

**Arabidopsis AUGMIN Subunit8 Is a Microtubule Plus-End Binding Protein That Promotes Microtubule Reorientation in Hypocotyls**

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In plant cells, cortical microtubules provide tracks for cellulose-synthesizing enzymes and regulate cell division, growth, and morphogenesis. The role of microtubules in these essential cellular processes depends on the spatial arrangement of the microtubules. Cortical microtubules are reoriented in response to changes in cell growth status and cell shape. Therefore, an understanding of the mechanism that underlies the change in microtubule orientation will provide insight into plant cell growth and morphogenesis. This study demonstrated that AUGMIN subunit8 (AUG8) in Arabidopsis thaliana is a novel microtubule plus-end binding protein that participates in the reorientation of microtubules in hypocotyls when cell elongation slows down. AUG8 bound to the plus ends of microtubules and promoted tubulin polymerization in vitro. In vivo, AUG8 was recruited to the microtubule branch site immediately before nascent microtubules branched out. It specifically associated with the plus ends of growing cortical microtubules and regulated microtubule dynamics, which facilitated microtubule reorientation when microtubules changed their growth trajectory or encountered obstacle microtubules during microtubule reorientation. This study thus reveals a novel mechanism underlying microtubule reorientation that is critical for modulating cell elongation in Arabidopsis.

**INTRODUCTION**

Microtubules function in many fundamental cellular processes, such as cell motility, morphogenesis, intracellular transport, and division (Lodish et al., 2007). In plant cells, cortical microtubules provide tracks for cellulose-synthesizing enzymes and guide the orientation of cellulose microfibrils (Paredez et al., 2006; Crowell et al., 2009; Gutierrez et al., 2009; Bringmann et al., 2012; Li et al., 2012). Load-bearing microfibrils, in turn, are proposed to restrict cell expansion in a direction that is perpendicular to their predominant orientation (Yuan et al., 1994; Wasteneys, 2004; Szymanski and Cosgrove, 2009; Crowell et al., 2010; Lloyd, 2011; Baskin and Gu, 2012). Hence, the orientation of cortical microtubules plays an essential role in the spatial regulation of cell expansion and the modulation of cell growth rate in plants and is critical for plant development and morphogenesis (Smith and Oppenheimer, 2005; Ehrhardt and Shaw, 2006; Paradze et al., 2006; Lucas and Shaw, 2008; Wasteneys and Ambrose, 2009; Lloyd, 2011). In the cells of rapidly elongating tissues, such as hypocotyls and roots, cortical microtubules are arranged in parallel and ordered perpendicularly (i.e., transversely) to the axis of maximal elongation (Ehrhardt and Shaw, 2006; Lucas and Shaw, 2008; Lloyd, 2011). The formation of a radial microtubule star array was recently proposed to be a key step in the reorientation to a transverse microtubule array and marks the onset of cell growth in hypocotyls (Sambade et al., 2012). As the cell ceases to elongate, cortical microtubules change their orientation to become parallel to the longitudinal axis of the cell (Ehrhardt and Shaw, 2006; Lucas et al., 2011). In puzzle-like leaf pavement cells, parallel transverse microtubules are found primarily at the indentation regions in cells that undergo localized expansion. This ordered pattern of microtubule arrays is periodic and transient. Randomly oriented microtubules are eventually dominant throughout the entire cell (Fu et al., 2002, 2005). Therefore, such a transition of cortical microtubule orientation is a major factor in the regulation of cell elongation and morphogenesis (Paradez et al., 2006; Ehrhardt, 2008). Understanding how developmental, hormonal, and environmental signals modulate the orientation of cortical microtubules is necessary for elucidating developmental mechanisms in plants.

Microtubule dynamics and polymer interactions have been implicated in the regulation of microtubule organization and orientation. Polymerization-biased dynamic instability at the plus end and slow depolymerization at the minus end contribute to sustained microtubule treadmilling. The dynamic minus end allows the location and orientation of the older microtubule lattice to be erased as the growing plus end explores new cellular space (Shaw et al., 2003). The extension of the plus end drives interactions with other microtubules, resulting in changes in...
polymerization behavior (Shaw et al., 2003) that depend on the angle of collision (Dixit and Cyr, 2004). At angles of <40°, the growing end is likely to follow the encountered microtubule, reorienting the direction of growth and forming a polymer bundle. Induced catastrophe has been observed at greater angles (Dixit and Cyr, 2004). These behaviors were hypothesized to have self-organizing properties to establish specific cortical microtubule arrays (Dixit et al., 2006; Ehhardt and Shaw, 2006; Ehhardt, 2008). However, how microtubule dynamics are regulated and impact microtubule reorientation when the rate of cell elongation declines is poorly understood.

Several microtubule-associated proteins (MAPs) that affect microtubule dynamics have been shown to regulate cell growth in hypocotyls. For example, MICROCTIBLE-DESTABILIZING PROTEIN25, a calcium regulatory protein, regulates hypocotyl cell elongation by destabilizing microtubules (Li et al., 2011). Another microtubule-destabilizing factor, MICROCTIBLE-ASSOCIATED PROTEIN18, also plays a role in regulating directional cell growth in hypocotyls (Wang et al., 2007). ROP-INTERACTIVE CRIB MOTIF CONTAINING PROTEIN1 is a MAP that acts in the ROP6 GTPase signaling pathway to promote the formation of transverse microtubules in both leaf pavement cells and hypocotyl cells (Fu et al., 2005, 2009). TORTOFILIA1/SPIRAL2 (SPR2) promotes microtubule polymerization and suppresses the pause state of microtubule ends (Fabri and Schäffner, 1994; Buschmann et al., 2004; Yao et al., 2008). Microtubule polymerization/depolymerization mainly occurs at the plus ends of microtubules in vivo (Bisgrove et al., 2004). Several microtubule plus-end tracking proteins, such as END BINDING1 (EB1) and SPIRAL1 (SPR1), play important roles in microtubule dynamics and organization, which in turn regulate directional cell expansion (Chan et al., 2003; Sedbrook et al., 2004; Bisgrove et al., 2008). Previous studies indicated that EB1 stimulated microtubule polymerization (Van Damme et al., 2004). Bisgrove et al. (2008) reported that Arabidopsis thaliana eb1 mutants exhibited defects in root growth. SPR1 was proposed to be involved in root cell polar expansion by affecting microtubule dynamic instability in Arabidopsis (Nakajima et al., 2004; Sedbrook et al., 2004). However, no studies to date have linked plus-end tracking proteins to microtubule reorientation.

In this study, we demonstrated that AUGMIN subunit8 (AUG8) is a novel microtubule plus-end binding protein in Arabidopsis. It specifically binds to the growing ends of microtubules and promotes cortical microtubule reorientation by regulating the dynamics of microtubule plus ends. It is also recruited to the branch site at the side wall of an existing microtubule, immediately before the formation of nascent microtubule branches, which might also contribute to microtubule reorientation. Therefore, AUG8 plays an important role in the modulation of directional cell growth in plants.

