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Involvement of COP1 in ethylene- and light-regulated hypocotyl elongation

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Abstract Ethylene and light act through specific signal transduction mechanisms to coordinate the development of higher plants. Application of 1-aminocyclopropane-1-carboxylic acid (ACC, an ethylene precursor) suppresses the hypocotyl elongation of *Arabidopsis* seedlings in dark, but stimulates it in light. However, the mechanisms of opposite effects of ethylene on hypocotyl elongation in light and dark remain unclear. In the present study, we investigated the key factors involved in the opposite effects of ethylene on hypocotyl elongation in *Arabidopsis* seedlings. The effects of ACC on hypocotyl elongation of IAA-insensitive mutants including *tir1-1*, *axr1-3,* and *axr1-12* seedlings were reduced in light but not in dark. The DR5 promoter, a synthetic auxin-response promoter, was used to quantify the level of IAA responses. There was a marked increase in DR5-GFP signals in response to ACC treatment in hypocotyls of *DR5-GFP* seedlings in light, but not in dark. CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) is an important downstream component of light signaling. ETHYLENE-INSENSITIVE3 (EIN3, an ethylene-stabilized transcription factor) directly regulates ETHYLENE-RESPONSE-FACTOR1 (ERF1). The *cop1-4* mutant treated with ACC and *cop1-4*/EIN3ox plants developed long hypocotyls in darkness. Expression of *ERF1* in the *cop1-4* mutant was induced by ACC treatment in dark, but the expression of *ERF1* in the wild type was not affected.

Taken together, ethylene-promoting hypocotyl via IAA is mediated by light, and COP1 has a significant impact on the transcription of some genes downstream of EIN3. Thus, COP1 plays a crucial role in the opposite effects of ethylene on hypocotyl elongation.

Abbreviations

ACC	1-Aminocyclopropane-1-carboxylic acid
COP1	CONSTITUTIVELY PHOTOMORPHOGENIC 1
HY5	LONG HYPOCOTYL 5
LMN	Low nutrient medium
EIN3	ETHYLENE-INSENSITIVE3
ERF1	ETHYLENE-RESPONSE-FACTOR 1
IAA	Indole-3-acetic acid
NPA	1-N-naphthylphthalamic acid
PIF1	PHYTOCHROME INTERACTING FACTOR 1

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Introduction

Light, as one of the most influential environmental factors, not only provides the source of energy for plant life, but also acts as a signal that affects plant growth and development throughout the entire life cycle (Smith 2000; Sullivan et al. 2003; Lee et al. 2007). A microarray analysis using *Arabidopsis thaliana* indicated that photomorphogenesis involves a regulation change in the expression of up to 30 % of the genes in its genome (Ma et al. 2001). Light signals are perceived by multiple photoreceptors and are transduced to downstream regulators, where they regulate the photomorphogenic development in a quantitative manner. Two key downstream components, CONSTITUTIVELY PHOTOMORPHOGENIC 1

(COP1) and LONG HYPOCOTYL 5 (HY5), act antagonistically in *Arabidopsis* seedling development (Osterlund et al. 2000). In darkness, COP1-regulated genes were estimated to account for 20 % of the gene content (Ma et al. 2002). The light repression of COP1 involves a quantitative reduction of the COP1 abundance in the nucleus depending on light intensity (von Arnim and Deng 1994). Multiple photoreceptors negatively regulate COP1 activity (Osterlund et al. 1999). COP1 may act as a putative E3 ubiquitin ligase within the nucleus, interacting directly with the transcription factor HY5 and targeting its degradation via the 26S proteasome (Osterlund et al. 2000; Schwechheimer and Deng 2000). Therefore, it is hypothesized that COP1 modulates gene expression by targeting key transcription factors for degradation in the nucleus (Yamamoto et al. 1998). The gene expression profile of dark-grown *cop1* mutants is very similar to that of light-grown wild-type seedlings (Ma et al. 2002). Light can release COP1 from the nucleus, but the total amount of cellular COP1 does not change. Exclusion of COP1 prevents the degradation of the positive signaling factors, such as HY5 and HYH, and allows photomorphogenesis to occur (Osterlund et al. 2000; Holm et al. 2002). Thus, HY5 is believed to be one of the central modulators for the coordination of light signals and the regulation of appropriate gene expression (Lee et al. 2007). The phenotype of *hy5* seedlings includes defects in light inhibition of hypocotyl elongation and light-induced chlorophyll accumulation. The *cop1-4* seedlings develop short hypocotyls and expanded cotyledons in dark, whereas their hypocotyls are of the same length as the wild type in light (Ang and Deng 1994).

The phytohormone ethylene plays significant roles in physiological processes throughout the life cycle of plants. Molecular and genetic analyses have revealed many details of ethylene signaling pathways, from perception to transcriptional regulation (Li and Guo 2007). Ethylene is perceived by proteins encoded by a gene family containing five membrane-bound receptors, which can be divided into two types (ETR1, ERS1, and ETR2, ERS2, EIN4) (Chen et al. 2005). CTR1 inhibits various downstream signaling components including EIN2 and EIN3/EIL1. Once ethylene inactivates the receptor CTR1, ethylene signaling is activated (Li and Guo 2007; Zhu and Guo 2008). The abundance of the EIN3 protein rapidly increases in ethylene treatment. In the absence of ethylene, EIN3 is targeted by SCFEBF1/EBF2 complexes and degraded by the 26S proteasome (Potuschak et al. 2003; Guo and Ecker 2003; An et al. 2010). By binding to specific promoter elements (EBS, EIN3 binding sites), EIN3 regulates the expression of primary target genes, such as ERF1, which bind to secondary target genes and lead to changes in the morphological phenotype. Overexpression of ERF1 can rescue only a subset of *ein3* phenotypes, suggesting that EIN3 regulates other target genes to mediate ethylene responses (Solano et al. 1998). Ethylene-responsive element binding factors (ERFs) are members of a novel family of plant-specific transcription factors. Five different ERF proteins have been described (gene products of *AtERF1* to *AtERF5*). The *AtERF* genes are differentially regulated by ethylene (Fujimoto et al. 2000).

Light and ethylene signals can be coordinated to affect the formation of the apical hook in *Arabidopsis* seedlings (Lehman et al. 1996). A well-known example of light and ethylene interaction is *hookless1*. The *hookless1* (*hls1*) mutant was initially isolated as one that could not form an exaggerated hook under ethylene treatment (Lehman et al. 1996). The level of HLS1 protein is increased in response to ethylene treatment in etiolated seedlings of the wild type and decreased upon light exposure, even in the presence of the ethylene precursor, ACC (Li et al. 2004). Both light and ethylene pathways function in this interaction, but the individual roles and the cross talk between them need to be further studied. The mechanisms of ethylene and light interaction are still a mystery. The most typical and research-focused ethylene response is the triple response, in which dark-grown seedlings under ethylene treatment exhibit shortened hypocotyls and roots, thickened hypocotyls, and an exaggerated apical hook. However in light, ethylene induces marked elongation of the hypocotyl of *Arabidopsis* seedlings (Smalle et al. 1997). It is still unknown how and why ethylene has opposite effects on the elongation of *Arabidopsis* hypocotyls in dark and light. In this study, the interaction of light and ethylene signals on the elongation of seedling hypocotyls was investigated. We found that ethylene affected hypocotyl elongation by two distinct pathways in the light and in dark. Ethylene-promoting IAA pathway to elongate the hypocotyl is mediated by light. EIN3 elevates the expression of *YUCCA1* and *YUCCA5*, which are necessary for hypocotyl development (Cheng et al. 2007), and up-regulates IAA in promoting hypocotyl elongation in light. COP1 serves as a critical nuclear regulator to inhibit hypocotyl elongation induced by ACC. Here, we also provide evidence that EIN3 cannot induce high levels of *ERF1* transcripts in dark, probably because of COP1 in the nucleus.

