The Microtubule-Associated Protein END BINDING1 Modulates Membrane Trafficking Pathways in Plant Root Cells

by

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Abstract

EB1 protein preferentially binds to the fast growing ends of microtubules where it regulates microtubule dynamics. In addition to microtubules, EB1 interacts with several additional proteins, and through these interactions modulates various cellular processes. Arabidopsis thaliana eb1 mutants have roots that exhibit aberrant responses to touch/gravity cues. Columella cells in the centre of the root cap are polarized and play key roles in these responses by functioning as sensors.I examined the cytoarchitecture of mutant columella cells to determine whether there were subcellular defects that might be correlated with aberrant responses to touch/gravity. No structural differences between mutant and wild type were found. However, by applying the lipophilic dye FM4-64 and actin disturbing drugs, I found that EB1 modulates membrane trafficking pathways possibly through an effect on the actin cytoskeleton. My results suggest that EB1 may affect root responses to touch/gravity signals by modulating membrane trafficking pathways in root cells.

Keywords: End Binding1 (EB1); microtubules; columella cells; membrane trafficking pathways; actin cytoskeleton

To My Dear Mom and Dad, Shamsi & Isa

Thanks for Your Help and Support, and

to My True Love, Negar

I Love You

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List of Acronyms

MT	Microtubule
EB1	End Binding 1
LCSM	Laser Confocal Scanning Microscope
GFP-MBD	Green Fluorescent Protein- Microtubule Binding Domain
AF	Actin Filament
MAP	Microtubule Associated Protein
FM4-64	N-(3- triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridiniumdibromide
Lat B	Latrunculin B
Jas	Jasplankinolide

1. Introduction

Global warming has negatively impinged on agriculture yields through changes in soil conditions, water availability, and vulnerability of plants to disease and pathogens (Rosenzweig et al., 2001). These negative impacts together with an ever-increasing global demand for food and other plant products have motivated scientists to enhance plant yields by improving their tolerance to environmental stresses (Osakabe et al., 2013). Producing transgenic plants with optimized tolerance to environmental stimuli requires an understanding of mechanisms underlying plant responses to various environmental cues (Smith and Smet, 2012; Osakabe et al., 2013).

As sessile organisms that cannot freely move from their ever-changing habitats, plants have evolved an impressive repertoire of mechanisms that they use to rapidly respond to a myriad of developmental and environmental stimuli. To display a fine-tuned and punctual response to stimuli, plants are able to sense diverse stimuli, initiate and synchronize signal transduction pathways, transmit the integrated signal to the site of response, and display a coordinated response (Salisbury, 1993; Kiss, 2000). Each plant organ displays distinct responses to environmental signals (Paul et al., 2013). It is therefore important to understand the mechanisms underlying responses of each organ to diverse stimuli to obtain a comprehensive knowledge on plant responses to environmental stimuli.

Roots play an undeniably important role in plant growth and development as they are primary sites of water and nutrient absorption and they anchor the plant in the soil. The growing root continuously encounters a variety of environmental cues as it penetrates through the soil. Roots are exquisitely sensitive to many different cues in the environment. Roots can respond to environmental cues by altering their direction of growth to take advantage of their surroundings or to bypass obstacles in their path of growth. Directional growth responses of plants that result in curvature or bending of plant organs towards or away from environmental stimuli are called positive and negative

tropisms respectively. Bending is accomplished by changing the rate of cell elongation on one side of the root or stem compared to the other side (Esmon et al., 2005). Primary roots predominantly grow down in response to gravity (positive gravitropism); however, additional tropic signals may modulate this direction (Massa and Gilory, 2003). The direction and trajectory of root growth through the soil is determined via integration of root responses to gravity (gravitropism) and numerous other signals (tropisms) including light (phototropism), touch (thigmotropism), water (hydrotropism), temperature (thermotropism), and chemicals (chemiotropism; Esmon et al., 2005). In the past two centuries, a significant amount of scientific literature on root responses to environmental stimuli has been published. Many of these papers are focused on the mechanisms regulating root gravitropism. The development of scientific tools in model plants such as *Arabidopsis thaliana* in which advanced techniques in genetics, cell biology, and biochemistry can be applied has facilitated investigation of root responses to gravity and other environmental signals.

Arabidopsis thaliana, a small flowering plant in the family Brassicaceae, has been used as a model plant to study the interaction between plant roots and their surrounding environments (Scheres and Wolkenfelt, 1998). The *A. thaliana* root is small and has simple cellular organization which makes it a well-suited model to study root responses to stimuli. This small plant also has a rapid life cycle and produces abundant seeds via self-fertilization. These natural features of *A. thaliana* coupled with its ease of cultivation facilitate the rapid production of many generations in a short time. The genome of *A. thaliana* is small and been completely sequenced. Transgenic plants can be easily generated as *A. thaliana* can be effectively transformed with *Agrobacterium tumefaciens*. *Agrobacterium*-mediated transformations as well as chemical and irradiation-induced mutagenesis have provided a large number of *A. thaliana* mutant lines which are available at The Arabidopsis Information Resource (TAIR). The biological features of *A. thaliana* coupled with its accessible genetic and genomic resources make it an excellent model plant to study root responses to environmental stimuli at both the organ and cellular level (Somerville and Koornneef, 2002; Koornneef and Meinke, 2010).

Of the various environmental cues that the plant responds to, gravity is a ubiquitous and constant signal that directs root growth down in the soil. When the root encounters an obstacle in the soil, the root alters its direction of growth and is able to

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manoeuvre around or bypass it (Massa and Gilory, 2003). To understand how root growth is altered, a common assay is used. In this widely-accepted assay, plants are grown on tilted hard-agar plates. In this case, the root constantly attempts to grow downward (in the direction of the gravity vector), but it hits the impenetrable agar surface and its growth is redirected (Okada and Shimura, 1990). This interaction between the root and the impenetrable surface repeatedly occurs. These contacts and responses result in various root growth patterns including skewing, waving and looping (Migliaccio and Piconese, 2001). Phenotypic analyses of root growth patterns in *A. thaliana* wild type and mutant plants growing on the surface of tilted hard-agar plates provide valuable information about the genes that regulate root responses to touch and gravity (Okada and Shimura, 1990).

Several mutants of *A. thaliana* with impaired root responses to touch and gravity have been characterized. Many of these mutants have defects in the processes that regulate auxin transport. In addition, mutations in signaling molecules or the cytoskeletal system could result in aberrant root responses to touch and gravity. These possible deficiencies that correlate with aberrant root responses to touch and gravity are discussed here. Polar transport of the plant hormone auxin plays a key role in root responses to environmental signals (see sub-section 1.1.2 for details). The direction of auxin flow is mainly regulated by the polar localization of its cellular influx AUXIN RESISTANT1 (AUX1) and efflux PIN FORMED (PIN) carrier proteins. The localization of auxin transporters and their relative abundance at the plasma membrane is controlled through their trafficking (see section 1.2 for details; Bennett et al., 1996; Peer et al., 2011). In a group of mutants defective in root responses to touch and gravity, polar auxin transport is disrupted. Roots of *aux1* and *pin2* mutant plants growing on tilted agar plates form more loops compared to wild type, suggesting that auxin transporters are required for proper root responses to touch and gravity (Mirza, 1987; Okada and Shimura, 1990; Vaughn et al. 2011). In addition, mutations in signalling molecules that regulate PIN trafficking such as the PINOID (PID) protein kinase, protein phosphatase 2A (PP2A), ROP GTPases, and ARF-GEF GNOM result in impaired responses to gravity (reviewed in Yang, 2008) which could, in turn, affect root responses to mechanical cues.

Mutations in some components of the cytoskeletal system also alter root responses to touch and gravity. Mutations in the *DISTORTED1 (DIS1)* gene, which encodes an actin-binding protein in *A. thaliana*, result in impaired root responses to gravity (Reboulet et al., 2010). DIS1 protein binds to the existing actin filaments (AFs) and stimulates the formation of new branch filaments (Amman and Pollard, 2001). Mutations in tubulins, the subunits of microtubules (MTs), as well as some of microtubule associated proteins (MAPs), which cause roots to twist, also affect root responses to touch and gravity. Instead of cortical arrays, MTs in elongating root cells of twisted roots have oblique helical arrays. It is thought that this arrangement of MTs causes roots to twist (Liu and Hashimoto, 2011).

Many, but not all, skewing mutants have twisted roots with oblique helical MT arrays in elongating root cells (Vaughn and Masson, 2011). For instance, *A. thaliana* plants carrying T-DNA insertions in the gene encoding the microtubule-associated protein END BINDING1 (EB1) have skewed roots, while MT in elongating root cells have cortical arrays similar to wild type (Bisgrove et al., 2008; Gleeson et al., 2012). The mechanism by which EB1 exerts its function in root responses to touch and gravity is still unknown. In my thesis, I investigated possible functions of EB1 in roots at the cellular level. In the next sections, I will first discuss what is known about how roots respond to touch and gravity. Then in the second section I will concentrate on the role of membrane trafficking in the regulation of root responses to touch and gravity. In the last section of the introduction, I will focus on the cytoskeletal system and its role in membrane trafficking and root responses to touch and gravity.

1.1. Root Responses to Gravity and Touch

1.1.1. Perception

Growing roots contain five zones; the root cap, the zone of cell division (meristem), the transition zone, the elongation zone, and the zone of maturation (differentiation zone) (Fig. 1; Verbelen et al., 2006).The first portion of the growing root that meets the surrounding environment is called the root cap. The thimble-shaped root cap consists of outer peripheral and inner columella cells (Blancaflor et al., 1998). The

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columella cells are covered and protected by a layer of secretory peripheral cells. The secretory cells produce and discharge a water-soluble polysaccharide mucilage. The hydrated mucilage lubricates the root, which reduces friction between the growing root and the soil thereby enhancing penetration of the root through the soil (Iijima et al., 2004). The root cap has been identified as the most important part of the root in the perception of environmental stimuli including gravity and touch. Removal of the root cap temporarily reduces the root response to gravity until the apical meristem generates a new root cap (Juniper et al., 1966).

The sensory function of the root cap is attributed to its centrally located columella cells. This knowledge has come from studies in which ablating columella cells abolished root curvature in response to a gravitropic stimulus (Blancaflor et al., 1998). The sensory columella cells are positioned in three horizontal tiers with four cells in each tier. The tiers are termed S1, S2, and S3 depending on their proximity to the meristem (Fig. 1- b). The outer layer of columella cells, the S3 tier, occasionally slough off while cells derived from the root apical meristem, the columella initials, expand and differentiate into new columella cells (Dolan et al., 1993; Iijima et al., 2008). Out of the tiers, the ablation of the S2 tier cells exerts the strongest inhibitory effect on root response to gravity; therefore, the central S2 tier of columella cells are thought to be the main sensory cells of the root (Blancaflor et al., 1998; Leitz et al., 2009).
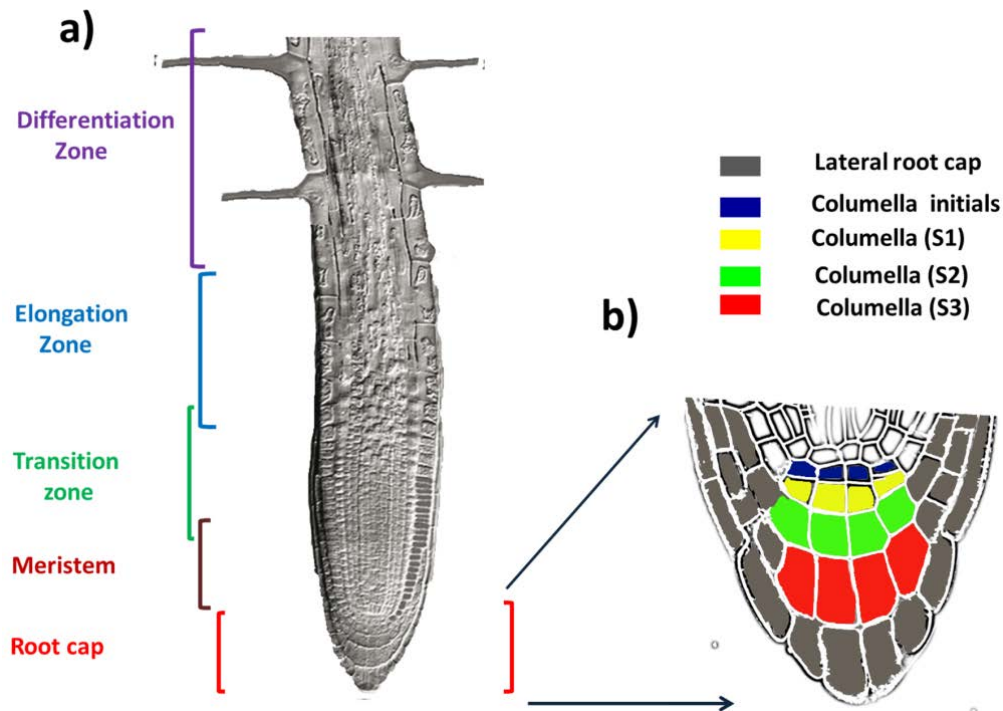
Figure 1.1.   Structure of the *Arabidopsis* root and root cap
Growing roots contain five zones; the root cap, meristem, transition zone, elongation zone, and differentiation zone (a). The sensory columella cells are positioned in three horizontal tiers with four cells in each tier. The tiers are termed S1 (yellow), S2 (green), and S3 (red) depending on their proximity to the meristem. The columella initials (blue) expand and differentiate into new columella cells. The columella cells are protected by lateral root cap cells (gray; b). Part (a) of this figure has been modified from Grieneisen et al., 2007

The columella cells are highly polarized; nuclei are located at the proximal ends of the cells while the endoplasmic reticulum (ER) is mainly positioned at the distal ends of the cells (Zheng & Staehelin 2001; Driss-Ecole et al., 2003). A randomly organized AF network, parallel array of cage-like cortical MTs, and small vacuoles reflect other structural features of columella cells. Starch-filled amyloplasts called statoliths are located at the bottom of the columella cells, where they have settled in response to gravity. The cytoskeleton plays a key role in setting up and maintaining polarity in columella cells (Hilaire et al., 1995). Columella cells treated with MTs and AFs disturbing drugs lose their cellular polarity (Hensel, 1986; Hilaire et al., 1995). It is postulated that the intracellular organization of columella cells facilitates their ability to sense touch and gravity cues (Zheng & Staehelin 2001).

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How do columella cells perceive touch and gravity? Two hypotheses have been put forward to explain the mechanism by which gravity is perceived by columella cells. The starch-statolith hypothesis proposes that the starch-filled amyloplasts (statoliths) in sensory columella cells (statocytes) are responsible for sensing gravity (Sack, 1997). The gravity-induced sedimentation of amyloplasts within the sensory columella cells triggers a signal transduction pathway through activating putative receptors located on the ER or plasma membrane (Kiss, 2000). This hypothesis is supported by the analysis of root responses to gravity in starchless or reduced-starch mutant plants. These plants still can respond to gravity, albeit with a delay (Kiss et al., 1996; MacCleery and Kiss, 1999; Fitzelle and Kiss, 2001). However, in some cases, the hypothesis cannot explain the behavior of roots in response to gravity. For instance, starchless mutant plants display delayed gravitropic responses (Caspar and Pickard, 1989). Therefore, another explanation has been proposed. The protoplast pressure hypothesis suggests that the weight of entire cytoplasm acts as the gravity sensor (Chen et al., 1999). This hypothesis was put forward to explain gravity-mediated polarity of cytoplasmic streaming in large internodal cells of *Chara* algae. It proposes that gravity-mediated position of the protoplast compresses the plasma membrane at the bottom of the cell against the wall and exerts tension between the plasma membrane and the cell wall on the upper side. This induced pressure differential could activate putative stretch-sensitive channels in the plasma membrane and trigger signal transduction pathways within the cells (reviewed in Staves, 1997).

Although many studies have focused on the mechanism of gravity perception in sensory columella cells, the molecular mechanism of mechanosensing has received less attention. As the growing root penetrates downward in the direction of gravity into the soil, it encounters obstacles. To avoid the obstacles, the root detects and navigates around the obstacles and follows a new trajectory. It has been hypothesized that mechanical cues activate putative mechanosensors at the plasma membrane via conformational changes in the cell wall and the plasma membrane. The activation of mechanosensors in turn facilitates ion fluxes into the cell. The transitory changes in ion concentrations in the cytoplasm initiates signal transduction cascades inside the cell (Monshausen et al., 2009).

