The Microtubule Plus-End Binding Protein EB1 Functions in Root Responses to Touch and Gravity Signals in *Arabidopsis*

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Microtubules function in concert with associated proteins that modify microtubule behavior and/or transmit signals that effect changes in growth. To better understand how microtubules and their associated proteins influence growth, we analyzed one family of microtubule-associated proteins, the END BINDING1 (EB1) proteins, in *Arabidopsis thaliana* (EB1a, EB1b, and EB1c). We find that antibodies directed against EB1 proteins colocalize with microtubules in roots, an observation that confirms previous reports using EB1-GFP fusions. We also find that T-DNA insertion mutants with reduced expression from EB1 genes have roots that deviate toward the left on vertical or inclined plates. Mutant roots also exhibit extended horizontal growth before they bend downward after tracking around an obstacle or after a 90° clockwise reorientation of the root. These observations suggest that leftward deviations in root growth may be the result of delayed responses to touch and/or gravity signals. Root lengths and widths are normal, indicating that the delay in bend formation is not due to changes in the overall rate of growth. In addition, the genotype with the most severe defects responds to low doses of microtubule inhibitors in a manner indistinguishable from the wild type, indicating that microtubule integrity is not a major contributor to the leftward deviations in mutant root growth.

INTRODUCTION

Since plants are sessile, they respond to changes in the environment by modifying their growth. Changes in growth are usually effected by altering patterns of cell expansion and cell division, and microtubules are key players in both processes. In addition, the microtubule arrays found in plants are strikingly different from the arrays found in other eukaryotes. Higher plant cells lack centrosomes, and microtubules in expanding interphase cells are found beneath the plasma membrane in parallel arrays that encircle the cell. Cell elongation is perpendicular to the microtubules, which are thought to regulate expansion by influencing cell wall properties, while the wall mechanically constrains the direction of expansion (reviewed in Wasteneys and Fujita, 2006). Plant microtubules also behave differently from those of other eukaryotes in mitosis. Prior to entering mitosis, a preprophase band of microtubules, actin, and associated proteins assembles in the cortex at the position of the future division site. Upon entry into mitosis, the preprophase band gives way to the mitotic spindle and the cell cortex becomes devoid of microtubules. Without centrosomes, plant spindles are barrel-shaped with unfocused poles and no astral microtubules. As cells exit anaphase, the spindle breaks down and a unique cytokinetic structure, the phragmoplast, assembles in the center of the cell. The phragmoplast is a cylindrical array composed of actin filaments and opposing sets of parallel microtubules that control cell plate deposition. As cytokinesis proceeds, the phragmoplast and cell plate expand centrifugally and fuse to the mother cell wall at the site marked by the preprophase band. After cytokinesis, cortical microtubules reappear and are organized into parallel arrays (for reviews, see Wasteneys, 2002; Gardiner and Marc, 2003).

How microtubules are regulated and how they influence growth are key questions in plant cell biology. Microtubules are dynamic structures that are usually growing or shrinking within the cell. The dynamic nature of microtubules provides the flexibility that allows rearrangements into different arrays. Microtubules also function in concert with a fleet of microtubule-associated proteins that modify microtubule dynamics and influence microtubule interactions with other subcellular structures (Niethammer et al., 2007). To understand how microtubules and their associated proteins influence plant growth, we are analyzing one family of microtubule-associated proteins, the END BINDING1 (EB1) proteins in *Arabidopsis thaliana*.

EB1 belongs to a group of microtubule-associated proteins that are known as microtubule plus-end tracking proteins because they preferentially accumulate at the rapidly growing or plus ends of microtubules. Although EB1 has been intensively studied in animal and fungal cells, how it functions remains enigmatic (Vaughan, 2005). Recent work has shown that EB1 proteins bind to microtubule plus ends at the seam that joins the tubulin protofilaments into a tube-shaped structure (Sandblad et al., 2006). In addition to microtubules, EB1 also interacts with several other proteins, including many of the known microtubule plus-end tracking proteins. This observation has led to the proposal that EB1 is an integrator of protein complex assembly.
on microtubules (Vaughan, 2005; Lansbergen and Akhmanova, 2006). In some cases, EB1 facilitates delivery of its interacting partners to specific subcellular sites. Examples include the axonal targeting of a voltage-gated potassium channel in neuronal cells (Gu et al., 2006), the delivery of DPhoGEF2 (a guanine nucleotide exchange factor) to target sites in the cell cortex in Drosophila melanogaster (Rogers et al., 2004), the melanophilin-dependent transfer of melanosomes from microtubule plus ends to actin at the distal ends of melanocytes (Wu et al., 2005), and the delivery of connexin to adherens junctions in animal cells (Shaw et al., 2007). EB1 proteins are also involved in microtubule searching of the cytoplasm for specific capture sites (Su et al., 1995; Morrison et al., 1998; Tirnauer et al., 1999, 2002a; Bloom, 2000; Miller et al., 2000; Tirnauer and Bierer, 2000; Nakamura et al., 2001). Capture sites often contain F-actin, and EB1 proteins can therefore link the microtubule and actin cytoskeletons in specific cellular domains (Goode et al., 2000; Carvalho et al., 2003). EB1-mediated microtubule search and capture is thought to facilitate mitotic spindle alignment and assembly, microtubule binding to chromosomes, and cargo delivery to specific sites within the cell (Bloom, 2000; Bienz, 2001; Hayles and Nurse, 2001; Schroer, 2001; Schuyler and Pellman, 2001; Segal and Bloom, 2001; Tirnauer et al., 2002a, 2002b; Galjart and Perez, 2003; Green et al., 2005). While bound to microtubules, EB1 proteins also influence microtubule dynamics. Recent in vitro analyses indicate that EB1 affects dynamics by suppressing shortening of the microtubule plus ends (Manna et al., 2008).

Although EB1 has been the object of intense scrutiny in yeast and cultured animal cells, analyses in multicellular systems are just beginning. So far, eb1 mutants have been described in Dictyostelium discoideum and D. melanogaster, and in both organisms the phenotypes are surprisingly mild. Rehberg and Graf (2002) found that a Dictyostelium eb1 null mutant was viable but had defects in spindle formation that slowed the progression into metaphase. Mutants were able to overcome many of the spindle defects after prolonged cultivation. In the first genetic study of EB1 in a developing metazoan, Elliott et al. (2005) showed that hypomorphic Dm Eb1 mutants did not display any obvious defects in spindle assembly or mitosis but did have neuromuscular defects.