Materials and methods

Plant materials and growth conditions

The *Arabidopsis thaliana* ecotype Columbia (Col-0) and ethylene-insensitive mutants, *etr1-3* and *ein2-1*, were provided by Prof. Ning Li (The Hong Kong University of Science and Technology). Arabidopsis seeds of cop1-4/EIN3ox, *EIN3ox* and *COP1ox* were provided by Prof. Hongwei Guo (Peking University). *Arabidopsis* seeds of *cop1-4*, *hy5*, *hy5hyh*, and *hyh* mutants were provided by Prof. Xingwang Deng (Peking University). *DR5::GUS*

seeds were provided by Prof. Ben Scheres (Utrecht University). *Arabidopsis* seeds of *tir1-1*, *axr1-3*, and *axr1-12* mutants were purchased from the Arabidopsis Biological Resource Center (Columbus, OH, USA). *DR5::GFP* seeds were purchased from the European Arabidopsis Stock Centre. Seeds were surface sterilized for 20 min in 20 % (v/v) commercial bleach and 0.1 % Triton X-100, rinsed four times with autoclaved distilled water, chilled for 2 days at 4 °C, and then sown on agar medium in Petri dishes. Seeds on agar plates were transferred to a growth chamber and maintained at 23 °C under continuous white light (approximately, 60–70 μmol m^{-2} s^{-1}) or in darkness for 7 days. All experiments were carried out using low nutrient medium (LMN) (Smalle et al. 1997), alone or supplemented with 1-aminocyclopropane-1-carboxylic acid (Sigma).

Hypocotyl length measurement

At indicated growth and treatment time points, at least 20 seedlings were laid horizontally on an agar plate and digital images were acquired. The hypocotyl length was measured using a standard 5-mm scaled ruler with the ImageJ software. The statistical significance of differences among mean values was analyzed using the SPSS 18.0 software.

Cell length measurement

Cells from the middle part of hypocotyls were photographed under a digital microscope. Cell length was measured using the ImageJ software with a 100-μm scaled ruler. A total of ten plants were measured for each treatment, and 20 cells were measured per plant.

Localization of DR5-GFP reporter proteins and quantitative analysis

Confocal laser-scanning micrographs were obtained with a Leica TCS SP2 AOBS under a 488-nm argon laser line for excitation of green fluorescent protein (GFP) fluorescence. Emissions were detected between 505 and 580 nm. Using a 20× water immersion objective (NA = 1.25, pinhole), confocal scans were performed with the pinhole at 1 Airy unit. Each image represents either a single focal plane or a projection of individual images taken as a z-series. The fluorescence intensity of the membrane DR5-GFP signal was quantified with the ImageJ software. At least ten seedlings were analyzed per treatment.

Histochemical GUS staining and quantitative fluorometric GUS assays

Whole seedlings ($n = 50$–100 seedlings), roots, and shoots were homogenized in extraction buffer as described elsewhere (Oono et al. 1998). After centrifugation to remove cell debris, the GUS activity in the supernatant was measured with 1 mM 4-methyl umbelliferyl β-D-glucuronide as the substrate at 37 °C. For the histochemical GUS assay, seedlings were washed three times with buffer A (100 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.5 mM $K_4Fe(CN)_6$, 0.5 mM $K_3Fe(CN)_6$, and 0.1 % Triton X-100) and then incubated in the staining buffer (buffer A containing 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc), the substrate for histochemical staining) at 37 °C until sufficient staining was developed.

Quantitative RT-PCR analysis

Total RNA was extracted from fresh tissues of 100 plants using Trizol reagent. The complementary DNA (cDNA) was then synthesized using the Takara Primescript RT Reagent kit (Takara) reverse transcriptase at 37 °C for 15 min. Then, quantitative real-time RT-PCR analysis (SYBR Premix, Takara) was carried out to analyze gene expression. The sequences of the PCR primers used in this study are listed in Table S1.

Statistical analysis

Each experiment was repeated at least three times. Values were expressed as mean $\pm$ SE. All comparisons were done using Student's *t* test for independent samples. In all cases, the confidence coefficient was set at 0.01.

Results

Opposite effects of ethylene on hypocotyl elongation

To confirm the effects of ethylene on the light-mediated hypocotyl elongation, we measured the hypocotyl length of *Arabidopsis* seedlings treated with different ACC concentrations under light and darkness. In the presence of 100 μM ACC, the hypocotyl length of Col-0 seedlings in light was approximately twice as long as those of the control (no ACC treatment). In contrast, in the presence of 10 μM ACC, the hypocotyl length of Col-0 seedlings in dark was approximately half of the control (Fig. 1). These results showed that, depending on the light condition, ethylene can induce opposite effects on the elongation of *Arabidopsis* hypocotyls. The sensitivity of ethylene in the hypocotyl elongation is different in light and dark.

It has been reported that high concentration of indole-3-acetic acid (IAA) promotes hypocotyl elongation in light (Smalle et al. 1997; Gray et al. 1998; Boerjan et al. 1995). To test whether ethylene affects the action of IAA in *Arabidopsis* hypocotyl elongation, we examined the
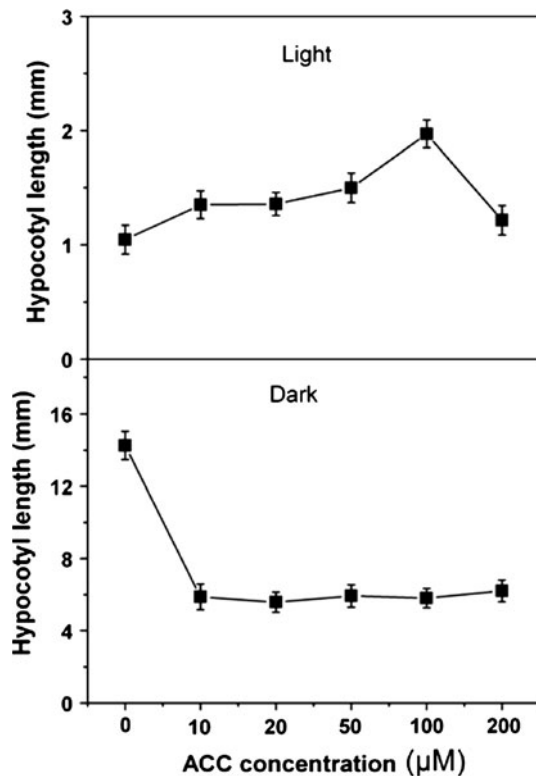
Fig. 1 Ethylene stimulates hypocotyl elongation in light, but suppresses it in dark. Col-0 seedlings were treated with different concentrations of ACC (0, 10, 20, 50, 100, or 200 μM) in continuous white light or darkness for 7 days. The hypocotyl lengths were measured as previously described. *Error bars* represent SE of the mean ($n = 20$)

