Microtubule bundling plays a role in ethylene-mediated cortical microtubule reorientation in etiolated hypocotyls

Qianqian Ma¹, Jingbo Sun¹, and Tonglin Mao*

State Key Laboratory of Plant Physiology and Biochemistry; Department of Plant Sciences, College of Biological Sciences, China Agricultural University, Beijing 100193, China

¹These authors contributed equally to this work.

*Corresponding author:
Tonglin Mao
Telephone: +8610-62732330. FAX: +8610-62732330. E-mail: maotonglin@cau.edu.cn

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Summary statement
This study reveals that microtubule bundling plays a role in ethylene signaling regulation of cortical microtubule reorientation to mediate etiolated hypocotyl cell elongation in Arabidopsis.
Abstract

The gaseous hormone ethylene is known to regulate plant growth under etiolated conditions (the "triple response"). Although organization of cortical microtubules is essential for cell elongation, the underlying mechanisms that regulate microtubule organization by hormone signaling, including ethylene, are ambiguous. In the present study, we demonstrate that ethylene signaling participates in regulation of cortical microtubule reorientation. In particular, regulation of microtubule bundling is important for this process in etiolated hypocotyls. Time-lapse analysis indicated that selective stabilization of microtubule bundling structures formed in various arrays is related to ethylene-mediated microtubule orientation. Bundling events and bundle growth lifetimes were significantly increased in oblique and longitudinal arrays, but decreased in transverse arrays in wild-type cells in response to ethylene. However, the effects of ethylene on microtubule bundling were partially suppressed in a microtubule-bundling protein WDL5 knockout mutant (wdl5-1). This study suggests that modulation of microtubule bundles formed in certain orientations plays a role in reorienting microtubule arrays in response to ethylene-mediated etiolated hypocotyl cell elongation.
Introduction

Ethylene is a gaseous hormone that plays crucial roles in plant growth, development, and stress responses. One of the most widely documented ethylene responses is the triple response, which includes a short, thickened hypocotyl when dark-grown Arabidopsis thaliana seedlings are treated with ethylene or its biosynthetic precursor 1-aminocyclopropane-1-carboxylic acid (ACC) (Bleecker et al., 1988; Ecker, 1995). The ethylene signal is detected by a family of five membrane-bound receptors (ETR1, ERS1, ETR2, ERS2, and EIN4) in Arabidopsis, while the redundant nuclear-localized transcription factors ETHYLENE-INSENSITIVE 3/EIN3-like 1 (EIN3/EIL1) mediate ethylene signaling. Altered cortical microtubule organization has been shown when exogenous ethylene or ACC is applied to plant cells (Soga et al., 2010a; Polko et al., 2012). Although the microtubule-associated protein WDL5 was recently identified as a participant in ethylene signaling-mediated etiolated hypocotyl elongation (Sun et al., 2015), evidence demonstrating that ethylene signaling is involved in regulation of microtubule reorientation is lacking.

Numerous studies have shown that cortical microtubule orientation is associated with the growth status of plant cells, especially in etiolated hypocotyl cells (Le et al., 2005; Crowell et al., 2011). As such, a parallel array of cortical microtubules is dominantly transversely oriented to the hypocotyl longitudinal growth axis in rapidly growing etiolated hypocotyl cells, while microtubules are longitudinally oriented when cell elongation stops (Le et al., 2005; Wang et al., 2012). Mutation or overexpression of many microtubule-associated proteins (MAPs) alters cortical microtubule orientation and results in abnormal hypocotyl cell elongation. For example, overexpression of a microtubule plus-end binding protein AUGMIN subunit 8 (AUG8) longitudinally orients cortical microtubules and inhibits etiolated hypocotyl cell elongation (Cao et al., 2013). Many factors are capable of altering cortical microtubule orientation in growing cells, such as the phytohormones gibberellic acid (GA) and auxin (Shibaoka, 1993; Vineyard et al., 2013). Recent studies have shown that blue light, auxin and brassinosteroid (BR) signaling participate in regulation of cortical microtubule reorientation in hypocotyl and root cells (Wang et al., 2012;
Lindeboom et al., 2013; Chen et al., 2014). Whether other signaling processes also participate in regulation of cortical microtubule reorientation in plant cells in response to complicated developmental and environmental cues remains unclear.

