Ethylene promotes hyponastic growth through interaction with \textit{ROTUNDIFOLIA3/CYP90C1} in \textit{Arabidopsis}

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Abstract

Upward leaf movement, called hyponastic growth, is employed by plants to cope with adverse environmental conditions. Ethylene is a key regulator of this process and, in \textit{Arabidopsis thaliana}, hyponasty is induced by this phytohormone via promotion of epidermal cell expansion in a proximal zone of the abaxial side of the petiole. \textit{ROTUNDIFOLIA3/CYP90C1} encodes an enzyme which was shown to catalyse C-23 hydroxylation of several brassinosteroids (BRs) – phytohormones involved in, for example, organ growth, cell expansion, cell division, and responses to abiotic and biotic stresses. This study tested the interaction between ethylene and BRs in regulating hyponastic growth. A mutant isolated in a forward genetic screen, with reduced hyponastic response to ethylene treatment, was allelic to \textit{rot3}. The cause of the reduced hyponastic growth in this mutant was examined by studying ethylene-BR interaction during local cell expansion, pharmacological inhibition of BR synthesis and ethylene effects on transcription of BR-related genes. This work demonstrates that \textit{rot3} mutants are impaired in local cell expansion driving hyponasty. Moreover, the inhibition of BR biosynthesis reduces ethylene-induced hyponastic growth and ethylene increases sensitivity to BR in promoting cell elongation in \textit{Arabidopsis} hypocotyls. Together, the results show that ROT3 modulates ethylene-induced petiole movement and that this function is likely BR related.

Key words: \textit{Arabidopsis}, brassinosteroids, cell expansion, ethylene, hyponasty, \textit{ROTUNDIFOLIA3}.

Introduction

Upward leaf movement, referred to as hyponastic growth, is an active response of several plant species to adverse environmental conditions such as flooding, elevated temperatures, and dense canopies (Ballaré \textit{et al.}, 1997; Cox \textit{et al.}, 2003; Koini \textit{et al.}, 2009; Keuskamp \textit{et al.}, 2010). It is defined as an increase in petiole angle, which is an outcome of unequal growth rates between two (ad- and abaxial) sides of the organ. Ethylene-induced hyponasty has been given much attention due to the discovery that ethylene is a key player in the regulation of hyponastic response in \textit{Arabidopsis} and that exogenous ethylene application on its own rapidly induces plants to reorient their leaves (Cox \textit{et al.}, 2003; Millenaar \textit{et al.}, 2005). The cellular mechanism of this nastic movement consists of enhanced longitudinal cell expansion at the abaxial side of the petiole which requires the reorientation of cortical microtubules and recruitment of cell-wall-modifying proteins in a...
region proximal to the rosette (Cox et al., 2004; Polko et al., 2012b). A number of studies have contributed to today’s understanding of the regulation and significance of ethylene-induced hyponasty. In Arabidopsis, its control involves abscisic acid, jasmonic acid, and salicylic acid action (Benschop et al., 2007; Millenaar et al., 2009; Peña-Castro et al., 2011; Van Zanten et al., 2012). The role of another class of hormones, brassinosteroid (BRs), is currently not well understood.

BRs are plant steroid hormones involved in many, generally growth-promoting, processes. Initially purified from Brassica napus pollen (Mitchell et al., 1970; Grove et al., 1979), they are widespread among the entire plant kingdom and present in various organs (Rao et al., 2002). Highest BR concentrations are found in young tissues, reproductive organs, seeds, and fruits (Symons et al., 2008). Developmental processes controlled by BRs include, for example, cell elongation and division, cell cycle progression, sex determination, seed germination, seedling gravitropism, vascular differentiation, and fruit ripening (Gonzalez-Garcia et al., 2011; Vandenbussche et al., 2011; Yang et al., 2011; Hartwig et al., 2011; Jiang et al., 2012). Therefore, BRs are an integral part of several signalling pathways during biotic and abiotic stress responses (Krishna, 2003; Albrecht et al., 2012; Belkhadir et al., 2012). The perception of BRs starts at the plasma membrane, where they bind to the receptor-like kinase BRASSINOSTEROID INSENSITIVE (BRI1). This initiates a signal transduction cascade leading to activation of several transcription factors controlling, for example, ion exchange, water uptake, and cell-wall modifications, eventually leading to cell expansion (Zhang et al., 2005; Clouse, 2011; Xie et al., 2011).