response of IAA-insensitive mutants [*transport inhibitor response 1-1* (*tir1-1*), *auxin resistant 1-3* (*axr1-3*), and *auxin resistant 1-12* (*axr1-12*)] and ethylene-insensitive mutants [(*ethylene-response 1-3* (*etr1-3*) and *ethylene-insensitive 2-1* (*ein2-1*)]. The results showed that the hypocotyl elongation of *etr1-3* and *ein2-1* was not affected by ACC treatment in both light and dark (Fig. 2). This result demonstrated that ethylene signaling pathway is involved in this process. Meanwhile, the hypocotyl elongation of *tir1-1*, *axr1-3,* and *axr1-12* mutants in light was almost not affected by ACC treatment. However, the hypocotyl elongation of *tir1-1*, *axr1-3,* and *axr1-12* in dark was inhibited by ACC (Fig. 2). Taken together, these results suggest that ethylene affects the hypocotyl elongation via IAA in light, but not in dark.

Effect of ethylene on IAA in hypocotyl elongation is mediated by light

It has been reported that ethylene up-regulates IAA in roots (Ruzicka et al. 2007; Swarup et al. 2007). How ethylene mediates IAA in hypocotyl elongation is not clear. The DR5 promoter, which is composed of tandem elements
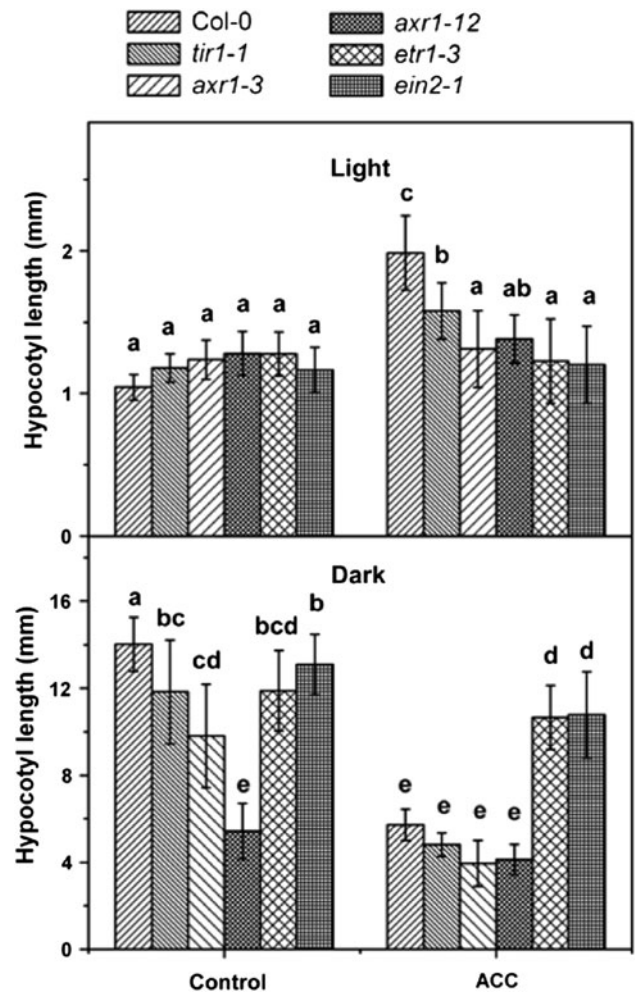

Fig. 2 IAA is required for the effects of ethylene on hypocotyl elongation in light. Untreated seedlings and those treated with 100 μM ACC (Col-0, *tir1-1*, *axr1-3*, *axr1-12*, *etr1-3*, and *etr2-1*) were grown for 7 days in constant white light at 22 °C or in darkness. Hypocotyl lengths were measured as previously described. *Error bars* represent SE of the mean ($n = 20$). Within each set of experiments, *bars with different letters* were significantly different at the 0.01 level

taken from the primary auxin-responsive GH3 promoter, can quantify the level of IAA responses (Ulmasov et al. 1997). As shown in Fig. 3a, b, ethylene induced DR5:GFP expression in the stele and epidermis in hypocotyls of light-grown *DR5:GFP* seedlings, but not in dark-grown seedlings. In particular, DR5-GFP was also induced by ACC in shoot meristems in light, but not in dark (Fig. 3a–c). Although a strong DR5:GFP signal was observed on the convex side of the apical hook, the GFP signals in the stele, epidermis, and shoot meristems of dark-grown seedlings remained weak. In light, the DR5:GUS staining in ACC-treated seedlings was virtually strong in shoots, especially in petioles, cotyledon tips, and shoot apex (Fig. 3d, e). In contrast, the staining pattern of DR5:GUS in dark indicates an auxin maximum in the middle of the hypocotyls (Fig. 3d). However, in the presence of ACC in dark,

