Research Article

Ethylene-induced microtubule reorientation is essential for fast inhibition of root elongation in Arabidopsis

Running title: Microtubule reorientation in root ethylene response

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Edited by: Jia Li, Lanzhou University, China

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1111/jipb.12666]

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Received: February 8, 2018; Accepted: May 9, 2018
Abstract
Microtubule reorientation is a long-standing observation that has been implicated in regulating the inhibitory effect of ethylene on axial elongation of plant cells. However, the signaling mechanism underlying ethylene-induced microtubule reorientation has remained elusive. Here, we reveal, by live confocal imaging and kinetic root elongation assays, that the time courses of ethylene-induced microtubule reorientation and root elongation inhibition are highly correlated, and that microtubule reorientation is required for the full responsiveness of root elongation to ethylene treatment. Our genetic analysis demonstrated that the effect of ethylene on microtubule orientation and root elongation is mainly transduced through the canonical linear ethylene signaling pathway. By employing pharmacological and genetic analyses, we demonstrate further that the TIR1/AFBs-Aux/IAAs-ARFs auxin signaling pathway, but not the ABP1-ROP6-RIC1 auxin signaling branch, is essential for ethylene-induced microtubule reorientation and root elongation inhibition. Together, these findings offer evidence for the functional significance and elucidate the signaling mechanism for ethylene-induced microtubule reorientation in fast root elongation inhibition in Arabidopsis.

Keywords: auxin; ethylene; microtubule reorientation; root elongation
INTRODUCTION
Exposure of plant tissue to ethylene or its biosynthesis precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), causes shortening and swelling of the treated tissue. This effect of ethylene had historically been attributed to an induction of transverse-to-longitudinal reorientation of cellulose microfibrils in the cell wall of these fast elongating organs (Apelbaum and Burg 1971; Eisinger and Burg 1972; Lang et al. 1982). Subsequent to the discovery of microtubules, a coalignment hypothesis was proposed that microtubules might provide directional information for the deposition of cellulose microfibrils (Ledbetter and Porter 1963).

Based on this notion, it was speculated that the influence of several plant hormones, including ethylene, on the directional cell expansion might be due to a putative induction of microtubule reorientation (Steen and Chadwick 1981; Lang et al. 1982). By comparing the orientation of microtubules and cellulose microfibrils in stem parenchyma cells of pea (Pisum sativum), with or without ethylene treatment, Steen and Chadwick (1981) determined that ethylene treatment indeed induced the reorientation of both microtubules and newly-deposited microfibrils from predominantly transverse to longitudinal. Similar effects of ethylene treatment on microtubule orientation were also observed in other plant species, including Arabidopsis (Roberts et al. 1985; Le et al. 2004; Le et al. 2005), suggesting a conserved mechanism of action.

Although the first observation of ethylene-induced microtubule reorientation was made almost 40 years ago, little progress has been made on deciphering the underlying molecular mechanism. This might be due to a limited understanding of the ethylene signaling mechanism during previous studies. In Arabidopsis, genetic methods have established a largely linear pathway for ethylene signaling (reviewed by Merchante et al. 2013). Briefly, perception of ethylene by a group of endoplasmic reticulum (ER)-located receptors activates the ER membrane protein ETHYLENE
INSENSITIVE 2 (EIN2). Upon activation, EIN2 is endo-cleaved and its C-terminal end functions both through repressing EIN3-BINDING F-BOX 1/2 mRNA translation in the cytosol (Li et al. 2015; Merchante et al. 2015) and through activating ETHYLENE INSENSITIVE 3/EIN3-LIKE 1 (EIN3/EIL1) in the nucleus (Ju et al. 2012; Qiao et al. 2012; Wen et al. 2012). EIN3/EIL1 are key transcription factors that are essential for ethylene-responsive gene expression (Guo and Ecker 2003; An et al. 2010). However, very limited information is available concerning the cellular machineries in distinctive ethylene-regulated responses downstream of EIN3/EIL1 (Hu et al. 2017). With regards to ethylene-induced microtubule reorientation, it was recently reported that EIN3 directly regulates gene expression of the microtubule-stabilizing protein WAVE-DAMPENED2-LIKE5 (WDL5), to regulate cortical microtubule organization during ethylene regulation of Arabidopsis etiolated hypocotyl elongation (Sun et al. 2015).

