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Research

Ethylene-induced differential petiole growth in *Arabidopsis thaliana* involves local microtubule reorientation and cell expansion

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Summary

- Hyponastic growth is an upward petiole movement induced by plants in response to various external stimuli. It is caused by unequal growth rates between adaxial and abaxial sides of the petiole, which bring rosette leaves to a more vertical position. The volatile hormone ethylene is a key regulator inducing hyponasty in *Arabidopsis thaliana*. Here, we studied whether ethylene-mediated hyponasty occurs through local stimulation of cell expansion and whether this involves the reorientation of cortical microtubules (CMTs).
- To study cell size differences between the two sides of a petiole in ethylene and control conditions, we analyzed epidermal imprints. We studied the involvement of CMT orientation in epidermal cells using the tubulin marker line as well as genetic and pharmacological means of CMT manipulation.
- Our results demonstrate that ethylene induces cell expansion at the abaxial side of the- petiole and that this can account for the observed differential growth. At the abaxial side, ethylene induces CMT reorientation from longitudinal to transverse, whereas, at the adaxial side, it has an opposite effect. The inhibition of CMTs disturbed ethylene-induced hyponastic growth.
- This work provides evidence that ethylene stimulates cell expansion in a tissue-specific manner and that it is associated with tissue-specific changes in the arrangement of CMTs along the petiole.

Introduction

The capacity to adjust to changes in the environment is a fundamental property of living organisms. To cope with environmental changes, plants have evolved plasticity in a range of metabolic, physiological and morphological processes, which allows them to survive unfavorable conditions (Lambers *et al.*, 1998). Plant organ movements are an adaptation to mainly abiotic stresses, and have been given much attention since Darwinian times. Some species, such as the rain tree (*Samanea saman*) (Satter *et al.*, 1974), the sensitive plant (*Mimosa pudica*) (Allen, 1969) and the runner bean (*Phaseolus coccineus*) (Mayer *et al.*, 1985), possess specialized structures, pulvini, which allow rapid collapse and closing movements (Kang, 1979). Tropic movements position organs directionally to the environmental stimulus, for example, phototropism and gravitropism, in which plants bend their stems towards the light or away from the gravitational vector, respectively (Firn & Digby, 1980; Morita & Tasaka, 2004; Iino, 2006).

Nastic movements occur without a directional external stimulus. Among the best described examples is upward leaf movement, called hyponastic growth, which is a rapid reaction of plants in response to several external stimuli (Kang, 1979; Van Zanten *et al.*, 2010). A number of plant species, including the model species *Arabidopsis thaliana* (*Arabidopsis*), use hyponasty as part of an escape mechanism from unfavorable conditions, such as complete submergence (Pierik *et al.*, 2005; Voesenek *et al.*, 2006), proximity of neighbors (Ballaré & Scopel, 1997; Keuskamp *et al.*, 2010), low light intensity (Millenaar *et al.*, 2005) and supraoptimal temperatures (heat) (Koini *et al.*, 2009; Van Zanten *et al.*, 2009b).

The volatile phytohormone ethylene is one of the main factors controlling hyponastic growth in *Arabidopsis* (Cox *et al.*, 2003, 2004; Van Zanten *et al.*, 2010), and has been used as a tool to unravel the underlying control mechanisms. When plants are exposed to ethylene, it induces a marked hyponastic growth response and, accordingly, ethylene accumulation is required for

the induction of submergence-induced hyponastic growth (Millenaar *et al.*, 2005). However, ethylene is not involved in the control of low light-induced hyponastic growth (Millenaar *et al.*, 2009) and even acts antagonistically to heat-induced hyponasty (Van Zanten *et al.*, 2009a).

Cox *et al.* (2003) have shown that hyponasty in flooded semi-aquatic *Rumex palustris* is driven by differential cell expansion on the abaxial side of the petiole. *Rumex palustris* belongs to a group of flood-tolerant species which respond to high concentrations of ethylene by cell expansion (Voesenek *et al.*, 2006), and can only respond positively to high ethylene concentrations once a minimal hyponastic leaf angle has been achieved (Cox *et al.*, 2003; Heydarian *et al.*, 2010). Most plant species, including *Arabidopsis*, are not flood tolerant (Peeters *et al.*, 2002; Vashisht *et al.*, 2011) and typically show growth inhibition by high concentrations of exogenous ethylene (Abeles *et al.*, 1992; Pierik *et al.*, 2006, 2007).

Despite the increasing insight into the physiological and hormonal regulation of ethylene-induced hyponasty, we still lack fundamental knowledge about the anatomical and morphological changes that lead to the upward petiole movement in *Arabidopsis*.

A clear correlation between the arrangement of cortical microtubules (CMTs) and the direction of cellular expansion has been demonstrated in a variety of plant species, and it is well known that growth-stimulating hormones, such as auxins or gibberellins, promote a transverse alignment of CMTs which favors cell elongation (reviewed in Shibaoka, 1994; Inada & Shimmen, 2000; Wiesler *et al.*, 2002; Foster *et al.*, 2003; Li *et al.*, 2011). Although the functional correlation between CMT alignment and directional cell expansion is complex and still debated, it is probably also associated with the CMT-mediated delivery of cellulose synthases and other proteins to the plasma membrane (Gutierrez *et al.*, 2009; Fujita *et al.*, 2011). In pea (*Pisum sativum*), ethylene induces reorientation of CMTs from transverse to longitudinal, allowing lateral cell expansion of epidermal cells and arresting longitudinal growth (Yuan *et al.*, 1994). However, it has been shown that the ethylene precursor, 1-aminocyclopropane-l-carboxylic acid, can promote the transverse orientation in light-grown *Arabidopsis* seedlings on low-nutrient medium (Le *et al.*, 2005). If hyponasty in *Arabidopsis* involves a local stimulation of cell expansion in abaxial epidermal cells, a stimulation of transverse CMTs would be predicted.

