YFP-PI would alter in the *xxt1-xxt2* background and this is what was found (Figure 6.8, pg. 192).

In addition, Prusicki (2014) and Martinière *et al.* (2012) both showed that in protoplasts just after cell wall removal, plasma membrane proteins have a different mobility to that found in plant cells with complete cell walls. This suggests that cell walls are necessary for correct protein mobility, but since the entire cell wall was removed it is impossible to determine whether any particular cell wall component had more influence over plasma membrane protein mobility than any other.

Lastly, it has been shown in zebrafish that genetic mutants can compensate to ‘buffer’ the effects of losing a gene, whereas animals that have a gene knock-down induced have much more severe phenotypes (Rossi *et al.*, 2015). It is possible the reason that the *xxt1-xxt2* plants lack a severe phenotype in general and the reason that there are variable responses in mobility amongst the proteins investigated, is due to the compensatory effort made by the plants. It is possible that an inducible RNAi line for *xxt1-xxt2* would have a very different cell wall to the T-DNA derived lines used in this study, although it may be more difficult in *A. thaliana* as it seems to display many instances of gene redundancy.

6.5.2.5 Comparison between the different marker proteins in wild-type

The MFRs of the plasma membrane marker proteins in wild type differ from each other, which is interesting considering that three of them consist just of a transmembrane-

associating domain and either GFP or YFP. It could be expected that they would have the same mobility, which is not what was found by Martinière *et al.* (2012) nor by this study. GFP-LTI6b and YFP-PI share similar MFR values: $74 \pm 7.6\%$ and $75.66 \pm 7.4\%$, respectively; but the first has two transmembrane domains and the latter binds PtdIns4P (Section 6.4.2.1, pg. 178 and Section 6.4.2.4, respectively). Martinière *et al.* (2012) also imaged GFP-LTI6b transiently in *Nicotiana tabacum* and found an MFR of $72.4 \pm 2.7\%$ with an $R^2 = 0.84$ (mean $\pm$ SEM), compared to an MFR of $91.4 \pm 3.6\%$ in *A. thaliana*. This difference between the MFR for the same protein in two different plants is intriguing (Martinière *et al.*, 2012). It suggests that the plasma membrane is different in the two species (*A. thaliana* and *N. tabacum*), or that transient expression alters the plasma membrane-cell wall environment somehow.

The MFR of GFP-PAP is lower than the others ($67.83 \pm 12.2\%$), meaning that the protein population is relatively more immobile (Section 6.4.2.2). This was unexpected as GFP-PAP is predicted to have the weakest association with the plasma membrane (prenylation; Section 6.5.2.1, pg. 202) (Martinière *et al.*, 2012). Lastly, the MFR for MAP-GFP found by this study was $86.54 \pm 10.7\%$: this was the largest percentage of mobile protein between the wild-type backgrounds. This may be the reason that almost no differences were detected between the protein in wild-type and in the *xxt1-xxt2* mutant: because it was so mobile already, if there were differences they were difficult to detect. It would be interesting to track single molecules of this protein in the wild-type background and in the *xxt1-xxt2* mutant to determine if there were any alterations in the behaviour of single molecules.

6.5.2.6 Recovery rates of proteins in *xxt1-xxt2*

It is important to note that in this study the half-times generally remained the same when the wild types were compared to their respective mutant lines, so that although the amount of protein being immobilised in the membrane might have changed, the time required for MFR did not alter. The only changes in half-times were seen in GFP-LTI6b and MAP-GFP (one mutant line each; Section 6.4.2.1, pg. 178 and Section 6.4.2.3, respectively). In both cases the half-time was slightly decreased in the mutant relative to the wild-type and these are also the two markers predicted to have the strongest association with the plasma membrane (Section 6.5.2, pg. 201).

Perhaps the disturbance in the plasma membrane-cell wall interface somehow affects protein turnover in the membrane. These proteins recover slower as their lateral diffusion through the membrane is impaired, possibly because they are more intimately connected with lipids and proteins that are affected by a lack of xyloglucan in the cell wall.

6.5.3 Mobility of plasma membrane resident proteins in a cell wall mutant background with the addition of isoxaben

The FRAP results from the previous experiment to measure dynamics of GFP-PAP and YFP-PI show large differences between wild type and xyloglucan mutant lines (Section 6.4.2.2, pg. 179 and Section 6.4.2.4). Mobility results for GFP-LTI6b and MAP-GFP were somewhat inconclusive. These latter pair of constructs were tested again with a slightly different experiment: FRAP data were collected much faster, but over a shorter time scale and in addition, both wild type and mutant were treated with isoxaben (see Section 1.3.3.1, pg. 25). Isoxaben causes the depletion of cellulose synthase complexes (CSCs; see Section 1.3.3, pg. 23) from the plasma membrane which means that cellulose production is adversely affected (Brabham and Debolt, 2012).

In this second experiment, there were no differences between any of the MAP-GFP conditions. Either MAP-GFP moved too fast to accurately measure any differences that exist at this resolution, or the cell wall has no influence over its mobility.

MAP-GFP contains two lipid modifications (Section 6.4.1, pg. 177). The first is myristoylation and the second modification is palmitoylation (S-acylation); this allows the protein to associate directly with the plasma membrane (Hurst and Hemsley, 2015). S-acylation is reported to associate with membranes with a strength that is equivalent to a transmembrane domain, but unlike a transmembrane domain the acyl group can be added and removed: it is thought for functional regulation (Hurst and Hemsley, 2015). During this study only one kind of recovery was observed (Figure 6.7, pg. 191 and Figure 6.12, pg. 196); this suggests that the S-acylation of the protein was not reversed during the course of the experiment. It might be possible that MAP-GFP is trafficked to the membrane and then the S-acyl group is immediately removed. However, S-acyltransferases are found in certain membranes rather than in the cytoplasm (Zeng *et al.*, 2007), and this would be

expected to result in a two-phase fluorescence recovery in FRAP experiments. Additionally, S-acylation is thought to require some kind of prior attachment to a membrane (Hurst and Hemsley, 2015), so in this case the N-myristoylation has probably already occurred, as there is no transmembrane domain or protein-protein interaction.

In the first set of FRAP experiments GFP-LTI6b WT had an increased half-time compared to the *xxt1-xxt2* line 8 mutant, whereas in this second FRAP experiment GFP-LTI6b WT mock had a decreased half-time compared to the *xxt1-xxt2* line 8 mutant ($p = 0.0283$; Section 6.4.3.1, pg. 182). The WT (untreated) appeared slower in the first experiment and WT mock-treated was faster in the second. In the second experiment both mock conditions were incubated with DMSO which is known to interfere with membranes, so it is possible this might change protein mobility. Indeed, even the incubation in $\frac{1}{2}$ MS may affect the mobility of plasma membrane proteins, as it changed the dynamics of the actin cytoskeleton in the temperature stress experiments in Chapter 4 (see Section 4.4.2, pg. 92).

In the second FRAP experiment, both WT and mutants were treated with isoxaben. The MFR and half-time of GFP-LTI6b WT mock was decreased compared to the isoxaben-treated WT, so the recovery of the protein in the WT mock was slower, but a greater proportion of protein moved within the membrane. However, Xiao *et al.* (2016) showed that cellulose synthesis was reduced in *xxt1-xxt2* mutants with a 20% reduction in crystalline cellulose and cellulose microfibrils were aligned in a highly parallel manner. Very speculatively, this suggests that treating *xxt1-xxt2* plants might not have a dramatic effect and indeed the GFP-LTI6b line mock has a tiny increase in half-time compared to the isoxaben treated ($p = 0.0473$; Figure 6.10, pg. 194).

6.5.4 Single molecule tracking of plasma membrane proteins with a perturbed cell wall

In this study, three fluorescently tagged plasma membrane proteins were used to investigate the influence of the cell wall on the mobile behaviour of single molecules. Single molecule tracking can be used to examine the relationship between different molecules of the same protein and to extract information on the rates of movement versus time (Martin-Fernandez *et al.*, 2013).

TIRF is the preferred method of visualising and tracking single molecules because of the high signal-to-background ratio and limited depth of excitation (Martin-Fernandez *et al.*, 2013). Under control conditions paGFP-LTI6b moves roughly ten times faster than pPIN1::PIN1-GFP and pFLS2::FLS2-GFP (Figure 6.14, pg.198; Figure 6.15, pg. 199; and Figure 6.13, pg. 197).

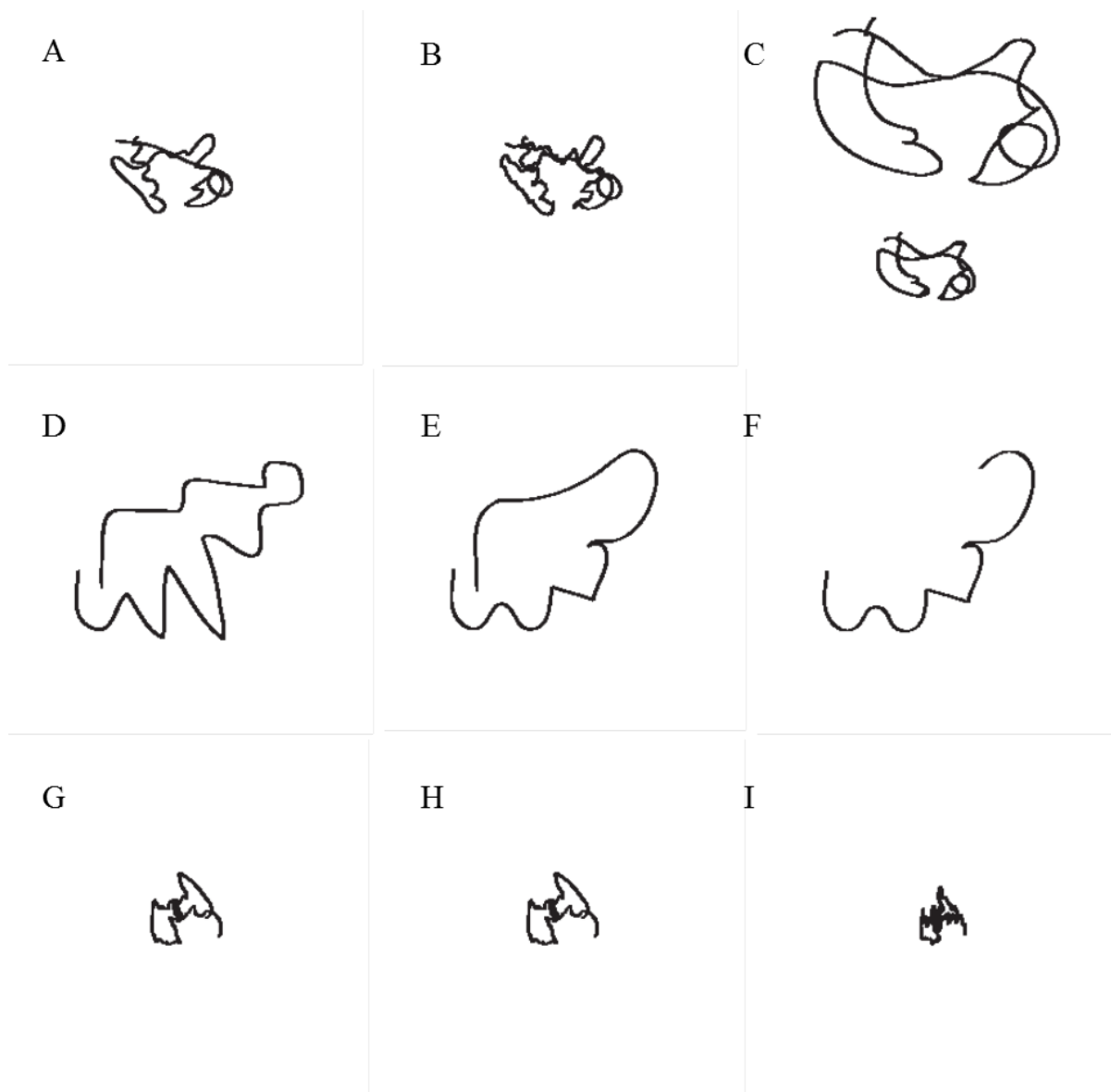


Figure 6.16 Schematic showing single molecule tracks relative to pFLS2::FLS2-GFP mock. The average diffusion rate and average area of the tracks indicate the kind of movement that was recorded for the proteins and conditions listed below:

A) pFLS2::FLS2-GFP 1h mock ($D = 0.005 \mu\text{m}^2/\text{s}$, $L = 0.25 \mu\text{m}^2$);

B) pFLS2::FLS2-GFP 1h cellulase ($D = 0.004 \mu\text{m}^2/\text{s}$, $L = 0.1024 \mu\text{m}^2$);

C) pFLS2::FLS2-GFP 1h pectinase ($D = 0.015 \mu\text{m}^2/\text{s}$, $L = 0.36 \mu\text{m}^2$);

D) paGFP-LTI6b 1h mock ($D = 0.06 \mu\text{m}^2/\text{s}$, $L = 1.1449 \mu\text{m}^2$);

E) paGFP-LTI6b 1h cellulase ($D = 0.08 \mu\text{m}^2/\text{s}$, $L = 1.2544 \mu\text{m}^2$);

- F) paGFP-LTI6b 1h pectinase ($D = 0.08 \mu\text{m}^2/\text{s}$, $L = 0.81 \mu\text{m}^2$);
 G) pPIN1::PIN1-GFP 1h mock ($D = 0.007 \mu\text{m}^2/\text{s}$, $L = 0.0625 \mu\text{m}^2$);
 H) pPIN1::PIN1-GFP 1h cellulase ($D = 0.005 \mu\text{m}^2/\text{s}$, $L = 0.0441 \mu\text{m}^2$);
 I) pPIN1::PIN1-GFP 1h pectinase ($D = 0.004 \mu\text{m}^2/\text{s}$, $L = 0.0256 \mu\text{m}^2$);

paGFP-LTI6b moves in an area approximately five times larger than the area that pPIN1::PIN1-GFP moves in, while pFLS2::FLS2-GFP moves in an area approximately twice the size of the area that pPIN1::PIN1-GFP diffuses within (Figure 6.14, pg.198; Figure 6.15, pg. 199; and Figure 6.13, pg. 197).

The proteins used in this study were chosen because of their different sizes and means of associating with the plasma membrane. FLS2 is involved in flagellin perception: it is a receptor kinase of 1173 amino acids, containing one transmembrane domain and an extracellular domain made up of 28 tandem leucine-rich repeats (Gomez-Gomez *et al.*, 2001). In this study, a stable *A. thaliana* line was used that expresses FLS2-GFP driven by its endogenous promoter (Robatzek *et al.*, 2006). A previous study that used the same line found that a large proportion of the protein remains immobile in the membrane during FRAP (Martinière *et al.*, 2012).

When treated with 1.5% cellulase for 1 h, both the speed of diffusion and the area within which pFLS2::FLS2-GFP was free to diffuse were decreased (Figure 6.13, pg. 197). This suggests that cellulose is required to create an environment in which FLS2 can move more freely. Conversely, when treated with 1.5% pectinase for 1 h the speed of diffusion dramatically increases (along with variation in the data) and the area within which each molecule moves is also increased (Figure 6.16, pg. 209). This suggests that pectins within the cell wall are involved in constraining pFLS2::FLS2-GFP.

LTI6b is made up of only 54 amino acids with two predicted helical transmembrane domains and only four amino acids in the apoplast. In a previous section it was tagged with GFP; here the photoactivatable GFP was used (Figure 6.4, pg. 188) (Martinière *et al.*, 2012). The individual molecules of paGFP-LTI6b had a diffusion rate of $0.0063 \pm 0.002 \mu\text{m}^2\text{s}^{-1}$ (Figure 6.14, pg.198). When treated with 1.5% cellulase for 1 h the diffusion rate of paGFP-LTI6b molecules was increased, as was the area in which the proteins were free to diffuse (Figure 6.14, pg.198). However, when paGFP-LTI6b was treated with 1.5%

pectinase for 1 h, the diffusion rate of paGFP-LTI6b molecules increased but the area within which proteins were freely diffusing was reduced (Figure 6.16, pg. 209).

Martinière *et al.* (2012) treated paGFP-LTI6b with isoxaben and found that paGFP-LTI6b became more confined, which is the opposite of what was found in this study with the application of cellulase. Indeed, given the other data detailed in (Martinière *et al.*, 2012), the expected result would be that paGFP-LTI6b should be less constrained with a perturbed cell wall, which is what was seen in cells treated with cellulase in this study. Cells treated with pectinase reacted the same as those published by (Martinière *et al.*, 2012) when they treated with isoxaben, in that the constrained area was reduced. This is confusing as although cellulase treatment gave the expected result, it is in contrast to the results from (Martinière *et al.*, 2012).

Li *et al.* (2011) used single molecule tracking to examine the mobility of GFP-LTI6a, a protein that has a very similar sequence to LTI6b and has the same kind of membrane association. They found that 51% of GFP-LTI6a molecules in the plasma membrane were undergoing Brownian diffusion, that approximately 26% exhibited directed diffusion while 22% had restricted diffusion (Li *et al.*, 2011). Perhaps the next step for this study would be to try to define these populations for the proteins examined and then to establish how the populations exhibiting different movements change when treated with cellulase and pectinase.

pPIN1::PIN1-GFP consists of 622 amino acids (67 kDa) with 10 transmembrane domains (Galweiler *et al.*, 1998, Ganguly *et al.*, 2010). Although it has been shown that both PIN1 and PIN2 somehow interact with the cell wall, for example, if a cell containing PIN1-GFP or PIN2-GFP is plasmolysed, signal is seen not only in Hechtian strands, but at the cell wall itself (Feraru *et al.*, 2011). Compared to mock both area and diffusion rate of pPIN1::PIN1-GFP under its endogenous promoter were reduced after enzyme treatments (Figure 6.15, pg. 199). Therefore both the diffusion rate of the molecules and the area that they were free to diffuse within was reduced when either cellulose or pectins were partially degraded (Figure 6.16, pg. 209). PIN proteins are very tightly regulated in space and time because of their involvement in creating auxin gradients (Ganguly *et al.*, 2010). It was expected that they should be very tightly associated when in the plasma membrane, so it is interesting that they are affected by the digestion of both cellulose and pectin. .

6.5.4.1 *The effect of cellulose digestion on the mobility of plasma membrane-resident proteins*

The three proteins detailed above (paGFP-LTI6b, pFLS2::FLS2-GFP and pPIN1::PIN1-GFP) all show different amounts of movement in mock treatments (Figure 6.16, pg. 209). Even though pFLS2::FLS2-GFP is the biggest protein, pPIN1::PIN1-GFP has the least and most restricted kind of movement. Possibly it has the most controlled localisation because of its involvement in creating auxin gradients (Ganguly *et al.*, 2010) (Figure 6.16, pg. 209). When treated with cellulase the three proteins react in different ways: pFLS2::FLS2-GFP moves in a slightly more convoluted manner; paGFP-LTI6b moves in a more direct manner; and pPIN1::PIN1-GFP moves in a more convoluted manner and in a reduced area (Figure 6.16, pg. 209).

Cellulose microfibrils are the major structural component in primary cell walls; the digestion of these would seriously weaken the cell wall (see Section 1.3.1.1, pg. 20). However, the digestion of cellulose seems to have less of an impact on the way the protein moves and more upon the size of the area that it is moving within (Figure 6.16, pg. 209). Although none of the proteins had their mobility altered in the same way by cellulose digestion, this treatment was sufficient to affect even the movement of paGFP-LTI6b, which is known to be a very mobile protein.

6.5.4.2 *The effect of pectin digestion on the mobility of plasma membrane-resident proteins*

Pectins are extremely complex carbohydrates that form a gel-like matrix in the cell wall. They can push cellulose microfibrils apart within the wall or, conversely, ‘lock’ them in place once expansion has ceased (see Section 1.3.1.3, pg. 20) (Cosgrove, 2005). When treated with pectinase the three proteins react in almost the same way: pFLS2::FLS2-GFP moves in a more direct manner, but with a big variation in area; paGFP-LTI6b moves in a more direct manner and in a reduced area; and although pPIN1::PIN1-GFP moves in a much more convoluted manner it also moves in a much reduced area (Figure 6.16, pg. 209). Compared to the digestion of cellulose, the digestion of pectin has a bigger impact, on the way the protein moves and the area in which it moves, but again the proteins reacted differently (Figure 6.16, pg. 209). This treatment was sufficient to affect the mobility of three very different and unrelated plasma membrane proteins, which suggests a broad influence from the cell wall, and especially from pectin, over proteins in the plasma membrane.

When pectin was digested, all three proteins were affected in the amount of space that they can move within (Figure 6.16, pg. 209). It is possible that pectin digestion has differential effects on protein mobility, indeed, it has been shown that different cellulose biosynthesis inhibitors cause different symptoms *in planta* (Tateno *et al.*, 2016). This suggests that the cell wall does have an influence over the dynamics of plasma membrane proteins, but that each protein has its own relationship with the various components of the cell wall.

6.5.5 Fluorescence Recovery After Photobleaching vs single molecule tracking

The single molecule tracking results show different membrane proteins are affected differently by alterations in cell wall components, which is also the conclusion from the two FRAP experiments detailed in earlier sections of this study (Sections 6.4.2, pg. 178 and 6.4.3, pg. 181). Both FRAP and TIRF can be considered 'global' measurements due to their spatial resolution being measured in μm (Barrantes, 2014), but TIRF allows a better spatial and temporal resolution. Although single particle tracking can underestimate the diffusion coefficient and it tends to estimate the track length as being shorter than it is, this last issue can be avoided by making sure a large number of molecules are tracked (Rose *et al.*, 2015), as was done in this study. There is currently no perfect method of single particle tracking, though there are many available and many are optimised for certain kinds of data (Chenouard *et al.*, 2014).

6.6 Conclusions

In this chapter it was shown that the dynamics of plasma membrane-resident proteins respond in different ways to treatments that affect the cell wall interface, depending on their type of association with the plasma membrane.

The cell wall mutant line *xtt1-xtt2* was transformed with different plasma membrane-resident proteins tagged with fluorescent proteins and the mobility of these proteins was examined in both wild type *A. thaliana* and in *xtt1-xtt2* using FRAP. This showed a mixed response based on the membrane association of the protein under investigation, suggesting specific and defined relationships between proteins in the plasma membrane and the cell wall.

Single molecule tracking was used to analyse the movement and behaviour of three different plasma-membrane proteins under conditions of cell wall perturbation. That all three proteins show different responses to different disturbances in the cell wall suggests that both the affected components within the cell wall (cellulose or pectins) and the manner in which the protein is anchored within the cell wall, feed into how the movement alters. This suggests that the cell wall influences plasma membrane proteins in a specific and complex manner.

Chapter Seven

General Discussion with Future Work

7.1 General summary of results

The work described in this thesis provides novel insights into the influence of the cell wall on both the organisation and dynamics of the actin cytoskeleton and on the mobility of plasma membrane-resident proteins. Chapter 3 detailed the two image analysis tools developed during this study for the purpose of quantifying the bulk remodelling of the actin cytoskeleton.

In Chapter 4, these tools were used to compare the dynamics of the actin cytoskeleton under conditions of cell wall disruption and also to discover if the actin cytoskeleton has a general abiotic stress response. It was found that plasmolysed cells had a reduction in the bulk remodelling index of the actin cytoskeleton, which means that the network had become more static when the cell wall was physically separated from the protoplasm. This was demonstrated with both GFP-FABD2 and GFP-Lifeact, fluorescent markers for the actin cytoskeleton in plants. The two types of differently marked networks were analysed with the two image analysis tools developed specifically for this study to quantify actin cytoskeleton dynamics. Whilst stressing cells with hot or cold temperatures did not cause a reduction in actin dynamics, treating cells with isoxaben, a drug that interferes with cellulose production, did cause a reduction in the bulk remodelling index of the actin cytoskeleton.

In Chapter 5, these tools were then used to compare the dynamics of the actin cytoskeleton in protoplasts immediately after cell wall removal and after 24 h of cell wall re-growth. The bulk remodelling index of the actin cytoskeleton in protoplasts appeared to be

different between the two time points when marked with GFP-FABD2, but no differences were seen when GFP-Lifeact expressing protoplasts were analysed.

Lastly, Chapter 6 examined the cell wall-plasma membrane interface by measuring plasma membrane protein mobility in a cell wall mutant line and by tracking single plasma membrane proteins in cells with perturbed cell walls. Four plasma membrane-resident proteins were examined using Fluorescence Recovery After Photobleaching (FRAP) in the *xxt1-xxt2* mutant background, which has no detectable xyloglucan. There were different changes in the mobility of the plasma membrane-resident proteins, suggesting that different membrane associations are differentially affected by alterations in the cell wall. Lastly, individual molecules of three different plasma membrane proteins were tracked in cells either treated with cellulase or pectinase and showed different responses. Again, these proteins reacted in different manners to the different treatments, suggesting that not only is the type of membrane association important, but that they are affected differently by the various carbohydrates in the cell wall.

7.2 Methods to quantify the dynamics of the actin cytoskeleton

Pairwise image subtraction was developed to measure ‘bulk remodelling’, a deliberately broad term to cover all kinds of alteration that the actin cytoskeleton undergoes (e.g. polymerising, severing, annealing, etc.; Section 1.2.2, pg. 8). This method appears to work well for networks marked with GFP-FABD2 (see Section 3.4.3.1, pg. 60 and Section 4.4.1.2, pg. 86). Additionally, this method was modified slightly so that the mean fluorescence intensity was measured, as opposed to the total amount of fluorescence (see Section 3.4.3.2). This allowed examination of organisational changes in the actin cytoskeleton.