RESULTS

AUG8 Regulates Hypocotyl Cell Elongation

In our screen for T-DNA insertional mutations that enhance a pollen tube phenotype induced by ROP1 overexpression (Hwang et al., 2008), we identified the ROP1 enhancer2 (REN2) gene (At4g30710; http://www.Arabidopsis.org, accessed January 7, 2013; M. Zheng, Y. Fu, and Z. Yang, unpublished data), which encodes a 70-kD protein with unknown function that belongs to the previously identified QWRF protein family in Arabidopsis (Pignocchi et al., 2009; Albrecht et al., 2010). Members of this family share a highly conserved QWRF amino acid sequence and a DUF566 domain of unknown function. One member of this protein family, ENDOSPERM DEFECTIVE1, has been shown to be a MAP (Pignocchi et al., 2009). Furthermore, the localization of another QWRF protein family member, peroxisomal-targeted SNOWY COTYLEDON3, depends on functional microtubules (Albrecht et al., 2010). Thus, we predicted that REN2 might be functionally related to microtubules. Indeed, a T-DNA insertion mutant allele (SALK_043608) of REN2 displayed a hypocotyl elongation phenotype. Hotta et al. (2012) recently detected REN2, which they referred to as AUG8, by mass spectrometry after purifying the Arabidopsis augmin complex, which has been reported to regulate γ-tubulin localization in mitotic spindles (Goshima et al., 2008). This further supports our prediction. Therefore, we renamed REN2 as AUG8 in this study.

When grown in the dark, the hypocotyls of 5-d-old AUG8 knockout mutant (aug8, SALK_043608) seedlings were longer (13.4 ± 1.4 mm [mean ± sd], n = 32) than those of wild-type seedlings (10.8 ± 0.8 mm, n = 31; Figures 1A and 1B; see Supplemental Figure 1 online). Additionally, epidermal cells of aug8 hypocotyls exhibited left-handed twisting cell files (Figure 1C), a phenotype that is usually due to defects in microtubule function (Ishida et al., 2007). The transformation of AUG8<sub>aug8</sub>, AUG8<sub>fl</sub> and AUG8-GFP (for green fluorescent protein) into aug8 complemented the mutant hypocotyl phenotypes in five individual lines (two lines shown in Figure 1 and Supplemental Figure 1 online displayed straight epidermal cell files, and the mean hypocotyl lengths were 11.4 ± 0.7 mm [n = 31] and 11.3 ± 0.4 mm [n = 35], respectively), demonstrating that the loss of AUG8 function was responsible for the aug8 mutant phenotypes.

To further reveal the physiological function of AUG8, AUG8-overexpressing lines were generated. Eight individual lines displayed elevated AUG8 expression and shorter hypocotyl lengths compared with the wild type (two lines designated as AUG8-OX-1 and AUG8-OX-2 in Figure 1 and Supplemental Figure 1 online; the mean hypocotyl lengths were 8.6 ± 0.6 mm [n = 30] and 9.2 ± 0.5 mm [n = 32], respectively). We carefully examined hypocotyl epidermal cells in the wild type, aug8, AUG8-OX-1, and complementary COM-1 lines using scanning electron microscopy. Although the twisted growth pattern of aug8 hypotocyt epidermal cells made measuring their lengths difficult, aug8 cells still appeared to be longer than wild-type cells (Figure 1C). By contrast, AUG8-OX-1 hypocotyl epidermal cells were much shorter (429 ± 20 µm, n = 100) than wild-type cells (575 ± 28 µm, n = 100), whereas no difference was detected between COM-1 (588 ± 35 µm, n = 100) and wild-type hypocotyl cells (Figures 1C and 1D).

AUG8 Regulates Microtubule Reorientation

The cell shape phenotypes were typical of cells with altered cortical microtubule organization (Nakajima et al., 2004; Sedbrook et al., 2004; Ishida et al., 2007; Wang et al., 2007; Bisgrove et al.,
AUG8 Participates in Hypocotyl Cell Elongation.

Five-day-old dark-grown seedlings of the wild type (Col), aug8, AUG8-OX-1, AUG8-OX-2, and two complementary lines that express AUG8pro:AUG8-GFP in an aug8 background (COM-1 and COM-2), showing their etiolated hypocotyls.

Five-day-old dark-grown aug8 seedlings had longer hypocotyls (13.4 ± 1.4 mm, n = 32, P < 0.05, Student’s t test), and AUG8-OX-1 (8.6 ± 0.6 mm, n = 30) and AUG8-OX-2 (9.2 ± 0.5 mm, n = 32) seedlings had shorter hypocotyls (Student’s t test, P < 0.05) than the wild type (10.8 ± 0.8 mm, n = 31). The lengths of hypocotyls of COM-1 (11.4 ± 0.7 mm, n = 31) and COM-2 (11.3 ± 0.4 mm, n = 35) seedlings were similar to those of the wild type (P > 0.05, Student’s t test).

Scanning electronic microscopy images of wild-type, aug8, AUG8-OX-1, and COM-1 etiolated hypocotyls. Hypocotyl epidermal cells of aug8 exhibited twisted cell files, whereas no such twisted cell files were present in the wild type or AUG8-OX-1. The epidermal cells of aug8 hypocotyls were longer than those of the wild type, whereas AUG8-OX-1 epidermal cells (429 ± 20 µm, n = 100) were much shorter than those of the wild type (575 ± 28 µm, n = 100; P < 0.05, Student’s t test).

No obvious difference was found between the epidermal cells of COM-1 (588 ± 35 µm, n = 100) and the wild type (P > 0.05, Student’s t test).

Bars = 5 mm in (A) and 10 µm in (C). The data are expressed as mean ± SD.

AUG8 Localizes to the Plus Ends of Growing Microtubules

To gain insight into the mechanism by which AUG8 promotes microtubule polymerization, we monitored AUG8 colocalization with microtubules in vitro using immunofluorescent microscopy. To visualize microtubules, rhodamine-labeled tubulin was mixed with nonlabeled tubulin and polymerized into microtubules in the presence of His-AUG8. His-AUG8 was visualized by staining with anti-His antibody and a fluorescent secondary antibody. AUG8 formed a dot-like structure at the ends of microtubules, usually in association at only one end of each microtubule (Figure 3E). Similar associations were not observed when the protein was denatured by boiling (Figure 3F) or when the samples were stained with secondary antibody alone (Figure 3G). These observations suggest that AUG8 might be a MAP that binds to an end of microtubules.

AUG8 Promotes Microtubule Reorientation

The above observations and previous reports about other members of the QWRF family suggest that AUG8 might be a MAP. To test this hypothesis, a cosedimentation assay was performed and showed that AUG8 directly associated with microtubules in vitro. Escherichia coli–expressing His-tagged AUG8 was purified (see Supplemental Figure 2 online) and incubated with preformed taxol-stabilized microtubules before high-speed centrifugation. We found that His-AUG8 cosedimented with microtubules (Figure 3A). Before reaching saturation, the mass of His-AUG8 protein in the pellets increased as higher concentrations of the recombinant protein were added (Figure 3B), indicating that AUG8 bound directly to microtubules. We then added various concentrations of His-AUG8 (0, 0.25, 0.5, 1, 2, and 2.5 µM) to a solution that contained 20 µM tubulin, and tubulin polymerization was monitored turbidimetrically. The presence of AUG8 increased turbidity in a dose-dependent fashion, indicating an increase in microtubule mass (Figure 3C). To further determine whether the increase in turbidity was attributable to increased microtubule polymerization, high-speed centrifugation was performed to examine the amount of tubulin in the pellets following an in vitro microtubule polymerization assay. Increasing amounts of tubulins were detected in the pellets when AUG8 was present. The increase was also dose dependent (Figure 3D), suggesting that AUG8 promoted microtubule assembly in vitro.