In contrast to etiolated hypocotyl elongation, ethylene regulation of root growth is largely dependent on the presence of auxin (reviewed by Hu et al. 2017). Forward genetic studies of ethylene signaling lead to the isolation of several root-specific ethylene resistant mutants that were later identified as being defective in auxin biosynthesis (Stepanova et al. 2005; Stepanova et al. 2008), transport (Pickett et al. 1990; Chen et al. 1998) or nuclear signaling (Wilson et al. 1990; Nagpal et al. 2000). Together, these findings suggested that the TIR1/AFBs-Aux/IAAs co-receptor-mediated auxin signaling pathway works downstream of ethylene signaling in regulating root growth. However, whether this were also the case for ethylene-induced microtubule reorientation, in the root, has not been tested. Interestingly, it was recently reported that the ABP1-ROP6-RIC1 auxin signaling branch is responsible for auxin-induced microtubule reorientation and fast inhibition of cell expansion, in both hypocotyl and roots (Chen et al. 2014).

In this study, we sought to clarify the molecular mechanism underlying ethylene-regulated root elongation. We first analyzed the dynamic progress of ethylene-induced microtubule reorientation and demonstrated its importance in regulating ethylene-induced fast inhibition of root elongation. We revealed that
functional components of the linear ethylene-signaling pathway, including ETR1, EIN2 and EIN3/EIL1, are essential during this process. Furthermore, we show that the TIR1/AFBs-Aux/IAAs-ARFs auxin signaling pathway, but not the ABP1-ROP6-RIC1 signaling branch, is specifically required for ethylene-induced microtubule reorientation during the fast inhibition of root elongation.

RESULTS

Ethylene induces progressive microtubule reorientation in the root elongation zone

In order to explore the signaling mechanism underlying the long-standing observation of ethylene-induced reorientation of microtubules, we visualized microtubule structure in root epidermal cells of *Arabidopsis* seedlings, by employing a transgenic microtubule marker GFP-MBD (green fluorescent protein fused microtubule binding domain of mammalian microtubule associated protein 4), which has been widely used to monitor microtubule dynamics and reorientation in diverse biological processes (Marc et al. 1998; Hamant et al. 2008). The middle elongation zone, located at approximately 600 to 800 μm shootward from root tip and representing the fastest growth region (Le et al. 2004), was imaged and microtubule orientation therein was quantified.

Microtubule reorientation could be visually detected within 15 min of ethylene treatment in GFP-MBD/Col-0, but a significant global reorientation took place from 30 to 60 min after ethylene treatment (Figure 1A), a finding consistent with a previous report using an immuno-fluorescent method (Le et al. 2004). Quantitative measurements revealed a similar progressive reorientation process (Figure 1B). Here, 120 min ethylene treatment was sufficient to induce complete reorientation of microtubules from a predominantly transverse to a longitudinal orientation, and was therefore used for further analysis (Figure 1A, B).

Ethylene-induced microtubule reorientation is required for full responsiveness of root elongation to ethylene treatment
In an attempt to explore the biological significance of microtubule reorientation, after ethylene treatment, we next examined the kinetics of root elongation in Arabidopsis seedlings. Interestingly, we established that the time course of ethylene-induced root elongation inhibition closely resembled that of microtubule reorientation, with a significant drop in growth rate within 15 min and a ~60% growth inhibition after 60 min ethylene treatment (Figure 2A). However, a bending away from the growth surface was observed in all ethylene-treated wild-type roots, which might be caused by swelling of the root tip after ethylene treatment. This bending resulted in growing out of the focal plane and made it impossible to measure the maximal growth inhibition. Nevertheless, since the time courses for microtubule reorientation and root elongation inhibition were quite comparable, an intrinsic correlation between these two processes was, therefore, inferred.

The requirement of microtubule reorientation for ethylene-induced root elongation inhibition, was next investigated either by pharmacologically disrupting microtubule stability, using the microtubule depolymerizing agent oryzalin, or by analyzing katanin (lue1) and sprial1-2 mutants that exhibit compromised reorientation abilities (Bouquin et al. 2003; Nakajima et al. 2004). After a 12 h exposure on plates in which the medium contained 0.1 μM oryzalin, typical fragmented decoration of microtubule structures was observed in GFP-MBD/Col-0 roots, confirming the depolymerizing effect of oryzalin (Figure 2B). Further, severer depletion of microtubule structure was observed when 0.2 μM oryzalin was applied (Figure 2B). Interestingly, the ability of ethylene to induce transverse-to-longitudinal reorientation of microtubules was indeed impaired by the application of oryzalin (Figure 2B, C), indicating the requirement of proper stability of microtubules for the normal reorientation process. Meanwhile, through root elongation kinetics we showed that application of oryzalin also attenuated the responsiveness of root elongation to ethylene treatment, in a dose-dependent manner (Figure 2A).