We tested whether ethylene-mediated hyponasty in *Arabidopsis* occurs through the local stimulation of cell expansion, and whether this involves the reorientation of CMTs. As the epidermal cell layer has an important role in both driving and limiting plant growth (Savaldi-Goldstein *et al.*, 2007), we focused our study on this tissue, on both the abaxial and adaxial sides of the petiole.

Our data demonstrate that ethylene primarily induces hyponastic petiole growth by driving longitudinal cell expansion specifically in a restricted abaxial region, proximal to the shoot. The analysis of CMT orientation revealed that ethylene-induced reorientation from longitudinal to transverse also occurs predominantly at this proximal abaxial side of the petiole and the disruption of CMTs prevented ethylene-induced hyponasty. Together with evidence from a geometrical model, these data show that localized cell expansion drives hyponasty in *Arabidopsis*.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana (L.) Heynh. Col-0 (N1092) was obtained from the Nottingham *Arabidopsis* Stock Centre (NASC), *35S::TUA6:GFP* (Ueda *et al.*, 1999) was a gift of Douglas Muench (University of Calgary, Calgary, AB, Canada) and *mor1-1* seeds (Whittington *et al.*, 2001) were provided by David Collings (University of Canterbury, Canterbury, Kent, UK). Seeds were dark stratified for 4 d at 4°C on filter papers. Germinated seedlings were transferred into pots containing a fertilized mixture of soil and perlite (RHP, 's-Gravenzande, the Netherlands) in a 1 : 2 ratio and grown in a controlled growth chamber (20°C; relative humidity, 70%; photosynthetically active radiation (PAR), 200 μmol m^{-2} s^{-1}; 9 h photoperiod) as described in Millenaar *et al.* (2005). Each day at the start of the photoperiod, plants were automatically watered until saturation.

Ethylene treatment and petiole angle measurements

For all experiments, 30-d-old plants in stage 3.9 (Boyes *et al.*, 2001) were used. To allow acclimation, plants were transferred to the experimental setup (Microclima 1750 growth cabinet; Snijders Scientific, Tilburg, the Netherlands), with similar conditions to the growth chambers (described above), 1 d before the start of the experiment. Ethylene (Hoek Loos, Amsterdam, the Netherlands) application started (t = 0 h) 1.5 h after the beginning of the photoperiod to prevent the effects of diurnal leaf movements. The experimental setup was flushed with 1.5 ppm ethylene, which is a saturating concentration for hyponastic growth (Fig. 1), with a continuous flow of 75 l h^{-1}. For the dose–response experiment, pure ethylene and air were mixed at different flow rates to achieve concentrations between 10 ppb

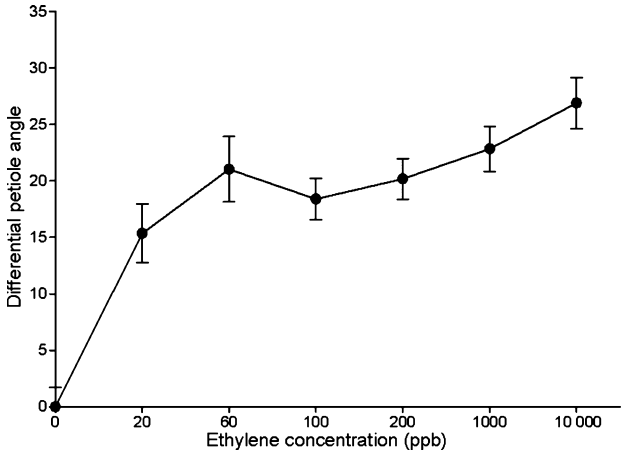

Fig. 1 Dose–response curve for *Arabidopsis thaliana* petiole hyponasty in ethylene (means ± SE).

and 10 ppm. The concentrations were checked on a gas chromatograph (GC995, Synspec, Groningen, the Netherlands). Plants were placed in glass cuvettes (18 l, eight plants per cuvette) and subjected to different concentrations of ethylene.

For petiole angle measurements, side pictures were taken with a digital camera (Canon PowerShot A530, Amstelveen, the Netherlands) at the start of the experiment and after 6 h of ethylene/control treatment. Petiole angles were measured relative to the horizontal plane by drawing a straight line from the petiole base to the leaf base using ImageJ software (Abramoff *et al.*, 2004). The differential angle was calculated as the difference between the angle of ethylene-treated petioles and the control at the same time point (Benschop *et al.*, 2007).

Epidermal cell length measurements and calculations

To obtain epidermal imprints, petioles of 1 cm or 5 mm in length were covered with polyvinylsiloxane paste (type 3) (Coltene Whaledent, Langenau, Germany). After solidification, the imprint was removed and brushed with one layer of transparent nail polish. When dried, the film was placed on a microscopic slide and observed under an Olympus BX50WI microscope. Pictures were taken with an Olympus DP70 camera and the photographs obtained were merged in Paint (Microsoft). Cell length measurements were performed with the use of a custom made macro in KS400 software (Carl Zeiss Vision, Oberkochen, Germany). Cell width measurements were performed on the first 3 mm of the petiole using ImageJ software.