AUG8 Binds to Microtubules and Promotes Microtubule Assembly in Vitro

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Quantiﬁcation of microtubules in aug8 and wild-type cells (n > 30 cells of each line). To quantify the microtubule (MT) density in the cell, a ﬁxed line (10 µm) was drawn perpendicularly to the orientation of most cortical microtubules, and the number of microtubules across this line was counted. Five measurements were performed per cell. The data are expressed as mean ± s.d.

Figure 2. Microtubule Organization in Hypocotyl Cells.

(A) Cortical microtubules were visualized in cells of the upper part of aug8, AUG8-OX-1, and wild-type etiolated hypocotyls expressing GFP-tubulin. Bar = 5 µm.

(B) Cartoon deﬁning the angle of orientation (θ). Transverse: 90° ≥ θ > 67.5° or −90° ≤ θ < −67.5°. Oblique: 67.5° ≥ θ > 22.5° or −22.5° ≥ θ > −67.5°. Longitudinal: 22.5° ≥ θ > −22.5°. This cartoon was modiﬁed from a previously reported deﬁnition (Crowell et al., 2011).

(C) Microtubules displayed a transverse orientation in 90% of aug8 hypocotyl epidermal cells (n = 122) and 59% of wild-type hypocotyl epidermal cells (n = 114) but primarily displayed random orientations in 78% of AUG8-OX-1 hypocotyl epidermal cells (n = 129).

(D) Quantiﬁcation of microtubules in aug8 and wild-type cells (n > 30 cells of each line). To quantify the microtubule (MT) density in the cell, a ﬁxed line (10 µm) was drawn perpendicularly to the orientation of most cortical microtubules, and the number of microtubules across this line was counted. Five measurements were performed per cell. The data are expressed as mean ± s.d.

showed that AUG8-GFP exhibited comet-like structures in cells that appeared to move fast along certain tracks in various directions, with some disappearing after a short time, suggesting that AUG8 associates with the plus ends of microtubules (see Supplemental Movie 1 and Supplemental Figure 3A online). To verify this observation, we studied hypocotyl epidermal cells that expressed both AUG8-GFP and mCherry-tagged EB1, a well-known microtubule plus-end binding protein conserved in animal, fungal, and plant cells (Chan et al., 2003; Vaughan, 2005). Indeed, the mCherry-EB1-labeled mobile structures partly overlapped and moved together with AUG8-GFP-labeled comet-like structures. The colocalization of AUG8-GFP and mCherry-EB1 demonstrated that AUG8 associates with the microtubule plus end (Figure 4A; see Supplemental Figure 3B and Supplemental Movie 2 online). We then determined the relative position of AUG8 and EB1 by plotting the normalized ﬂuorescence intensity along the microtubule ends (Kirk et al., 2007). AUG8 was localized closer to the microtubule end. Quantitative analysis conﬁrmed that the peak maximal positions of AUG8-GFP and mCherry-EB1 were clearly separated, with an average distance of 0.16 ± 0.04 µm (n = 13; see Supplemental Figure 3C online). Live imaging of hypocotyl cells that coexpressed AUG8-GFP and mCherry-tubulin revealed that AUG8 was only associated with the plus ends of fast-growing microtubules (Figure 4B; see Supplemental Movie 3 online). We found that AUG8 disappeared from the microtubule end when the microtubules stopped growing and began to shrink but reappeared at the microtubule end when growth resumed (Figure 4B; see Supplemental Movie 3 online). Kymographic analysis conﬁrmed that AUG8 tracks the plus end during microtubule growth (Figure 4C; see Supplemental Movie 3 online). AUG8 was associated with the growing ends of all of the 168 growing microtubules observed in 29 cells from ﬁve individual seedlings (approximately ﬁve to seven cells were observed per seedling) (see Supplemental Movie 3 online). No AUG8 was detected at the ends of another 160 microtubules that were shrinking (see Supplemental Movie 3 online). Given these results, we conclude that AUG8 is a novel microtubule plus-end binding protein that speciﬁcally binds to growing microtubules.

AUG8 Is Expressed in Various Organs and Tissues

To further elucidate the function of AUG8 in Arabidopsis, transgenic lines that expressed β-glucuronidase (GUS) driven by the native promoter of AUG8 (AUG8pro::GUS) were generated to analyze the AUG8 expression pattern in Arabidopsis. AUG8 was constitutively expressed in various organs/tissues (Figures 5A and 5B), which is consistent with the microarray database (https://www.genevestigator.com/gv/index.jsp; accessed January 7, 2013). Notably, the expression level of AUG8 varied during hypocotyl development. In etiolated hypocotyls, the expression of AUG8 corresponded to hypocotyl elongation. In etiolated hypocotyls of 3-d-old dark-grown seedlings, when fast growth was dominant, low GUS activity was detected (Figure 5B). However, in etiolated hypocotyls of 5- to 6-d-old dark-grown seedlings, when the elongation of hypocotyls was slow, GUS activity increased (Figure 5B). A similar pattern was detected by RNA in situ hybridization (see Supplemental Figure 4 online). Additionally, quantitative real-time PCR showed that the AUG8 expression level was much higher in the hypocotyls of light-grown seedlings, in which growth is strongly inhibited by light,
than in etiolated hypocotyls (Figure 5C). These results indicate that the expression of AUG8 is suppressed in rapidly elongating hypocotyls but upregulated when hypocotyl elongation slows or stops. Interestingly, we also noticed an uneven expression pattern of AUG8 in most hypocotyls (Figure 5B). The expression of AUG8 gradually increased from the basal to apical regions of hypocotyls. To verify this result, quantitative real-time PCR was performed using 6-d-old etiolated hypocotyls. We confirmed that AUG8 was expressed more in the apical part than in the middle part of hypocotyls, whereas the lowest expression level was detected in the basal region of hypocotyls (Figure 5D). These results suggest that AUG8 plays an important role in modulating hypocotyl growth.

**AUG8 Promotes Reorientation of Cortical Microtubules**

Because the expression level of AUG8 increased when cell elongation slowed down, a process that is associated with the reorientation of cortical microtubules (Ehrhardt and Shaw, 2006), we suspected that AUG8 plays a role in modulating cortical microtubule reorientation. To test this hypothesis and track microtubule reorientation in live cells, we used a 100-W incandescent...
Figure 4. AUG8 Specifically Binds to the Plus End of Growing Microtubules in Vivo.