Cumulative fluorescence intensity was developed in collaboration with Dr Axel Poulet, who wrote an ImageJ plug-in to sum the cumulative fluorescence at each pixel for every time-frame in a time-series (see Section 3.4.3.3). This method appears to be effective in determining the amount of change in networks decorated with GFP-Lifeact (see Section 4.4.1.5).

7.2.1 Image analysis tools – next steps

These two techniques could be used as a ‘starting point’ in investigations into the remodelling of the actin cytoskeleton, as they would provide baseline data about relative rates of change in different experimental situations. This type of analysis could then be followed up with methods that analyse specific remodelling events, such as annealing or severing. In addition to optimisation of the analysis tools developed herein, the actin network analysis techniques detailed below may be employed to complement the data gained from *Pairwise image subtraction* and *Cumulative fluorescence intensity*.

7.2.1.1 *Optimisation of image analysis tools already developed*

The methods developed during the course of this study seem to support the qualitative observations of actin cytoskeleton morphology and dynamics that were made. However, it would be good to have a different and independent set of conditions in which the changes of the cytoskeleton are already known in order to obtain extra validation for the accuracy of these methods. Ideally, both methods developed during this study would have been effective in analysing both markers used during this study; although FABD2 and Lifeact present alternate morphologies of the actin cytoskeleton under control conditions (see Section 4.4.1.1, pg. 85 and Section 4.4.1.4).

To allow analysis of both markers by both tools, perhaps the imaging parameters need to be changed. If the time-series were collected using a smaller ROI and more quickly, then it is possible they could be suitable. The FABD2 marked networks will appear less dense with higher resolution and quicker collection time might reduce the amount of non-remodelling movement observed in Lifeact marked networks. This could be easily achieved with a confocal microscope such as the Zeiss 880 [Zeiss].

It would also be useful to adapt *Pairwise image subtraction* so that instead of reporting proportions, it returned values per μm per second. This could be done as long as all imaging was carried out using the same scale and imaging duration.

7.2.1.2 *Persistency mapping to quantify actin network stability*

Persistency mapping investigates actin cytoskeleton dynamics by quantifying the amount of network that remains stationary for a given amount of time. This takes advantage of a

protocol developed by Sparkes *et al.* (2009) to measure persistency in the endoplasmic reticulum in plant cells. It uses the ‘Image Calculator’ function in ImageJ (Rasband, 1997-2016) to calculate the proportion of a network that remains stationary during a defined time period which can be compared between conditions (Sparkes *et al.*, 2009). While this method was developed for a membranous cell component, it would be interesting to investigate whether this would work for cytoskeletal networks and how the data might complement the analysis tools developed in this study.

7.2.1.3 *Co-localisation to quantify actin dynamics*

To complement the *Cumulative fluorescence intensity* method (but unconnected to it), it would be interesting to use co-localisation analysis to quantify change in the actin cytoskeleton. This study could not find an appropriate available co-localisation tool for the actin cytoskeleton. In order to develop an effective tool, it would be best to collaborate with an image analyst specifically on the problem of measuring dynamic change. A plugin for ImageJ (Rasband, 1997-2016) could then be developed that measures the amount of co-localisation between actin filaments in subsequent time-frames, rather than between different colour channels of the same still image.

7.2.1.4 *Total Internal Reflection Fluorescence microscopy to analyse the behaviour of single filaments*

This study struggled to find image analysis tools that were applicable to datasets with a relatively large field of view and variable fluorescence intensities (see Section 3.1, pg. 54). However, there are several excellent published methods that measure the structure and dynamics of single filaments (Staiger *et al.*, 2009, Smertenko *et al.*, 2010). The next step would be to use Total Internal Reflection Fluorescence (TIRF) microscopy to track single filaments and measure their remodelling to discover if their individual dynamics reflect what is seen on the cellular level (see Section 1.7.1.2, pg. 37).

7.2.1.5 *Applicability of the image analysis tools to the microtubule cytoskeleton and endoplasmic reticulum*

The image analysis tools developed in this study may not be as effective with the slower dynamics of the microtubule cytoskeleton, but that is something that could be tested. It is also possible that they could be used to investigate the dynamics of the endoplasmic reticulum; however, they may not be as accurate because of the cisternal sheets that form within the endoplasmic reticulum, but it would be interesting to establish if this is the case.

7.3 Interactions between the actin cytoskeleton and the microtubule cytoskeleton

It is known that the actin and microtubule cytoskeletons interact (see Section 1.2.5.2, pg. 17). Given this, an important next step for this investigation would be to discover if the dynamics of the microtubule cytoskeleton alter in a similar manner when the cell wall-plasma membrane interface is disrupted. It would also be instructive to investigate the interaction between the two cytoskeletons, i.e. to what degree are the observed alteration in the organisation and dynamics of the actin and microtubule cytoskeleton related during cell wall perturbation. For example, do actin filaments move differently around microtubules?

The links between the microtubule cytoskeleton and the cell wall are more defined compared to the actin cytoskeleton, so it is possible that this is the direct cause, or at least part of the reason for the reduction in bulk remodelling seen in the actin cytoskeleton when the cell-plasma membrane interface is disrupted. This relationship could be investigated by treating with drugs such as oryzalin, or colchicine (microtubule depolymerising agents), or taxol (microtubule stabilising agents) and then disrupting the cell wall-plasma membrane interface, to determine if any extra effects can be measured in the actin network. In addition, it would also be interesting to investigate the relationship between the actin cytoskeleton and endoplasmic reticulum under cell wall disrupting conditions as they are also tightly connected.

7.4 Disruption of the cell wall-plasma membrane interface

Four different approaches were used to disrupt the cell wall-plasma membrane interface: plasmolysis (via mannitol), disruptive substances (isoxaben and the digestive enzymes cellulase and pectinase), protoplasting (complete cell wall removal), and an *A. thaliana* cell wall mutant line (*xxt1-xxt2*) that lacks detectable xyloglucan, an abundant hemicellulose. Given the previous work on this area, all four approaches seemed logical extensions to what had already been established. These approaches were used to determine the extent of

the influence of the cell wall over intracellular components, as they are all quite different to each other, in an attempt to construct a more complete picture of the cell wall-plasma membrane-actin cytoskeleton continuum.

7.4.1 Plasmolysis vs isoxaben-treatment: the effect on actin cytoskeletal dynamics

The bulk remodelling index of the actin cytoskeleton marked with GFP-FABD2 was reduced in plasmolysed cells compared to control cells (see Section 4.4.1, pg. 85). GFP-FABD2 expressing cells were also treated with isoxaben, a herbicide that interferes with cell wall biosynthesis and maintenance (Tateno *et al.*, 2016). Again, the bulk remodelling index of the actin cytoskeleton was reduced in both isoxaben-treated 1 h cells compared to mock 1 h cells and in isoxaben-treated 4 h cells compared to mock 4 h cells (see Section 4.4.3).

These approaches complement each other because plasmolysis is an unspecific, naturally occurring phenomenon, whilst isoxaben is a drug that acts in a specific manner. Plasmolysis is the separation of the cell wall from the protoplasm due to shrinking of the vacuole (see Section 1.4.1.1, pg. 28; although it should be noted that actin filaments can be found in Hechtian strands), whilst isoxaben causes the cellulose synthase complexes (CSCs) to be removed from the plasma membrane (see Section 1.3.3.1). Both treatments disrupt the cell wall-plasma membrane interface and both treatments were shown by this study to reduce the bulk remodelling index of the actin cytoskeleton. It is interesting that they can have such different modes of action and yet have the same effect on the dynamics of the actin cytoskeleton. This does suggest that the cell wall has some kind of organisational influence over the actin cytoskeleton, whether direct or indirect. It might be interesting to look at the dynamics of the actin cytoskeleton in *A. thaliana* that are resistant to isoxaben, to determine whether this effect is due to the mode of action of the drug or whether it is due to interference with the CSCs. It would also be interesting to try to measure the viscosity of the cytoplasm when the cell is plasmolysed and how this affects the cytoskeleton and motile organelles. In addition, it would also be interesting to test isoxaben in a concentration gradient manner, to try to determine the lowest dose to which a

cell responds. Lastly, it would also be instructive to measure the dynamics of single actin filaments under these conditions as well.

When the mean fluorescence intensity was measured, plasmolysed cells had a reduction in fluorescence intensity (GFP-FABD2) compared to the control and so did both isoxaben-treated 1 h cells compared to mock 1 h cells and in isoxaben-treated 4 h cells compared to mock 4 h cells. Again, this shows that disruption of the cell wall-plasma membrane, using two very different methods, caused an alteration in the organisation of the actin cytoskeleton.

7.4.1.1 Biological function of actin bundles during plasmolysis

The actin bundles observed in plasmolysed cells in this study and in other studies have been compared by some to the actin-based stress fibres seen in certain animal cells (Wojtaszek *et al.*, 2007, Lang *et al.*, 2014). The suggestion is that these bundles are involved in regulating the shape and volume of the protoplast during plasmolysis (Komis *et al.*, 2002, Wojtaszek *et al.*, 2007).

This study has shown that not only was the organisation of the actin cytoskeleton altered during plasmolysis, but that its dynamics were also reduced. However, this study did not begin to explore the possibility that actin bundles formed during plasmolysis are similar to stress fibres, so there are many possible experiments. Myosins are known to be components of stress fibres in mammalian cells (Blanchoin *et al.*, 2014), so plasmolysed cells could be treated with BDM (a ‘dirty’ myosin inhibitor) or with dominant-negative non-functional myosin mutants, to determine if actin bundles still form and if they remain immobile. Cytoplasmic streaming could be investigated in these cells, as the streaming motion is caused by the action of myosins trafficking organelles on the actin cytoskeleton (see Section 1.2.3). It would also be interesting to use mutant lines for the three actin genes expressed in vegetative cells (actin 2, 7 and 8) to try to determine if the actin bundles have a specific composition of actin protein or proteins.

In mammalian cells, cofilin is known to sever stress fibres, often when there is a loss of tension (Hayakawa *et al.*, 2011). There are *A. thaliana* lines available that lack cofilin; these could be transformed with actin cytoskeleton markers and then plasmolysed to determine if there is a difference in the rate that bundles are severed in the mutant

compared to a control. Komis *et al.* (2002) treated plant cells with Cytochalasin-B before plasmolysis; an experiment like this could be used to measure and quantify any shape changes in the protoplasm in 3D in a time-series during both plasmolysis and re-hydration.

7.4.1.2 *Transcriptional changes during plasmolysis*

Some studies have also investigated changes in the regulation of genes during water stress (Bray, 2004). This kind of data could be used to examine whether there are changes in transcription for proteins known to bind actin, particularly those such as myosins and cofilin.

The protoplasting procedure used in this study was based on Yoo *et al.* (2007) (see Section 2.4, pg. 45) and the authors state it is not suitable for long-term experiments, such as dedifferentiation or cell division. Yoo *et al.* (2007) state that the short incubation time for digestion means that the cells do not become as stressed as during a long incubation: this stress is considered important for dedifferentiation. Dedifferentiated protoplasts have been shown to have a decrease in the number and size of their chromocentres (Tessadori *et al.*, 2007). Chromocentres are composed of condensed chromatin, whilst a greater proportion of decondensed chromatin implies an up-regulation of gene activity (Tessadori *et al.*, 2007).

The protoplasts used in this study were not checked to determine the number or size of their chromocentres; this would be an interesting next step, especially as regards transcriptional changes (Tessadori *et al.*, 2007). For example, an analysis of genome-wide changes in gene expression to determine the number of genes up- and down-regulated in response to cell wall removal, perhaps using both dedifferentiated and non-differentiated protoplasts.

7.4.2 Protoplasts: actin cytoskeletal dynamics

The bulk remodelling index was found to be decreased in both samples of GFP-FABD2 expressing protoplasts imaged at 24 h after cell wall removal compared to their respective 0 h. This is the opposite result to what was expected, given the data obtained from

plasmolysed and isoxaben-treated cells. However, the 0 h isoxaben-treated cells had a reduced bulk remodelling index compared to mock 0 h cells, which is expected.

As the isoxaben result above was expected, it is hard to understand why the 24 h sample had a reduced bulk remodelling index compared to 0 h. Perhaps it is because although the cell wall is influential, at 0 h the actin cytoskeleton should be heavily involved in cell wall regrowth and this may involve much remodelling activity. Possibly a more appropriate choice of time points would have been 24 h and 48 h, because at 24 h the cell wall is well established, but not yet complete (Alex Martinière and John Runions, personal communication). It is also possible to check viability using a viability assay such as Evans blue which stains damaged or dead cells.

7.4.3 Actin cytoskeleton dynamics: FABD2 vs Lifeact

Plasmolysed plant cells stably-expressing GFP-Lifeact had a reduction in actin cytoskeletal dynamics compared to control cells, which is the same result as found with cells that stably-expressed GFP-FABD2. Similar results were found with GFP-Lifeact and GFP-FABD2 when cells were temperature stressed. This suggests that GFP-FABD2 and GFP-Lifeact generally react the same way.

7.4.4 Golgi body motility as a proxy for actin cytoskeletal dynamics

Golgi body motility was used as a proxy for actin cytoskeletal dynamics and it was found that in plasmolysed cells compared to control cells: average velocity was reduced; average displacement rate was reduced; the meandering index, a measurement of movement complexity (a higher value denotes more directional movement), was found to be reduced; and the number of time frames that Golgi bodies were present in the field of view was increased.

It has been shown that Golgi bodies in *A. thaliana* hypocotyl cells move slower and less directionally on fine actin filaments compared to bundles and their motility in *N. tabacum*

during a transient assay was also reduced when the actin cytoskeleton resembled a dense mesh with fewer bundles (Akkerman *et al.*, 2011, Martinière *et al.*, 2011a).

However, this study found that Golgi body motility was reduced and the Golgi bodies moved in a more confused manner in plasmolysed cells which had previously been found to display a greater degree of actin bundling and a reduced bulk remodelling index. This suggests that not all actin bundles are constructed in the same manner. For example, in mammalian cells stress fibres are antiparallel actin filaments, whilst other actin strands can be comprised of parallel actin filaments (Blanchoin *et al.*, 2014). This type of structure has never been seen in plant cells, however, perhaps during plasmolysis, actin bundles have a slightly different structure and this could be sufficient to affect Golgi body motility.

7.4.4.1 Golgi body motility in protoplasts

Golgi body motility was examined in protoplasts and after 24 h of cell wall regrowth both mock-treated and isoxaben-treated were compared to their respective 0 h. It was found that they had: an increase in the average velocity; an increase in the average displacement rate; an increase in the meandering index; a decrease in the number of time-frames that the Golgi bodies were present in the field of view; and a decrease in the length of the tracks. Additionally, when the 24 h isoxaben-treated cells were compared to the 24 h mock-treated there was a decrease in the average displacement rate and the meandering index of Golgi bodies. A decreased displacement rate means the Golgi bodies have, on average, taken a longer time to move a shorter distance and a decreased meandering index means the Golgi bodies have moved in a more complicated fashion.

Therefore, Golgi bodies were moving more after 24 h of cell wall regrowth; this is probably the reason that they remained in the field of view for a decreased number of time-frames and also why the track length was reduced.

7.4.4.2 Golgi bodies as part of cell wall regeneration

Golgi bodies are an essential part of cell wall production, not just for protein glycosylation, but hemicellulose and pectin manufacture too. Due to this functional connection to the cell wall, any future work should include some tracking of mitochondria or peroxisomes, as they are not so intimately linked to cell wall growth. Taking this into account, it is notable that in protoplasts the actin cytoskeleton remodelled less at 24 h compared to 0 h. Conversely, Golgi bodies moved more after 24 h of cell wall regrowth; this suggests that

perhaps the differences seen in Golgi body motility were due to cell wall regeneration. This is very interesting and deserves some follow up work, although proving a link between localised carbohydrate production and Golgi body motility seems complex. Perhaps the amount of different carbohydrates produced could be measured at different time-points during cell wall regeneration, or perhaps antibodies could be used to localise the different types of carbohydrates in the wall. The respective caveats here are that this does not specifically link to dynamics and that antibodies are sometimes not very specific and may not be able to bind to carbohydrates bound into the cell wall.

7.4.4.3 How Golgi body motility affects cell wall deposition?

Crowell *et al.* (2009) tracked individual insertion events of CSCs into the plasma membrane and found that each one coincided with a Golgi body that had ‘paused’ below that position under the plasma membrane. However, Sampathkumar *et al.* (2013) carried out a larger scale study and showed that although Golgi bodies are involved in approximately two-thirds of insertion events, they do not have to be ‘paused’.

AtKinesin-13A has been shown to localise to Golgi bodies in *A. thaliana*; the mutant of this gene has trichomes with four branches rather than three and the Golgi bodies inside, rather than being loosely distributed, were found in aggregates, although their velocity was recorded as being the same as wild type (Lu *et al.*, 2005). The mutant of another kinesin in *A. thaliana*, Fragile Fiber1, which is important for the delivery of polysaccharides to the cell wall, has been shown to have altered Golgi body structure (Zhu *et al.*, 2015). Although that study did not look at Golgi body movement, kinesins are motor proteins, which suggests that dynamics are part of the function of this protein. These results all imply the importance of Golgi body motility for its function in cell wall production.

7.4.4.4 Organelle analysis as a proxy for actin remodelling

Volocity [PerkinElmer] is a versatile program. For example, it is possible to take all the measurements per time frame for each individual Golgi body. This means that it is possible to monitor how the velocity or meandering index changes between each time frame, so it is possible to detect whether and how often Golgi bodies pause in their tracks, as done by (Crowell *et al.*, 2009). It would also be possible to calculate, for example, velocity variance per Golgi body, so they could be binned and sub-populations defined by specific types of movement could be identified and categorised. Finally, Golgi bodies

show a large variation in the amount and kind of movement; Sparkes *et al.* (2009) used cumulative distribution frequencies coupled with Kolmogorov-Smirnov statistical analysis to compare the distributions, any future work with motility should also try to include this kind of analysis.

In addition to monitoring Golgi body motility, it would also be useful to analyse the mobile behaviour of other cellular components such as peroxisomes, nuclei and chloroplasts, as their mobility is also dependent on the actin cytoskeleton (Gao *et al.*, 2016). This study never looked at the nucleus, but it is possible that nucleus mobility could be used as a proxy for the structure and dynamics of the actin cytoskeleton and its shape is partly determined by the actin cytoskeleton (Kandasamy *et al.*, 2010). Lastly, it would also be interesting to examine cytoplasmic streaming and whether this is affected when the cell wall-plasma membrane interface is disturbed.

7.4.5 The absence of a discernible general stress response

Holweg (2007) showed that organelles had reduced velocity when plant cells were cold-treated and other studies have shown that severe heat treatments can fragment the actin cytoskeleton (Muller *et al.*, 2007, Malerba *et al.*, 2010). In this study no differences in actin cytoskeletal dynamics were found in cold-treated cells compared to mock-treated and while heat-treated cells appeared to have a different network organisation, there was not a consistent change in dynamics of the actin cytoskeleton. It would also be interesting to try a pressure stress as well, perhaps something as simple as extra pressure or a thin wire under the coverslip. However, care would be needed that a pathogen response or damage response were not initiated.

7.4.6 Actin cytoskeleton dynamic alterations vs changes in plasma membrane protein mobility

FRAP on different plasma membrane proteins showed that they were affected differentially when the cell wall was disturbed, either by mutations (the *xt1-xt2* mutant) or with isoxaben; perhaps due to the heterogeneous nature of both the cell wall and the

plasma membrane. It is known in animal cells that the actin cytoskeleton is involved in ‘corralling’ plasma membrane proteins (Gowrishankar *et al.*, 2012). Any changes in plasma membrane protein mobility could hence indicate a disruption in actin cytoskeleton organisation. Indeed, there was a reduction in mean fluorescence intensity of the GFP-FABD2 tagged actin cytoskeleton in isoxaben-treated cells compared to mock-treated, suggesting an alteration in the organisation of the actin cytoskeleton.

The FRAP results are different to the more consistent reduction in actin cytoskeleton dynamics seen with GFP-FABD2 and GFP-Lifeact in plasmolysed cells and GFP-FABD2 in isoxaben-treated cells. Both GFP-FABD2 and GFP-Lifeact mostly seem to label the same network, whereas the four plasma membrane-resident proteins (GFP-LTI6b, MAP-GFP, GFP-PAP and YFP-PI – all with the same localisation pattern) examined all had different membrane associations. If these different associations help define protein mobility, then perhaps this is the reason for their different reactions when the cell wall-plasma membrane interface is perturbed.

7.4.6.1 *Single molecule tracking vs FRAP and actin remodelling*

FRAP and single molecule tracking were discussed in Chapter 6 (see Section 6.5.5, pg. 213). Single molecule tracking showed that different proteins with different membrane associations respond differently when the cell wall-plasma membrane interface is disturbed. This is the same result as was found with FRAP on plasma membrane proteins, but again different to the situation with actin cytoskeleton dynamics.

7.4.6.2 *Single molecule tracking using xxt1-xxt2*

Single molecule tracking in this study was only carried out using 'wild type' background *A. thaliana* lines over-expressing fluorescent proteins. Xiao *et al.* (2016) showed that the microtubule cytoskeleton was disturbed in the *xxt1-xxt2* mutant, so it is a good candidate to investigate the plasma membrane-cell wall interface. It would be very interesting to compare the mobility of individual fluorescent proteins in a 'wild type' background and in the *xxt1-xxt2* mutant (also with cellulose and pectin mutants). It was not possible to do this during this study because low fluorescence of the same tagged protein is required in both the 'wild type' and its respective *xxt1-xxt2* mutant. In addition, it would be very interesting to investigate the actin cytoskeleton in this line, to discover if it is similarly affected

compared to the microtubule cytoskeleton (Xiao *et al.*, 2016) (see Appendix Figure E.1, pg. 299).

7.4.7 Investigation of the cell wall-plasma membrane continuum

Continuing from the data discussed above, another important next step would be to continue characterising the proteins that link the cell wall, plasma membrane and actin cytoskeleton. Martinière *et al.* (2011a) investigated the formin, AtFH1; other formins have been examined, although not necessarily in the context of the cell wall-plasma membrane-actin cytoskeleton continuum. Another family of proteins that are thought to link from the cell wall through the plasma membrane to interact with the actin cytoskeleton is phospholipase D (PLD; although a connection to the cell wall would need to be tested): there are 12 proteins and they are part of one of the pathways that produce phosphatidic acid (PA) (Munnik and Testerink, 2009). PA has been shown, in its capacity as a small signalling molecule, to cause changes in the actin cytoskeleton, while some PLDs have been shown to actually bind actin (Pleskot *et al.*, 2010, Pleskot *et al.*, 2013).

Another facet to the continuum that was not addressed during this study is how much of the influence of the cell wall over the plasma membrane and actin cytoskeleton is via biochemical means, such as linker proteins, like those mentioned above, or potential cell wall integrity sensors, such as Wall-Associated Kinase4 or Theseus1 (Humphrey *et al.*, 2007). The other cause of cell wall influence could be a mechanical effect, as the cell wall physically constrains the protoplasm that is pressed against it by turgor pressure from the vacuole. This mechanical pressure could be somehow sensed by cellular components: for example, in mammalian cells the actin cytoskeleton is involved in sensing tension (Hayakawa *et al.*, 2011).

7.4.7.1 A cell wall-plasma membrane-actin cytoskeleton continuum model

The cell wall-plasma membrane-actin cytoskeleton continuum could be considered as a series of interactions between its constituents (Figure 7.1). The actin cytoskeleton is involved in trafficking macromolecules to the cell wall and in corralling proteins in the plasma membrane (see Section 1.2.1, pg. 6 and Section 1.5.3, pg. 32). The plasma membrane contains proteins that interact with the actin cytoskeleton and proteins that are

bound into the cell wall (see Section 1.1.2, pg. 3). The cell wall interacts with the plasma membrane via cell wall proteins and structures such as the CSCs (see Section 1.3.1.4, pg. 21 and Section 1.3.3). Lastly, this study has shown that the cell wall also has an influence on the dynamics of the actin cytoskeleton (see Section 4.5.2, pg. 132).

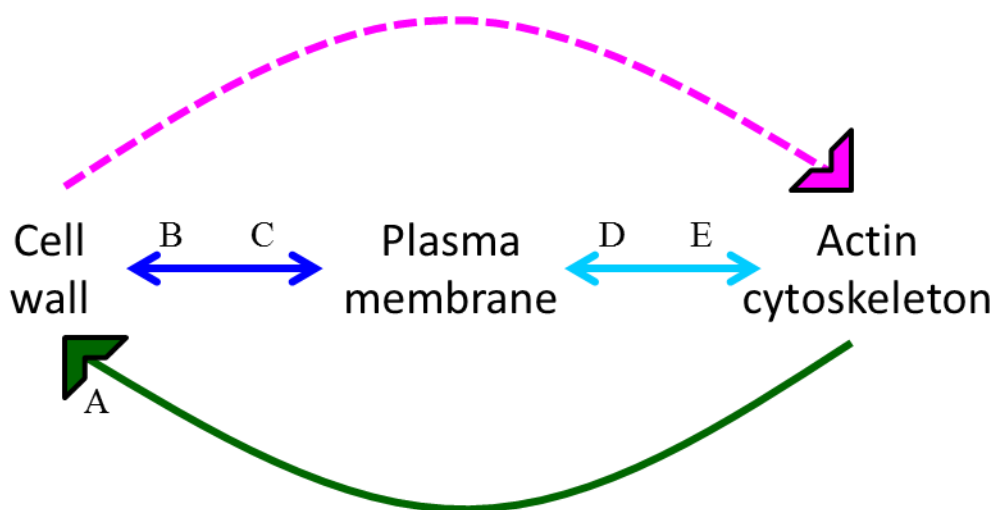


Figure 7.1 Schematic showing the interactions in the continuum.