1.1.2.   Signal Transductions and Response

Although the sensory role of columella cells in gravitropic responses has been well established, the mechanism by which mechanical signals are translated to biochemical signals is still unclear (Strohm et al., 2012). Both gravity and mechanical stimuli trigger rapid changes in the cytosolic levels of several signaling molecules including calcium ions (Ca^{2+}), protons (H^+) and reactive oxygen species (ROS) which are correlated with root responses to touch and gravity (Joo et al., 2001; Perrin et al., 2005; Strohm et al., 2012). The transitory elevation of cytosolic Ca^{2+} is thought to initiate a cascade of signal transduction pathways, which would modulate transcriptional and translational processes required for root responses to touch and gravity (Kimbrough et al., 2004). In addition, the high concentration of cytoplasmic Ca^{2+} might stimulate influx of H^+ from the extracellular space into the cytoplasm as well as the production of ROS in the apoplast (Monshausen et al., 2009). The high concentration of cytoplasmic H^+ influences enzymatic activities as well as expression of regulatory genes that control root responses to touch and gravity. The high concentration of apoplastic ROS strengthen the cell wall and improve its tolerance against mechanical strain during bending responses to touch/gravity (reviewed in Apel and Hirt, 2004; Monshausen et al., 2009). Root bending in response to touch and gravity requires the propagation of signals from the site of perception, the root cap, to the elongation zone where the response, root bending, occurs.

As a biochemical transmitter, the plant hormone auxin (indole-3-acetic acid; IAA) plays a key role in root responses to environmental signals (Schrader et al., 2003). Auxin is polarly transported cell-to-cell from its main site of biosynthesis in the shoot apex through the vascular tissue to the root tip (Müller and Leyser, 2011; Peer et al., 2011). In columella cells, the central and basipetal direction of the auxin stream is redirected laterally and acropetally through epidermis and cortex cells. In vertically-oriented roots, auxin is uniformly distributed around the root in cells of the root cap and the outer cell layers of elongation zone. In roots that have been reoriented horizontally, amyloplasts are displaced to the new bottom side of columella cells. This displacement of amyloplasts somehow triggers signaling events that result in an uneven distribution of auxin across the root. Auxin flows more to the lover flank than to the upper flank of the root (Yoder et al., 2001; Friml et al., 2002). This asymmetric distribution of auxin results

in downward curvature of the roots. The higher concentration of auxin in the lower flank of the root reduces the cell growth in its elongation zone and induces the bending of the root toward the new gravity vector (Rashotte et al., 2001; Ottenschläger et al., 2003). The auxin flow in root cells depends on polar localization of auxin carrier proteins at the plasma membrane. Endocytosis and subcellular trafficking of auxin transporters mediate their asymmetric localization and as a result the differential distribution of auxin in root cells. Therefore, membrane trafficking plays a key role in signal transduction pathways in root responses to touch and gravity (Strohm et al., 2012).

1.2. Membrane Trafficking Pathways and Root Responses to Touch and Gravity

The flow of membrane between the plasma membrane and endomembrane compartments coupled with intracellular membrane trafficking, herein called the membrane trafficking pathways, facilitate transportation of macromolecules to their intracellular and extracellular destinations (Cheung and Vries, 2008). Membrane trafficking pathways connect the plasma membrane and the endomembrane system, which comprises endosomes, Golgi apparatus, ER, and the vacuole (Fig. 1.2; Contento and Bassham, 2012). As intermediaries of membrane trafficking, membrane-enclosed vesicles are formed and pinched off from donor membranes, transported and fused to acceptor membranes. Exchange of materials between the plasma membrane and endomembrane compartments is mediated through different routes of membrane trafficking pathways (Fig. 1.2): the endocytic pathway, the exocytic pathway, and transcytosis.

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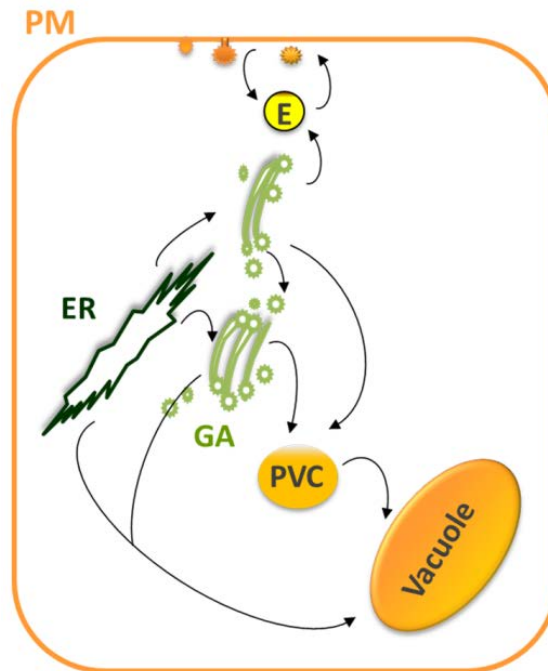
Figure 1.2. Membrane trafficking pathways in plant cells
Membrane trafficking pathways connect the plasma membrane (PM) and the endomembrane system, which comprises endosomes (E; yellow), Golgi apparatus (GA; light green), the endoplasmic reticulum (ER; dark green), and the vacuolar apparatus (the prevacuolar compartment (PVC) and the vacuole)

The retrograde endocytic pathway internalizes vesicles containing extracellular milieu and plasma membrane associated components. After fission from the plasma membrane and internalization, vesicles fuse to early endosomes and are then either recycled back to the plasma membrane or transported to the Trans Golgi (TNG) or vacuoles for sorting or degradation (Robinson et al., 1998; Samaj et al., 2012b). Due to the unique features of plant cells, i.e., rigid cell wall and high turgor pressure, the existence of endocytosis in plant cells was questioned for a long time. However, a great deal of studies in the past decade has repeatedly confirmed that this event occurs in both aerial and under-ground plant cells (Samaj et al., 2005). Endocytosis is categorized into two different pathways: clathrin-mediated and clathrin-independent endocytic pathways (reviewed in Miaczynska and Stenmark et al., 2008). In the well-characterized clathrin- mediated pathway, clathrin and its associated adaptor proteins mediate the formation of receptor-containing coated pits at the plasma membrane. Accessory and regulatory proteins facilitate the invagination of coated pits to form coated vesicles. Clathrin- independent pathways do not use clathrin, instead, they exploit a variety of

mechanisms to select cargos and form endocytic vesicles. Similar to animal cells both clathrin-mediated and clathrin-independent pathways have been reported in plant cells (reviewed in Mayor and Pagano, 2007; Miaczynska and Stenmark et al., 2008). However, the well-characterized clathrin-mediated pathway is most widely studied in plants.

The anterograde secretory or exocytic pathway transports cargo from the ER to Golgi apparatus, vacuoles and the plasma membrane for sorting, modification, degradation, and recycling (Connerly, 2010). In plants, the role of this pathway in tip-growth and in the final stage of cell division, cytokinesis, in plants has been widely studied. In tip-growing plant cells and cytokinesis, exocytosis deliver Golgi-derived vesicles containing new membrane and cell wall materials to the growing tips and the new cell plate, respectively (Bednarek and Falbel, 2002). This pathway plays a role in trafficking of auxin transporters in root cap cells (Kleine-Vehn et al., 2010).

In the third membrane trafficking pathway, transcytosis, the macromolecular cargos are translocated from one side of the plasma membrane cell to other sides (Peer, 2011). Membrane trafficking performs an integral role in variety of cellular processes, including signal transduction pathways and cell morphogenesis , which endow rooted plants with the ability to monitor their surroundings and re-adjust their growth in response to different environmental stresses (Peer, 2011).

The role of membrane trafficking in polar auxin transport is an interesting and highly active area of plant research. Polar auxin transport is important in plant responses to environmental signals (Friml et al., 2002; Nakayama et al., 2012). Polar auxin flow is controlled mainly by the localization of auxin transporters to the ends or sides of the cells through which auxin is transported. Auxin transporters are located in the plasma membrane and facilitate the flow of auxin into and out of the cell. Auxin enters the cell by diffusion and the AUX1 auxin influx carrier proteins (Zažímalová et al., 2010). In the relatively acidic environment of the apoplast, auxin is mainly protonated (IAAH) in which this neutral form of auxin easily enters the cell via passive diffusion. On the other hand, the charged form of auxin (IAA^-) is actively transported from the intracellular space into the cell through AUX1 receptors. Auxin exits the cell only through the PIN auxin carrier proteins. In the neutral environment of cytoplasm, auxin is deprotonated (IAA^-) in which

this charged form of the auxin molecule is not able to cross the plasma membrane and exits cells via the PIN efflux carrier proteins (Raven, 1975; Estelle, 1998; Michniewicz et al., 2007).The polar localization of auxin transporters at the plasma membrane of columella cells as well as epidermis and cortex cells direct the flow of auxin in the root. Out of eight members of the PIN family in *Arabidopsis*, PIN3 and PIN2 play key roles in the differential distribution of auxin during gravity stimulation (Friml et al., 2002; Kleine-Vehn et al., 2010; Ambrose et al., 2013; Kakar et al., 2013). Auxin transporters are not fixed at the plasma membrane. In response to different environmental signals, auxin carrier proteins cycle between the plasma membrane and endomembrane compartments by the endocytic and exocytic pathways. They are also translocated from one side of the plasma membrane to other side by transcytosis (Friml et al., 2002; Dhonukshe et al., 2008; Kleine-Vehn et al., 2010).

In vertically-oriented roots, PIN3 is symmetrically dispersed around the plasma membrane of columella cells and directs auxin flow evenly in all directions through the root cap cells. Also, PIN2 is mainly localized at the shootward side of the plasma membrane in root epidermal cells and directs auxin flow equally towards the shoot. In roots that have been reoriented horizontally, PIN3 relocates to the new basal side of columella cells via transcytosis. This new localization of PIN3 redirects auxin flow preferentially to the lower side of the root cap, leading to a high concentration of auxin on this side. This higher concentration of auxin is needed to be directed from the root cap towards the shoot. In the lower side of the root, PIN2 retains in the plasma membrane (its endocytosis is inhibited) and this improves the movement of auxin flow on this side. The low concentration of auxin in the upper side is also directed towards the shoot. In this side, PIN2 is more degraded in vacuoles (fewer PIN2 is recycled back to the plasma membrane) because it directs the low amount of auxin in this side. Membrane trafficking pathways mediate the polar localization of auxin carriers via recycling, degradation or translocation processes (reviewed in Kleine-Vehn and Friml, 2008).

Several signaling molecules such as ROP GTPases and ARFs as well as a number of the cytoskeletal elements have been shown to be involved in regulation of membrane trafficking pathways (Samaj et al., 2004). However, the exact regulatory mechanism of these pathways is yet to be identified. Next, some of the tools used to

study membrane trafficking will be described and then the role of the cytoskeleton in root responses to touch and gravity will be discussed.

1.2.1.   Tools to Study Membrane Trafficking

Much of our knowledge on membrane trafficking has been obtained using state-of-the-art live cell imaging techniques to monitor the trafficking of fluorescent probes in combination with applying genetic and pharmaceutical approaches. The advent of the membrane-selective fluorescent FM-dyes in the late 1980s has provided a unique experimental probe to study membrane trafficking (Griffing, 2008). Among FM-dyes, FM4-64 with distinct features has been wildly used for studying membrane trafficking in plant cells. The dye fluoresces intensely and has a high photostability. In addition, its emission spectrum does not overlap with GFP, which facilitates using the dye to label cells expressing GFP tagged proteins (Bolte et al., 2004). Due to the amphiphilic nature of FM-dyes, these dyes are not able to cross the plasma membrane bilayer. The dyes are non-fluorescent in aqueous media and they become intensely fluorescent only after inserting into the outer leaflet of the membrane (Bolte et al., 2004; Ivanov et al., 2008). Upon incorporation into the plasma membrane, the dye molecules are internalized into the cell via endocytosis, from where they either are recycled back to the membrane or transported to different endosomes (Helling et al., 2006). The vacuolar membrane is the final destination of FM4-64; about one hour after applying FM4-64 to *A. thaliana* roots, the dye stains vacuolar membranes in root cells. Therefore, the dye has widely been applied to study endocytosis as well as the vacuolar morphology in various eukaryotes including plants (Vida and Emr, 1995; Ueda et al., 2001; Bolte et al., 2004; Tamura et al., 2010).

Advanced imaging techniques such as confocal laser scanning microscopy (CLSM) accompanied with subcellular targeting methods including fluorescent proteins have enhanced the ability to visualize and examine vesicular transport in living cells. There are several key features that make CLSM a valuable and efficient tool for a wide range of studies in cell biology. The pinhole apparatus eliminates out-of-focus light and provides high optical resolution and signal-to-noise images. This feature facilitates acquisition of typical stack of optical sections or a Z-series without the need for physical sectioning of thick samples. In addition, CLSM affords the ability to acquire

multidimensional images (x, y, z, and time). Furthermore, the versatile advanced image analysis software enables data acquisition through quantitative image analyses (Claxton et al., 2006; Zhang et al., 2013).

The pharmacological approach is a commonly used method to study membrane trafficking pathways in plant cells. A variety of drugs that disturb proteins involved in membrane trafficking pathways have been identified by chemical screening methods. The advantage of the pharmacological approach is that drugs can be applied at different times and with different doses. This can be an advantage in cases where mutations in relevant genes are lethal (review in Samaj et al., 2012b). However, it should be noted that a specific drug can have multiple targets in the cell. This can confound the interpretation of results since membrane trafficking pathways are complex and interconnected.

1.3. The Cytoskeleton and Root Responses to Touch and Gravity

MTs and AFs as well as their associated proteins comprise the major structural components of the cytoskeletal system in plants. MTs and AFs are highly dynamic biopolymers. The intrinsic dynamic properties of MTs and AFs enable them to undergo rapid reorganization in response to developmental and environmental signals. The arrangement of MTs and AFs are regulated by their binding proteins. Cytoskeletal binding proteins also facilitate the interaction of MTs and AFs with other cellular components. Through these interactions, the cytoskeleton could modulate a variety of cellular processes. These biopolymers have been implicated in a myriad of cellular activities including membrane trafficking pathways, cell division and elongation, and generating / maintaining cell polarity. MTs and AFs fulfill their cellular functions independently or in a coordinated manner (Petrasek et al., 2009). A role for the cytoskeletal system in root responses to touch and gravity stimuli has been reported (Bisgrove et al., 2008; Gleeson et al., 2012); however, the mechanisms underlying its role and possible interactions between different cytoskeletal elements in these responses have yet to be established.

1.3.1.   The Actin Cytoskeleton

The actin cytoskeleton coupled with a plethora of actin binding proteins mediates a variety of cellular activities involved in plant responses to developmental, biotic and abiotic stimuli (Blancaflor, 2012). ATP bound globular actin (ATP-G-actin) monomers are polymerized into AFs in a polar manner with two distinct ends: fast- growing barbed ends and slow -growing pointed ends (Littlefield et al., 2001). During polymerization, the ATP-G-actin monomer is added to the fast growing end of AFs. The ATP bound to the polymerized actin monomers is hydrolyzed into ADP+Pi. Then Pi is slowly released from AFs leaving behind the ADP-G-actin monomer within AFs. During depolymerization, the ADP-G-actin monomer is disassembled from AFs and nucleotide exchange of ADP for ATP generates the ATP-G-actin monomer, which can then be added to the barbed ends of AFs (Fig. 1.3; Chaudhry, et al., 2007).
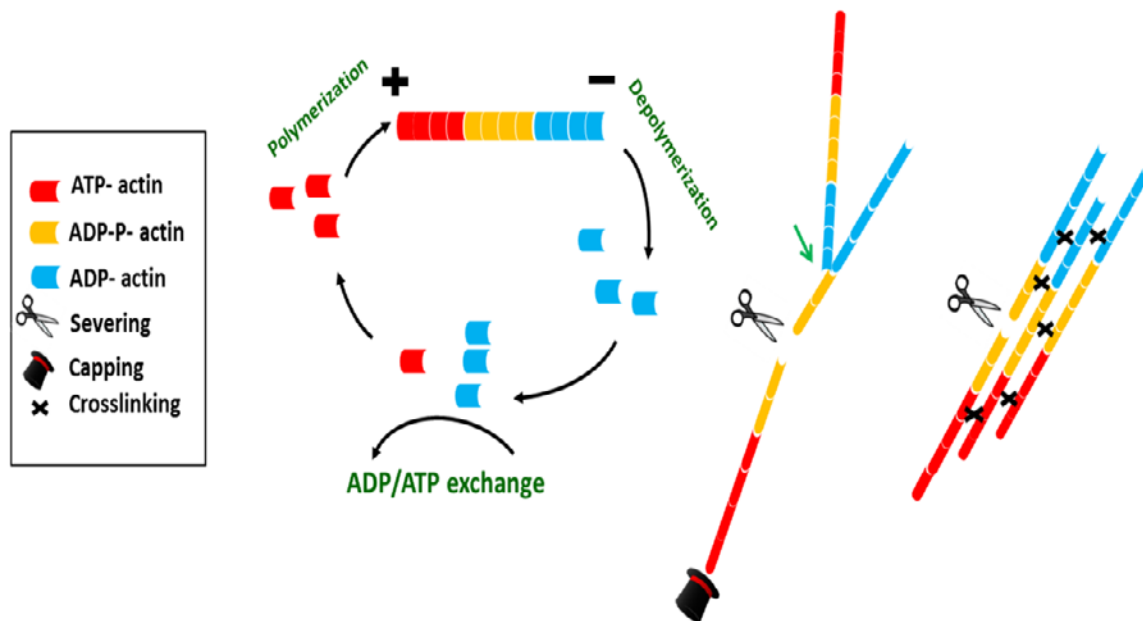
Figure 1.3.   Actin filament dynamics and organization in plant cells
ATP-actin monomers (red) are polymerized into AFs in a polar manner with two distinct ends: barbed ends (+) and pointed ends (-). The ATP bound to the polymerized actin monomers is hydrolyzed into ADP+Pi and generates the ADP-P-actin monomer (yellow) within AFs. Then Pi is slowly released from AFs leaving behind the ADP-actin monomer (light blue) within AFs. During depolymerization , the ADP-actin monomer is disassembled from AFs and nucleotide exchange of ADP for ATP generates the ATP-actin monomer. Actin binding proteins regulate AF assembly, disassembly, and organization by facilitating filament formation, branching (green arrow), severing (scissors), capping (hat), and cross-linking (cross).

