© 2016. Published by The Company of Biologists Ltd | Journal of Cell Science (2016) 129, 2043-2051 doi:10.1242/jcs.184408

RESEARCH ARTICLE

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

Qianqian Ma*, Jingbo Sun* and Tonglin Mao‡

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 that have formed in certain orientations plays a role in reorienting microtubule arrays in response to ethylene-mediated etiolated hypocotyl cell elongation.

KEY WORDS: Ethylene, Cortical microtubule, Orientation, Hypocotyl growth, *Arabidopsis*

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 results in a short thickened hypocotyl when *Arabidopsis thaliana* seedlings that have been grown in the dark 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*, and the redundant nuclear-localized transcription factors ETHYLENE-INSENSITIVE 3 (EIN3) and EIN3-like 1 (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 has been 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, whereas 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 protein that binds to microtubule plus ends, 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 and auxin (Shibaoka, 1993; Vineyard et al., 2013). Recent studies have shown that blue light, auxin and brassinosteroid 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 the 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 the regulation of cortical microtubule reorientation. For example, γ-tubulin complexes are required for reorientation of cortical microtubules through 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 in order to sever nucleated microtubules and at crossovers sites in order 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, whereas 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 have 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 the orientation of 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.

State Key Laboratory of Plant Physiology and Biochemistry, Department of Plant Sciences, College of Biological Sciences, China Agricultural University, Beijing 100193, China.
*These authors contributed equally to this work

‡Author for correspondence (maotonglin@cau.edu.cn)

Received 4 December 2015; Accepted 30 March 2016

Journal of Cell Science (2016) 129, 2043-2051 doi:10.1242/jcs.184408

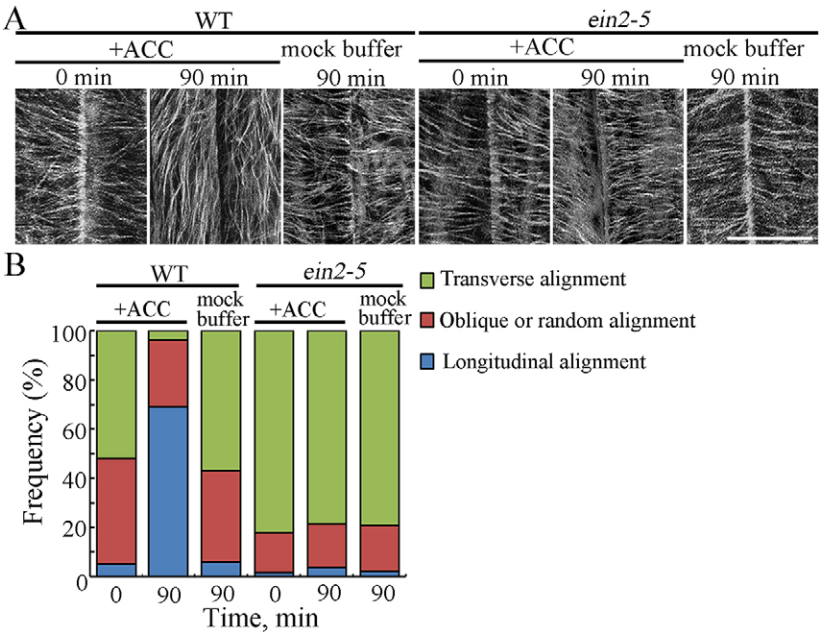

Fig. 1. Ethylene signaling regulates orientation of cortical microtubules in etiolated hypocotyl epidermal cells. (A) Etiolated hypocotyl epidermal cells from wild-type (WT) and *ein2-5* mutants with a YFP–tubulin background were treated with or without ACC for 0 and 90 min after growth for 96 h, and 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.

RESULTS

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

Although treatment with exogenous ethylene or ACC 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, and ACC was applied.

Confocal microscopy analysis 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. Multiple physiological processes are regulated by cross-talk between the responses to ethylene and auxin (Muday et al., 2012; Bours et al., 2015). We found that 1-naphthalene acetic acid (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 had 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 with 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 the 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 (Fig. 3A, B). After treatment with 10 µM oryzalin for 3 min, more microtubules were observed in the wild-type cells that had been pretreated with ACC for 90 min than those that had not. This effect was even more obvious when the duration of oryzalin treatment was increased to 8 min. However, cortical microtubules in *ein2-5* cells exhibited similar sensitivity to oryzalin treatment when pretreated or not with ACC for 90 min (Fig. 3A,B), suggesting that the ethylene signaling pathway leads to increased microtubule stability, 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

