THE MICROTUBULE ASSOCIATED PROTEIN END BINDING 1 AND ROOT RESPONSES TO MECHANICAL/GRAVITY STIMULI

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Abstract

Do microtubules influence growth responses to environmental stimuli in plants? Microtubules (MTs) have numerous roles in plant development and these functions are assisted by Microtubule Associated Proteins (MAPs). To further explore MT function we study a MAP called END BINDING 1 (EB1). Previous analysis of *eb1* mutants indicates root defects in responses to mechanical stimulation (MS) and/or gravity. To determine whether EB1 activity contributes to root responses to MS or gravity or both, two approaches were taken. First, I analyze the effects of altering the type and amount of MS perceived by the root. Second, I analyzed double mutants between *eb1b* and plants carrying mutations in genes associated with responses to MS and gravity. Results from both approaches suggest that EB1 has a role in root responses to MS and an indirect role in responses to gravity.

Keywords: End Binding 1 (EB1); microtubules; gravity response; mechanical stimulation; root growth

Dedication

To my husband and family, who have been endless sources of prayers and encouragement throughout the duration of my graduate studies. And, to my son, who arrived with perfect timing.

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1: Introduction

Plants come in various shapes and sizes. Plant cells are non-motile therefore the diversity of flowers, leaves, stems and roots is largely dependent on the position of cell division and direction of cell expansion. Microtubules (MTs) are key regulators of both cell division and cell expansion. MTs are highly conserved, long tubule-shaped filaments that can alter their organization during various stages of the cell cycle and in different cell types (Wasteneys, 2002). During plant cell division, MTs arrange from the preprophase band, which marks the future plane of cell division, into mitotic spindles, which separates the chromosomes and then into the phagmoplast, which helps in forming the cell plate (Wasteneys, 2002). During cell expansion, MTs are arranged in a cortical array just within the cortex of the cell. In this conformation MTs guide cellulose synthase complexes for the deposition of cellulose. Cellulose constrains turgor-driven cell expansion in the perpendicular direction to the cortical array. MTs can alter their conformations by undergoing growth through polymerization and shrinking through depolymerisation (Akhmaova & Steinmetz, 2009). This dynamic ability allows the MTs to grow in one direction and then regrow in a new direction in search of possible target sites such as the plasma membrane or chromosome kinetochores (Akhmaova & Steinmetz, 2009). MT functions are assisted by a group of microtubule associated proteins (MAPs). MAPs can bind the ends or along the length of MTs. Some MAPs are motor proteins which facilitate the transport of vesicles and proteins. Some MAPs can alter the rates of MT dynamics by making the MT more stable or by promoting

polymerization. While other MAPs mediate interactions between MTs and other proteins, organelles and cellular structures including the endoplasmic reticulum, actin filaments and plasma membrane (Hamada, 2007). Studying MAPs can further our understanding of the MT function. Our lab is interested in how MTs affect growth and development in plants. We do this by studying a MAP which binds to the plus end of MTs called END BINDING 1 (EB1). Based on animal and fungal data, EB1 is thought to function by recruiting proteins to the plus ends of MTs. These specialized complexes are modified to suit the needs of the cell (Akhmanova et al., 2009). When *eb1b* mutants were analyzed in *Arabidopsis*, their roots displayed phenotypes that may be attributed to defects in responding to mechanical stimulation (MS) and/or gravity (Bisgrove et al., 2008). I am interested in understanding how EB1 and MTs are involved in growth responses to MS and gravity. The goal of this thesis was to elucidate whether EB1 has a role in root responses to MS or gravity or both.

Gravitropism refers to growth that aligns plant organs with respect to the gravity vector. In roots this response consists of three stages; gravity detection, signal transduction/transmission and differential growth that results in bend formation. The primary site of gravity detection is the columella cells of the root cap. In the past 20 years, two mechanisms have been proposed for how columella cells perceive the root's orientation within the field of gravity; they are called the starch-statolith hypothesis and the protoplast pressure model. The starch-statolith hypothesis proposes that when the root tip gets deflected out of the plane of gravity the starch filled amyloplasts, located on the lower side of columella cells, will sediment to the "new" bottom of the cell (Sack et al., 1991; Kiss, 2000). The sedimentation of amyloplasts onto receptive surfaces, such as

the plasma membrane or ER, is thought to exert a force or pressure that activates signalling channels (Yoder et al., 2001; Blancaflor and Masson, 2003; Perrin et al., 2005; Leitz et al., 2009; Stanga et al., 2009; Morita, 2010). Some support for this model comes from studying mutants with reduced amounts of starch. *phosphoglucomutase -1 (pgm-1)* mutants lack the enzyme for starch biosynthesis resulting in less starch accumulation in the amyloplasts. *pgm-1* roots exhibit reduced rates of amyloplast sedimentation and delays in responding to gravity (Caspar and Pickard, 1989; Sæther and Iversen, 1991). The second model called the protoplast-pressure model proposes that the weight or downward "slouching" of the protoplast in response to gravity will shift to the "new" bottom of the cell when the root tip gets deflected out of the plane of gravity (Staves et al., 1995; Staves et al., 1997). The slouching of the protoplast may exert pressure on the lower plasma membrane or tension on the upper plasma membrane which would then be detected by gravireceptors in the plasma membrane and initiate signal transduction events (Staves et al., 1997). Both of these models propose mechanisms by which signal transduction events can be initiated. Cytoplasmic Ca^{+2} and pH are potential signalling molecules used in signal transduction since the levels of both are observed to increase within columella cells shortly after gravity stimulation (Scott and Allen, 1999; Fasano et al., 2002; Monshausen et al., 2008; Morita, 2010).

The site of gravity perception, the root cap, is spatially distinct from the site of gravity response, the root elongation zone. Roots use auxin to transfer the gravity signal from the root cap to the elongation zone. Auxin is a plant hormone that gets transported in a directional manner from cell to cell. Protonated auxin enters the cell through passive diffusion while deprotonated auxin enters by auxin influx carriers, such as AUX1

(Blakeslee et al., 2005). Once inside the cell, all auxin will become deprotonated due to the neutral pH of the cytoplasm. The deprotonated form of auxin can only exit the cell through auxin anion efflux carriers called PIN -FORMED (PIN) proteins (Friml et al., 2002). The localization of PIN proteins within specialized cells directs the flow of auxin (Blakeslee et al., 2005). When the root is growing vertically, auxin is transported down the middle of the root to the columella cells where it is redirected laterally and flows evenly back up the sides to the elongation zone, resulting in even cell expansion across the root. In contrast, during gravitropism, changes in the distributions of PIN proteins alters auxin transport by directing a greater amount of auxin towards the lower flank of the root cap which is then transported to the elongation zone resulting in higher levels of auxin in cells on the lower flank of the root (Rashotte et al., 2000; Friml et al., 2002; Ottenschläger et al., 2003). Auxin is thought to influence cell expansion by regulating gene expression of many proteins and enzymes including ones that modify the extensibility of the cell wall (Muday and Rahman, 2008). Asymmetrically distributed auxin has been closely linked with decreasing the rate of cell expansion along the lower flank relative to the upper flank (Rashotte et al., 2001). This process is called differential growth and results in the formation of a downward bend, thus completing the gravitropic response (Rashotte et al., 2000; Yamamoto, 2003).