In plants, several groups have reported subcellular localization patterns of green fluorescent protein (GFP) fusions to At EB1 proteins (Chan et al., 2003, 2005; Mathur et al., 2003; Van Damme et al., 2004a, 2004b; Abe and Hashimoto, 2005; Dhonukshe et al., 2005; Dixit et al., 2006). These analyses show that plant EB1-GFP fusions colocalize with microtubules and exhibit microtubule plus end tracking ability. In addition, Van Damme et al. (2004b) have shown that overexpression of one EB1 family member increased microtubule polymerization rates, indicating that EB1 proteins can influence microtubule dynamics in plant cells.

Complete analysis of the role of EB1 proteins in plants requires analysis of mutant phenotypes. The existence of Arabidopsis eb1 mutants has been reported in a review article (Kaloriti et al., 2007). Here, we find that Arabidopsis eb1 mutants have root growth defects. Mutant roots deviate toward the left when grown on vertically oriented or inclined agar plates. Mutant roots also exhibit delayed responses to gravity after tracking around an obstacle in their path or after a 90° clockwise rotation of the root. These delays suggest that EB1 plays a role in initiating downward bends in response to gravity and/or touch signals, and this may be the reason for the enhanced leftward deviations in root growth observed in mutants. We also find that EB1 antibodies colocalize with microtubules in roots, an observation that confirms previous reports using At EB1-GFP fusions.

RESULTS

Surveys of the Arabidopsis genome reveal three EB1 genes, designated EB1a (At3g47690), EB1b (At5g62500), and EB1c (At5g67270) (Figure 1A; Chan et al., 2003; Gardiner and Marc, 2003; Mathur et al., 2003; Meagher and Fechheimer, 2003; Bisgrove et al., 2004). Each gene is predicted to code for 31.7-, 32.7-, and 35.8-kD proteins that are 54 to 63% similar and 37 to 41% identical to human EB1 (National Center for Biotechnology Information accession number AAC09471) at the amino acid level. Each predicted protein also contains two domains that are conserved in EB1 proteins from diverse organisms. Microtubule binding has been mapped to the calponin homology domain near the N terminus, while the coiled-coil EB1 domain is involved in protein–protein interactions (Bu and Su, 2003; Figure 1B). The Arabidopsis EB1 family members are closely related to each other. At the amino acid level, EB1a is 78% identical to EB1b, and EB1c is more divergent; it is 49% identical to EB1a and EB1b. Most of the sequence identity maps to the conserved calponin homology and EB1 domains of the proteins.

To determine where EB1 genes are expressed, several organs were assayed for the presence or absence of transcripts. RT-PCR experiments revealed that EB1 genes are expressed in multiple plant tissues (Figure 1C). All three EB1 genes are expressed in cotyledons, leaves, flowers, siliques, and roots, and two family members, EB1a and EB1c, were detected in stems. Coexpression of multiple EB1 genes in the same tissues raises the possibility that the genes could have overlapping functions. Curiously, cDNA from some organs yielded more PCR product than others. Although these experiments were not quantitative in nature, this observation raises the possibility that expression from EB1 genes varies across different organs. Lower expression levels could be due to either a uniform reduction in expression throughout the organ or a restriction of expression to a subset of cells within the organ. Hence, the weaker PCR bands observed in roots could be due to a confinement of expression to actively elongating or dividing cells.

EB1 Proteins Colocalize with Microtubules in Roots

Previous reports have shown that GFP-tagged At EB1 proteins colocalize with microtubules and track microtubule plus ends in plant cells (Chan et al., 2003, 2005; Mathur et al., 2003; Van Damme et al., 2004a, 2004b; Abe and Hashimoto, 2005; Dhonukshe et al., 2005; Dixit et al., 2006). We used an alternative approach to assess EB1 localization. Affinity-purified polyclonal antibodies were generated against bacterially expressed full-length EB1c, the most divergent of the three family members (Figure 1B). Protein gel blot analysis of bacterially expressed proteins showed that the antibodies recognized both EB1c and
EB1a (Figure 2A). Although EB1b was not tested, because EB1a shares 78% sequence identity with EB1b and only 49% identity with EB1c, the antibodies most likely cross-react with all three EB1 family members. The antibodies also detected a major band around 35 kD in protein extracts from wild-type seedlings (Figure 2B). Since the three EB1 proteins are very close in size, this band is most likely an unresolved triplet.

EB1 localization was examined in wild-type root tips where the antibodies labeled microtubules in preprophase bands, mitotic spindles, and phragmoplasts (Figure 3). Localization to mitotic and cytokinetic arrays in plant cells has previously been reported for At EB1 fusions to GFP (Chan et al., 2003, 2005; Mathur et al., 2003; Van Damme et al., 2004a; Dhonukshe et al., 2005). EB1 antibodies labeled microtubules throughout these arrays, a pattern that could reflect either EB1 localization along the lengths of microtubules or preferential localization to the ends of short microtubules that are distributed throughout the arrays. In the preprophase band, EB1 appears to be more abundant toward the center of the band. Changes in microtubule dynamics have been observed during preprophase band formation, and one model proposes that dynamic microtubules are recruited into the preprophase band by a search and capture mechanism (Dhonukshe and Gadella, 2003; Vos et al., 2004). Preferential localization to the center of the preprophase band suggests that EB1 proteins could play a role in this recruitment process. The EB1 antibodies labeled microtubules in the mitotic spindle, as has previously been observed for EB1 proteins in other eukaryotic cells (Berrueta et al., 1998; Morrison et al., 1998; Rehberg and Graf, 2002; Rogers et al., 2002; Tirnauer et al., 2002b), including plants (Chan et al., 2003, 2005; Van Damme et al., 2004a). In the phragmoplast, EB1 antibodies label more abundantly in the midzone, suggesting that EB1 proteins are biased toward microtubule plus ends. In many interphase cells, the nuclear region labeled intensely and a more diffuse, punctuate pattern was observed in the cytoplasm. Perinuclear and cytoplasmic microtubules were also visible in these cells. Although the significance of nuclear-associated EB1 is not clear, a transient perinuclear array of microtubules (the radial array) has been detected in plant cells that are exiting cytokinesis (Hasezawa et al., 1991), and it is possible that EB1 proteins are associated with these microtubules. Nuclear labeling of GFP-tagged EB1a has also been reported in cytokinetic Arabidopsis suspension cells (Chan et al., 2003, 2005). In addition, nuclear labeling has also been reported for an At EB1c-GFP fusion in interphase Arabidopsis and BY-2 cells (Dixit et al., 2006). Although not visible in this photograph, occasional EB1 puncta were observed in association with the cortical microtubules of interphase root tip cells. Previous studies with At EB1-GFP fusions report numerous EB1 puncta in the