Fig. 3 Effects of ACC on IAA accumulation in light or in darkness. **a** *DR5-GFP* seedlings treated or untreated with 100 μM ACC were grown for 7 days in constant white light or darkness at 23 °C. *Bar* 100 μm. **b** Relative fluorescence intensity of *DR5-GFP* in the seedlings treated as described in **a**. The fluorescence intensity of *DR5-GFP* seedlings in light was adjusted to 1. *Error bars* represent SE of the mean (*n* = 10). **c** *DR5-GFP* expression patterns in the shoot meristems of seedlings treated as shown in **a**. The image is a projection of individual images taken as a z-series of meristems. *Bar* 50 μm. **d** *DR5::GUS* seedlings treated as described in **a**. *Bar* 1 mm. **e** Quantitative determination of GUS activity in *DR5::GUS* lines treated as described in **a**. The GUS activity was determined fluorometrically by the fluorogenic substrate, 4-methyl umbelliferyl β-D-glucuronide. GUS activity in ACC-treated seedlings in dark was adjusted to 100 % value. **f** Col-0 seedlings treated as described in **a**, and shoots were collected from each treatment for RNA analyses by qRT-PCR
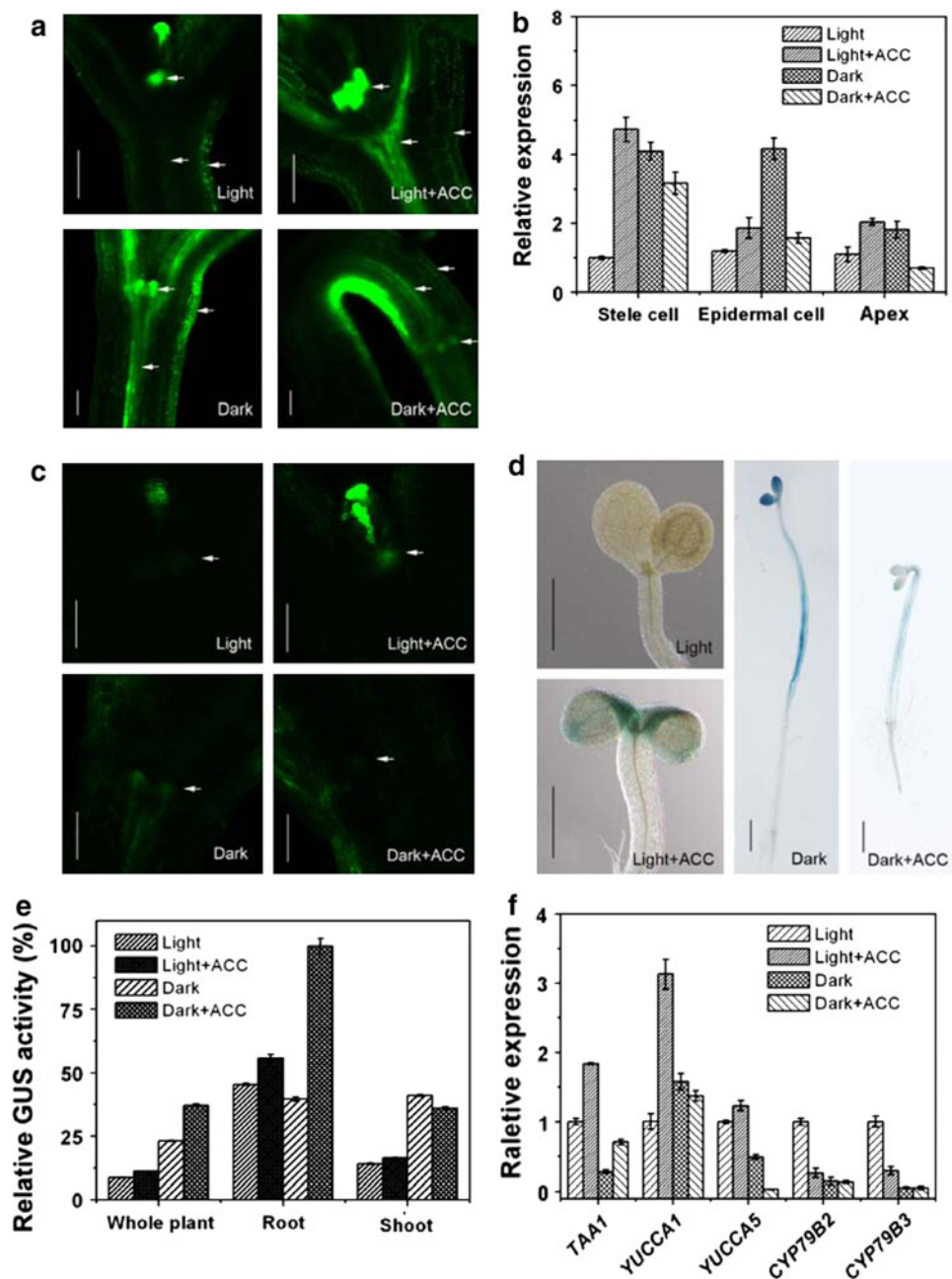

DR5:GUS exhibited obvious staining in the apical hook, but showed only a very faint signal in hypocotyls (Fig. 3d, e). In whole plants and roots, ACC up-regulated DR5:GUS, no matter the seedlings were light grown or dark grown (Fig. 3e). These results showed that the effect of ethylene on IAA in hypocotyl elongation is mediated by light.

To understand how ethylene mediates IAA, some key genes of main auxin biosynthesis pathways were investigated. *TAA1*, *YUCCA*, and *CYP79B2/B3* are the key genes of the IPA pathway, the YUC pathway, and the IAOx and glucosinolate pathway, respectively (Zhao 2010). Ethylene exerts opposite effects on the expression in some of the tested genes (Fig. 3f). Among them, the expression of

TAA1, *YUCCA1*, and *YUCCA5* was induced by ethylene in light. In dark, the expression of *TAA1* was up-regulated but the expression of *YUCCA1* and *YUCCA5* was down-regulated by ethylene (Fig. 3f). The expression of *CYP79B2* and *CYP79B3* markedly decreased in response to ACC treatment and a very low level of expression in dark was observed. Ethylene increased the level of IAA response in hypocotyls in light, but decreased it in dark (Fig. 3b, d). These results indicated that the changes in expression levels of *YUCCA1* and *YUCCA5* were consistent with the IAA level in hypocotyls.

To test whether ethylene also affects the IAA transport in hypocotyl elongation, we used the IAA polar transport

Fig. 4 Effects of ACC on IAA transport in darkness or in light. Col-0 seedlings (7-days-old) were grown under constant white light or in darkness on LNM or LNM supplemented with 100 μM ACC, 100 μM ACC + 1 μM NPA, or 1 μM NPA. **a** Hypocotyl lengths were measured as previously described. *Error bars* represent SE of the mean ($n = 20$). Within each set of experiments, *bars with different letters* were significantly different at the 0.01 level. **b** DR5-GFP expression pattern in plants treated as described above. *Bar* 100 μm. **c** Shoots were collected from each treatment for RNA analyses by qRT-PCR
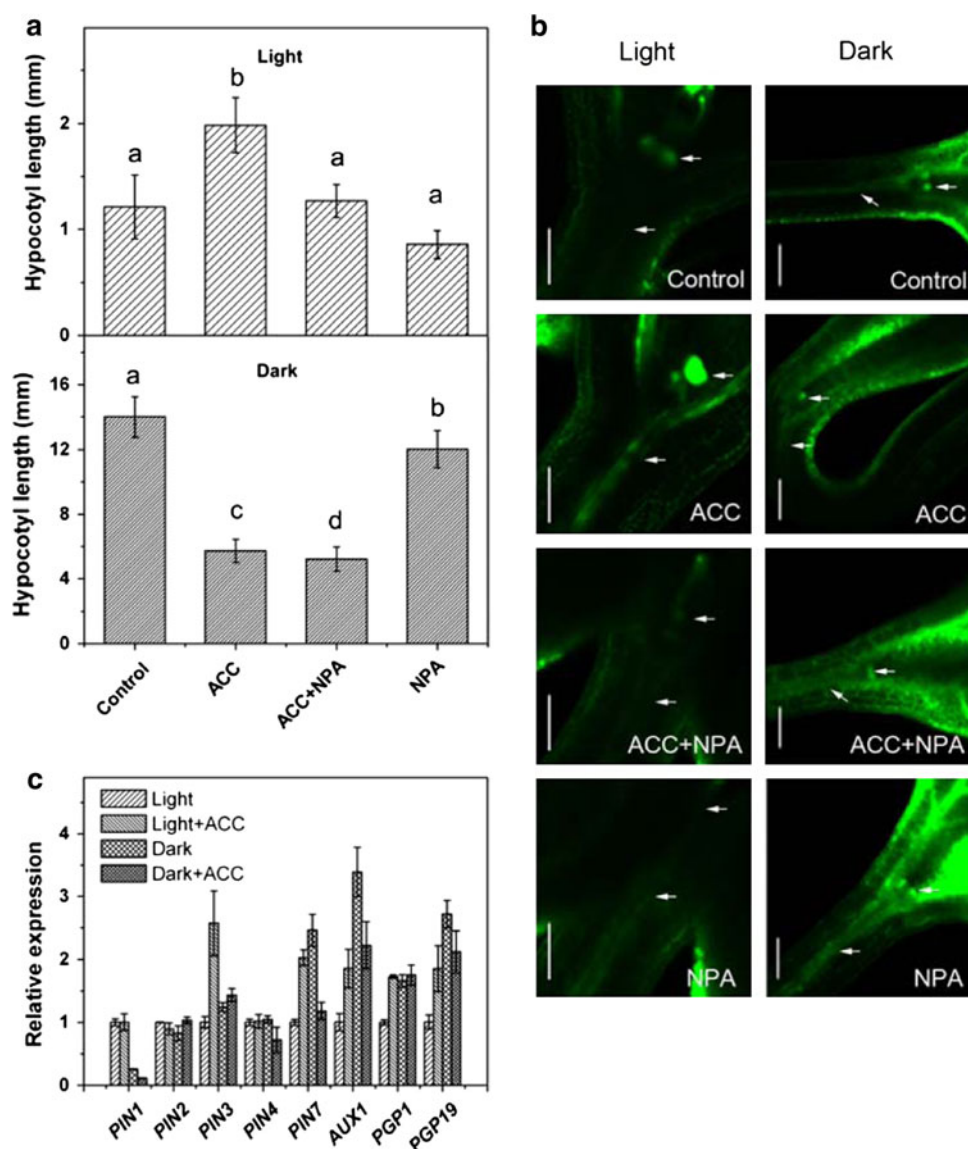
inhibitor 1-*N*-naphthylphthalamic acid (NPA). NPA reduced the ethylene-induced elongation of hypocotyls in light, but had almost no effects in dark (Fig. 4a). NPA had a significant impact on DR5-GFP signals in the stele and shoot meristems in light-grown seedlings. The result showed that NPA affected the IAA distribution in light. But in dark-grown seedlings, IAA distribution was not affected by NPA (Fig. 4b). NPA also inhibited IAA accumulation induced by ACC on the convex side of the apical hook in dark-grown seedlings. These results suggested that IAA transport might contribute to ethylene-induced hypocotyl elongation in light. We also analyzed the expression of some genes related to IAA transport. Ethylene had almost no effects on the expression of *PIN1*, *PIN2*, or *PIN4*. *PIN1* encodes the most important IAA transport protein. *PIN1* is almost not expressed in dark. The expression of *PIN7*,