In agreement with the effect of oryzalin, we also observed that the inhibitory effect of ethylene treatment, on root elongation, was compromised in two microtubule-related mutants, katanin and sprial1-2 (Figure 2D). Although not
explored here, regarding how KATANIN and SPRIAL1 are being regulated by ethylene in roots, these results collectively suggest the necessary role of proper microtubule reorientation for full root responsiveness to ethylene treatment.

**ETR1, EIN2 and EIN3/EIL1 are essential for ethylene-induced microtubule reorientation and root elongation inhibition**

Previous genetic analyses have established a largely linear ethylene signaling pathway (Merchante et al. 2013). In order to evaluate the contribution of ethylene signaling components in the process of microtubule reorientation, we examined ethylene response of microtubules in corresponding mutants. As expected, microtubule configuration in the ethylene-insensitive receptor mutant, *etr1-1*, did not exhibit an obvious response to ethylene treatment (Figure S1). This indicates that ethylene induces microtubule reorientation through a signal transduction pathway initiated from ethylene perception.

In previous growth kinetic studies of etiolated hypocotyl, an EIN2-dependent but EIN3/EIL1-independent fast growth inhibition by ethylene has been documented (Binder et al. 2004; Men et al. 2012). Likewise, time course results indicated that *ein2-5* was completely insensitive to ethylene treatment, in both microtubule orientation and root elongation (Figure 3A, C, E), while a modest response was observed for both processes in *ein3-1 eil1-1* (Figure 3B, D, F). Nevertheless, wild-type-like complete reorientation of microtubules, into a predominantly longitudinal array, was never observed in the mutant, suggesting a major contribution of EIN3/EIL1 during the ethylene-induced microtubule reorientation (Figure 3B, D, F). Together, these results demonstrate that the effect of ethylene, on microtubule orientation and root elongation, is mainly transduced through the canonical linear ethylene signaling pathway.

**Disruption of auxin biosynthesis and transport abolishes ethylene-induced microtubule reorientation and root elongation inhibition**

Auxin is a well-recognized morphogen that controls root growth and development,
and the inhibitory effect of long-term ethylene treatment on Arabidopsis root elongation is strongly dependent on auxin (Hu et al. 2017). In order to test the necessity of endogenous auxin, during the fast process of ethylene-induced microtubule reorientation, we used the specific chemical inhibitor of TAA1/TARs, l-kynurenine (Kyn), to block auxin biosynthesis in roots (He et al. 2011). Kyn application led to insensitivity of GFP-MBD/Col-0 to ethylene treatment in terms of microtubule configuration (Figure S2A, B). Furthermore, this inhibitor reversed the longitudinal orientation of microtubules in elongation zone epidermal cells of the ethylene over-producing mutant eto1-2 to predominant transverse (Figure S2C, D). These results indicate that endogenous auxin biosynthesis, mediated by TAA1/TARs, is required for ethylene-induced microtubule reorientation.

Polar transport is an essential part for auxin action during plant development. We therefore next tested whether auxin transport is also required for ethylene-induced microtubule reorientation. For this we analyzed the microtubule configuration in the auxin influx carrier mutant aux1-7. In agreement with the requirement of auxin biosynthesis, this mutant did not show any detectable change in microtubule configuration, even after 120 min ethylene treatment (Figures 4A, B, S2E, S2F). Meanwhile, we examined the root elongation kinetics of aux1-7 and established that it was almost ethylene insensitive (Figure 4C), supporting the necessity of AUX1-mediated auxin transport in ethylene-induced microtubule reorientation and fast root elongation inhibition.