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**Figure 1. Expression Analysis of EB1 Genes.**

(A) EB1 genes, with introns designated as lines and exons as boxes. Horizontal arrows mark the positions of the PCR primers used in RT-PCR experiments (primers are not drawn to scale). Thin black arrows indicate the a1, b1, and c1 primer pairs, thick black arrows represent the a2, b2, and c2 primer pairs, and gray arrows represent a3, b3, and c3 primer pairs (corresponding to EB1a, EB1b, and EB1c, respectively). Scale is in nucleotides.

(B) Predicted EB1 proteins contain conserved calponin homology and EB1 domains (gray boxes). Vertical arrows in (A) and (B) designate the sites of T-DNA insertions. Black arrows mark the insertion sites in plants from the Ws genetic background, while the gray arrow represents the insertion site of the eb1b-2 allele in the Col-0 background.

(C) RT-PCR analyses using RNA from wild-type (Ws) plants indicate that EB1 genes are coexpressed in multiple plant organs. (D) RT-PCR analyses using RNA isolated from flowers indicate that full-length transcripts are undetectable in each homozygous eb1 mutant line (left panel). However, partial transcripts corresponding to sequences 5' of the insertion site are detected (right panel). In (C) and (D), the PCR primer pairs used in each analysis are indicated to the left of the appropriate lanes.
cortex of elongating interphase cells (Chan et al., 2003; Van Damme et al., 2004b; Dhonukshe et al., 2005; Dixit et al., 2006). Why interphase cells in the root tip would have fewer cortical EB1 puncta than other cell types is not clear. Perhaps there is a shift toward EB1 accumulation on cortical microtubules as cells exit the cell cycle and enter the elongation zone of the root.

EB1c-Enriched Antibodies Preferentially Label Microtubule Plus Ends

We also investigated the labeling patterns associated with a pool of antibodies more specific for EB1c, the original antigen. To enrich for EB1c-specific antibodies, a column containing bacterially expressed EB1a protein was used to remove antibodies that recognize epitopes common to EB1a and EB1c from the pool. When used as a probe in protein gel blot analyses, the anti-EB1c enriched pool clearly detected a band at \(35\) kD from plants carrying intact versions of the \(EB1c\) gene (Figure 4A). Bands were barely detectable in samples from mutants carrying the \(eb1c-1\) allele, suggesting that the pool is significantly enriched for EB1c-specific antibodies. When used on dividing Arabidopsis cells, the EB1c labeling was biased toward the midzones of spindles and phragmoplasts, where the plus ends of microtubules are more concentrated (Figures 4B to 4I). Scans of fluorescence intensity across the phragmoplast confirmed that EB1c labeling was most intense in the midline of the phragmoplast, between opposing sets of microtubules (Figure 4E).

Identification of Plants Carrying T-DNA Insertions in \(EB1\) Genes

To assess the effects of altering \(EB1\) expression on plant growth and development, we isolated plants carrying T-DNA insertions in each of the three \(EB1\) genes from the BASTA population at the Wisconsin Knockout Facility (Krysan et al., 1999; Weigel et al., 2000). The alleles were designated \(eb1a-1\), \(eb1b-1\), and \(eb1c-1\). Each line was backcrossed to wild-type Wassilewskija (Ws) plants three times, and the genotypes of F1 and F2 progeny were verified by PCR using primers specific for \(EB1\) genes and the T-DNA insert (see Methods). The PCR fragments were sequenced to determine the positions of the T-DNA insertions in each gene (Figures 1A and 1B). To test for the presence of T-DNA insertions at additional sites in the...
genome, F2 progeny from backcrosses to Ws plants were grown on Basta-containing agar plates. Since Basta resistance is conferred by the T-DNA insert, F2 plants from a line with an insertion at a single site will segregate 3 resistant to 1 sensitive, while lines with multiple unlinked insertions will have a higher number of Basta-resistant F2 plants. For all three lines, F2 progeny from backcrosses exhibit 3:1 segregation for Basta resistance. These lines, therefore, carry either a single T-DNA insertion or multiple closely linked insertions. The eb1b-2 allele was identified using the SIGnAL T-DNA Express Arabidopsis gene mapping tool at the Salk Institute website (http://signal.salk.edu/cgi-bin/tdnaexpress). The WiscDsLox331A08 line in the Columbia (Col-0) genetic background was found to carry an insertion in the EB1b gene; it was designated as eb1b-2, and the corresponding seeds were obtained from The Arabidopsis Information Resource (TAIR; Garcia-Hernandez et al., 2002; Alonso et al., 2003). Progeny from plants heterozygous for the eb1b-2 allele also segregate 3 resistant to 1 sensitive when grown on Basta-containing agar plates.

Insertional lines were also analyzed for the presence of EB1 transcripts by RT-PCR. First, PCR primers located on opposite sides of the T-DNA insertion site were used (a2, b2, and c2 primer pairs; Figure 1). We were unable to detect transcripts corresponding to genes carrying T-DNA insertions, indicating that EB1 gene activity is disrupted in mutants (Figure 1D). We also analyzed mutants for the presence of partial transcripts using primer pairs located 5′ of the T-DNA insertion site (a3, b3, and c3 primers; Figure 1). In every case, transcripts corresponding to sequences 5′ of the T-DNA insertion were detected, indicating that transcription from mutant genes is not completely abolished. The locations of the T-DNAS within the genes predict that if truncated proteins were translated from the partial transcripts, the proteins would be missing at least part of the conserved EB1 domain. The eb1a-1 insertion is located in the middle of the EB1

Figure 4. An Anti-EB1c-Enriched Pool of Antibodies Labels Microtubules in a Spindle and the Phragmoplast.