Journal of Cell Science (2016) 129, 2043-2051 doi:10.1242/jcs.184408

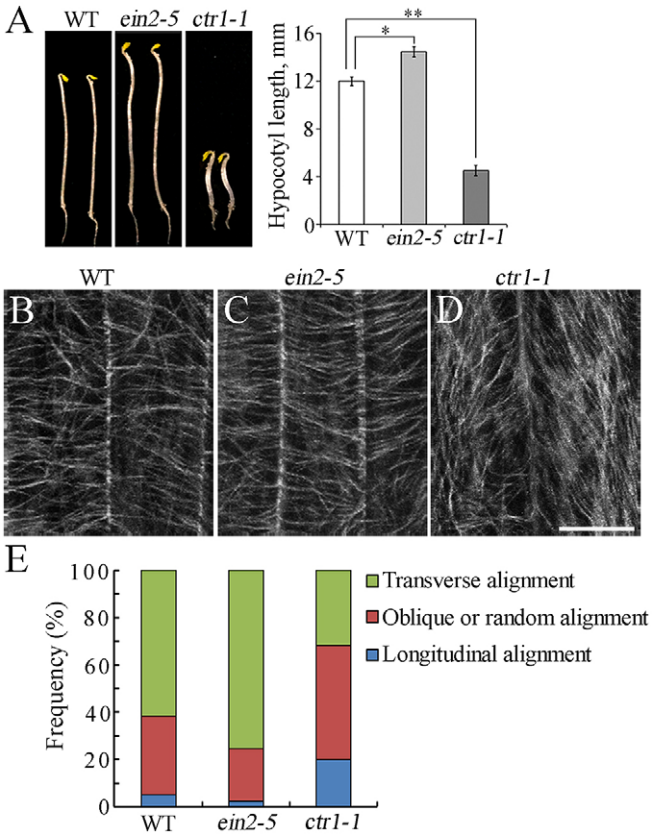

Fig. 2. Cortical microtubule orientation was altered in etiolated hypocotyl epidermal cells from the *ctr1-1* mutant. (A) Wild-type (Columbia ecotype; WT), *ein2-5* and *ctr1-1* mutant seedlings were grown on MS medium in the dark for 5 days. *Ein2-5* seedlings showed longer etiolated hypocotyls, whereas *ctr1-1* seedlings exhibited much shorter etiolated hypocotyls compared with the wild type (data passed Shapiro-Wilk normality tests, see Table S1 for *P*-values). Significance was determined using a Student's two-tailed *t*-test (see Table S2 for exact *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±s.d. (B–D) Cortical microtubules in epidermal cells from the upper region of etiolated hypocotyls from wild-type, *ein2-5* and *ctr1-1* seedlings with a YFP–tubulin background were observed with confocal microscopy after growth in the dark for 4 days. (E) Frequency of different microtubule orientation patterns in etiolated hypocotyl epidermal cells from wild-type, *ein2-5* and *ctr1-1* seedlings (*n*>160 cells from over 45 seedlings for each sample). Scale bar: 20 µm.

ethylene response mutant *ctr1-1*. Many microtubules were disrupted in wild-type epidermal cells after treatment with 5 µM oryzalin for 5 min, whereas 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. 3C,D), indicating that cortical microtubules are more stable in *ctr1-1* compared to those in 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 could be prone to stabilization as a result of ACC-regulated microtubule orientation in etiolated hypocotyl cells. To evaluate the characteristics of individual microtubules within 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 that had been 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 that had not been exposed to ACC (Table 1). In contrast, microtubule dynamics from oblique and longitudinal arrays were significantly altered following ACC treatment in comparison to those following treatment 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}). The 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 that had been treated with ACC. Thus, cortical microtubules from oblique and longitudinal arrays are prone to growth and stabilization, whereas 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 regulates 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 treatment with ACC. 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 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 that had been 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 treatment with ACC, 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 has shown that the microtubule-associated protein WDL5 participates in ethylene-signaling-mediated etiolated hypocotyl cell elongation and that ethylene-regulated cortical microtubule reorientation is 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 that in wild-type cells after treatment with ACC for 50 min (Fig. 4F–I). The effect of ethylene on microtubule bundling was further confirmed using super-resolution structured illumination microscopy (SIM) (Fig. S3). In addition to skewness, the Fibriltool ImageJ plugin was used to quantify the orientation (average orientation of microtubules in the cell) and anisotropy (whether microtubules are well ordered, where