The ability of actin to polymerize and depolymerize endows AFs with the capability to undergo reorganization in response to different signals (Henty-Ridilla et al., 2013). Actin binding proteins regulate AF assembly, disassembly, and organization by facilitating filament formation, stabilization, destabilization, severing (breaking AFs), capping (preventing further polymerization), and cross-linking (bundling AFs) (Fig. 1.3; reviewed in Gopinathan et al., 2007). Two distinct configurations of the actin cytoskeleton have been reported in plant cells: extremely dynamic mesh-like actin networks (or actin meshworks) and relatively more stable bundled actin networks (or actin bundles) (Volkmann and Baluska, 1999; Ananthakrishnan and Ehrliche, 2007).

The dynamic mesh-like actin networks are usually located in the cortex of plant and animal cells in close association with the plasma membrane (Thomas, 2012). In yeast, animal, and plant cells, the cortical actin and its regulatory proteins such as the actin-related protein (Arp) 2/3 complex are involved in endocytosis (reviewed in Samaj et al., 2012a). In addition, the mesh-like actin networks are involved in the polar growth of tip-growing plant cells as well as in the local growth of the puzzle-shaped leaf pavement cells. AF bundles in plant cells are thought to function as passive highways. AF associated motor proteins myosins travel along AFs during cytoplasmic streaming and mediate long range movement of vesicles/endosomes and subcellular organelles (Kuroda, 1990; Smertenko et al., 2010).

Distinctive AF organizations in tip-growing cells including pollen tubes and root hairs have received much attention in studying the role of the actin cytoskeleton in plant cells. In a tip-growing cell, the shank contains AF bundles, the sub-apex has less bundled AFs and the apex has more dynamic mesh-like actin networks. These AF arrangements are involved in movement of endosomes containing the plasma membrane and cell wall materials to the growing tip as well as in retrieval of excess secreted materials into the cell (reviewed in Ovecka et al., 2005). Endosomal movements in the shank and the sup-apex area of root hairs are mediated by actin motor proteins, myosins, which use AFs as tracks. In addition to actin meshworks, the tip in a tip-growing cell contains a high population of exocytic and endocytic vesicles as well as endosomes. Treatment with a low concentration of the actin destabilizing drug Latrunculin B (Lat B) inhibited the movement of vesicles and endosomes in the apex of the tip-growing cell; however, disrupting the ATPase activity of myosins failed to inhibit

the movement of endosomes in this region, suggesting a role for the dynamic actin meshwork in membrane trafficking (Voigt et al., 2005; Wang et al. 2006).

In addition to tip-growing cells, the actin cytoskeleton has been implicated in membrane trafficking in other aerial and underground plant cells. To date, most experiments aimed at investigating the role played by the actin cytoskeleton in plant cells have been based on pharmacological approaches. Treatment with high concentration of Lat B that destabilizes both actin meshworks and actin bundles inhibits endocytosis in root epidermal cells (Konopka et al. 2008). On the other hand, relatively low concentration of Lat B induces the formation of small intracellular aggregates in these cells (Samaj et al., 2012a). In addition, high concentration of the actin stabilizing drug Jasplakinolide (Jas) inhibits endocytosis in root cells (Dhonukshe et al., 2008). Yet, the exact role of the actin cytoskeleton in membrane trafficking pathways is unknown. Nagawa et al. (2012) have proposed that the cortical AFs could act as an inhibitory barrier to endocytosis in plant cells. This proposal was put forward based on their observation that overexpression of ROP2 GTPase and its effector Rac4 increased the accumulation of mesh-like cortical actin networks and inhibited endocytosis in leaf epidermal pavement cells. Treatment ROP2 GFPase or Rac4 overexpressed lines with a low concentration of Lat B, which reduces the accumulation of cortical actin and has no effects on actin bundles, restored endocytosis in these cells. Also, stabilization of cortical actin with a relatively low concentration of Jas in these cells inhibited endocytosis. On the other hand, Bloch et al. (2005) have reported that treatment with a relatively low concentration of Jas has no effect on endocytosis in root hairs.

The actin cytoskeleton also has been proposed to play a role in perception of gravity in sensory columella cells (reviewed in Blancaflor, 2012). Columella cells contain cortical mesh-like actin networks coupled with the organized cytoplasmic actin bundles (Collings et al., 2001). Treatment with the actin destabilizing drug Lat B significantly promotes the gravitropic responses (bending) in roots that have been reoriented horizontally (Mancuso et al., 2006). This observation has led to two proposals about the possible role of actin in gravitropsm. The first hypothesis proposes that the cytoplasmic network of AFs in columella cells acts as barrier against the movement of amyloplsts in these sensory cells. The destabilization of AFs may promote amyloplast sedimentation to the new bottom of the columella cells in reoriented roots and enhance gravity

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perception in root sensory cells. Another hypothesis suggests that destabilization of the cortical actin in columella cells with Lat B could promote root responses to gravity by affecting the trafficking of PIN3 in columella cells (Blancaflor, 2012). In vertically oriented roots, PIN3 is equally distributed around the columella cells and directs auxin flow evenly in all directions through the root cap cells. In roots that have been reoriented horizontally, PIN3 relocates to the new lower side of columella cells. This new localization of PIN3 redirects auxin flow preferentially to the lower side of the root cap (Friml et al., 2002). This high concentration of auxin in the lower side is then directed to the shoot via PIN2, which results in a higher concentration of auxin in the elongation zone on the lower side of the root. The hypothesis suggests that destabilization of cortical AFs in columella cells might promote relocalization of PIN3 and enhance root responses to gravity (Blancaflor, 2012).

1.3.2. Microtubules

After more than half a century of studies on the MT cytoskeleton, our understanding about the roles of MTs in plant cells is extending beyond cell division and expansion (Wasteneys 2013). The hallmark of MTs is that they are intrinsically dynamic polymers; they continuously undergo polymerization (growing), depolymerization (shrinking), pausing (no significant growth or shrinkage), and rebuilding (rescue; Mitchison and Kirschner, 1984). MTS are filamentous polymers of α/β-tubulin heterodimers. These tubulin subunits are GTP-binding proteins; the GTP bound to α-tubulin does not hydrolyze, while the GTP bound to β-tubulin can be hydrolyzed to GDP (Tian et al., 1999). The longitudinal assembly of dimers in a head-to-tail fashion results in the formation of linear protofilaments. The lateral interaction of these protofilaments leads to the formation of a cylindrical MT with two distinctive ends: plus (fast -growing) and minus (slow- growing) ends (Kirschner and Mandelkow, 1985; Wu et al., 2009). The GTP bound states of tubulin dimers assemble onto the sheet-like structures at the fast growing ends of MTs (polymerization). The sheet-like structure rapidly zips up in a cylindrical MT. After GTP-bound heterodimers bind to the MT, GTP in β-tubulin is hydrolyzed to GDP (depolymerization; Kirschner and Mandelkow, 1985). At the plus end of the MT, GTP-bound heterodimers are added at a higher rate than the rate which the hydrolysis of GTP occurs. This generates a GTP cap at this end. In a polymerizing MT,

the GTP cap stabilizes the MT and induces the association of GTP-bound subunits with the growing ends of the MT. Further back in the MT lattice, GDP-bound subunits are less tightly bound to MTs. During depolymerization, GDP-bound subunits are disassembled from MTs. When the incorporation rate of GTP-bound subunits in the MT is slower than the hydrolysis rate of GTP, the GTP cap is lost. This results in a rapid dissociation of GDP tubulin subunits from the MT and a rapid shrinkage of MT. This transition of MTs from growth to shrinkage is called catastrophe (Dhamodharan, 1995). When new GTP-bound subunits are added to the shrinking MT, a GTP cap is formed and this switches the MT from shrinkage to growth (rescue; Fig. 1.4).
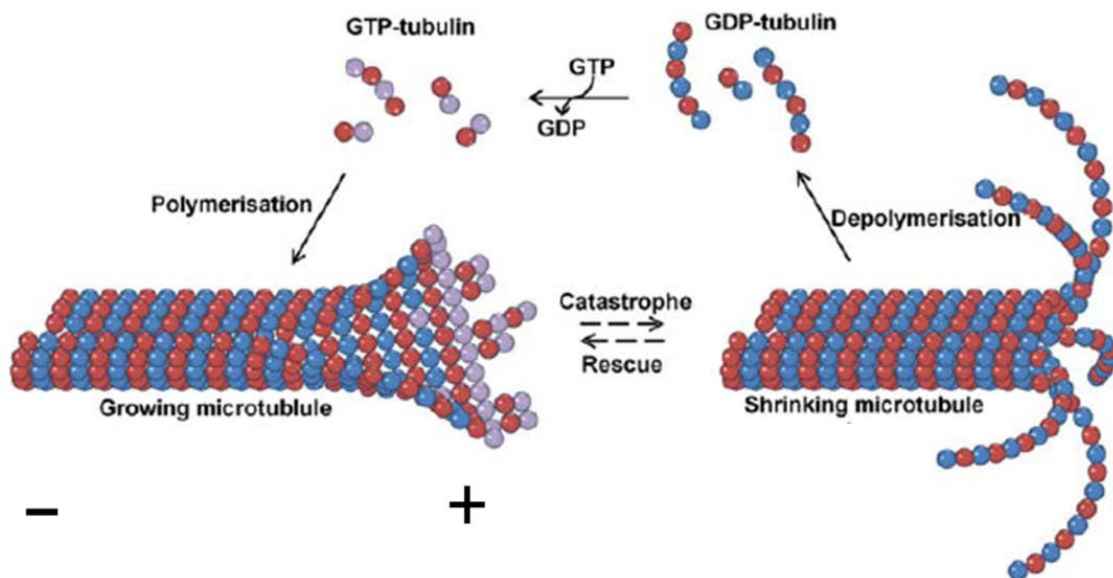
Figure 1.4.   Assembly, disassembly and dynamic instability of microtubules
Microtubules are intrinsically dynamic polymers; they continuously undergo polymerization (growing), depolymerization (shrinking), pausing (no significant growth or shrinkage), and rebuilding (rescue). The GTP bound states of tubulin dimers (red- pink beads) assemble onto the sheet-like structures at the fast growing ends of MTs (+) and this generates a GTP cap at this end (polymerization). Polymerization rate at the plus end of the MT (+) is faster than the minus end (-). After GTP-bound heterodimers bind to the MT, GTP in β-tubulin is hydrolyzed to GDP (red-blue beads). GDP-bound subunits are disassembled from MTs (depolymerization). Transition from growth to shrinkage is known as catastrophe and transition from shrinkage to growth is known as rescue. This figure has been modified from Calligaris et al., 2010.

The dynamic instability of MTs underlies their ability to display diverse arrangements and perform various functions during different stages of the plant cell cycle. In interphase of somatic plant cells, MTs display a cortical array that facilitates cell expansion via modification of the cell wall (Bisgrove, 2008). In elongating cells, MTs

beneath the plasma membrane guide the direction of cellulose synthetase and as a result the orientation of cellulose microfibrils. This means that cellulose microfibrils are oriented parallel to MTs and perpendicular to the long axis of the cell. Cellulose microfibrils are resistant to stretching along their length and this feature constrains direction of cell expansion. The isotropic outward turgor pressure supplies the force that increases the distance between the adjacent cellulose microfibrils, resulting in anisotropic cell expansion. Prior to mitosis, the unique MT arrays, the so- called preprophase band (PPB), encircles the nucleus. The PPB somehow defines the future division site. In metaphase of mitosis, the PPB is replaced by the mitotic spindle. MTs in spindles are arranged in a bipolar manner; their minus ends are at the cell poles, whereas their plus ends are towards the center of the cell. The mitotic spindle mediates the alignment of chromatids at the center of the cell and their subsequent segregation to the two daughter cells. As the cell enters cytokinesis, the spindle MTs rearrange into a distinctive arrangement, the so-called phragmoplast, composed of two set of MTs with opposite polarity between daughter nuclei (Wasteneys, 2002). The phragmoplast contributes to the delivery of Golgi-derived vesicles towards the forming cell plate.

The dynamic properties and activities of MTs are modulated by a fleet of MT-associated proteins (MAPs) that bind directly to MTs. Some MAPs preferentially accumulate at the minus or the plus ends of MTs, while others associate with the MT lattice (Liu et al., 2011). In animal cells, the minus ends of MTs are anchored at MT organizing centers (MTOCs), centrosomes, which are comprised of centrioles and pericentriolar material (PCM; Rusan and Rogers, 2009). The minus end of the MT is embedded in PCM and it contains proteins such as γ-tubulin that are involved in MT nucleation (Oakley, 1999). MTs grow outwards from centrosomes through the cytoplasm into the cortex of the cell. Plant cells lack centrosome-like MT organizing centers, but they have some of the proteins found in PCM of animal cells. For example, γ-tubulin is dispersed in the cytoplasm and it binds to the minus ends of MTs in plant cells. Several MAPs such as MICROTUBULE ORGANIZATION 1 bind to the MT lattice and regulate MT organization and function (Kawamura et al., 2006). Furthermore, Microtubule motor proteins, kinesins, use MTs as tracks to carry cargo within the cell in a directional manner: towards plus ends (N-kinesins) or towards minus ends(C-kinesins). Another group of MAPs preferentially bind and track the MT plus ends; these ones are

known as MT plus end-tracking proteins or +TIPs (Akhmanova and Steinmetz, 2008). The localization of +TIPs at the active ends of MTs allows them to interact with other cellular components and structures and, therefore, modulate a variety of cellular activities including signal transduction pathways. Out of +TIPs, the End Binding 1(EB1) family is thought to be a key regulator of MT functions, since it regulates MT dynamics and recruits different types of cargo proteins to the plus ends of microtubules (Akhmanova and Steinmetz, 2008).

The role of MTs in root responses to touch and gravity is not well understood. Since MTs in interphase plant cells are associated with the plasma membrane, it has been postulated that cortical MTs in roots might facilitate the perception of environmental stimuli including touch and gravity through activation of putative microtubule-gated membrane ion channels or interaction with potential stretch-activated membrane ion channels (Nick, 2008). The fact that MTs regulate cell expansion by determining the orientation of cellulose microfibrils raises another possibility about the role of MTs in root responses to gravity and mechanical cues. The hypothesis proposes that gravity and mechanical signals reorganize MT arrays in roots and this reorganization induces root bending. In support of this idea is the observation that during root bending MTs on the outer side of the root MTs rearrange from parallel to transverse arrays, whereas on the inner side of the root MTs retains their parallel array. The different orientations of MTs on the two sides of the root could alter the direction of cellulose microfibrils deposition and the direction of root growth, resulting in root bending. However, whether the different MT orientations on the two sides of roots are responsible for root bending or whether MT rearrangements are induced by root bending is still controversial (reviewed in Bisgrove, 2008). There is evidence that supports the idea that MTs reorient after root bending. In horizontally-oriented maize roots, the early stage of root bending occurred before microtubule reorientation (Blancaflor and Hasenstein, 1997; Bisgrove, 2008). MT dynamics and functions are regulated by MAPs; therefore, detailed studies of MAPs are fundamental in our understanding about the role of MTs in root responses to touch and gravity. In *A. thaliana*, EB1 plays a role in root responses to touch and gravity (Bisgrove et al., 2008; Gleeson et al., 2012); however, the mechanism underlying its role in these responses is unknown.