(A) Protein gel blot analysis of extracts from mutant and wild-type plants probed with the anti-EB1c pool of antibodies (top panel). Labeling is reduced or absent in samples from seedlings carrying the eb1c-1 allele. Probing with anti-tubulin antibodies reveals that all lanes contain approximately equivalent amounts of protein (bottom panel). Arrows mark the positions of bands corresponding to At EB1c (~35 kD) and tubulin (~50 kD).

(B) to (E) Labeling with EB1c antibodies is biased toward microtubule plus ends in a metaphase spindle from an Arabidopsis root tip cell and in a phragmoplast from an Arabidopsis suspension cell. Both cells were double-labeled with anti-EB1c–enriched antibodies (B) and (F) and anti-tubulin antibodies (C) and (G) and were imaged by confocal microscopy. The spindle was also labeled with 4′,6-diamidino-2-phenylindole to visualize chromosomes at the metaphase plate (E). Merged images (D) and (H) and single labels are false colored with EB1c in green and microtubules in red. Intensity scans across the phragmoplast (E) show that EB1c is concentrated toward the midzones, near the plus ends of microtubules. The white line marks the position of the intensity scan.
domain, the *eb1b-1* and *eb1b-2* insertions fall in the calponin homology domain, and in *eb1c-1*, the T-DNA is located 3’ of the calponin homology domain (Figure 1B). These mutations, therefore, may not be completely devoid of gene function.

**Root Growth Is Affected in *eb1* Mutants**

Plants homozygous for *eb1a-1, eb1b-1, eb1b-2, or eb1c-1* are all fertile, with leaves, flowers, and stems similar in appearance to wild-type organs at the gross morphological level. Double and triple mutant combinations were generated with the *eb1a-1, eb1b-1, and eb1c-1* lines in the Ws genetic background. In every case, plants were fertile and obvious defects in aerial structures were not apparent (data not shown). To assess possible root growth defects, three analyses were conducted. First, seedlings were grown on agar plates that were oriented vertically. Under these conditions, *eb1* mutants exhibited root growth that skewed toward the left of the plate when viewed from above the agar surface (Figure 5). The most pronounced skewing was observed in *eb1b-1* mutants. All mutant combinations that were homozygous for *eb1b-1* showed skewing that was significantly different from wild-type roots (*P* ≤ 0.01) when the average angle at which roots deviated from the vertical direction was measured. Roots of *eb1a-1* and *eb1c-1* mutants also skewed to the left, but less dramatically than *eb1b-1*, and only *eb1c-1* mutants had skewing angles significantly different from the wild type (*P* ≤ 0.01).

Second, on agar plates inclined by 45°, all *eb1* genotypes exhibited leftward-oriented root growth that was significantly different from wild-type plants (*P* ≤ 0.01). On inclined plates, root skewing was again most pronounced in genotypes homozygous for *eb1b-1* (Figure 5). Double and triple mutant combinations skewed about the same amount as single mutants. Although skewing is reduced in the Col-0 genetic background, *eb1b-2* roots also exhibit significantly more leftward deviation in growth than Col-0 (*P* = 0.002; Figure 6). Third, on inclined plates containing a higher agar concentration (1.6% rather than 0.8%) all *eb1* genotypes had roots that skewed toward the left (data not shown). Plants homozygous for *eb1b-1* again showed the most dramatic defects; they tended to form leftward (clockwise) oriented loops more often than wild-type roots (Figure 7). Closer examination of loops revealed that they were associated with epidermal cell files that were twisted into left-handed helices (Figure 7B). Occasionally, loops with twisted epidermal cell files were also observed in wild-type roots. The amount of twisting in mutant and wild-type loops appeared identical, and mutant loops were not associated with excessive twisting of epidermal cell files. These analyses suggest that *EB1* family members influence root growth. Individual homozygous mutants all had a root skewing phenotype, indicating that intact *EB1* family members were not able to compensate for mutated genes. *eb1b-1* mutants had the most severe defects, suggesting that either *EB1b* plays a larger role in root growth or the *eb1b-1* allele retains less function than the *eb1a-1* and *eb1c-1* alleles.

Because *eb1b-1* mutants exhibited the strongest phenotype, we chose this line to assess root growth in heterozygous plants. Thirty-five F2 progeny from crosses between wild-type and *ateb1b-1* plants were analyzed on inclined 0.8% agar plates and then genotyped either by PCR or by testing the F3 progeny from individual F2 plants for Basta resistance. We found that homozygous *eb1b-1* roots exhibited the most pronounced leftward skewing; on average their roots skewed 44° (C.I. = 4.5°) to the left of a vertical vector. Heterozygous roots skewed significantly less than homozygotes (*Student’s*’ *t* test, *P* = 0.01) with an average skewing angle of 36° (C.I. = 6°). Skewing in wild-type roots (31°, C.I. = 9°) was slightly, but not significantly, less than heterozygous plants (*Student’s*’ *t* test, *P* = 0.24). Therefore, *eb1b-1* is recessive.