The direction of the arrow shows what is being influenced. Known interactions are solid lines, whilst the one investigated during the course of this study is a dotted line.

A) Sampathkumar *et al.* (2013); B) Kohorn and Kohorn (2012); C) Martinière *et al.* (2012); D) Gowrishankar *et al.* (2012); and E) Pleskot *et al.* (2010) and Martinière *et al.* (2011a).

7.4.7.2 A cell wall-plasma membrane interface model

This study tried to generate a very general and hypothetical model to describe what happens to the mobility of plasma membrane proteins when the cell wall is disturbed. Three of the four proteins analysed in the *xtt1-xtt2* mutants were relatively more immobile in the mutants, without a change in the rate of recovery. When single molecules of paGFP-LTI6b, pFLS2::FLS2-GFP and pPIN1::PIN1-GFP were tracked in perturbed cell wall conditions, their mobility was more affected when pectin composition of the cell wall was disturbed, compared to mock and cellulose disturbance. This suggests that proteins are differentially affected by carbohydrates and that the association of the protein with the plasma membrane also determines this reaction.

The possibility of mechanical ‘control’ from the cell wall was mentioned above and is probably relevant here: the loss of cell wall integrity affecting proteins differently,

depending on which structural element was compromised. Perhaps those proteins that are tightly bound into the membrane and perhaps have chemical connections with the cell wall are more affected when pectins are removed, whereas proteins that have evolved to be held in place by mechanical constraints (turgor pressure from the vacuole that presses them against the cell wall) are more affected when cellulose, the main structural support in the cell, is removed. Lastly, perhaps those proteins that are very loosely associated with the plasma membrane are corralled by the actin and microtubule cytoskeleton, which itself is affected by perturbations in the cell wall-plasma membrane interface.

7.4.8 The large variation in data reflects a diverse population of cells of the same type

All of the discussions about the actin cytoskeleton are linked by the huge variation in the data analysed. Indeed, one of the most interesting things observed during the course of this study was the propensity for variation in the morphology of the actin cytoskeleton. It is not only possible for two neighbouring cells to have completely different networks, but even for the same cell to contain regions of differently arranged network (see Appendix Figure E.2). Developmentally there may be differences between two neighbouring cells, but in the case of a mature leaf, cell division would have occurred a relatively long time ago. Possibly there are differences between cells in the work-load of the secretory machinery, perhaps different structural functions or unknown stress responses.

Regardless of any investigations into the cause of differences between neighbouring cells, a thorough categorisation should be carried out to determine the amount of variation for each marker in different cell types and at different developmental time points.

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Appendix

Appendix A

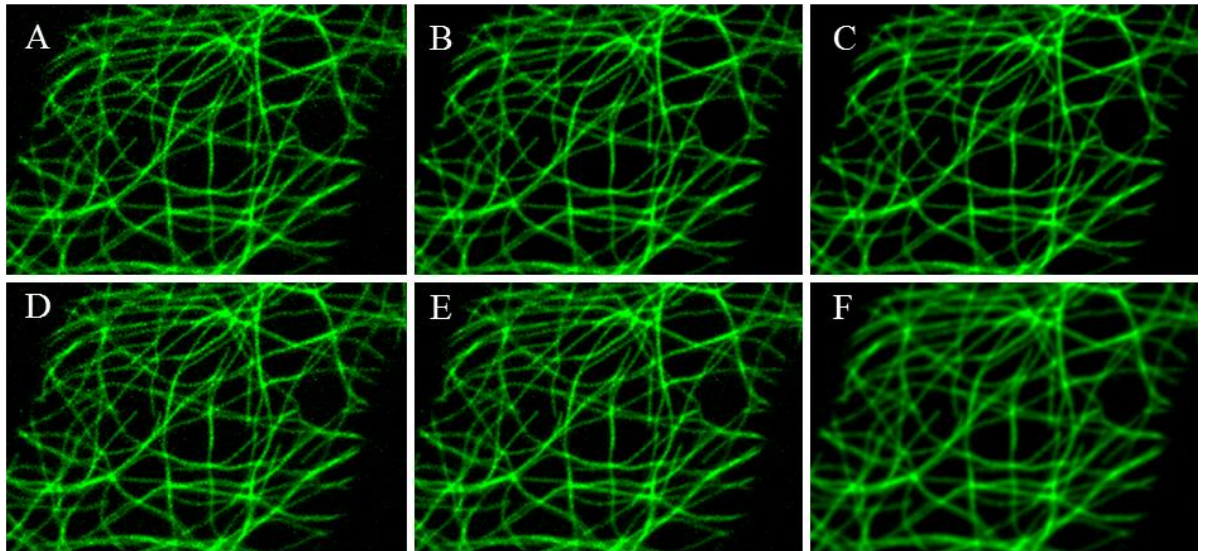


Figure A.1 GFP-MAP4 tagged microtubule cytoskeleton example to show the difference between the Median filter and Gaussian blur in ImageJ (Rasband, 1997-2016).

A) GFP-MAP4 no filter; B) GFP-MAP4 Median filter of 0.2 pixels; C) GFP-MAP4 Median filter of 2.0 pixels. D) GFP-MAP4 no filter; E) GFP-MAP4 Gaussian Blur 0.5 pixels; F) GFP-MAP4 Gaussian Blur 0.5 pixels.

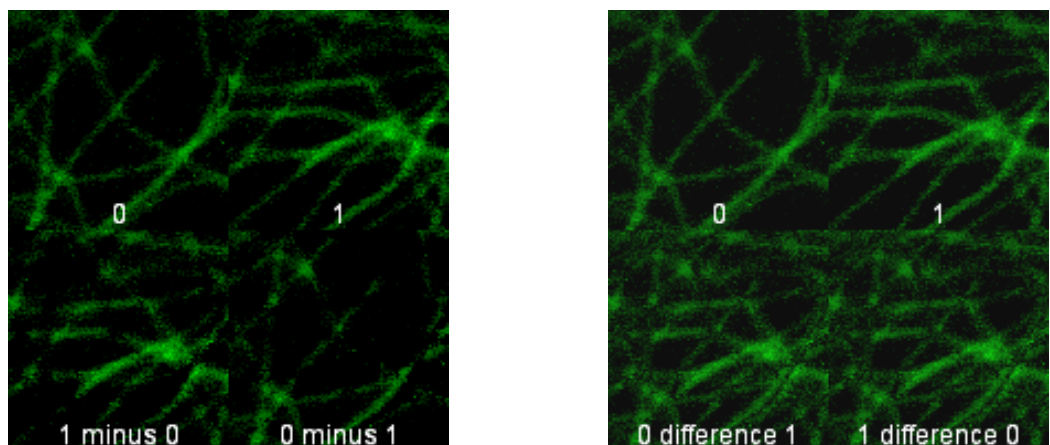


Figure A.2 GFP-MAP4 tagged microtubule cytoskeleton example to show the difference between the ‘Subtract’ and ‘Difference’ functions in Image Calculator in ImageJ (Rasband, 1997-2016).

On the left GFP-MAP4 time-frames (0 and 1) subtracted. On the right GFP-MAP4 difference between time-frames (0 and 1).

A.1 Pairwise Image Subtraction of GFP-FABD2 marked actin cytoskeleton during plasmolysis with different parameters

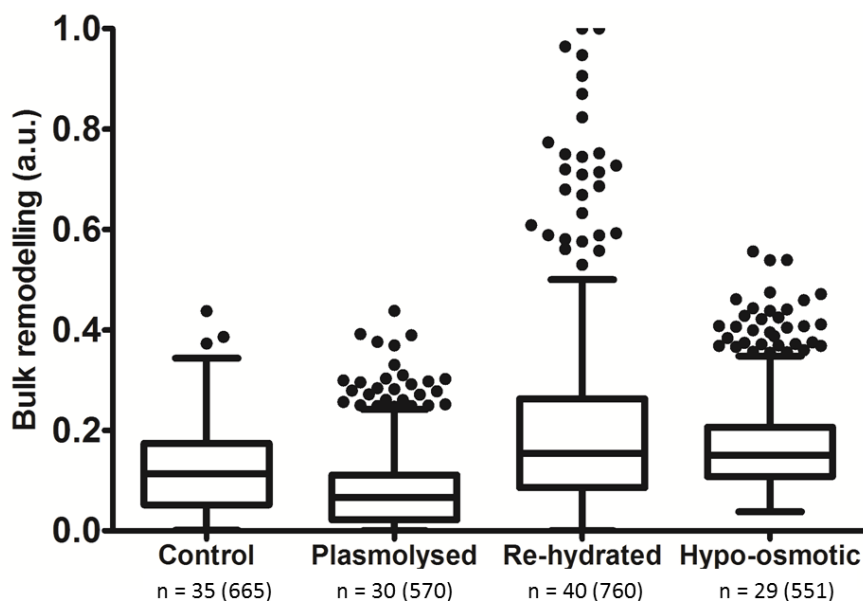


Figure A.3 *Pairwise image subtraction analysis on cells with different treatments expressing GFP-FABD2.*

Leaf epidermal pavement cells from a transgenic line of *A. thaliana* that stably-expressed GFP-FABD2 under four experimental conditions (control, plasmolysed, re-hydrated and hypo-osmotic) and analysed using the *Pairwise image subtraction* method with the parameters: minimum fluorescence intensity = 30 and median filter = 0.2 (see Section 3.4.3.1, pg. 60). The results are detailed in Table A.1.

The GFP-FABD2 dataset from Chapter 4 (see Section 4.4.1.2, pg. 86) was processed again using the parameters: minimum fluorescence intensity of 30 and a median filter of 0.2 (Section 3.4.3.1). The conditions were then compared using an unpaired t-test with Welch's correction (Figure A.3). The bulk remodelling index of the GFP-FABD2 marked actin cytoskeleton calculated was the mean and standard deviation see Table A.1. The bulk remodelling index of the actin cytoskeleton was significantly reduced in the plasmolysed sample compared to the control ($p < 0.0001$), although the variances were not significantly different ($p < 0.1258$). The bulk remodelling index of the actin cytoskeleton was significantly increased in both the re-hydrated and the hypo-osmotic cells compared to the control ($p < 0.0001$). The variances between control and re-hydrated and also between control and hypo-osmotic cells were significantly different ($p < 0.0001$ and $p = 0.0025$, respectively). The mean of the re-hydrated cells was bigger than expected. This occurred because the second image had more fluorescent pixels than the first image, thus giving a ratio value greater than '1'.

Table A.1 Actin cytoskeleton bulk remodelling visualised with GFP-FABD2.

Measured using the *Pairwise image subtraction* method with the parameters: minimum fluorescence intensity = 30 and median filter = 0.2 (see Section 3.4.3.1).

	Control	Plasmolysed	Re-hydrated	Hypo-osmotic
Number of values	665	570	760	551
	0.001217	0.0000	0.0000	0.03784
Minimum	0.05149	0.02198	0.08613	0.1083
25% Percentile	0.1135	0.06638	0.1540	0.1507
Median	0.1743	0.1108	0.2630	0.2060
75% Percentile	0.4377	0.4380	1.833	0.5561
Maximum	0.1186	0.08137	0.1970	0.1700
Mean	0.07866	0.07393	0.1837	0.08894
Std. Deviation	0.003050	0.003096	0.006665	0.003789
Std. Error	0.1126	0.07529	0.1839	0.1625
Lower 95% CI	0.1246	0.08745	0.2101	0.1774
Upper 95% CI	665	570	760	551

The GFP-FABD2 dataset from Chapter 4 (see Section 4.4.1.2) was processed again using the parameters: minimum fluorescence intensity of 1 and a Gaussian blur of 0.5 (Section 3.4.3.1). The conditions were then compared using an unpaired t-test with Welch's correction (Figure A.4). The bulk remodelling index of the GFP-FABD2 marked actin cytoskeleton was calculated as the mean and standard deviation, see Table 8.2. The bulk remodelling of the actin cytoskeleton was significantly reduced in the plasmolysed sample, compared to the control ($p < 0.0001$) and the variances were significantly different ($p < 0.02$). The bulk remodelling index of the actin cytoskeleton was significantly increased in the re-hydrated sample compared to the control ($p < 0.0001$) and the variances were significantly different ($p < 0.0001$). There was no difference between the control and the hypo-osmotic sample ($p = 0.1698$), although the variances were significantly different ($p < 0.0001$).

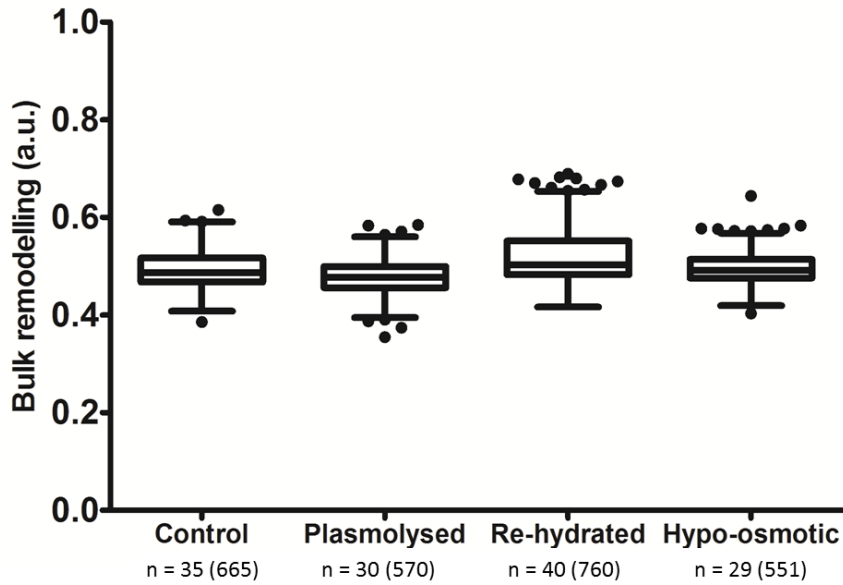


Figure A.4 *Pairwise image subtraction* analysis on cells with different treatments expressing GFP-FABD2.

Leaf epidermal pavement cells from a transgenic line of *A. thaliana* stably-expressed GFP-FABD2 under four experimental conditions and analysed using the *Pairwise image subtraction* method with the parameters: minimum fluorescence intensity = 1 and Gaussian blur = 0.5 pixels (see Section 3.4.3.1). The results are detailed in Table A.2.

Table A.2 Actin cytoskeleton bulk remodelling visualised with GFP-FABD2.

Measured using the *Pairwise image subtraction* method with the parameters: minimum fluorescence intensity = 1 and Gaussian blur = 0.5 pixels (see Section 3.4.3.1).

	Control	Plasmolysed	Re-hydrated	Hypo-osmotic
Number of values	665	570	760	551
Minimum	0.3859	0.3548	0.4164	0.4034
25% Percentile	0.4676	0.4559	0.4828	0.4759
Median	0.4866	0.4770	0.5029	0.4915
75% Percentile	0.5169	0.4991	0.5515	0.5138
Maximum	0.6152	0.5846	0.6891	0.6439
Mean	0.4925	0.4774	0.5185	0.4951
Std. Deviation	0.03664	0.03350	0.04955	0.02965
Std. Error	0.001421	0.001403	0.001798	0.001263
	0.4897	0.4747	0.5150	0.4927
Lower 95% CI	0.4953	0.4802	0.5220	0.4976
Upper 95% CI	665	570	760	551

A.2 Cumulative fluorescence intensity analysis with GFP-FABD2

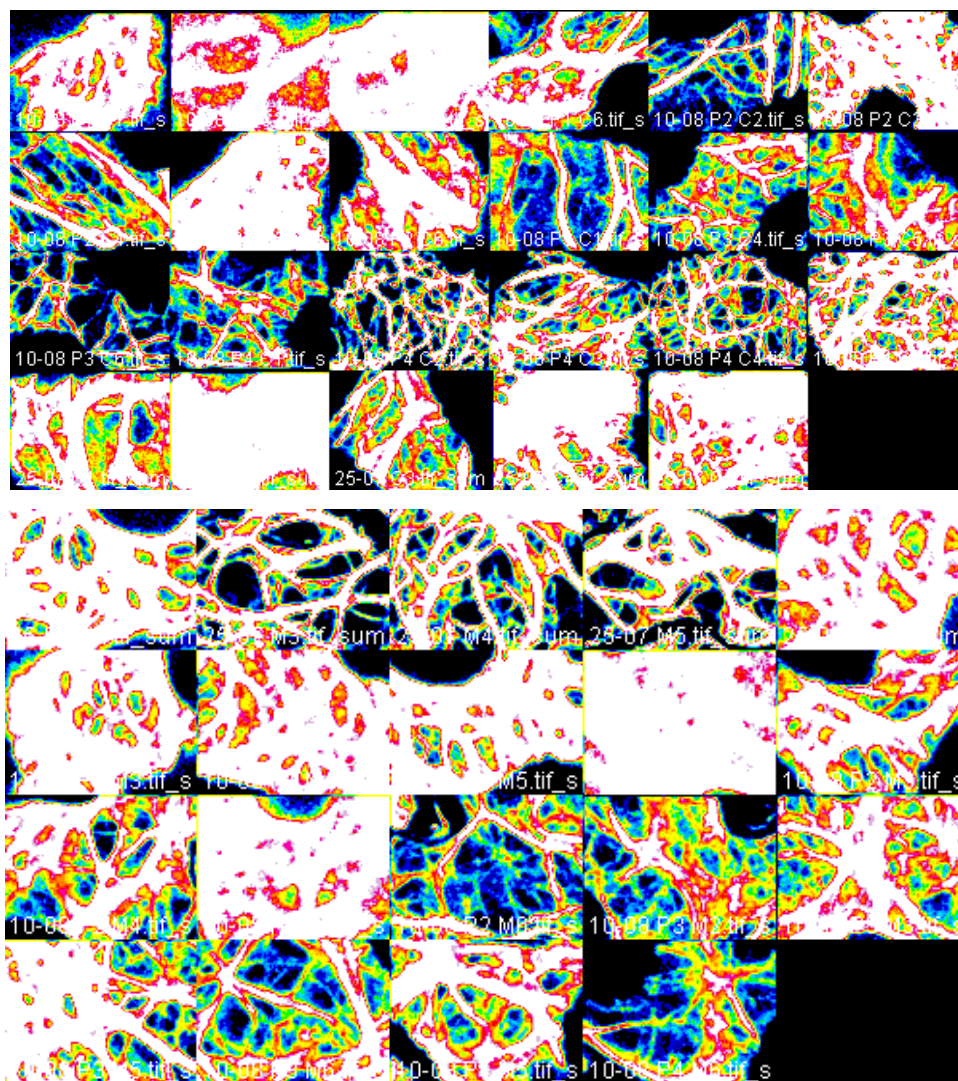


Figure A.5 Visual results from the *Cumulative fluorescence intensity* analysis on cells with different treatments stably-expressing GFP-FABD2.

Heat map shows the fluorescence intensity value per pixel, where white represents stationary filaments and darker colours represent lots of movement.

Top panel of 23 different control cells that had no treatment; lower panel of 19 cells plasmolysed in 0.6 M mannitol for 30 min. These heat maps were constructed as described in Section 3.4.3.3, pg. 63.

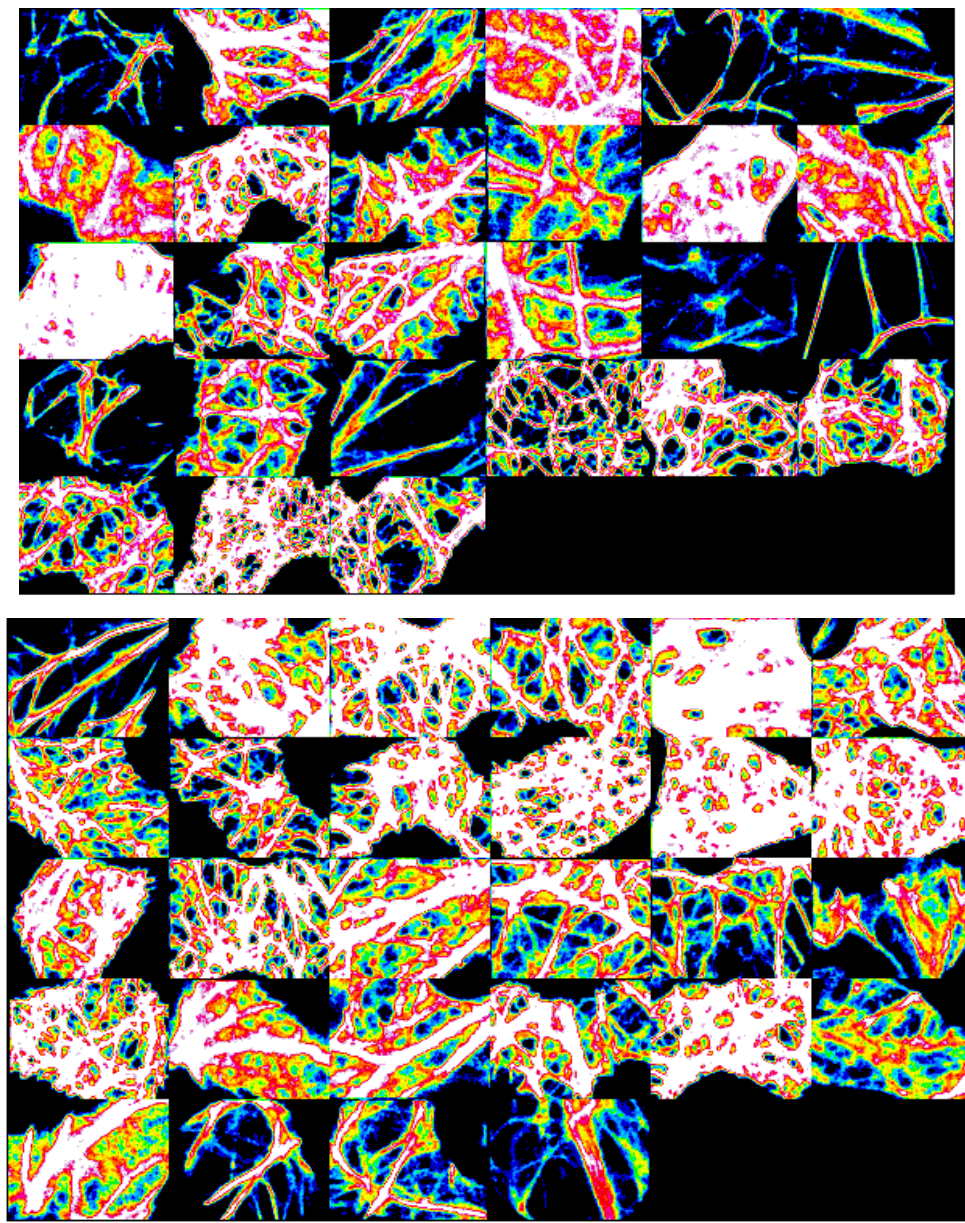


Figure A.6 Visual results from the *Cumulative fluorescence intensity* analysis on cells with different treatments stably-expressing GFP-FABD2.

Heat map shows the fluorescence intensity value per pixel, where white represents stationary filaments and darker colours represent lots of movement.

Top panel of 27 different re-hydrated cells plasmolysed in 0.6 M mannitol for 30 min and then incubated in dH₂O for a further 30 min; lower panel of 28 cells incubated in dH₂O for a further 30 min. These heat maps were constructed as described in Section 3.4.3.3.

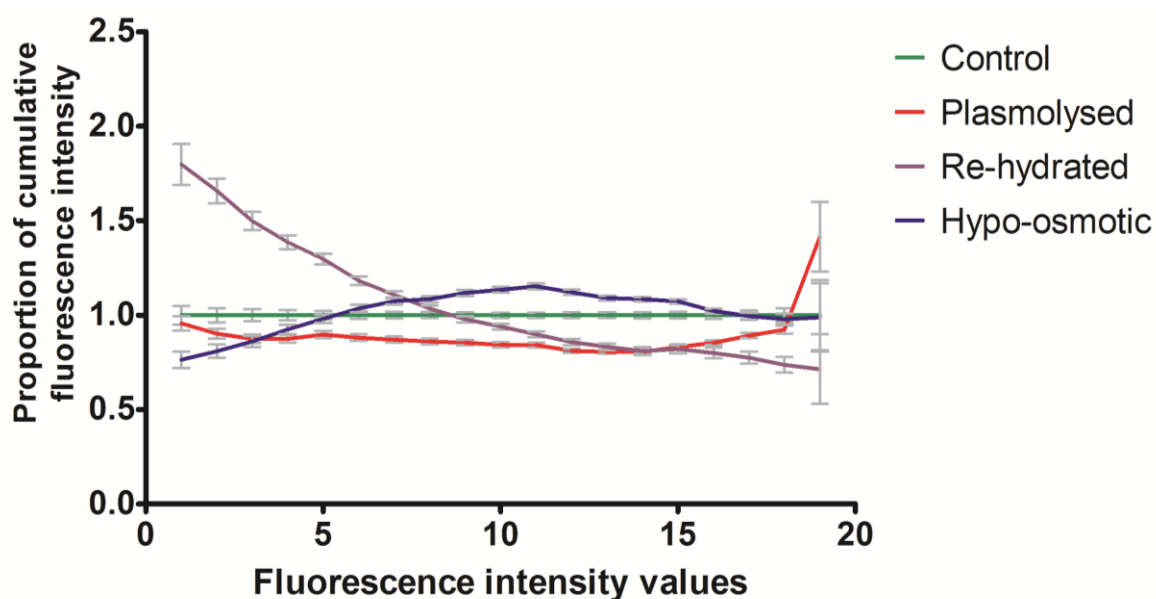


Figure A.7 Results from *Cumulative fluorescence intensity* analysis of cells expressing GFP-FABD2 with different treatments.