1.3.3. EB1

EB1 is an evolutionarily conserved protein with homologues found in eukaryotes ranging from yeast to humans and plants. Studies on the protein in animals, fungi, and plants have revealed that it binds directly to the fast growing ends of MTs where it regulates growth and shrinkage rates of the MTs (Vitre et al., 2008). In addition to MTs, EB1 in animal cells interacts with a large number of additional proteins in the cell. Through these interactions, EB1 influences a variety of cellular processes (Akhmanova and Stehbens, 2008). Studies aimed at disclosing the mechanism underlying EB1 interaction with other proteins have characterized two groups of proteins as putative EB1 binding partners: proteins containing the short polypeptide motif SxIP (serine-any amino acid-isoleucine-proline; Honnappa et al., 2009), and proteins with Cytoskeleton-Associated Protein-Glycin-rich (GAP-Gly) domains which bind to EEY motif of EB1 (Weisbrich et al., 2007). Plant EB1 proteins lack the EEY motif and proteins with the CAP-Gly domains are not found in plant cells. This indicates that this mode of interaction between EB1 and other proteins might not exist in plants (Akhmanova and Stehbens, 2008; Liu et al., 2011). Identifying putative EB1 binding proteins in plants would reveal possible signaling pathways regulated by EB1 proteins. The *A. thaliana* genome encodes a myriad of proteins with SxIP motif (personal communication with S. Squires). Whether *Arabidopsis* EB1 proteins interact with the SxIP motif –containing proteins is still unknown.

Since EB1 regulates MT dynamics and it interacts with other proteins, it has been proposed to be a master regulator of MT plus end (Liu et al., 2011). The *Arabidopsis thaliana* genome contains three EB1 genes: *EB1a, EB1b, and EB1c* (Chan et al., 2003; Mathur et al., 2003). EB1 proteins are dimeric proteins with two conserved domains: a calponin homology (CH) domain at the N-terminus and a dimerization domain at the C-terminus (Fig. 1.5). The CH domain is responsible for the interaction of EB1 with the fast growing ends of MTs (Slep et al., 2005). Several hypotheses have been put forward to explain the mechanism by which EB1 recognizes the plus ends of MTs. One explanation is that EB1 might preferentially bind to the extended tubulin sheet structure at the fast growing ends of MTs rather than to the tubular wall of MTs. Another hypothesis proposes that EB1 identifies the GTP bound state of β-tubulins confined to the plus ends of MTs. Nevertheless, the exact mechanism of the plus end tracking behavior of EB1 is

still unknown (Buey et al., 2011). The C-terminal of EB1s contains an alpha-helical coiled coil, a flexible tail and EB-homology (EBH) domain (Komarova et al., 2005; Slep et al., 2005). The coiled coil region and the flexible tail determine EB1s dimerization, whereas EBH is responsible for interaction of EB1s with additional proteins (Komarova et al., 2005). While C-terminal tails in EB1a and EB1b are acidic, the tail in EB1c is basic and directs EB1c to the nucleus. In addition, in vitro studies have revealed that EB1a and EB1b are able to form heterodimers with each other but not with EB1c. The fact that EB1c does not dimerize with EB1a and EB1b and its location in the nucleus led to the proposal that EB1c might have a role in spindle formation in plant cells (Komaki et al., 2010). T-DNA insertion mutants with reduced expression of the three *EB1* genes have been identified in *A. thaliana* (Bisgrove et al., 2008). Analyses of these mutant plants are providing valuable information about the function of EB1 proteins in plant cells.
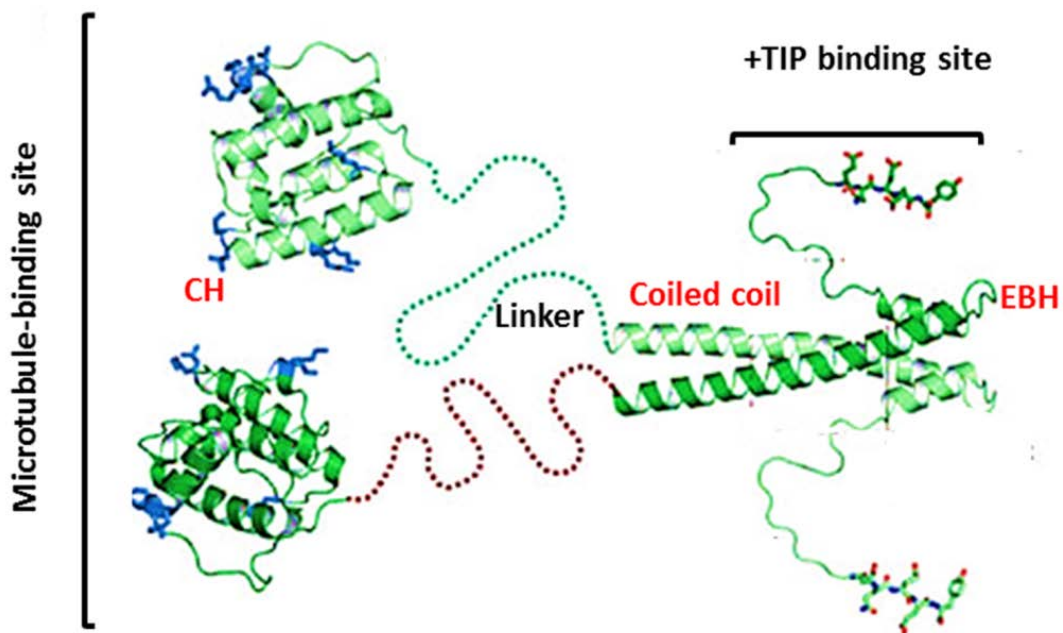
Figure 1.5.   Schematic illustration of structural organization of homodimeric EB1 proteins
EB1 proteins are dimeric with two conserved domains: a calponin homology (CH) domain at the N-terminus and a dimerization domain at the C-terminus. The CH domain mediates the interaction of EB1 with the fast growing ends of MTs. The C-terminus of EB1 contains a coiled coil, a flexible tail (linker) and an EB-homology (EBH) domain. The coiled coil region and the linker are responsible for dimerization, whereas the EBH domain mediates interactions between EB1 and its cargo proteins. This figure has been modified from Akhmanova and Steinmetz (2008)

By studying the role of EB1 proteins in plant growth and development, our lab has found that these proteins modulate root responses to touch and gravity (Bisgrove et al., 2008; Gleeson et al., 2012; Squires et al., 2013). Plants carrying T-DNA insertions in each of the *EB1* genes have roots that exhibit exaggerated responses to touch and gravity. When grown on the surface of reclined agar plates, roots of *eb1* mutants skew more to the left and form more loops than wild type. *A. thaliana eb1b-1* and *eb1* triple mutants have the same phenotypic defects in their responses to touch/gravity cues, suggesting that *eb1b-1* has the main role in these responses (Bisgrove et al. 2008). The fact that *eb1b* mutant roots skew more and form more loops than wild type suggests that the wild type protein acts as a repressor of the response (Bisgrove et al., 2008; Gleeson et al., 2012).

1.4. Thesis Objectives

In my thesis, I investigated whether EB1b, as a master regulator of +TIPs, could alter root responses to touch/gravity cues through an effect on the subcellular organization of columella cells or on membrane trafficking pathways in root cells. This investigation involved two sets of experiments:

1.   Mutant root caps were examined for possible subcellular defects that might be correlated with an aberrant response to these stimuli. I examined the arrangement of MTs, the distribution of the ER, and the position of nuclei in columella cells.

2.   Membrane trafficking pathways were studied in root cells. I applied a membrane trafficking marker to investigate the role of EB1 in membrane trafficking pathways in root cells of wild type and eb1 mutants.

2. Materials and Experimental Approaches

2.1. Plant Material and Growth Conditions

Wild type and the *eb1b-1* mutant *A. thaliana* lines used in our study were of Wassilewskija (Ws) ecotype. The *eb1b-1* mutant, carrying a T-DNA insertion in the *EB1b* gene, has been previously characterized (Bisgrove et al., 2008; Gleeson et al., 2012; Squires and Bisgrove, 2013). Seeds were sterilized according to the vapour-phase protocol (Clough and Bent, 1998). Seeds were put in eppendorf tubes with open lids and the tubes were placed on an eppendorf tube rack in a glass jar. 100 ml bleach was poured in a plastic beaker and then 3 ml concentrated hydrogen chloride was added to the bleach afterwards. The beaker was placed inside of the glass jar and its lid was tightly sealed. Seeds were incubated for 45 minutes (min) inside of the glass jar.Sterilized seeds were placed on hallf- strength Murashige and Skoog medium (MS, Sigma-Aldrich) supplemented with 1% (w/v) sucrose, 0.1% (w/v) 2-(N-morpholino) ethanesulfonic acid (MES), 1% (w/v) agar (Phytablend, Caisson Laboratories Inc.), with the final pH adjusted to 5.8. Plates containing sterilized seeds were incubated at 4ºC in the dark for 3-4 days to break seed dormancy. Then the petri dishes were sealed with micropore tape (3M Health Care) and placed in a growth chamber in a vertical position for 3-5 days. Parameters for the growth chamber were 16 h light/ 8 h dark and 20ºC.

GFP-MBD (Columbia-0 (Col-0); Marc et al., 1998; Granger and Cyr, 2001) and DR5rev::GFP (Col-0; Friml et al., 2003; Palme et al., 2006) seeds were obtained from The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org/). GFP-MBD and DR5rev::GFP plants were crossed to the *eb1b-1* mutant plants and homozygous lines were identified by analyzing the F3 generation (personal communication with Dr. Bisgrove; Squires and Bisgrove, 2013). EB1 rescued and overexpressed lines were generated and characterized by others in the lab. These lines were generated by transforming *A. thaliana eb1b-1* mutants with wild type *EB1b* gene under control of its

endogenous promoter pEB1b:EB1b (Gleeson et al., 2012; C. Chen, S. Squires and L. Vita, unpublished data).

2.2. Confocal Laser Scanning Microscopy

Fluorescence images were captured by a Nikon Eclipse Ti inverted laser scanning confocal microscope system (CLSM). Images were acquired using a 60x water immersion objective lens (NA 1.2). GFP fluorophores were excited at 488nm and the emission collected at 525-550nm. For FM4-64 detection, excitation was 561 nm and the emission was collected at 575 nm. All image analyses were performed using Nikon Advanced Research analysis software. All confocal setup parameters were kept constant in all experiments in which the uptake of FM4-64 was monitored.

2.3. Quantification of Cell Polarity Parameters

3-4-day-old *A. thaliana* wild type and the *eb1b* mutant seedlings expressing fluorescent reporter proteins were used to determine the spatial location, distribution, and arrangement of subcellular compartments and structures. The distribution of ER was determined by calculating the ratio of the circumference of the distal ER to the circumference of the corresponding cell. To examine nuclear position, two distances were measured: the distance of the center of nucleus to the proximal cell wall (Y), and the distance of the center of nucleus to the outer cell wall (X). The nuclear position was defined as the ratio of X to the cell length and Y to the cell width.

2.4. FM4-64 Staining

FM4-64 (N-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide; Molecular probes, Invitrogen Life technologies) was stored as a 2mM stock solution in Dimethyl sulfoxide (DMSO) at 4ºC and in the dark. To monitor endocytosis, 3-4-day-old seedlings were incubated in liquid MS medium containing 2μM FM4-64 for 5 min in the dark at room temperature, rinsed twice for 1min each time in liquid MS medium. The dye was washed out at this step to remove all the

dye molecules that have not been incorporated into the plasma membrane. This allowed monitoring of the amount of the dye that is lost from the plasma membrane during endocytosis. Seedlings were then mounted on slides in liquid MS medium for 3 min. The slides were covered with cover glasses and imaged by CLSM beginning 6 min afterwards. Therefore, the cell had an overall time of 15 min to endocytose the dye. FM4-64 was 15 min.

To stain the vacuolar membrane, 4-5-day-old seedlings were incubated in 5 µM FM4-64 for 10 min in the dark at room temperature, rinsed twice for 1min each time in liquid MS medium and then incubated in liquid MS medium for 2 hours.

2.5. Quantification of FM4-64 Uptake

To analyze the uptake of FM4-64 through endocytosis, confocal images consisted of 6 sequential optical z sections taken at 0.5µm intervals were compressed. To quantify FM4-64 uptake, the mean fluorescence intensity of the cytoplasm excluding the plasma membrane and the nucleus (MFC) and the mean fluorescence intensity of the corresponding plasma membrane (MFP) were measured. FM4-64 uptake was defined as the ratio of MFC to MFP. Nikon Advanced Research analysis software was used to create compressed images as well as measuring the fluorescence intensity.

2.6. Pharmaceutical Treatments

Brefeldin A (BFA) (Sigma-Aldrich) was prepared as a 1.5 mM stock solution in ethanol and stored at 4ºC in the dark. For BFA treatment, 3-4-day-old seedlings were incubated in liquid medium containing 25 µM BFA for 30 min. Then BFA treated seedlings were stained with 5 µM FM4-64 containing 25 µM BFA for 5 min in the dark at room temperature for 30 min and imaged with CLSM.

For BFA washout experiments, two sets of seedlings were used: one set as a control line and the other as BFA washed-out set. Both sets of seedlings were placed in liquid medium containing 25 µM BFA for 30 min. The seedlings were stained with 5 µM FM4-64(with 25 µM BFA in the control lines) for 5 min in the dark at room temperature.

The seedlings then were incubated in liquid medium (with 25 µM BFA in the control set) for 2 hours and then imaged with CLSM.

Oryzalin (Sigma-Aldrich) was dissolved in DMSO to yield a stock solution of 10mM and then stored at 4ºC in the dark. To examine the effects of oryzalin on FM4-64 uptake, 3-4-day-old wild type and *eb1b* mutants grown on agar medium containing 50 nM oryzalin were used. Latrunculin B (Sigma-Aldrich) was stored at -20ºC as a 5 mM stock solution in DMSO. To assess the effects of Lat B on FM4-464 uptake, 3-day-old wild type and *eb1b* mutants grown on agar medium supplemented with 10 nM Lat B were used. A 100 µg/ml stock solution of Jasplakkinolide (Jas; Sigma-Aldrich) was prepared in DMSO and stored at -20ºC in the dark. To investigate the effects of Jas on FM4-64 uptake, 3-4-day-old seedlings grown on 100 nM Jas were used. In seedlings treated with oryzalin, Lat B, and Jas, FM4-64 uptake was monitored based on the processes described in section 2.4, except, liquid MS medium used in all the processes (staining, rinsing, and mounting) contained the same concentration of drugs.

2.7. Amyloplast Staining and Visualization

Amyloplasts were visualized by staining their starch with Lugol's iodine solution (1% (w/v) iodine and 2% (w/v) potassium iodide). 3-4-day-old seedling were immersed in 1% Lugol's solution diluted with MS liquid for 2-3 min in the dark and rinsed in liquid MS medium for 2 min. The stained seedlings were mounted on slides in a clearing solution that contained 8:3:1(w/v/v) chloral hydrate, liquid MS medium, and glycerol respectively. Slides were vertically positioned for 15 min and imaged immediately with CLSM.

2.8. Statistical Analysis

In all experiments, a Student's T-test (using Excel) was used for pair-wise comparisons of means. Means, standard deviations, and P values were all calculated using Excel. All FM4-64 uptake experiments were repeated at least three times and

each replicate contained at least one root for each genotype/treatment. In each root, at least eight cells were analyzed.

Experiments aimed at investigating subcellular architecture of columella cells and amyloplast distributions in these cells were repeated as follows: MT arrays were examined in six independent seedlings for each genotype. Distal ER distribution was examined in eight seedlings for each genotype from four independent experiments. Nuclear positions were assessed in four independent experiments having two seedlings for each genotype. Amyloplast distribution was visualized in two seedlings for each genotype.

3.   Results

In my thesis I studied the role of EB1 in root responses to touch and gravity at the cellular level. The Arabidopsis thaliana genome has three *EB1* genes: *EB1a*, *EB1b*, and *EB1c*. Plants carrying T-DNA insertion with reduced expression of each of these genes has been identified (Bisgrove et al., 2008). These mutants have defects in responding to touch/gravity stimuli. Roots of the mutant plants grown on reclined agar plates skew and form more loops than wild type seedlings. *A. thaliana eb1b-1* and *eb1* triple mutants demonstrate the same phenotypic defects in responding to touch/gravity cues (Bisgrove et al. 2008; Gleeson et al., 2012). Consequently, my work is focused on *eb1b-1* mutants.

