EIN3/EIL1 cooperate with PIF1 to prevent photo-oxidation and to promote greening of *Arabidopsis* seedlings

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The ability to switch from skotomorphogenesis to photomorphogenesis is essential for seedling development and plant survival. Recent studies revealed that COP1 and phytochrome-interacting factors (PIFs) are key regulators of this transition by repressing the photomorphogenic responses and/or maintaining the skotomorphogenic state of etiolated seedlings. Here we report that the plant hormone ethylene plays a crucial role in the transition from skotomorphogenesis to photomorphogenesis by facilitating greening of etiolated seedlings upon light irradiation. Activation of EIN3/EIL1 is both necessary and sufficient for ethylene-induced enhancement of seedling greening, as well as repression of the accumulation of protochlorophyllide, a phototoxic intermediate of chlorophyll synthesis. EIN3/EIL1 were found to induce gene expression of two key enzymes in the chlorophyll synthesis pathway, protochlorophyllide oxidoreductase A and B (PORA/B). ChIP and EMSA assays demonstrated that EIN3 directly binds to the specific elements present in the *PORA* and *PORB* promoters. Genetic studies revealed that EIN3/EIL1 function in cooperation with PIF1 in preventing photo-oxidative damage and promoting cotyledon greening. Moreover, activation of EIN3 reverses the blockage of greening triggered by *cop1* mutation or far-red light irradiation. Consistently, EIN3 acts downstream of COP1 and its protein accumulation is enhanced by COP1 but decreased by light. Taken together, EIN3/EIL1 represent a new class of transcriptional regulators along with PIF1 to optimize de-etiolation of *Arabidopsis* seedlings. Our study highlights the essential role of ethylene in enhancing seedling development and survival through protecting etiolated seedlings against photo-oxidative damage.

chlorophyll synthesis | ethylene | photooxidation

The first step in the life cycle of a plant is seedling establishment after seed germination. Upon light irradiation, germinating seedlings undergo photomorphogenesis, including cotyledon opening and greening (i.e., chlorophyll biosynthesis), which allows seedlings to become photosynthetically competent and autotrophic (1). In darkness, a variety of chlorophyll precursors, including protochlorophyllide, pre-exist in the etioplasts. Once the cotyledon is exposed to light, the rate-limiting enzyme protochlorophyllide oxidoreductase (POR) is photoactivated and catalyzes the formation of chlorophyllide from protochlorophyllide, eventually leading to the synthesis of chlorophyll in plastids (2, 3). Three isoforms of POR have been identified in the Arabidopsis genome, i.e., PORA, PORB, and PORC, with PORA/B being the major iosforms in young seedlings (4, 5). Nonetheless, if protochlorophyllide cannot be converted promptly to chlorophyll, for instance, because of a lack or reduction of POR activity, it accumulates and produces large amounts of reactive oxygen species (ROS) upon light irradiation, causing photo-oxidative damage. Under this circumstance, cotyledon bleaching or even seedling death occurs (3, 5–7).

Light is the primary signal affecting the cotyledon greening process of etiolated seedlings. For instance, far-red light is known to block cotyledon greening by dramatically reducing the protein accumulation of PORA/B (8, 9). Despite its established role as a repressor of photomorphogenesis, COP1 was reported to be required for cotyledon greening, as etiolated *cop1* seedlings show extremely low levels of PORA/B accumulation and thereby are impaired in normal chlorophyll synthesis when exposed to light (10, 11). Recent studies revealed a critical role of phytochrome-interacting factors (PIFs) in modulating seedling photomorphogenesis, including cotyledon greening (12, 13). PIFs are a class of basic helix-loop-helix (bHLH) transcription factors that function as negative regulators of distinct phytochrome-mediated responses (14, 15). PIF1 and PIF3 were recently shown to facilitate seedling greening partly by repressing the accumulation of protochlorophyllide in the dark (12, 16, 17). In addition, PIF1 was found to induce *PORC* gene expression by directly binding to its promoter sequence (18). Consequently, the dark-grown *pif1* seedlings accumulate high amounts of protochlorophyllide but low levels of PORC, leading to photooxidation and bleaching of the cotyledons upon light exposure (16, 18).

Ethylene is a gaseous hormone that plays important roles in plant growth, development, and stress responses. Ethylene has been reported to enhance seedling development and cotyledon greening when plants are subjected to adverse conditions, such as high salinity or excess glucose (19–21). Molecular and genetic analysis uncovered a largely linear signaling pathway from hormone perception to transcriptional regulation in plant's responses to ethylene (22). Upon binding to its receptors, ethylene inactivates the receptor/CTR1 module and in turn alleviates its inhibitory effect on the downstream signaling components, which include EIN2 and EIN3/EIL1 (23, 24). EIN3 was identified as a plant-specific transcription factor and its protein level rapidly increases upon ethylene treatment. In the absence of an ethylene signal, EIN3 protein is targeted by $SCF^{EBF1/EBF2}$ complexes and degraded by the 26S proteasome (25, 26). By binding to specific promoter elements (EBS, *E*IN3 *b*inding *s*ites), EIN3 regulates the expression of many target genes, such as *ERF1*, which leads to changes in morphological phenotype (27).