Several important regulators have been identified as playing different roles in regulation of cortical microtubule reorientation. For example, γ-tubulin complexes are required for reorientation of cortical microtubules via nucleation of nascent microtubules as branches diverge by approximately 40° from existing microtubules (Murata et al., 2005). The microtubule severing protein katanin is prone to localization at nucleation sites to sever nucleated microtubules and at crossovers sites to sever overlapping microtubules to create new arrays during reorientation (Lindeboom et al., 2013; Zhang et al., 2013). Previous models have demonstrated that microtubules in their original orientation are prone to depolymerization, while other microtubules build new arrays (Soga et al., 2010b). Although much is known about the roles of the γ-tubulin complex and katanin in regulating microtubule reorientation, questions remain about the underlying mechanisms regarding regulation of microtubule stability in different orientations.

In this study, we demonstrated that ethylene signaling is involved in regulation of cortical microtubule reorientation. Further analyses showed that the effects of ethylene on microtubule bundling events and lifetime are related to variously oriented microtubule arrays. The results of this study suggest that regulation of microtubule bundling plays a role in modulation of cortical microtubule reorientation in response to ethylene in etiolated hypocotyl cells.
**Results**

**Ethylene signaling participates in regulation of cortical microtubule reorientation in etiolated hypocotyls**

Although exogenous ethylene or ACC treatment can alter cortical microtubule orientation (Le et al., 2005; Sun et al., 2015), the underlying molecular mechanism regulating cortical microtubule orientation remains unknown. To learn whether this regulation is required for ethylene signaling, the ethylene-insensitive mutant *ein2-5* from a critical positive regulator of ethylene signaling, ETHYLENE-INSENSITIVE2 (EIN2), was generated with a yellow fluorescence protein (YFP)-tubulin background. ACC treatment was applied.

Confocal microscopy showed that most cortical microtubules exhibited a transverse orientation in epidermal cells from the upper region of etiolated hypocotyls in wild-type and *ein2-5* seedlings in the absence of ACC and in mock buffer. However, after treatment with 100 µM ACC for 90 min, the majority of transverse cortical microtubules were longitudinally oriented relative to the longitudinal hypocotyl growth axis in epidermal cells from wild-type hypocotyls. In contrast, cortical microtubule arrays remained predominately transverse despite treatment with ACC for 90 min in *ein2-5* mutant cells (Fig. 1A,B), suggesting that alteration of cortical microtubule orientation from transverse to longitudinal was significantly suppressed in *ein2-5* cells in response to ACC. Ethylene cross-talks with auxin in multiple physiological processes (Muday et al., 2012; Bours et al., 2015). We found that NAA altered microtubule orientation in wild-type cells, although this effect was significantly suppressed in *ein2-5* etiolated hypocotyl cells (Fig. S1A-F). This suggests that ethylene signaling is crucial for regulating microtubule reorientation in plant cells.

The relationship between cortical microtubule orientation and ethylene signaling was evaluated in the ethylene-insensitive mutant *ein2-5*, which exhibits longer etiolated hypocotyls, and the constitutive ethylene response mutant *ctr1-1*, which has
much shorter etiolated hypocotyls (Fig. 2A). Cortical microtubules in epidermal cells of 4-day-old etiolated hypocotyls from ein2-5 and ctr1-1 mutants with a YFP-tubulin background were observed using confocal microscopy. Parallel arrays of cortical microtubules were mostly transversely oriented relative to the longitudinal hypocotyl growth axis in epidermal cells from the upper region of wild-type etiolated hypocotyls (Fig. 2B,E). Transverse orientation was even more obvious in ein2-5 cells (Fig. 2C,E). In comparison, random, oblique, or longitudinal cortical microtubules were observed in most ctr1-1 etiolated hypocotyl cells (Fig. 2D,E). This evidence demonstrates that ethylene signaling plays an important role in regulation of microtubule orientation.