To allow calculations of average cell sizes relative to the distance along the petiole, each cell was assigned to a 200-μm class, depending on its position relative to the most proximal part (close to the stem) of the petiole (see Fig. 2a for details).

Disruption and visualization of CMTs

To inhibit the expansion of cells driving hyponastic growth, oryzalin (Sigma Aldrich, Zwijndrecht, the Netherlands) solution (200 μM in water and 0.2% dimethyl sulfoxide (DMSO); containing 0.1% Tween 20) was applied 24 h before the start of the experiment at the abaxial side of each petiole. To genetically disrupt the organization of CMTs, the heat-inducible *mor1-1* mutant was used (Whittington *et al.*, 2001). Plants were treated with ethylene at noninductive (20°C) and inductive (30°C) temperatures and, after 6 h, compared with controls at the corresponding temperatures.

The arrangement of CMTs was studied with *35S::TUA6:GFP* reporter lines (Ueda *et al.*, 1999). Four-week-old plants were subjected to ethylene/control treatment and, after 5 h, CMTs of petiole epidermal cells were visualized using an inverted confocal laser scanning microscope (Leica CS SPII, ×63 C-apochromat objective, excitation wavelength of 488 nm, collecting at 505–530 nm for green fluorescence protein (GFP) emission). Petioles were divided into four parts depending on their distance from the base, and the abaxial and adaxial sides were observed separately. CMT areas at least twice as long as the cell width were taken into account (Himmelspach & Nick, 2001). The CMTs were grouped into categories relative to the long cell axis: transverse (0°), oblique 30°, oblique 60°, longitudinal (90°) and randomly oriented, according to Himmelspach & Nick (2001).

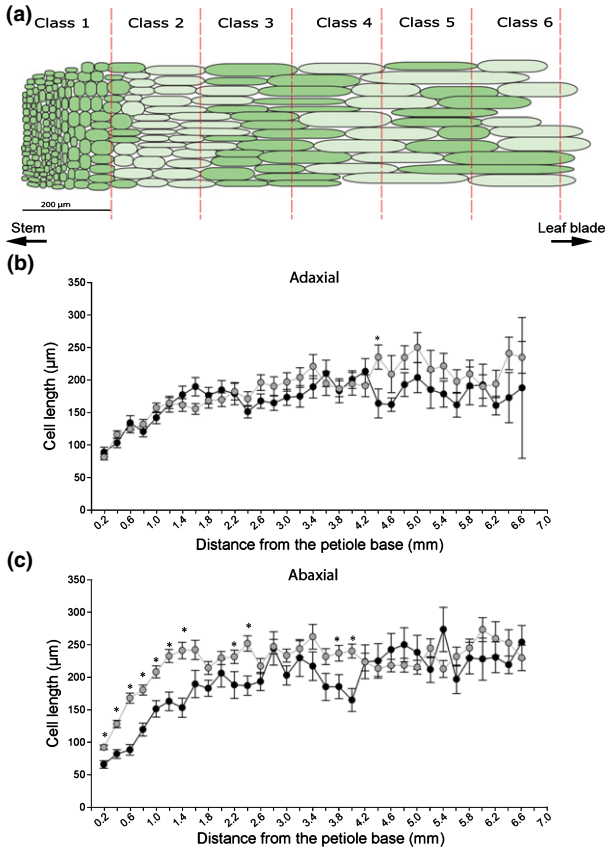

Fig. 2 (a) Schematic representation of the classes used for cell length calculations in the petiole epidermis. Cells are classified depending on their distance from the base of the petiole. Each class consists of 200 μm. (b, c) Epidermal cell length of a 1-cm *Arabidopsis thaliana* petiole. Data points represent the means ± SE of cell lengths of the first 6.6 mm at the adaxial (b) and abaxial (c) sides of a petiole after 10 h of ethylene (gray circles) and control (black circles) treatment. Asterisks indicate significant differences relative to the cell lengths of control-treated petioles ($P < 0.05$), $n = 17$.

Analysis of At1g20190 (*EXPA11*) expression

Col-0 petioles of 8–12 mm in length were harvested, divided into quarters (Fig. 3a) and snap frozen in liquid nitrogen. RNA was isolated using the RNeasy extraction kit (Qiagen, Venlo, the Netherlands) and genomic DNA was removed with on-column DNase digestion (Qiagen); 1 μg of total RNA was employed for cDNA synthesis conducted with random hexamer primers using the SuperScript III RNase H Reverse Transcriptase kit (Invitrogen, Breda, the Netherlands). Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed using the MyiQ Single-Color Detection System (Bio-Rad, Veenendaal, the Netherlands) with iQ SYBR Green Supermix Fluorescein (Bio-Rad) and gene-specific primers (At1g20190, 5′-TGCTTTGCCTAACAACAACG-3′, 5′-TCGCTCAGGGAGAAAAGAAA-3′). Relative mRNA values were calculated using the $2^{-\Delta\Delta Ct}$