(A) The colocalization of AUG8-GFP and mCherry-EB1 in a hypocotyl epidermal cell indicates that AUG8 was associated with the plus ends of microtubules. (B) A time series of a hypocotyl epidermal cell expressing mCherry-TUA5 and AUG8-GFP. Closed arrowheads indicate the AUG8-GFP signal (green) associated with the growing end of a microtubule (red). AUG8-GFP disappeared from the microtubule end during shrinkage but reappeared when microtubule growth resumed. Open arrowheads indicate the plus end of a microtubule that started shrinking at 6 s. Bars = 5 µm in (A) and (B). (C) Kymographs of a microtubule are indicated by the closed arrowhead in (B), showing that the AUG8-GFP signal tracked the microtubule growing end.

light source attached to a microscope to induce microtubule reorientation in cells located at the upper part of etiolated hypocotyls from 3-d-old seedlings that expressed GFP-tubulin. Images were acquired at 10-min intervals using spinning-disc confocal microscopy during microtubule reorientation. In wild-type cells, the orientation of cortical microtubules shifted from transverse to longitudinal at 43 ± 4 min (mean ± sD; n = 10), with randomly oriented microtubules observed −30 min after exposure to light (Figure 6). In AUG8-OX-1 cells, the reorientation of cortical microtubules was complete 48 ± 8 min (n = 8) after light exposure, with randomly oriented microtubules observed at −30 min (Figure 6). A Student’s t test revealed no significant difference in the timing of microtubule reorientation between AUG8-OX-1 and wild-type cells. However, in aug8 cells, transverse cortical microtubules were still dominantly present 30 min after light exposure, and disordered microtubules occurred −40 min after light exposure. Although microtubules still displayed a transverse orientation 60 min after light exposure in eight of the 18 observed cells, microtubules in another 10 cells displayed a longitudinal alignment; however, this was dramatically delayed, with an average time of 67 ± 4 min (n = 10) after light exposure (Figure 6). These observations indicate that AUG8 participates in the reorientation of cortical microtubules in hypocotyl cells.

To further elucidate the role of AUG8 in this process, we analyzed the orientation angles of microtubules at different times during microtubule reorientation induced by light. We grouped microtubules into 10° bins according to their angles relative to the longitudinal cell axis. We then calculated the percentage of microtubules that fell into each group and determined the distribution pattern of the orientation angles (see Supplemental Figure 5 online). With regard to total microtubules, including both growing and nongrowing microtubules, the majority of microtubule orientation angles changed from 90° (i.e., perpendicular to the growth axis) to 0° (i.e., parallel to the growth axis) after light exposure, with a transitional step that occurred −30 min after light exposure, in which microtubules were randomly oriented (see Supplemental Figure 5 online). Interestingly, AUG8-associated microtubules (i.e., growing microtubules) appeared to lead the shift in orientation angles. Figure 7 presents data collected from a representative cell that coexpressed AUG8-GFP and mCherry-tubulin. We compared the distribution pattern of AUG8-associated microtubules, indicated by a distribution histogram that showed the percentage of AUG8-associated microtubules in groups with different orientation angles, to total microtubules, indicated by a distribution histogram that showed the percentage of total microtubules in different groups. Before exposure to light, most AUG8-associated microtubules were transversely oriented, and the distribution pattern of the orientation angles was similar to that of total microtubules (Figure 7A), indicating that the growth trajectory of growing microtubules was transverse to the cell elongation axis. However, more AUG8-associated microtubules were observed with angles that were close to longitudinal alignment during the microtubule reorientation process (Figures 7B and 7C). A striking difference was detected 30 min after light exposure, when total microtubules were randomly oriented. A considerable number of AUG8-associated microtubules were oriented at an angle that was less than 20° (Figure 7C), indicating that the shift in the orientation of growing microtubules occurred before the shift in the orientation of total cortical microtubules. Microtubule reorientation was complete 45 min after light exposure, and no significant difference was observed with regard to orientation angles between total microtubules and AUG8-associated microtubules (Figure 7D). Altogether, we propose that microtubule reorientation is attributable to changes in the growth trajectory of newly polymerized microtubules.
instability were analyzed in live cells located at the upper part of hypocotyls from aug8 and wild-type seedlings, respectively (Table 1). Four-day-old etiolated seedlings that expressed GFP-tubulin were exposed to light for 30 min, and time-series images were acquired using spinning-disc confocal microscopy.

We measured the growth rates, shrinkage rates, and transition frequencies between the microtubule growth, shrinkage, and pause phases. Microtubules with clearly visible leading ends were selected for all of the measurements. This included a total of 92 microtubules from 16 cells of 10 wild-type seedlings and 85 microtubules from 15 cells of 10 aug8 seedlings (Table 1). The results showed that the plus ends of individual microtubules in aug8 cells displayed different features from those in wild-type cells. The average growth rate of microtubule leading ends was $5.45 \pm 1.13 \mu \text{m/min (n = 10)}$ in aug8 cells and $6.19 \pm 0.75 \mu \text{m/min (n = 10)}$ in wild-type cells. A Student’s t test revealed no significant difference between microtubule growth rates in aug8

AUG8 Regulates the Dynamics of Microtubule Plus Ends

The above results imply that newly formed microtubules grow in a new trajectory to lead the reorientation of the entire cortical microtubule array. It has been known that new microtubules in plant cells are primarily formed through branching at an angle from existing microtubules (Nakamura et al., 2010; Kirik et al., 2012). The augmin complex was reported to be associated with microtubule branching (Goshima et al., 2008; Uehara et al., 2009; Petry et al., 2013). Therefore, we investigated whether AUG8 regulates microtubule branching from existing microtubules (Murata et al., 2005; Nakamura et al., 2010; Kirik et al., 2012). The formation of microtubule branches in cells that coexpressed AUG8-GFP and mCherry-tubulin indicated that AUG8 appeared at the branch site immediately before nascent microtubules branched out (see Supplemental Figure 6 and Supplemental Movie 4 online). We then compared the rates of microtubule branch formation between the wild type and aug8 mutants. An average rate of $72 \pm 27$ events per 1000 $\mu \text{m}^2$ per hour was observed in aug8 cells, which was significantly reduced compared with wild-type cells, which had an average of $207 \pm 63$ events per 1000 $\mu \text{m}^2$ per hour ($P < 0.001$, Student’s t test; seven cells were observed, respectively). These results indicate that AUG8 also promotes microtubule branching in plant cells.

Next, we determined the effect of AUG8 on the dynamics of individual microtubules. The parameters of microtubule dynamic

Figure 5. Histochemical GUS Staining of AUG8pro-GUS Transgenic Plants.

(A) GUS staining of a 10-d-old transgenic Arabidopsis plant harboring an AUG8pro-GUS construct.
(B) Seedlings of AUG8pro-GUS were grown in the dark for 3, 4, 5, and 6 d. GUS staining showed that AUG8 expression was promoted when the elongation of hypocotyls decreased. Bar = 10 mm.
(C) Quantitative real-time PCR analysis of AUG8 in hypocotyls of 6-d-old dark- or light-grown wild-type seedlings. The expression of AUG8 was higher in hypocotyls of light-grown seedlings than in those of dark-grown seedlings.
(D) Quantitative real-time PCR analysis of AUG8 in different parts of hypocotyls. The expression of AUG8 gradually increased from the basal to apical regions of hypocotyls. The difference between different parts was significant ($P < 0.001$, Student’s t test).