**Regulation of microtubule stability is important for ethylene-mediated microtubule reorientation**

Although exogenous ethylene or ACC stabilizes cortical microtubules in plant cells (Steen and Chadwick, 1981; Sun et al., 2015), evidence demonstrating that ethylene signaling is involved in regulation of microtubule stability is lacking. To investigate the underlying mechanisms regarding ethylene-regulated microtubule reorientation, cortical microtubule stability in cells from ein2-5 etiolated hypocotyls was evaluated using the microtubule-disrupting drug oryzalin. Although the number of cortical microtubules was not obviously different before oryzalin treatment, it was significantly different after oryzalin treatment in wild-type and ein2-5 epidermal cells pretreated with 0 μM and 100 μM ACC. After 10 μM oryzalin treatment for 3 min, more microtubules were observed in the wild-type cells that were pretreated with ACC for 90 min, compared with that of unpretreated cells. This effect was even more obvious when the duration of oryzalin treatment increased to 8 min. However, cortical microtubules in ein2-5 cells exhibited similar sensitivity to oryzalin treatment when pretreated with ACC for 0 min or 90 min (Fig. 3A,B), suggesting that ethylene increases microtubule stability through its signaling pathway, which is related to ethylene-regulated cortical microtubule reorientation in etiolated hypocotyl cells. In agreement with this hypothesis, the effect of ACC on regulation of microtubule
reorientation was partially hindered in the presence of the microtubule-stabilizing agent taxol in etiolated hypocotyl cells (Fig. S2A-E).

The relationship between the stability of cortical microtubules and ethylene signaling was further evaluated in the constitutive ethylene response mutant *ctr1-1*. Many microtubules were disrupted in wild-type epidermal cells after treatment with 5 μM oryzalin for 5 min, while microtubules in *ctr1-1* cells were largely unaffected. Increasing the oryzalin concentration and duration of treatment (10 μM oryzalin for 10 min) resulted in disruption of the majority of cortical microtubules in wild-type cells. However, cortical microtubules remained relatively unaffected in *ctr1-1* cells (Fig. 3 C, D), indicating that cortical microtubules are more stable in *ctr1-1* compared to wild-type cells. This suggests that increased microtubule stability is associated with ethylene signaling, which is important for ethylene-mediated alteration of cortical microtubule orientation in etiolated hypocotyl cells.

Cortical microtubules may be prone to stabilization in ACC-regulated microtubule orientation in etiolated hypocotyl cells. To evaluate the characteristics of individual microtubules from various arrays, dynamic parameters of transverse, oblique and longitudinally oriented microtubules were analyzed in the presence of ACC. Microtubules with clearly visible leading plus ends (identified by growth rate) were selected for measurement in etiolated hypocotyl cells treated with ACC or mock buffer. The catastrophe frequency was increased and the rescue frequency was decreased in individual microtubules from transverse arrays in the ACC-treated cells compared to cells without ACC (Table 1). In contrast, microtubule dynamics from oblique and longitudinal arrays were significantly altered following ACC treatment compared with mock buffer. The catastrophe frequency of individual microtubules was much lower in ACC-treated cells (0.012 s⁻¹) than in cells without ACC (0.038 s⁻¹). Duration of the microtubule growth phase was obviously increased (from 56.4% to 79.7%) and the shrinkage phase was significantly decreased (from 27.4% to 9.1%) in cells treated with ACC. Thus, cortical microtubules from oblique and longitudinal arrays are prone to growth and stabilization, while microtubules from transverse arrays are prone to depolymerization and destabilization, demonstrating that cortical
microtubule stability at certain orientations varies in response to ethylene. This evidence suggests that complex regulation of array stability is important for ethylene-regulated microtubule reorientation.

**Ethylene promotes microtubule bundling in etiolated hypocotyl cells**

Microtubule stability is related to ACC-regulated microtubule reorientation, although the regulatory mechanisms regarding this process remain unknown. Microtubule bundles are thought to increase microtubule stability and affect microtubule dynamics in plant cells (Ehrhardt and Shaw, 2006; Bratman and Chang, 2008). We propose that microtubule bundling may regulate the stability of various microtubule orientations in response to ethylene.