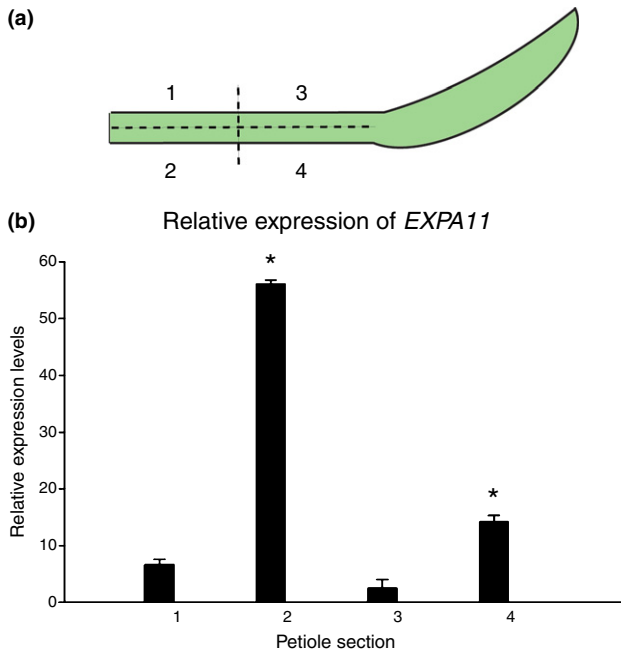

Fig. 3 (a) Schematic representation of petiole quarters used for studies of *EXPANSIN11* (*EXPA11*) expression. (b) Expression of *EXPA11* on ethylene treatment. *Arabidopsis thaliana* petioles were divided into four quarters and expression measurements were performed separately on each quarter. Data points represent the fold change of *EXPA11* expression (means ± SE) after 6 h of ethylene exposure relative to the control conditions and relative to β-*Tubulin-6*. Asterisks indicate significant differences between the two treatments ($P < 0.05$).

method (Livak & Schmittgen, 2001) with β-*Tubulin-6* (At5g12250, 5′-ATAGCTCCCCGAGGTCTCTC-3′, 5′-TCCATCTCGTCCATTCCTTC-3′) as an internal reference gene.

A mathematical model to predict petiole angles from cell length data

In the model, the petiole is divided into a series of consecutive sections, denoted '*s*'. Each section is represented by an arc with a constant radius (see Supporting Information Fig. S1). As the relative size of the sections determines the spatial resolution of the model, we used sections equal in size to the classes of the cell length data (i.e. 200 μm). Each arc can be defined by its radius, which is calculated relative to the center of the petiole cross-section, and its angle. Both can be calculated from the adaxial and abaxial section length and the petiole thickness:

$$r_s = \frac{d \cdot (i_s + j_s)}{j_s - i_s} \quad \text{Eqn 1}$$

$$\theta_s = \frac{i_s}{r_s - (d/2)} \quad \text{Eqn 2}$$

(r_s, arc radius; θ_s, arc angle; d, petiole diameter; i_s, adaxial arc length; j_s, abaxial arc length); a positive value of r_s indicates an upward bend. The model seeks to link observed changes in cell length under ethylene treatment to the observed changes in petiole shape. To do so, we used the observed petiole shape under air treatment as a reference shape. A detailed description is given in Methods S1, but, basically, given the known petiole shape under air treatment and a measured petiole thickness of d = 700 μm, we can straightforwardly calculate the adaxial and abaxial arc lengths for each segment i_s and j_s. Moreover, the measured cell length data supply us with the information needed to calculate, under air treatment, the average number of cells spanning each segment along both the adaxial and abaxial sides. Using this reconstruction as the starting point, we then need to predict the petiole shape under ethylene treatment. To do so, we calculate a predicted change in length for each adaxial and abaxial section, using the measured changes in mean cell length as a result of ethylene treatment (see Methods S1 and Figs S6–S8 for details and a discussion on the role of cell division). Once i_s and j_s have been estimated, Eqns 1 and 2 allow us to calculate, for each segment, the corresponding arc angle. The predicted petiole shape can be calculated from the arc angle in the following way. The relative x and y coordinates of the end-point of each arc are given by:

$$x_s = r_s \cdot \sin(\theta_s) \quad \text{Eqn 3}$$

$$y_s = r_s - r_s \cdot \cos(\theta_s) \quad \text{Eqn 4}$$

To correct for the curvature of the previous petiole sections, the x, y coordinates of each section are rotated using the cumulative angle of all previous sections. The initial angle (θ_0) at which the petiole emerges from the shoot is also taken into account:

$$\phi_s = \sum_{t=0}^{s-1} \theta_t \quad \text{Eqn 5}$$

$$x'_s = [\cos(\phi]_s) \cdot x_s - \sin(\phi_s) \cdot y_s \quad \text{Eqn 6}$$

$$y'_s = [\sin(\phi]_s) \cdot x_s + \cos(\phi_s) \cdot y_s \quad \text{Eqn 7}$$

(ϕ_s, cumulative rotation until section s; x'_s and y'_s, its rotated x, y coordinates). Finally, the absolute x and y coordinates of each section are calculated:

$$x''_s = \sum_{t=1}^{s} x'_t \quad \text{Eqn 8}$$

$$y''_s = \sum_{t=1}^{s} y'_t \quad \text{Eqn 9}$$

where x''_s and y''_s are the absolute coordinates of the end-points of section s. These coordinates of each section can be used to plot

the predicted petiole shape. Additional details about the calibration of the model are given in Methods S1.