BRs interact with other phytohormones, including auxin, and often share the same molecular targets (Goda et al., 2002, 2004; Nakamura et al., 2003, 2006; Halliday, 2004; Nemhauser et al., 2004; Vert et al., 2008). Relatively few studies, however, have focused on a functional interdependence of these two hormones. Exceptions are the crosstalk between auxin and BRs in responses to light cues (De Grauwe et al., 2005; Kozuka et al., 2010; Keuskamp et al., 2011). Mutant and pharmacological analyses demonstrated that exogenous application of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) induces expression of BR-related genes, suggesting a shared signalling and response network between these three hormones. Ethylene and brassinosteroids have been shown to affect one another in various ways (Arteca et al., 1983; Schlaghaufer and Arteca, 1985), and brassinopride, an inhibitor of BR action, promotes ethylene synthesis (Gendron et al., 2008). Des Lauriers and Larsen (2010) showed that FERONIA (FER), a gene responsible for male–female gametophyte interactions during pollen tube reception (Huck et al., 2003), functions as a signalling hub in the inhibitory interaction between BRs and ethylene during hypocotyl elongation in darkness. Interestingly, BR also acts in a crosstalk with ethylene during thermotolerance and salt stress (Divi et al., 2010).

A forward genetic screen was recently performed, which resulted in isolation of activation-tagged lines affected in petiole hyponasty in Arabidopsis (Polko et al., 2012a). This yielded several lines with reduced hyponastic growth in response to ethylene treatment. The current study shows that one of the isolated lines is allelic to a mutant in ROTUNDIFOLIA3 (ROT3). ROT3 encodes cytochrome P450 (CYP90C1) and has been shown to be involved in polar elongation of cells, particularly during leaf and flower development (Kim et al., 1998, 1999). Despite the weak phenotype of rot3 mutants, further studies indicated that ROT3 together with CYP90D1 catalyse C-23 hydroxylation step during synthesis of bioactive BRs (Ohnishi et al., 2006).

This isolation of ROT3 from the screen inspired the study of the interaction between ethylene and BRs in regulating hyponastic growth. This report shows that rot3 mutants have decreased petiole hyponasty in ethylene and that this reduction is reflected on a cellular level. Furthermore, it is demonstrated that inhibition of BR biosynthesis reduces ethylene-induced hyponasty and that ethylene increases sensitivity to BR in promoting hypocotyl cell elongation. The conclusion is that ROT3 controls differential cell expansion during hyponastic growth downstream of ethylene action.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana Col-0 (N1902), Col-7 (N3731), activation tagged lines (N21991, N23153 (Weigel et al., 2000), and rot3-1 (N3727) were obtained from the Nottingham Arabidopsis Stock Centre (NASC, stock numbers between brackets). Seeds were dark stratified for 4 days at 4 °C on moist filter papers and thereafter kept in light for 3 days. Seedlings were subsequently transferred to pots containing a fertilized mixture of soil and perlite (RHP, s-Gravenzande, The Netherlands) in a 1:2 ratio and grown in a growth chamber under controlled conditions (20 °C, 70% relative humidity, 200 mol m–2 s–1 photosynthetic active radiation, 9 h photoperiod) as described by Millenaar et al. (2005). For hypocotyl experiments, seeds were surface sterilized in an ethanol/bleach solution (8:2), sown on plates containing 8 g l–1 agar and 0.22 g l–1 Murashige and Skoog medium (both Duchefa, Haarlem, The Netherlands), dark stratified for 4 days at 4 °C, and transferred to the indicated growth cabinets.