To test this hypothesis, we quantified the extent of microtubule filament bundling in etiolated hypocotyl cells in response to ACC treatment. Skewness is a measure of the degree of asymmetry of a distribution and the skewness of the fluorescence intensity distribution is generally considered to be an indicator of filament bundling in the cells (Higaki et al., 2010). Thus, we measured the skewness to evaluate microtubule bundling in the etiolated hypocotyl cells in response to ACC. More microtubule bundles were present in wild-type hypocotyl cells treated with ACC for 30 min (Fig. 4B), as indicated by the increase in mean skewness compared with that of untreated wild-type cells (Fig. 4A) or cells treated with mock buffer for 50 min (Fig. 4E). By increasing the duration of ACC treatment, the effects of ACC on microtubule bundling were more pronounced in treated wild-type cells at 50 and 90 min (Fig. 4C,D,I). A previous study showed that the microtubule-associated protein WDL5 participates in ethylene signaling-mediated etiolated hypocotyl cell elongation and that ethylene-regulated cortical microtubule reorientation was partially hindered in *WDL5* loss-of-function mutant (*wdl5-1*) cells (Sun et al., 2015). Thus, if bundling is involved in ethylene-mediated microtubule reorientation, we predicted that the ACC effect on microtubule bundling would be suppressed in *wdl5-1* cells. In agreement with our hypothesis, microtubule bundling was obviously decreased in *wdl5-1* cells compared with wild-type cells after ACC treatment for 50 min (Fig. 4F,G,H,I).
effect of ethylene on microtubule bundling was further confirmed using superresolution structured illumination microscopy (SIM) (Fig. S3). In addition to skewness, Fibriltool was used to quantify the orientation (average orientation of microtubules in the cell) and anisotropy (whether microtubules are well ordered, where 0 represents no order and 1 represents perfectly ordered) of cortical microtubules (Boudaoud et al., 2014). We found that transverse cortical microtubules were well ordered before ACC treatment, but had no order after ACC treatment for 30 min. The cortical microtubules regained order in the longitudinal orientation after ACC treatment for 50 min, which was more pronounced after 90 min in wild-type cells, but not in wdl5-1 cells in which cortical microtubules were progressing from ordered transverse to isotropic arrays after ACC treatment for 50 min (Fig. 4A-H, below). This evidence suggests that microtubule bundling plays a role in regulating microtubule stability and reorientation in response to ethylene.

**Bundling stability is related to various arrays in ethylene-regulated microtubule orientation**

Forming stable polymer bundles during ethylene-regulated microtubule orientation raises a fundamental question—how do the newly created stable structures participate in regulation of microtubule organization? To answer this question, we investigated the characteristics of bundles in various arrays (transverse, oblique, and longitudinal) in wild-type and wdl5-1 seedlings in response to ethylene. The percentage of cortical microtubule bundling events (orientation bundling events/total bundling events) and bundle growth lifetimes were evaluated in ethylene-regulated orientation.

Microtubule bundling events from transverse arrays were significantly decreased in the presence of ACC compared with events in the presence of mock buffer in wild-type and wdl5-1 cells. In contrast, bundling events were increased in oblique arrays, and a similar response was even more pronounced in longitudinal arrays when treated with ACC for 50 min (Fig. 5A,B,C). This result is consistent with data showing that microtubules from transverse arrays are prone to depolymerization and
that microtubules from oblique/longitudinal arrays are prone to growth in the presence of ACC.

Bundling structures have been shown to be required for maintaining timelines for diverse biochemical activities, such as cell wall biosynthesis (Tian et al., 2004). We hypothesized that selective bundling stability in various arrays may be important for ethylene-regulated microtubule orientation. We measured bundle growth lifetimes to evaluate bundle structure stability in various orientations in response to ACC. Bundle growth lifetimes from transverse arrays were significantly decreased in ACC-treated wild-type cells compared to cells treated with mock buffer, indicating that bundle structures become unstable in transverse arrays in response to ACC (Fig. 5D, Movie 1 and 2). Unlike the effects of ACC treatment on bundling events, bundle growth of transverse arrays was less sensitive to ACC treatment in wdl5-1 cells (Fig. 5D, Movie 3 and 4), which is consistent with the observation that more transverse arrays were found in wdl5-1 cells in the presence of ACC compared with wild-type cells (Sun et al., 2015).

Bundle growth lifetimes from oblique and longitudinal arrays were dramatically increased in ACC-treated wild-type cells compared to cells treated with mock buffer (Fig. 5D). This explains the observation that the number of microtubule bundles was increased in the presence of ACC. However, ACC effects on bundle growth lifetimes were obviously reduced in oblique arrays than in longitudinal arrays (Fig. 5D, left panel), indicating that bundle structures from oblique arrays are less stable compared to structures in longitudinal arrays. In addition, bundle growth lifetimes from oblique arrays were not much different in wdl5-1 cells in the presence and absence of ACC (Fig. 5D, right panel), demonstrating that the effect of ACC on bundling stability in longitudinal arrays was obviously lower in wdl5-1 cells than in wild-type cells. These data suggest that forming diverse stabilizing bundle structures may facilitate different microtubule functions during alteration of microtubule orientation in response to multiple signals.
Discussion

Understanding the molecular mechanisms regarding hormonal regulation of cortical microtubule orientation is essential for elucidating developmental mechanisms in plants. In this study, we demonstrated that ethylene signaling participates in reorienting cortical microtubules from a transverse to longitudinal orientation in etiolated hypocotyl cells. Moreover, microtubule bundling is likely related to regulation of cortical microtubule orientation in response to ethylene.