Journal of Cell Science (2016) 129, 2043-2051 doi:10.1242/jcs.184408

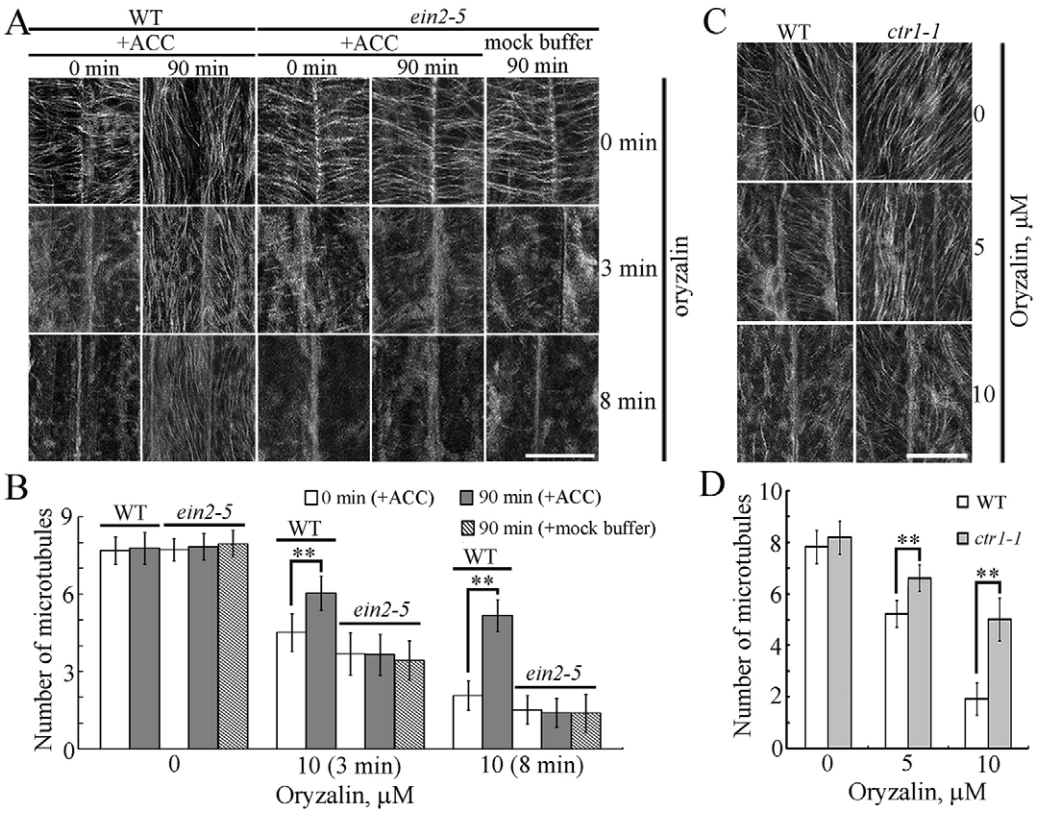

Fig. 3. Ethylene signaling regulates the stability of cortical microtubules in ethylene-mediated microtubule reorientation. (A) Cortical microtubules were observed in wild-type (WT) and *ein2-5* etiolated hypocotyl epidermal cells untreated (0 min) or pretreated with ACC for 90 min, or with mock buffer 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 ImageJ 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 (or not) with 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 ImageJ software (n>50 cells from each sample). Data passed Shapiro-Wilk normality tests (see Table S1 for P-values). Significance was determined using a Student's two-tailed t-test (see Table S2 for exact P-values); **P<0.01. Vertical scale represents the number of cortical microtubules across a fixed line (~10 µm) that was vertical to the orientation of the majority of cortical microtubules in the cell. Error bars represent the mean±s.d. Scale bars: 20 µm.

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 treatment with ACC but had no order after 30 min of treatment with ACC. The cortical microtubules regained order in the longitudinal orientation after treatment with ACC for 50 min, which was more pronounced after 90 min in wild-type cells, but not in *wdl5-1* cells, in which cortical microtubules progressed 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 divided by total bundling events) and bundle growth lifetimes were evaluated in the context of ethylene-regulated orientation.

Microtubule-bundling events in 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 they were exposed to ACC for 50 min (Fig. 5A–C). This result is consistent with data showing that microtubules from transverse arrays are prone to depolymerization and that microtubules from oblique or 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 might 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 that had been treated with mock buffer, indicating that bundle structures become unstable in transverse arrays in response to ACC (Fig. 5D; Movies 1 and 2). Unlike the effects of ACC treatment on bundling events, bundle growth of transverse arrays was less sensitive to treatment with ACC in *wdl5-1* cells (Fig. 5D; Movies 3 and 4), which is consistent with the previous observation that more transverse arrays are found in *wdl5-1* cells in the presence of ACC than in wild-type cells under the same conditions (Sun et al., 2015).