**eb1b Mutants Exhibit Defects in Response to Gravity/Touch Stimuli**

Roots growing on inclined agar plates respond to a combination of touch and gravity stimuli, and mutants with altered responses to these stimuli often exhibit skewed root growth and/or loop formation (Okada and Shimura, 1990; Rutherford and Masson, 1996). To investigate the responses of *eb1* mutants to touch and/or gravity, we monitored the ability of roots to navigate around an obstacle placed in their path. Plants were grown on vertically oriented plates. After 9 d, a cover slip was inserted into the agar just in front of the root tip, plates were rotated to position root tips parallel with the gravity vector, and roots were observed after they had navigated around the barrier. As previously described (Massa and Gilroy, 2003), wild-type roots tracked across the cover slip, and when they reached the edge, they curved downward in response to gravity (Figure 8A). *eb1* mutants also grew across the cover slip. However, when they reached the edge, many roots did not bend down but instead continued to grow horizontally before forming a downward curvature (Figure 8B). On average, wild-type Ws roots formed a bend within 0.25 mm (C.I. = 0.04) of the edge of the cover slip (Figure 8C). *eb1a-1* and *eb1c-1* roots grew 0.37 (C.I. = 0.11) and 0.35 (C.I. = 0.05) mm, respectively, beyond the edge of the cover slip, a distance that is significantly greater than wild-type plants (*P* ≤ 0.01 by *Student’s*’ *t* test). *eb1b-1* and triple mutants continued to grow horizontally for a much longer distance than any of the other genotypes. *eb1b-1* roots grew an average of 0.69 mm (C.I. = 0.21), and triple mutants grew ~0.72 mm (C.I. = 0.27) before forming downward bends. Delays were also observed in *eb1b-2* roots. Wild-type Col-0 plants formed bends ~0.2 mm (C.I. = 0.03) from the edge of the cover slip, while *eb1b-2* roots continued to grow horizontally for ~0.36 mm (C.I. = 0.13) before they bent down.

To assess whether mutant roots are also delayed in their response to a change in the gravity vector, seedlings on vertical agar plates were rotated to reorient the root tip horizontally. Roots were photographed prior to rotation and again after gravitropic bends were completed. The two images were superimposed in Photoshop, and the distance from the root tip before reorientation to the downward bend that formed afterwards was measured. Triple and *eb1b-1* mutants were chosen for these analyses since they exhibited the longest delays in the obstacle navigation assay described above. When roots were rotated in the clockwise direction, the left side of the root was positioned on the top, and both *eb1b-1* and triple mutants exhibited significant delays before forming downward bends (*P* = 0.05 and 0.01, respectively, by *Student’s*’ *t* test; Figure 8D). Wild-type roots grew ~0.43 mm (C.I. = 0.14) before bending down, while *eb1b-1* and
triple mutants grew 0.64 (C.I. = 0.39) and 0.89 (C.I. = 0.42) mm, respectively. By contrast, rotating roots in the counterclockwise direction positioned the left side of the root on the bottom, and mutant roots were able to bend down with kinetics similar to wild-type plants.

**Overall Elongation Is Normal in eb1 Roots**

To determine whether eb1 mutants have general defects in root expansion, we analyzed root growth and root tip morphology. Mutant root tips appeared morphologically normal, and root
widths and elongation rates were similar to the wild type (Figure 9). Mutant roots grew at a slightly faster rate than the wild type, but statistical analysis indicated that this difference was not significant (P > 0.01). Only eb1c-1 roots grown on inclined agar plates were slightly, but significantly (P < 0.01), thinner than the wild type. The fact that root widths and growth rates were normal in eb1 mutants indicates that the primary defect is not in the regulation of overall root expansion. In addition, observations at the cellular level failed to reveal any obvious defects in cell size, shape, or organization in mutant roots (data not shown).

**Microtubule Stability in eb1 Roots**

Since directional biases in root growth are sometimes associated with changes in microtubule stability (for example, Ishida and Hashimoto, 2007; Ishida et al., 2007), we assessed the sensitivities of eb1 mutants to the microtubule inhibitors oryzalin and taxol. Treating Arabidopsis seedlings with low doses of microtubule inhibitors is known to alter microtubule dynamics (Nakamura et al., 2004). Adding oryzalin, a microtubule destabilizing agent, to the growth medium inhibited root elongation in a

![Figure 6](image1.png)

**Figure 6.** In the Col-0 Genetic Background, eb1b-2 Mutant Roots Also Deviate toward the Left.

- **(A) and (B)** When grown on inclined 0.8% agar plates, Col-0 roots (A) exhibit a slight deviation toward the left, and this leftward skewing is enhanced in the eb1b-2 allele (B).
- **(C)** The average root skewing angle was determined for the wild type (Ws and Col-0, open bars) as well as for eb1b-1 and eb1b-2 mutants (gray bars). Angles are reported in degrees, and C.I.s are calculated at P = 0.01. n = 79 (9 to 27 seedlings per genotype), and asterisks denote average angles that are significantly different from the wild type (P ≤ 0.01) by Student’s t test.

![Figure 7](image2.png)

**Figure 7.** Mutant Roots Form Clockwise Oriented Loops and Coils on Inclined Plates with a High Concentration of Agar (1.6%).

- **(A)** A triple mutant has formed a clockwise coil (seedling on the left), while the wild-type seedling (right) has not.
- **(B)** Epidermal cell files in the coil of a triple mutant root are twisted into left-handed helices.
- **(C)** A representative experiment showing a percentage of cells forming loops and/or coils. Greater than 75% of roots homozygous for eb1b-1 formed loops and/or coils, while the same structures were not observed in the other genotypes. In this experiment, n = 160 (15 to 23 seedlings per genotype). Although the proportions of roots that form loops varies between experiments, eb1b mutants always form loops at much higher frequencies than the other genotypes.
dose-dependant manner for all of the genotypes tested (Figure 10A). At each concentration of oryzalin, root elongation was inhibited by about the same amount in eb1a-1, eb1b-1, eb1b-2, Ws, and Col-0, indicating that these genotypes are all equally sensitive to the drug. Root elongation in eb1c-1 and triple mutants, by contrast, was more severely inhibited at 0.2 and 0.1 μM oryzalin, respectively, indicating that these genotypes are more sensitive to oryzalin treatment. In addition to decreasing root elongation, 0.1 μM oryzalin also increased the amount of leftward skewing exhibited by eb1a-1, eb1b-1, eb1b-2, Ws, and Col-0 roots. For each mutant, root skewing was increased by about the same amount as it was in the corresponding wild-type plant. By contrast, oryzalin did not increase root skewing in eb1c-1 or triple mutants. In 0.1 μM oryzalin, eb1c-1 roots skewed about the same amount as they did in DMSO controls. Triple mutants skewed less in oryzalin than they did in DMSO, although these roots were also much shorter and may not have grown enough to exhibit much leftward deviation in growth. Taxol, a drug that stabilizes microtubules, also inhibited root elongation and increased leftward skewing. In taxol, however, all of the genotypes responded in a similar fashion (Figure 10B). Analysis of variance was used to statistically test for genotypic differences in root skewing responses to either oryzalin or taxol treatment (Zar, 1974). This analysis revealed significant differences only between the wild type and eb1c-1 or triple mutants in oryzalin (P = 0.0182 for eb1c-1 and P = 0.0007 for triple mutants). It is important to note that the responses of eb1b-1, the genotype that exhibits the most skewing, were statistically indistinguishable from those of wild-type plants. We also observed cortical microtubules in wild-type and triple mutant roots fixed and labeled with antitubulin antibodies and were unable to detect differences in microtubule organization (Figure 10C). Thus, we were unable to correlate the eb1 root skewing phenotype with a change in the integrity of cortical microtubules.