Results

Epidermal cells at the proximal abaxial side of the petiole elongate on ethylene exposure

Differential petiole growth depends on unequal growth rates between the abaxial and adaxial sides of an organ (Kang, 1979). In the case of hyponasty, this can be achieved by an arrest of longitudinal cell expansion at the adaxial side of the petiole, by enhancement of cell elongation at the abaxial side of the petiole, or a combination of both. To determine which of these events occurs during ethylene-induced hyponasty, we first performed a comparative analysis of cell lengths using epidermal imprints of both the adaxial and abaxial parts of 1-cm-long petioles. To this aim, petioles of plants subjected for 10 h to a saturating concentration of 1.5 ppm ethylene (Fig. 1) and control treated plants were used, as the hyponastic growth response is strongest at this time point (Millenaar *et al.*, 2005). No difference in cell length (see Fig. 2a for experimental details) was found at the adaxial surface between ethylene and control treatments (Fig. 2b). By contrast, cell length at the abaxial side was increased significantly on ethylene treatment in approximately the first 3–4 mm from the base (Fig. 2c), which suggests that expansion does not occur along the entire petiole, but is restricted to the region proximal to the shoot. Moreover, we observed a significant reduction in lateral expansion in this region at the abaxial side of the petiole and widening of the cells of the adaxial epidermis (Fig. S2a,b). Interestingly, in the case of younger petioles (5 mm), the pattern of cell expansion differs. The increase in longitudinal cell size occurs abaxially along the entire organ (Fig. S3b). This implies that the cells involved in hyponastic growth differ between developmental stages.

Cell wall loosening proteins are well-known downstream target genes involved in cell elongation in many organ types and species (Cosgrove, 2005). We examined the expression of *EXPANSIN11* (*EXPA11*) in the petiole as a marker to identify the region of elongation in ethylene-exposed petioles. This gene has been shown in a genome-wide gene expression profiling study to be differentially expressed in petioles on ethylene treatment (Millenaar *et al.*, 2006). To assess the differential expression of *EXPA11* along petiole segments, we analyzed distinct parts of the petiole (Fig. 3a). A significant increase in expression between ethylene- and control-treated plants was observed in sections located on the abaxial side of the petiole (sections 2 and 4) (Fig. 3b). Strikingly, the highest upregulation was noted in the proximal abaxial region, where ethylene-induced cell elongation was observed (Fig. 2c).

Taken together, these data support the hypothesis that cell expansion at the proximal abaxial petiole side, rather than inhibition of cell growth at the adaxial side, drives ethylene-induced hyponastic growth.

Ethylene induces the local reorientation of CMTs in petiole epidermis

A transverse arrangement of CMTs is associated with cell elongation, whereas a longitudinal pattern promotes lateral expansion (Yuan *et al.*, 1994). To study the involvement of CMTs in ethylene-induced hyponastic growth, 24 h before the exposure of plants to ethylene, we applied oryzalin, which prevents the polymerization of tubulin (Morejohn *et al.*, 1987). After 6 h of ethylene treatment, oryzalin-treated plants showed a significantly lower leaf angle than mock-treated plants ($P < 0.05$). The calculation of the differential angle change (which is the difference between the mock and treated plants at the same time point) confirmed that oryzalin inhibits the hyponastic growth response to ethylene (Fig. 4a). The ± 50% reduction in the response also persisted after 10 h of ethylene exposure when, in control plants, the differential response is at its maximum (data not shown).

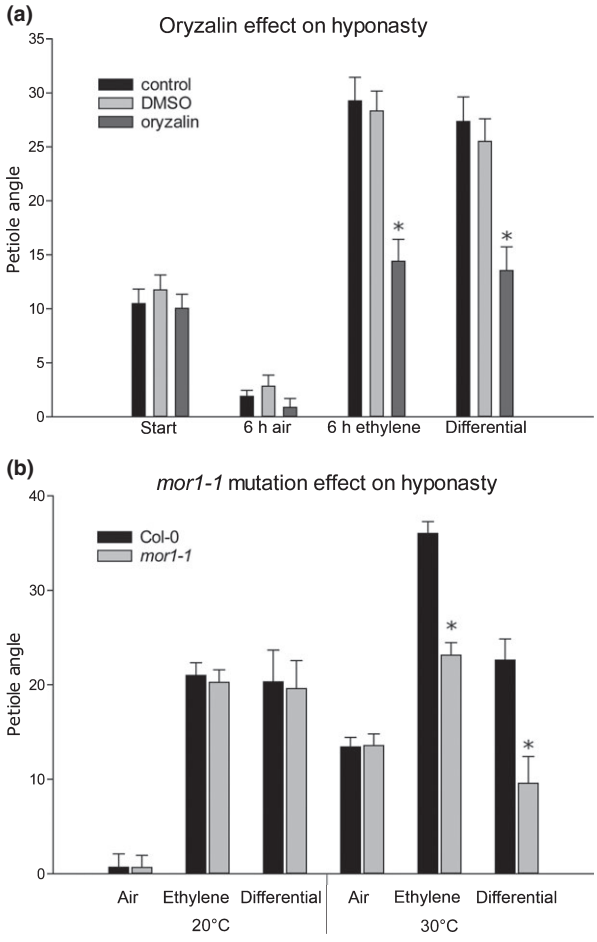

Fig. 4 (a) Effect of 200 μM oryzalin on petiole angle in control and 6 h ethylene treatments. (b) Effect of the heat-inducible *mor1-1* mutation on hyponasty. Asterisks indicate significant differences ($P < 0.05$) between the angles of DMSO and untreated petioles (a) or between *Arabidopsis thaliana* Col-0 and *mor1-1* plants (b) ($P < 0.05$). Data points represent means ± SE of petiole angles, n = 40–63. [correction added after online publication 4th October 2011: Fig. 4 has been updated to include a key in both parts (a) and (b) of the figure. Previously this key was erroneously omitted from the figure.]