Hormone signaling participates in regulation of cortical microtubule reorientation

Cortical microtubule orientation is closely interrelated with plant cell growth (Le et al., 2005; Crowell et al., 2011). Recent studies have shown that auxin and brassinosteroid (BR) signaling participates in the regulation of cortical microtubule reorientation, which is essential for modulation of plant cell growth (Wang et al., 2012; Chen et al., 2014). In this study, we provide evidence demonstrating that ethylene signaling is involved in regulating microtubule reorientation from transverse arrays to oblique or longitudinal arrays in etiolated hypocotyl cells. In addition, auxin-induced alteration of microtubule orientation was obviously suppressed in ein2-5 etiolated hypocotyl cells, suggesting that auxin regulation of microtubule reorientation may be due to ethylene signaling in etiolated hypocotyls. This is consistent with previous studies that have shown that auxin stimulates ethylene production, resulting in inhibition of etiolated hypocotyl elongation (Vandenbussche et al., 2005; Arteca et al., 2008). Cortical microtubule orientation is also regulated by other hormones. For example, exogenous applied GAs are capable of aligning cortical microtubules transversely to the long axis of growing cells (Shibaoka, 1993; Vineyard et al., 2013), although it is unclear whether this signaling is specifically involved in regulation of orientation. Thus, this evidence suggests that microtubule effects on plant cell growth may be controlled by different signals in response to multiple developmental and environmental cues.
Light signaling is also capable of reorienting cortical microtubules from a transverse orientation to oblique and longitudinal arrays and inhibits hypocotyl cell growth (Sambade et al., 2012; Lindeboom et al., 2013). Although mutation or overexpression of some MAPs, such as AUG8 and WDL3, resulted in abnormal responses to light-regulated microtubule orientation (Cao et al., 2013; Liu et al., 2013), no MAPs have been identified to be directly targeted and regulated by light signaling pathways. Previous studies have shown that the microtubule-associated proteins MDP40 and WDL5 are directly targeted and regulated by BR and ethylene signaling pathways (Wang et al., 2012; Sun et al., 2015). Thus, modulation of microtubule orientation via upstream signaling pathways likely occurs through direct targeting and regulation of MAP expression.

A previous study showed that BR signaling is involved in regulation of cortical microtubule reorientation from oblique or longitudinal arrays to transverse arrays in etiolated hypocotyl cells (Wang et al., 2012). We propose that alteration of microtubule orientation from oblique or longitudinal arrays to transverse arrays may be required for hormone-induced cell elongation, whereas reorientation from transverse to oblique or longitudinal arrays may be necessary for hormone-inhibited cell elongation. Future studies will be necessary to demonstrate whether similar mechanisms involving changes in microtubule orientation are exploited by other environmental and developmental cues to mediate plant cell growth and cell morphogenesis through microtubules.

**Microtubule bundling plays a role in ethylene-mediated cortical microtubule reorientation in etiolated hypocotyls**

Pharmacological and dynamic assays in the present study showed that microtubules from transverse arrays are unstable and prone to depolymerization, whereas microtubules from other arrays are stable and prone to polymerization in response to ACC in etiolated hypocotyl cells. In particular, the microtubule-stabilizing agent taxol significantly decreased the effect of ACC on regulation of microtubule reorientation. These evidences demonstrate that regulation of microtubule stability is associated
with ethylene-mediated cortical microtubule reorientation. Our findings are in agreement with previous studies showing that altered expression of microtubule stabilizers or destabilizers disturbs microtubule orientation in response to signals. For example, decreasing expression of the microtubule-destabilizing protein MDP40 partially hindered BR-regulated microtubule reorientation in etiolated hypocotyl cells (Wang et al., 2012).