3.1. Cytoarchitechture of Root Cap Columella Cells

Columella cells in the center of the root cap function as sensory cells in roots. These specialized cells are highly polarized, and it has been postulated that their distinctive structure facilitates their function in mechano- and gravity-sensing (Sack, 1991; Blancaflor et al., 1998; Chen et al., 1999, Zheng and Staehlin 2001). Since roots of *eb1b* mutant plants exhibit aberrant responses to touch/gravity stimuli (Bisgrove et al., 2008; Gleeson et al., 2012), I investigated the possibility that the abnormal response might be due to structural defects in columella cells. My first series of experiments was aimed at examining the cytoarchitecture of columella cells in wild type and *eb1b-1* mutant roots. Specifically, the arrangement of MTs, ER distribution, nuclear positioning and the distribution of amyloplasts were assessed.

3.1.1.   Microtubule Organization

To determine whether *eb1b* mutants display MT organizational defects in root-tip cells, transgenic plants expressing a MT reporter, the MT binding domain (MBD) of the

mammalian microtubule-associated protein 4 (MAP4) fused to green fluorescent protein (GFP), (GFP-MBD; Marc et al., 1998; Granger and Cyr, 2001) were examined by CLSM. The MBD binds to MTs and enables in vivo visualization of MT arrays in plant cells (Marc et al., 1998). Parallel arrays of cortical MTs were observed in columella cells of wild type roots (Fig. 3.1- a), which is consistent with previously published reports (Hensel, 1984; Hilaire et al., 1995; Baluska et al., 1996; Yoder, 2001). MT arrays in mutants were also organized in parallel arrays that closely resembled the arrays observed in wild type plants (Fig. 3.1- b). In summary, there are no defects in MT organization in *eb1b-1* mutants.
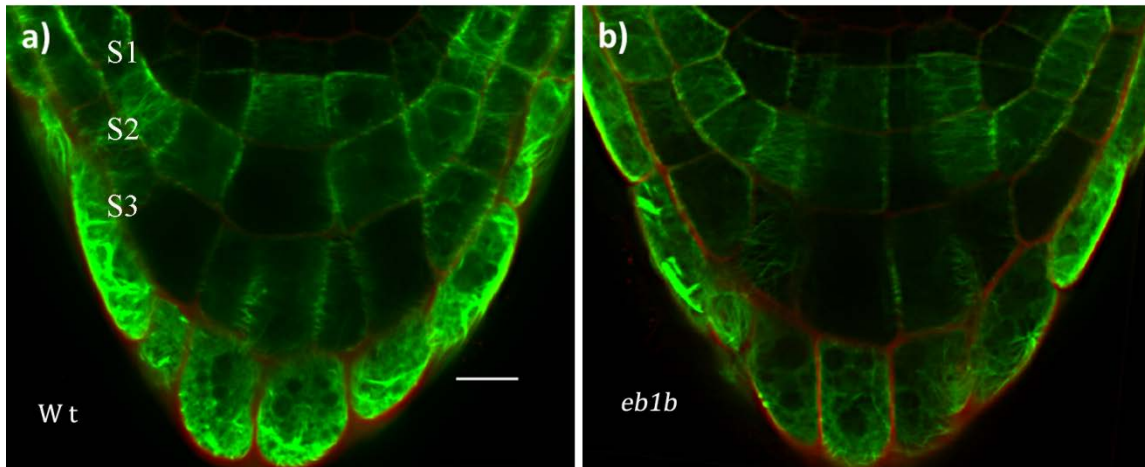
Figure 3.1.   Examination of microtubule arrays in *A. thaliana* wild type and *eb1b* mutant root cap cells
Three-old-day *A. thaliana* seedlings expressing MBD-GFP (green) were used to examine the arrangements of MTs in columella cells. Parallel arrays of MTs were observed in the cortex of columella cells in both wild type (a) and the *eb1b* mutant (b). Roots were stained with FM4-64 to visualize the plasma membrane (red). Similar results were obtained from six independent seedlings for each genotype. Each image is a single optical section. Scale bar =50 μm.

3.1.2.   The ER is Localized at the Distal Ends of *eb1b* Mutant Columella Cells

To examine the distribution of ER in columella cells, we used transgenic plants expressing the widely used auxin-responsive DR5*rev*::GFP reporter. This construct includes GFP to which an ER retention signal is attached, so GFP is targeted to the ER (Friml et al., 2003; Palme et al., 2006). Localization of the ER to the distal ends of columella cells was quantified by calculating the ratio of the circumference of the distally-

localized ER to the circumference of the corresponding cell (Fig. 3.2- a). In columella cells of wild type, the ER was mainly located at the cortex of the cells and more concentrated at distal ends, as previously reported (Sack, 1991; Chen et al., 1999). The ER in *eb1b* columella cells also exhibited the same localization pattern as those of wild type. The comparison of the average ratios for *eb1b* and wild type revealed statistically indistinguishable values (Student's T-test, P=0.99), suggesting that ER localization is equivalent in columella cells of both genotypes (Fig. 3.2- b).
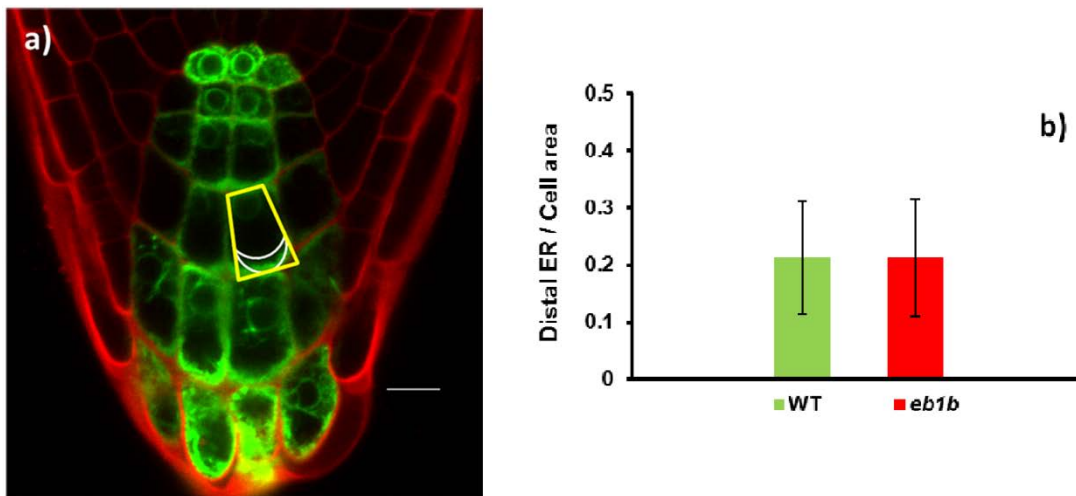
Figure 3.2. **Examination of the distribution of ER in wild type and *eb1b* mutant columella cells**
The ER in columella cells (green) was visualized in 3-4-day-old wild type seedlings expressing the auxin-sensitive reporter DR5rev::GFP with an ER retention signal (a). The relative distribution of ER was quantified by determining the ratio of the circumferences of the distal ER in the central S2 columella cells (white crescent-shaped area) to the circumference of the corresponding cell (yellow trapezoid-shaped area). ER localization is equivalent in columella cells of both genotypes (Student's T-test, P=0.99) (b). The plasma membrane was stained with FM4-64 (red). Confocal microscopic images were taken from eight seedlings for each genotype in four independent experiments. Size bar in (a) = 50 μm and the black bars in (b) represent standard deviations (SD).

3.1.3. Nuclei Display the Same Position in Columella Cells in Wild Type and *eb1b* Mutants

To visualize nuclei, transgenic plants expressing the auxin reporter construct DR5*rev*::GFP with a nuclear localization signal were used (Heisler et al., 2005). Nuclear position was quantified by calculating two ratios: 1) the ratio of the distance of the nucleus from the proximal cell wall to the cell length: 2) the ratio of the distance of nucleus from the outer cell wall to the cell width (Fig. 3.3- a). Nuclei in wild type

columella cells were positioned at the proximal outer corner of the cells, which is consistent with previously published reports (Sack, 1991; Chen et al., 1999). Nuclei in *eb1b* mutant columella cells displayed the same localization pattern as those of wild type. Comparisons of mutant and wild type columella cells revealed no detectable differences in nuclear position between the two genotypes (Student's T-test, P1= 0.47 and 0.86) (Fig. 3.3- b).

Figure 3.3.   Assessment of nuclear position in wild type and *eb1b* mutant columella cells
Three-old-day seedlings expressing DR5rev::GFP were used to visualize nuclei (green) in wild type columella cells (a). The membrane-selective dye FM4-64 was applied to roots to visualize the plasma membrane (red). Nuclear position is equivalent in columella cells of both genotypes (Student's T-test, P= 0.47 and 0.86) (b). Data shown is the average of four independent experiments having 2 seedlings for each genotype. Size bar in (a) = 50μm and error bar in (b) represents (SD).

3.1.4.   Amyloplast Distribution in Columella Cells

The sedimentation of starch-filled amyloplats to the bottom of central columella cells is a key factor in gravi-sensing (Sack, 1991; Kiss, 2000). It is, therefore, possible that the altered responses of *eb1b* mutant roots to touch/gravity cues result from aberrant positioning of sedimented amyloplasts in columella cells. This possibility was addressed by observing amyloplast position in gravity-oriented wild type and mutant columella cells stained with Lugol's iodine solution to visualize starch.

As expected, the specialized starch-containing plastids in wild type cells were concentrated at the distal ends of the sensory columella cells (Fig. 3.4- a). However, amyloplasts in *eb1b* mutant cells were more scattered throughout the sensory cells (Fig. 3.4- b).
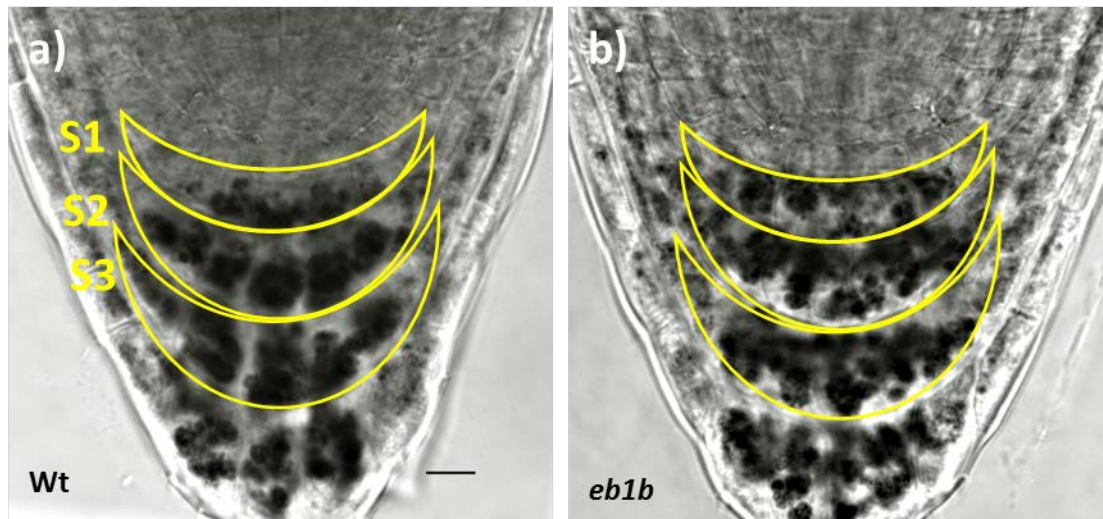
Figure 3.4.   Examination of amyloplast distribution in wild type and *eb1b* mutant columella cells
Amyloplasts were visualized by staining starch with Lugol's iodine solution (black). Amyloplasts in wild type columella cells are more concentrated at the bottoms of the cells (a) compared to those of the *eb1b* mutant (b). Different tiers of columella cells (S1, S2, and S3) are shown by yellow crescents. Scale bar is 50µm and applies to both confocal images. Similar results were obtained from two independent seedlings for each genotype.

2.5. EB1b has a Role in Membrane Trafficking in *A. Thaliana* Root Cells

To assess whether columella cells of *eb1b-1* mutants have defects in membrane trafficking, uptake of the lipophilic styryl dye, FM4-64, was analyzed. Due to the amphiphilic nature of FM4-64, the plasma membrane prevents its internalization by diffusion. The dye is fluorescent only after inserting into the plasma membrane (Bolte et al., 2004; Ivanov et al., 2008). Endocytosis internalizes the dye into the cell in which it is either recycled back to the plasma membrane or transported to TNG and the vacuole membrane (Helling et al., 2006).

The uptake of FM4-64 into columella cells was assessed five min after exposure of roots to the dye; no FM4-66 labeled compartments were detected in either wild type or *eb1b* mutants. Fifteen min after applying the dye, FM4-64 labeled compartments in columella cells of *eb1b* mutants were clearly visible, whereas no internalization of FM4-64 was observed in wild type columella cells (Fig. 3.5). After monitoring the internalization of the dye for 30 min, FM4-64-labeled compartments were clearly detected in root cap cells of both genotypes, although; the uptake of the dye in *eb1b* mutants was more than wild type.
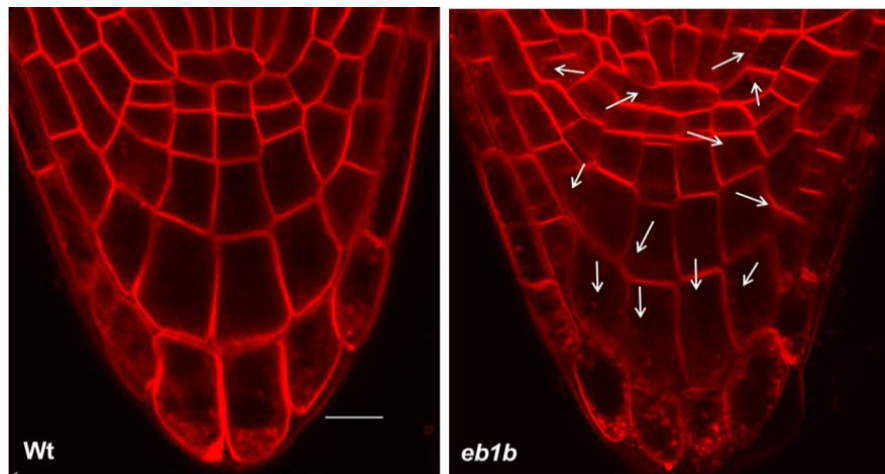
Figure 3.5.   FM4-64 uptake in wild type and *eb1b* mutant columella cells
FM4-64 uptake in root cap cells of 3-day-old *A. thaliana eb1b* mutants (b) was detectable as punctuate structures 15 min after exposure to the dye, while in wild type no noticeable FM4-64-positive compartments were found (a). Images are single confocal sections (0.5 µm). Size bar is 50µm.

Visualization of FM4-64 uptake in columella cells is difficult for two reasons: 1) Columella cells are located deep inside the root, which makes it very difficult to monitor FM4-64 internalization in these cells; 2) The root cap is surrounded with mucilage secreted by peripheral root cap cells. This mucilage could limit the accessibility of columella cells to the dye. Therefore, FM4-64 uptake was also analyzed in cells of the root transition zone. In general, uptake of the dye occurred more rapidly in these cells than it did in the root cap. In contrast to root cap cells, five min after exposure to FM4-64, the dye was detectable inside of small subcellular compartments in both wild type and *eb1b* mutant cells. However, there were more FM4-64 labeled compartments in *eb1b* mutants compared to wild type (Fig. 3.6- a - b). The internalization of the dye was

monitored for 15 min after applying the dye. Confocal images obtained at that time were used to assess the relative uptake of FM4-64. To quantify FM4-64 uptake, the ratio of the mean fluorescence intensity of the cytoplasm to the mean fluorescence intensity of the plasma membrane was calculated. Statistical analyses revealed that FM4-64 uptake in *eb1b* mutants was significantly higher than in wild type (Student's T-test, $P<0.0001$).