Although ethylene is well known to regulate seedling growth and development in the etiolated condition (the "triple response"), a possible role of ethylene in modulating the de-etiolation process has not been identified. Here we present evidence to demonstrate that ethylene is essential for the proper establishment of green seedlings, and that EIN3/EIL1 represent a new class of regulators functioning in conjunction with PIF1 and COP1 to optimize de-etiolation of Arabidopsis seedlings.

Results

Ethylene Application Rescues the Greening Defect of *cop*-Like Seedlings. Previous studies revealed that overexpression of the CRY2 carboxyl-terminus (CCT2) or CRY2-GFP fusion protein results in

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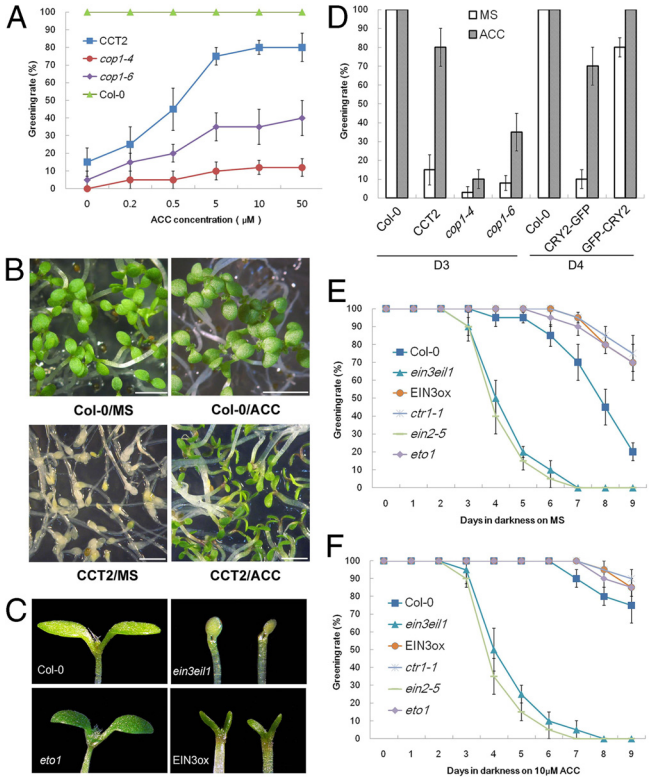

Fig. 1. Ethylene facilitates greening of etiolated seedlings via an EIN3/EIL1-dependent pathway. (*A*) Greening rate of 3-day-old etiolated seedlings followed by 2 days of white light irradiation (WL) on MS medium supplemented with indicated concentrations of ACC. (*B*) Greening rate of 3- or 4-day-old etiolated seedlings followed by 2 days of WL with or without 10-μM ACC treatment. Cotyledon phenotype of 3-day-old (*C*) or 5-day-old (*D*) etiolated seedlings followed by 2 days of WL. (*E* and *F*) Greening rates of various etiolated seedlings grown in darkness for indicated number of days before exposure to WL for 2 days on MS medium with or without 10 μM ACC. Error bars represent standard error of at least three independent experiments.

a *cop*-like phenotype in dark-grown seedlings (28, 29). Interestingly, when 3-day-old CCT2 or 4-day-old CRY2-GFP etiolated seedlings were transferred to white light, we found that most of their cotyledons were unable to turn green (Fig. 1 *B* and *C*). When CCT2 or CRY2-GFP seedlings were grown in darkness for 5 days or longer, none of the etiolated seedlings developed green cotyledons [supporting information (SI) Fig. S1]. Conversely, a GFP-CRY2 overexpressing line that failed to show a *cop*-like phenotype (29) exhibited a normal cotyledon greening process (Fig. 1*B* and Fig. S2). The same greening defect was also found in the *cop1* mutant (Fig. 1 *A* and *B* Fig. S2), as previously documented (10). These results indicate that dark-grown *cop*-like seedlings are impaired in cotyledon greening when exposed to light.