However, stable microtubule arrays from various orientations in the same cell raise an important question regarding regulation of microtubule dynamics when new arrays are being formed. A previous study showed that γ-tubulin complex and katanin gene transcript levels were transiently increased by ACC treatment, indicating that these components may play a role in ethylene-regulated microtubule reorientation (Soga et al., 2010a). In the present study, we considered underlying mechanisms from a different point of view than previous studies. We provide several lines of evidence supporting the notion that regulation of microtubule bundling plays a role in ethylene signaling-mediated microtubule reorientation from transverse to longitudinal in etiolated hypocotyls. First, microtubule stability was significantly increased in ACC-treated wild-type cells, but not in ACC-treated ein2-5 cells. Second, the frequency and lifetime of microtubule bundling were increased for oblique and longitudinal arrays, but were significantly decreased for transverse arrays in ethylene-regulated reorientation. Third, microtubule bundling events were obviously decreased in wdl5-1 cells in response to ethylene, in which alteration of cortical microtubule orientation has been shown to be partially hindered in the presence of ACC (Sun et al., 2015).

Our findings are in agreement with previous studies showing that regulation of cortical microtubule orientation by diverse signals is disturbed in cells from loss-of-function mutants or in seedlings that overexpress microtubule-bundling proteins. For example, the microtubule-bundling protein WDL3 was previously shown to participate in light-inhibited hypocotyl elongation and light altered microtubule orientation from transverse to oblique and longitudinal arrays, the formation of which were partially hindered in cells from WDL3 RNAi seedlings (Liu
et al., 2013). In addition, the present study may provide an explanation as to how microtubule-bundling proteins, such as WDL3 and WDL5, influence hypocotyl cell elongation in response to light and ethylene signals (Liu et al., 2013; Sun et al., 2015). Future studies will investigate the role of bundling in regulating microtubule orientation in response to multiple environmental and developmental signals.

Variations in the stability of microtubule bundles from transverse, oblique and longitudinal arrays indicate that this characteristic may play a role in regulation of microtubule orientation. A decreased number of bundling events and less stable bundle structures provide explanations for the prior observation that transverse microtubules are prone to disruption when oriented from transverse to oblique and longitudinal (Soga et al., 2010b; Lindeboom et al., 2013). Stable, organized longitudinal arrays generally orient cellulose fibrils and cellulose fibril arrays in cell wall inhibition (Tian et al., 2004; Bashline et al., 2014). We hypothesize that formation of less stable bundling structures in oblique arrays may be important for maintaining oblique arrays before reorienting into longitudinal arrays. Future studies will be necessary to provide additional genetic and cellular evidence to test this hypothesis in other cell types regarding signaling-regulated microtubule reorientation.
Materials and Methods

Plant materials and growth conditions

All plant materials used in this study were from the Arabidopsis thaliana Columbia (Col) ecotype background. Seeds were sterilized and placed on MS medium (Sigma-Aldrich) with 1% agar and 3% sucrose (w/v). For hypocotyl measurement, plates were placed at 22°C in the light for 12 h after stratification at 4°C for 2 d and then transferred to the dark for 5 d. Mutants ein2-5 (Alonso et al., 1999), ctr1-1 (Kieber et al., 1993), wdl5-1 (Sun et al., 2015) and 35S:Tubulin5A-YFP transgenic plants (Kirik et al., 2012) were used in this study.

Confocal imaging

Four-day-old etiolated hypocotyls from wild-type, ein2-5, ctr1-1 and wdl5-1 with a 35S:Tubulin5A-YFP background grown on MS medium were used. Seedlings were transferred to glass slides and gently covered with glass coverslips. We obtained images from one seedling per group in three minutes.

ACC treatment

Four-day-old etiolated hypocotyls from wild-type, ein2-5, ctr1-1 and wdl5-1 with a 35S:Tubulin5A-YFP background grown on MS medium were used. Seedlings were treated with ACC at a concentration of 100 μM for 0, 30, 50 and 90 min. Cortical microtubules were observed using a Zeiss 510 META confocal microscope (objective 40 ×, 1.4 numerical aperture). YFP was excited at 488 nm, and emissions were collected through 505-530 nm filters. At least 100 cells from each treatment were used.

Quantitative analyses of microtubule arrays in etiolated hypocotyl cells

To quantify the extent of microtubule bundling in etiolated hypocotyl cells, skewness was measured as previously described (Higaki et al., 2010; Li et al., 2012). Data were analyzed using Image J software (http://rsbweb.nih.gov/ij/). To analyze bundling events and bundle growth lifetimes, wild-type and wdl5-1 hypocotyl cells with
YFP-tubulin backgrounds from 4-day-old seedlings were treated with 100 μM ACC or mock buffer for 50 min, respectively.