Similar to gravity, root responses to MS can also result in a bend formation. Unfortunately, the mechanisms involved in responding to MS are not as well understood as they are for gravitropism. However, it has been observed that roots growing into an impenetrable obstacle, such as a rock in the soil, are able to maneuver around it. It has been speculated that as the root grows the tip is pushed against the obstacle, theoretically

increasing the pressure applied to the root cells, resulting in a curvature or buckling at the weakest part which happens to be the elongation zone (Thompson and Holbrook, 2004). During root buckling the strain imposed upon the cells may then activate mechanosensitive ion channels through deformations in the plasma membrane. Once activated, these channels would release an abundance of signalling molecules into the cytoplasm thereby initiating signal transduction events. This idea is based on studies of mechanosensitive ion channels in animals, yeast and bacteria (Monshausen et al., 2008). Homologues to the bacterial mechanosensitive channels of small conductance (MscS) have been identified in *Arabidopsis*. The functionality of these channels in response to MS is under investigation (Haswell and Meyerowitz, 2006; Haswell et al., 2008)
In terms of signalling molecules, Ca^{+2}, pH and reactive oxygen species (ROS) are all speculated to play a role. Cells under mechanical strain exhibit increases in cytoplasmic Ca^{+2} followed by increases in extracellular pH and ROS production (Legué, 1997; Monshausen et al., 2009). It is speculated that Ca^{+2} influences the pH by activating H^{+}-ATPase channels (Monshausen et al., 2009). The cytoplasmic alterations in Ca^{+2} and pH affect gene transcription and protein activity (Lapous et al., 1998; Apel and Hirt, 2004). For example, calmodulin and calmodulin –like genes are significantly up-regulated in response to increased cytoplasmic Ca^{+2} levels (Polisensky and Braam, 1996; Braam et al., 1997). In addition, increases in extracellular pH and ROS production are proposed to strengthen the cell wall in the region where buckling occurs to help the root withstand the strain (Monshausen et al., 2009).

How might MTs be involved in root responses to gravity and MS? The discovery that *eb1* mutants have defects responding to gravity and/or touch stimulation provides us

with a tool to explore this question. The purpose of this work was to determine whether EB1 affects responses to MS, gravity, or both. I altered the mechanical stimulus given to the root and analyzed genetic interactions between *eb1b* and plants carrying mutations in genes associated with responses to MS or gravity. My results suggest that EB1b is involved in root responses to MS and indirectly involved in root responses to gravity.

2: Materials and Methods

2.1 Plants and Growth Conditions

Ws, Col-0, *pgm-1*, *arg1-3* and *tch3-1* seeds were obtained from The *Arabidopsis* Information Resource (TAIR; http://www.Arabidopsis.org/). The *eb1b-1* allele was previously characterized in Bisgrove et al, (2008). Seeds were sterilized using the vapor phase method (Clough and Bent, 1998) and placed on the surface of 0.8% or inside 1.0% agar plates (Phytablend, Caisson laboratories Inc.). Agar medium also contained half-strength Murashige and Skoog (MS) medium (Sigma-Aldrich) supplemented with 0.5 g MES and 1% sucrose per liter and was brought to a pH of 5.8. Plates containing seeds were placed in the dark at 4°C for 3 d and then transferred to a growth chamber set at 20°C with a 16-h-light/8-h-dark cycle where they were grown for 7-9 d.

2.2 Phenotypic and Statistical Analyses

Seedlings were photographed using QCapture Pro software and a Qimaging Retiga 4000R digital camera mounted on an Olympus SZX16 stereo microscope. Measurements were made using either Photoshop or ImageJ software (http://rsbweb.nih.gov/ij/index.html). Statistical analyses were performed in JMP 7 (Tukey's test to compare average means) and graphs were made in SPSS 17.0.

2.3 Extraction of nucleic acids

DNA was extracted from leaves using a slightly modified protocol from the one

outlined in Dellaporta et al. (1983). Plant tissue was frozen using either liquid N2 or overnight storage in a -80°C freezer and then ground up using a drill and pestle. Next, 500µl of buffer (200mM Tris-CL pH 8, 250mM NaCL, 25mM EDTA, 0.5% SDS) was added to each sample and centrifuged slowly (2000-3000 rpm) for 10 minutes. Supernatant was added to 500µl of cold isopropanol, mixed, and centrifuged again for 5-10 minutes at 4°C. Pellet was washed with 500µl of cold 70% ethanol and centrifuged at 13000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 50µl EB (Qiagen) (Dellaporta et al., 1983). RNA was extracted from 7-11 day old whole seedlings using the RNeasy kit (Qiagen). RNA was reverse transcribed using RevertAid Minus First Strand cDNA Synthesis kit (Fermentas) and the oligo(dT) primers provided in the kit. The resulting cDNA was subjected to PCR amplification using the following primers: U (forward) 5'-CCGTGATGTTTTCCCT-3', U (reverse) 5'-CGGAGCTCATTCACGG-3', F (forward) 5'-CCTCGGTAAAAACCGGACA-3', F (reverse) 5'-ACAGCGCTTCGAACAAATCT-3', D (forward) 5'-AAGGTCAGGGTCAAGTGCAG-3', D (reverse) 5'-ACAGCGCTTCGAACAAATCT-3'

2.4 Genotyping

Progeny from crosses to T-DNA insertional mutants were genotyped by PCR using *Taq*DNA polymerase (Invitrogen) and the following primers: LBa1 5'-TGGTTCACGTAGTGGGCCATCG-3' (T-DNA insertion for SALK lines), *arg1-3* AT1G68370.1F 5'-CGATTGAAGCACT-CTGTGCCA-3', *arg1-3* AT1G68370.1R 5'-TCTGTTCCGCCTTCTTCTCCC-3', *tch3-1* AT2G41100F2 5'-CCTCGGTAAAAAC-CGGACA-3', *tch3-1* AT2G41100R2 5'-ACAGCGCTTCGAACAAATCT-3', *pgm-1*

AT5G51820F 5'-TTGGATGATTTACAATGCTGAAAGA-3', *pgm-1* AT5G51820R 5'-TCAGTGATCACGAAGGAAAAACTT-3'.

The T-DNA insertion in *eb1b-1* mutants contains a gene for BASTA resistance. To confirm the *eb1b-1* mutant allele during double mutant analyses seeds were grown on agar plates containing 25mg/L glufosinate-ammonium (Pestanal: Sigma-Aldrich). Derived Cleaved Amplified Polymorphic Sequences (dCAPS) was used to genotype progeny from crosses to *pgm-1,* since this allele carries a point mutation in the PGM gene. dCAPS Finder 2.0 software generates a list of possible restriction enzymes that can detect a single nucleotide polymorphism or point mutation in either the wild-type or mutant sequences (http://helix.wustl.edu/dcaps/dcaps.html; (Neff et al., 2002). To detect the difference between wild type (Col-0) and *pgm-1* genotypes, primers were used to PCR amplify the PGM gene from both wild type and *pgm-1*. The PCR products were subjected to a restriction digest using the BspCNI restriction enzyme (recognition site: CTCAG $(N)_9$). Only *pgm-1* contained the BspCNI restriction site due to the point mutation.