Journal of Cell Science (2016) 129, 2043-2051 doi:10.1242/jcs.184408

Table 1. Microtubule dynamic parameters in epidermal cells from wild-type etiolated hypocotyls treated with or without ACC

	Transverse		Oblique or longitudinal	
Dynamic parameters	+mock buffer	+ACC	+mock buffer	+ACC
Growth rate (μm/min)	7.41±1.38	7.60±1.01	7.26±1.26	8.32±1.10**
Shrinkage rate (μm/min)	13.12±3.67	13.03±3.09	15.59±5.96	12.45±5.41*
Catastrophe (events/s)	0.022	0.038*	0.038	0.012**
Rescue (events/s)	0.031	0.020*	0.022	0.017
Time in growth phase (%)	72.1%	66.8%	56.4%	79.7%
Time in pause phase (%)	16.2%	17.6%	16.2%	11.2%
Time in shrinkage phase (%)	11.7%	15.6%	27.4%	9.1%

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–tubulin background) etiolated hypocotyls treated with or without ACC. Data passed Shapiro-Wilk normality tests (see Table S1 for *P*-values). Significance was determined using Student's two-tailed *t*-test (see Table S2 for exact *P*-values); **P*<0.05, ***P*<0.01. Values are expressed as the means (±s.d. for growth rate and shrinkage rates).

Bundle growth lifetimes from oblique and longitudinal arrays were dramatically increased in ACC-treated wild-type cells compared to cells that had been treated with mock buffer (Fig. 5D). This explains the observation that the number of microtubule bundles was increased in the presence of ACC. However, the effects of ACC on bundle growth lifetimes were obviously more 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 from 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 could 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 to be 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 signaling participates in