Ethylene and green shade treatment

All experiments used 30-day-old plants in stage 3.9 (Boyés et al., 2001). To allow acclimation, 1 day before the start of the treatment, plants were transferred to the experimental set up with similar conditions to the growth chambers (Microclima 1750 growth cabinet, Snijders Scientific, Tilburg, The Netherlands). The treatments always started 1.5 h after the beginning of the photoperiod. The set up was flushed with 1.5 µl l–1 ethylene (Hoek Loos, Amsterdam, The Netherlands) with a continuous flow of 75 l h–1. For green shade experiments, Lee 122 Fern Green (Lee Hampshire, UK) filters were applied. This reduced the photosynthetic active radiation to 65 µmol m–2 s–1, red/ far red light to 0.19, and blue light to <2 µmol m–2 s–1.

The control experiment was carried out under white light (140 µmol m–2 s–1 photosynthetic active radiation and R/FR of 2.1). For ethylene treatment of seedlings, after 4 days in 4 °C plates were opened and placed in the experimental set up flushed with 5 µl l–1 ethylene at a continuous flow of 75 l h–1. High humidity (95%) prevented the agar plates from drying out. Hypocotyl length was measured using ImageJ software (Abrâmoff et al., 2004).

Petiole angle measurements and camera set up

Side pictures were taken (Canon PowerShot A530) at the start of the experiment, after 6 h of ethylene or control treatment, or in case of green shade experiment, after 24 h of treatment. Petiole
angles were measured relative to the horizontal plane and the rosette base, using ImageJ software (Abrámoff et al., 2004). The differential angle was calculated as a difference between the angle of ethylene-treated petioles and the control at the same time point (Benschop et al., 2007). For petiole kinetics measurements, a custom-built digital camera system was used (Millenaar et al., 2005). Plants were placed in glass cuvettes and side pictures were taken every 10 min for 24 h in constant light. Petiole angles were measured using a custom-made macro (KS400, Carl Zeiss Vision, Hallbergmoos, Germany).

Epidermal cell length measurements and calculations

Cell length measurements were performed on epidermal imprints as described in Polko et al. (2012b) with the use of a custom-made macro in KS400 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 meristem) of the petiole (Polko et al., 2012b).

Pharmacological treatments

For the hypocotyl elongation experiments, the Murashige and Skoog medium was supplemented with ACC (Sigma Aldrich, Zwijndrecht, The Netherlands), brassinazole (Brz, TCI Europe, Hanover, Germany), or 24-epibrassinolide (24-epiBL, Sigma Aldrich) to reach the following concentrations: ACC 0.01, 0.1, 1, 10, and 100 μM; Brz 0.5 μM; 24-epiBL 0.001, 0.01, 0.1, and 1 μM. For Brz treatment of petioles, 100 μM Brz containing 0.1% DMSO and 0.1% Tween 20 was applied with a brush on the abaxial side of each wild-type Columbia (Col-0) petiole. The control treatment contained 0.1% DMSO and 0.1% Tween 20. Solutions were applied 24, 20, and 16 h before the start of the experiment. The abaxial sides of petioles were brushed with the solution and side pictures were taken at the beginning of the experiment and after 6 h of control or ethylene exposure. BR level measurements were performed as described in the Supplementary Methods S1 (available at JXB online).

Gene expression studies

Petioles of 8–12-mm length were harvested and snap frozen in liquid nitrogen. RNA was isolated using the RNeasy extraction kit (Qiagen, Venlo, The Netherlands). Genomic DNA was removed using on-column DNase digestion (Qiagen). For the analysis of gene expression in different petiole quarters, petioles were divided in four sections from which RNA was isolated. Subsequently, 1 μg total RNA was used 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 PCR was performed using the MyiQ Single-Color Detection System (Bio-Rad, Veenendaal, The Netherlands) with iQ SYBR Green Supermix Fluorescein (Bio-Rad), ROTUNDIFOLIA-3 (At4g36380), and BRASSINOSTEROID-6 OXIDASE (At5g38970) specific primers. Relative mRNA values were calculated using the 2−ΔΔCt method (Livak and Schmittgen, 2001) with β-Tubulin-6 (At5g12250) as an internal reference gene. Primer sequences are listed in Supplementary Table S1.