Figure 6. AUG8 Promotes Light-Induced Microtubule Reorientation.

The cells in the upper part of etiolated hypocotyls of 3-d-old wild-type, AUG8-OX-1, and aug8 seedlings were observed. Microtubules were visualized using transgenically expressed GFP-tagged tubulin. The cortical microtubules in wild-type cells shifted their orientation from transverse to longitudinal 40 min after exposure to light, with randomly oriented microtubules appearing at ~30 min. In AUG8-OX-1 cells, the reorientation of cortical microtubules was completed 48 ± 8 min ($n = 8$) after exposure to light, which was not significantly different from our observations in the wild type (43 ± 4 min, $n = 10$, $P > 0.05$, Student’s t test). Randomly oriented microtubules in AUG8-OX-1 cells were also observed at ~30 min (middle row of images). In aug8 cells, transverse cortical microtubules were still dominantly observed 30 min after exposure to light. Randomly oriented microtubules occurred at ~40 min and were persistent up to 50 min after exposure to light. Further observations revealed that complete microtubule reorientation in aug8 took an average of $67 \pm 4$ min ($n = 10$), which was significantly slower than in the wild type ($P < 0.05$, Student’s t test). The data are expressed as mean ± so. Bar = 5 $\mu \text{m}$. 
and wild-type cells. However, the average shrinkage rate in aug8 cells (7.63 ± 2.36 μm/min, n = 10) was significantly lower than that of wild-type cells (11.45 ± 1.81 μm/min, n = 10, P < 0.01, Student’s t test). The catastrophe and rescue frequencies of individual microtubules in aug8 cells (0.015 s⁻¹ for catastrophe and 0.014 s⁻¹ for rescue) were much higher than those of wild-type cells (0.007 s⁻¹ for catastrophe and 0.009 s⁻¹ for rescue). Moreover, microtubules in wild-type cells spent more time in the growth phase (71.8%) compared with microtubules in aug8 cells (50.7%). We found that the microtubule dynamicity was significantly reduced in mutant cells (5.59 ± 1.10 μm/min) compared with wild-type cells (7.32 ± 1.54 μm/min; P < 0.01, Student’s t test; Table 1). These results indicate that AUG8 indeed regulates the dynamics of microtubule plus ends.

Additionally, because newly polymerized microtubules adopt growth paths that are different from those of previous transverse microtubules, one might expect that more microtubule-microtubule encounters occur during the process of microtubule reorientation. Microtubule-microtubule encounters have been previously shown to contribute to microtubule self-organization in an angle-dependent manner (Dixit and Cyr, 2004). In this previous study, shallow-angle encounters led to microtubule zippering, whereas steep-angle encounters led to catastrophic collisions or crossover, with a cutoff angle of ~40°. Therefore, pairs of microtubules with an encounter angle of >40° were selected to count the occurrences of catastrophic collisions in aug8 and wild-type cells during microtubule reorientation. We found that 58% of microtubules (n = 106) underwent catastrophic collisions in aug8 cells compared with 38% (n = 89) in wild-type cells. This observation indicated that AUG8 plays a protective role during microtubule reorientation, facilitating crossover but not catastrophic collision in the event of a microtubule-microtubule encounter.

Altogether, our findings demonstrate that AUG8 specifically associates with the plus ends of growing microtubules and promotes microtubule reorientation by regulating the dynamic instability of microtubule plus ends.

**DISCUSSION**

**AUG8 Is Required for Microtubule Reorientation**

Microtubule reorientation processes have been known to exist for a long time. Yuan et al. (1994) found that transverse-to-longitudinal reorientation is attributable to an increase in discordant microtubules in nontransverse alignment, followed by neighboring microtubules adopting the new alignment. However, the mechanism that underlies the transition of microtubule orientation is poorly understood. This study uncovers an important role for a novel microtubule plus-end binding protein, AUG8, in the modulation of microtubule reorientation. Microtubule reorientation is initiated when elongating cells change to a nongrowing state. We found that individual newly formed growing microtubules grew along a new trajectory, leading the transverse cortical microtubule array to a longitudinal orientation within ~45 min (Figures 6 and 7). This implies that the changes in the growth trajectory of growing microtubules play a major role in microtubule reorientation during the decline of cell elongation. Loss of
function of AUG8 causes a defect in light-induced microtubule reorientation in etiolated hypocotyls, indicating that AUG8 is required in this process. The analysis of the formation of microtubule branches and dynamic instability of microtubules indicated that AUG8 is required for new microtubule formation but does not affect the growth rates of microtubule leading ends (Table 1). In light-grown hypocotyls, AUG8 might have an indirect effect on microtubule shrinkage, catastrophe, and rescue. Altogether, AUG8 specifically protects the growing microtubules and eventually promotes the accomplishment of transverse-to-longitudinal reorientation that signals growth cessation.

### Table 1. In Vivo Transition Rates for Individual MTs

<table>
<thead>
<tr>
<th>Dynamic Parameter</th>
<th>aug8</th>
<th>Col</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transitions (events/min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kg-g</td>
<td>2.14</td>
<td>3.68</td>
</tr>
<tr>
<td>Kg-s</td>
<td>0.50</td>
<td>0.23</td>
</tr>
<tr>
<td>Kg-p</td>
<td>0.47</td>
<td>0.47</td>
</tr>
<tr>
<td>Kg-g</td>
<td>0.36</td>
<td>0.32</td>
</tr>
<tr>
<td>Kg-p</td>
<td>0.39</td>
<td>0.17</td>
</tr>
<tr>
<td>Kg-s</td>
<td>0.38</td>
<td>0.20</td>
</tr>
<tr>
<td>Kg-g</td>
<td>0.33</td>
<td>0.09</td>
</tr>
<tr>
<td>Kg-p</td>
<td>0.47</td>
<td>0.2</td>
</tr>
<tr>
<td>Kg-s</td>
<td>0.82</td>
<td>0.50</td>
</tr>
<tr>
<td>% Time in phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catastrophe</td>
<td>0.015</td>
<td>0.007</td>
</tr>
<tr>
<td>Rescue</td>
<td>0.014</td>
<td>0.009</td>
</tr>
<tr>
<td>Growth</td>
<td>50.7%</td>
<td>71.8%</td>
</tr>
<tr>
<td>Shrinkage</td>
<td>29.1%</td>
<td>15.7%</td>
</tr>
<tr>
<td>Pause</td>
<td>20.2%</td>
<td>12.5%</td>
</tr>
<tr>
<td>Growth rate (µm/min)</td>
<td>5.45 ± 1.13</td>
<td>6.19 ± 0.76</td>
</tr>
<tr>
<td>Shrinkage rate (µm/min)</td>
<td>7.63 ± 2.36</td>
<td>11.45 ± 1.81</td>
</tr>
<tr>
<td>Dynamics (µm/min)</td>
<td>5.59 ± 1.10</td>
<td>7.32 ± 1.54</td>
</tr>
</tbody>
</table>

K is the rate of transitions between dynamic states in events/min; g, growth; s, shrinkage; p, pause. Selected microtubules of wild-type (n = 92 MTs from 10 seedlings) and aug8 cells (n = 85 MTs from 10 seedlings) were analyzed in this assay. For the statistical analysis of catastrophe and rescue frequencies, phase transition events of wild-type cells (n = 1721 events) and aug8 cells (n = 2111 events) were measured. The dynamics was calculated according to a previously described method, in which the sum of the length grown and shortened was divided by the total time a particular microtubule was observed (Toso et al., 1993; Abe and Hashimoto, 2005). The values for growth rate and dynamicity are expressed as mean ± SD.