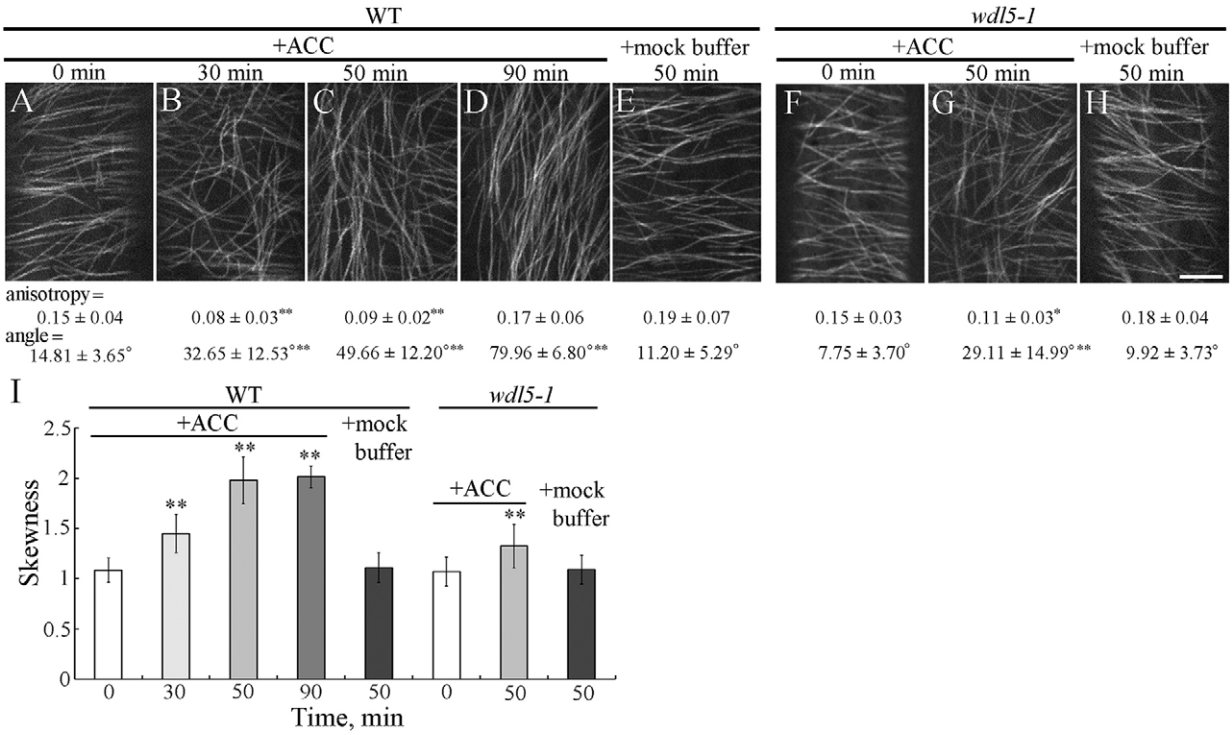

Fig. 4. Ethylene increases cortical microtubule bundling in etiolated hypocotyl cells. (A–E) Cortical microtubules were observed in wild-type etiolated hypocotyl epidermal cells that had been treated with ACC for 0, 30, 50 and 90 min or mock buffer for 50 min. (F–H) Cortical microtubules were observed in *wdl5-1* etiolated hypocotyl epidermal cells that had been treated (or not) with ACC for 50 min, or mock buffer for 50 min. The FibriTool plugin was used to quantify the anisotropy and orientation of cortical microtubules (*n*>45 cells from 15 seedlings for each sample). In A–H, values beneath the figures are mean±s.d. (I) Quantitative analysis of microtubule bundling (skewness) in wild-type and *wdl5-1* cells that had been treated with ACC compared to cells that had not been treated with ACC (*n*>15 cells were quantified for each analysis). Data passed Shapiro-Wilk normality tests (see Table S1 for *P*-values). Significance was determined using a Student's two-tailed *t*-test (see Table S2 for exact *P*-values); **P*<0.05, ***P*<0.01. Error bars represent the mean±s.d. Scale bar: 20 μm.