FM4-64 uptake was also monitored in transgenic *eb1b* mutants transformed with a wild type copy of the *EB1b* gene under the control of the endogenous *EB1b* promoter. Previous analyses have shown that root responses to touch/gravity cues in these seedlings are equivalent to those of wild type plants, indicating that the transgene is capable of rescuing the root growth defects observed in mutants (Gleeson et al., 2012). To investigate whether FM4-64 uptake is also restored to wild type levels, the rescued line (RL) was monitored 15 min after exposure to the dye. FM4-64 uptake in RL root cells closely resembled that of wild type (Fig. 3.6- c). Statistical analyses also confirmed that FM4-64 uptake rate in RL root cells was not significantly different from wild type (Student`s T-test, $P=0.92$; Fig. 3.6- e). To obtain more insights into potential roles for EB1 in FM4-64 trafficking, uptake of the dye was also monitored in an *EB1b* overexpressing line (OE). The OE line was generated through transformation of *eb1b-1* mutants with wild type *EB1b* gene under control of its endogenous promoter pEB1b:EB1b (Gleeson et al., 2012). The OE line has approximately 2-fold greater expression of *EB1b* gene than wild type measured by qPCR (L. Vita, unpublished data). The relative FM4-64 uptake in OE line was significantly higher than in wild type and lower than in *eb1b* mutants (Fig. 3.6- d- e). Taken together, these results suggest a role for EB1b in membrane trafficking in root cells.
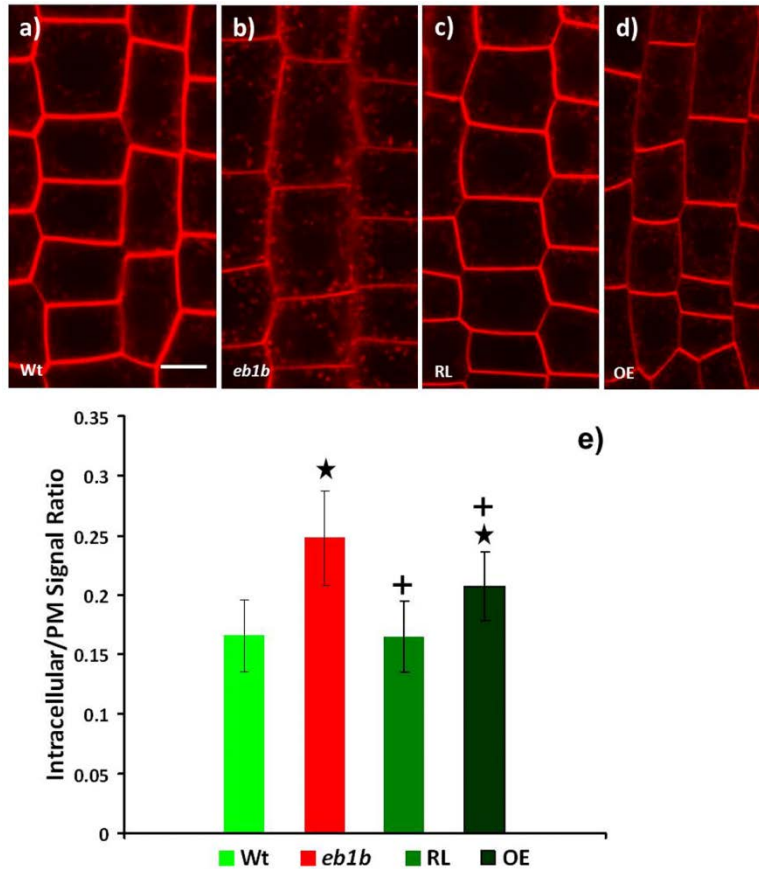
Figure 3.6.   FM4-64 uptake in wild type and *eb1b* mutant transition zone cells
Uptake of FM4-64 in cells in the transition zone of roots in 3-4-day-old seedling was observed 15 min after loading with the dye. FM4-64 uptake in wild type (a) *eb1b* mutants (b) RL (c) and OE (e) is shown. FM4-64 uptake rate in *eb1b* mutants was statistically higher than in wild type, RL, and OE lines (e) (Student's T-test, $P < 0.005$). Images shown here are single confocal sections (0.5 μm). Compressed images consisted of 6 sequential optical z sections taken at 0.5 μm intervals, were used to quantify the uptake of FM4-64. Scale bar in (a) indicates 50 μm and applies to all images. The results are mean values obtained from three independent experiments. Averages that are significantly different (Student's T-test, $P < 0.05$) from wild type or *eb1b* are denoted by Symbols * and + respectively.

3.1.5.   The Role of Eb1 in Membrane Trafficking Depends on Intact Microtubules

EB1 is a microtubule-associated protein, therefore; I investigated the effects of MT disruption on FM4-64 uptake. The MT disturbing drug oryzalin was applied to partially depolymerize MTs. Oryzalin is a dinitroaniline herbicide that directly binds to plant tubulin and inhibits the assembly of MTs (Morejohn et al., 1987).

I first established optimized concentrations of the drug that partially depolymerize MTs. Based on previously published reports (Baskin et al., 2004; Nakamura et al., 2004) two different concentrations of the drug were selected: 50 nM, and 200 nM. Root morphology in 3-day-old wild type seedlings grown on agar medium containing aforementioned concentrations of oryzalin as well as 0 nM oryzalin (control) were examined. Roots in control seedlings show normal root morphology with slight radial expansion in the meristem, as previously reported (Baskin et al., 1994; Fig. 3.7- a). Seedlings grown on 50 nM oryzalin still display normal root morphology with slight radial expansion in the meristem as well as the elongation zone (Fig. 3.7- b). However, in seedlings grown on 200 nM oryzalin, both the meristem and the elongation zone of roots undergo significant radial expansion. In addition, anisotropic expansion of cells in the elongation zone was impaired (Fig. 3.7- c). I also assessed the effects of the aforementioned concentrations of oryzalin (0 nM, 50 nM, and 200 nM) on MT arrays in root cells of plants expressing GFP-MBD. MTs displayed parallel cortical arrays in expanding root cells in *A. thaliana* control seedlings consistent with previously published reports (Wasteneys, 2002; Fig. 3.7- d). MTs in expanding root cells of seedlings grown on 50 nM oryzalin were partially depolymerized, but the intact MTs retain their ordered cortical array (Fig. 3.7- e). MT depolymerization was more severe in 200 nM oryzalin (Fig. 3.7- f). The degree of MT depolymerization and the impairments to anisotropic expansion of root cells in seedlings treated with 200 nM oryzalin indicate that this concentration has a severe effect on roots. Based on these results, I chose 50 nM oryzalin to investigate the effects of partial disruption of MTs on FM4-64 uptake.
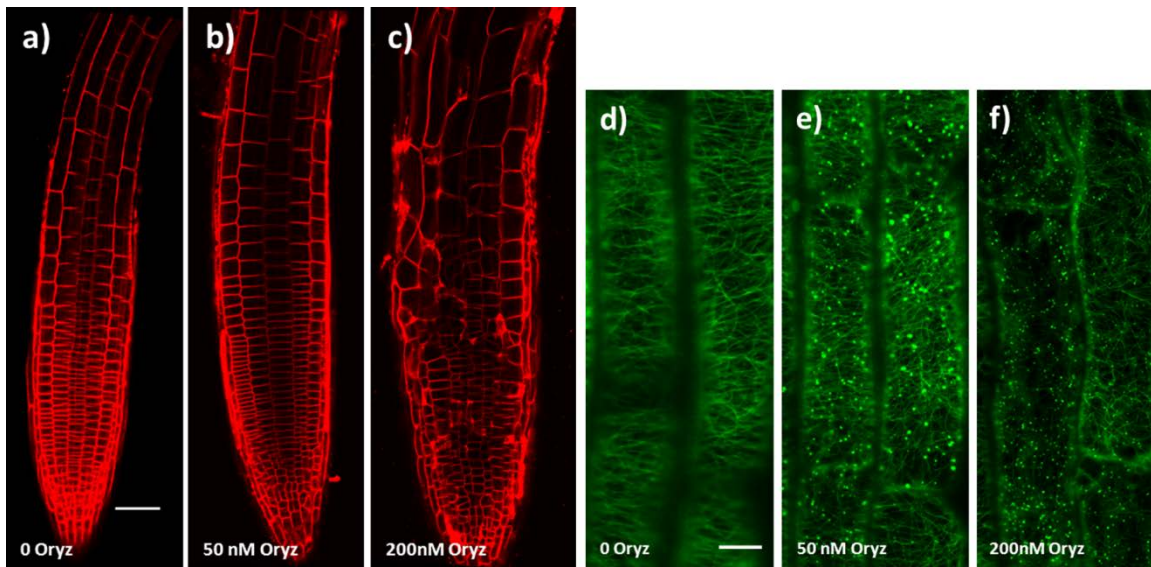
Figure 3.7.   Effects of different concentration of the microtubule depolymerizing drug oryzalin on cell expansion and MT arrays in the elongation zone of the root
Confocal microscopic images of root morphology in wild type seedlings treated with 0 nM (a) 50nM (b), and 200nM oryzalin (c). Mt arrays in the elongation zone of roots in wild type seedlings expressing GFP-MBD construct treated with 0 nM (e) 50 nM (e), and 200 nM oryzalin (f). Scale bars in (a) and (d) indicate 50μM and apply to all images.

To assess the effects of the MT disturbing drug oryzalin on FM4-64 uptake, 3-4-day-old wild type and *eb1b* mutants grown on agar medium containing 0nM (control) and 50nM oryzalin were stained with FM4-64 and the uptake of the dye was monitored in cells of the root transition zone 15 min later. FM4-64 uptake in untreated wild type and *eb1b* mutants were in line with my previous observations; FM4-64 uptake in *eb1b* mutants was significantly higher than wild type (Fig. 3.8- a) –b)- e). FM4-64 uptake in wild type treated with 50 nM oryzalin was statistically higher than control cells (Student's T-test, P< 0.0001; Fig. 3.8). However, statistical analyses showed no significant difference between FM4-64 uptake in *eb1b* mutants grown on 0 nM and 50 nM oryzalin (Student's T-test, P=0.35). These results suggest that the role of EB1 in membrane trafficking depends on intact MTs.
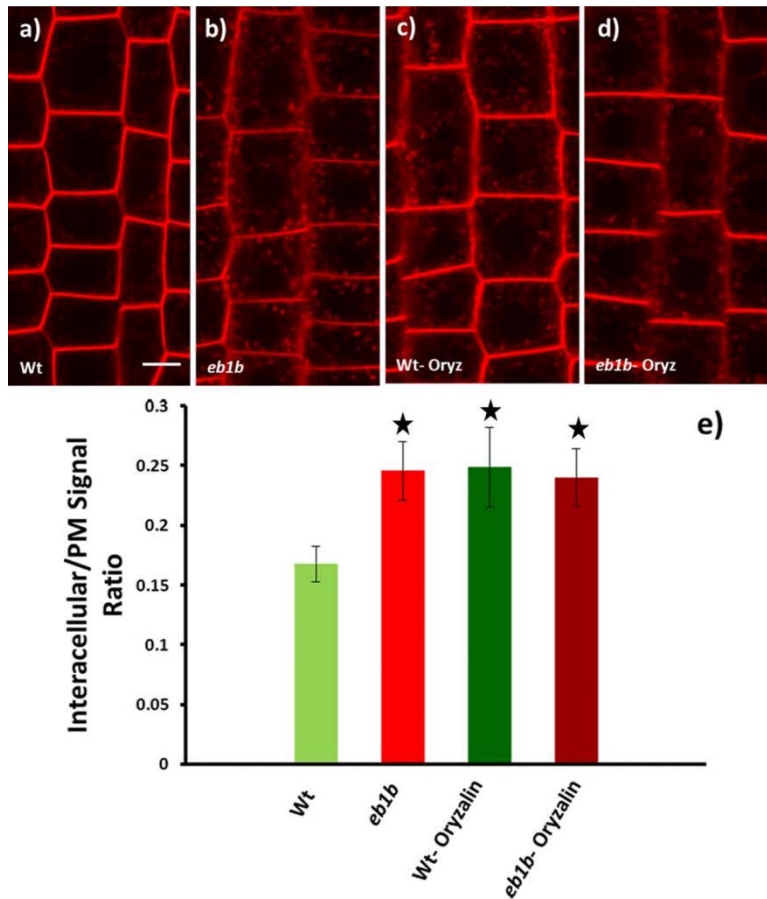
Figure 3.8.   Effects of the microtubule depolymerizing drug oryzalin on FM4-64 uptake in wild type and *eb1b* mutants

FM4-64 uptake in root cells of 3-day-old seedlings; wild type grown on 0 nM (a) and 50 nM oryzalin(c), *eb1b* mutants grown on 0 nM (a) and 50 nM oryzalin(c). FM4-64 uptake in root cells in wild type and *eb1b* mutants (e). FM4-64 uptake in wild type significantly increases upon treatment with 50nM oryzalin (Student's T-test, P< 0.0001); however, there is no statistical difference between FM4-64 uptake in *eb1b* mutants treated with 50 nM oryzalin and untreated lines (Student's T-test, P=0.35). Scale bar in (a) is 50μM and apply to all images. The results are mean values obtained from three independent experiments. Averages that are significantly different (Student's T-test, P < 0.05) from wild type or *eb1b-1* are denoted by symbols * and + respectively.

3.1.6.   The Effect of Actin Disturbing Drugs on FM4-64 Uptake

EB1 has been reported by several research groups as a mediator of MTs and the actin cytoskeleton in animal cells (Schober et al., 2009; Schober et al., 2012). In plants, it has been postulated that EB1 might mediate cross-talk between MTs and the actin cytoskeleton; however, functional evidence for this hypothesis is still missing (Petrášek and Schwarzerová, 2009). I investigated the effects of actin disruption on FM4-64 uptake

in wild type and *eb1b* mutants. The actin disturbing drug Lat B was applied to partially depolymerize the actin cytoskeleton in root cells. The drug binds to G-actin monomers and prevents their polymerization into AFs (Morton et al., 2000; Desouza et al., 2012).

The effect of Lat B on root responses to touch/gravity has been studied in our lab (Dr. R. Young and H. Kim). Roots in plants grown on 10 nM Lat B have the same length as control plants (0 nM Lat B); however, plants grown on 100 nM Lat B display very short roots. Therefore, the two aforementioned concentrations of Lat B were selected for optimization of Lat B treatments. The uptake of FM4-64 was examined in transition cells in seedlings treated with the selected concentrations of Lat B. Fifteen min after applying FM4-64, the dye was visible in punctuate spots in control root cells (0 nM Lat B) consistent with previously published reports (Kitakura et al., 2011; Fig. 3.9- a). Similarly, FM4-64 labeled compartments were observed in root cells treated with 10 nM Lat B (Fig. 3.9- b). Upon treatment with 100 nM Lat B, FM4-64 was aggregated in large compartments in root cells, suggesting that this concentration has a severe effect on FM4-64 trafficking (Fig. 3.9- c). Therefore, I chose 10 nM Lat B for performing FM4-64 uptake experiments.
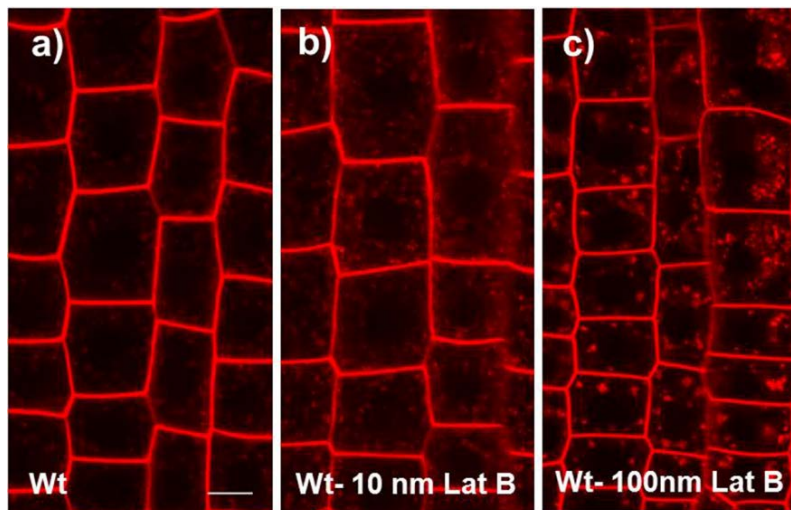
Figure 3.9.   Effects of different concentration of the actin depolymerizing drug Lat B on FM4-64 uptake in wild type root cells
3-day-old wild type seedlings treated with different concentrations of Lat B, 0 nM (a) 10 nM (b) and 100 nM (c). FM4-64 labeled compartments are visible in cells treated with 0 nM and 10 nM Lat B. FM4-64 is aggregated in large compartments in cells treated with 100 nM Lat B. Scale bar is 50µm in (a) and applies to all images.