The TIR1/AFBs-Aux/IAAs auxin signaling module, but not the ABP1-ROP6-RIC1 auxin signaling branch is required for root ethylene responses

The aforementioned results in this study promoted us to address the question concerning which of the two auxin signaling pathways participates in ethylene-induced microtubule reorientation (Mockaitis and Estelle 2008). We therefore used a specific chemical inhibitor of the TIR1/AFBs-Aux/IAAs signaling module, auxinole (Hayashi et al. 2012), and a dominant negative IAA7 mutant, axr2-1
(Wilson et al. 1990; Nagpal et al. 2000), to examine the necessity for the TIR1/AFBs-Aux/IAAs co-receptor. Complete ethylene insensitivity, in microtubule reorientation and fast root elongation inhibition, was observed in auxinole incubated wild-type seedlings (Figure 5A, C, E), and only marginal responses of microtubule reorientation and root elongation inhibition were observed in axr2-1 (Figure 5B, D, F). These findings collectively indicate an essential role for the TIR1/AFBs-Aux/IAAs signaling module in root ethylene responses. Furthermore, we investigated an arf7 arf19 (nph4-1 arf19-4) double mutant (Wilmoth et al. 2005). This line exhibited reduced ethylene-sensitivity in kinetic analysis of root ethylene response (Figure S3C), and compromised microtubule reorientation after ethylene treatment (Figure S3A, B). Taken together, these results demonstrate the essential role for the TIR1/AFBs-Aux/IAAs-ARF7/19 signaling module, in ethylene-induced microtubule reorientation.

To test the requirement for the ABP1-ROP6-RIC1 auxin signaling branch, for root ethylene responses, we next analyzed microtubule orientation and root elongation in abp1-5 and abp1-TD1 mutants. Wild-type-like microtubule reorientation and fast root elongation inhibition were induced by ethylene treatment in both the point mutant abp1-5 (Figure 6A, E) and the null mutant abp1-TD1 (Figure 6B, F). ROP6 and RIC1 have been recently documented as essential downstream components to transduce the auxin signal from ABP1 to microtubules, in root epidermal cells (Chen et al. 2014). Therefore, we also examined rop6-1 and ric1-1 mutants and observed wild-type-like responses of microtubule reorientation and fast root elongation inhibition in both mutants (Figure 6C, D, G, H). These results indicate that the ABP1-ROP6-RIC1 signaling pathway is not required in root ethylene responses.

**DISCUSSION**

**Biological significance of microtubule reorientation during ethylene-induced inhibition of plant cell elongation**

During the past decades, accumulated evidence had suggested microtubules as being mediators of developmental cues and environmental factors to modulate plant cell
growth (Chan 2012). With the identification of the molecular mechanism of multiple hormone signaling pathways, a mechanistic relationship between hormone signaling, microtubule function and plant cell growth has been the subject of intensive studies (Fu et al. 2005; Fu et al. 2009; Lin et al. 2013; Locascio et al. 2013; Wang et al. 2015). In this study, we used the Arabidopsis root elongation zone as a model system to analyze the gaseous hormone ethylene-triggered microtubule reorientation, its biological significance and the signaling mechanism that underlies this process.

In pea stem tissue, microtubules have been demonstrated to mediate the inhibitory effect of ethylene on cell elongation, because disruption of microtubular structure, either by low temperature or colchicine, could abolish the effect of ethylene (Steen and Chadwick 1981). A contradictory conclusion that microtubule reorientation is a result, rather than the cause or the mediator of ethylene-induced growth inhibition was also proposed, mainly based on the observation that complete microtubule reorientation lagged that of growth inhibition in the Arabidopsis root elongation zone (Le et al. 2004).

However, caution should be taken for accepting the latter conclusion, since microtubule reorientation initiates much earlier before maximal growth inhibition was reached. In this study, we carefully compared time courses of ethylene-induced microtubule reorientation and fast root growth inhibition and showed there was no significant lag between each other. Our data therefore suggest an intrinsic correlation between these two processes. Furthermore, our quantitative measurements of root elongation rate suggest that the retardation of elongation, per se, is not sufficient to induce microtubule reorientation. This became clear as dominant transverse orientation was still observed in root epidermal cells of seedlings treated with low concentrations of oryzalin or auxinole, whilst root elongation rate was significantly reduced (Figures 2A, 5E).