Time series images 120 s in length (with 4-s intervals) were obtained under a spinning disc confocal microscope system (Yokogawa) using an Olympus IX81 microscope equipped with an Andor iXon charge-coupled device camera (Andor Technology). An Olympus objective (100 ×, 1.4 numerical aperture) was used. YFP was excited at 488 nm, and emissions were collected through 525 ± 5.5 nm filters. To distinguish the various bundles from transverse, oblique and longitudinal arrays, we measured their degrees using Image J software and calculated bundling events (a growing plus end of an individual microtubule associated into a bundle or interacting to form a bundle with another microtubule) and the bundle growth lifetime (the time from which a growing plus end of an individual microtubule associated into a bundle until it dissociated from the bundle). All data were processed using Excel software (Microsoft Office 2003).

**SIM microscopy**

Four-day-old etiolated hypocotyls from wildtype and *wdl5-1* with a 35S:Tubulin5A-YFP background grown on MS medium were used. All samples were examined with T1-E+N-SIM+A1 type superresolution structured illumination microscopy. The light source for SIM included a diode laser at 488 nm. Images were captured with an electron-multiplying (EM)-CCD camera (Andor iXON3 EMCCD; 1024 × 1024 px, cooled at -70°C, 16 bit) at typical exposure times of 200 ms with gain values of 75. The high-performance SIM setup included three rotations and five phases of the grated pattern for each image layer. Up to seven (average of three) Z-stacks were acquired per image with a slice thickness of 200 nm for the 100 ×, NA 1.49 objectives.
NAA treatment
Four-day-old etiolated hypocotyls from wildtype and ein2-5 with a 35S:Tubulin5A-YFP background grown on MS medium were used. Seedlings were treated with NAA at a concentration of 100 nM for 0 and 90 min. Cortical microtubules were observed under spinning disc confocal microscopy with an Andor iXon charge-coupled device camera (Andor Technology).

Taxol and ACC treatment
Four-day-old etiolated hypocotyls from wildtype with a 35S:Tubulin5A-YFP background grown on MS medium were used. Seedlings were pretreated with taxol at 1 μM for 30 min, then treated with 0.5 μM taxol plus 100 μM ACC or mock buffer for 90 min. Cortical microtubules were observed under spinning disc confocal microscopy with an Andor iXon charge-coupled device camera (Andor Technology).

Quantification of cortical microtubules
Image J software was used to quantify cortical microtubule density. A vertical line oriented to the majority of cortical microtubules with a fixed length (~10 μm) was drawn, and the density of cortical microtubules across the line was measured. Four repeated measures were performed for each cell, and at least 30 cells from each treatment were used. Values were recorded and significance was analyzed using the paired Student’s t test.
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Author contributions
T.M. designed the project. Q.M. and J.S. performed specific experiments and analyzed the data. T. M. wrote, revised and edited the manuscript.