To assess the effects of Lat B on FM4-64 uptake, 3-day-old wild type and *eb1b* mutants grown on agar medium supplemented with 0 nM (control) and 10 nM Lat B were stained with FM4-64 and uptake of the dye was monitored in transition zone root cells. FM4-64 uptake in *eb1b* mutants was higher than wild type, which is in consistent with my previous results (Fig. 3.10). FM4-64 uptake was significantly higher in wild type grown on 10 nM Lat B compared to wild type controls. This result was in line with previously published reports (Du et al., 2011; Nagawa et al., 2012). In contrast, FM4-64 uptake was dramatically reduced in *eb1b* mutants treated with 10 nM Lat B (Fig. 3.10- e). Statistical analyses of FM4-64 uptake revealed that uptake of FM4-64 in *eb1b* mutants treated with 10 nM Lat B closely resembled that of untreated wild type (Fig. 3.10- g), suggesting that the actin cytoskeleton in wild type is more destabilized than in *eb1b* mutants.
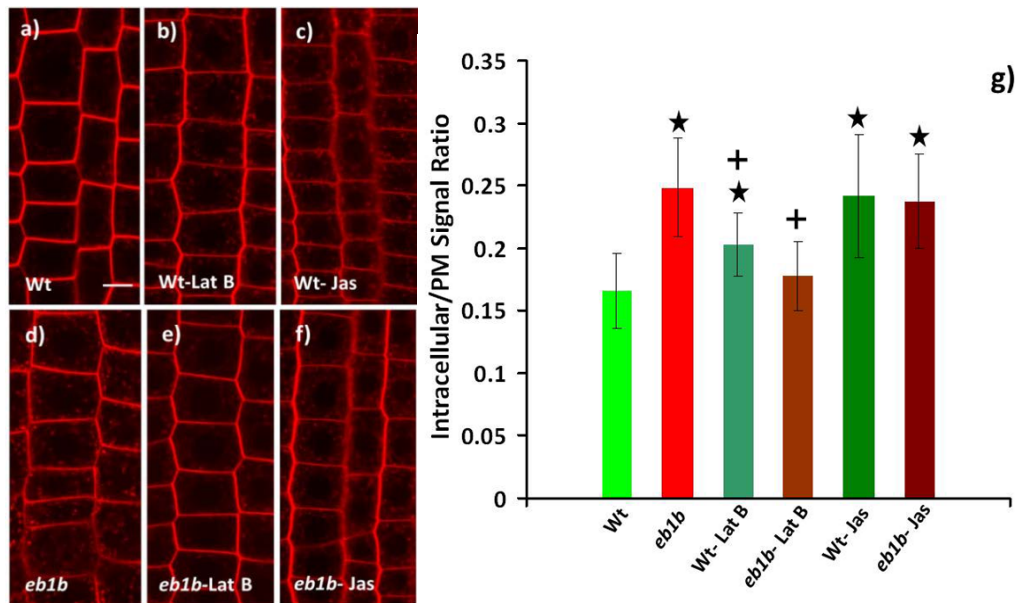
Figure 3.10. Effects of actin disturbing drugs on FM4-64 uptake in wild type and *eb1b* mutant root cells

FM4-64 uptake in root cells of 3--day-old seedlings treated with actin disturbing drugs, Wild type (a, b, c) and *eb1b* mutants (d,e,f). Wild type seedlings treated with 0 nM Lat B and 0 nM Jas (a), 10 nM Lat B (b), and 100 nM Jas (c). *eb1b* mutant seedlings treated with 0 nM Lat B and 0 nM Jas(d), 10 nM Lat B (e), and 100 nM Jas (f). Statistical analyses of FM4-64 uptake reveals that treatment of wild type with Lat B shows an increase in uptake of the dye, but FM4-64 uptake decreases in *eb1b* mutants treated with Lat B. Treatment with Jas give rises to an increase in FM4-64 uptake in wild type; however, it does not have any effect on FM4-64 uptake in eb1b mutants (h). The results are mean values obtained from three independent experiments. Error bars indicate (SD). Scale bar in (a) is 50µM and apply to all images. Averages that are significantly different (Student's T-test, $P < 0.05$) from wild type or *eb1b-1* are denoted by symbols * and + respectively.

I also investigated the effects of the actin stabilizing drug Jasplankinolide (Jas) on FM4-64 uptake in wild type and *eb1b* mutants. Jas stabilizes and promotes the polymerization of AFs by binding to F-actin (Bubb et al., 1994). Previous studies on the effects of the drug on the actin cytoskeleton in live cells suggests that treatment of cells with 100-150 nM Jas preferentially disrupts cortical AFs, but treatment with 400 nM and higher results in bundling of AFs (Ou et al., 2002). Based on this report, I chose 100 nM for partial disruption of AFs.

To investigate the effects of Jas on FM4-64 uptake, 3-4-day-old seedlings grown with or without Jas were stained with FM4-64 and uptake of the dye in root cells was observed 15 min after exposure to the dye. In consistent with our previous results, root cells in *eb1b* mutants had higher FM4-64 uptake than wild type. Wild type cells treated with 100 nM Jas showed a dramatic increase in FM4-64 uptake. FM4-64 uptake was not statistically different in *eb1b* mutants treated with 0 nM and 100 nM Jas, suggesting that EB1 in wild type counteracts with Jas and destabilizes the actin cytoskeleton. These results are in line with our previous results performed with Lat B.

3.1.7. Reversible Effects of BFA on FM4-64 Uptake in Root Cells

To gain additional insights into the function of EB1 on membrane trafficking, I disrupted membrane trafficking with brefeldin A (BFA) and monitored FM4-64 uptake in cells of transition root zone. BFA is a drug that is commonly used to study endomembrane trafficking pathways in plants. It is thought to primarily disrupt a GTPase exchange factor (GEF) that regulates trafficking between the Golgi, recycling endosomes, and the plasma membrane (Spano et al., 1999; Ritzenthaler et al., 2002; Nie et al., 2003; Naramato et al., 2010). In BFA treated *Arabidopsis* root cells, disruption of membrane trafficking results in the formation of large compartments, so called BFA-compartments. I chose to apply 25 µM BFA because this concentration has been shown to have only slight or no effect on Golgi/ER morphology (Grebe et al., 2003; Lam et al., 2009). Higher concentrations of the drug (50-100 µM) disrupt the ER (Robinson et al., 2008; Lam et al., 2009).

3-4-old-day seedlings were incubated in BFA for 30 min and stained with FM4-64 in the presence of BFA for 30 min then visualized with CLSM. BFA-induced FM4-64

positive compartments were observed in wild type, consistent with previously published reports (Fig. 3.11- a; Spano et al., 1999; Ritzenthaler et al., 2002). FM4-64 was also aggregated into BFA-induced compartments in *eb1b* mutants; however, they appeared to be more abundant and larger in mutant cells (Fig. 3.11- b). This is consistent with the idea that there could be more membrane internalization in *eb1b* mutants. To determine whether the larger and more abundant BFA compartments in mutants could be due to altered FM4-64 uptake, I quantified the relative amount of internalized FM4-64 by calculating the ratio of the mean fluorescence intensity of the cytoplasm to the mean fluorescence intensity of the plasma membrane. Consistent with my previous result I found that there was more FM4-64 uptake in mutants than in wild type, suggesting that mutants internalize more dye than wild type even in the presence of BFA. In fact, BFA had no effect on internalization of FM4-64 in either genotype, since uptake of the dye in BFA- treated and untreated cells was the same (compare Fig. 3.11- c and Fig. 3.6- e).
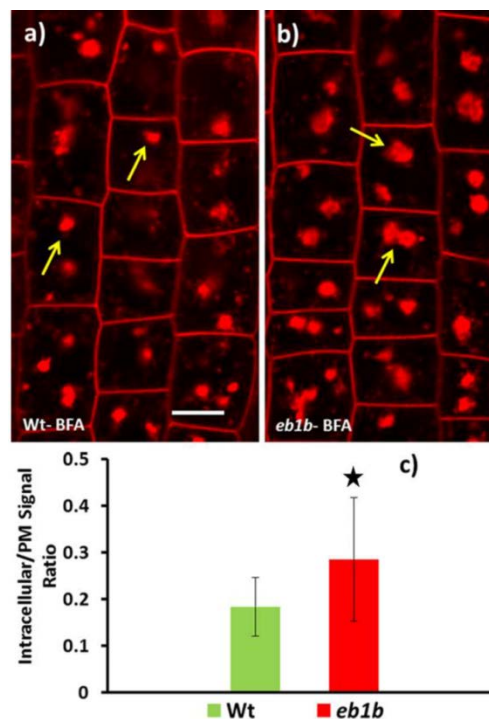
Figure 3.11. Effects of BFA on FM4-64 uptake in wild type and *eb1b* mutant root cells
BFA-induced compartments are more abundant and larger in root cells of *eb1b* mutants (b) compared to wild type (a). 4-day-old seedlings were pretreated with BFA, stained with FM4-64 coupled with BFA for 30 min., and visualized using CLSM. FM4-64 uptake and accumulation in *eb1b* mutants is statistically higher than wild type (Student's T-test, P< 0.0001) (c). Results represented are average of three independent experiments. Error bar indicates (SD). Scale bar in (a) is 50 µm and applies to both images.

When FM4-64 is internalized in the cell via endocytosis, it first accumulates in the endosomal compartments that are visible in the light microscope. The dye is then either recycled back to the plasma membrane or it travels to the vacuole (Bolte et al., 2004). FM4-64 is visible in vacuolar membranes, tonoplasts, in root cells after incubation in the dye for more than 60 min (Vida et al., 1995). I observed FM4-64-labeled tonoplasts in cells of the transition and elongation zones of wild type and *eb1b* mutants. However, *eb1b* mutants appeared to have more membrane in their vacuoles than wild type (Fig. 3.12).
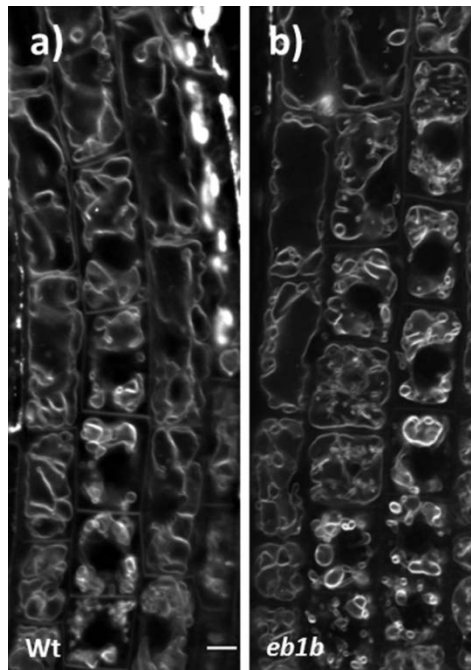
Figure 3.12. The vacuolar morphology in wild type and *eb1b* mutant cells of the transition and elongation zones
Wild type (a) and *eb1b* mutants (b) were stained with FM4-64 and incubated for 2 hours in liquid MS. The dye stained the vacuole membrane. However, in *eb1b* mutants the vacuoles appeared to have more membrane compared to wild type. Scale bar in (a) is 50 μm and applies to both images.

To assess the effect of BFA on membrane accumulation in vacuoles, I examined the ability of root cells to recover from BFA treatments. Upon 30 min pretreatment with BFA and subsequent staining with FM4-64 in the presence of BFA, wild type and *eb1b* mutant seedlings were incubated in liquid medium containing BFA for 2 hours as observed in my previous experiments FM4-64 was detected in large BFA compartments in root cells of both genotypes (Fig. 3.13- a)- b). In BFA washout experiments, wild type

and the mutant seedlings pretreated with BFA were stained with FM4-64 and placed in liquid medium for 2 hours. The BFA compartments disappeared in both genotypes, suggesting that the effect of BFA on membrane trafficking was reversible. In addition, more vacuole-associated membranes were again observed in mutants compared to wild type (Fig. 3.13- c) - d). Taken together, these observations suggest that both genotypes were able to recover from BFA equally.
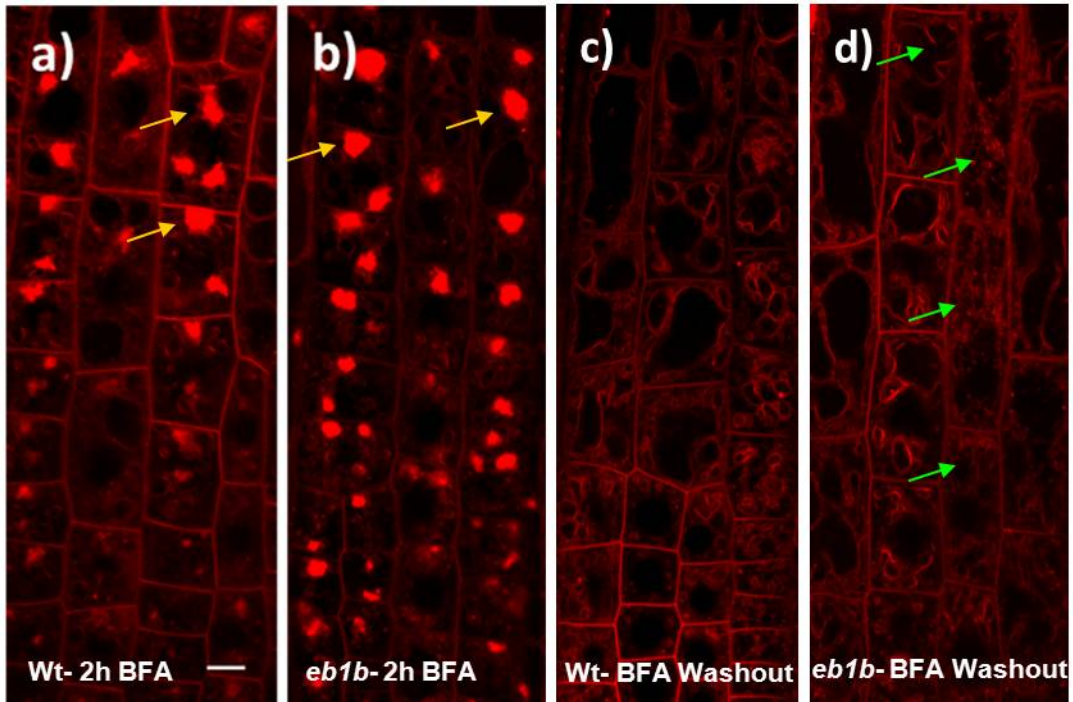
Figure 3.13. Effects of long-term BFA-treatment/wash out on FM4-64 trafficking
4-day-old wild type (a) and *eb1b* mutant (b) root cells treated with BFA and stained with FM4-64 display large BFA compartments (bright spots). In BFA washout experiments, BFA pretreated seedlings were stained with FM4-64, and incubated in liquid medium. Confocal microscopic image of transition zone root cells show that BFA-induced compartments have disappeared in both wild type (c) and *eb1b* mutant root cells (d). However, aberrant vacuole-like structures were observed in mutant cells. Scale bar in (a) represents 20 µm and applies to all confocal images.

4.   Discussion

Roots of the *eb1b* mutant plants exhibit defective responses to touch/gravity stimuli (Bisgrove et al, 2008; Gleeson et al., 2012). However, the mechanism by which EB1 contributes in these responses is still unclear. In my thesis, I studied the role of EB1 in root cells at the cellular levels to gain insights into how the function of this protein inside of root cells could modulate root responses to touch and gravity. By examining the subcellular architecture and membrane trafficking in the sensory columella cells, I found that EB1 has a role in membrane trafficking in these cells. I extended my study to root cells in the transition zone and found that the role of EB1 in membrane trafficking is not limited to columella cells and, additionally, this role of EB1 depends on intact MTs. To obtain further insights into the link between EB1 and membrane trafficking, I investigated the effects of actin disturbing drugs on membrane trafficking in wild type and *eb1b* mutants. The results show that EB1 modulates membrane trafficking through an effect on the actin cytoskeleton. I propose that EB1 might affect root responses to touch and gravity by regulating the trafficking of membrane transporters, receptors, and ion channels which are involved in these responses.

4.1. EB1 Modulates Membrane Trafficking in Root Cells

FM4-64 labeled compartments in columella cells in *eb1b* mutants were detected earlier than wild type upon staining with the lipophilic dye FM4-64, suggesting that the internalization of FM4-64 in *eb1b* mutants is faster than wild type. In addition, the observation of more FM 4-64 labeled puncta in *eb1b* mutants than wild type shows that there is a higher uptake of the dye in *eb1b* mutants. These results support the conclusion that EB1 has a role in FM4-64 trafficking in columella cells. FM4-64 is a marker of the endocytic pathway and membrane trafficking in plant cells (Bolte et al., 2004; Van Gisbergen et al., 2008). Therefore, these results indicate that EB1 modulates membrane trafficking in columella cells. I also propose that this role of EB1 in columella

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cells appears to be relatively specific, since I could not detect any structural or architectural differences between mutant and wild type columella cells.