Journal of Cell Science (2016) 129, 2043-2051 doi:10.1242/jcs.184408

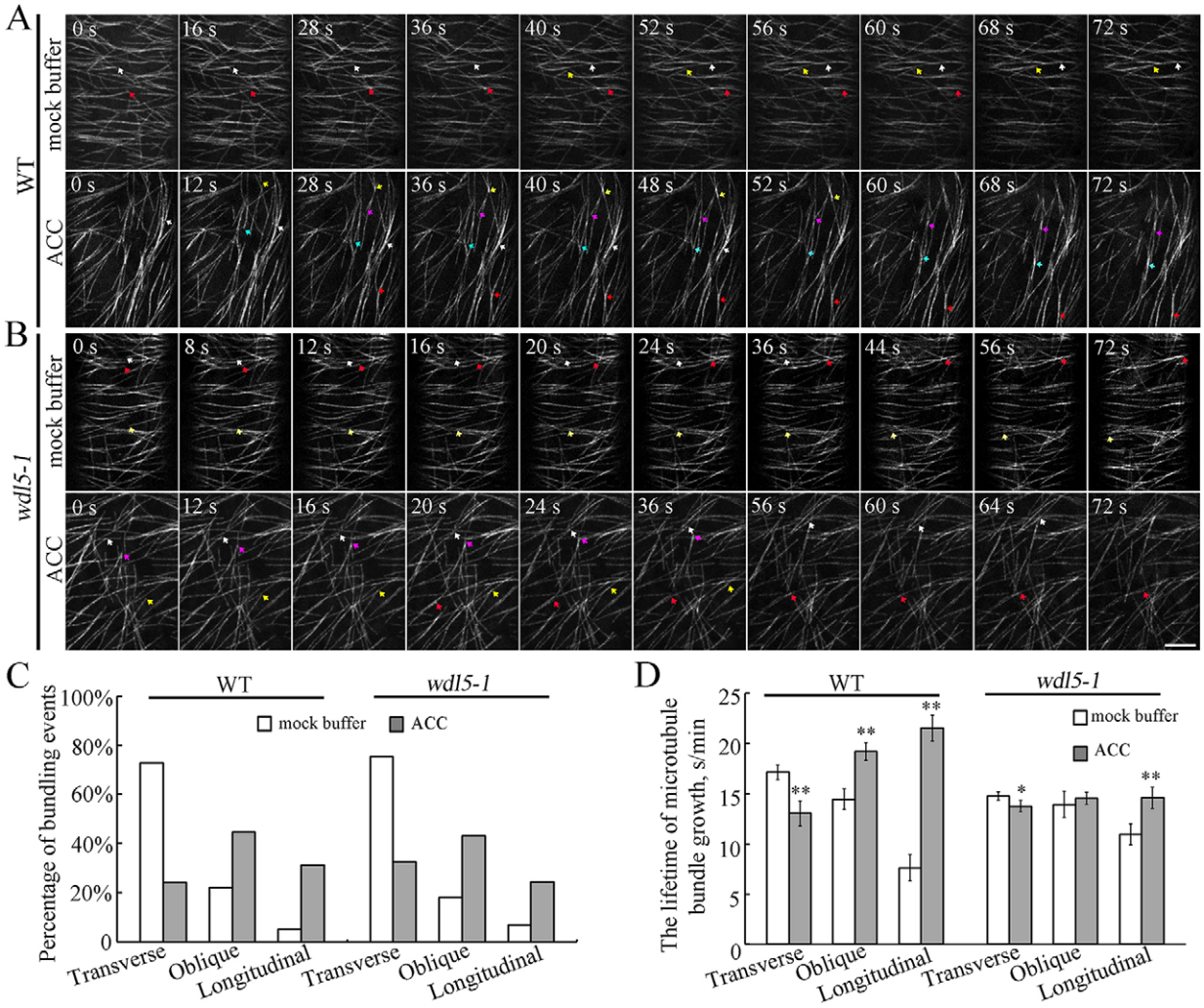

Fig. 5. Ethylene increases microtubule-bundling frequency in etiolated hypocotyl cells. (A) Time-lapse images of cortical microtubules in wild-type (WT) etiolated hypocotyl epidermal cells that had been treated with ACC or mock buffer for 50 min. See Movies 1 and 2 for the entire series. (B) Time-lapse images of cortical microtubules in *wdl5-1* etiolated hypocotyl epidermal cells that had been treated with ACC or mock buffer for 50 min. See Movies 3 and 4 for the entire series. Arrows indicate microtubule-bundling events. Each colored arrow represents a bundling event. (C) Quantification of the frequency of microtubule-bundling events, using ImageJ software, from transverse, oblique and longitudinal arrays in etiolated hypocotyl epidermal cells from the wild type treated with mock buffer (n=339) or ACC (n=407), and *wdl5-1* treated with mock buffer (n=327) or ACC (n=414) for 50 min. (D) Quantification of microtubule bundle growth lifetimes, using ImageJ software, from transverse, oblique and longitudinal arrays in etiolated hypocotyl epidermal cells from the wild-type and *wdl5-1* plants with or without ACC treatment for 50 min (n>20 cells from 10 seedlings for each sample). Data passed Shapiro-Wilk normality tests (see Table S1 for P-values). Significance was determined using a Student's two-tailed t-test (see Table S2 for exact P-values); *P<0.05, **P<0.01. Error bars represent the mean± s.d. Scale bar: 20 µm.

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 is 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 and Arteca, 2008). Cortical microtubule orientation is also regulated by other hormones. For example, exogenous applied gibberellic acids 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 might 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, results 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 brassinosteroid and ethylene signaling pathways (Wang et al., 2012;

Journal of Cell Science (2016) 129, 2043-2051 doi:10.1242/jcs.184408

Sun et al., 2015). Thus, modulation of microtubule orientation through upstream signaling pathways is likely to occur through direct targeting and regulation of the expression of MAP genes.

A previous study has shown that brassinosteroid signaling is involved in the 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 is required for hormone-induced cell elongation, whereas reorientation from transverse to oblique or longitudinal arrays might 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 in order 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 data 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 hinders brassinosteroid-regulated microtubule reorientation in etiolated hypocotyl cells (Wang et al., 2012).

However, stable microtubule arrays of various orientations in the same cell raise an important question regarding regulation of microtubule dynamics when new arrays are being formed. A previous study has shown that the gene transcript levels of the γ-tubulin complex and katanin are transiently increased by treatment with ACC, indicating that these components play a role in ethylene-regulated microtubule reorientation (Soga et al., 2010a). In the present study, we considered underlying mechanisms from a point of view different from that taken by 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 upon 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 has been 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 are partially hindered in cells from *WDL3* RNA interference (RNAi) seedlings (Liu et al., 2013). In addition, the present study might 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 might play a role in the regulation of microtubule orientation. A decreased number of bundling events and less-stable bundle structures provide explanations for the previous 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 during cell wall inhibition of cell growth (Tian et al., 2004; Bashline et al., 2014). We hypothesize that formation of less-stable bundling structures in oblique arrays are important for maintaining oblique arrays before reorientation 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 Murashige–Skoog (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 days and then transferred to the dark for 5 days. 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* plants with a *35S*:Tubulin5A–YFP background that had been 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 3 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.

ACC treatment

Four-day-old etiolated hypocotyls from wild-type, *ein2-5*, *ctr1-1* and *wdl5-1* plants with a *35S*:Tubulin5A–YFP background that had been grown on MS medium were used. Seedlings were treated with ACC at a concentration of 100 µM for 0, 30, 50 and 90 min.

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 ImageJ 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.

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 the angles (degrees) using ImageJ software and calculated bundling events (a growing plus end of an individual microtubule incorporated 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 incorporated 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 wild-type and *wdl5-1* plants with a *35S*:Tubulin5A–YFP background that had been grown on MS medium were used. All samples were examined with T1-E+N-SIM+A1 type super-resolution SIM. 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 pixels, 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 wild-type and *ein2-5* plants with a *35S*:Tubulin5A–YFP background that had been 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 wild-type plants with a *35S*:Tubulin5A–YFP background that had been 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

ImageJ 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 measurements were performed for each cell, and at least 30 cells from each treatment condition were used. Values were recorded, and significance was analyzed using the paired Student's *t*-test.