3: Results

3.1 Sensitivities of wild type and *eb1b-1* mutants to altered plate angles.

To assess the influence of mechanical (or touch) stimulation on the *eb1b-1* phenotype, root growth was analyzed in seedlings grown on the surface of tilted agar plates (Fig 1). Roots growing along the surface of an agar plate are thought to be mechanically stimulated when the root tip encounters the agar surface (Okada and Shimura, 1990). As plates are reclined more horizontally (at a higher angle), the root tip will encounter the agar surface more frequently as it attempts to penetrate the agar in response to gravity. These additional interactions with the agar surface increase the amount of touch stimulation perceived by the growing root. On plates oriented at 20°, both wild type (Wassilewskija or Ws) and *eb1b-1* roots formed loops, although the proportion of roots that made loops was significantly higher for *eb1b-1* than it was for wild type (P=0.006; Fig 1 a, c). At 35°, both wild type and *eb1b-1* formed more loops than they did at 20° (Fig 1 b, d). However, increasing the plate angle had a greater effect on *eb1b-1* seedlings, since the change in the proportion of loops was significantly greater for this genotype than it was for wild type seedlings (P=0.036; Fig 1e). This shows that *eb1b-1* seedlings are more sensitive to increasing the plate angle than wild type plants.

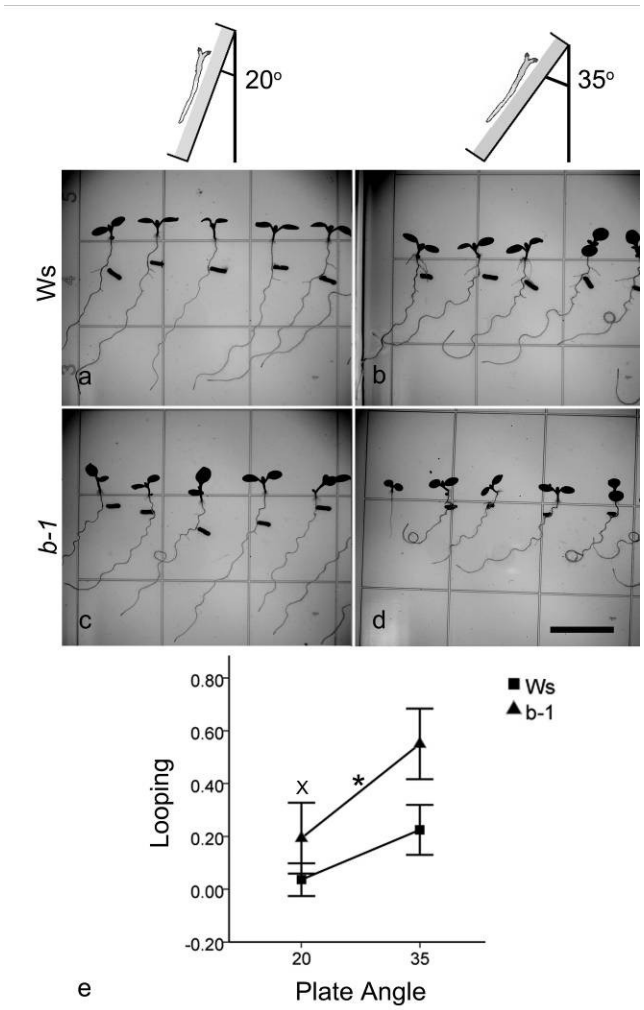

Figure 1 *eb1b-1* roots are more sensitive than wild type to growth on reclined plates.

Both wild type (**a, b**) and *eb1b-1* (**c, d**) seedlings have roots that skew or form loops when grown on plates reclined at either 20º (**a,c**) or 35º (**b,d**). Size bar in (**d**) is 1 cm and applies to a-d. For each genotype the proportion of roots that made loops was quantified after 7 days of growth on plates reclined at 20º or 35º (**e**). Data points denote the average proportions of loops made by Ws (squares, n=94) and *eb1b-1* (triangles, n=103) in 6 experiments. Bars represent 95% CIs and X denotes a significant difference between the proportion of loops made by *eb1b-1* and Ws seedlings on plates reclined to 20º (P=0.006; Tukey's Test). The asterisk indicates a significant difference in the response of wild type and *eb1b-1* seedlings to increasing the plate angle from 20º to 35º (P= 0.036; Tukey's Test).

3.2 Wild type and *eb1b-1* roots have similar growth responses when grown inside the agar

Our analysis of seedlings growing on the surface of tilted plates indicated that *eb1b-1* mutants are more sensitive than wild type plants to MS imposed by the agar surface. To determine whether mutant roots also have growth defects when given more evenly distributed MS, roots grown inside the agar medium were analyzed. Seeds were embedded in the agar and the plates were oriented vertically, allowing the roots to grow through the agar rather than on the surface. Under these conditions, both wild type and *eb1b-1* roots grew straight down (Fig 2 c, d) rather than skewing off to one side as they do when grown on the surface (Fig 2 a, b) (Bisgrove et al., 2008). The fact that *eb1b-1* roots are indistinguishable from wild type when grown inside the agar contrasts with the differences observed when seedlings were grown on the surface. This indicates that the *eb1b-1* mutant skewing phenotype is dependant on type of mechanical stimulus perceived.

To address the possibility that mutants may have defects responding to gravitropic stimulation while inside the agar, roots were observed after a 90° clockwise rotation. The roots responded to the change in gravity by bending downwards. Each seedling was photographed before and 2 days after the reorientation of the root, the images were superimposed and the distance was measured from the position of the root tip before the 90°rotation to the completion of a gravitropic bend (Fig 2e-h). To ensure that this assay could adequately detect gravitropic delays, *pgm-1,* a mutant with known gravity defects, was analyzed. *PGM* encodes an enzyme in the starch biosynthesis pathway. *pgm-1* mutant statocytes have starch depleted amyloplasts, a defect that results in slower amyloplast sedimentation rates and delays in gravitropic bending (Caspar and Pickard,

1989; Sæther and Iversen, 1991). Inside the agar, *pgm-1* mutants formed a bend after a mean distance of 2.68 mm while wild type roots bent within a mean distance of 1.20 mm (Fig 2 f, h). The distance to form a bend was significantly greater for *pgm-1* mutants than it was for wild type Col-0 roots ($P<0.0001$; Fig 2j), indicating that the assay does detect gravitropic defects. In contrast to *pgm-1,* analysis of *eb1b-1* roots revealed a response that was statistically indistinguishable from wild type plants (Fig 2e, g). Wild type Ws roots formed bends within a mean distance of 1.30 mm and *eb1b-1* within a mean distance of 1.38 mm. These distances that were not significantly different from one another ($P=0.230$; Fig 2i). In summary, when *eb1b-1* mutants were grown inside the agar, their root growth patterns and gravitropic responses resemble wild type seedlings. This suggests that EB1b activity is influenced by the type of mechanical stimulation perceived by the root such that EB1b activity does not appear to be involved in root skewing and gravitropic responses when MS is evenly distributed.