I also found that cells in the transition zone of *eb1b* mutant roots take up more FM4-64 than transition zone cells in wild type roots. This result demonstrates that the role of EB1b in membrane trafficking is not confined to columella cells. Another line of evidence that indicates EB1b has a role in membrane trafficking comes from my examination of transgenic *eb1b* mutant plants transformed with wild type copies of the *EB1b* gene. When *EB1b* is expressed at levels equivalent to wild type (Gleeson et al., 2012), I observed that the internalization of FM4-64 in transition cells was equivalent to wild type. I also found that partial depolymerisation of MTs increased FM4-64 uptake in wild type root cells but had no effect on *eb1b* mutants. This indicates that, as expected for a MAP, EB1b depends on intact MTs to exert its function in membrane trafficking pathways.

The finding that FM4–64 uptake was more in *eb1b* mutant root cells compared to wild type raised three different possibilities about the role of EB1 in membrane trafficking in wild type root cells (Fig. 1.4): EB1 might negatively regulate endocytosis (route 1 in Fig. 1.4), EB1 might promote exocytosis (route 2 in Fig. 1.4), and EB1 might promote vesicle trafficking from endosomes/TNG to vacuoles (route 3 in Fig. 1.4).

To examine these possibilities, I first treated wild type and *eb1b* mutants with a low concentration of the endomembrane trafficking disturbing drug BFA. It has been reported that the low concentration of this drug has a reversible inhibitory effect on trafficking between the Golgi, recycling endosomes, and the plasma membrane (Spano et al., 1999; Ritzenthaler et al., 2002; Nie et al., 2003; Naramato et al., 2010).
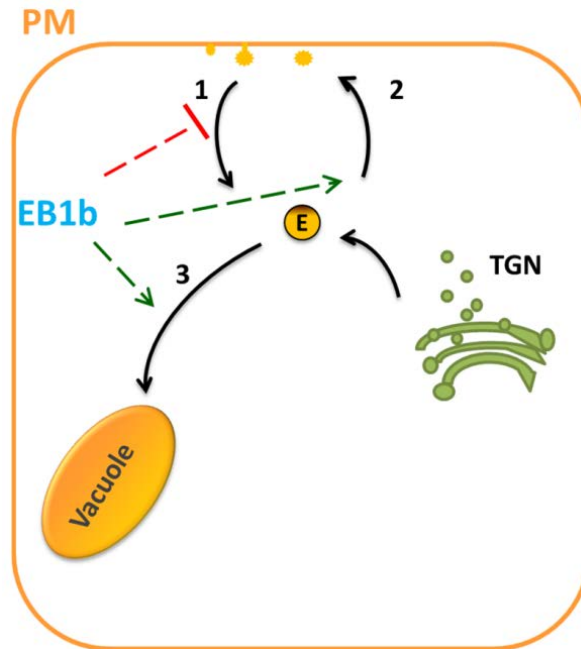
Figure 4.1.   Potential mechanisms for EB1 in regulating membrane trafficking pathways in plant root cells
Three possibilities by which EB1 could affect membrane trafficking in wild type root cells: EB1 might negatively regulate endocytosis (route 1), EB1 might promote exocytosis (route 2), and EB1 might promote vesicle trafficking from endosomes/TNG to vacuoles (route 3).

Treatment with BFA induced the formation of BFA compartments in both genotypes; however, the compartments appeared to be larger and more abundant in *eb1b* mutants than wild type, supporting the idea that there may be defects in membrane uptake in *eb1b* mutants. In addition, FM4-64 uptake in BFA treated root cells was the same as in untreated cells, even though the BFA compartments that formed in mutants were larger and more abundant than the ones that formed in wild type. Since BFA has no effect on internalization of the dye in either genotype, the presence of BFA compartments must be due to an equivalent effect of BFA on exocytosis in both genotypes. This suggests that the additional FM4-64 uptake in *eb1b* mutants is due to more endocytotic membrane internalization rather than a defect in exocytosis. Therefore these results suggest that EB1 might not have any effects on exocytosis (route 2 in Fig 4.1 might not be affected by EB1).

Next, I assessed the possible effects of EB1 on vesicle trafficking from endosomes/TGN to vacuoles. I examined the vacuolar morphology in wild type and *eb1b* mutant root cells. It appears that the extra internalization of membranes in *eb1b* mutants

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is redirected to the vacuoles, since they have vacuoles with extensive membranes compared to wild type, suggesting this pathway might not be affected by EB1. I also examined the recovery of BFA compartments and the vacuolar morphology in wild type and *eb1b* mutant root cells after washing out BFA. After BFA washout, the BFA compartments disappeared and vacuoles in *eb1b* mutants look similar to vacuoles in untreated mutants. Since the mutants are capable of recovering, it suggests that the membrane trafficking pathways required for BFA recovery are functioning normally in the mutants (routes 2 and 3 might not be affected by EB1). Taken together, these results suggest that the primary defect in membrane trafficking in *eb1b* mutants is endocytosis (route 1 in Fig. 4.1).

The level of *EB1* expression appears to be important in regulating membrane trafficking in root cells, since reducing the level (in *eb1b* mutants) increases the internalization of membranes and wild type expression level of *EB1* (wild type and rescued lines) restores internalization of membranes. Another line of evidence to support this assumption is that internalization of FM4-64 in overexpressing lines was more than wild type. This higher FM4-64 uptake in overexpressing lines might be due to the enhanced levels of EB1 on MTs, which could block access of other proteins involved in modulating membrane trafficking to MTs. Aberrant accumulation of EB1 along the entire length of MTs in EB1 overexpressing lines has already been reported (Mathur et al., 2003).

How could EB1b as a microtubule-associated protein (MAP) modulate the endocytic pathway? I found that EB1b exerts its role in membrane trafficking through an effect on the actin cytoskeleton. Treatment of *eb1b* mutants with a low concentration of the actin destabilizing drug Lat B reduces internalization of FM4-64 to the wild type level, suggesting that the actin cytoskeleton in the *eb1b* mutant is more stable than wild type. Based on this result, I propose that EB1 has a destabilizing effect on the actin cytoskeleton (Fig. 4.2). In support of this idea I found that stabilizing the actin cytoskeleton with a low concentration of Jas increases FM4-64 uptake in wild type to the level of *eb1*b mutants, while the drug had no effect on FM4-64 uptake in *eb1b* mutants.
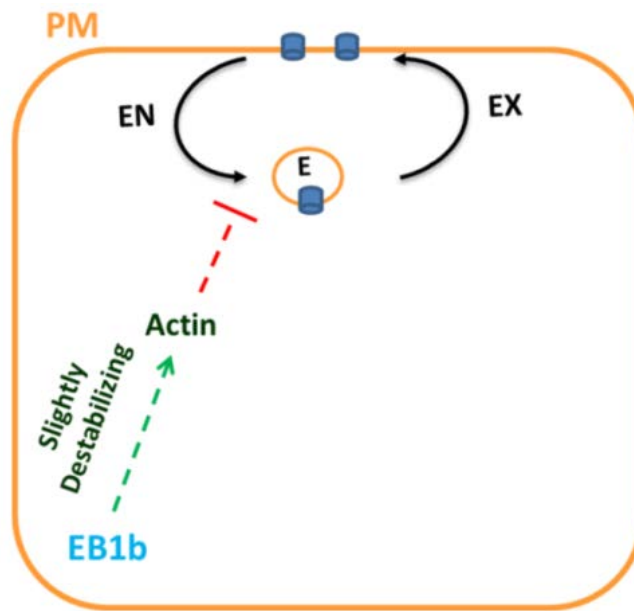
Figure 4.2.   Schematic diagram summarizing the putative role of EB1 on actin filaments and membrane trafficking pathways
I propose that EB1 regulates endocytosis in plant root cells through a destabilizing effect on the actin cytoskeleton. Figure is not to scale. PM= the Plasma Membrane, EN=endocytosis, EX=exocytosis, E=endosome, Dark blue structures =membrane proteins

Taken together, these results indicate that the dynamic state of the actin cytoskeleton modulates FM4-64 uptake and suggest that EB1 plays a role in maintaining the proper balance of actin polymerization/depolymerisation. A fine-tuned balance appears to be operating during FM4-64 uptake, because in wild type EB1 destabilizes actin and further destabilization with Lat B increases FM4-64 uptake rather than decreasing it. A dynamic actin cytoskeleton is thought to play a role in endocytosis and subsequent trafficking of endocytic vesicles in yeast, animal, and plant cells (reviewed in Samaj et al, 2012a). EB1 is known to have a role in the regulation of actin dynamics and activities during cell migration (Schober et al., 2009; Schober et al., 2012).

4.2. How might the Role of EB1 in Membrane Trafficking be Correlated to Root Responses to Mechanical Cues

One way that EB1 could modulate root responses to touch and gravity is through an effect on endocytosis. I found that EB1 has an inhibitory effect on endocytosis, this would mean that in *eb1b* mutants there would be more internalization of receptors, ion

channels, or transporters that are involved in root responses to touch and gravity. For instance, PIN transporters are one of the major determinants in the polar transportation of auxin, which is known to have a key role in root responses to environmental cues (Zazimalova et al., 2010). In *eb1b* mutants, there would be more internalization of these transporters and this is likely to affect their ability to relocalize in response to a cue. Recent reports have shown that some MAPs are involved in polar PIN localization at the plasma membrane (Nagawa et al., 2012; Ambrose et al., 2013; Kakar et al., 2013). I also found that EB1 affects endocytosis through the actin cytoskeleton. Intact actin arrays are important for PIN recycling and localization (Nagawa et al., 2012), supporting the idea that EB1b might regulate PIN trafficking through an effect on the actin cytoskeleton.

Alternatively, EB1 could modulate root responses to gravity by affecting amyloplasts sedimentation. My results suggest that EB1 has a destabilizing effect on the actin cytoskeleton. This means that in *eb1b* columella cells actin filaments would be more stable. Given that it has been reported that actin filaments form a network that restricts amyloplast sedimentation through the cytoplasm of columella cells (Yamamoto and Kiss, 2002; Hou et al., 2003), it is plausible that a more stable array of actin in *eb1b* columella cells would be more restrictive than in wild type. Amyloplast sedimentation would be reduced in *eb1b* mutants and this would slow down their responses to gravity. This could in turn affect root responses to mechanical cues. The root bends away when it encounters an obstacle and must be redirected towards gravity after bypassing the obstacle; if response to gravity is delayed in *eb1b* mutants it could result in an exaggerated response to the mechanical cue.

5.   Conclusion and Future Prospects

The aim of this thesis was to investigate the role of EB1 in root cells that might be correlated to root responses to touch and gravity. To sum up, my major finding is that EB1b modulates membrane trafficking in root cells through destabilization of the actin cytoskeleton. I also found that, surprisingly, there are no defects in the cytoarchitecture of sensory columella cells in *eb1b* mutants, suggesting that the role of EB1 in columella cells might be limited to endomembrane organization. I propose that the role of EB1 in membrane trafficking is the mechanism by which it affects root responses to touch/gravity cues.

I found that EB1 regulates endocytosis in root cells. Although both clathrin-dependent and clathrin-independent endocytotic events have been reported in plant cells, the clathrin mediated pathway is the prominent pathway for endocytosis in plant cells (reviewed in Chen et al., 2011). This raises the question of whether EB1 affects the clathrin-dependent pathway in root cells. One way to answer this question is by monitoring FM4-64 uptake in plants expressing a clathrin marker in which clathrin light chain is tagged with GFP (CLC-GFP; Konopka et al., 2008; Robert et al., 2010). FM4-64 uptake would be monitored in wild type and *eb1b* mutant plants expressing CLC-GFP. Quantitative analysis of the co-localization between FM4-64 labeled compartments and CLC-GFP in the cytoplasm in wild type and *eb1b* mutant root cells would provide insights into the possible effects of EB1 on the clathrin-dependent pathway.

I propose that EB1 affects root responses to touch and gravity by modulating trafficking of membrane proteins including transporters, receptors, and ion channels. The importance of PIN trafficking on root responses to various environmental stimuli has been well established (reviewed in Kleine-Vehn and Friml, 2008). PIN trafficking plays a key role in directional cell-to-cell movement of auxin in root cells. Polar transport and differential distribution of auxin is important in root responses to environmental cues including gravity and mechanical cues (Raven, 1975; Estelle, 1998; Friml et al., 2002;

Michniewicz et al., 2007). In roots, PIN3 in columella cells and PIN2 in epidermal and cortical cells facilitate the transport of auxin in and across root cells. An interesting question that needs to be addressed is whether the role of EB1 in membrane trafficking could affect PIN trafficking in root cells. One way to answer this question would be to monitor PIN trafficking in wild type and *eb1b* mutant plants expressing PIN-GFP. Quantitative analysis of the abundance of GFP-tagged PIN at the plasma membrane and in the cytoplasm in wild type and *eb1b* mutant root cells would reveal whether EB1 affects PIN trafficking. These studies would assess the putative role of EB1 in auxin movement in and across root cells which could be correlated to the function of EB1 in root responses to touch and gravity.

Our results suggest that EB1 modulates membrane trafficking pathways. Membrane trafficking has been implicated in plant responses to a variety of biotic and abiotic stresses (Robatzek et al., 2006; Kleine-Vehn and Friml, 2008). Future research could reveal whether EB1 has a role in root responses to other environmental cues including pathogens. In plants, the membrane localized receptor-like kinase FLAGELLIN-SENSING 2 (FLS2) recognizes and binds to a segment of a peptide called flagellin secreted by pathogenic bacteria. After ligand binding, FLS2 is internalized and degraded. This FLS2 trafficking is important for plant immunity responses (Robatzek et al., 2006). It would be interesting to compare endocytosis of FLS2 in wild type and *eb1b* mutant root cells as well as plant immunity to pathogens in both genotypes. GFP-FLS2 plants (Robatzek et al., 2006; Beck et al., 2012) would be used to monitor localization of FLS2 at the plasma membrane and in the cytoplasm.

Another question that needs to be answered is whether EB1 activity is needed in the root cap, the elongation zone or both during root responses to touch and gravity. One way to answer this question would be to express EB1 protein in *eb1b* mutant root cap cells and disturb expression from the *EB1* gene in wild type root cap cells. A root cap specific promoter, RCP1 (Tsugeki and Fedoroff, 1999) could be used to drive expression from the *EB1b* gene solely in root caps of *eb1b* mutants. The same promoter could be used to drive expression of an RNAi construct to reduce EB1 protein levels specifically in root cap cells of wild type plants. Analyses of touch/gravity responses in these plants could shed light on in which zone of the root EB1 is required for proper root responses to touch and gravity. Measurement of FM4-64 uptake in root cap and cells in

the elongation zone of these plants would provide insights into a possible correlation between the function of EB1 in endocytosis and its role in root responses to touch and gravity.

I found that the role of EB1 in endocytosis depends on intact MTs. Another open question that needs to be addressed is whether EB1 activities in root responses to touch and gravity depend on intact MTs. It would be interesting to investigate the effects of the MT depolymerizing drug oryzalin on root responses to touch and gravity in wild type and *eb1*b mutants.

Another question that needs to be addressed is whether EB1 exerts its functions in membrane trafficking and root responses to touch and gravity through interaction with additional proteins or through regulation of MT dynamics. To answer this question, a truncated version of EB1 that is missing the domain that mediates interactions with other proteins (EBH) could be transformed into *eb1b* mutants. Also, a truncated EB1 gene that is missing CH domain could be transformed into *eb1b* mutants. Assessment of FM4-64 uptake in root cells of the transformed plants as well as root responses to touch and gravity in these plants would reveal whether EB1 activities in membrane trafficking and root responses to touch and gravity require an interaction with MTs or additional proteins.

Another line of investigation would be to identify proteins that interact with EB1 in plant cells. Several approaches could be used to identify these proteins. This includes yeast hybrid assay as well as protein pull-down assay followed by mass spectrometry. Based on studies in other organisms (Jiang et al., 2012), there is likely to be a large number of proteins capable of interacting with EB1 in plant cells. In this thesis I reported that EB1 affects actin dynamics in root cells. It would be interesting to examine this list of proteins that interact with the actin cytoskeleton. Indeed, in other organisms several EB1-binding proteins have been found that regulate actin dynamics and activities (Liu et al., 2011). Potential proteins that bind to EB1 and interact with the actin cytoskeleton in plant cells could be further investigated by fluorescently tagging these proteins and EB1 and examining their interaction using live cell imaging approaches.

Finally, I propose that more stabilized AFs in *eb1b* columella cells could affect amyloplast mobility and sedimentation in gravistimulated roots. In future investigations, it might be possible to measure the velocity of amyloplast sedimentation in gravi-stimulated roots in wild type and *eb1b* mutant.

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