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Microtubule dynamic parameters at the microtubule plus ends were quantified based on spinning disk confocal micrographs. Growth and shrinkage velocities were calculated based on 120 leading ends in the upper region of epidermal cells from wild-type *Arabidopsis* (YFP-TUA5 background) etiolated hypocotyls treated with or without ACC. Data passed Shapiro-Wilk normality tests (see Table S1 for P-values). Student’s two-tailed t-test (see Table S2 for P-values), *P < 0.05, **P < 0.01. Values are expressed as the mean ± SD.
Fig. 1. Ethylene signaling regulates orientation of cortical microtubules in etiolated hypocotyl epidermal cells. (A) Etiolated hypocotyl epidermal cells from wild-type and ein2-5 mutants with a YFP-tubulin background were treated with or without ACC for 0 and 90 min after growth for 96 h and cortical microtubules were observed. (B) Frequency of microtubule orientation patterns in etiolated hypocotyl epidermal cells from wild-type and ein2-5 mutants (n > 100 cells, at least 30 seedlings). Scale bar = 20 μm.
Fig. 2. Cortical microtubule orientation was altered in etiolated hypocotyl epidermal cells from the ctr1-1 mutant. (A) Wild-type (Columbia ecotype), ein2-5 and ctr1-1 mutant seedlings were grown on MS in the dark for 5 days. Ein2-5 seedlings showed longer etiolated hypocotyls, whereas ctr1-1 seedlings exhibited much shorter etiolated hypocotyls compared with the wildtype (Data passed Shapiro-Wilk normality tests (see Table S1 for P-values). Student’s two-tailed t-test (see Table S2 for P-values), *P < 0.05, **P < 0.01). The graph shows the average hypocotyl length measured from a minimum of 45 seedlings. Error bars represent the mean ± SD. (B), (C) and (D) Cortical microtubules in epidermal cells from the upper region of etiolated hypocotyls from wild-type (WT), ein2-5 and ctr1-1 seedlings with
a YFP-tubulin background were observed by confocal microscopy after growth in the dark for 4 days. (E) Frequency of different microtubule orientation patterns in etiolated hypocotyl epidermal cells from WT, ein2-5, and ctr1-1 seedlings ($n > 160$ cells from over 45 seedlings for each sample). Scale bar = 20 μm.
Fig. 3. Ethylene signaling regulates the stability of cortical microtubules in ethylene-mediated microtubule reorientation. (A) Cortical microtubules were observed in wild-type and ein2-5 etiolated hypocotyl epidermal cells pretreated with ACC for 0 and 90 min or mock buffer for 90 min after treatment with 10 μM oryzalin for 3 or 8 min. (B) Quantification of cortical microtubules in wild-type and ein2-5 hypocotyl epidermal cells using Image J software (n > 33 cells from each sample). (C) Cortical microtubules were observed in etiolated hypocotyl epidermal cells in wild-type and ctr1-1 seedlings after treatment with 0 and 5 μM oryzalin for 5 min and 10 μM oryzalin for 10 min. (D) Quantification of cortical microtubules in hypocotyl epidermal cells from wild-type and ctr1-1 mutants using Image J software (n > 50 cells from each sample). Data passed Shapiro-Wilk normality tests (see Table S1 for P-values). Student’s two-tailed t-test (see Table S2 for P-values), **P < 0.01. Vertical scale represents the number of cortical microtubules across a fixed line (~10 μm) vertical to the orientation of the majority of cortical microtubules in the cell. Error bars represent the mean ± SD. Scale bars = 20 μm.
Fig. 4. Ethylene increases cortical microtubule bundling in etiolated hypocotyl cells. (A)-(E) Cortical microtubules were observed in wild-type etiolated hypocotyl epidermal cells treated with ACC for 0, 30, 50 and 90 min or mock buffer for 50 min. (F)-(H) Cortical microtubules were observed in wdl5-1 etiolated hypocotyl epidermal cells treated with ACC for 0 and 50 min or mock buffer for 50 min. FibriTool was used to quantify the anisotropy and orientation of cortical microtubules ($n > 45$ cells from 15 seedlings for each sample). (I) Quantitative analysis of microtubule bundling (skewness) in wild-type and wdl5-1 cells treated with ACC compared to cells not treated with ACC ($n > 15$ cells were quantified for each analysis). Data passed Shapiro-Wilk normality tests (see Table S1 for P-values). Student’s two-tailed $t$-test (see Table S2 for P-values), *$P < 0.05$, **$P < 0.01$. Error bars represent the mean ± SD. Scale bar = 20 μm.
Fig. 5. Ethylene increases microtubule bundling frequency in etiolated hypocotyl cells. (A) Time-lapse images of cortical microtubules in wild-type etiolated hypocotyl epidermal cells treated with ACC or mock buffer for 50 min. See movies 1 and 2 for the entire series. (B) Time-lapse images of cortical microtubules in wdl5-1 etiolated hypocotyl epidermal cells treated with ACC or mock buffer for 50 min. See movies 3 and 4 for the entire series. Arrows indicate microtubule bundling events. Different bundling events are represented by different colored arrows. (C) Quantification of the frequency of microtubule bundling events from transverse, oblique and longitudinal arrays in etiolated hypocotyl epidermal cells from the wild-type with mock buffer (n = 339) and ACC treatment (n = 407), and wdl5-1 with mock buffer (n = 327) and ACC treatment (n = 414) for 50 min using Image J software. (D) Quantification of microtubule bundle growth lifetimes from transverse, oblique and longitudinal arrays in etiolated hypocotyl epidermal cells from the wild-type and wdl5-1 with or without ACC treatment for 50 min using Image J software (n > 20 cells from 10 seedlings for
each sample). Data passed Shapiro-Wilk normality tests (see Table S1 for P-values). Student’s two-tailed t-test (see Table S2 for P-values), $*P < 0.05$, $**P < 0.01$. Error bars represent the mean ± SD. Scale bar = 20 μm.