Bulk remodelling of the actin cytoskeleton marked with GFP-FABD2 under the four experimental conditions: control in green with values normalised to '1', plasmolysed in red, re-hydrated in purple and hypo-osmotic in blue. This graph was constructed using the analysis from the *Cumulative fluorescence intensity* method, the error bars are standard deviation. All conditions are expressed as a ratio compared to the control, hence the control itself is always one.

Cells used in this analysis are shown in Figure A.5 and Figure A.6.

Appendix B

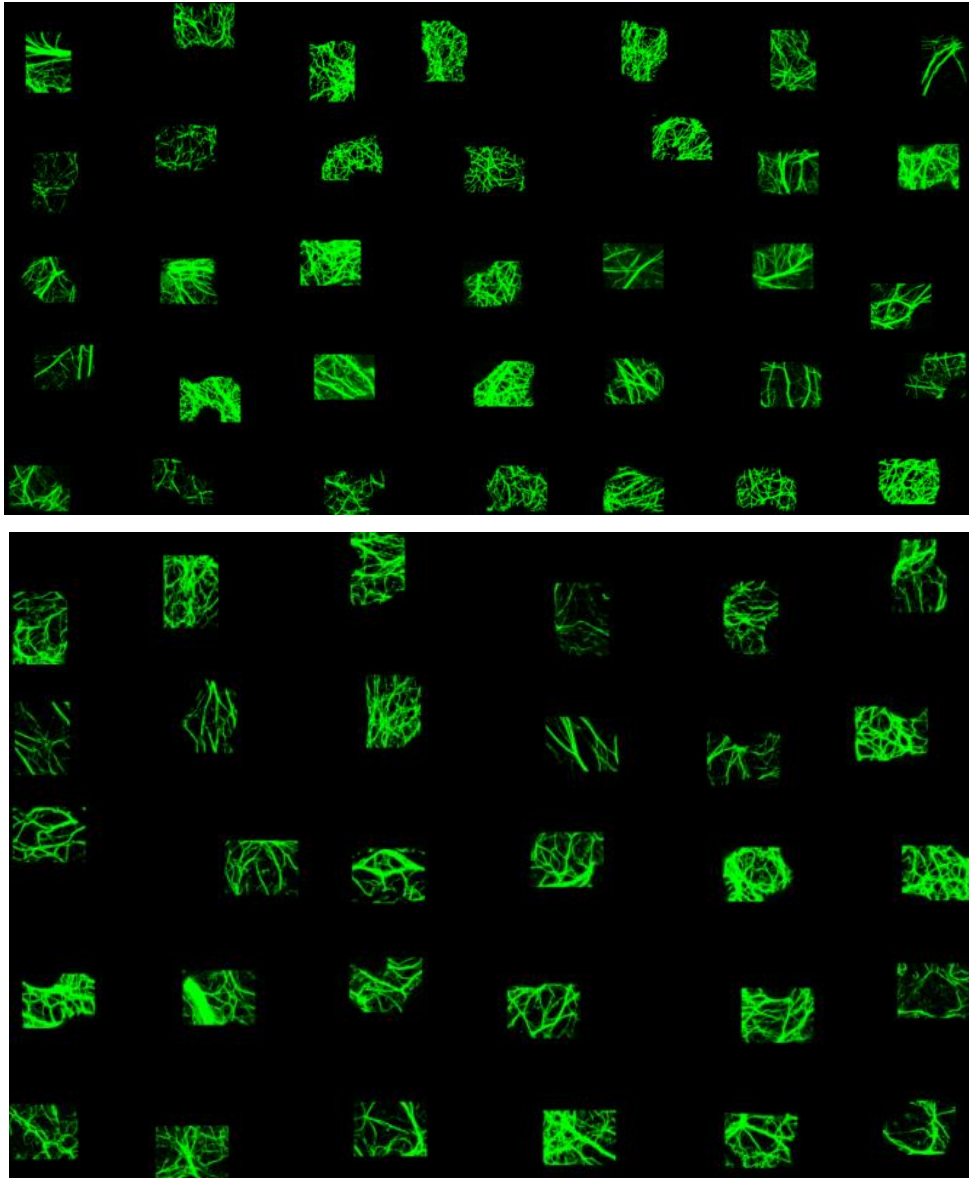


Figure B.1 Leaf epidermal pavement cells with different treatments expressing GFP-FABD2, only the maximum projection of the first Z-stack from each time-series analysed in Chapter 4 is shown here.

Top panel of 35 different control cells that had no treatment; lower panel of 30 cells plasmolysed in 0.6 M mannitol for 30 min.

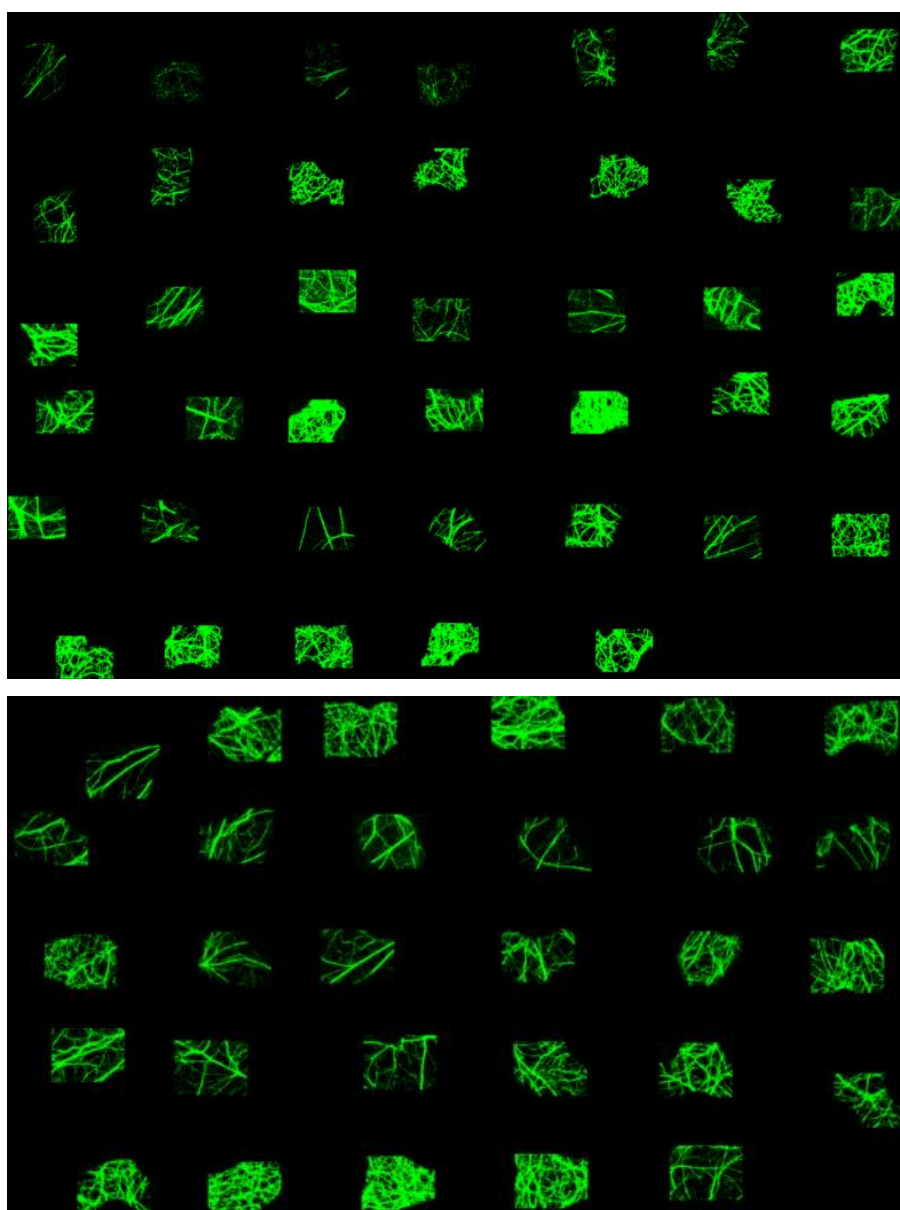


Figure B.2 Leaf epidermal pavement cells with different treatments expressing GFP-FABD2, only the maximum projection of the first Z-stack from each time-series analysed in Chapter 4 is shown here.

Top panel of 40 different re-hydrated cells plasmolysed in 0.6 M mannitol for 30 min and then incubated in dH₂O for a further 30 min; lower panel of 33 cells incubated in dH₂O for a further 30 min.

Table B.1 Actin cytoskeleton bulk remodelling visualised with GFP-FABD2.

Measured using the *Pairwise image subtraction* method (see Section 4.4.1.2, pg. 86).

	Control	Plasmolysed	Re-hydrated	Hypo-osmotic
Number of values	665	570	760	551
Minimum	0.4553	0.4060	0.4655	0.4668
25% Percentile	0.5154	0.4979	0.5327	0.5186
Median	0.5446	0.5208	0.5735	0.5493
75% Percentile	0.5880	0.5501	0.6656	0.5860
Maximum	0.7718	0.6833	0.9887	0.6996
Mean	0.5558	0.5273	0.6013	0.5558
Std. Deviation	0.05486	0.04412	0.08848	0.04867
Std. Error	0.002127	0.001848	0.003209	0.002074
Lower 95% CI	0.5516	0.5236	0.5950	0.5517
Upper 95% CI	0.5600	0.5309	0.6076	0.5598

Table B.2 Actin cytoskeleton bulk remodelling visualised with GFP-FABD2.

Measured using the *Pairwise image subtraction* measuring mean fluorescence intensity method (see Section 4.4.1.3).

	Control	Plasmolysed	Re-hydrated	Hypo-osmotic
Number of values	684	589	760	551
Minimum	0.1219	0.1104	0.1549	0.2180
25% Percentile	0.2167	0.1719	0.2405	0.3028
Median	0.2870	0.2148	0.3240	0.3398
75% Percentile	0.3929	0.2913	0.4296	0.4002
Maximum	0.6082	0.5323	0.7974	0.6240
Mean	0.3132	0.2423	0.3491	0.3558
Std. Deviation	0.1191	0.09364	0.1459	0.08073
Std. Error	0.004552	0.003859	0.005293	0.003439
Lower 95% CI	0.3043	0.2348	0.3387	0.3490
Upper 95% CI	0.3222	0.2499	0.3595	0.3625

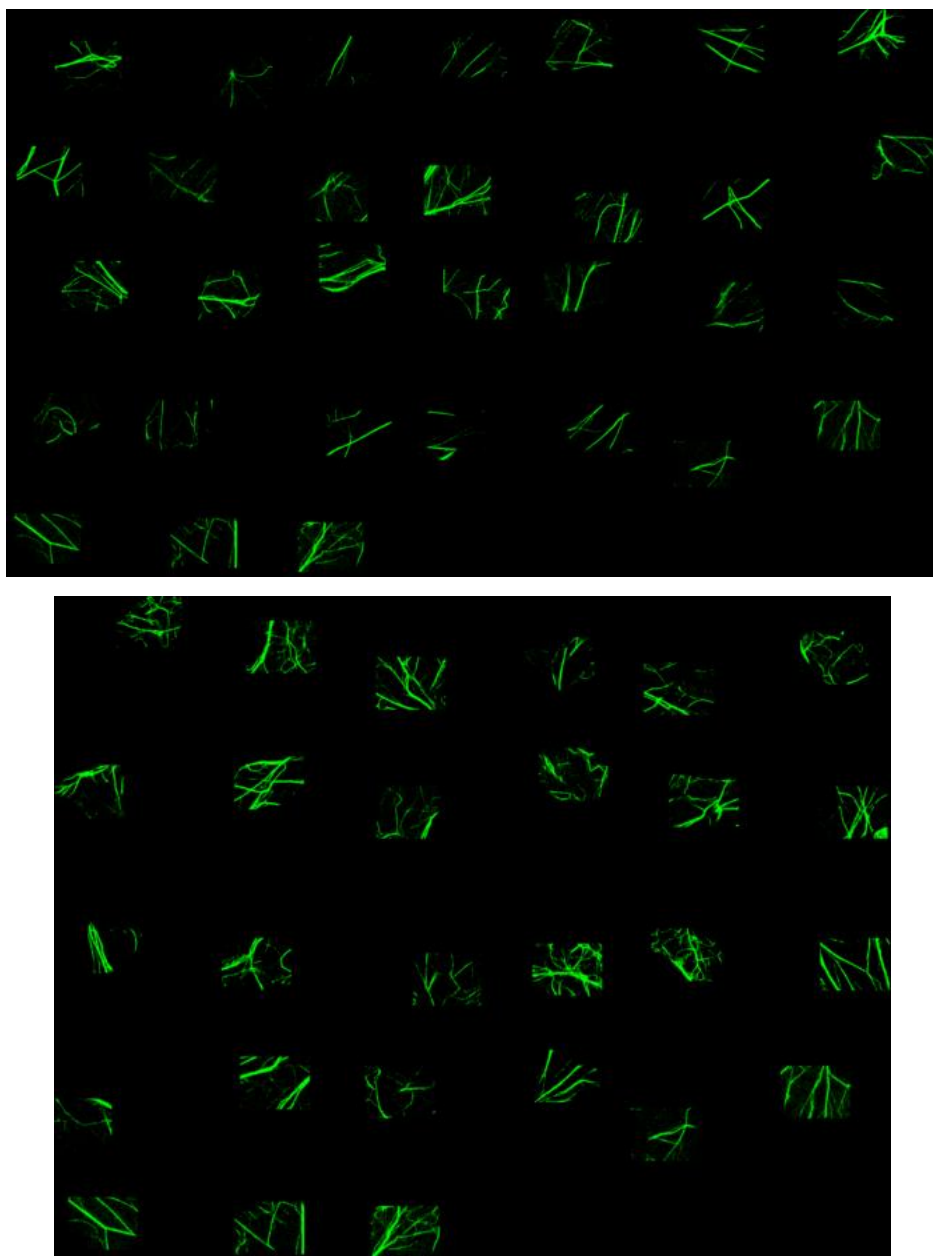


Figure B.3 Leaf epidermal pavement cells with different treatments expressing GFP-Lifeact, only the maximum projection of the first Z-stack from each time-series analysed in Chapter 4 is shown here.

Top panel of 31 different control cells that had no treatment; lower panel of 27 cells plasmolysed in 0.6 M mannitol for 30 min.

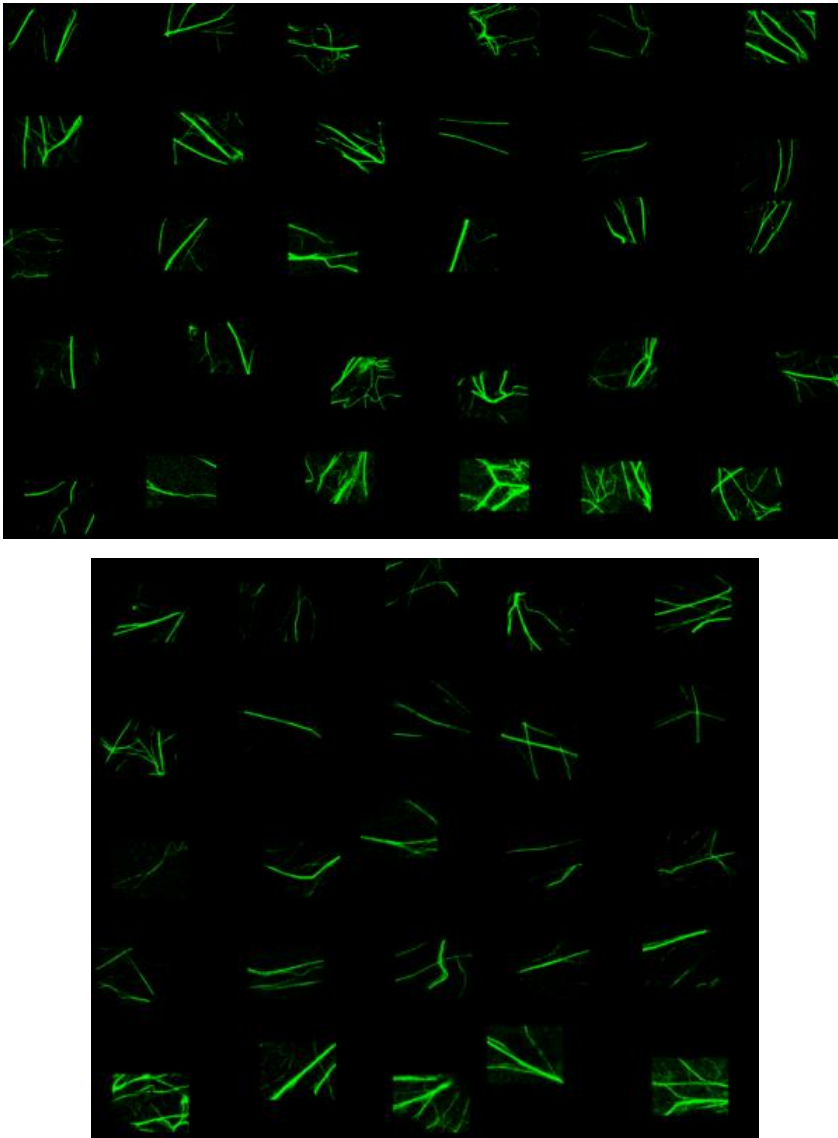


Figure B.4 Leaf epidermal pavement cells with different treatments expressing GFP-Lifeact, only the maximum projection of the first Z-stack from each time-series analysed in Chapter 4 is shown here.

Top panel of 30 different re-hydrated cells plasmolysed in 0.6 M mannitol for 30 min and then incubated in dH₂O for a further 30 min; lower panel of 25 cells incubated in dH₂O for a further 30 min.

Table B.3 Actin cytoskeleton bulk remodelling in cells with different treatments visualised with GFP-Lifeact.

Measured using the *Cumulative fluorescence intensity* method; values as mean $\pm$ standard deviation (see Section 3.4.3.3).

Fluorescence Intensity	Control	Plasmolysed	Re-hydrated	Hypo-osmotic
1	0.2695 $\pm$ 0.09911	0.2063 $\pm$ 0.07478	0.2007 $\pm$ 0.11160	0.2717 $\pm$ 0.14060
2	0.1647 $\pm$ 0.03782	0.1360 $\pm$ 0.03988	0.1338 $\pm$ 0.05890	0.1536 $\pm$ 0.05945
3	0.1209 $\pm$ 0.02862	0.1015 $\pm$ 0.02916	0.0994 $\pm$ 0.03314	0.1028 $\pm$ 0.03930
4	0.0880 $\pm$ 0.02181	0.0825 $\pm$ 0.02142	0.0794 $\pm$ 0.02296	0.0746 $\pm$ 0.02662
5	0.0683 $\pm$ 0.01434	0.0686 $\pm$ 0.01458	0.0699 $\pm$ 0.02128	0.0594 $\pm$ 0.02228
6	0.0553 $\pm$ 0.01502	0.0592 $\pm$ 0.01094	0.0625 $\pm$ 0.02068	0.0505 $\pm$ 0.01718
7	0.0472 $\pm$ 0.01579	0.0490 $\pm$ 0.00780	0.0531 $\pm$ 0.01877	0.0430 $\pm$ 0.01610
8	0.0401 $\pm$ 0.01590	0.0421 $\pm$ 0.00948	0.0463 $\pm$ 0.01913	0.0392 $\pm$ 0.01698
9	0.0319 $\pm$ 0.01356	0.0366 $\pm$ 0.01204	0.0410 $\pm$ 0.01973	0.0341 $\pm$ 0.01890
10	0.0256 $\pm$ 0.01214	0.0320 $\pm$ 0.01361	0.0368 $\pm$ 0.02011	0.0294 $\pm$ 0.02114
11	0.0207 $\pm$ 0.01177	0.0289 $\pm$ 0.01424	0.0316 $\pm$ 0.02151	0.0256 $\pm$ 0.02195
12	0.0166 $\pm$ 0.01060	0.0248 $\pm$ 0.01408	0.0279 $\pm$ 0.02255	0.0230 $\pm$ 0.02210
13	0.0136 $\pm$ 0.01020	0.0212 $\pm$ 0.01421	0.0240 $\pm$ 0.02248	0.0198 $\pm$ 0.02160
14	0.0100 $\pm$ 0.00820	0.0193 $\pm$ 0.01404	0.0207 $\pm$ 0.02314	0.0163 $\pm$ 0.01943
15	0.0074 $\pm$ 0.00759	0.0167 $\pm$ 0.01318	0.0178 $\pm$ 0.02225	0.0133 $\pm$ 0.01718
16	0.0058 $\pm$ 0.00719	0.0157 $\pm$ 0.01365	0.0156 $\pm$ 0.02079	0.0111 $\pm$ 0.01505
17	0.0044 $\pm$ 0.00674	0.0151 $\pm$ 0.01403	0.0135 $\pm$ 0.01718	0.0096 $\pm$ 0.01404
18	0.0042 $\pm$ 0.00750	0.0150 $\pm$ 0.01367	0.0120 $\pm$ 0.01500	0.0092 $\pm$ 0.01472
19	0.0059 $\pm$ 0.01500	0.0298 $\pm$ 0.03793	0.0138 $\pm$ 0.01871	0.0140 $\pm$ 0.02665

Table B.4 Actin cytoskeleton bulk remodelling extrapolated via the proxy of Golgi body track velocity ($\mu\text{m/s}$).

Visualised with ST-GFP and measured using Volocity (see Section 4.4.1.6, pg. 90).

	Control	Plasmolysed	Re-hydrated	Hypo-osmotic
Number of values	910	848	774	626
Minimum	0.1150	0.0845	0.1330	0.1600
25% Percentile	0.3813	0.2303	0.4020	0.4170
Median	0.5830	0.3880	0.6180	0.6365
75% Percentile	0.8483	0.6568	0.9330	0.9363
Maximum	3.680	3.790	3.870	3.510
Mean	0.6856	0.5201	0.7651	0.7427
Std. Deviation	0.4485	0.4387	0.5432	0.4529
Std. Error	0.01487	0.01506	0.01952	0.01810
Lower 95% CI	0.6564	0.4905	0.7268	0.7071
Upper 95% CI	0.7148	0.5497	0.8035	0.7782

Table B.5 Actin cytoskeleton bulk remodelling extrapolated via the proxy of Golgi body displacement rate ($\mu\text{m/s}$).

Visualised with ST-GFP and measured using Volocity (see Section 4.4.1.6).

	Control	Plasmolysed	Re-hydrated	Hypo-osmotic
Number of values	910	848	774	626
Minimum	0.00126	0.000813	0.000249	0.0
25% Percentile	0.05358	0.0227	0.06243	0.06208
Median	0.1400	0.06745	0.1590	0.1710
75% Percentile	0.3580	0.2038	0.3793	0.3593
Maximum	3.660	3.780	3.590	3.500
Mean	0.2839	0.2090	0.3431	0.3130
Std. Deviation	0.3927	0.3999	0.4915	0.4274
Std. Error	0.01302	0.01373	0.01767	0.01708
Lower 95% CI	0.2583	0.1820	0.3084	0.2794
Upper 95% CI	0.3094	0.2359	0.3777	0.3465

Table B.6 Actin cytoskeleton bulk remodelling extrapolated via the proxy of Golgi body meandering indices.

Visualised with ST-GFP and measured using Volocity (see Section 4.4.1.6).

	Control	Plasmolysed	Re-hydrated	Hypo-osmotic
Number of values	910	848	774	626
Minimum	0.00391	0.00529	0.00116	0.0
25% Percentile	0.1230	0.07265	0.1338	0.1235
Median	0.2560	0.1740	0.2805	0.2665
75% Percentile	0.5048	0.3985	0.5210	0.4855
Maximum	0.9950	0.9990	0.9940	0.9970
Mean	0.3305	0.2762	0.3517	0.3357
Std. Deviation	0.2529	0.2608	0.2651	0.2587
Std. Error	0.008385	0.008956	0.009529	0.01034
Lower 95% CI	0.3141	0.2586	0.3330	0.3154
Upper 95% CI	0.3470	0.2937	0.3704	0.3560

Table B.7 Actin cytoskeleton bulk remodelling extrapolated via the proxy of Golgi body time span, that is, how long they are present in the field of view.