Results

Line decreased initial decreased ethylene decreased low light angle 1 (ddd1) has reduced hyponasty due to impaired cell expansion under ethylene treatment

Arabidopsis line ddd1 was isolated in a forward genetic screen of 35S-GmMV promoter-tagged plants (Weigel et al., 2000), based on its decreased petiole angle phenotype (Polko et al., 2012a). Due to its constitutively reduced petiole angles and decreased responsiveness to both 6 h ethylene and 6 h low light, the line was initially named decreased initial angle decreased ethylene angle decreased low light angle 1 (ddd1). In the vegetative stage, the distinctive phenotype of ddd1 included compact rosettes, broader leaf blades, and shorter petioles compared to Col-0 plants (Fig. 1A, B). Detailed analysis of ddd1 leaf movement kinetics, using time-lapse photography, confirmed that hyponastic growth upon ethylene and low-light treatment was reduced and that this was the case throughout the duration of the experiment (24 h) (Fig. 1C–E and Supplementary Fig. S1A). Moreover, induction of the response appears to be moderately delayed by about 1 h. In addition, supra-optimal temperature (heat) also resulted in a reduced response in ddd1 (Supplementary Fig. S1B), suggesting that a general genetic determinant of hyponastic growth is affected in this line. To check whether reduced hyponasty in ddd1 could be attributed to mechanical constraints imposed by its compact phenotype and short petioles, its response to spectral (green) shade was assessed. Spectral shade mimics dense canopy conditions leading to hyponasty and petiole elongation (Pierik et al., 2005). Despite the initial difference between petiole angles of Col-0 and ddd1 at the start of the experiment, the 24-h green shade exposure resulted in a comparable increase in petiole angles of both genotypes (Fig. 2A–E). This implies that ddd1 has the potential to show hyponastic growth and indicates that the reduced response to ethylene, low light, and heat is not due mechanical constraints.

Ethylene-induced hyponasty depends on unequal growth rates between two sides of the petiole and is driven by local stimulation of cell expansion at the abaxial side of the organ (Polko et al., 2012b). First, the current study tested whether the substantially reduced hyponasty in ddd1 was visible at a cellular level. To do so, cell lengths were measured in epidermal imprints of 1-cm-long petioles after 10 h of ethylene and control treatment. Wild-type plants, treated with ethylene, showed increased cell lengths in the proximal ~2 mm at the abaxial side of the petiole compared to control (Polko et al., 2012b). However, this study observed no difference in cell lengths after ethylene treatment in ddd1 (Fig. 3A, B), indicating that the reduced hyponastic response is accompanied by a lack of local cell expansion in response to ethylene.

The ddd1 line has reduced levels of ROT3 expression which does not affect ethylene sensitivity

To identify the genetic nature of the ddd1 phenotype, Illumina sequencing was employed (Polko et al., 2012a). This revealed that the T-DNA insertion is located on chromosome 2 in the fourth exon of ROTUNDIFOLIA3 (ROT3, At4g36380). Accordingly, ROT3 transcript levels were substantially decreased in ddd1 (Polko et al., 2012a). To confirm that the insertion in ROT3 is causal to the ddd1 phenotype, the previously described rot3-1 mutant (Kim et al., 1998) was examined. rot3-1 has a deletion of at least 1 kb in the promoter region and the first intron (Kim et al., 1998). Analysis of petiole angles upon ethylene application showed that the rot3-1 mutant had a significantly reduced hyponastic
response, comparable to *ddd1* (Fig. 4B). Moreover, *rot3-1* has compact rosettes, broad leaves, and shorter petioles, clearly resembling *ddd1* (Fig. 4C, D). To confirm the genetic interrelation between *ddd1* and *rot3-1*, this study performed a genetic complementation test by crossing *ddd1* and *rot3-1*. All characteristic phenotypes remained in the F1 heterozygous offspring (Fig. 4C-E), indicating that *ddd1* and *rot3-1* are allelic. It was therefore concluded that ROT3 is required to mediate the effect of ethylene on cell expansion to induce hyponastic growth and, thus, subsequent experiments were carried out using the *rot3-1* line.