**DISCUSSION**

The ability of a plant to direct root growth through the soil is vitally important for survival, and roots change the direction of their growth in response to a myriad of signals. Gravity, gradients of moisture and nutrients, as well as rocks and other impediments in the soil trigger bends that redirect root growth in a more favorable direction. Here, we report that roots of eb1 mutants exhibit delays in the initiation of downward bends after growing around an obstacle or after a 90° clockwise reorientation of the root. The fact that bend initiation is delayed suggests that mutants are slow to perceive, transmit, and/or respond to signals that induce downward root bending. eb1 roots also have a greater tendency to form loops when grown on tilted agar plates, a phenotype that has also been associated with gravitropic defects (Okada and Shimura, 1990; Mullen et al., 1998b; Ferrari et al., 2000; Rashotte et al., 2001).

In the literature, the stability and organization of microtubules has been associated with directional biases in root growth (Furutani et al., 2000; Hashimoto, 2002; Thitamadee et al., 2002; Sedbrook et al., 2004; Abe and Hashimoto, 2005; Ishida et al., 2007; Ishida and Hashimoto, 2007). For the eb1 alleles described here, however, microtubule defects do not appear to
be linked to the deviations in root growth. This conclusion is based on the fact that the genotype with the strongest leftward growth bias, \( eb1b-1 \), responds to microtubule inhibitors in a manner that is statistically indistinguishable from wild-type plants. Of the \( eb1 \) single mutant lines examined, only \( eb1c-1 \) roots are more sensitive to microtubule disruption. Why this is the case is not clear, although the observation does indicate that \( EB1 \) family members influence microtubules in different ways. Others have reported different effects of individual \( EB1 \) homologs on microtubules. Van Damme et al. (2004b) found that overexpression of \( EB1a:GFP \) increased microtubule polymerization rates and overexpression of \( EB1b:GFP \) did not. Mathur et al. (2003) report that overexpressed mouse \( EB1:GFP \) labeled entire microtubules and reduced their growth rates, but overexpression of \( At \) \( EB1b:GFP \) did not.

Delays in bend initiation could explain the enhanced leftward deviations in root growth that occur when \( eb1 \) mutants are grown on the surface of an agar plate. On inclined or vertically oriented agar surfaces, \( Arabidopsis \) roots grow in a waving pattern that is thought to be produced through a combination of normal root tip circumnutation movements and growth responses to touch and gravity signals (Okada and Shimura, 1990; Rutherford and Masson, 1996). The tilted agar surface seems to act as a barrier; a root growing vertically downward touches the surface of the agar, triggering an obstacle avoidance response that bends the root away from the downward trajectory. The agar surface also impedes movement of the root tip, causing the root to bend as cells behind the tip continue to elongate (Thompson and Holbrook, 2004). In either case, the root senses that its growth direction is no longer vertical and responds by reorienting the direction of growth downwards until the tip once again contacts the agar surface, triggering another cycle of bending (Okada and Shimura, 1990; Rutherford and Masson, 1996; Sedbrook, 2004). We find that when \( eb1 \) root tips encounter an obstacle and rotate away from the gravity vector, they undergo prolonged horizontal growth rather than immediately bending down. If similar delays occurred each time a mutant root tip encountered the agar surface, they would, over time, produce a root that deviated from the vertical more than the wild type.

Although bend initiation is delayed in mutant roots after encountering obstacles or after clockwise reorientations, there is no delay when roots are rotated in the counterclockwise direction. Why the delay would depend on which way the root is rotated is not clear, but it may be related to the directional growth bias that is present in wild-type plants. \( Ws \) roots have a growth bias that causes their tips to rotate more toward the left as they circumnutate down the plate (for reviews, see Migliaccio and Piconese, 2001; Oliva and Dunand, 2007). Clockwise reorientations place the left flanks of roots on top. Because of the leftward growth bias, these roots tend to circumnutation upwards and their tips would hit the agar surface before they could circle around

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**Figure 9.** Root Elongation and Morphology Is Not Altered in \( eb1 \) Mutants.

(A) and (B) Wild-type (A) and triple mutant (B) root tips are morphologically similar.

(C) Average root widths, measured at the base of the elongation zone where root hair emergence begins, are similar for all genotypes, regardless of whether they are grown on vertically oriented (white bars) or inclined (gray bars) agar plates. The asterisk indicates a slight, but significant (\( P < 0.01 \) by Student’s \( t \) test), difference between \( eb1c-1 \) and the wild type. On vertically oriented plates, \( n = 217 \) (19 to 39 seedlings per genotype), and on inclined plates \( n = 239 \) (27 to 33 seedlings per genotype).

(D) When grown on vertically oriented plates, the elongation rate of mutants did not deviate significantly from the wild type (white bar), although mutant roots grew at slightly faster rates. Light-gray bars denote single \( eb1 \) mutants, dark-gray bars indicate double mutants, and the triple mutant is shown in black. \( n = 238 \) (16 to 40 seedlings measured per genotype). Widths are reported in millimeters, elongation rates in millimeters/day, and C.I.s are calculated at \( P = 0.01 \).
into the downward direction. Contact with the agar provides a touch stimulus that is thought to modulate gravitropism and bend the root away from the surface (Massa and Gilroy, 2003). If mutants have trouble responding to gravity after contact with the agar surface, they would exhibit delays in downward bend formation. Wild-type roots, on the other hand, have no problems responding to gravity and they would form downward bends without delay. By contrast, counterclockwise reorientations position the left flanks of roots on the bottom. This time circumnutation rotates the tips downwards without interference from the agar, and both mutants and wild-type plants form downward bends without delay.