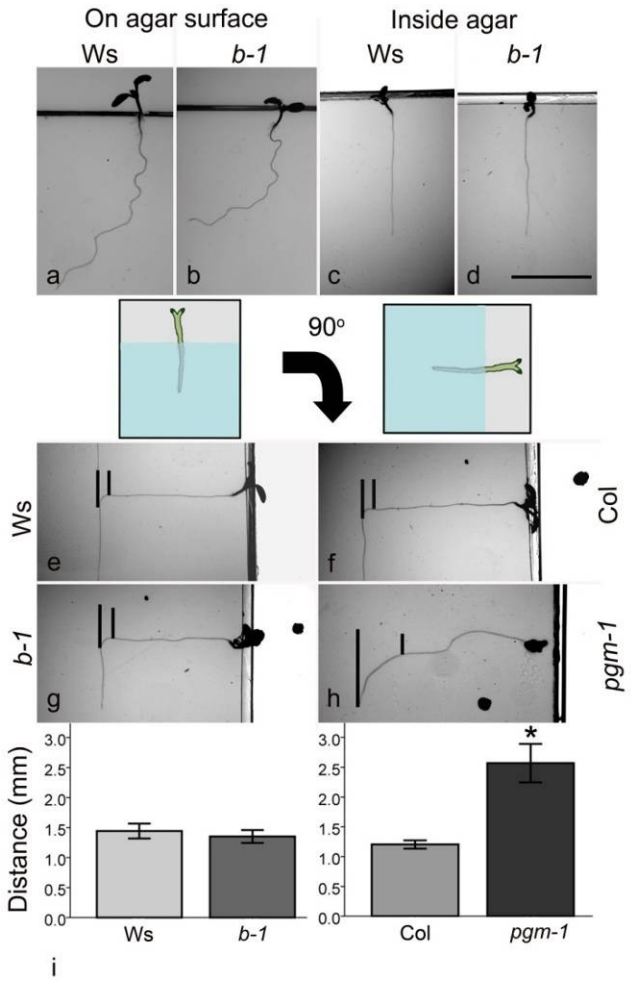

Figure 2 Growth and gravitropic responses of roots penetrating through an agar medium

On the surface of vertically oriented plates, wild type Ws (**a**) roots skew less than *eb1b-1* (**b**) roots. Inside the agar both Ws (**c**) and *eb1b-1* (**d**) roots grow straight down. Seedlings shown are 7 days old. Size bar in (**d**) represents 1 cm and applies to a-h. When 7 day old seedlings are rotated by 90º in the clockwise direction roots respond by bending down (**e-h**). The responses of Ws (**e**), Col-0 (**f**), *eb1b-1* (**g**) and *pgm-1* (**h**) were assessed by marking root tip position at the time of rotation and the location where root growth became reoriented parallel with the new gravity vector (indicated by vertical lines in the photographs). Average distances and 95% CIs are reported for seedlings from 4 experiments for Ws and *eb1b-1* (n=83) and 3 experiments for Col-0 and *pgm-1* (n=50). Asterisk denotes a response that is significantly different from wild type.

3.3 Double Mutant Analyses

Results from the first approach suggests that EB1b has a primary defect in root responses to MS. To determine whether genetic data supports this finding, *eb1b-1* mutants were crossed to plants carrying mutations in genes associated with responses to MS and gravity. Three different genotypes were chosen for crosses to *eb1b-1*. Two mutants, *pgm-1* and *arg1-3*, have defects responding to gravity while the third genotype carries a T-DNA insertion in *TCH3,* a gene that is up- regulated in response to MS, (Caspar and Pickard, 1989; Sedbrook et al., 1999; Braam, 2005). As discussed above, *PGM* encodes an enzyme that is required for starch biosynthesis. Without starch, the amyloplasts sediment at a slower rate in response to gravity resulting in delays in gravitropic bending (Caspar and Pickard, 1989; Sæther and Iversen, 1991). ARG1 is also involved in root responses to gravity and encodes a DnaJ-like protein (Sedbrook et al., 1999). Although its precise role in gravitropism is not well understood, *arg1pgm* double mutant analysis revealed enhanced gravitropic delays suggesting that ARG1 functions in a pathway that is genetically distinct from PGM (Guan et al., 2003). ARG1 is proposed to have a role in gravity signalling (Harrison and Masson, 2008). In response to a change in gravity, *arg1* mutants have defects in relocalizing PIN3 (an auxin efflux carrier). PIN3 directs the flow of auxin in the columella cells therefore the lateral distribution of auxin is disrupted in the root cap. This results in delays in forming a bend upon gravity stimulation (Harrison and Masson, 2008).

TCH3, on the other hand has been implicated in plant responses to MS due to an up-regulation of *TCH3* expression in plants stimulated by rain, wind and wounding. TCH3 encodes a calmodulin-like protein containing six EF-hand domains (Braam and Davis,

1990; Sistrunk et al., 1994; Chehab et al., 2009). TCH3 localizes to several areas in the plant including cells of the root cap and elongation zone, which correspond to regions of mechanical perception and response (Antosiewicz et al., 1995). To our knowledge *tch3* mutants have never been analyzed. The *Arabidopsis* Information Resource (TAIR) identified a mutant line carrying a T-DNA insertion in the final exon of *TCH3*. I crossed this mutant (*tch3-1*) to *eb1b-1* and the F3 progeny were analyzed for root growth defects.

The mutants exist in two different genetic backgrounds (*eb1b-1* is in Ws while, *pgm-1*, *arg1-3* and *tch3-1* are all in Col-0). To ensure that differences in root growth responses were attributed to the genotypes rather than differences in genetic background, homozygous wild-type, as well as single and double mutants in the Ws/Col-0 background were isolated from the progeny of the crosses.

Seedlings were grown on the surface of either vertically oriented or reclined plates and two measurements were made. (1) The angle between the gravity vector and the root tip (skewing angle or ɵ, Fig 3 a) was measured on seedlings growing on vertically oriented plates. A vertical plate orientation was chosen to measure skewing angles because roots rarely form loops under these conditions. (2) The proportion of roots with loops was determined from seedlings grown on plates oriented at 45°. At this plate orientation many of the roots form loops.