Ethylene sensitivity and cell expansion can be studied using the so-called triple response assay, which in *Arabidopsis* consists of inhibition of hypocotyl elongation, exaggerated apical hook, and swelling of the hypocotyl in darkness (Bleecker et al., 1988; Guzmán and Ecker, 1990). To test whether impaired hyponasty of *rot3-1* is due to a generally reduced responsiveness to ethylene, this study tested the mutants’ hypocotyl elongation in presence of exogenous ACC. In the absence of ACC, *rot3-1* has significantly shorter hypocotyls in darkness, confirming interference of ROT3 with cell expansion. Upon growth inhibition by ACC, the reduction of hypocotyl lengths follows the wild-type pattern and remained significantly shorter up to 1 µM. At higher concentrations *rot3-1* hypocotyl lengths became similar to wild type (Fig. 5). This result suggests that general sensitivity to ethylene is unaffected in *rot3-1* and that effects on hyponasty through ROT3 probably occur downstream of ethylene signalling.

Brassinosteroid action is required for ethylene-induced hyponasty

*ROT3* encodes a member of the cytochrome P450 family, CYP90C1, an enzyme involved in the conversion of
typhasterol to castasterone. The latter is the direct precursor of the bioactive BRs (Kim et al., 2004, 2005). To test whether BR biosynthesis plays a role in regulating ethylene-induced hyponasty, this study tested whether perturbation of BR synthesis reflects on the petiole angle. Brz is an inhibitor of cytochrome CYP450 function specifically in the steroid synthesis pathway (Asami et al., 2000). Its application resulted in reduced petiole angles in both control treatment and upon ethylene treatment. In addition, the ethylene-induced increase in petiole angle was significantly reduced by Brz (Fig. 6, $P < 0.05$). These results indicate that presence of BR modulates ethylene-induced hyponastic growth. To assess whether BR levels change in response to ethylene exposure in petioles, the concentrations of brassinolide and its direct precursors – castasterone and typhasterol – were measured in the petioles, after 6 h of ethylene exposure and in control conditions. No measurable difference in any of the active BRs was observed (Fig. S2) which indicates that total BR levels do not change upon ethylene treatment and that the control of ethylene-induced hyponastic growth by BRs cannot be attributed to putative ethylene-mediated effects on BR synthesis via ROT3. Accordingly, ethylene did not induce transcription of neither $ROT3$ nor another BR biosynthesis-related gene, $BRASSINOSTEROID-6 OXIDASE (BR6OX)$ (Fig. 7A–C). In fact, 3 and 6 h after start of ethylene exposure,
the expression of *ROT3* and *BR6OX* declined. This was significant for *ROT3* in quarter 2, which corresponds with the abaxial side proximal to the rosette where hyponastic growth is induced (Polko et al., 2012b). Since BR application to petioles in absence of ethylene does not affect petiole angles (data not shown) or general petiole growth, this study tested whether ethylene is capable of interfering with BR responsiveness/sensitivity using an alternative system that involves cell expansion: ethylene-induced hypocotyl elongation in light-grown *Arabidopsis* seedlings (Smalle et al., 1997). A range of 24-epiBL concentrations was applied to the medium and the effects on hypocotyl elongation under control and high ethylene conditions were examined. In addition, to separate effects of ethylene on biosynthesis from effects on BR responsiveness, a Brz treatment was included to inhibit endogenous BR biosynthesis. 24-epiBL application stimulated hypocotyl elongation in a concentration range between 0.01 and 1 µM, but was much more effective in the low concentration range, between 0.001
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and 0.1 µM, in the presence of ethylene as compared to control air. Application of Brz alone substantially inhibited hypocotyl elongation in ethylene-exposed seedlings, which suggests a role for brassinosteroids in this process (Fig. 8, De Grauw et al., 2005), consistent with observations described earlier for hyponasty. Exogenous 24-epiBL eliminated this response leading to comparable lengths of hypocotyls in ethylene and ethylene in presence of Brz.

**Discussion**

Hyponastic growth induced by ethylene is driven by local longitudinal expansion of cells at the abaxial side of the petiole (Polko et al., 2012b). The current report presents a modulating role of ROT3 and, tentatively brassinosteroids, in this system. This work was initiated by the identification of line *ddd1*, a mutant in the BR-associated gene *ROT3* (Polko et al., 2005).