AUX1, and *PGP19* was induced by ACC in light, but weakened in dark. The expression of *PIN3* and *PGP1* was strongly stimulated by ACC only in light-grown seedlings (Fig. 4c). These results indicated that the effect of ethylene on IAA transport is mediated by light in hypocotyls.

Taken together, these results demonstrated that the effect of ethylene on IAA biosynthesis, transport, and distribution in the hypocotyl was also mediated by light.

COP1 plays a key role in the opposite effects of ethylene on hypocotyl elongation

The *Arabidopsis HY5* gene has been defined as a positive regulator of photomorphogenesis. The *HY5* gene product is a basic leucine zipper-type transcription factor that is involved in a wide range of light-regulated gene expressions
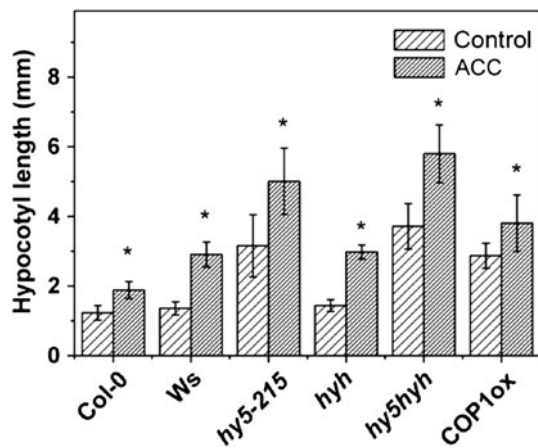
Fig. 5 HY5 has no effect on ethylene-induced hypocoytl elongation. Col-0, Ws, *hy5-215*, *hyh*, *hy5hyh*, and COP1ox seedlings treated or not treated with 100 μM ACC were grown for 7 days under constant white light at 22 °C. Hypocotyl lengths were measured as previously described. *Error bars* represent SE of the mean ($n = 20$). *Asterisks* denote significant differences ($P < 0.01$, according to Student's *t* test) between indicated ACC treatment and controls

(Chattopadhyay et al. 1998; Lee et al. 2007). To test whether the opposite effects of ethylene on hypocotyl elongation depend on light signaling, we used some mutants defective in light signaling. COP1 acts as a light-inactive repressor of photomorphogenic development and negatively regulates *HY5*. It has been shown that *hy5-215* exhibits a skotomorphogenic (etiolated) feature in light and *COP1ox* partly suppresses the photomorphogenic development (McNellis et al. 1994). On the contrary, the morphology of *cop1* seedlings in dark is the same as that of light-grown wild-type seedlings. In our study, ACC still promoted hypocotyl elongation of *hy5-215*, *hyh*, *hy5hyh*, and *COP1ox* in light (Fig. 5), but the extent of ACC-induced elongation decreased in *hy5-215*, *hy5hyh*, and *COP1ox*. The ACC-induced hypocotyl elongation in Col-0, *hy5-215*, *hy5hyh*, *hyh*, and *COP1ox* were 2.0-, 1.6-, 1.6-, 2.0-, and 1.3-fold, respectively.

The hypocotyl length of *cop1-4* mutant seedlings under ACC treatment was 2.0 times longer than that of the control in dark. In wild-type seedlings treated with ACC, the hypocotyl length was just half of the control in dark. Hypocotyl elongation appears to be caused by longitudinal cell expansion instead of cell division (Smalle et al. 1997; Gray et al. 1998). Therefore, we measured the cell length of mutant and wild-type seedlings under various treatments to confirm that cell expansion, and not cell division, is responsible for hypocotyl elongation. The length of longitudinal cell expansion and hypocotyls was consistent (Fig. 6k). These results indicated that COP1 is a key factor in ethylene-induced opposite effects on hypocotyl elongation of *Arabidopsis*.

Interaction of COP1 and EIN3 in hypocotyl elongation

COP1 regulates genes by directly interacting with transcription factors, such as HY5 and PIF (Feng et al. 2008). EIN3 is the most important transcription factor in the ethylene signaling pathway (Zhong et al. 2009). To elucidate whether COP1 interacts with EIN3, we first examined the phenotype of EIN3-overexpressing plants in Col-0 (*EIN3ox*) and *cop1-4* (*cop1-4/EIN3ox*) backgrounds. The hypocotyl length of *EIN3ox* plants was about 1.6-fold longer than that of the wild type in light, and about 0.5-fold shorter than that of the wild type in dark. The hypocotyl length of *cop1-4/EIN3ox* plants was still significantly longer than those of the *cop1-4* mutant in dark (Fig. 6j). These results showed that EIN3 plays a key role in the ethylene-affected hypocotyl elongation.

To know whether auxins are necessary in ethylene-triggered *cop1-4* hypocotyl elongation in dark, we studied whether NPA affects *cop1-4* mutant in dark. NPA significantly inhibited the hypocotyl elongation induced by ACC (Fig. 6l). The result indicated that ACC induced hypocotyl elongation in *cop1-4* dark-grown seedlings via IAA, and that IAA transport plays a role in this process. It is worth noting that NPA did not completely inhibit the ACC-induced hypocotyl elongation, but there was also a slight elongation of NPA–ACC-treated Col-0 in light (Fig. 4a). Thus, there are apparently some factors independent of auxins effective in the ACC-induced hypocotyl elongation.