How might EB1 proteins function in the initiation of root bending? A bend is initiated when cells across the root begin elongating at different rates. In roots that have been reoriented into a horizontal plane, cell elongation increases on the upper flank and decreases on the lower flank, causing the root to bend down (Mullen et al., 1998a). Changes in elongation rates are thought to be effected via alterations in the extensibility of the cell wall (McQueen-Mason et al., 2007). The fact that eb1 roots exhibit extended horizontal growth before they initiate a bend suggests that there are delays in triggering changes in elongation rates across the root. However, overall elongation appears to be normal since mutant roots are the same lengths and widths as wild-type plants. One possibility is that EB1 proteins are involved in relaying the signals that effect changes in cell elongation rates across the root. In animal and fungal cells, EB1 facilitates the delivery of certain proteins, including signaling molecules and ion channels, to specific places in the cell (Rogers et al., 2004; Vaughan, 2005; Wu et al., 2005; Gu et al., 2006; Lansbergen and Akhmanova, 2006; Shaw et al., 2007). By analogy, At EB1 could target proteins that trigger changes in cell expansion rates to their sites of action in elongating root cells.

The root growth phenotype observed for eb1 mutants is rather mild given the roles proposed for EB1 proteins in cultured animal and fungal cells. However, it is important to note that budding and fission yeast lines carrying null mutations in EB1 genes are viable even though each organism carries only one EB1 homolog in its genome (Chen et al., 2000; Miller et al., 2000). In addition, mild eb1 phenotypes have also been reported for both of the nonplant multicellular organisms that have been examined. In third day, the position of the root tip was marked and the seedlings were allowed to continue growth for a total of 7 d, at which time root skewing angles and the amount of growth between day 3 and day 7 were measured. For all genotypes tested, root growth decreased with increasing concentrations of oryzalin or taxol (top panels in [A] and [B]), although eb1c-1 and triple mutants were more sensitive to oryzalin than were the other genotypes. Low concentrations of oryzalin or taxol in the medium (0.1 and 0.5 μM, respectively) increased root skewing angles in all genotypes except eb1c-1 and the triple mutant (bottom panels in [A] and [B]). Open circles denote wild-type plants (either Ws or Col-0), gray symbols indicate single mutants, and black circles designate triple mutants. 

Figure 10. Microtubule Integrity in eb1 Roots. (A) and (B) Several eb1 genotypes were germinated on agar plates containing different concentrations of oryzalin (A) or taxol (B). On the
Dictyostelium, a null eb1 mutant was viable but its cells progressed through metaphase more slowly than the wild type (Rehberg and Graf, 2002). In addition, hypomorphic Drosophila EB1 mutants form fully viable larvae that pupate and develop into morphologically normal adults with neuromuscular defects (Elliott et al., 2005).

Why Arabidopsis eb1 mutants exhibit mild phenotypes is not clear. Perhaps there are other microtubule-associated proteins that overlap functionally with EB1 in plant cells. This is the case in Schizosaccharomyces pombe, where spindle defects were unveiled in cells carrying mutations in both the S. pombe EB1 homolog (MINICHROMOSOME ALTERED LOSS3) and MICROTUBULE OVEREXTENDED1 (MOE1), another microtubule-associated protein (Chen et al., 2000). A MOE1 homolog is present in the Arabidopsis genome (Bisgrove et al., 2004), and analyses of eb1 moe1 double mutants could provide additional information about AT EB1 functions. It is also possible that the mutant lines reported here do not carry null alleles, since all of the mutants express partial transcripts that could encode proteins with some functional capabilities. Whatever the reason for the mild phenotypes in Arabidopsis, these alleles do reveal a role for EB1 proteins in root responses to gravity and/or touch signals, a result that may not have been apparent in lines with more severe defects.

**METHODS**

**Plants and Growth Conditions**

Wild-type Ws seeds were obtained from TAIR (http://www.Arabidopsis.org/), and eb1 alleles were identified by screening the BASTA population at the Wisconsin Knockout Facility according to protocols on the website (http://www.biotech.wisc.edu/Arabidopsis/default.htm; Krysan et al., 1999; Weigel et al., 2000). For phenotypic analyses, seeds were sterilized using the vapor phase method outlined in Clough and Bent (1998) and placed on either 0.8% Phytablend agar plates containing half-strength Murashige and Skoog (MS) medium (Sigma-Aldrich) with 0.5 g MES per liter and a pH of 5.8. Seeds were vernalized in the dark at 4°C for 3 to 7 d and then grown at 20°C under either constant light or a 16-h-light/8-h-dark cycle. Drug sensitivities were assayed by germinating seedlings on 0.8% Phytablend agar plates containing half-strength MS and one of 25 mg/L glucosinate-amaromnon (Perstan; Sigma-Aldrich), pacitaxel, or oryzalin. For protein isolation, seeds were germinated for 3 d in the dark with shaking (200 rpm) in half-strength MS liquid medium supplemented with 1% sucrose. To analyze root growth, seedlings were photographed using a Toshiba 4.0 megapixel digital camera, and measurements were made using either Photoshop or ImageJ. Statistical analyses were performed in Excel (Student’s t tests) or JMP 6 (analysis of variance testing).

**PCR Analyses**

RNeasy and DNeasy kits (Qiagen) kits were used to extract RNA and DNA from plant organs. RNA was reverse transcribed using the Promega reverse transcription system and the oligo(dT) primers provided in the kit. The product from the reverse transcription reaction was diluted 100-fold and used as a template in PCR amplification using Ex Taq polymerase (TaKaRa). The primers used for PCR amplification were as follows: a1 forward 5′-CAAGCTCGAGATGTAGAGA-3′, a1 reverse 5′-TCCAGTGTCTCGAGTTTCC-3′, a2 forward 5′-CGTATACCTCTCCGAG-3′, b2 reverse 5′-TTGTAGAACATCTCTCCCA-3′, c2 forward 5′-ATTGGGATGGATGATTGTCG-3′, c2 reverse 5′-AAGGCTGTTGCTGCTAGA-3′, a3 forward 5′-GTCGAAAGCCGTTGCAAG-3′, c3 reverse 5′-TGAGATCCAGACGCTCC-3′, b3 forward 5′-TTGTTTTCGTTTGCTACCCATACC-3′, b3 reverse 5′-CACAATATTGGCTGACGAG-3′, c3 forward 5′-GCGGTAGAGAGAGGAGAGAGAGAGG-3′, and c3 reverse 5′-GCTGCGCTCCATACCAAGA-3′. The identities of amplification products were verified by excising and sequencing bands from agarose gels.