Visualised with ST-GFP and measured using Volocity (see Section 4.4.1.6).

	Control	Plasmolysed	Re-hydrated	Hypo-osmotic
Number of values	910	848	774	626
Minimum	4.000	6.000	5.000	6.000
25% Percentile	17.00	30.00	21.00	22.00
Median	30.00	56.50	35.00	35.00
75% Percentile	52.00	105.8	65.00	65.00
Maximum	200.0	200.0	200.0	200.0
Mean	45.52	75.54	52.59	53.73
Std. Deviation	44.14	58.18	47.17	47.14
Std. Error	1.463	1.998	1.696	1.884
Lower 95% CI	42.64	71.62	49.26	50.03
Upper 95% CI	48.39	79.46	55.91	57.43

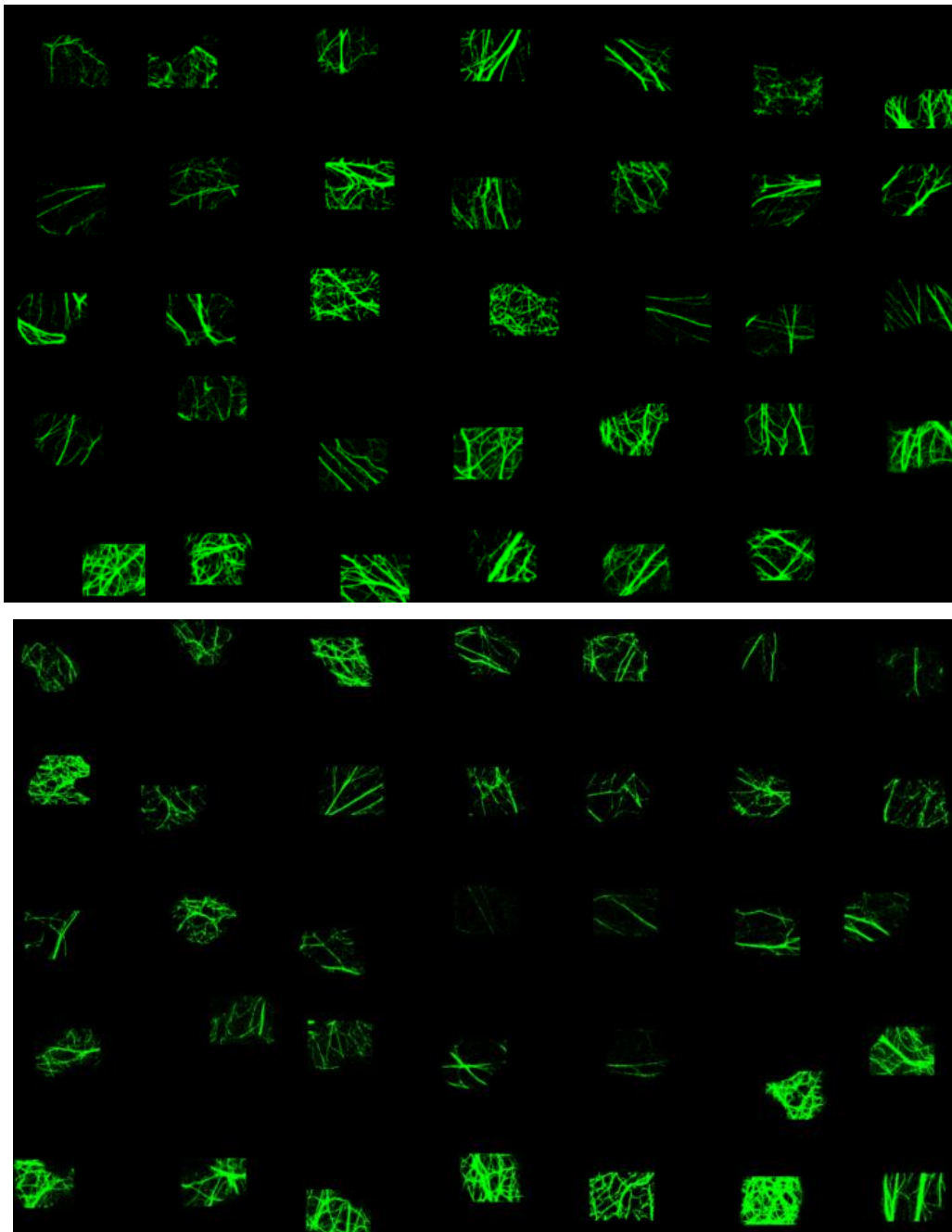


Figure B.5 Leaf epidermal pavement cells with different treatments expressing GFP-FABD2, only the maximum projection of the first Z-stack from each time-series analysed in Chapter 4 is shown here.

Top panel of 34 different control cells incubated in $\frac{1}{2}$ MS at room temperature for 30 min; lower panel of 35 cells incubated in $\frac{1}{2}$ MS at room temperature for 1 h.

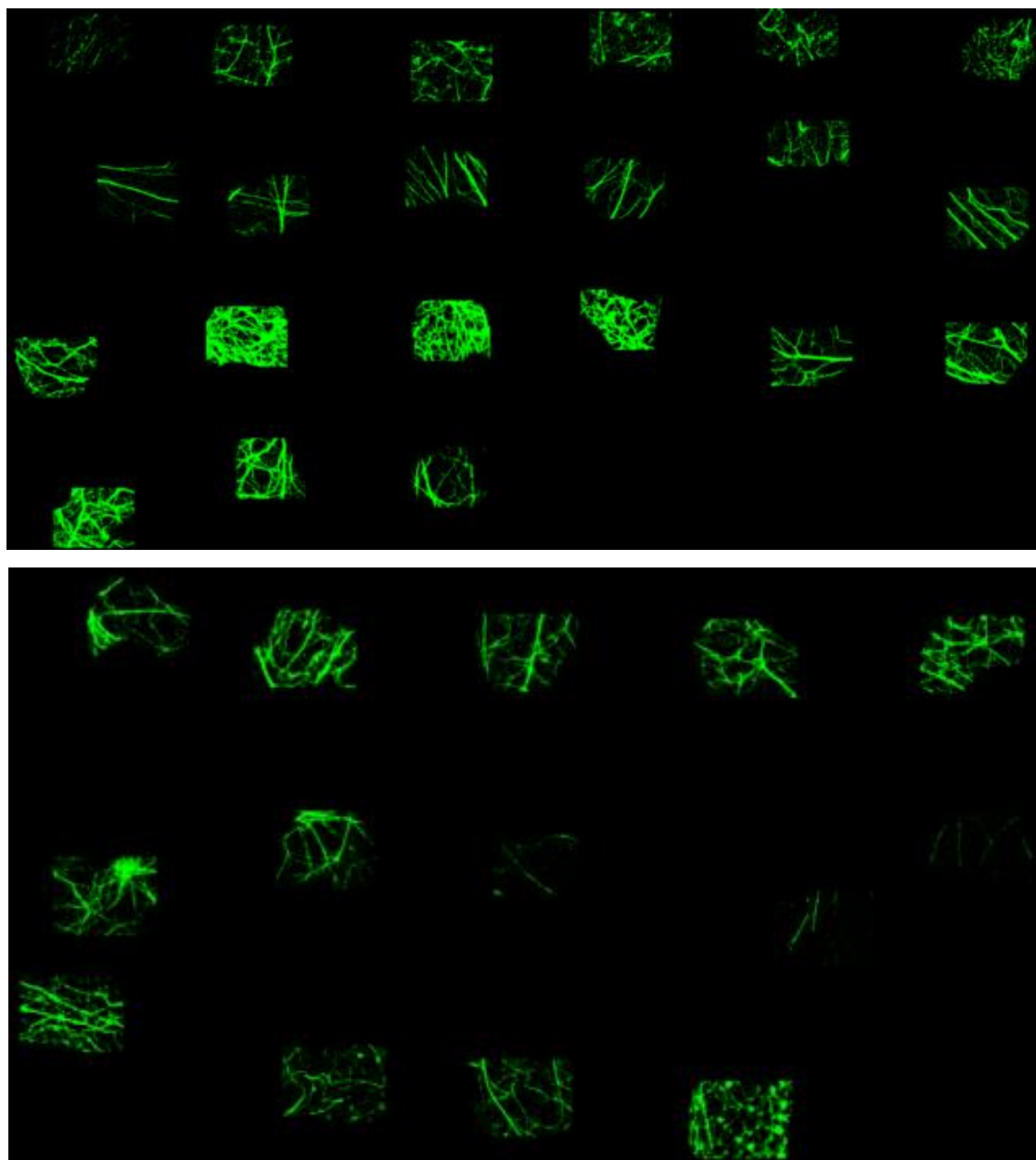


Figure B.6 Leaf epidermal pavement cells with different treatments expressing GFP-FABD2, only the maximum projection of the first Z-stack from each time-series analysed in Chapter 4 is shown here.

Top panel of 21 different control cells incubated in $\frac{1}{2}$ MS at 37°C for 30 min; lower panel of 14 cells incubated in $\frac{1}{2}$ MS at 37°C for 1 h.

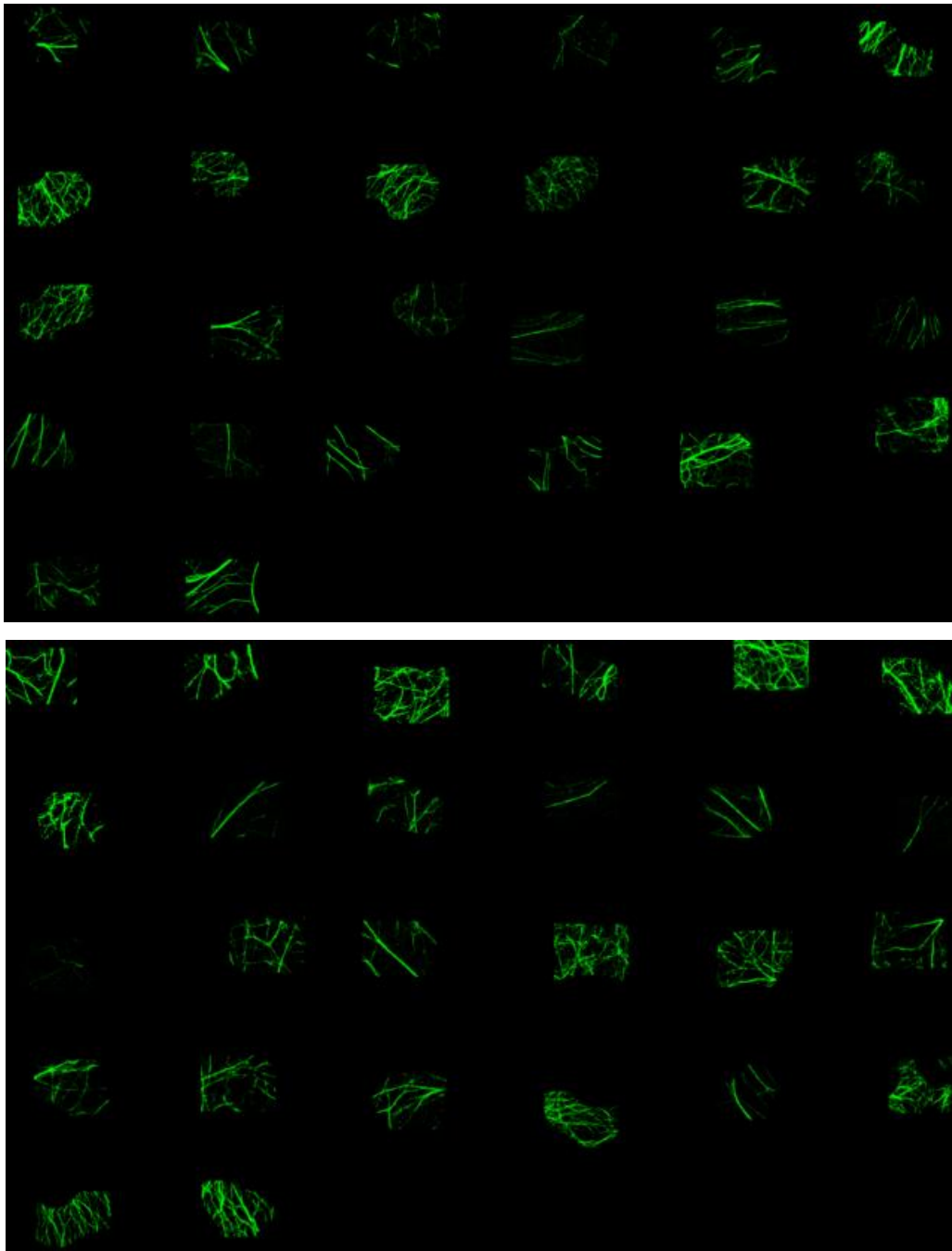


Figure B.7 Leaf epidermal pavement cells with different treatments expressing GFP-FABD2, only the maximum projection of the first Z-stack from each time-series analysed in Chapter 4 is shown here.

Top panel of 26 different control cells incubated in $\frac{1}{2}$ MS at 4°C for 30 min; lower panel of 26 cells incubated in $\frac{1}{2}$ MS at 4°C for one h.

Table B.8 Actin cytoskeleton bulk remodelling cells expressing GFP-FABD2 that have been temperature stressed.Measured using the *Pairwise image subtraction* method (see Section 4.4.2.2, pg. 93).

	Mock 30 min	Mock 1 h	37°C 30 min	37°C 1 h	4°C 30 min	4°C 1 h
Number of values	646	665	418	266	494	494
Minimum	0.4825	0.4192	0.4931	0.4373	0.5067	0.4663
25% Percentile	0.5811	0.5917	0.5815	0.6091	0.5963	0.5893
Median	0.6241	0.6487	0.6416	0.6377	0.6464	0.6498
75% Percentile	0.6641	0.7038	0.6781	0.6819	0.6968	0.6921
Maximum	1.037	1.364	1.051	1.119	0.9145	1.280
Mean	0.6295	0.6548	0.6382	0.6595	0.6498	0.6509
Std. Deviation	0.07563	0.08943	0.07369	0.09044	0.07553	0.09647
Std. Error	0.002976	0.003468	0.003604	0.005545	0.003398	0.004340
Lower 95% CI	0.6236	0.6480	0.6312	0.6486	0.6431	0.6424
Upper 95% CI	0.6353	0.6616	0.6453	0.6704	0.6565	0.6594

Table B.9 Actin cytoskeleton bulk remodelling cells expressing GFP-FABD2 that have been temperature stressed.Measured using the *Pairwise image subtraction* measuring mean fluorescence intensity method (see Section 4.4.2.3).

	Mock 30 min	Mock 1 h	37°C 30 min	37°C 1 h	4°C 30 min	4°C 1 h
Number of values	646	665	437	266	494	494
Minimum	0.2220	0.1988	0.2277	0.3237	0.2307	0.2876
25% Percentile	0.3657	0.4243	0.4202	0.4561	0.4061	0.4228
Median	0.4687	0.5558	0.5223	0.4935	0.5144	0.5281
75% Percentile	0.5514	0.6430	0.6203	0.6578	0.6102	0.5998
Maximum	0.8553	0.9575	2.140	0.9212	0.8219	0.9759
Mean	0.4728	0.5409	0.5313	0.5455	0.5069	0.5278
Std. Deviation	0.1291	0.1606	0.1660	0.1478	0.1345	0.1452
Std. Error	0.005080	0.006230	0.007943	0.009061	0.006053	0.006531
Lower 95% CI	0.4628	0.5286	0.5156	0.5277	0.4950	0.5150
Upper 95% CI	0.4828	0.5531	0.5469	0.5634	0.5188	0.5406

Table B.10 Actin cytoskeleton remodelling in temperature stressed cells expressing GFP-Lifeact (see Section 4.4.2.5).Measured using *Cumulative fluorescence intensity*; values as mean $\pm$ standard deviation.

Fluorescence Intensity	Mock 30 min	Mock 1 h	37°C 30 min	37°C 1 h	4°C 30 min	4°C 1 h
1	0.2884 $\pm$ 0.1348	0.2929 $\pm$ 0.0830	0.3041 $\pm$ 0.0974	0.2783 $\pm$ 0.1079	0.2882 $\pm$ 0.0703	0.3337 $\pm$ 0.1031
2	0.1575 $\pm$ 0.0456	0.1784 $\pm$ 0.0369	0.1583 $\pm$ 0.0481	0.1606 $\pm$ 0.0353	0.1704 $\pm$ 0.0302	0.1888 $\pm$ 0.0413
3	0.1102 $\pm$ 0.0275	0.1227 $\pm$ 0.0237	0.1048 $\pm$ 0.0251	0.1122 $\pm$ 0.0223	0.1197 $\pm$ 0.0268	0.1267 $\pm$ 0.0271
4	0.0862 $\pm$ 0.0222	0.0875 $\pm$ 0.0164	0.0755 $\pm$ 0.0205	0.0839 $\pm$ 0.0173	0.0882 $\pm$ 0.0190	0.0866 $\pm$ 0.0178
5	0.0693 $\pm$ 0.0201	0.0659 $\pm$ 0.0164	0.0566 $\pm$ 0.0155	0.0658 $\pm$ 0.0153	0.0670 $\pm$ 0.0147	0.0642 $\pm$ 0.0194
6	0.0543 $\pm$ 0.0202	0.0501 $\pm$ 0.0157	0.0460 $\pm$ 0.0124	0.0521 $\pm$ 0.0156	0.0533 $\pm$ 0.0116	0.0489 $\pm$ 0.0211
7	0.0433 $\pm$ 0.0187	0.0392 $\pm$ 0.0147	0.0370 $\pm$ 0.0116	0.0422 $\pm$ 0.0172	0.0419 $\pm$ 0.0119	0.0363 $\pm$ 0.0200
8	0.0350 $\pm$ 0.0175	0.0319 $\pm$ 0.0142	0.0317 $\pm$ 0.0105	0.0352 $\pm$ 0.0165	0.0345 $\pm$ 0.0118	0.0272 $\pm$ 0.0176
9	0.0299 $\pm$ 0.0168	0.0255 $\pm$ 0.0133	0.0269 $\pm$ 0.0111	0.0288 $\pm$ 0.0161	0.0283 $\pm$ 0.0110	0.0209 $\pm$ 0.0161
10	0.0254 $\pm$ 0.0156	0.0203 $\pm$ 0.0125	0.0225 $\pm$ 0.0116	0.0250 $\pm$ 0.0131	0.0230 $\pm$ 0.0106	0.0150 $\pm$ 0.0118
11	0.0214 $\pm$ 0.0138	0.0162 $\pm$ 0.0113	0.0187 $\pm$ 0.0107	0.0217 $\pm$ 0.0116	0.0195 $\pm$ 0.0107	0.0115 $\pm$ 0.0103
12	0.0179 $\pm$ 0.0132	0.0139 $\pm$ 0.0112	0.0159 $\pm$ 0.0103	0.0182 $\pm$ 0.0107	0.0162 $\pm$ 0.0095	0.0089 $\pm$ 0.0095
13	0.0141 $\pm$ 0.0111	0.0117 $\pm$ 0.0108	0.0144 $\pm$ 0.0099	0.0151 $\pm$ 0.0093	0.0120 $\pm$ 0.0077	0.0069 $\pm$ 0.0084
14	0.0110 $\pm$ 0.0095	0.0098 $\pm$ 0.0103	0.0127 $\pm$ 0.0098	0.0129 $\pm$ 0.0086	0.0099 $\pm$ 0.0073	0.0053 $\pm$ 0.0067
15	0.0094 $\pm$ 0.0100	0.0090 $\pm$ 0.0108	0.0124 $\pm$ 0.0109	0.0098 $\pm$ 0.0070	0.0075 $\pm$ 0.0063	0.0044 $\pm$ 0.0062
16	0.0076 $\pm$ 0.0094	0.0076 $\pm$ 0.0116	0.0129 $\pm$ 0.0154	0.0085 $\pm$ 0.0069	0.0062 $\pm$ 0.0065	0.0040 $\pm$ 0.0064
17	0.0065 $\pm$ 0.0089	0.0069 $\pm$ 0.01074	0.0117 $\pm$ 0.0131	0.0070 $\pm$ 0.0067	0.0053 $\pm$ 0.0071	0.0035 $\pm$ 0.0060
18	0.0057 $\pm$ 0.0091	0.0051 $\pm$ 0.0081	0.0122 $\pm$ 0.0157	0.0065 $\pm$ 0.0082	0.0043 $\pm$ 0.0065	0.0034 $\pm$ 0.0067

19	0.0070 ±	0.0056 ±	0.0257 ±	0.0166 ±	0.0047 ±	0.0038 ±
	0.0174	0.0118	0.0429	0.0497	0.0089	0.0088

Table B.11 Actin cytoskeleton bulk remodelling visualised with GFP-FABD2 treated with isoxaben.

Measured using the *Pairwise image subtraction* method (see Section 4.4.3.2).

	Mock 1 h	Isoxaben 1 h	Mock 4 h	Isoxaben 4 h
Number of values	570	494	589	741
Minimum	0.5126	0.5106	0.4699	0.4971
25% Percentile	0.5825	0.5663	0.5645	0.5599
Median	0.6122	0.6002	0.6058	0.5884
75% Percentile	0.6605	0.6485	0.6447	0.6310
Maximum	0.8614	0.7900	0.7928	0.7785
Mean	0.6222	0.6103	0.6087	0.5967
Std. Deviation	0.05656	0.05670	0.05847	0.04860
Std. Error	0.002369	0.002551	0.002409	0.001786
Lower 95% CI	0.6176	0.6053	0.6039	0.5932
Upper 95% CI	0.6269	0.6153	0.6134	0.6002

Table B.12 Actin cytoskeleton bulk remodelling visualised with GFP-FABD2 treated with isoxaben.

Measured using the mean fluorescence intensity from the *Pairwise image subtraction* (see Section 4.4.3.3).

	Mock 1 h	Isoxaben 1 h	Mock 4 h	Isoxaben 4 h
Number of values	570	494	589	760
Minimum	0.2964	0.2479	0.2064	0.2220
25% Percentile	0.3895	0.3684	0.3580	0.3347
Median	0.4601	0.4269	0.4322	0.3940
75% Percentile	0.5384	0.5391	0.5244	0.4543
Maximum	0.7960	0.7156	0.7710	0.6593
Mean	0.4717	0.4542	0.4438	0.4004
Std. Deviation	0.1000	0.1223	0.1203	0.08612
Std. Error	0.004190	0.005502	0.004955	0.003124
Lower 95% CI	0.4634	0.4434	0.4340	0.3943
Upper 95% CI	0.4799	0.4650	0.4535	0.4065

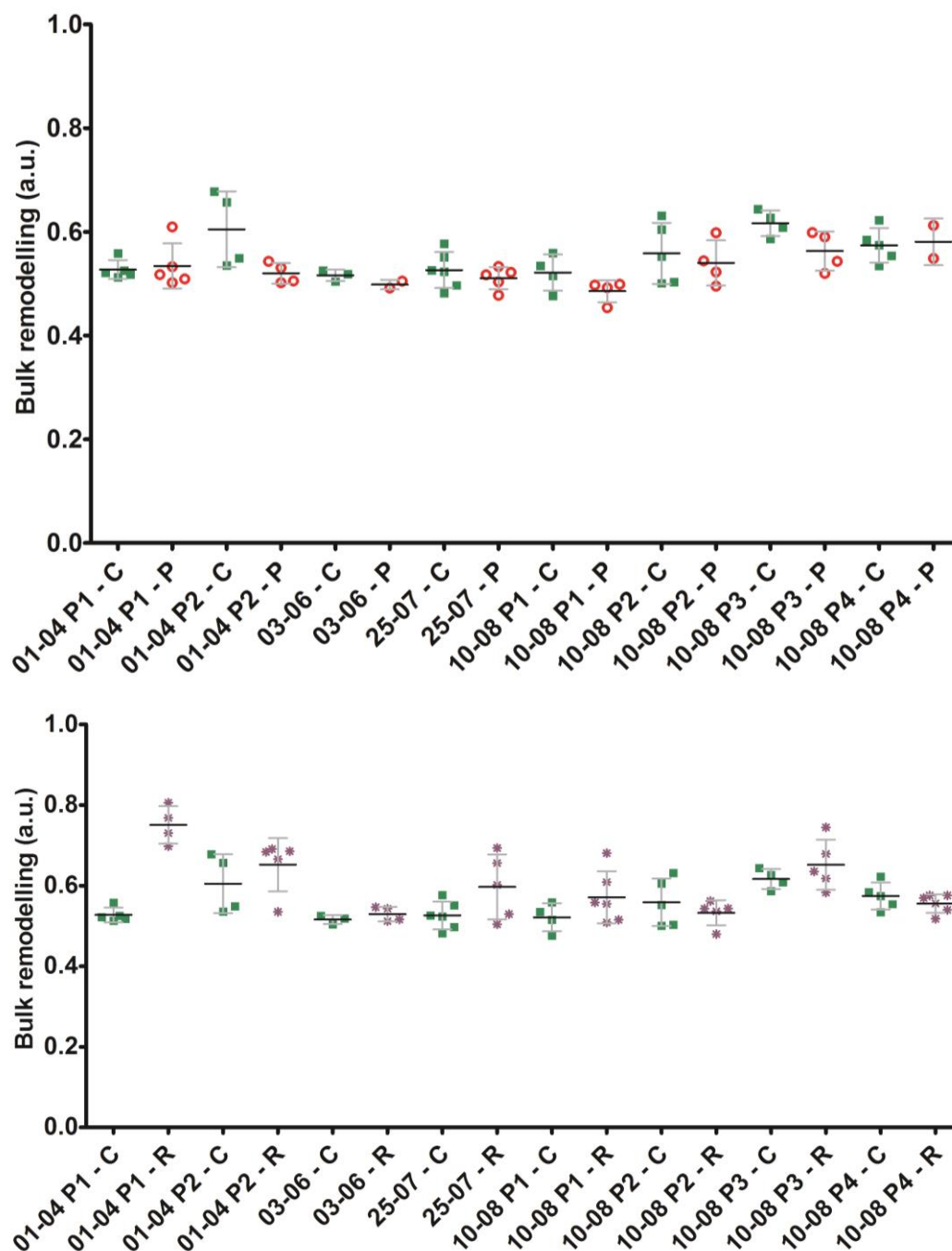


Figure B.8 Average bulk remodelling index of stably-expressing GFP-FABD2 control cells (from the 19 measurements) grouped per plant the cells originated from and plotted next to treated cell averages taken from the same plant.