3.3.1 **Analyses of *eb1b-1/pgm-1***

To determine possible genetic interactions between EB1b and PGM, single and double mutants were analyzed. On vertical plates, both *eb1b-1* and double mutant roots skewed more than wild type and *pgm-1* ($P<0.0001$; Fig 3). While, *pgm-1* root skewing angle was statistically indistinguishable from wild type ($P=0.3833$). As would be

expected, the skewing angles of double mutant roots were statistically indistinguishable from the *eb1b-1* single mutant (P=0.2439). When seedlings were grown on 45° plates all genotypes formed loops. The *eb1b-1* mutants formed more loops than both wild type (P<0.0001) and *pgm-1* (P=0.0112). In contrast to the root growth on vertical plates, *pgm-1* roots looped significantly more than wild type when grown on 45° plates. Since *pgm-1* roots display a mutant phenotype, analyzing the response of the double mutants may indicate a genetic interaction between EB1b and PGM. If the double mutants display an additive phenotype it would suggest no genetic interaction between EB1b and PGM. When double mutants were grown on 45° plates they formed a slightly higher proportion of loops compared to *eb1b-1*. Statistical analysis provides some evidence to suggest a difference in the average proportion of loops formed by double mutants when compared to *eb1b-1* roots (P=0.0685). This slight enhancement of loop formation may be an indication of an additive phenotype suggesting that EB1b and PGM do not genetically interact to influence root skewing and looping.

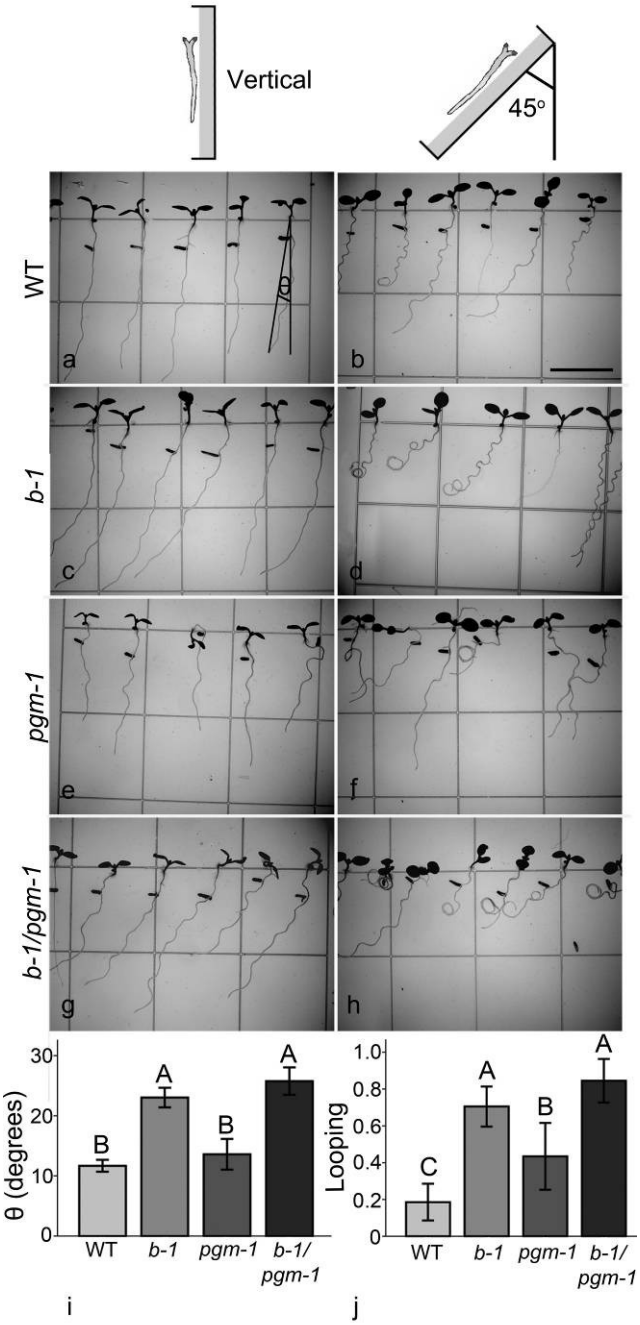

Figure 3 Phenotypic analyses of *eb1b-1/pgm-1* double mutants

Wild type (**a, b**) *eb1b-1* (**c,d**), *pgm-1* (**e, f**) and *eb1b-1/pgm-1* (**g, h**) roots were analyzed on vertically oriented (**a, c, e** and **g**) and reclined (**b, d, f** and **h**) plates. Size bar in (**b**) represents 1 cm and applies to a-h. Skewing angles (ɵ, shown in **a**) were measured from roots grown on vertically oriented plates and the average angle for each genotype was determined (**i**). The average proportion of roots that formed loops (looping) was determined from seedlings grown on plates reclined at 45° (**j**). Skewing angles and proportions of roots with loops represent the averages from 5 experiments (n for each genotype ranged from 74-113 roots). The error bars are 95% CIs. A, B, and C refer to statistically different averages ($P<0.05$, Tukey's statistical test).

3.3.2 **Analyses of *eb1b-1/arg1-3***

To further explore whether EB1b may interact with a protein involved in gravity response, *eb1b-1* was crossed to *arg1-3* and the root skewing and looping phenotypes were analyzed from the progeny of this cross. The *eb1b-1* roots again skewed more and formed more loops than did wild type or *arg1-3* roots ($P<0.0001$; Fig 4). However, in contrast to *pgm-1*, *arg1-3* roots skewed less than wild-type. In addition, the *eb1b-1/arg1-3* mutants displayed skewing angles that were intermediate between the single mutant parents. Double mutant roots skewed significantly more than *arg1-3* and significantly less than *eb1b-1* ($P<0.0001$; Fig 4 e). This phenotype can be accounted for by an additive effect of both mutations, since *eb1b-1* and *arg1-3* have opposing effects on root skewing angle. Alternatively, when seedlings were grown on plates oriented at 45°, the proportion of loops formed by *arg1-3* mutants was indistinguishable from wild-type. Under these same conditions, double mutants formed a reduced proportion of looping compared to *eb1b-1* ($P=0.0018$; Fig 4 f). The double mutant phenotype suggests that *arg1-3* is masking or suppressing the effects *eb1b-1* on root skewing and looping. Since the double mutant phenotype can not be explained by the independent effects of each mutation this interaction is said to be epistatic where *arg1-3* is epistatic to *eb1b-1* (Roth et al., 2009). This interaction may be specific to growth on inclined plates which are responding to increased touch and gravity stimuli.