### AUG8 Might Play a General Role in Various Microtubule Array Organizations and Transitions in Different Cell Types

A previous study showed that the entire microtubule array undergoes marked reorientation in light-grown hypocotyls that grow slowly. This rotary movement of the intact cortical microtubule array is believed to account for consecutive wall layers with different orientations observed in slow-growing Arabidopsis hypocotyls (Chan et al., 2007). Longer term live-cell imaging revealed that stabilized and parallel microtubules grow along common trajectories during array rotation (Chan et al., 2007). Additionally, sequential microtubule array transitions (i.e., longitudinal to star, star to longitudinal, and star to transverse) are also observed in light-grown hypocotyl cells that precede the burst of growth (Sambade et al., 2012). This microtubule array transition was recently suggested to be caused by physiological changes that result in decreased microtubule catastrophes or increased nucleation or stability (Sambade et al., 2012). In aug8 cells, microtubule catastrophe increased, and microtubules spent less time in the growth phase compared with microtubules in wild-type cells, indicating that AUG8 is required for maintaining microtubule elongation. Together with the higher expression level of AUG8 in light-grown hypocotyls (Figure 5C), the altered microtubule dynamic features in aug8 cells suggest that AUG8 plays a general role in the regulation of rotary movements and sequential realignment of cortical microtubule arrays by regulating the dynamics of cortical microtubule plus ends.

AUG8 appeared to be recruited to the microtubule branch sites immediately before new microtubules branched out (see Supplemental Figure 6 and Supplemental Movie 4 online), and the rate of microtubule-branchoing events was dramatically reduced in aug8 cells compared with wild-type cells. This suggests that the ability of AUG8 to promote microtubule reorientation might be partially attributable to its promotion of new microtubule branching. Hotta et al. (2012) recently detected AUG8 by mass spectrometry after purifying the Arabidopsis augmin complex. Augmin proteins have been shown to play a role in pollen mitosis (Ho et al., 2011), and our study indicates an additional role during interphase. The augmin complex has also been shown to be associated with microtubule branching in other systems (Goshima et al., 2008; Uehara et al., 2009; Petry et al., 2013). One interesting question is whether AUG8 promotes microtubule reorientation by acting on the augmin complex. Further investigations are needed to address this issue.

Our analysis of the AUG8 expression pattern in Arabidopsis revealed that AUG8 is constitutively expressed in various organs/tissues (Figures 5A and 5B; see Supplemental Figures 7A to 7D online), indicating that AUG8 might play a general role in regulating microtubule reorientation in different cell types that transition from rapid to slow elongation, such as those in stems and roots. Microtubule reorientation was also shown to correspond to cell elongation status in root cells (Baluska et al., 1992; Sugimoto et al., 2000; Granger and Cyr, 2001). Furthermore, AUG8 expression levels varied in different root cells. As shown in Supplemental Figure 7 online, AUG8 expression was suppressed in the lower part of the root elongation zone where cells elongate rapidly. This expression pattern mirrors that in hypocotyls, implying that AUG8 may also regulate microtubule reorientation in
roots. We observed the roots of aug8, AUG8-OX-1, and AUG8-OX-2 and compared them with those of the wild type. We found that aug8 roots were significantly longer than those of the wild type and skewed to the left (viewed from above the plate), whereas the overexpression of AUG8 produced significantly shorter roots that skewed to the right. Similar to hypocotyls, twisting left-handedness was observed in aug8 roots. However, AUG8-OX-1 and AUG8-OX-2 roots exhibited right-handed twisted growth (see Supplemental Figure 7 online). These observations demonstrate that AUG8 plays a broad role in regulating plant growth and development. Additionally, evidence from aug8 mutant cells and AUG8-overexpressing cells suggests a rate-limiting role for AUG8 in some aspects of microtubule organization. The transcription of AUG8 appeared to be temporally and spatially regulated in hypocotyls and roots, and we propose that the regulation of AUG8 is important for microtubule dynamics and is likely at the transcriptional level in addition to the posttranscriptional level. The transcriptional regulation of some MAPs is known to be of central importance for microtubule dynamics (Nakajima et al., 2004; Li et al., 2011).

The correlation between microtubule stability and twisted growth has been suggested previously, based on a pharmacological analysis (e.g., with microtubule-disrupting drug treatment) and the observation of various microtubule-related mutants (e.g., lefty, phs1-1, and mort1) and transgenic plants overexpressing microtubule-stabilizing MAPs or mutated tubulin. Left-handed twisted growth has been suggested to be correlated with destabilizing microtubules, whereas right-handed twisted growth has been suggested to be correlated with stabilizing microtubules (Whittington et al., 2001; Thitamadee et al., 2002; Hamada et al., 2004; Nakamura et al., 2004; Naoi and Hashimoto, 2004; Abe and Hashimoto, 2005; Ishida et al., 2007). An interesting question for future research is whether the regulation of microtubule plus end dynamics by AUG8 is also involved in microtubule stability.

**Microtubule Plus-End Binding Proteins and Microtubule Reorientation**

Microtubule reorientation is critical for changes in cell growth status. Light-induced AUG8-dependent microtubule reorientation in etiolated hypocotyl cells signals the inhibition of growth. Previously described microtubule array rotations in light-grown hypocotyls could occur during growth (Chen et al., 2007), whereas the radial-star array (i.e., a key transitional step to the transverse array) forecasts the burst of growth in light-grown seedlings (Sambade et al., 2012). These results indicate that the initiation of microtubule reorientation is complex. We provide evidence that AUG8 directly binds to the plus end of a growing microtubule, with no bias against its growth direction (see Supplemental Movie 3 online). Therefore, we suspect that other key factors, such as other MAPs, and not AUG8 are involved in the initiation of microtubule reorientation. Once microtubule reorientation is initiated, AUG8 promotes the completion of microtubule reorientation. Accordingly, determining the mechanisms that underlie the onset of microtubule reorientation induced by various developmental signals, such as light and growth-promoting endogenous hormones (e.g., gibberellins), will be interesting in future studies.