Top panel shows control cells as green squares and plasmolysed cells as red hollow circles.

Lower panel shows control cells as green squares and re-hydrated cells as maroon stars.

The black line represents the mean per plant and the grey lines show the standard deviation per plant. The groups are labelled with the date and plant number.

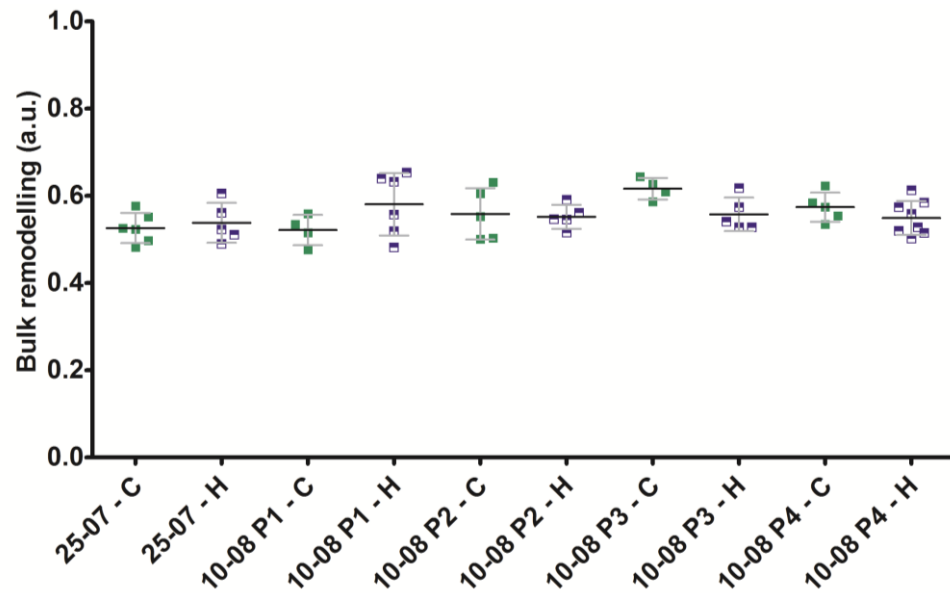


Figure B.9 Average bulk remodelling index of stably-expressing GFP-FABD2 control cells (from the 19 measurements) grouped per plant the cells originated from and plotted next to hypo-osmotic cell averages taken from the same plant.

Shows control cells as green squares and hypo-osmotically treated cells as purple hollow circles. The black line represents the mean per plant and the grey lines show the standard deviation per plant. The groups are labelled with the date and plant number.

Table B.13 Actin cytoskeleton bulk remodelling extrapolated via the proxy of Golgi body track length (μm).

Visualised with ST-GFP and measured using Volocity (see Section 4.4.1.6).

	Control	Plasmolysed	Re-hydrated	Hypo-osmotic
Number of values	910	848	774	626
Minimum	0.956	1.04	1.3	1.95
25% Percentile	5.115	7.095	7.4475	7.925
Median	8.385	10.785	10.97	11.095
75% Percentile	13.8075	15.215	15.79	15.855
Maximum	42.47	41.89	40.62	45.64
Mean	10.2451	11.7207	12.3445	12.917
Std. Deviation	6.94828	6.30554	6.9183	7.11873
Std. Error	0.230333	0.216533	0.248673	0.284522
Lower 95% CI	9.79311	11.2957	11.8563	12.3583
Upper 95% CI	10.6972	12.1457	12.8326	13.4758

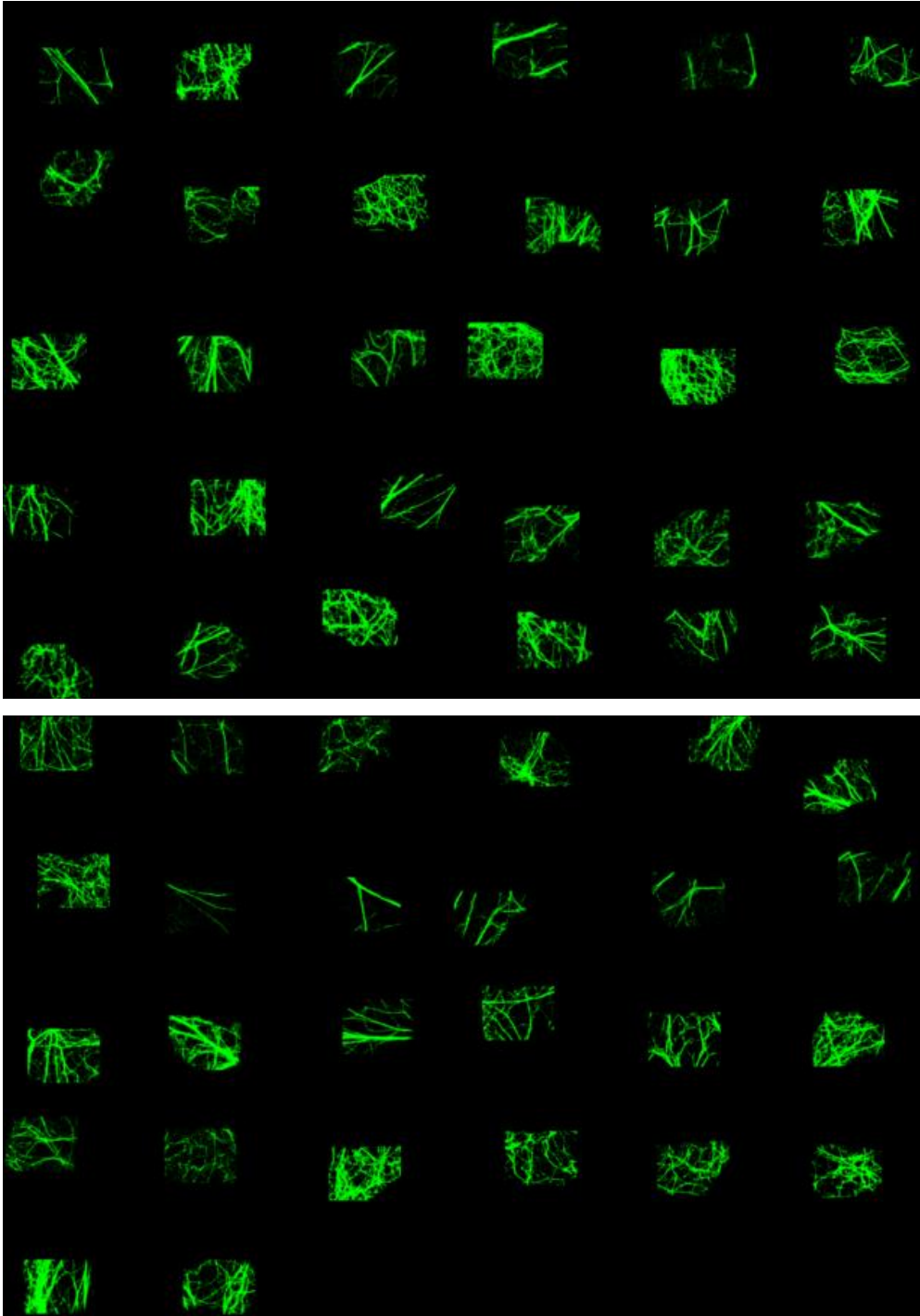


Figure B.10 Leaf epidermal pavement cells with different treatments expressing GFP-FABD2, only the maximum projection of the first Z-stack from each time-series analysed in Chapter 4 is shown here.

Top panel of 30 different control cells incubated in $\frac{1}{2}$ MS with DMSO at room temperature for 1 h; lower panel of 26 cells incubated in $\frac{1}{2}$ MS with 20 μ M isoxaben at room temperature for 1 h.

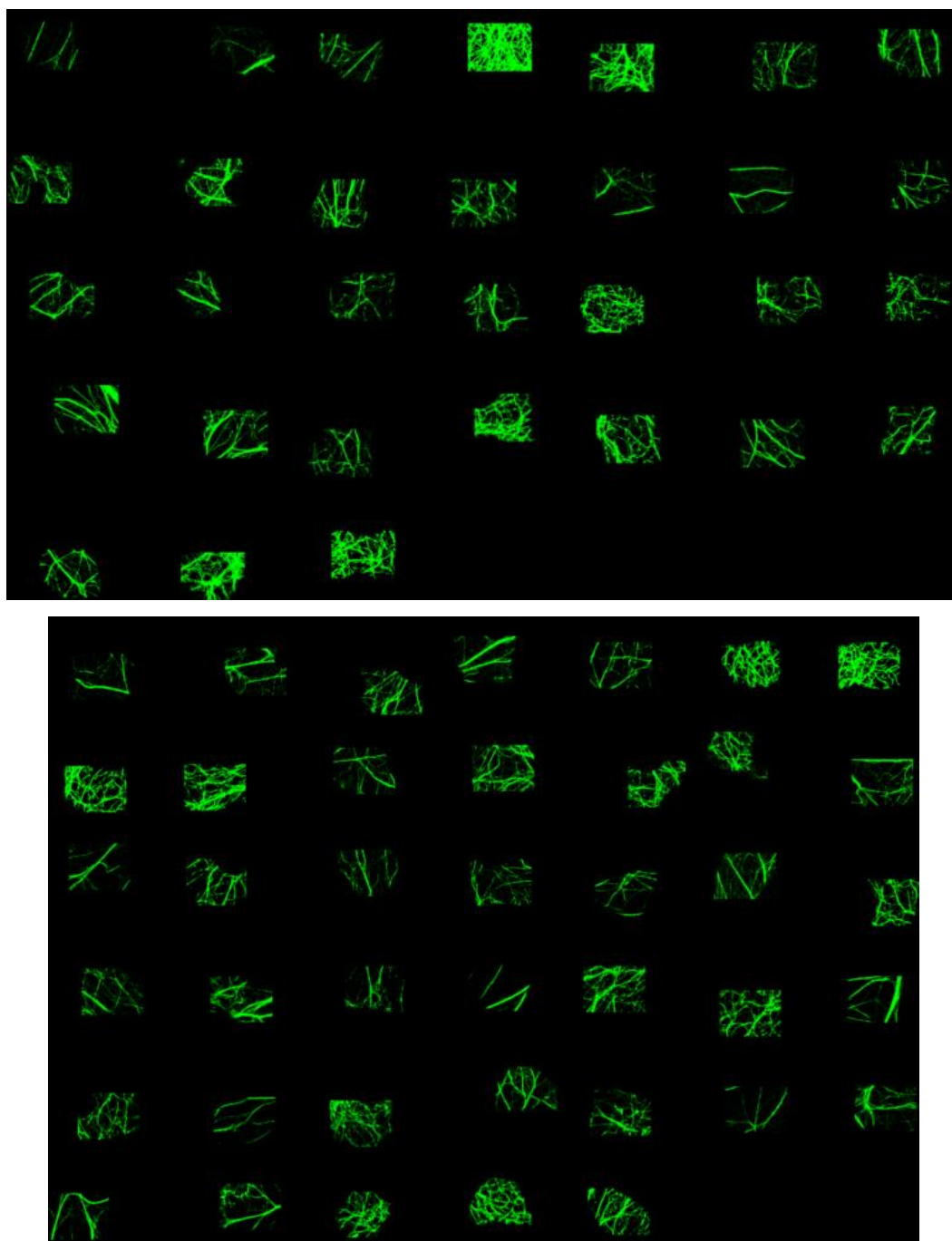


Figure B.11 Leaf epidermal pavement cells with different treatments expressing GFP-FABD2, only the maximum projection of the first Z-stack from each time-series analysed in Chapter 4 is shown here.

Top panel of 31 different control cells incubated in $\frac{1}{2}$ MS with DMSO at room temperature for 4 h; lower panel of 40 cells incubated in $\frac{1}{2}$ MS with 20 μ M isoxaben at room temperature for 4 h.

Appendix C

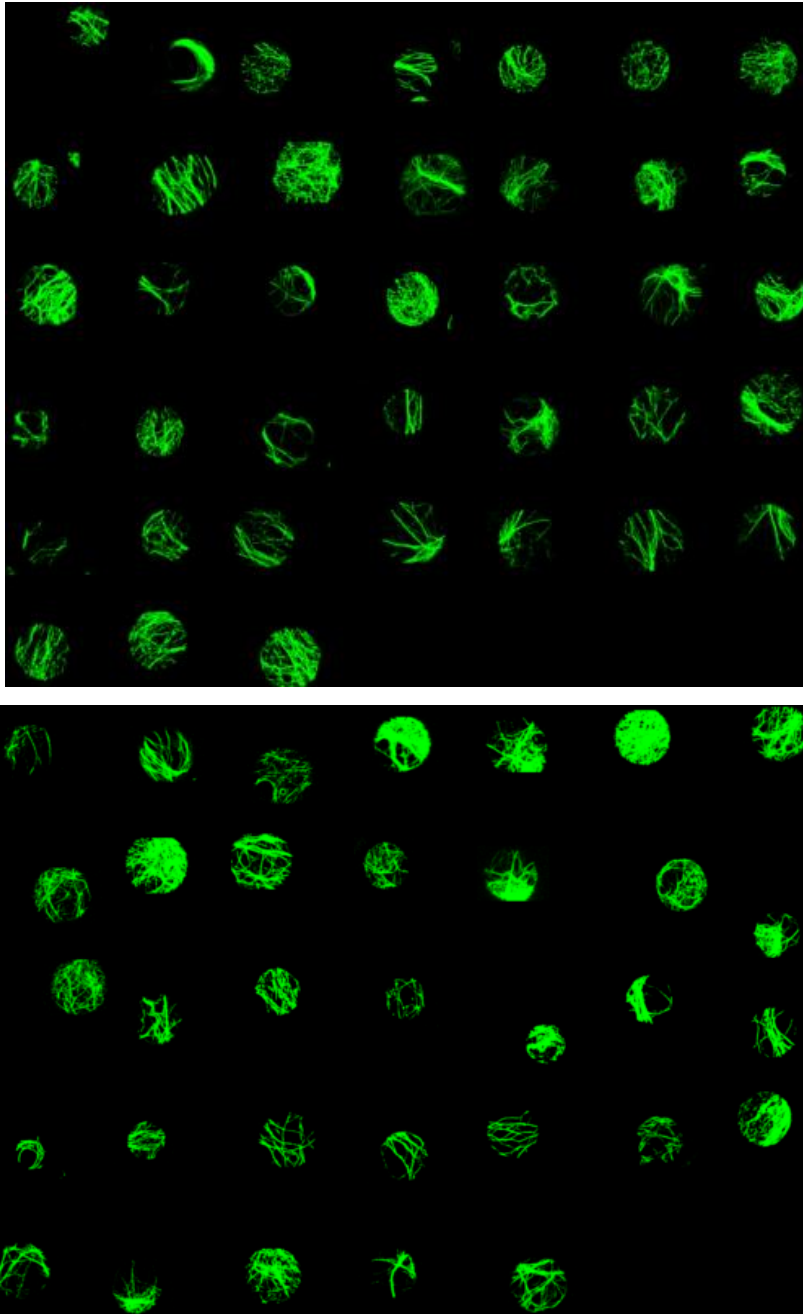


Figure C.1 Protoplasts expressing GFP-FABD2 at different time-points, only the maximum projection of the first Z-stack from each time-series analysed in Chapter 5 is shown here.

Top panel of 31 different cells incubated with DMSO at 0 h; lower panel of 40 cells incubated with DMSO at 24 h.

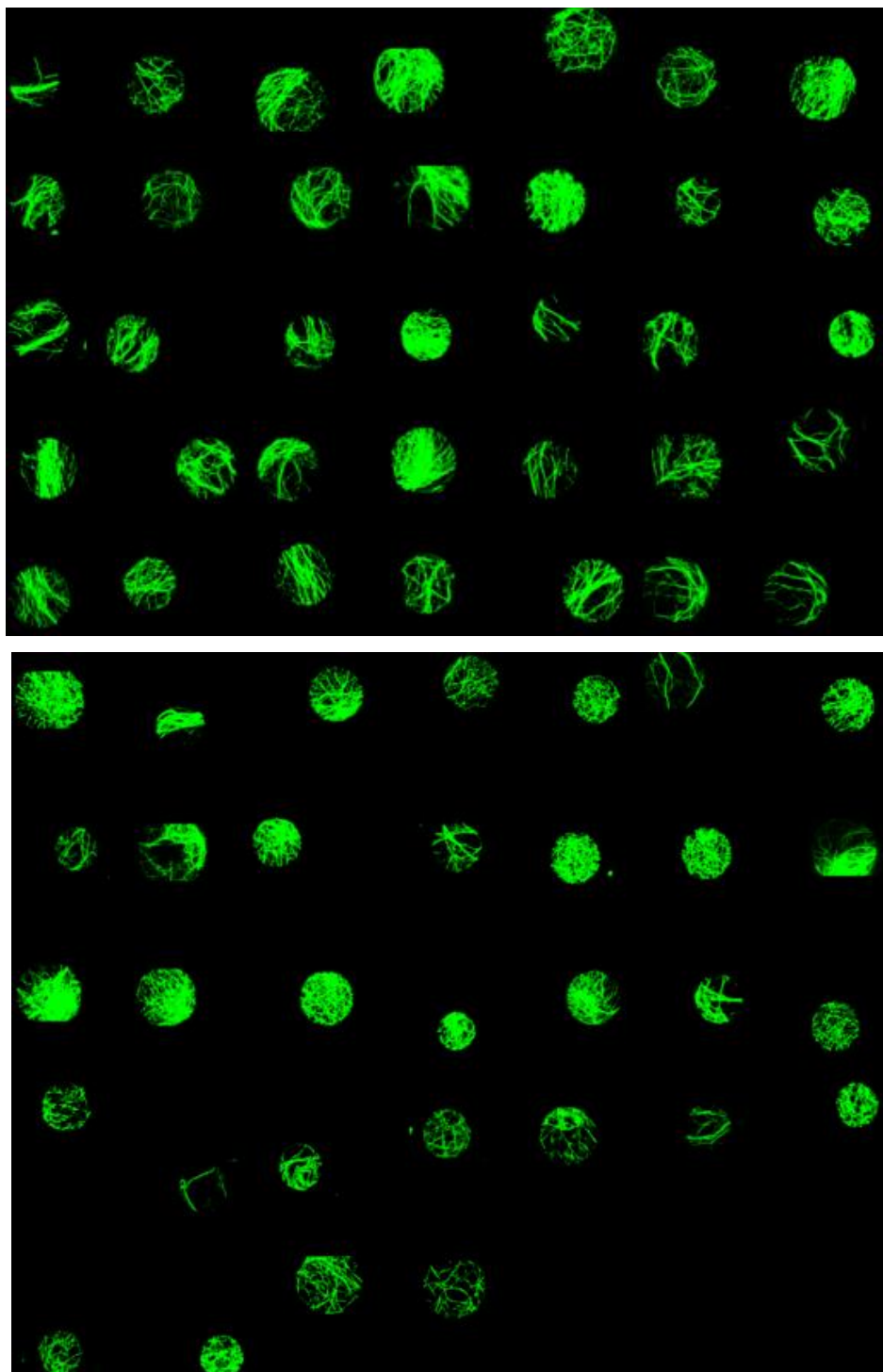


Figure C.2 Protoplasts expressing GFP-FABD2 at different time-points, only the maximum projection of the first Z-stack from each time-series analysed in Chapter 5 is shown here.

Top panel of 35 different cells incubated with 20 μ M isoxaben at 0 h; lower panel of 32 cells incubated with 20 μ M isoxaben at 24 h.

Table C.1 Actin cytoskeleton bulk remodelling in protoplasts visualised with GFP-FABD2.Measured using *Pairwise image subtraction* (see Section 5.4.1.2, pg. 149).

	Mock	Isoxaben	Mock	Isoxaben
	0 h	0 h	24 h	24 h
Number of values	722	665	627	608
Minimum	0.4437	0.4497	0.4319	0.4311
25% Percentile	0.5062	0.4928	0.4920	0.4890
Median	0.5270	0.5167	0.5160	0.5152
75% Percentile	0.5503	0.5519	0.5436	0.5444
Maximum	0.7288	0.6816	0.7217	0.7216
Mean	0.5299	0.5241	0.5233	0.5185
Std. Deviation	0.03884	0.03994	0.04463	0.04198
Std. Error	0.001446	0.001549	0.001782	0.001703
Lower 95% CI	0.5271	0.5210	0.5198	0.5151
Upper 95% CI	0.5328	0.5271	0.5268	0.5218

Table C.2 Actin cytoskeleton bulk remodelling in protoplasts visualised with GFP-FABD2.Measured using the *Pairwise image subtraction* measuring mean fluorescence intensity (see Section 5.4.1.3).

	Mock	Isoxaben	Mock	Isoxaben
	0 h	0 h	24 h	24 h
Number of values	722	665	627	589
Minimum	0.1263	0.1343	0.1127	0.1496
25% Percentile	0.1999	0.1969	0.2024	0.2076
Median	0.2551	0.2673	0.2660	0.2455
75% Percentile	0.3065	0.3208	0.3290	0.3156
Maximum	0.7974	0.5245	0.5587	0.6373
Mean	0.2671	0.2706	0.2745	0.2712
Std. Deviation	0.09029	0.08569	0.09378	0.09105
Std. Error	0.003360	0.003323	0.003745	0.003752
Lower 95% CI	0.2605	0.2641	0.2671	0.2638
Upper 95% CI	0.2737	0.2771	0.2818	0.2785

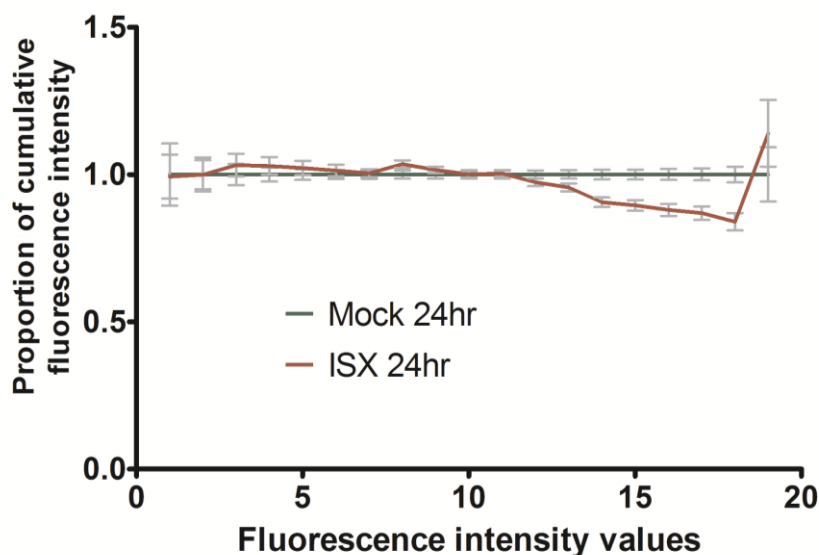


Figure C.3 Results from *Cumulative fluorescence intensity* analysis of protoplasts expressing GFP-Lifeact with different treatments.

Bulk remodelling of the actin cytoskeleton marked with GFP-Lifeact under mock condition at 24 h after cell wall removal and 20 μ M isoxaben treated sample at 24 h. For the graph the mock 24 h intensities have all been set to one and the other condition is shown as a proportion of that (see Figure 5.8).

Table C.3 Actin cytoskeleton bulk remodelling extrapolated via the proxy of Golgi body track velocity (μ m/s).

Visualised with ST-GFP and measured using Volocity (see Section 5.4.3.1).

	Mock 0 h	Isoxaben 0 h	Mock 24 h	Isoxaben 24 h
Number of values	294	475	706	773
Minimum	0.1270	0.1370	0.1480	0.1140
25% Percentile	0.2100	0.2420	0.2940	0.2955
Median	0.2800	0.3170	0.3820	0.3830
75% Percentile	0.4243	0.4140	0.4945	0.5015
Maximum	1.310	0.8020	1.120	1.070
Mean	0.3338	0.3436	0.4089	0.4093
Std. Deviation	0.1676	0.1384	0.1575	0.1525
Std. Error	0.009772	0.006352	0.005927	0.005486
Lower 95% CI	0.3146	0.3311	0.3973	0.3986
Upper 95% CI	0.3531	0.3561	0.4205	0.4201

Table C.4 Actin cytoskeleton bulk remodelling extrapolated via the proxy of Golgi body displacement rate ($\mu\text{m/s}$).