**Fig. 4.** Mutations associated with the *ROT3* locus. (A) Schematic illustration of the insertion sites within the *ROT3* locus. (B) Hyponastic growth phenotypes in *rot3* mutants. Data points represent means ± SE of petiole angles (*n* = 15). Asterisks represent significant differences between mutants and Col-0 (*P* < 0.05). (C–D) The phenotypes of *ddd1* (C), *rot3-1* (D), and F1 of a cross between the two lines (*ddd1* and *rot3-1*) (E).
Polko et al. (2012a), which has profoundly reduced hyponastic responses and lacks differential cell expansion in response to ethylene treatment. Most described BR-related mutants, including det2 (Chory et al., 1991), cpd (Szekeres et al., 1996), most bri1 alleles (Clouse et al., 1996), and bin2-1 (Kim et al., 2009), exhibit severely hampered development with hardly distinguishable aerial organs and are therefore unsuitable for studies on hyponastic leaf growth. Although ROT3 has been shown to be involved in BR biosynthesis (Ohnishi et al., 2006), the rot3 phenotype is not nearly as severe as that of the mutants mentioned above. This relatively mild phenotype of ddd1 and rot3, however, enabled the study a putative BR-ethylene interaction during hyponastic growth, which would have been impossible using other dwarfed BR-deficient mutants.

Early publications discussing the function of ROT3/CYP90C1 mostly focused on its role in regulating polar elongation of cells (Kim et al., 1998) and shape control of leaves and flower organs (Kim et al., 1999). Later work by Bancos et al. (2002) indicated that ROT3 is involved in BR biosynthesis. ROT3 catalyses the conversion of typhasterol to castasterone (Kim et al., 2005; Ohnishi et al., 2006). Castasterone is a direct precursor of brassinolide and serves as a biologically active form of BR (Grove et al., 1979; Suzuki et al., 1993). In

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**Fig. 5.** Inhibition of hypocotyl elongation in darkness by 1-aminocyclopropane-1-carboxylic acid (ACC). Data points represent mean hypocotyl length of Col-0 and rot3-1 seedlings (n = 70–120). Asterisks indicate significant differences between the two treatments (P < 0.05).

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**Fig. 6.** Effect of brassinazole on petiole hyponasty of Col-0 in ethylene. (A) Data points show means ± SE of petiole angles (n = 15). Asterisks indicate significant differences (P < 0.05) between brassinazole and mock treatment. (B, C) Visual appearance of plants after mock (B) and brassinazole (C) treatment (this figure is available in colour at JXB online).
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Fig. 7. brassinosteroid-related gene expression upon ethylene treatment. (A) Schematic representation of petiole quarters used for studies of gene expression. Petioles were divided into four quarters and expression measurements were performed separately for each quarter. (B, C) Expression of ROT3 (B) and BR6OX (C) after 3 and 6 h of ethylene exposure. Data points represent the fold change of gene expression (mean ± SE) after 3 and 6 h of ethylene exposure relative to the control conditions and relative to β-Tubulin-6. Asterisks indicate significant differences between results from ethylene and control treatments (P < 0.05) (this figure is available in colour at JXB online).

Fig. 8. hypocotyl elongation in ethylene in the presence of different concentrations of brassinolide with ethylene and control treatment. Data points show hypocotyl lengths of Col-0 seedlings (n = 40–50). brz, brassinizole.

contrast to Kim et al. (2005), who did not detect changes in BR levels in rot3cyp90c1 mutants, Ohnishi et al. (2006) generated convincing biochemical data indicating that CYP90C1 and CYP90D are required for the conversion of typhasterol to castasterone.

Previously characterized rot3 mutants showed an identical phenotype to ddd1, consisting of shorter petioles and relatively large leaf laminas. Kim et al. (1998, 1999) showed that ROT3 is responsible for polar elongation of cells, specifically in leaves and flower organs. Mutant rot3-1 displayed a significant and similar reduction in the hyponastic response as ddd1. One of the explanations for the decreased hyponasty in ddd1 is that its distinctive compact phenotype could mechanically interfere with the upward petiole movement. The finding that, upon spectral shade treatment ddd1 has a response similar to wild-type plants, suggests that reduced hyponasty is unlikely due to biophysical constraints. Therefore, it is proposed that the reduced hyponasty in ddd1 is more likely a consequence of interference with the signal transduction pathway from ethylene towards differential petiole growth. Because hyponasty in ddd1 is not completely absent, this study cannot exclude that minor changes in cell expansion dynamics still occurred, beyond the resolution of the measurement, which could be responsible for the residual movement.