In addition to time course comparisons, artificial manipulation of microtubule orientation, by transgenic strategies, has been demonstrated to be sufficient to alter elongation rate (Li et al. 2011; Liu et al. 2013; Locascio et al. 2013); these findings support the notion that microtubules mediate in growth regulation. Our data strongly
support the hypothesis that proper reorientation of the microtubule array is necessary for full inhibition of root elongation by ethylene. Support of this notion came also from the genetic study that etiolated hypocotyls of the *wdl5* loss-of-function mutant showed compromised ethylene sensitivity (Sun et al. 2015). Nevertheless, compromised root elongation inhibition, by ethylene treatment, is still observed in both the *katanin* and *spirial1* mutants and in oryzalin-treated wild-type seedlings. This suggests that putative cellular processes other than microtubule reorientation, such as biochemical modification of cell wall constituents, might be involved in the root ethylene response.

Accompanied with its function in elongation inhibition, transverse-to-longitudinal reorientation of microtubules may also facilitate the radial expansion of root cells (Chan 2012). During mechanical impedance of root tissue, a substantial amount of ethylene is produced (Yamamoto et al. 2008). The promotion of radial swelling, mediated by longitudinal microtubule orientation, might play an important role in modifying the bending resistance of the root tip and facilitating its exploration of underground resources. Future studies should explore the physiological significance of ethylene-induced microtubule reorientation in plant tissues under natural growth conditions.

**Auxin signaling is essential during ethylene-induced microtubule reorientation and root elongation inhibition**

Our genetic and pharmacological evidence convincingly demonstrated that not only the key components of the linear ethylene signaling pathway, namely ETR1, EIN2 and EIN3/EIL1, but also biosynthesis, transport and signaling of auxin are required for ethylene-induced microtubule reorientation and fast root elongation inhibition. Similar to previous reports on hypocotyls (Binder et al. 2004; Men et al. 2012), minor ethylene responses were found in root tissue in an EIN3/EIL1-independent manner, suggesting that other EIN3-like transcription factors may work downstream of EIN2, or that EIN2 may directly interact with the auxin signaling pathway to transduce ethylene signaling.
Our differential analysis also demonstrates that the ABP1-ROP6-RIC1 signaling branch is not involved in this process, albeit the contribution of auxin is indisputable. This contrasts with recent findings in which the ABP1-ROP6-RIC1 auxin signaling branch was proposed to be responsible for auxin-induced microtubule reorientation and cell expansion inhibition in both hypocotyl and root of Arabidopsis (Chen et al. 2014).

Strikingly, similar dispensability of ABP1 for auxin signaling and plant development has been reported by other investigators, which is mainly based on normal auxin responsiveness and wild-type-like developmental phenotype of the new null abp1 alleles (Gao et al. 2015). In addition, multiple background mutations were reported in the abp1-5 allele (Enders et al. 2015) and this could well account for the auxin-insensitive phenotypes that have been observed by other investigators. Although the impact of such background mutations, on ethylene sensitivity, was not explicitly addressed in our study, we also observed wild-type-like ethylene-induced microtubule reorientation and root elongation inhibition in the abp1-TD1 null allele, suggesting at least ABP1 is not essential for these processes. However, our results pinpointed the essential role for TIR1/AFBs-Aux/IAAs-ARFs signaling during this process.

In canonical auxin signaling, perception of auxin by TIR1/AFBs-Aux/IAAs co-receptors results in degradation of Aux/IAAs transcriptional repressor and consequent de-repression of ARF transcription factors and induction of downstream gene expression and auxin responses (Mockaitis and Estelle 2008). Our results, obtained by the usage of auxinole and the employment of the axr2-1 line, indicate that proper degradation of Aux/IAAs transcriptional repressor is necessary to maintain root ethylene sensitivity. Consistent with a previous report (Li et al. 2006), we also detected residual ethylene responsiveness in arf7 arf19 double mutants, indicating other ARFs may redundantly function with ARF7/19 downstream of TIR1/AFBs-Aux/IAAs co-receptors.

Readout of auxin signaling could be regulated, either indirectly by altering the cellular auxin level, or directly through interacting with, or regulating the auxin
signaling pathway components. In previous studies, long-term ethylene treatment has been shown to increase the auxin response level in the root tip both by inducing auxin biosynthesis and promoting polar auxin transport (Stepanova et al. 2005; Ruzicka et al. 2007; Swarup et al. 2007; Stepanova et al. 2008). Here, we provide further evidence that these mechanisms also operate within the 2 h timeframe of ethylene-induced microtubule reorientation and fast root elongation inhibition.