Visualised with ST-GFP and measured using Volocity (see Section 5.4.3.1).

	Mock	Isoxaben	Mock	Isoxaben
	0 h	0 h	24 h	24 h
Number of values	294	475	706	773
Minimum	0.00118	0.00119	0.00133	0.000687
25% Percentile	0.0182	0.0202	0.03538	0.03225
Median	0.03935	0.0415	0.07445	0.0669
75% Percentile	0.08338	0.0733	0.1383	0.1220
Maximum	1.240	0.7170	1.050	0.7160
Mean	0.07583	0.06138	0.1095	0.09382
Std. Deviation	0.1223	0.07392	0.1168	0.09416
Std. Error	0.007132	0.003392	0.004395	0.003387
Lower 95% CI	0.06180	0.05471	0.1009	0.08718
Upper 95% CI	0.08987	0.06804	0.1181	0.1005

Table C.5 Actin cytoskeleton bulk remodelling extrapolated via the proxy of Golgi body meandering indices.

Visualised with ST-GFP and measured using Volocity (see Section 5.4.3.1).

	Mock	Isoxaben	Mock	Isoxaben
	0 h	0 h	24 h	24 h
Number of values	294	475	706	773
Minimum	0.0036	0.00362	0.00902	0.00235
25% Percentile	0.06958	0.0707	0.1018	0.08735
Median	0.1385	0.1270	0.1965	0.1710
75% Percentile	0.2533	0.2210	0.3313	0.3070
Maximum	0.9420	0.9490	0.9880	0.9410
Mean	0.1925	0.1741	0.2441	0.2220
Std. Deviation	0.1794	0.1556	0.1847	0.1798
Std. Error	0.01046	0.007139	0.006953	0.006468
Lower 95% CI	0.1719	0.1601	0.2305	0.2093
Upper 95% CI	0.2131	0.1881	0.2578	0.2347

Table C.6 Actin cytoskeleton bulk remodelling extrapolated via the proxy of Golgi body time span, that is, how long they are present in the field of view.
Visualised with ST-GFP and measured using Volocity (see Section 5.4.3.1).

	Mock	Isoxaben	Mock	Isoxaben
	0 h	0 h	24 h	24 h
Number of values	294	475	706	773
Minimum	13.00	20.00	11.00	12.00
25% Percentile	35.00	34.00	28.00	25.00
Median	64.00	54.00	41.00	38.00
75% Percentile	124.3	120.0	72.00	69.00
Maximum	200.0	200.0	200.0	200.0
Mean	87.00	81.81	59.52	59.08
Std. Deviation	63.68	60.04	46.27	50.42
Std. Error	3.714	2.755	1.741	1.814
Lower 95% CI	79.69	76.39	56.10	55.52
Upper 95% CI	94.31	87.22	62.94	62.64

Table C.7 Actin cytoskeleton bulk remodelling extrapolated via the proxy of Golgi body track length (μm).
Visualised with ST-GFP and measured using Volocity (see Section 5.4.3.1).

	Mock	Isoxaben	Mock	Isoxaben
	0 h	0 h	24 h	24 h
Number of values	294	475	706	773
Minimum	1.370	1.960	1.350	1.520
25% Percentile	5.998	5.460	5.300	4.810
Median	9.010	8.990	7.750	7.230
75% Percentile	14.39	15.25	11.97	12.15
Maximum	35.42	51.39	38.88	43.59
Mean	10.69	11.39	9.739	9.578
Std. Deviation	6.216	7.871	6.502	6.947
Std. Error	0.3625	0.3612	0.2447	0.2499
Lower 95% CI	9.974	10.68	9.259	9.088
Upper 95% CI	11.40	12.10	10.22	10.07

C.1 Analysis of bulk actin cytoskeleton remodelling under conditions of cell wall removal and isoxaben treatment using TIRF microscopy

C.1.1 Methods of TIRF imaging at the University of Exeter

Imaging was performed by Dr Mike Deeks and Dr Stefan Sassmann from the University of Exeter because of the requirements of the facility. Protoplasts were made as detailed previously (Section 2.4, pg. 45). Cells were imaged every 0.1 s for 30 s using a custom built Olympus microscope [Olympus Corporation, Tokyo, Japan] with a Visitron TIRF system [Visitron Systems GmbH, Puchheim, Germany].

Protoplasts were made from the epidermal pavement leaf cells of mature GFP-FABD2 and GFP-Lifeact stably-expressing *A. thaliana* plants and were then imaged by Dr Mike Deeks and Dr Stefan Sassmann on a TIRF microscope.

C.1.2 Qualitative assessment of GFP-FABD2 and GFP-Lifeact at 0 h after cell wall removal and later

The field of view for a protoplast visualised using TIRF microscopy is often quite limited because they are spherical and TIRF microscopes have an imaging depth of around 200 to 300 nm (see Section 1.7.1.2, pg. 37). This means that the area of actin cytoskeleton that can be analysed is smaller in than that obtained from confocal microscopy. Using a TIRF microscope, protoplasts expressing GFP-FABD2 and GFP-Lifeact were imaged and 100 consecutive time-frames (approximately 10 s of the movie) were selected and used for *Cumulative fluorescence intensity* analysis (see Section 3.4.3.3, pg. 63). Figure C.4 and Figure C.5 show the image output from the *Cumulative fluorescence intensity* analysis method. Using a TIRF microscope, this kind of analysis and this size field of view make GFP-FABD2 and GFP-Lifeact look similar and also appear to have the same amount of remodelling (Figure C.4 and Figure C.5). It was difficult to compare confocal data with the data presented in this section as they are so different. However, this is preliminary data with very limited sample sizes and there seem to be few differences between time after cell wall removal or between the two markers (number of cells shown in Figure C.4 and Figure C.5). Lastly, no statistical comparisons were made because of the small sample sizes for both markers and all time points.

C.1.3 Pairwise image subtraction of GFP-FABD2 marked actin cytoskeleton in protoplasts 0h and 24h after cell wall removal using TIRF microscopy

As mentioned above, the sample sizes are very small, so no definitive conclusions can be drawn from the data presented here. However, the two methods of analysis used on the confocal data previously (see Section 3.4.3.1) were attempted to see if they worked on the TIRF data collected during this study. Using *Pairwise image subtraction* the data from the GFP-FABD2 expressing cells looked noisy, but there seems to be a trend for more movement in the GFP-Lifeact expressing protoplasts as the cell wall regenerates over time (Figure C.6).

C.1.4 Cumulative fluorescence intensity of GFP-Lifeact marked actin cytoskeleton in protoplasts 0 h, 24 h and 48 h after cell wall removal using TIRF microscopy

Using *Cumulative fluorescence intensity* analysis the GFP-FABD2 expressing cells appear to have more movement at 0 h after cell wall removal (Figure C.7). The proportion of fluorescence at the lowest fluorescence intensity values is higher for the cells at 0 h than the cells at 24 h after cell wall removal (Figure C.7). Using *Cumulative fluorescence intensity* analysis the GFP-Lifeact expressing cells appear to have both more and less movement at 24 h after cell wall removal compared to 0 h and 48 h after cell wall removal (Figure C.7).

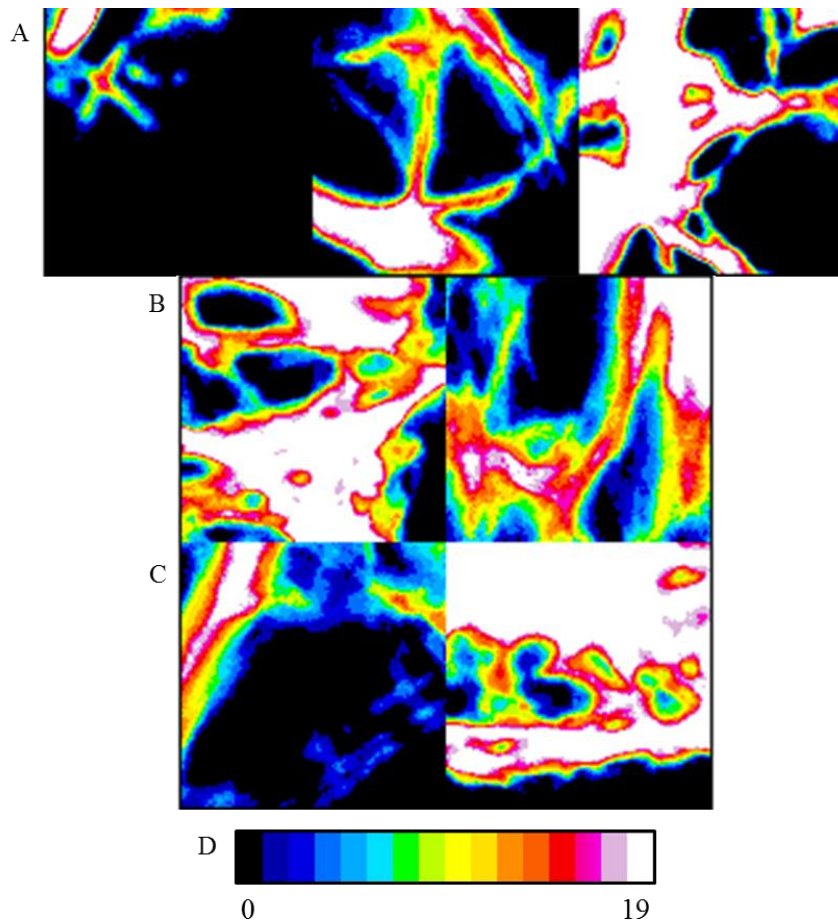


Figure C.4 Protoplasts expressing GFP-FABD2 imaged using a TIRF microscope and analysed using *Cumulative fluorescence intensity*.

In each image 100 time-frames (over a total of approximately 10 s of each movie) are summed. The colours correspond to fluorescence intensity values for each pixel from '0' to '19', blue shades are smaller values and red and white are larger values.

Row A is 0 h and rows B and C are protoplasts after 24 h of cell wall regrowth.

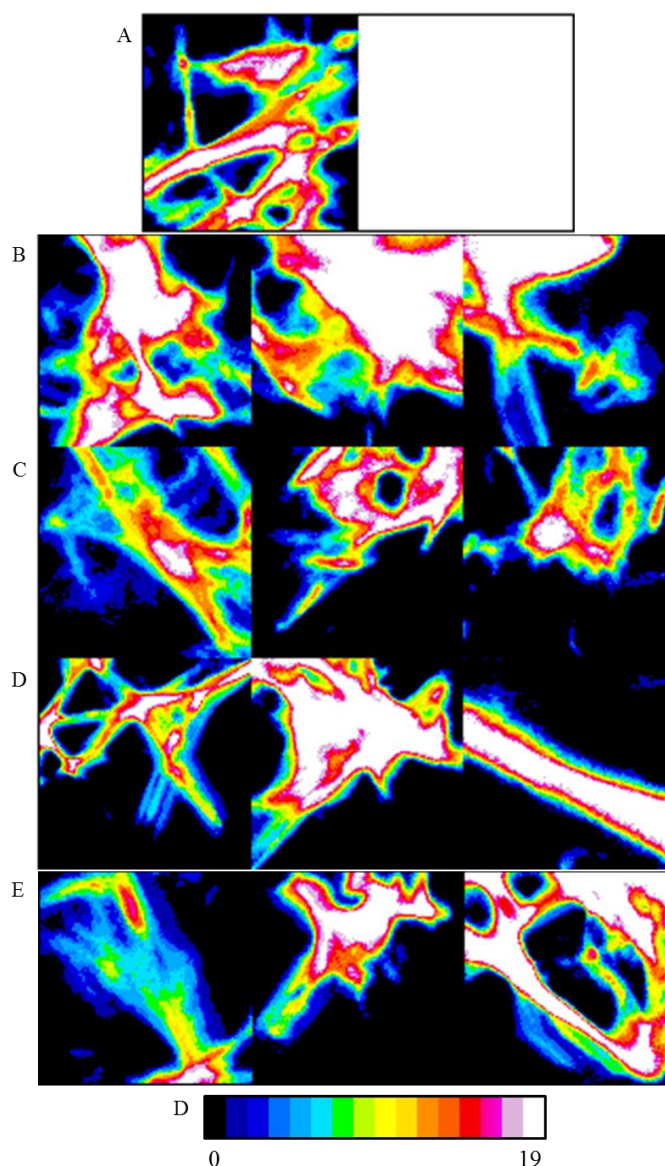


Figure C.5 Protoplasts expressing GFP-Lifeact imaged using a TIRF microscope and analysed using *Cumulative fluorescence intensity*.

In each image 100 time-frames (over a total of approximately 10 s of each movie) are summed. The colours correspond to fluorescence intensity values for each pixel from '0' to '19', blue shades are smaller values and red and white are larger values.

Row A is 0 h; rows B, C and D are 24 h; and row E are protoplasts after 48 h of cell wall regrowth.

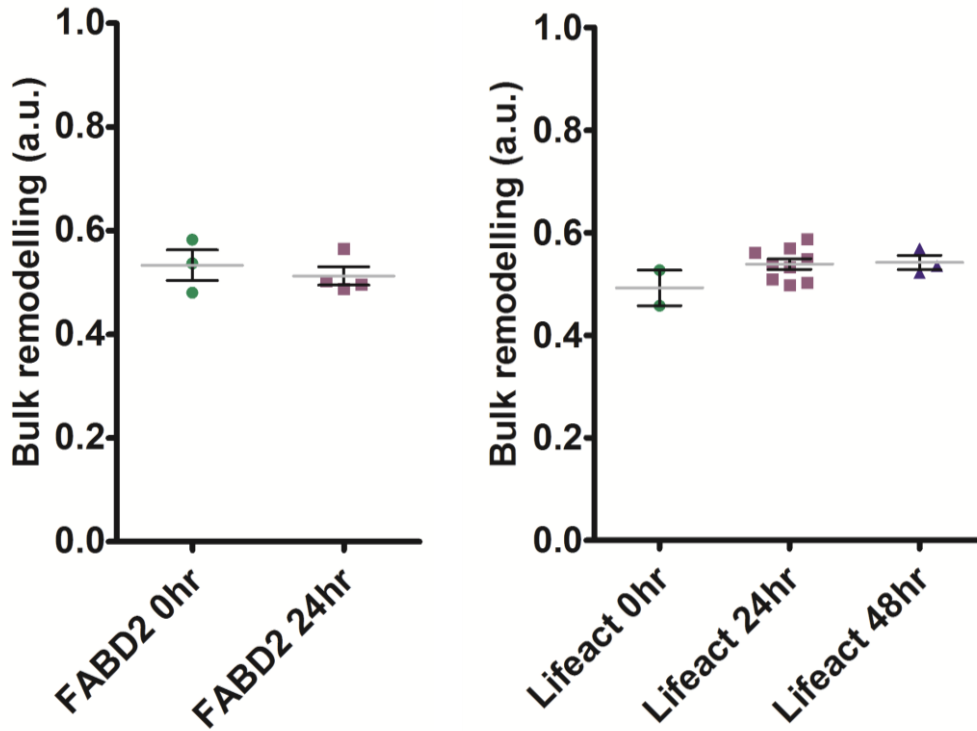


Figure C.6 *Pairwise image subtraction* analysis on protoplasts expressing cytoskeletal markers and visualised using TIRF.

The graphs show the data for protoplasts originating from epidermal pavement leaf cells from two transgenic lines of *A. thaliana* one stably expressing GFP-FABD2 and one stably expressing GFP-Lifeact both under mock condition at 0 h and 24 h after cell wall removal. Each symbol represents the average value of one cell and the error bars are standard deviation. They have been analysed using the *Pairwise image subtraction* method.

Using TIRF microscopy to image the actin cytoskeleton in protoplasts, the GFP-Lifeact tagged cytoskeleton looks similar to the GFP-FABD2 tagged cytoskeleton, in contrast to the confocal where they look different (Figure C.4 and Figure C.5). However, the sample sizes are too small to draw any conclusions about changes in cytoskeletal behaviour between the different conditions (0 h, 24 h and 48 h after cell wall removal).

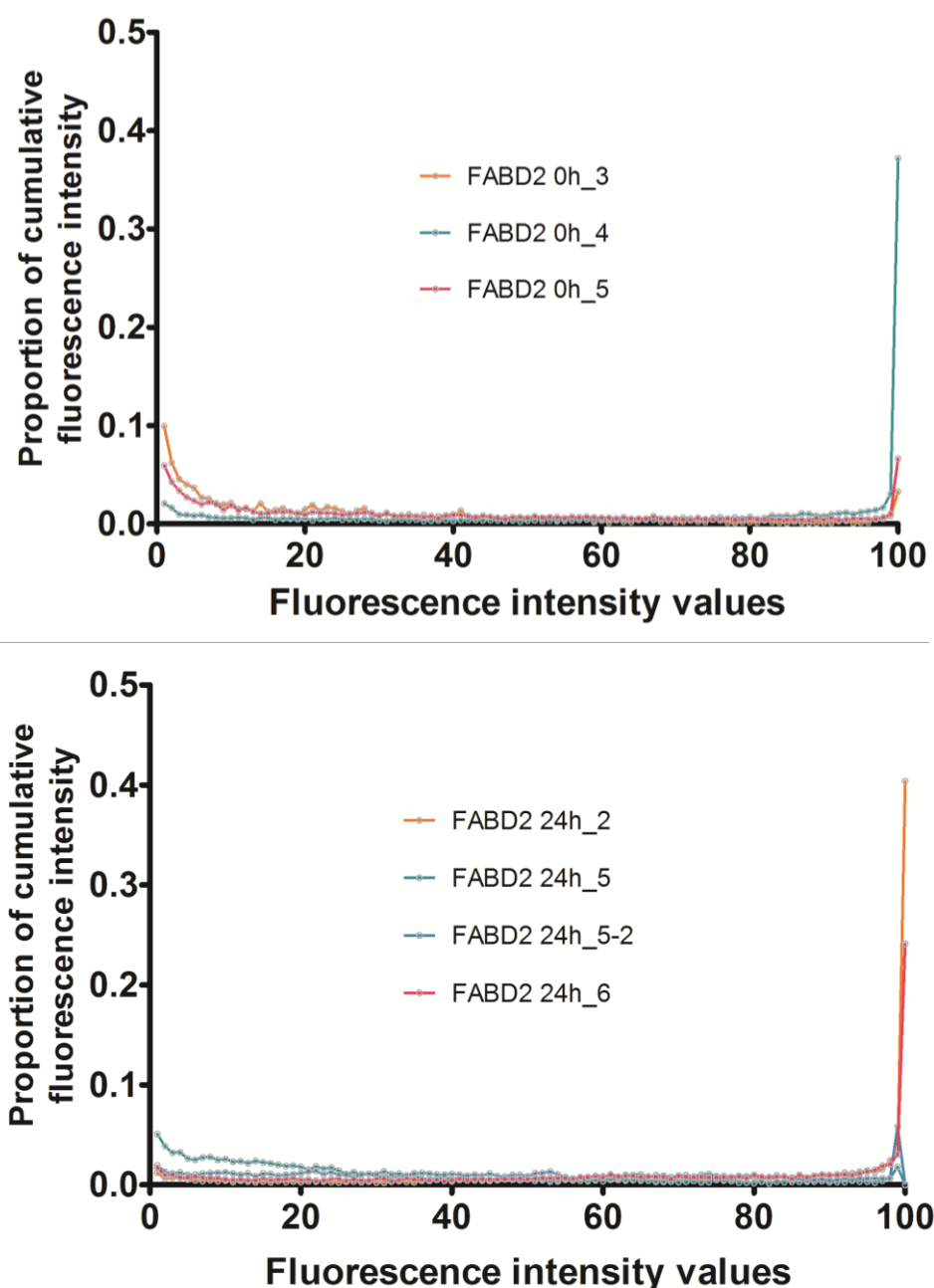


Figure C.7 Line graphs of the results from *Cumulative fluorescence intensity* analysis on protoplasts expressing GFP-FABD2 and visualised using TIRF.

Line graphs representing the bulk remodelling of the actin cytoskeleton marked with GFP-FABD2 under mock condition at 0 h (top graph) and 24 h (bottom graph) after cell wall removal. This graph was made using the proportional fluorescence intensities derived from the *Cumulative fluorescence intensity* method.

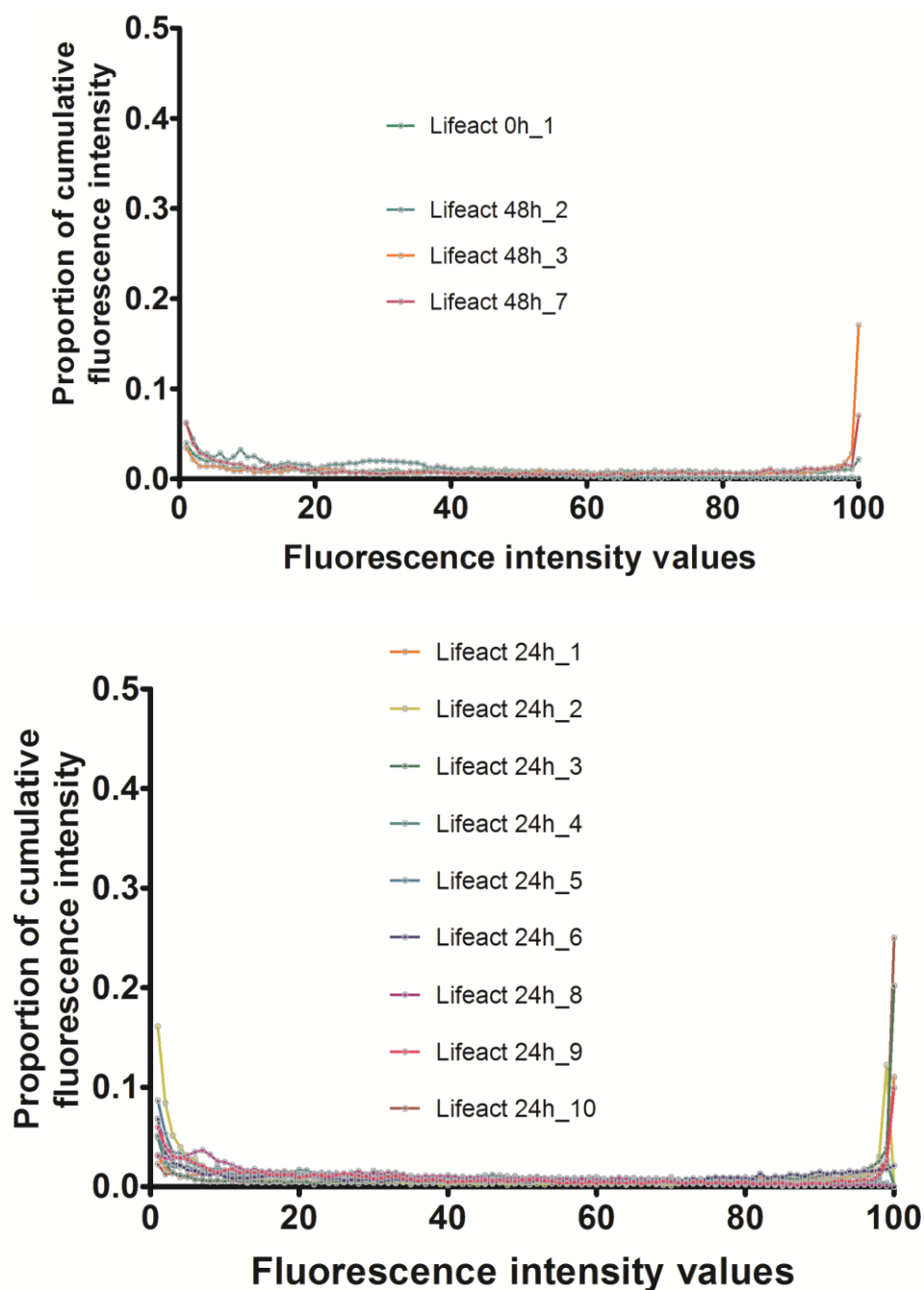


Figure C.8 Line graphs of the results from *Cumulative fluorescence intensity* analysis on protoplasts expressing GFP-Lifeact and visualised using TIRF.

Curves representing the bulk remodelling of the actin cytoskeleton marked with GFP-Lifeact under mock condition at 0 h (top graph), 24 h (bottom graph) and 48 h (top graph) after cell wall removal. This graph was made using the proportional fluorescence intensities derived from the *Cumulative fluorescence intensity* method.