Acknowledgements

The authors thank Dr Shuhua Yang (China Agricultural University) for generously providing the ethylene-related *Arabidopsis* mutant seeds.

Competing interests

The authors declare no competing or financial interests.

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.

Funding

This research was supported by grants from the National Basic Research Program of China (from the Ministry of Science and Technology of the People's Republic Of China) [grant number 2012CB114200 to T.M.]; the National Natural Science Foundation of China [grant numbers 31471272 and 31222007 to T.M.]; and Program for New Century Excellent Talents in University (from the Ministry Of Education Of The People's Republic Of China) [grant numbers NCET-12-0523 and 2016QC103 to T.M.].

Supplementary information

Supplementary information available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.184408/-/DC1

References

Alonso, J. M., Hirayama, T., Roman, G., Nourizadeh, S. and Ecker, J. R. (1999). EIN2, a bifunctional transducer of ethylene and stress responses in *Arabidopsis*. *Science* **284**, 2148-2152.

Arteca, R. N. and Arteca, J. M. (2008). Effects of brassinosteroid, auxin, and cytokinin on ethylene production in *Arabidopsis thaliana* plants. *J. Exp. Bot.* **59**, 3019-3026.

Bashline, L., Lei, L., Li, S. and Gu, Y. (2014). Cell wall, cytoskeleton, and cell expansion in higher plants. *Mol. Plant* **7**, 586-600.

Bleecker, A. B., Estelle, M. A., Somerville, C. and Kende, H. (1988). Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. *Science* **241**, 1086-1089.

Boudaoud, A., Burian, A., Borowska-Wykręt, D., Uyttewaal, M., Wrzalik, R., Kwiatkowska, D. and Hamant, O. (2014). FibrilTool, an ImageJ plug-in to quantify fibrillar structures in raw microscopy images. *Nat. Protoc.* **9**, 457-463.

Bours, R., Kohlen, W., Bouwmeester, H. J. and van der Krol, A. (2015). Thermoperiodic control of hypocotyl elongation depends on auxin-induced ethylene signaling that controls downstream PHYTOCHROME INTERACTING FACTOR3 activity. *Plant Physiol.* **167**, 517-530.

Bratman, S. V. and Chang, F. (2008). Mechanisms for maintaining microtubule bundles. *Trends Cell Biol.* **18**, 580-586.

Cao, L., Wang, L., Zheng, M., Cao, H., Ding, L., Zhang, X. and Fu, Y. (2013). *Arabidopsis* AUGMIN subunit8 is a microtubule plus-end binding protein that promotes microtubule reorientation in hypocotyls. *Plant Cell* **25**, 2187-2201.

Chen, X., Grandont, L., Li, H., Hauschild, R., Paque, S., Abuzeineh, A., Rakusová, H., Benkova, E., Perrot-Rechenmann, C. and Friml, J. (2014). Inhibition of cell expansion by rapid ABP1-mediated auxin effect on microtubules. *Nature* **516**, 90-93.

Crowell, E. F., Timpano, H., Desprez, T., Franssen-Verheijen, T., Emons, A.-M., Höfte, H. and Vernhettes, S. (2011). Differential regulation of cellulose orientation at the inner and outer face of epidermal cells in the *Arabidopsis* hypocotyls. *Plant Cell* **23**, 2592-2605.

Ecker, J. R. (1995). The ethylene signal transduction pathway in plants. *Science* **268**, 667-675.

Ehrhardt, D. W. and Shaw, S. L. (2006). Microtubule dynamics and organization in the plant cortical array. *Annu. Rev. Plant Biol.* **57**, 859-875.

Higaki, T., Kutsuna, N., Sano, T., Kondo, N. and Hasezawa, S. (2010). Quantification and cluster analysis of actin cytoskeletal structures in plant cells: role of actin bundling in stomatal movement during diurnal cycles in *Arabidopsis* guard cells. *Plant J.* **61**, 156-165.

Kieber, J. J., Rothenberg, M., Roman, G., Feldmann, K. A. and Ecker, J. R. (1993). CTR1, a negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a member of the raf family of protein kinases. *Cell* **72**, 427-441.

Kirik, A., Ehrhardt, D. and Kirik, V. (2012). *TONNEAU2/FASS* regulates the geometry of microtubule nucleation and cortical array organization in interphase *Arabidopsis* cells. *Plant Cell* **24**, 1158-1170.

Le, J., Vandenbussche, F., De Cnodder, T., Van Der Straeten, D. and Verbelen, J.-P. (2005). Cell elongation and microtubule behavior in the *Arabidopsis* hypocotyl: responses to ethylene and auxin. *J. Plant Growth Regul.* **24**, 166-178.