In addition, the positive feedback regulation of ethylene signaling, by auxin, may partially account for the ethylene-insensitive phenotypes observed here in some auxin-related mutants and during chemical treatments. Indeed, endogenous auxin has been demonstrated to be required for both proper accumulation and transcriptional activity of EIN3 protein (Stepanova et al. 2007; He et al. 2011). Combined with multilevel regulation of auxin accumulation and signaling components, by ethylene signaling, this positive feedback loop would ultimately lead to a robust reaction system for both fast, short-term, and sustained, long-term root ethylene responses. Biological significance and the molecular mechanism by which auxin signaling positively regulates EIN3 protein turnover and transcriptional activity, will be an interesting direction for future research.

MATERIAL AND METHODS

Plant Materials and Chemicals
Col-0 and WS-0 ecotype were used in this study. Transgenic marker lines and mutants used were: 35S::GFP-MBD/Col-0 (Hamant et al. 2008), katanin (lue1) (Bouquin et al. 2003), sprial1-2 (Nakajima et al. 2004), etr1-1 (Hua and Meyerowitz, 1998), ein2-5 (Alonso et al. 1999), ein3-1 eil1-1 (Alonso et al. 2003), eto1-2 (Wang et al. 2004), aux1-7 (Rahman et al. 2001), axr2-1 (Nagpal et al. 2000), arf7 arf19 (nph4-1 arf19-4) (Wilmuth et al. 2005), abp1-5 (Enders et al. 2015), abp1-TD (Gao et al. 2015), rop6-1 and ric1-1 (Fu et al. 2009). All of the GFP-MBD marker lines with ethylene- or auxin-related mutant background were generated by genetic crossing between 35S::GFP-MBD/Col-0 and the corresponding mutants, and homozygous F3 or F4 generations were used. GFP-TUA5/rop6-1 and GFP-TUA5/ric1-1 were described
previously (Fu et al. 2009). Auxin signaling inhibitor, auxinole, was kindly provided by Ken-Ichiro Hayashi (Hayashi et al. 2012). All other chemicals were purchased from Sigma-Aldrich. Stock solutions were prepared at indicated concentration and filter-sterilized using 22-nm sterilized filters: Kyn (50 mM in DMSO), auxinole (200 mM in DMSO), IAA (100 mM in ethanol) and oryzalin (10 mM in DMSO). All chemicals were directly supplemented in growth medium before plate preparation.

**Plant Growth Conditions**
Sterilized seeds were cold-treated at 4°C for 2 d before being sown on growth medium, which contained full-strength Murashige and Skoog salts and 15 g L\(^{-1}\) sucrose and solidified with 5 g L\(^{-1}\) phytagel (Sigma, product No. P8169), pH 5.8. Seeds were grown on the surface of medium in vertically set 9 cm square plates that were sealed with Parafilm and incubated at 22°C with a 16 h light/8 h dark cycle for 5 d before imaging.

**Root elongation kinetic assays**
Root elongation kinetic assays were carried out by a commercial seedling phenotyping platform (DYNAPLANT) with high throughput (up to 300 roots/5 min) and a spatial resolution of 1.2 μm/pixel (http://www.yph-bio.com/DynaPlant.asp). 4.5-d-old seedlings were transferred carefully onto new plates of growth medium with respective chemicals 12 h prior to analysis to saturate incubation. Plates were docked onto the platform and subjected to 5-min interval imaging. All images were automatically analyzed using DYNAPLANT software.

**Confocal Imaging and Quantification**
Before confocal imaging, 5-d-old seedlings were incubated in ethylene-free air or mixed air containing 10 μl L\(^{-1}\)/(10 ppm) ethylene for the indicated periods. Sampled seedling were quickly mounted on cover slides with liquid growth medium and imaged within 5 min.
For in vivo microtubule observation of GFP-MBD and tubulin-GFP expressing seedlings, a ZEISS 710 confocal microscopy was used with a 40× water-immersion, 1.2 numerical aperture, C-Apochromat objective. GFP was excited with the 488-nm argon laser and detected at 493-598 nm. Serial confocal optical sections were taken at a 1 μm step size. For each Z-stack, imaging was completed within 1 min.

MicroFilament Analyzer software (Jacques et al. 2013) was used to quantify the orientation of cortical microtubules. Microtubule orientation was measured as the angle between a cortical microtubule and the longitudinal axis of an epidermal cell. Transverse oriented microtubules are assigned as 90 degree, whereas longitudinal are 0 degree. More than 2000 orientation reads were automatically extracted by the software from images of 10-12 epidermal cells, in 3 independent roots at each time point. Results for each genotype or treatment condition were reproduced at least twice.