Appendix D

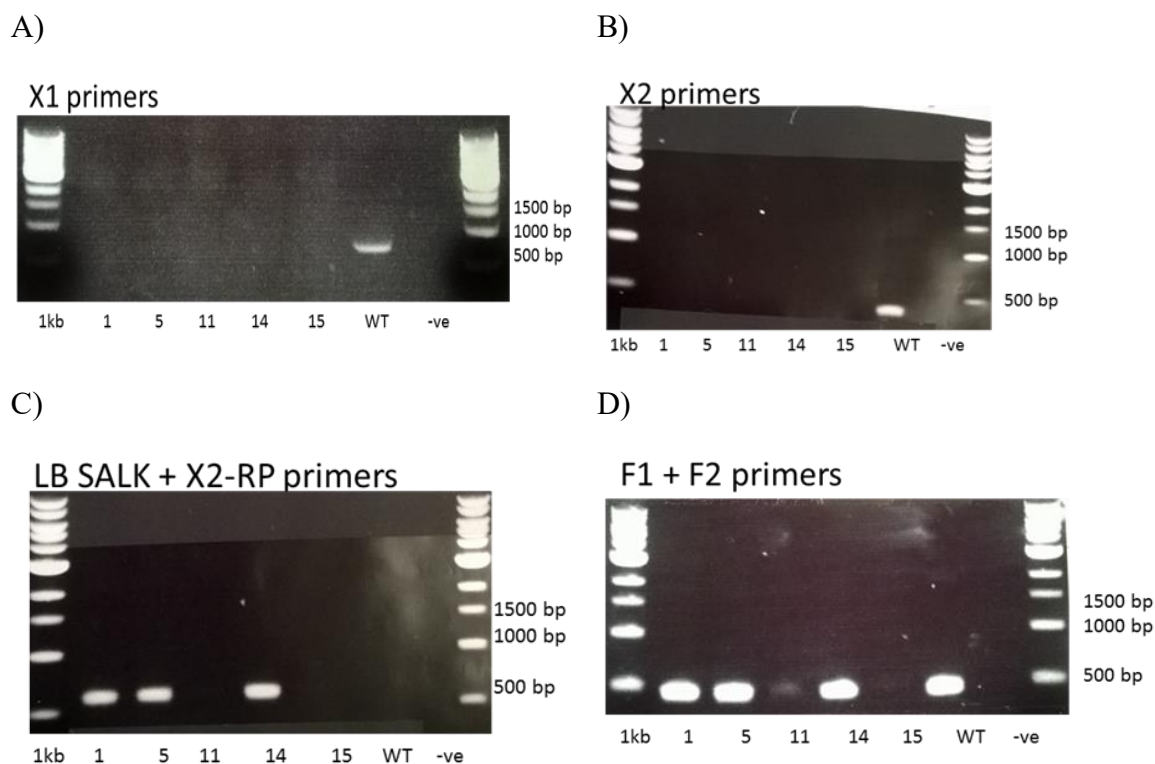


Figure D.1 PCR amplifications from genomic DNA.

- A) 700 bp band indicates *xxt1* wild type (WT) genomic DNA. Only lane 7 contains a band, confirming WT and suggesting that the five double-mutant plants (1, 5, 11, 14, and 15) do not have an intact *xxt1* gene.
- B) 400 bp band indicates *xxt2* WT genomic DNA. Only lane 7 contains a band, confirming WT and suggesting that the five plants (1, 5, 11, 14, and 15) are homozygous for lacking *xxt2*.
- C) 500 bp band indicates the presence of the SALK T-DNA insert for *xxt2*. Lanes 2, 3 and 5 (plants 1, 5 and 14) confirm the SALK insertion, while lanes 4, 6 and 7 (plants 11, 15 and WT respectively) do not; this is expected with the WT (lane 7).
- D) As no band was seen in gel C) for plants 11 and 15, the presence or absence of genomic DNA was queried using primers for an unrelated gene. 500 bp band indicates *AtFH1* WT genomic DNA. Lanes 2, 3, 5 and 7 confirm WT (plants 1, 5, 14 and WT, respectively), however, lane 4 (plant 11) shows a faint band and would need to be repeated. Lane 6 (plant 15) shows no band, so more genomic DNA would need to be extracted and the PCRs repeated.

Table D.1 Mean values and global fit values of fluorescence recovery after photobleaching for GFP-LTI6b in WT and in two different *xxt1-xxt2* lines.

	WT	WT	Line 1	Line 1	Line 8	Line 8
		global fit		global fit		global fit
Number of values	40	40	35	35	34	34
MFR (%)	74.52	74.60	68.69	68.05	72.64	72.21
MFR Std. Deviation	7.630		8.551		10.33	
MFR 95% Confidence Incidence		73.83 to 75.38		67.11 to 69.00		71.23 to 73.20
Half-time (s)	4.586	4.851	4.335	4.253	4.070	4.178
Half-time Std. Deviation	0.8532		1.432		1.205	
Half-time 95% Confidence Incidence		4.603 to 5.128		3.949 to 4.608		3.878 to 4.528
R²		0.8922		0.8141		0.8160

* The global fit values are reported in addition to the mean to check how well the data fits non-linear regression analysis.

Table D.2 Mean values and global fit values of fluorescence recovery after photobleaching for GFP-PAP in WT and in two different *xxt1*-*xxt2* lines.

	WT	WT	Line 1	Line 1	Line 2	Line 2
		global fit		global fit		global fit
Number of values	26	26	42	42	26	26
MFR (%)	67.83	67.17	86.32	85.97	82.79	81.19
MFR Std. Deviation	12.23		8.197		9.678	
MFR 95% Confidence Incidence		65.66 to 68.68		85.31 to 86.63		79.88 to 82.49
Half-time (s)	1.577	1.387	1.655	1.586	1.440	1.339
Half-time Std. Deviation	0.9603		0.5685		0.4193	
Half-time 95% Confidence Incidence		1.143 to 1.763		1.484 to 1.703		1.159 to 1.584
R²		0.4619		0.8276		0.6160

Table D.3 Mean values and global fit values of fluorescence recovery after photobleaching for MAP-GFP in WT and in two different *xxt1-xxt2* lines.

	WT	WT	Line 3	Line 3	Line 4	Line 4
		global fit		global fit		global fit
Number of values	48	48	19	19	37	37
MFR (%)	86.54	86.24	84.51	84.29	80.87	80.62
MFR Std. Deviation	10.74		7.876		7.379	
MFR 95% Confidence Incidence		85.44 to 87.03		83.32 to 85.26		79.95 to 81.30
Half-time (s)	1.822	1.787	1.593	1.549	1.425	1.369
Half-time Std. Deviation	0.5524		0.4616		0.4478	
Half-time 95% Confidence Incidence		1.658 to 1.938		1.403 to 1.730		1.267 to 1.487
R²		0.7569		0.8234		0.8125

Table D.4 Mean values and global fit values of fluorescence recovery after photobleaching for YFP-PI in WT and in one *xxt1-xxt2* line.

	WT	WT global fit	<i>xxt1-xxt2</i>	<i>xxt1-xxt2</i> global fit
Number of values	26	26	20	20
MFR (%)	75.66	75.27	60.38	59.86
MFR Std. Deviation	7.362		12.83	
MFR 95% Confidence Incidence		74.37 to 76.16		58.27 to 61.44
Half-time (s)	1.771	1.627	1.319	1.205
Half-time Std. Deviation	0.7580		0.9091	
Half-time 95% Confidence Incidence		1.474 to 1.816		0.9503 to 1.646
R²		0.7701		0.4380

Table D.5 Mean values and global fit values of fluorescence recovery after photobleaching for GFP-LTI6b in WT with and without isoxaben treatment.

Continued on the next page.

	WT mock	WT mock global fit	WT isoxaben treated	WT isoxaben treated global fit
Number of values	33	33	18	18
MFR (%)	50.70	50.61	43.82	44.07
MFR Std. Deviation	9.373		7.011	
MFR 95% Confidence Incidence		49.79 to 51.42		43.50 to 44.63
Half-time (s)	5.911	6.002	4.144	4.434
Half-time Std. Deviation	1.105		1.088	
Half-time 95% Confidence Incidence		5.786 to 6.234		4.270 to 4.612
R²		0.8563		0.8639

Continued from the previous page.

Mean values and global fit values of fluorescence recovery after photobleaching for GFP-LTI6b in one *xtt1-xtt2* line with and without isoxaben treatment.

	<i>xtt1-xtt2</i> mock	<i>xtt1-xtt2</i> mock global fit	<i>xtt1-xtt2</i> isoxaben treated	<i>xtt1-xtt2</i> isoxaben treated global fit
Number of values	41	41	34	34
MFR (%)	54.35	53.65	53.46	52.26
MFR Std. Deviation	7.064		10.96	
MFR 95% Confidence Incidence		52.97 to 54.32		51.35 to 53.17
Half-time (s)	6.732	6.628	5.911	5.706
Half-time Std. Deviation	1.990		1.416	
Half-time 95% Confidence Incidence		6.451 to 6.814		5.479 to 5.953
R²		0.9131		0.8113

Table D.6 Mean values and global fit values of fluorescence recovery after photobleaching for MAP-GFP in WT with and without isoxaben treatment.

	WT mock	WT mock global fit	WT isoxaben treated	WT isoxaben treated global fit
Number of values	40	40	45	45
MFR (%)	69.88	69.85	68.61	68.55
MFR Std. Deviation	7.679		8.793	
MFR 95% Confidence Incidence		69.90 to 70.10		68.26 to 68.85
Half-time (s)	2.087	2.2	2.268	2.345
Half-time Std. Deviation	0.5506		0.4707	
Half-time 95% Confidence Incidence		2.158 to 2.244		2.296 to 2.396
R²		0.8378		0.7975

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Mean values and global fit values of fluorescence recovery after photobleaching for MAP-GFP in one *xxt1-xxt2* line with and without isoxaben treatment.

	<i>xxt1-xxt2</i> mock	<i>xxt1-xxt2</i> mock global fit	<i>xxt1-xxt2</i> isoxaben treated	<i>xxt1-xxt2</i> isoxaben treated global fit
Number of values	35	35	32	32
MFR (%)	71.01	71.27	69.30	69.57
MFR Std. Deviation	7.393		5.649	
MFR 95% Confidence Incidence		70.94 to 71.61		69.25 to 69.88
Half-time (s)	2.283		2.371	
Half-time Std. Deviation	0.5246		0.4549	
Half-time 95% Confidence Incidence		2.403 to 2.517		2.469 to 2.579
R²		0.8177		0.8518

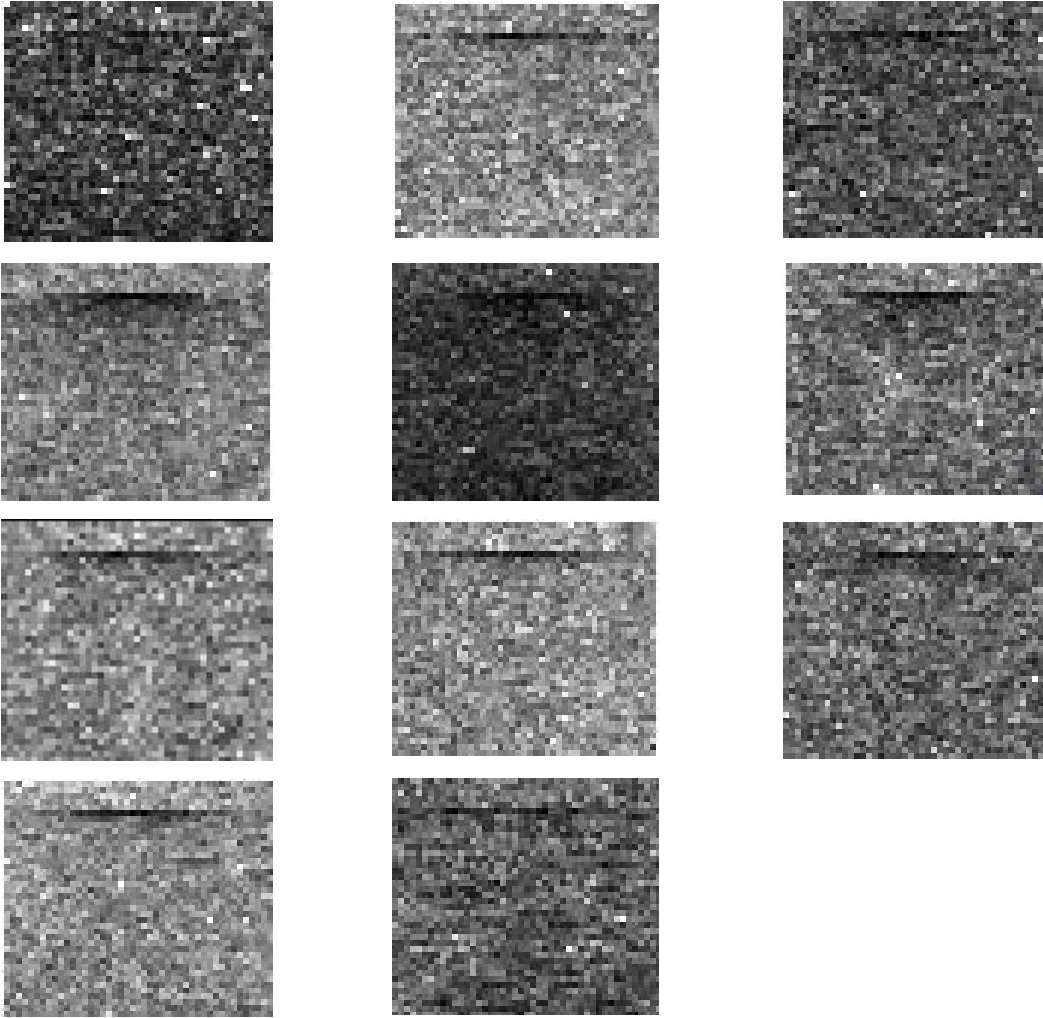


Figure D.2 Kymographs of plasma membrane-resident proteins during FRAP (bleach area has a radius of 1.2 μm) and the time of the movie is 75 s.

Kymographs show one line of pixels and how the fluorescence alters at those specific pixels over time. They allow the visualisation of movement in a still image, for example in all of the kymographs shown in this figure, an area was bleached and then the fluorescence recovers very quickly.

A) GFP-PAP_C-0_P8F1_24-11_Kymo; B) GFP-PAP_x1_P2F2_24-11_Kymo;
 C) GFP-PAP_x2_P2F2_17-11_Kymo; D) LTI6b-GFP_Ler_P1F1_25-11_Kymo;
 E) LTI6b-GFP_x1_P1F4_18-11_Kymo; F) LTI6b-GFP_x8_P2F2_25-11_Kymo;
 G) MAP-GFP_C-0_P1F4_24-11_Kymo; H) MAP-GFP_x3_P1F4_11-11_Kymo;
 I) MAP-GFP_x4_P1F3_24-11_Kymo; J) YFP-PI_C-0_P2F1_25-11_Kymo; and
 K) YFP-PI_x3_P1F3_12-11_Kymo.

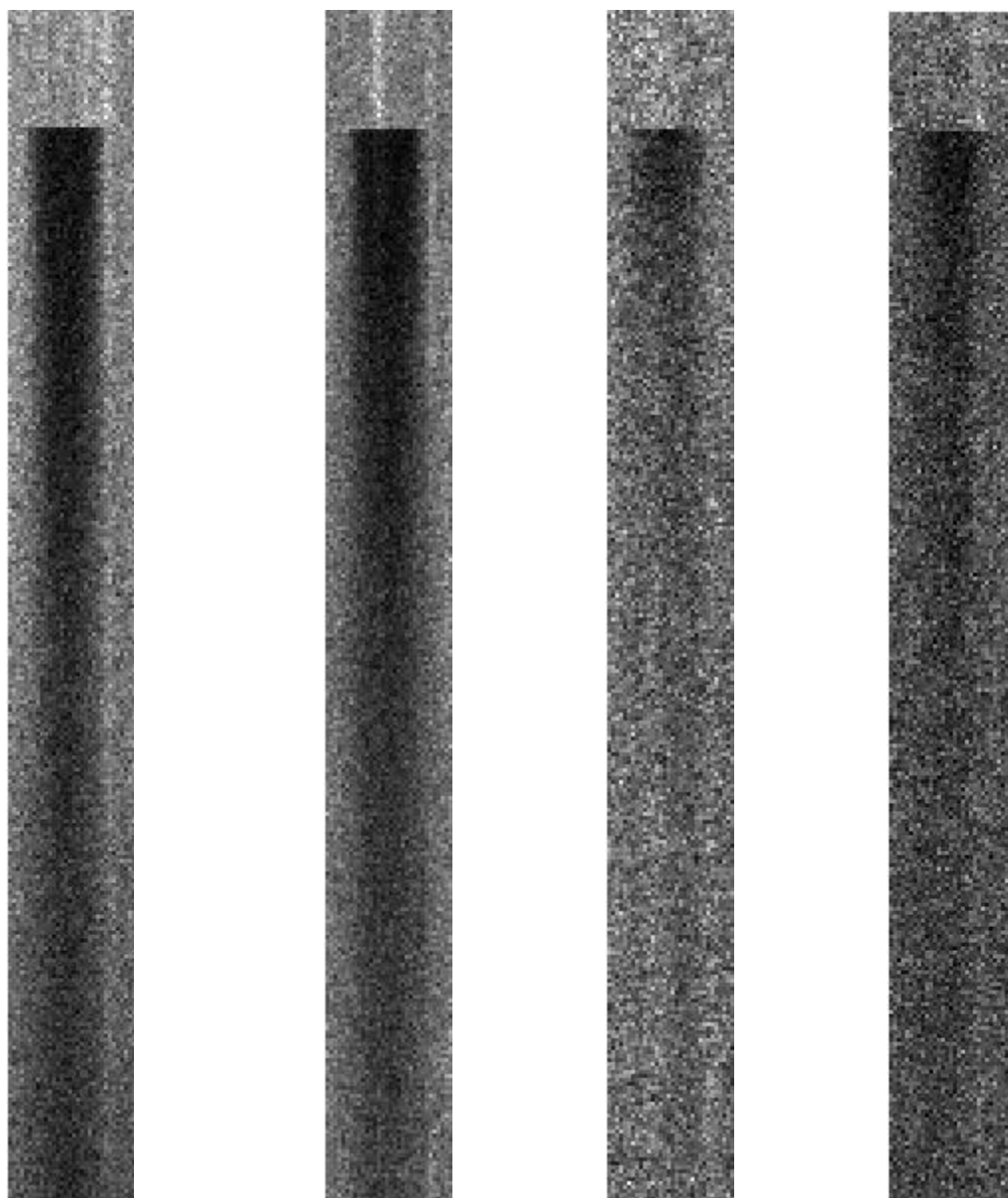


Figure D.3 Kymographs of plasma membrane-resident proteins during FRAP using the Zeiss 880 confocal microscope (bleach area has a radius of 25 pixels) and the time of the movie is 19 s.

These kymographs show that GFP-LTI6b took a relatively longer time to recover than MAP-GFP. They also appear to show that GFP-LTI6b fluorescence recovers from below, whilst MAP-GFP appears to be by lateral diffusion from outside the bleaching area.

A) LTI6b-GFP_Ler_DMSO_S1F1_Kymo;

B) LTI6b-GFP_x8_DMSO_S1F1_Kymo;

C) MAP-GFP_C-0_DMSO_S1F1_Kymo;

D) MAP-GFP_x4_DMSO_S1F1_Kymo

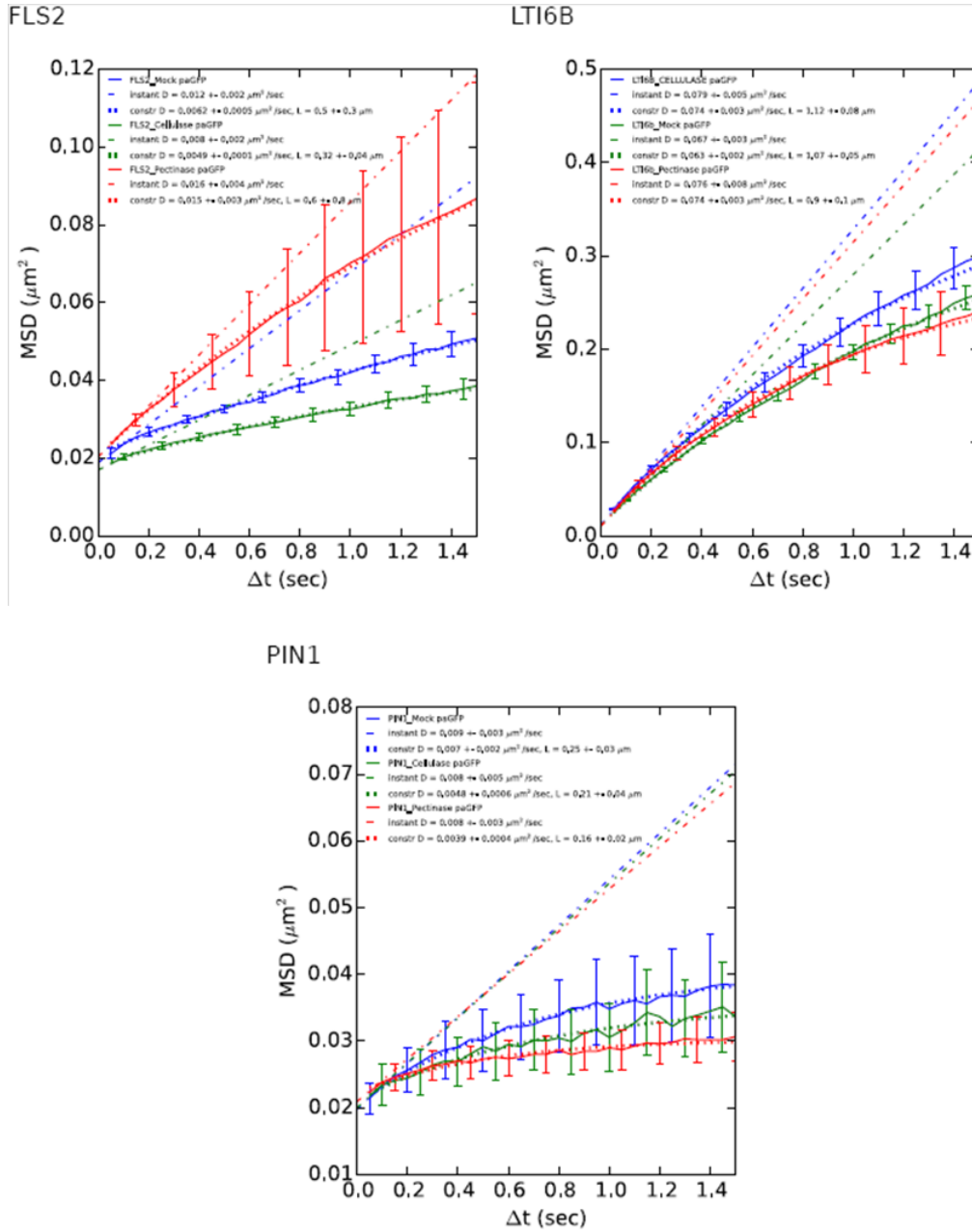


Figure D.4 MSD curves of plasma-membrane resident proteins from single-molecule tracking data (see Section 6.5.4, pg. 208).

(A) MSD of single pFLS2::FLS2-GFP molecules as a function of time.

(B) MSD of single paGFP-LTI6b molecules as a function of time.

(C) MSD of single pPIN1::PIN1-GFP molecules as a function of time.

Time zero on the graph denotes the first measurement.

Appendix E

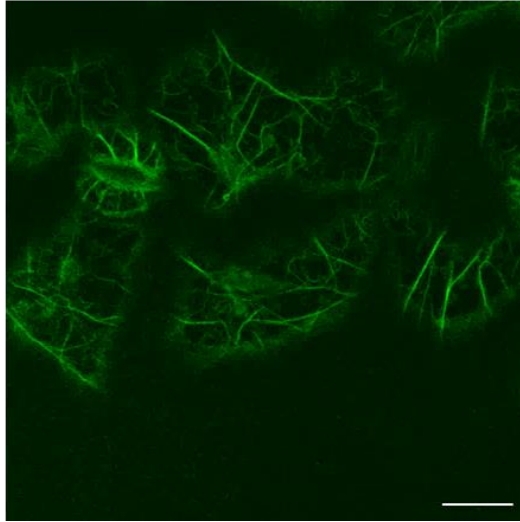


Figure E.1 GFP-Lifeact in green in *xtt1-xtt2*.

Only one seedling was recovered when *A. thaliana* F₁ seeds were treated with the appropriate antibiotic and it did not survive, no more seedlings were recovered.

Scale bar = 20 μ m.

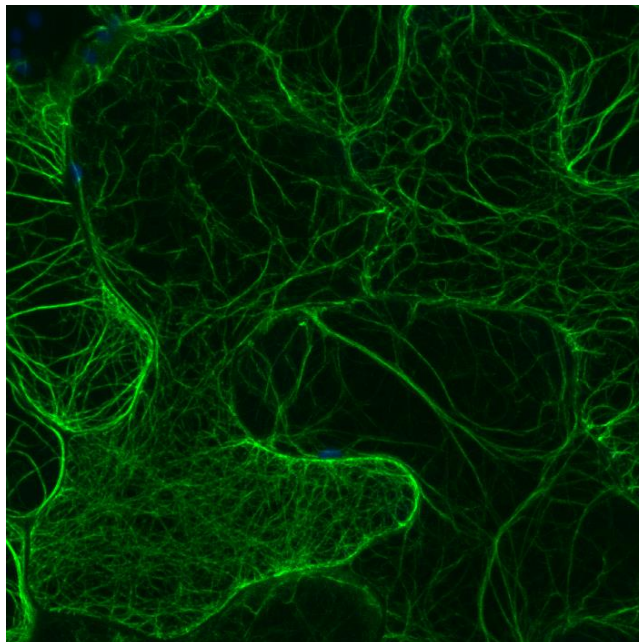


Figure E.2 Illustration of the contrasting morphologies.

Actin cytoskeleton morphology in leaf epidermal pavement cells stably-expressing GFP-FABD2 from *A. thaliana*.