Next, we analyzed the expression of *YUCCA1* and *YUCCA5*, two genes that promote IAA biosynthesis to affect hypocotyl elongation. As shown in Fig. 7, the expression of both *YUCCA1* and *YUCCA5* was enhanced in all ACC-treated seedlings and in *EIN3ox* plants that were not treated with ACC in light, but not induced in dark. Maybe due to the feedback inhibition, *YUCCA1* was inhibited in *EIN3ox* plants. These two genes were still induced in *cop1-4* plants treated with ACC or *cop1-4/EIN3ox* mutant seedlings untreated with ACC in dark. These results indicated that COP1 may regulate the transcriptional activity of *EIN3*. To confirm the role of COP1 in regulating the transcriptional activity of *EIN3*, we examined the expression of some direct downstream components of *EIN3*, including the positively regulated *ERF1* (Solano et al. 1998) and the negatively regulated *SALICYLIC ACID INDUCTION DEFICIENT2 (SID2)* (Chen et al. 2009). The expression of *ERF1* was elevated in all ACC-treated seedlings and in *EIN3ox* without ACC treatment in light, but was inhibited in dark (Fig. 7). Same as in Col-0 in light, *ERF1* was also induced in *cop1-4* by ACC and in the *cop1-4/EIN3ox* without ACC treatment in dark. The opposite expression pattern was observed in the expression of *SID2*. Its expression level in ACC-treated wild-type and *EIN3ox* seedlings was increased in dark, but
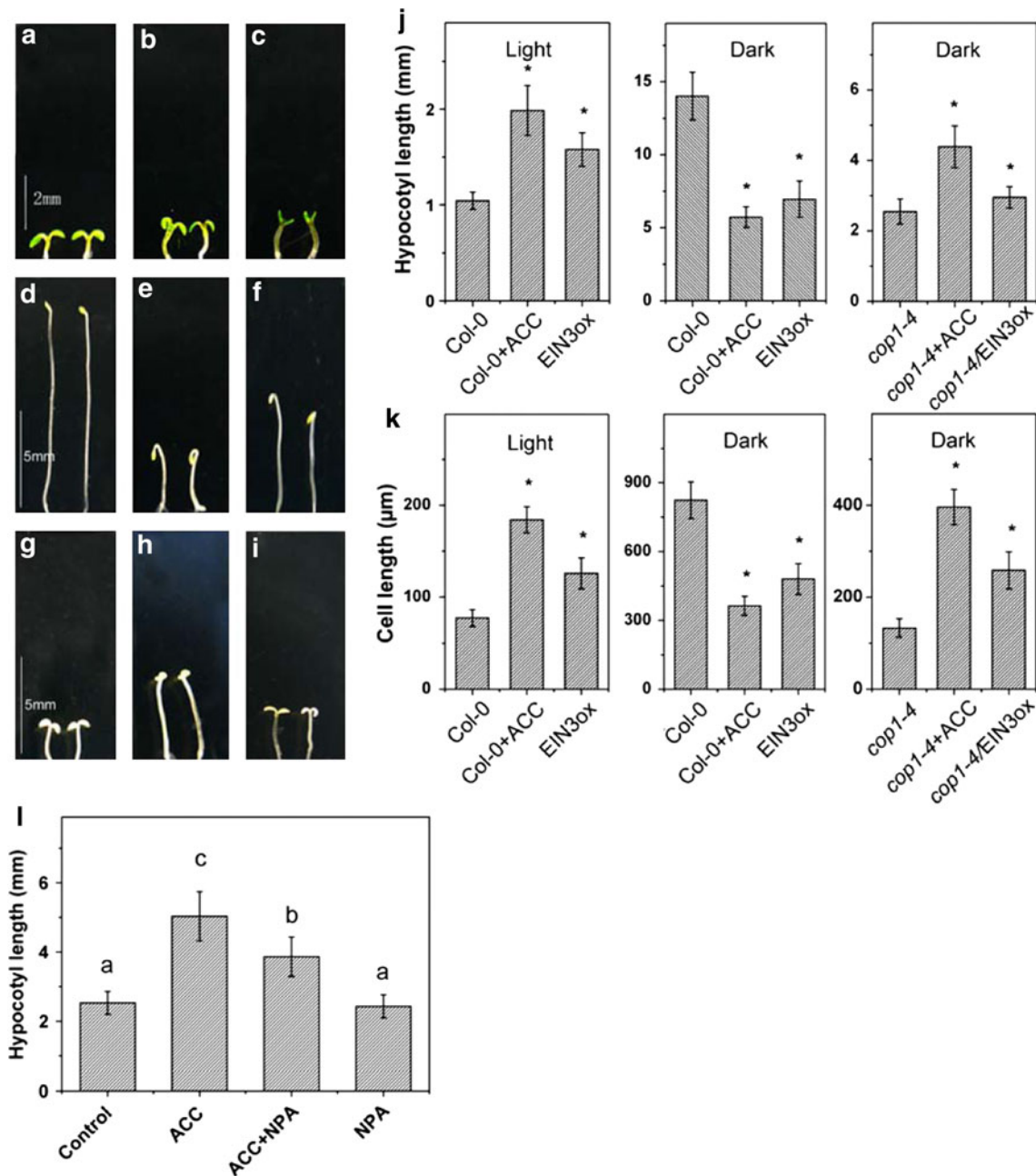
Fig. 6 COP1 may be a key factor in ethylene-induced opposite effects on hypocotyl elongation of *Arabidopsis*. **a–c** Col-0, Col-0 treated with 100 μM ACC, and *EIN3ox* in light. **d–f** Col-0, Col-0 treated with 100 μM ACC, and *EIN3ox* in dark. **g–i** *cop1-4*, *cop1-4* treated with 100 μM ACC, and *cop1-4/EINox* in dark. **j** Hypocotyl lengths were measured as previously described. *Error bars* represent SE of the mean (n = 20). *Asterisks* denote significant differences (P < 0.01, according to Student's t test) between indicated ACC treatment (or EIN3ox) and controls (23 °C). **k** Cell length was measured as previously described. *Error bars* represent SE of the mean (n = 50). *Asterisks* denote significant differences (P < 0.01, according to Student's t test) between indicated ACC treatment (or EIN3ox) and controls (23 °C). Images were captured under a digital microscope. **l** *cop1-4* (7-days-old) were grown in darkness on LNM, or on LNM supplemented with 100 μM ACC, 100 μM ACC + 1 μM NPA, or 1 μM NPA. Hypocotyl lengths were measured as previously described. *Error bars* represent SE of the mean (n = 20). Within each set of experiments, *bars with different letters* were significantly different at the 0.01 level

decreased in light. These results suggest that COP1 regulates the transcriptional activity of EIN3. To further test this hypothesis, we determined the time course of *ERF1* expression. The expression of *ERF1* in Col-0 treated with