T-DNA insertion allelic and wild-type plants were genotyped by PCR using Ex Taq polymerase and the following primers: JLI202 5′-CATTGTATTAATACGCTGGGACACTCATC-3′ (T-DNA insertion), At3g7690F 5′-ACCAGATTCCTCTCATGCTCGTTTTCCCA-3′, At3g7690R 5′-CCAAGCGATTGTGTCACCCTTCTACTTA-3′, and At5g67270F 5′-TGAAATGTTAAGACTCCGCGCTGCTCCTTTCT-3′, and At5g67270R 5′-CGGTTTTGCGTTTCTGTTTCT-3′.

**Preparation of EB1 Antibodies**

Glutathione S-transferase (GST) fusion protein constructs were made by PCR amplifying EB1 coding sequences from the cDNA clones 188D24 and U23125, corresponding to EB1c and EB1a, respectively, using Vent DNA polymerase (New England Biolabs). To make the GST-EB1c construct, the following primers were used: ateb5e 5′-CGGAATTCAGATTCCTG-3′, GGTACGACATTGGG-3′ and sp6 5′-ATTAGTGGACCATATTAG-3′. The amplified DNA fragment was digested with EcoRI and HindIII and then cloned into the pGEX-KG vector (Guan and Dixon, 1991) at the corresponding restriction enzyme sites. The GST-EB1a construct was amplified from U23125 using the following primers: II47690.5′-GGC-3′, At5g62500F 5′-TCGTCGTCGTTGCACTGTAACAAAAA-3′, At5g62500R 5′-TCTGATTGACCCCGCTGCTGCTTCCTTTCT-3′, and At5g67270R 5′-CGGTTTTGCGTTTCTGTTTCT-3′.

**Immunoblotting**

Protein extracts were prepared from plant tissues as described previously (Liu et al., 1996), separated by SDS-PAGE, and then transferred to nitrocellulose membranes prior to immunoblotting. The secondary antibody was alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad), and a colorimetric detection method using nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate substrate solution (Bio-Rad) was used.

**Immunofluorescence Microscopy**

Whole-mount roots were prepared using a modification of the freeze shatter protocol outlined in Wasteneys et al. (1997). Briefly, roots were stained with the water-soluble fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI) and mounted on glass slides in Mowiol (Calbiochem) under a coverslip. Roots were examined with an Axiophot microscope (Zeiss) equipped with epifluorescence optics and photographed with a color charge-coupled device camera. Images were processed with Adobe Photoshop.
fixed for 1 to 2 h at room temperature under a vacuum in PEM buffer (50 mM PIPES, 2 mM EGTA, and 2 mM MgSO4, pH 7.2) containing 1.5% (v/v) formaldehyde, 0.5% (v/v) glutaraldehyde, and 5% (v/v) DMSO. Fixed roots were rinsed two to three times with PEM and then placed on slides coated with poly-l-lysine (Sigma-Aldrich), a cover slip was clipped to the slide over the root, and the assembly was briefly immersed in liquid nitrogen. While still frozen, the eraser on the end of a pencil was used to apply pressure to the cover slip and roots, the cover slip was removed, and the roots were digested 30 min at room temperature in PEM, pH 5.5, containing 1% (w/v) cellulase Y-6 (ICN),pectolyase Y-23 (ICN), and 0.1 mM phenylmethyl sulfonyl fluoride (Sigma-Aldrich). Tissue was rinsed in PEM, pH 7.2, incubated 10 min in –20°C methanol, and then rehydrated in PBS (2.8 mM NaCl, 54 mM KCl, 200 mM Na2HPO4, 36 mM KH2PO4, and 2% [w/v] sodium azide, pH 7.3) and incubated for 20 min in PBS with 1 mg/mL sodium borohydride. Prior to antibody addition, roots were rinsed in PBS and then incubated for 30 min in incubation buffer (PBS with 50 mM glycine added). Antibodies (monoclonal DM1A; Sigma-Aldrich) to label microtubules as well as the secondary antibodies Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 564 goat anti-mouse (Molecular Probes) were applied overnight at room temperature in incubation buffer. Finally, roots were rinsed in incubation buffer, incubated for 20 min in –20°C methanol, mounted in 1 part benzyl alcohol:2 parts benzyl benzoate, and imaged using a Zeiss LSM 510 confocal microscope or an Olympus IMT-2 equipped with fluorescence and a Photometrics CoolSnap CCD camera.

Squashed root cells were prepared according to the method described by Palevitz (1988). Briefly, Arabidopsis thaliana roots were fixed in 4% paraformaldehyde in PEM buffer, followed by digestion with 1% cellulase in PEM. The Arabidopsis suspension cell line, generated in the laboratory of Luca Comai at the University of Washington, was provided to us by the laboratory of Richard Michelmore. The cells were grown in MS basal medium supplemented with 4.2 mg/L naphthylacetic acid and 0.02 mg/L kinetin at 21°C and processed as described previously (Liu et al., 1996). Both root squashes and tissue cultured cells were labeled with anti-EB1 and DM1a antibodies followed by fluorescein isothiocyanate–conjugated goat anti-rabbit IgG (Sigma-Aldrich) and Texas Red X–conjugated goat anti-mouse IgG (Molecular Probes). Images were collected under a Leica TCS SP2 confocal microscope (Leica Microsystems). All figures were assembled using the Photoshop 7.0 program (Adobe Systems).

Accession Numbers
Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: At4g17690, AT5g62500, AT5g67270, and NM_126127.

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**The Microtubule Plus-End Binding Protein EB1 Functions in Root Responses to Touch and Gravity Signals in *Arabidopsis***

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