In plants, several plus-end binding proteins, including EB1, SPR1, and CLASP, are known to be involved in the regulation of cell expansion (Chan et al., 2003; Mathur et al., 2003; Nakajima et al., 2004; Sedbrook et al., 2004; Ambrose et al., 2007, 2011; Bissgrove et al., 2008). More recently, the role of CLASP in the effects of microtubules on polar auxin transport has been revealed (Ambrose et al., 2013; Kakar et al., 2013), but no study of which we are aware has linked them to microtubule reorientation. CLASP and EB1 were reported to affect microtubule dynamics by suppressing the catastrophe of microtubule plus ends (Ambrose et al., 2007; Manna et al., 2008), which is similar to AUG8. In this study, we found that AUG8 is involved in microtubule reorientation. However, lacking AUG8 only delays and does not completely abolish microtubule reorientation associated with growth cessation. We suspect that other plus-end binding proteins, such as CLASP or EB1, might be involved in the regulation of microtubule reorientation. Notably, previous studies indicated that different plus-end binding proteins influence microtubule organization in different ways. For example, CLASP was found to accumulate at specific cell edges to overcome sharp edge-induced microtubule depolymerization. CLASP was proposed to generate microtubule ordering by modulating the location and degree of microtubule catastrophe (Ambrose et al., 2011). Van Damme et al. (2004) reported an increase in microtubule polymerization rate induced by overexpression of one EB1 family member, EB1a, whereas Chan et al. (2003) found that EB1a-GFP marks both the plus ends and minus ends of microtubules. Considering that AUG8 only binds to the plus ends of growing microtubules and that loss of function of AUG8 does not affect the microtubule growth rate, we propose that multiple plus-end binding proteins might be required for microtubule reorientation, with partially overlapping functions in the regulation of the dynamics of microtubule plus ends. Furthermore, SPR1 is a plant-specific microtubule plus-end binding protein. SPR1 was shown to be expressed in rapidly elongating tissues, and SPR1 overexpression led to enhanced resistance to a microtubule-disrupting drug and increased hypocotyl elongation (Nakajima et al., 2004), which are opposite to the phenotypes caused by AUG8 overexpression. These findings suggest that the regulatory mechanisms of plus-end binding proteins in the control of microtubule organization and orientation are complex. Therefore, different microtubule plus-end binding proteins may function differently but coordinately in the regulation of microtubule reorientation.

In summary, this study identified a microtubule plus-end binding protein, AUG8, in plants and demonstrated an important role for AUG8 in modulating microtubule reorientation, which signals the decline of cell elongation. Our observations also raise the intriguing possibility that AUG8 might function in various cell types and be involved in the organization and transition of different microtubule arrays, which is worthy of further investigation.

Kakar et al. (2013) recently linked microtubule orientation to PIN polarization and polar auxin transport, which is mediated by the microtubule plus-end binding protein CLASP. This suggested a mechanism by which oriented microtubules regulate plant cell growth by modulating polar auxin transport, in addition to guiding the deposition of cellulose fibrils. An interesting future
line of investigation would be to determine whether AUG8-mediated microtubule reorientation is also involved in the regulation of polar auxin transport and subsequent plant cell growth. Another important issue for future studies is to determine whether and how AUG8 interacts with other plus-end binding proteins to regulate microtubule organization and orientation. Such information will elucidate the regulatory mechanisms underlying microtubule dynamics and changes in microtubule arrays and provide insight into microtubule-based growth regulation in plants.

**METHODS**

**Plant Materials and Growth Conditions**

*Arabidopsis thaliana* ecotype Columbia (Col) was used in this study. The aug8 mutant (SALK_043608), ordered from the ABRC, is in the Col ecotype background. The PCR genotyping and sequencing results revealed that aug8 is a knockout mutant with a T-DNA insertion in the first exon of AUG8.

Seeds were germinated on 0.8% agar plates containing Murashige and Skoog medium (Sigma-Aldrich). Plants were then grown at 22°C in growth chambers. Dark-grown seedlings were prepared as described (Gendreau et al., 1997). Seeds in Petri dishes were imbibed at 4°C for 48 h and then exposed to white light for 4 h before being transferred to dark growth. The days of growth were counted beginning when the Petri dishes were transferred to the growth chambers.

*Arabidopsis* lines expressing AUG8-GFP were obtained by *Agrobacterium tumefaciens*-mediated transformation of AUG8-GFP into aug8 and then crossing with *Arabidopsis* lines expressing mCherry-TUA5 or mCherry-EB1 that harbored a 35S promoter to create double-labeled transgenic lines. To obtain the AUG8 overexpression lines, full-length coding sequence (CDS) of AUG8 was constructed in pSuper1300 with a hygromycin-resistant marker and then transformed into Col seedlings using the floral dip method. Additional resistance screening was applied to obtain homozygous AUG8-overexpressing lines. Eight lines showed elevated AUG8 expression and shorter hypocotyl lengths than the wild types. One of the lines (marked as AUG8-OX-1) was used for further analysis. To complement AUG8 function in the aug8 mutant line, AUG8+ AUG8-GFP was transformed into the aug8 mutant. Five lines showed complementation of transcript levels and hypocotyl lengths, and one of the lines (marked as COM-1) was used for further study.

**Plasmid Construction**

The cDNA sequence of AUG8 was obtained from The Arabidopsis Information Resource (AT4G30710.1; http://www.Arabidopsis.org; accessed January 7, 2013). For plant transformation, two primers of AUG8 (forward, 5'-GGATCCATGATGATGAAACAGACTACAAG-3' [with the BamHI site underlined]; reverse, 5'-GTCGACTGCGCCATGGAAACTGCT-3' [with the Sal site underlined]) were used to amplify AUG8 cDNA. A 200-bp fragment before the AUG8 CDS was amplified as a native promoter of AUG8.

Full-length AUG8 CDS was cloned into the pET30a(+) vector for the preparation of recombinant His-AUG8 protein (see below).

**PCR Analysis**

An RNA purification kit (BioTeke) was used to extract RNA from *Arabidopsis* seedlings. RNA was reverse transcribed using the Takara reverse transcription system and the oligo d(T)18 primers provided in the kit. For real-time PCR analysis, two primers of AUG8 (5'-CCACAAAGTC- GGAAAGATAAAAC-3' and 5'-ACTCTAACTCTGGGAAATGACG-3') were used to detect AUG8 expression, resulting in a 177-bp DNA product. EF1α (195 bp) was used as a control using the following primers: 5'-CACCCCTGTGTTCAAGCAATGA-3' and 5'-TGGCTCTCCGGAAATC- CAGAGATT-3'. Hypocotyls of 6-d-old seedlings that were grown under dark or light conditions were collected to detect AUG8 expression.