To test the involvement of CMTs in hyponastic growth genetically, we used a temperature-sensitive mutation in *MICROTUBULE ORGANIZATION1* (*MOR1*), which is required for CMT organization in plants by controlling their polymerization (Whittington *et al.*, 2001). The petiole angles of *mor1-1* in mock- and ethylene-treated plants are similar to those of wild-type plants at a noninductive temperature of 20°C, whereas, at an inductive temperature of 30°C, they are reduced significantly (Fig. 4b). Together, these experiments support the hypothesis that reorientation of CMTs is required for ethylene-induced hyponasty.

To gain further insight into the involvement of CMTs in ethylene-induced hyponastic growth, we investigated CMT arrangement in the epidermal cells at both the adaxial and abaxial sides of the petiole. Transgenic plants carrying a *35S::TUA6:GFP* reporter construct, which exhibits normal hyponasty in response to ethylene (Fig. S4), were subjected to ethylene and control treatments, and images of the epidermal cells were taken using confocal microscopy. Adaxial and abaxial sides were divided into four segments (Fig. 5a) and, subsequently, the CMT orientation was classified for each section. Fig. 5(b,c) shows typical CMT orientations in air and control conditions, whereas Fig. 5(d,e) illustrates the detailed frequency of CMTs in a transverse orientation (promoting cell expansion) and longitudinally (inhibiting cell expansion) (Wilms & Derksen, 1988; Wasteneys & Williamson, 1993). The complete coverage, including the intermediate (oblique 30°, 60°) and random orientations, is presented in Fig. S5.

The orientation of CMTs in control conditions is largely transverse at the adaxial side of the petiole and longitudinal at the abaxial side. On ethylene treatment, these proportions change significantly in the first three classes, with the adaxial side displaying the highest frequency of longitudinal CMTs, whereas the elongation-stimulating transverse orientation at the abaxial side increases from 5% to ± 30%. Moreover, in the case of the abaxial side, there is a reduction in the frequency of transverse CMTs towards the leaf lamina. The sections with the highest abundance of this type of CMT (1ab–2ab) co-localize with the region in which we detected cell expansion and elevated levels of *EXPA11* mRNA (Figs 2c, 3b).

To calculate whether ethylene-induced changes in longitudinal cell expansion can explain the observed hyponastic growth, we constructed a geometrical model that predicts petiole shape from the measured adaxial and abaxial cell lengths. Our model is related to that used by Cox *et al.* (2004), which calculates the