ACKNOWLEDGMENTS
We thank Dr. Ken-Ichiro Hayashi for kindly providing the auxinole, Dr. Mark Estelle for the axr2-1 mutant lines, Dr. Elliot M. Meyerowitz for the 35S::GFP-MBD/Col-0 line and Dr. Yunde Zhao for the abp1-TD1 mutant line. Thanks also to Dr. Peter Pimpl and Dr. Wenyang Li for help manuscript preparation. This work was supported by grants from the National Natural Science Foundation of China (31700239) to Y. W. and (91740203) to H.G., and China Postdoctoral Science Foundation (2012M510263 and 2014M560845) to Y. W..

AUTHOR CONTRIBUTIONS
Y. W. and H.G. designed the experiments. Y. W. and Y. J. performed experiments and analyzed the data. Y. F. assisted in experiments and discussed the results. Y. W. and H.G. wrote the manuscript.
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SUPPORTING INFORMATION

Figure S1. Ethylene receptor ETR1 is essential for ethylene-induced microtubule reorientation in Arabidopsis root elongation zone

(A–D) Microtubule configuration in air control (A and C) or 2 hour ethylene treated (B and D) samples of GFP-MBD/Col-0 (A and B) or GFP-MBD/etr1-1 (C and D). Scale bar, 20 μm.

Figure S2. Disruption of endogenous auxin biosynthesis and transport confers ethylene insensitivity in microtubule reorientation in root elongation zone

(A and D) Microtubule configuration in air control samples of 1.5 μM Kyn supplemented GFP-MBD/Col-0 (A) and GFP-MBD/eto1-2 (D). (B) Microtubule configuration in 2 hour ethylene treated samples of GFP-MBD/Col-0 supplemented with 1.5 μM Kyn. (C) Microtubule configuration in air control samples of GFP-MBD/eto1-2 without Kyn supplement. (E and F) Microtubule configuration
in air control samples of and GFP-MBD/aux1-7 (E) and 2 hour ethylene treated samples of GFP-MBD/aux1-7 (F). Scale bar, 20 μm.

**Figure S3.** Double mutation of ARF7 and ARF19 impairs ethylene-induced microtubule reorientation and fast root elongation inhibition

(A and B) Microtubule configuration in air control (A) and 2 hour ethylene treated (B) root samples of GFP-MBD/arf7 arf19. Wild-type-like complete reorientation of microtubules into a dominant longitudinal array was not observed in GFP-MBD/arf7 arf19, but mosaic silence of GFP-MBD transgene in GFP-MBD/arf7 arf19 roots prevents further quantification of microtubule orientation. Scale bar, 20 μm. (C) Time course of ethylene-induced root elongation inhibition in arf7 arf19. n=24 for Col-0 control, n=22 for arf7 arf19, Mean ± S.E.M.

**Figure legends**

**Figure 1. Quantification of microtubule orientation in Arabidopsis root epidermal cells**

(A) Microtubule configuration in epidermal cells located in the middle elongation zone of GFP-MBD/Col-0 roots after different periods of ethylene treatment. Inserted schematic on the left illustrates the orientation of the root and the relative location of the imaged cells. Numbers indicate time in min of ethylene treatment. Scale bar, 20 μm. (B) Distribution of microtubule orientation in epidermal cells located in the root middle elongation zone after the indicated periods of ethylene treatment. For each time point in (B) over 2000 orientation reads was pooled from more than 10 epidermal cells in 3 independent roots.

**Figure 2. Effect of microtubule depolymerizing agent oryzalin or mutation of microtubule-related
proteins KATANIN and SPRIAL1 on ethylene-induced microtubule reorientation and fast root elongation inhibition

(A) Time courses of ethylene-induced root elongation inhibition in Col-0 seedlings treated with DMSO control or oryzalin (n=20 for DMSO, 0.1 μM oryzalin and 0.2 μM oryzalin). Mean± S.E.M. (B) Microtubule configuration in middle elongation zone of GFP-MBD/Col-0 roots after different periods of ethylene treatment in DMSO controls or oryzalin application, as indicated above the images. Scale bar, 20 μm. (C) Quantification of microtubule orientation in middle elongation zone of GFP-MBD/Col-0 roots after 2 hour ethylene treatment with DMSO control or 0.1 μM oryzalin application. For each time point in (C) over 2000 orientation reads were pooled from more than 10 epidermal cells in 3 independent roots. Severe depletion of microtubule structure by 0.2 μM Oryzalin application and the consequent patchy fluorescent signal of GFP-MBD in (B) prevent quantitative measurement of microtubule orientation therein. (D) Time courses of ethylene-induced root elongation inhibition in Col-0 control, katanin and spiral1-2 seedlings (n=20 for Col-0 control, katanin, and spiral1-2). Mean± S.E.M.