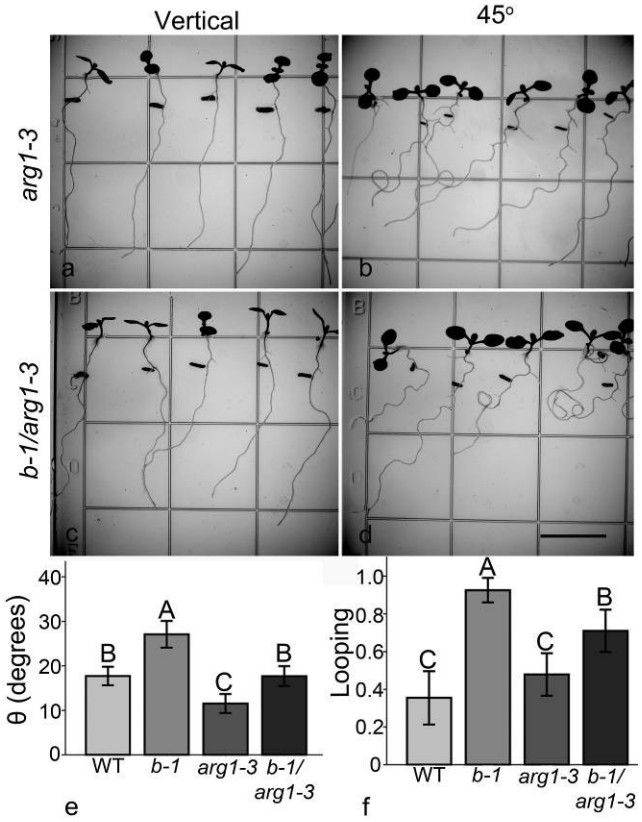

Figure 4 Phenotypic analyses of *eb1b-1/arg1-3* double mutants

Phenotypes of *arg1-3* (**a, b**) and *eb1b-1/arg1-3* double mutants (**c, d**) grown on vertically oriented (**a, c**) and reclined (**b, d**) plates. Size bar in (**b**) represents 1 cm and applies to a-d. Skewing angles (ɵ; e) and the average proportions of roots that formed loops (**f**) were measured as previously described. Skewing angles and proportions of roots with loops represent the averages from 5 experiments (n from each genotype ranged from 80-120 roots). The error bars are 95% CIs. A, B, and C refer to statistically different averages ($P<0.05$, Tukey's statistical test).

3.3.3 **Molecular Characterization of *tch3-1* mutants**

To assess whether EB1b has a role in root responses to mechanical stimuli, we investigated a possible genetic interaction between *eb1b-1* and a plant carrying a T-DNA insertion in a touch responsive gene, *TCH3*. *TCH3* is up regulated in response to MS (Chehab et al., 2009). We initially characterized two T-DNA insertional alleles. One line carries a T-DNA insertion in the predicted region of the promoter (SALK_098779.39.95.x). However, since RT-PCR analyses revealed the present of full-length transcripts in plants homozygous for the T-DNA insert this line was not analyzed any further (Fig 5c). The second line contained a T-DNA insertion in the final exon of *TCH3* (SALK_122731.26.30.x; Fig. 5a). The TAIR database lists several full length cDNAs corresponding to *TCH3.* These cDNAs vary with respect to the length of the coding sequence and the intron/exon structure (Fig 5a). To determine if SALK_122731.26.30.X (*tch3-1*) T-DNA insert disrupts the production of full length TCH3 RNA, RT-PCR analyses was performed. As a control to test for the presence of amplifiable cDNA in our samples, primers designed to recognize EB1b cDNA were used in PCR reactions. Bands of the appropriate size (500bp) were detected from both the Col-0 and the *tch3-1* mutant cDNA samples, indicating that cDNA sequences were successfully produced in our RT reactions (Fig 5b). Next, primers designed to amplify regions of the *TCH3* transcript located upstream (U), flanking (F) or downstream (D) of the T-DNA insertion site (Fig 5a) were used in PCR reactions. Bands were detected for Col-0 using each primer set (Fig 5b). However, bands were not detected from the *tch3-1* cDNA using the U and F primer sets indicating that neither a flanking nor an upstream region made mRNA. However, a band was detected with the D primer set (Fig 5b), indicating the presence of truncated transcript downstream of the T-DNA insertion. This

suggests that full-length transcripts are not present in *tch3-1* mutants, although a partial transcript downstream of the T-DNA insertion was detected. The downstream transcript corresponds to one of the six EF-hand domains present in TCH3 and a start codon is available in a correct reading frame, raising the possibility that this *tch3-1* allele may have partial function.

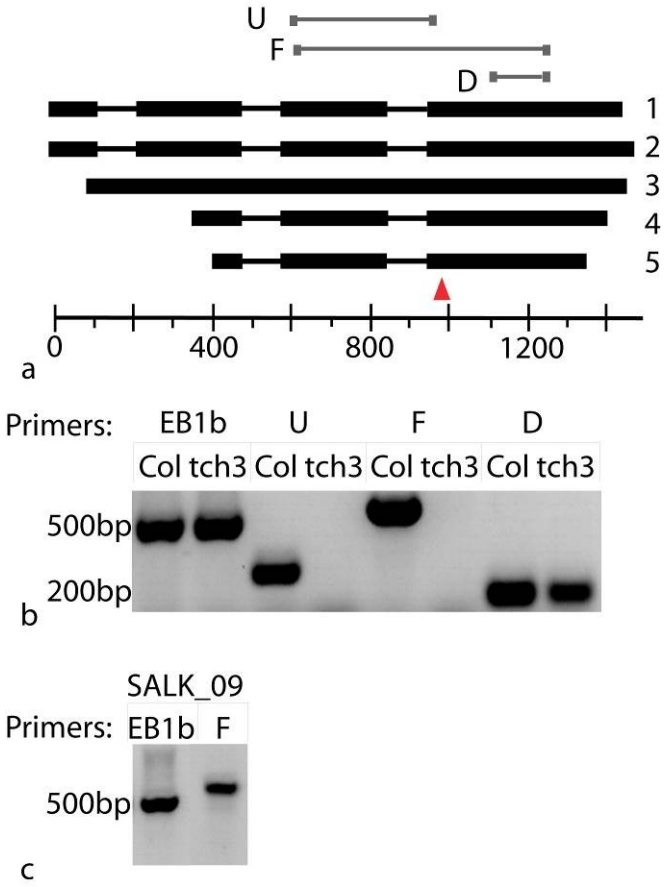

Figure 5 RT-PCR Analysis of *tch3-1* mutant cDNA

Multiple predicted transcripts, primer binding sites and T-DNA insertion site corresponding to the AT2G41100 (TCH3) gene (**a**). The transcripts (1-5) correspond to the following accession numbers reported by TAIR and NCBI: 1. AF424577, 2. AY120719, 3. BX820390, 4. BX818994, 5. BT000036. The introns are represented as black lines and exons as black boxes. Small grey boxes denote the primer pairs and the line connecting them represents the region amplified by RT- PCR. The arrowhead indicates the point of T-DNA insertion for the *tch3-1* (SALK_122731.26.30.x) allele. The scale is in nucleotides (**a**). RT-PCR analyses of Col-0, *tch3-1* (**b**) and SALK_098779.39.95.x (**c**) using RNA isolated from whole seedlings. The primer sets and cDNA sources are labeled along the top of the gel. Base pair number is indicated along the left margin. RT-PCR analysis of SALK_098779.39.95.x was performed by Jenine Suen.

3.3.4 **Gravitropic response of *tch3-1* when grown inside the agar**

When grown inside the agar it was observed that *tch3-1* grew similar to wild type. However, when given a gravitropic stimuli inside the agar after 7 days of growth (Fig 6 a, b), the *tch3-1* roots form a gravitropic bend after a shorter distance than wild type (1.08mm and 1.24mm respectively; P=0.008; Fig 6 c). In contrast to *arg1-3 and pgm-1*, *tch3-1* does not display gravitropic delays when grown inside the agar.