**Microtubule Polymerization and Cosedimentation Assay**

Porcine brain tubulin was purified as described (Castoldi and Poppow, 2003), pET30a(+)-AUG8 was transformed into the Escherichia coli strain BL21 (DE3) and was induced to express. The recombinant protein was purified with a nickel-nitrioltriacetic acid column (Qiagen) equilibrated with elution buffer (50 mM NaH2PO4, 300 mM NaCl and 250 mM imidazol, pH 8.0). The protein concentration was determined using a Bio-Rad protein assay kit. Protein samples were analyzed by SDS-PAGE. The microtubule polymerization and cosedimentation experiments were performed as described (Mao et al., 2005). For microtubule polymerization, concentrations of 0, 0.25, 0.5, 1, 2, and 2.5 μM recombinant His-AUG8 were added to 20 μM tubulin solution in PEM buffer (0.1 M PIPES, 1 mM EGTA, and 1 mM MgSO4, pH 6.9) that contained 1 mM GTP. The polymerization course was monitored turbidimetrically by absorbance at 350 nm, and 2.5 μM BSA was used as a negative control. For the cosedimentation assay, 0, 0.25, 0.5, 1, 2, and 2.5 μM recombinant His-AUG8 were added to taxol-stabilized microtubules (5 μM tubulin) in PEMT (100 mM PIPES, 1 mM EGTA, 1 mM MgCl2, and 20 μM taxol, pH 6.9). After incubation at 25°C for 30 min, the samples were centrifuged at 100,000g at 25°C for 20 min. Pellets and supernatants were analyzed by 10% SDS-PAGE and visualized by staining the gels with Coomassie Brilliant Blue R 250. His-NMAP65-1c and BSA were used as positive and negative controls, respectively.

**Immunofluorescence Assay**

Rhodamine-labeled tubulin was prepared according to Hyman (1991). Tubulin mixed with rhodamine-labeled tubulin (molar ratio, 1:4) was incubated with 50 nM His-AUG8 protein for polymerization for 15 min at 37°C and then cross-linked with 20 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Pierce Biotechnology) for 5 min at 37°C. The mixture was then centrifuged at 12,000g for 5 min, and the pellet was resuspended in PEM buffer preheated to 37°C. His-AUG8 was stained with an anti-His antibody (1:10,000) and secondary antibody conjugated with FITC (1:10,000). The solution was then centrifuged at 12,000g for 5 min, and the pellet was resuspended with PEM buffer preheated to 37°C. The samples were observed using spinning-disc confocal microscopy (see below).

**Analysis of GUS Activity in Etiolated Seedlings**

For AUG8+GUS construction, a 2019-bp fragment upstream of the initiation codon (ATG) of AUG8 was amplified and constructed into the pCAMBIA1391 vector. The construct was then transformed into *Arabidopsis* plants using *Agrobacterium* strain GV3101. GUS staining was performed according to Wang et al. (2007). Images were obtained using an Olympus microscope (SZX16) equipped with a color charge-coupled device camera (DP72).

**In Situ Hybridization Using Whole-Mount Seedlings**

The region of AUG8 cDNA that corresponded to nucleotides 471 to 940 was cloned and used as a probe. Digoxigenin-labeled sense and antisense probes were prepared by in vitro transcription according to the DIG RNA labeling kit (Roche). Three-, four-, five-, and six-day-old etiolated
seedlings were fixed, permeabilized, and hybridized as previously described (Hejátko et al., 2006). Images were obtained under an Olympus microscope (SZX16) equipped with a color charge-coupled device camera (DP72). The following primers were used: SP6 sense (5′-TCGGAAGGA-GAATCAGCA-3′) and T7 antisense (5′-GTCTATAGACAGTCAGTATC-GGC-3′).

Measurement of Hypocotyl Length

To measure hypocotyl length in wild-type, aug8, AUG8-OX-1, AUG8-OX-2, COM-1, and COM-2 plants, 3-d-old dark-grown seedlings with similar hypocotyl lengths were selected and grown for another 2 d in the dark. The images of hypocotyls of 5-d-old seedlings were taken with a G11 camera (Canon), and hypocotyl length was measured using ImageJ software (http://rsb.info.nih.gov/ij). To measure hypocotyl cell length, images of wild-type, aug8, AUG8-OX-1, and COM-1 etiolated hypocotyl cells were obtained with a scanning electron microscope (Hitachi TM3000), and the length was then measured using ImageJ software.

Spinning-Disc Confocal Microscopy

Imaging was performed on an Olympus IX81 inverted microscope equipped with a Yokogawa spinning-disc confocal head (Yokogawa Electric) and an Andor iXon charge-coupled device camera (Andor Technology). An Olympus objective (×100, 1.4 numerical aperture) was used. GFP and mCherry were excited at 488 and 561 nm, respectively. Images were acquired using Andor iQ software, version 1.1 (Andor Technology), and processed using ImageJ and Photoshop software (Adobe Systems).

For the microtubule reorientation assay, 3-d-old dark-grown seedlings expressing GFP-tubulin were used as described (Ueda and Matsuyama, 2000). Hypocotyl cells of wild-type, aug8, or AUG8-OX-1 lines with transverse and dynamic microtubules were selected for imaging. The cells were exposed to light using the 100-W incandescent light source of the microscope at full power, aligned for Kohler illumination. Images were acquired at 10-min intervals until the reorientation procedure was completed. The completion of microtubule reorientation was indicated by no obvious changes in microtubule alignment in the previous two images. Seedlings that expressed AUG8-GFP and mCherry-tubulin were used to measure microtubule orientation angles during reorientation. A time series was established over a short 10-s period at 1-s intervals and repeated every 15 min until the reorientation procedure was completed. The microtubule angles against the hypocotyl elongation axis were measured using the angle tool in ImageJ, All of the angle measurements were acute.

To quantify the density of cortical microtubules in aug8 and wild-type cells, the number of cortical microtubules across a fixed line (10 μm) drawn perpendicularly to the orientation of most cortical microtubules in the cell was counted using ImageJ software. Five repeated measurements were performed in each cell, and at least 30 cells from each line were analyzed. Excel software (Microsoft) was used for the statistical analysis.

AUTHOR CONTRIBUTIONS

L.C. and Y.F. contributed to project design. L.C. and L.W. performed the experiments and data analysis. M.Z. initiated this project. H.C., L.D., and L.C. and Y.F. contributed to project design. L.C. and L.W. performed the

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REFERENCES


Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Quantitative Real-Time PCR Analysis of the Transcript Level of AUG8 in 6-d-Old Seedlings.

Supplemental Figure 2. Purification of Recombinant His-AUG8 Protein.

Supplemental Figure 3. Colocalization of AUG8-GFP and mCherry-EB1 in Etiolated Hypocotyl Cell.

Supplemental Figure 4. In situ Hybridization for AUG8 mRNA in Whole-Mount Hypocotyls.

Supplemental Figure 5. Microtubules in Etiolated Hypocotyl Cells Undergo Reorientation after Light Induction.

Supplemental Figure 6. AUG8-GFP Is Recruited to the Microtubule Branch Site Immediately before a Nascent Microtubule Branches out.

Supplemental Figure 7. AUG8 Participates in Root Elongation.

Supplemental Movie 1. AUG8-GFP Exhibits a Comet-Like Structure in Cells.

Supplemental Movie 2. AUG8-GFP Colocalizes with mCherry-EB1 in Vivo.

Supplemental Movie 3. The Binding of AUG8 at Microtubule Plus Ends Is Associated with Microtubule Elongation.

Supplemental Movie 4. Recruitment of AUG8-GFP at the Microtubule Branch Site in an Etiolated Hypocotyl Cell.

Accession Number

The sequence data for AUG8 can be found in the Arabidopsis Genome Initiative database under accession number At4g30710.