100 μM ACC was significantly increased and reached a maximum at 1 h of treatment (to approximately 3.6-fold of the control) and then decreased in light (Fig. 8). However, in dark, the expression level of *ERF1* in Col-0 treated with
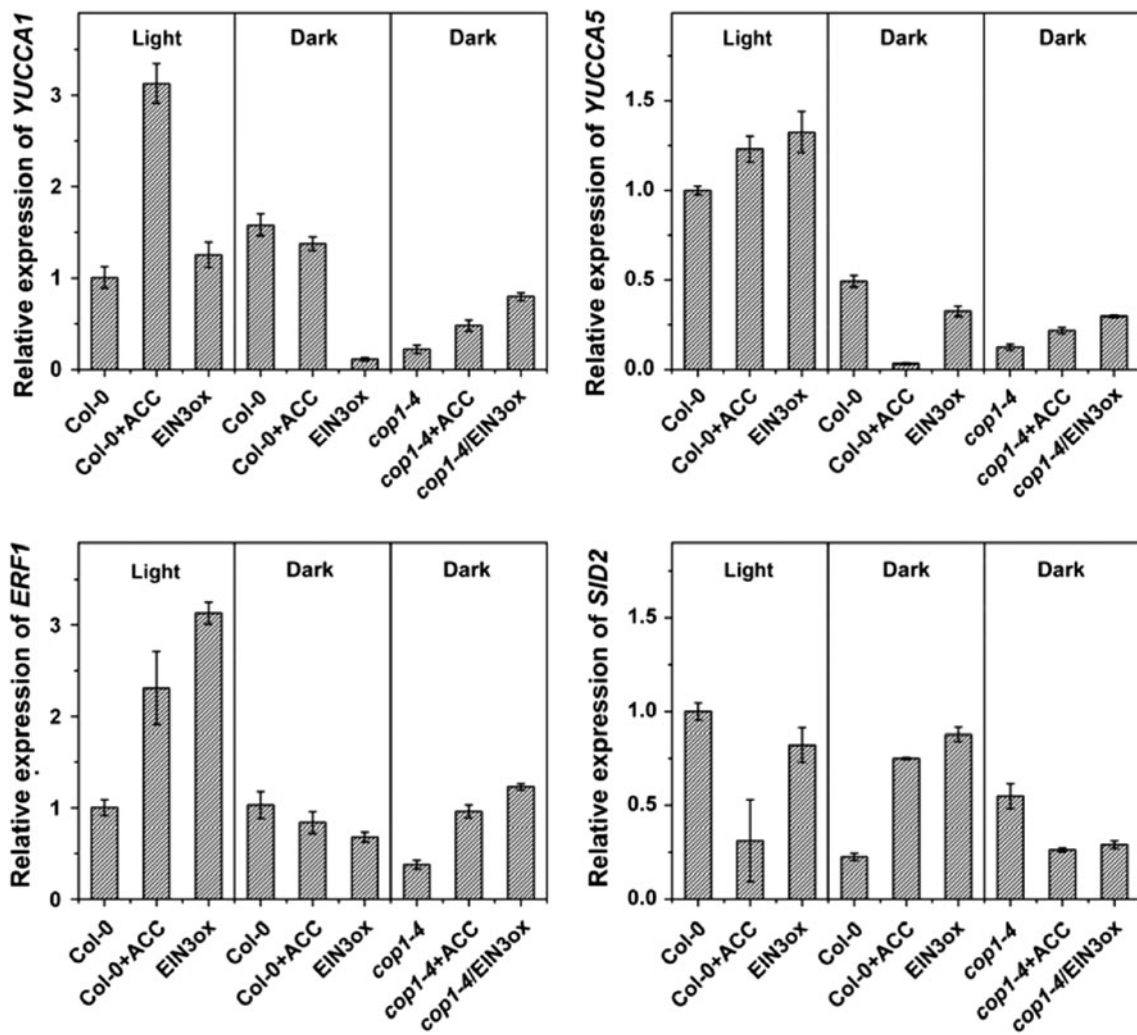
Fig. 7 Transcription of genes downstream of EIN3 is affected by COP1. Col-0 and mutant seedlings treated or not treated with 100 μM ACC were grown for 7 days in constant white light at 22 °C or in darkness. Shoots were collected from each treatment for RNA analyses by qRT-PCR

100 μM ACC decreased at 12 h of treatment (to approximately half of its expression level at 0 h). The expression pattern of *ERF1* in *cop1-4* in dark was similar to that of Col-0 in light, and a marked increase in the *ERF1* expression was observed at 3 h of treatment (Fig. 8). These results showed that COP1 also affects the transcription of EIN3 downstream genes.

Discussion

Hypocotyl elongation is a useful model for investigating the regulation of plant growth. Hypocotyl elongation is mainly controlled by environmental cues and hormones (Gray et al. 1998). This elongation is caused by cell expansion and is correlated with IAA levels (Davies 1995). Ethylene inhibits hypocotyl elongation in dark-grown *Arabidopsis* seedlings. In light, however, the ethylene
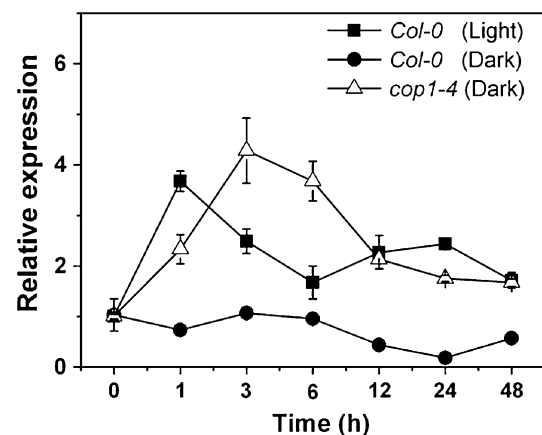

Fig. 8 COP1 inhibits ethylene-induced transcription of *ERF1*. Col-0 seedlings (7-days-old) were transferred to dark or light conditions and subjected to 100 μM ACC treatment for 1, 2, 6, 12, 24, and 48 h. Shoots were collected at each time point for RNA analyses by qRT-PCR

precursor ACC markedly induces hypocotyl elongation in *Arabidopsis* (Smalle et al. 1997). In the present study, we investigated the mechanism of the antagonistic effects of ethylene on hypocotyl elongation in light and dark, and provided evidence for the involvement of COP1 in this process.

The roles of auxin in hypocotyl development are different in light and dark. Auxin-overproducing mutant *sur1* and 35S::iaaM transgenic seedlings have long hypocotyls when grown in light, yet exhibit normal hypocotyl length in dark (Boerjan et al. 1995; Romano et al. 1995). Conversely, auxin-underproducing *iaaL* plants exhibit short hypocotyls in light, yet have normal hypocotyl length in dark (Romano et al. 1995). Together, IAA affects hypocotyl elongation in light, but has less effect in dark.