3.3.5 **Analysis of *b-1/tch3-1* double mutants**

In order to test whether EB1b interacts with a protein up regulated in response to MS *eb1b-1* was crossed to *tch3-1* and the progeny was analyzed. When grown on vertical plates, *tch3-1* skewed less and *eb1b-1* skewed more than wild-type. The double mutant skewed significantly less than *eb1b-1* single mutants ($P<0.0001$). Similarly, when roots were analyzed on 45° plates, the double mutants form a significantly reduced amount of loops compared to *eb1b-1* ($P<0.0001$) and a similar amount to *tch3-1* ($P= 0.3107$). The double mutants having reduced skewing and looping phenotypes compared to *eb1b-1* indicates that *tch3-1* is epistatic to *eb1b-1* suggesting that TCH3 and EB1b genetically interact to influence root skewing and looping.

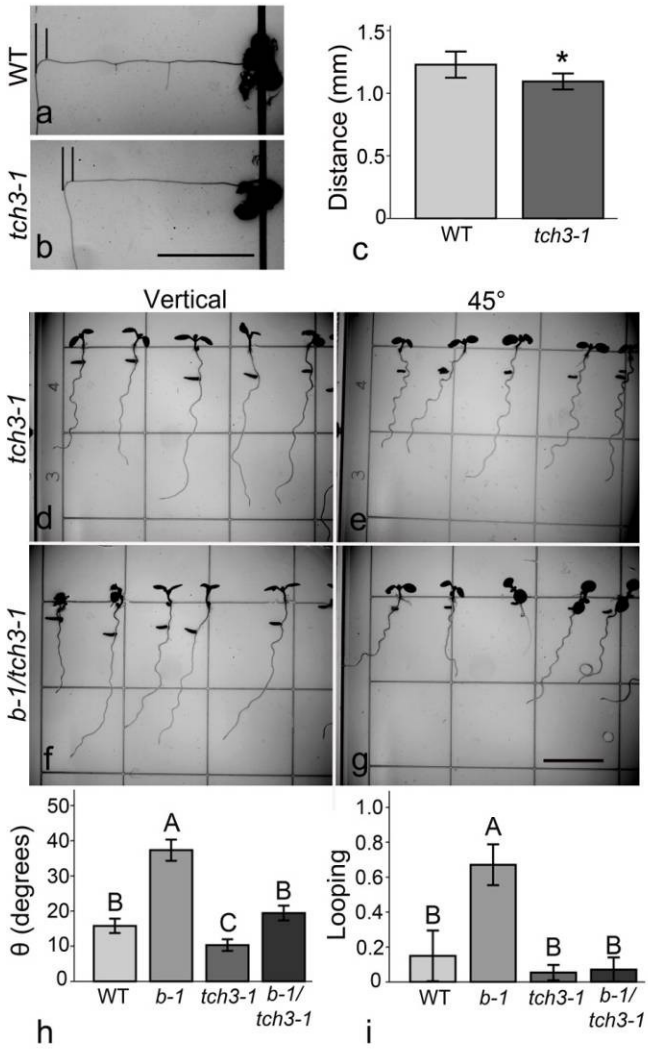

Figure 6 Phenotypic analysis of *tch3-1* and *b-1/tch3-1* mutants

7 day old seedlings are rotated by 90º in the clockwise direction and Col- 0 (**a**) and tch3-1 (**b**) roots respond by bending down. Gravitropic responses were measured (**c**) as described previously. Size bar in (**b**) represents 1 cm and applies to a and b. Average distances and 95% CIs are reported for seedlings from 3 experiments (Col-0, n= 80; tch3-1, n= 115). The asterisk denotes a significant difference in response compared to wild type. Phenotypes of *tch3-1* (**d, e**) and *eb1b-1/tch3-1* double mutants (**f, g**) grown on vertically oriented (**d, g**) and reclined (**e, g**) plates. Size bar in (**g**) represents 1 cm and applies to d-g. Skewing angles (ɵ; **h**) and the average proportions of roots that formed loops (**i**) were measured as previously described. Skewing angles and proportions of roots with loops represent the averages from 6 experiments (n from each genotype ranged from 119-148 for skewing data and 73-92 roots for looping data). The error bars are 95% CIs. A, B, and C refer to statistically different averages ($P<0.05$, Tukey's statistical test).

4: Discussion

Although EB1b was previously reported to be associated with root responses to touch and/or gravity signals, its relative contributions to each response was unknown (Bisgrove et al., 2008). I set out to determine whether EB1b functions in root responses to MS, gravity, or both. I began by assessing the response of *eb1b-1* roots to altered amounts of MS. This was accomplished by analyzing roots growing through agar or on the surface of agar plates tilted at varying degrees from a vertical alignment. Roots growing inside the agar will receive MS that is uniformly distributed. On the other hand, roots growing on the surface of the agar will perceive touch stimulation as the root tries to penetrate the agar in response to gravity. Increasing the angle at which the plates are tilted results in more touch stimulation. I also assessed possible genetic interactions by analyzing double mutants between *eb1b-1* and plants carrying mutations in genes associated with root responses to MS or gravity. My results support a model in which EB1b affects root responses to MS and indirectly influences gravity response.

4.1 EB1b has a role in root responses to mechanical stimulation

There are two lines of evidence to suggest EB1b has a role in root responses to MS. First, the *eb1b-1* phenotype depends on the type of MS perceived. In contrast to roots grown on the surface of the agar, *eb1b-1* roots are indistinguishable from wild type when grown inside the agar as neither genotype skews nor loops (Fig 2). Similarly, *eb1b-1* roots grown inside the agar respond equivalently to wild type when given a gravity

stimulus. These results indicate that *eb1b-1* mutants have root growth defects after perceiving asymmetric touch stimulation. The second line of evidence comes from *eb1b-1* root responses to increasing MS. *eb1b-1* roots are more sensitive to increasing the plate angle than wild type roots (Fig 1). This suggests that wild type EB1b activity results in the suppression of root looping in response to asymmetric MS.

4.2 EB1b and TCH3 in the same genetic pathway

To investigate possible genetic interactions between *EB1b* and a gene implicated to function in touch response, *eb1b-1* mutants were crossed to plants carrying T-DNA insertions in the *TCH3* gene (*tch3-1*). TCH3 is a calmodulin like protein containing 6 Ca^{2+} binding EF hand domains.TCH3 was chosen because its expression is rapidly up regulated in response to mechanical perturbation and is expressed in most plant organs including the root (Antosiewicz et al., 1995; Chehab et al., 2009). My molecular analyses of *tch3-1* indicate that the T-DNA insertion disrupts transcription of the gene. However, a partial transcript was detected, corresponding to sequences downstream of the T-DNA insertion. This partial transcript encodes a single EF-hand domain suggesting that *tch3-1* may not be a null mutant. Nevertheless, I found that the *tch3-1* mutants do have a mutant phenotype. They exhibited reduced skewing and looping on the surface of agar plates although only the skewing phenotype was significantly different from wild type (Fig 6). In addition, the reduction in loop formation of the double mutants suggests a role for TCH3 in promoting both looping and skewing. (This interpretation impinges on *tch3-1* being a recessive mutation, which remains to be determined). One explanation for the mild phenotype of *tch3-1* single mutants could be functional overlap with other genes.