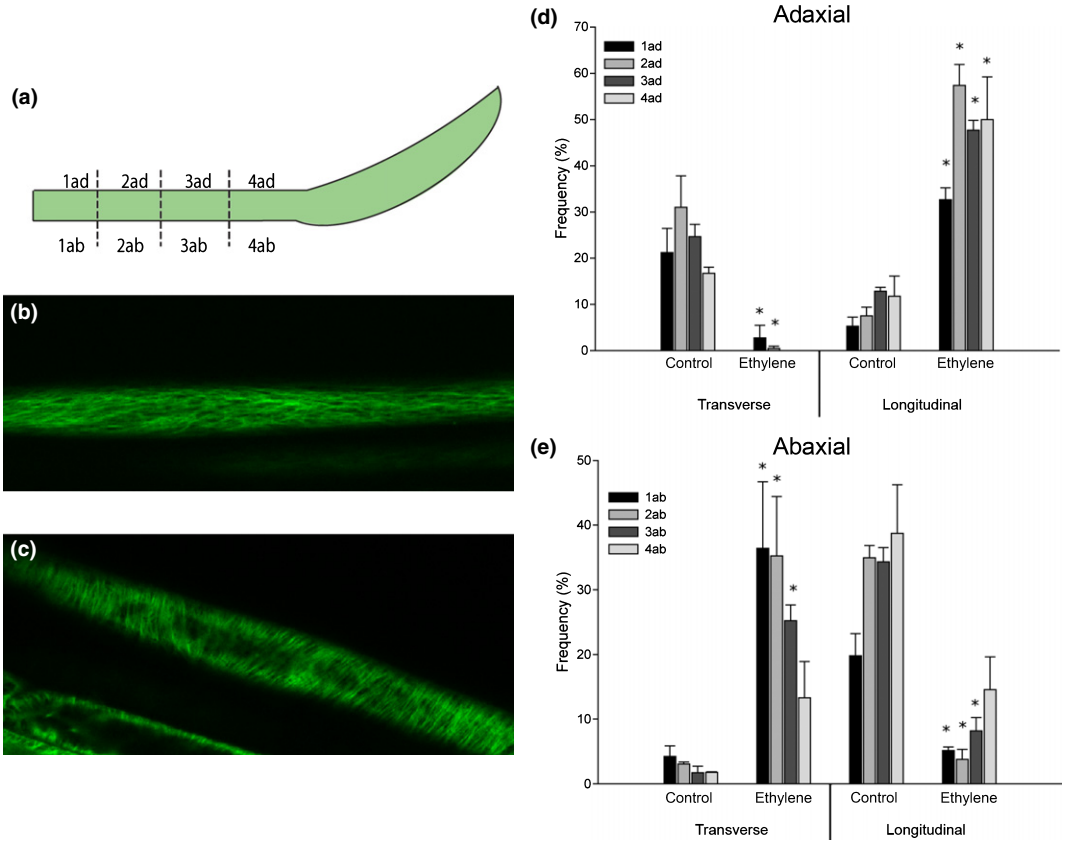

Fig. 5 (a) Schematic illustration of the experimental setup of cortical microtubule (CMT) visualization. Petioles were divided into four sections, and the adaxial (1ad, 2ad, 3ad, 4ad) were observed separately from the abaxial (1ab, 2ab, 3ab, 4ab) parts. The orientation of CMTs in petiole epidermal cells changes on 6–10 h of ethylene treatment. Confocal images of a longitudinal arrangement of CMTs at the abaxial side of an *Arabidopsis thaliana* petiole on control conditions (b) and a transverse orientation in ethylene (c). The frequency of CMT categories at the adaxial side (d) and abaxial side (e). Data points refer to the average number of areas representing either a transverse or a longitudinal class of CMT orientation ± SE, $n = 3$. Asterisks indicate significant differences ($P < 0.05$) between the frequency in control and ethylene-treated plants.

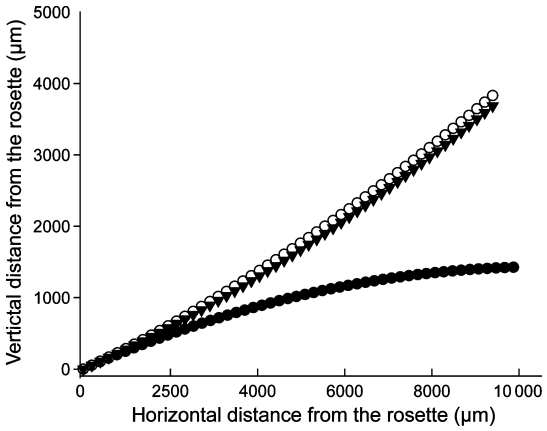

Fig. 6 Geometrical model predicting petiole curvature after ethylene treatment (ethylene predicted, open circles), which is consistent with the observed differential growth values (ethylene observed, closed triangles) on 6 h of ethylene exposure. Air, closed circles.

ratio between the petiole angles from the difference in adaxial and abaxial petiole lengths. To allow the prediction of local changes in petiole shape, we divided the petiole into sections and calculated the curvature for each of these sections separately. The results show that, if we normalize for the observed petiole elongation as a result of ethylene treatment (derived from Millenaar *et al.*, 2005), the hyponastic growth and resulting petiole shape can be correctly predicted from the measured cell lengths (Fig. 6). The prediction is accurate to within a single degree. As an overall conclusion, the model supports the hypothesis that hyponastic growth observed during ethylene treatment is caused by increased abaxial cell elongation.

Discussion

Ethylene-induced hyponastic leaf movement depends on unequal growth rates between the adaxial and abaxial sides of the petiole. In this work, we investigated what event drives this process. In accordance with the observation by Cox *et al.* (2003) that hyponasty is driven by differential cell expansion in submerged *Rumex palustris*, we found that epidermal cells at the abaxial side of *Arabidopsis* petioles elongate rapidly on ethylene treatment, whereas cells on the adaxial side do not. We did not assess the elongation of underlying mesophyll tissues because elongation growth of the epidermis is considered to be both required for and sufficient to drive and restrict the growth of underlying basal tissues (Savaldi-Goldstein *et al.*, 2007; Savaldi-Goldstein & Chory, 2008). In accordance, the prediction of the resulting leaf angle matched the observed hyponastic leaf movement measured after ethylene treatment, suggesting that the observed cell elongation is sufficient to drive hyponasty. Our noninvasive method of epidermal imprints allowed the study of changes in cell size without affecting the biomechanical integrity, which would be distorted on tissue excision.

The measurements performed on 1-cm petioles revealed that expansion takes place in the most proximal zone of the abaxial side of the petiole, approximately within the first 3–4 mm. By contrast, in younger (5-mm) petioles, hyponasty is triggered by elongation of all cells along the petiole, rather than in a restricted part of the organ (Fig. S3). The latter has been observed previously for ethylene-induced epinasty in the castor oil plant (*Ricinus* sp.), *Datura* sp., coleus (*Coleus* sp.) and *Hibiscus* sp. (Doubt, 1917). This observation is consistent with the concept that petiole epidermis cells lose the capacity to elongate on ethylene treatment when they become older.