Figure 3. EIN2 and EIN3/EIL1 are essential for ethylene-induced microtubule reorientation and fast root elongation inhibition

(A and B) Microtubule configuration in epidermal cells in the middle elongation zone of GFP-MBD/ein2-5 (A) and GFP-MBD/ein3-1 eil1-1 (B) roots after different periods of ethylene treatment. Scale bar, 20 μm. (C and D) Distribution of microtubule orientation in epidermal cells in the middle elongation zone of GFP-MBD/ein2-5 (C) and GFP-MBD/ein3-1 eil1-1 (D) roots after different periods of ethylene treatment. For each time point in (C) and (D) over 2000 orientation reads were pooled from more than 10 epidermal cells in 3 independent roots. (E and F) Time courses of ethylene-induced root elongation inhibition in ein2-5 (E, n=20) and ein3-1 eil1-1 (F, n=19) seedlings. Mean± S.E.M.

Figure 4. AUX1 auxin influx carrier is essential for ethylene-induced microtubule reorientation and fast root elongation inhibition

(A) Microtubule configuration in epidermal cells in the middle elongation zone of GFP-MBD/aux1-7 roots after different periods of ethylene treatment. Scale bar, 20 μm. (B) Distribution of microtubule
orientation in epidermal cells in the middle elongation zone of \textit{GFP-MBD/aux1-7} roots after different periods of ethylene treatment. For each time point in (B) over 2000 orientation reads were pooled from more than 10 epidermal cells in 3 independent roots. (C) Time course of ethylene-induced root elongation inhibition in \textit{aux1-7} (n=22) seedlings. Mean ± S.E.M.

Figure 5. TIR1/AFBs-Aux/IAAs auxin signaling module is essential for ethylene-induced microtubule reorientation and root elongation inhibition

(A and B) Microtubule configuration in epidermal cells in the middle elongation zone of \textit{GFP-MBD/Col-0} roots supplemented with auxinole (A) and \textit{GFP-MBD/axr2-1} (B) after different periods of ethylene treatment. Scale Bar, 20 μm. (C and D) Distribution of microtubule orientation in epidermal cells in the middle elongation zone of \textit{GFP-MBD/Col-0} roots supplemented with auxinole (C) and \textit{GFP-MBD/axr2-1} (D) after different periods of ethylene treatment. For each time point in (C) and (D) over 2000 orientation reads were pooled from more than 10 epidermal cells in 3 independent roots. (E and F) Time courses of ethylene-induced root elongation inhibition in \textit{Col-0} seedlings supplemented with auxinole (E, n=22) and \textit{axr2-1} (F, n=24). Mean ± S.E.M.

Figure 6. ABP1-ROP6-RIC1 auxin signaling pathway is not required during ethylene-induced microtubule reorientation and root elongation inhibition

(A–D) Microtubule configuration in epidermal cells in the middle elongation zone of \textit{GFP-MBD/abp1-5} (A), \textit{GFP-MBD/abp1-TD1} (B), \textit{GFP-TUA5/rop6-1} (C) and \textit{GFP-TUA5/ric1-1} (D) seedlings after different periods of ethylene treatment. Scale bar, 20 μm. (E–H) Time course of ethylene-induced root elongation inhibition in \textit{abp1-5} (E, n=22 for \textit{abp1-5}, n=24 for \textit{Col-0}, n=20 for \textit{WS-0}), \textit{abp1-TD1} (F, n=22 for \textit{abp1-TD1}, n=20 for \textit{WS-0}), \textit{rop6-1} (G, n=22 for \textit{rop6-1}, n=20 for \textit{WS-0}) and \textit{ric1-1} (H, n=15 for \textit{ric1-1}, n=20 for \textit{WS-0}) seedlings. Mean ± S.E.M.
Figure 3
Figure. 4
Figure 5
Figure 6