TCH2, another calmodulin- like protein is also up-regulated during touch stimulation (Braam and Davis, 1990; Chehab et al., 2009). The double mutant phenotype also suggests that *tch3-1* is epistatic to *eb1b-1* indicating that EB1b and TCH3 have a genetic interaction, supporting a role for EB1b in root responses to MS.

4.3 EB1b does not appear to have a direct role in root responses to gravity

EB1b does not appear to directly influence root responses to gravity since *eb1b-1* mutants grown inside the agar have a gravitropic response that is indistinguishable from wild type (Fig 2). Previous studies have shown that *eb1b-1* roots have gravitropic defects when grown on the surface of the agar (Bisgrove et al., 2008).This observation could be accounted for if there was cross talk between gravity and touch responses. In such a scenario enhanced touch stimulation on the surface of the agar would increase the down regulation of root responses to gravity. It has been reported that MS can down regulate root responses to gravity (Massa and Gilroy, 2003). The idea of cross talk is also consistent with the results from our double mutant analyses.

Two mutants with defects in root responses to gravity (*arg1-3* and *pgm-1*) were chosen for crossing to *eb1b-1*. ARG1 is a DNA-J like protein that appears to have a role in gravity signal transduction pathways (Harrison and Masson, 2008). It has been shown to function downstream of amyloplast sedimentation in a separate pathway from PGM (Sedbrook et al., 1999; Guan et al., 2003). ARG1 appears to have a role in root growth on agar surfaces since *arg1-3* mutants have reduced skewing and double mutants have significantly reduced looping relative to that seen in *eb1b-1.* The reduced looping

phenotype of the double mutants relative to *eb1b-1* observed on 45° plates indicates that *arg1-3* is epistatic to *eb1b-1*. This may suggest that EB1b and ARG1 have a genetic interaction in response to increased mechanical stimulation. Since ARG1 has proposed roles in gravity response and EB1b in touch response, this interaction may be a result of crosstalk between gravity and touch response pathways. As mentioned above there has been previously reported evidence to suggest crosstalk between these two pathways (Massa and Gilroy, 2003).

The second mutant with defects in gravity response to be crossed to *eb1b-1* was *pgm-1*.PGM is an enzyme involved in starch synthesis; *pgm-1* mutants accumulate less starch in their amyloplasts. This reduces the rate of amyloplast sedimentation and results in gravitropic delays (Caspar and Pickard, 1989; Sæther and Iversen, 1991). When *pgm-1* mutants were grown on the surface of the agar, root looping was significantly greater than wild type while skewing remained equivalent. The enhanced looping of *pgm-1* on 45° plates could be attributed to defects in detecting gravity such that the root forms a loop rather than bending downwards in response to gravity. One explanation for observing equivalent skewing angles of *pgm-1* and wild-type may be that root tips deviate less from the plane of gravity when grown on vertical plates reducing the need to detect gravity. Similar to *pgm-1*, *eb1b-1* mutants also have increased looping. The looping phenotype observed in the *eb1b-1/pgm-1* double mutants was additive, as would be expected if the two genes contributed individually to root looping and skewing. This result would then suggest that EB1b and PGM do not genetically interact, supporting a primary role of EB1b in root responses to MS since PGM is thought to function in gravity responses.

4.4 Roles for EB1

Taken together, these results are consistent with a model in which EB1b contributes to root responses to MS and gravitropism is then affected by cross talk between the two pathways. What roles might MTs and EB1 have in root responses to MS? One possibility is that EB1 assists in the detection of MS. In plants, it has been proposed that MTs are tethered to mechanosensory transmembrane proteins. Deformations in the plasma membrane would cause alterations in MTs that result in channel activation and the initiation of signal transduction (Nick, 2008). In support of this idea, cells treated with drugs that depolymerize MTs have increased calcium channel activity that could be linked to the initiation of signalling events (Thion et al., 1996; Thion et al., 1998). Another possibility is that MTs are involved in vesicle trafficking. MTs, in association with MAPs, can target the delivery of vesicles by anchoring to membrane surfaces (Akhmanova and Steinmetz, 2008).Through this activity MTs and EB1 may contribute to the localization of receptors or ion channels that are involved in mechanosensing or signal transduction (Bisgrove, In press). EB1 has been shown to target K^+ channels to axons in neurons (Gu et al., 2006). In addition, EB1 could interact with and localize/sequester proteins involved in signalling pathways (Sun et al., 2008; Akhmanova et al., 2009). For example, EB1 associates with a Rho-type guanine nucleotide exchange factor in *Drosophila* and proteins in kinase signalling pathways in mammalian cells (Rogers et al., 2004; Sun et al., 2008; Zhang et al., 2009).

EB1 could also have a role in localizing organelles within mechanosensory cells. One organelle thought to function as a receptive surface for perceiving mechanical signals is the endoplasmic reticulum (ER) (Leitz et al., 2009). In mechanosensitive

columella cells, the ER is held close to the cell cortex by MTs adjacent to the plasma membrane where it has the potential to initiate signal transduction through the rapid release of internal calcium stores (Hensel, 1984). In addition, GFP-EB1 localizes to membranes of ER and ER bodies in leaf epidermal cells (Mathur et al., 2003). Animal literature also implicates a potential role for EB1 in remodeling the ER through its association with an ER transmembrane protein called stomatal interaction molecule 1 (STIM1) (Grigoriev et al., 2008). EB1 could also affect cytoplasmic organization within cells through cross talk with the actin cytoskeleton since EB1 is known to bind proteins that modify the actin cytoskeleton (Bartolini et al., 2008; Minc et al., 2009; Schober et al., 2009; King et al., 2010; Takahashi et al., 2010). Disruption of the actin cytoskeleton would then result in a disorganized mechanosensory cell with defects in responding to stimulation.

In addition to possible functions in sensory cells, EB1 could also have a role in the responding cells located in the elongation zone of the root. EB1 localizes to the plus ends of MTs in cells of the elongation zone (Bisgrove et al., 2008; S. Squires personal communication). During MS the root buckles causing strain in the cells of the elongation zone. These cells are thought to respond by increasing the strength of their cell walls (Monshausen et al., 2009). One idea is that EB1 could be helping strengthen the cell walls in the elongation zone in order to resist mechanical strain. In support of this idea, MTs are known to have roles in the insertion of cell wall modifying enzymes into the plasma membrane (Robert et al., 2005; Roudier et al., 2005; Crowell et al., 2009).

Which of these processes EB1 may be functioning in is a topic for future investigations. Possible links between EB1 and signal detection, vesicle trafficking, ER,

regulation of the actin cytoskeleton, cytoplasmic organization in columella cell and wall modifications in cells of elongation zone are all areas for possible research.

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