Several lines of independent evidence strongly suggest that CMT reorganization is involved in ethylene-induced hyponastic growth. First, application of the drug oryzalin inhibited ethylene-induced hyponasty. Oryzalin has been successfully used previously to assess the role of CMTs in plant development in several other studies (Morejohn *et al.*, 1987; Baskin *et al.*, 1994). Microtubule depolymerization by oryzalin causes swelling of cells and prevents CMTs from orienting in a transverse direction, thereby arresting anisotropic growth (Grandjean *et al.*, 2004; Hamant *et al.*, 2008). The application of oryzalin at the abaxial side of the petiole resulted in a significant reduction in the petiole angle before the induction of hyponastic growth by ethylene treatment, and also reduced significantly the response to this hormone. As petiole inclination in response to ethylene was not completely absent, it can be concluded that, in addition to anisotropic growth, there may be other factors playing a role in the induction of hyponasty. A similar effect was observed in studies on apical meristem growth, where oryzalin only partially prevented organ outgrowth (Hamant *et al.*, 2008). The additional, more specific, genetic approach involving plants carrying the temperature-sensitive *mor1-1* mutation (Whittington *et al.*, 2001; Sugimoto *et al.*, 2003; Collings *et al.*, 2006) confirmed the role of CMT reorganization in the induction of hyponastic growth.

Subsequent visualization of CMT orientation demonstrated that two types of CMT reorientation event take place. At the adaxial side, ethylene changes the alignment of CMTs from transverse (stimulating elongation) to longitudinal. However, at the abaxial side of the petiole, CMTs reorient from longitudinal to transverse, thus indicating local cell expansion. The frequency of CMTs in the transverse category is highest in the region located at the petiole base (proximal) and decreases towards the leaf lamina (distal). This matches our observation that ethylene-induced cell elongation is strongest at the proximal abaxial side of the petiole and is probably sufficient for the occurrence of hyponasty.

As cell expansion requires primary cell wall-modifying agents which, according to the acid growth hypothesis, bring about wall loosening (Rayle & Cleland, 1970; Cosgrove, 1989), we used the expression of an expansin gene as another marker for cell expansion. A previous microarray study on whole petioles showed *EXPA11* to be the only cell wall-modifying enzyme differentially regulated in petioles on ethylene treatment (Millenaar *et al.*, 2005). The spatial analysis of *EXPA11* expression, which is most strongly induced in the proximal abaxial region, is consistent with our observations on cell expansion and CMT rearrangement, which also occur in the same region. It has been demonstrated previously that ethylene specifically regulates the transcription of one *EXPA* gene in petioles of submerged

R. palustris (Vreeburg *et al.*, 2005), which is associated with enhanced physiological cell wall loosening activity. It is possible that only a few specific members of this family are recruited by ethylene to adjust the plant phenotype, but control at levels other than transcription is certainly also possible. To obtain a more comprehensive understanding of the involvement of cell wall-modifying proteins in the regulation of ethylene-induced hyponasty in *Arabidopsis*, it will be crucial to test the expression profiles, as well as knockout mutant phenotypes, of more expansins and other cell wall-modifying agents in future studies.

High ethylene concentrations are frequently associated with inhibitory growth effects on terrestrial plants (reviewed in Pierik *et al.*, 2006). In *Arabidopsis*, this includes the triple response, characterized by an inhibition of hypocotyl and root elongation, and an exaggerated apical hook formation (Guzman & Ecker, 1990; Abeles *et al.*, 1992). In accordance, ethylene has been mostly implicated so far in the promotion of the longitudinal orientation of CMTs, thereby causing lateral expansion (Steen & Chadwick, 1981; Lang *et al.*, 1982; Roberts *et al.*, 1985; Soga *et al.*, 2010). Our results provide evidence of an opposite effect of high ethylene levels at the cellular level, and provide support for the dual role of ethylene in growth control (Pierik *et al.*, 2006, 2007 and references therein). Interestingly, the adaxial and abaxial sides of a petiole respond differently to ethylene treatment, making it an elegant example of asymmetric growth. This is in line with a growing number of studies, which indicate its stimulatory function: for example, *Arabidopsis* hypocotyl elongation in light (Smalle *et al.*, 1997; Le *et al.*, 2005; Pierik *et al.*, 2006), submergence escape by petiole elongation and hyponastic growth in *Rumex palustris* (Cox *et al.*, 2003, 2004; Vreeburg *et al.*, 2005), elongation growth in rice (Kende *et al.*, 1998) and shade avoidance by hyponasty and internode elongation in tobacco (Pierik *et al.*, 2004). It has been reported that ethylene can stimulate endoreduplication levels in *Arabidopsis* (Gendreau *et al.*, 1999; Dan *et al.*, 2003) and cucumber (Dan *et al.*, 2003) hypocotyls. Increased ploidy levels are also tightly associated with cell size in *Arabidopsis* petal tissue (Roeder *et al.*, 2010) and in pericarp and mesocarp in tomato fruit (Cheniclet *et al.*, 2005; Nafati *et al.*, 2011). Therefore, it is possible that local, tissue-specific, ethylene-induced stimulation of cell expansion might involve control through the endocycle.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Detailed description of reconstruction of the petiole shape for the geometrical model.

Fig. S2 Epidermal cell width data for 1-cm petioles.

Fig. S3 Epidermal cell length data for 5-mm petioles.

Fig. S4 Hyponasty in the *35S::TUA:GFP* line.

Fig. S5 Full coverage of cortical microtubule (CMT) orientation categories in epidermal cells in control conditions and on ethylene treatment.

Fig. S6 Reconstruction of the petiole shape in control conditions.

Fig. S7 Cell length data after the use of fitted functions.

Fig. S8 Reconstruction of the petiole shape in the null model.

Methods S1 Description of the calibration of the mathematical model.

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