Ethylene influences hypocotyl growth in the respective pathways in light and dark. Under normal light conditions, such as 60–70 µmol m^{-2} s^{-1}, ACC promotes hypocotyl elongation (Saibo et al. 2003; Vandenbussche et al. 2007), but inhibits hypocotyl elongation under low light conditions, such as 40 µmol m^{-2} s^{-1} (Collett et al. 2000). In continuous normal light and darkness, our results demonstrated similar patterns (Fig. 1). These observations indicate that the role of ethylene on hypocotyl elongation is dependent on light intensity. *ACC-related long hypocotyl 1* (*alh1*) mutant has long hypocotyls in light, but normal length yet thicker hypocotyls in dark with an exaggerated apical hook (Vandenbussche et al. 2003). This proves that the effects of ethylene on hypocotyl elongation may be through two respective pathways in light and dark. In light, ethylene increases the level of IAA responsiveness and eventually results in hypocotyl elongation. However, ethylene reduces the level of IAA responsiveness in hypocotyls in dark. On the other hand, ethylene does not affect the hypocotyl length via IAA (Fig. 2), and the ACC inhibition of hypocotyl length may be due to ethylene responses. Because auxin transport is required for hypocotyl elongation in light-grown seedlings but not in dark-grown seedlings (Jensen et al. 1998), ethylene may induce hypocotyl elongation by promoting IAA transport in light. But in darkness, ethylene reduces the auxin transport to maintain the apical hook (Vandenbussche et al. 2010; Zadnikova et al. 2010). More detailed mechanism of ethylene function in hypocotyl elongation in light and dark needs to be studied.

YUCCA flavin monooxygenases are key enzymes in auxin biosynthesis. Overexpression of each *YUCCA* gene leads to auxin overproduction and long hypocotyls and cotyledons in light. Although disruption of a single *YUCCA* gene causes no obvious developmental defects, all of the triple and quadruple mutants of the four *YUCCA* genes (*YUCCA1*, *YUCCA2*, *YUCCA4*, and *YUCCA6*) show dramatically decreased expression of the auxin reporter

DR5-GUS in tissues where *YUCCA* genes are expressed (Cheng et al. 2006). *OsYUCCA* knockdown plants showed severe dwarfism, resulting from inhibited shoot elongation and inhibited root formation/elongation (Yamamoto et al. 2007). In the present study, we found that the high level of DR5:GFP expression and the long hypocotyl were associated with high levels of *YUCCA* expression (Figs. 3, 6, 7). Seedlings of the *yuc1yuc4yuc10yuc11* quadruple mutants lack hypocotyls (Cheng et al. 2007). *YUCCA* is expressed in the shoot apex and vascular tissues (Cheng et al. 2006, 2007). Perhaps due to the fact that the YUC pathway may be downstream of TAA1 (Stepanova et al. 2011), higher *TAA1* but lower *YUCCAs* were not associated with higher level of IAA responses in hypocotyls. TAA1 plays a more important role in response to ethylene in roots (Stepanova et al. 2008). However, CYP79B was not induced by ACC (Fig. 3f). Therefore, YUCCA may play a crucial role in ethylene-affected hypocotyl elongation.

EIN3 regulates the expression of ethylene-responsive genes. Upon ethylene treatment, the EIN3 protein is quickly stabilized and accumulates in the nucleus (An et al. 2010). In estradiol-inducible *EIN3-FLAG* transgenic plants, the accumulation of EIN3-FLAG fusion proteins was induced by estradiol in a dose-dependent manner. The expression level of *ERF1* (a direct target of EIN3) was similarly induced by estradiol in a concentration-dependent manner in light-grown seedlings (An et al. 2010). In the present study, the accumulation of EIN3 indeed activated the *ERF1* transcription in light. However, we observed that in wild-type plants treated with ACC and in seedlings over-expressing EIN3, the level of *ERF1* did not increase in dark (Figs. 7, 8). Furthermore, we also observed that the expression of *YUCCA1* and *YUCCA5* was not increased in ACC-treated wild-type and *EIN3ox* plants in dark (Fig. 7). These results demonstrated that the regulation of *EIN3* was altered by light.

COP1, an important component of the light signaling pathway, regulates the expression of *EIN3*. In dark, COP1-regulated genes are estimated to account for more than 20 % of the genome. The genes that are regulated by HY5 in light largely overlap with those regulated by COP1 in dark (Ma et al. 2002). The *hy5-215* mutant and *COP1ox* show a skotomorphogenic feature, and normal development of its hypocotyls could be promoted by ethylene in light (Fig. 5). However, *COP1ox* inhibited most of the elongation induced by ACC in light, and *cop1-4* was induced by ACC in dark. This shows that COP1 play an important role in this process and that other factors may take part in this process. Overexpression of EIN3 resulted in the elongation of the *cop1-4* mutant hypocotyls in dark (Fig. 6). Likewise, *YUCCA1* and *YUCCA5* were elevated on *cop1-4* treated with ACC and *cop1-4/EIN3ox*. These results indicate that the effects of light on ethylene
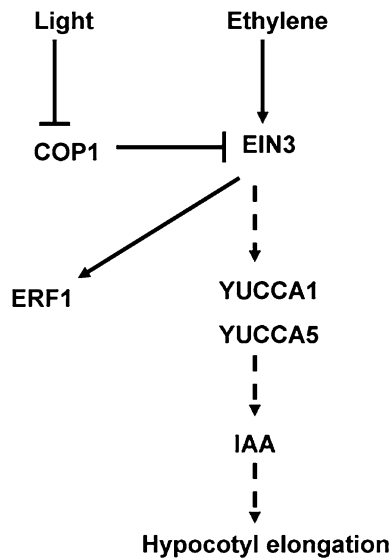
Fig. 9 Model illustrating the hypothetical cross talk of EIN3 and COP1

responses may be due to the interaction between COP1 and EIN3. COP1 can constantly degrade a number of transcription factors that are required for development in light, such as the bZIP transcription factors HY5 and HYH, but it allows accumulation of others that promote etiolated growth, such as PHYTOCHROME INTERACTING FACTOR 1 (PIF1), PIF3, and PIF4 (Alabadi et al. 2008). Similarly, COP1 positively regulates the accumulation of EIN3 protein, although the exact regulatory mechanism is yet to be identified (Zhong et al. 2009). COP1 may inhibit the transcription of *ERF1*, *YUCCA1*, and *YUCCA5* via its effects on EIN3 (Fig. 7). However, it is yet to be investigated whether the effects of COP1 on genes downstream of EIN3 are direct or indirect. In addition, the effects of ethylene on hypocotyl elongation and the quantitative reduction of the COP1 abundance in the nucleus are dependent on light intensity. COP1 not only regulates the amount of EIN3, but also significantly affects the transcription of EIN3 downstream genes.

Figure 9 shows a hypothetical model based on our results. In light, ethylene enhances the amount of EIN3 protein and induces the transcription of *YUCCA1* and *YUCCA5*, resulting in hypocotyl elongation via IAA. In dark, COP1 inhibits some genes downstream of EIN3, such as *ERF1*, *YUCCA1*, and *YUCCA5*.

In conclusion, the roles of ethylene in hypocotyl elongation are affected by light. In light, ethylene promotes the accumulation of IAA in hypocotyls, thus promoting hypocotyl elongation. In dark, ethylene inhibits hypocotyl elongation due to ethylene responses. EIN3 and COP1, as important components in the ethylene-signaling and light-signaling pathways, play important roles in the cross talk between these pathways. COP1 may modulate EIN3 and

affect the transcription of its downstream genes such as *ERF1*, *YUCCA1*, and *YUCCA5*. Thus, COP1 may be the key factor which determines the opposite effects of ethylene on hypocotyl elongation in dark and light.

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