Li, J., Henty-Ridilla, J. L., Huang, S., Wang, X., Blanchoin, L. and Staiger, C. J. (2012). Capping protein modulates the dynamic behavior of actin filaments in response to phosphatidic acid in *Arabidopsis*. *Plant Cell* **24**, 3742-3754.

Lindeboom, J. J., Nakamura, M., Hibbel, A., Shundyak, K., Gutierrez, R., Ketelaar, T., Emons, A. M. C., Mulder, B. M., Kirik, V. and Ehrhardt, D. W. (2013). A mechanism for reorientation of cortical microtubule arrays driven by microtubule severing. *Science* **342**, 1245533.

Liu, X., Qin, T., Ma, Q., Sun, J., Liu, Z., Yuan, M. and Mao, T. (2013). Light-regulated hypocotyl elongation involves proteasome-dependent degradation of the microtubule regulatory protein WDL3 in *Arabidopsis*. *Plant Cell* **25**, 1740-1755.

Muday, G. K., Rahman, A. and Binder, B. M. (2012). Auxin and ethylene: collaborators or competitors? *Trends Plant Sci.* **17**, 181-195.

Murata, T., Sonobe, S., Baskin, T. I., Hyodo, S., Hasezawa, S., Nagata, T., Horio, T. and Hasebe, M. (2005). Microtubule-dependent microtubule nucleation based on recruitment of gamma-tubulin in higher plants. *Nat. Cell Biol.* **7**, 961-968.

Polko, J. K., Van Zanten, M., van Rooij, J. A., Marée, A. F. M., Voesenek, L. A. C. J., Peeters, A. J. M. and Pierik, R. (2012). Ethylene-induced differential petiole growth in *Arabidopsis thaliana* involves local microtubule reorientation and cell expansion. *New Phytol.* **193**, 339-348.

Sambade, A., Pratap, A., Buschmann, H., Morris, R. J. and Lloyd, C. (2012). The influence of light on microtubule dynamics and alignment in the *Arabidopsis* hypocotyl. *Plant Cell* **24**, 192-201.

Shibaoka, H. (1993). Regulation by gibberellins of the orientation of cortical microtubules in plant cells. *Aust. J. Plant Physiol.* **20**, 461-470.

Soga, K., Yamaguchi, A., Kotake, T., Wakabayashi, K. and Hoson, T. (2010a). 1-aminocyclopropane-1-carboxylic acid (ACC)-induced reorientation of cortical microtubules is accompanied by a transient increase in the transcript levels of gamma-tubulin complex and katanin genes in azuki bean epicotyls. *J. Plant Physiol.* **167**, 1165-1171.

Soga, K., Yamaguchi, A., Kotake, T., Wakabayashi, K. and Hoson, T. (2010b). Transient increase in the levels of γ-tubulin complex and katanin are responsible for reorientation by ethylene and hypergravity of cortical microtubules. *Plant Signal Behav.* **5**, 1480-1482.

Steen, D. A. and Chadwick, A. V. (1981). Ethylene effects in Pea stem tissue: EVIDENCE OF MICROTUBULE MEDIATION. *Plant Physiol.* **67**, 460-466.

Sun, J., Ma, Q. and Mao, T. (2015). Ethylene regulates the *Arabidopsis* microtubule-associated protein WAVE-DAMPENED2-LIKE5 in etiolated hypocotyl elongation. *Plant Physiol.* **169**, 325-337.

Tian, G.-W., Smith, D., Glück, S. and Baskin, T. I. (2004). Higher plant cortical microtubule array analyzed in vitro in the presence of the cell wall. *Cell Motil. Cytoskeleton* **57**, 26-36.

Vandenbussche, F., Verbelen, J.-P. and Van Der Straeten, D. (2005). Of light and length: regulation of hypocotyl growth in *Arabidopsis*. *Bioessays* **27**, 275-284.

Vineyard, L., Elliott, A., Dhingra, S., Lucas, J. R. and Shaw, S. L. (2013). Progressive transverse microtubule array organization in hormone-induced *Arabidopsis* hypocotyl cells. *Plant Cell* **25**, 662-676.

Wang, X., Zhang, J., Yuan, M., Ehrhardt, D. W., Wang, Z. and Mao, T. (2012). *Arabidopsis* MICROTUBULE DESTABILIZING PROTEIN40 is involved in brassinosteroid regulation of hypocotyl elongation. *Plant Cell* **24**, 4012-4025.

Zhang, Q., Fishel, E., Bertroche, T. and Dixit, R. (2013). Microtubule severing at crossover sites by katanin generates ordered cortical microtubule arrays in *Arabidopsis*. *Curr. Biol.* **23**, 2191-2195.