Importantly, rot3-1 does not display a general difference in sensitivity to ethylene, since inhibition of hypocotyl elongation induced by the ethylene precursor ACC in dark was nearly identical between rot3-1 and Col-0. The fact that hypocotyl shortening at ACC concentrations ≥1 μM is the same in rot3-1 and Col-0 suggests that the inhibition effect reaches saturation. These data indicate that ROT3 is a positive regulator of ethylene-induced hyponastic growth, by mediating cell expansion tentatively via BR action, downstream of ethylene signalling. Indeed, pretreatment with Brz,
a chemical inhibitor of castasterone biosynthesis (Asami et al., 2000) reduced hyponasty, confirming that BR synthesis might contribute to the occurrence of hyponasty in ethylene-enriched environments. Since Brz had a negative effect on petiole angles at the start of the experiment, it is likely that it affects the circadian or diurnal leaf movements. The fact that the reduction of hyponasty was not complete in ddd1 and rot3-1 suggests that either BR biosynthesis was only partially inhibited by Brz or that other regulators are also involved. Many studies have demonstrated a synergistic relation between BRs and auxin (reviewed in Halliday, 2004). It is therefore possible that a crosstalk between these two hormones exists in the hyponastic response in Arabidopsis. Since this study did not detect any changes in BR levels upon ethylene exposure, it is possible that ethylene either induces their production in a site-specific manner or that it stimulates responsiveness to BRs rather than their biosynthesis. Importantly, this study found that ethylene application sensitizes the capacity of brassinolide-mediated cell expansion, since low brassinolide levels (0.001–0.01 μM) stimulated elongation in ethylene while being ineffective in control plants. The finding that BR may be involved in ethylene-induced hyponasty is consistent with earlier studies on dark-grown seedlings which demonstrated that apical hook formation in presence of ethylene also requires a functional BR pathway (De Grauwe et al., 2005). Despite the finding that BRs modulate the hyponastic growth response induced by ethylene, the region in which cell expansion occurs during hyponasty exhibited significantly lower expression of ROT3 and slightly reduced levels of BR6OX transcripts already after 3 h in ethylene. It is plausible that this reduction is an outcome of a negative feedback loop between increased levels of BRs, which would result in a decline in transcript abundance of genes involved in their biosynthesis. This is in agreement with a study by Tanaka et al. (2005), which illustrated a tight negative feedback regulation of BR homeostasis by expression dynamics of BR metabolism-related genes. Exogenous application of brassinolide resulted in a substantial decrease in transcript levels of ROT3 and BR6OX together with other BR biosynthesis genes such as DWARF5 (DW5) or CONSTITUTIVE PHOTOMORPHOGENIC DWARF (CPD) in Arabidopsis seedlings already after 2 h of hormone treatment. Even though the molecular mechanism of this negative feedback loop has not been fully recognized, a downstream component of BR signalling, BRASSINAZOLE-RESISTANT1 (BZR1) has been shown to bind to cis-regulatory elements of CPD and DWF4 (He et al., 2005), thereby negatively regulating their expression (Kim et al., 2006). Taken together, these data provide evidence for a positive role of BRs in the regulation of ethylene-induced hyponastic growth. Consistent with this conclusion, BR signalling has been shown to be crucial for organ growth by regulating cell expansion in the epidermis (Savaldi-Goldstein et al., 2007), where also hyponasty is likely controlled (Polko et al., 2012b). Future studies should elucidate what are the molecular mechanisms behind ethylene–BR interaction and whether ethylene might promote a local, tissue-specific synthesis of BRs.

Supplementary material
Supplementary data are available at JXB online.
Supplementary Methods S1.
Supplementary Table S1. Primer sequences used for PCR.
Supplementary Fig. S1. Kinetics of Col-0 and ddd1 petiole angle in low light and high temperature.
Supplementary Fig. S2. Quantification of BR levels in petioles at the start of the experiment and after 6 h with control and ethylene treatment.

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