Ethylene was reported to facilitate cotyledon greening and seedling survival when seedlings are stressed by excess salt or glucose (19, 20). So we determined whether ethylene treatment could rescue the greening defect of *cop*-like mutants or transgenic plants. When grown on medium supplemented with 10 μM ACC (the ethylene biosynthetic precursor), CCT2 and CRY2-GFP etiolated seedlings were able to turn green normally (Fig. 1 *B* and *C*). ACC facilitated the cotyledon greening of CCT2 seedlings in a concentration-dependent manner (Fig. 1*A*). We also found that excess ACC treatment markedly rescued a weak *cop1* mutant allele (*cop1–6*), although the greening rate of a stronger *cop1* allele (*cop1–4*) was only slightly improved by ACC application (Fig. 1*A*).

EIN3/EIL1 Are Required for Ethylene-Induced Greening of Etiolated Seedlings. To understand how ethylene enhances cotyledon greening of etiolated seedlings, we investigated the greening process of various ethylene response mutants after extended periods of dark treatment. Without ACC treatment, the greening rate of wild-type seedlings gradually declined with increasing length of dark treatment (Fig. 1*E*). After growing in the dark for 9 days, more than 80% of wild-type cotyledons failed to turn green. Ethylene dramatically enhanced the greening of wild-type seedlings, as ≈75% of 9-day-old etiolated seedlings developed green cotyledons in the presence of ACC (Fig. 1*F*). Not surprisingly, two ethylene-insensitive mutants, *ein2* and *ein3eil1* (but not the *ein3* or *eil1* single mutant, Fig. S1), displayed remarkable reduction of cotyledon greening, regardless of ACC treatment (Fig. 1 *E* and *F*). On the other hand, two constitutive triple-response mutants, *ctr1*, *eto1*, and the EIN3-overexpressing transgenic line (EIN3ox), showed increased greening rate under prolonged dark conditions when compared with wild-type seedlings (Fig. 1 *D–F*). Taken together, these results demonstrate that ethylene enhances cotyledon greening of etiolated seedlings by activating a signaling pathway involving EIN3/EIL1.

Levels of Protochlorophyllide and ROS Are Elevated in *ein3eil1* Seedlings. Previous studies suggest that failure of seedling greening is attributable to the photo-oxidative damage that is associated with elevated accumulation of ROS in cotyledons (7). To investigate whether this is the case for *cop* or *ein* mutants that show a defect in cotyledon greening, we determined the levels of ROS in these mutants. We found that the levels of ROS, indicated by H_2DCFDA fluorescence, were remarkably higher in *ein3eil1*, *cop1*, and CCT2 seedlings than that in the wild type after light irradiation (Fig. 2*A*). By contrast, the accumulation of chlorophyll, indicated by autofluorescence, was pronounced in the cotyledons of wild-type but not *ein3eil1*, *cop1*, or CCT2 seedlings (Fig. 2*A*).

Because excessive accumulation of protochlorophyllide and/or deprivation of POR activity contribute to ROS accumulation and photo-oxidative damage (6, 7), we next asked whether the level of protochlorophyllide is regulated by the ethylene signal. With ACC treatment, the level of protochlorophyllide, measured by the relative fluorescence at 634 nm (16, 18), was dramatically reduced in wild-type seedlings (Fig. 2*B*). Although EIN3ox seedlings accumulated low levels of protochlorophyllide, *ein3eil1* seedlings displayed a relatively high accumulation. Thus, the accumulation of protochlorophyllide in etiolated cotyledons is inversely correlated with the greening phenotype of various ethylene response mutants. These results support the hypothesis that excessive accumulation of protochlorophyllide and the resulting ROS formation accounts for the failure of cotyledon greening observed in etiolated *ein3eil1* and *cop*-like seedlings (Fig. 4*C*).

***PORA* and *PORB* Are Direct Target Genes of EIN3.** To further investigate how loss of EIN3/EIL1 function leads to excessive accumulation of protochlorophyllide and ROS, we next determined whether EIN3/EIL1 regulate the expression of various chlorophyll biosynthetic genes. From the microarray data, a number of chlorophyll biosynthetic genes showed altered expression in *ein3eil1* mutant seedlings (Fig. 2*C*). Of particular interest among these genes, *PORA* and *PORB* were greatly induced by EIN3/EIL1. Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) assay confirmed that both *PORA* and *PORB* were induced by ethylene in an EIN3/EIL1-dependent manner (Fig. 2*D*). Bioinformatics analysis of the promoter regions of the two genes revealed two putative EIN3-binding sites (EBS) (30, 31) in each promoter (Fig. 2*F*). To test the possibility of whether EIN3 directly binds to the promoters of both *POR* genes, we carried out an EMSA assay using the recombinant EIN3 DNA-binding domain (DBD, amino acids 1–314). A wild-type fragment of *PORA* or *PORB* promoter sequence was specifically bound by EIN3 DBD, whereas a mutant version of the *PORA* promoter fragment that contains mutations in the putative EBS failed to associate with EIN3 (Fig. 2*E*). To further confirm the binding of EIN3 to the promoter

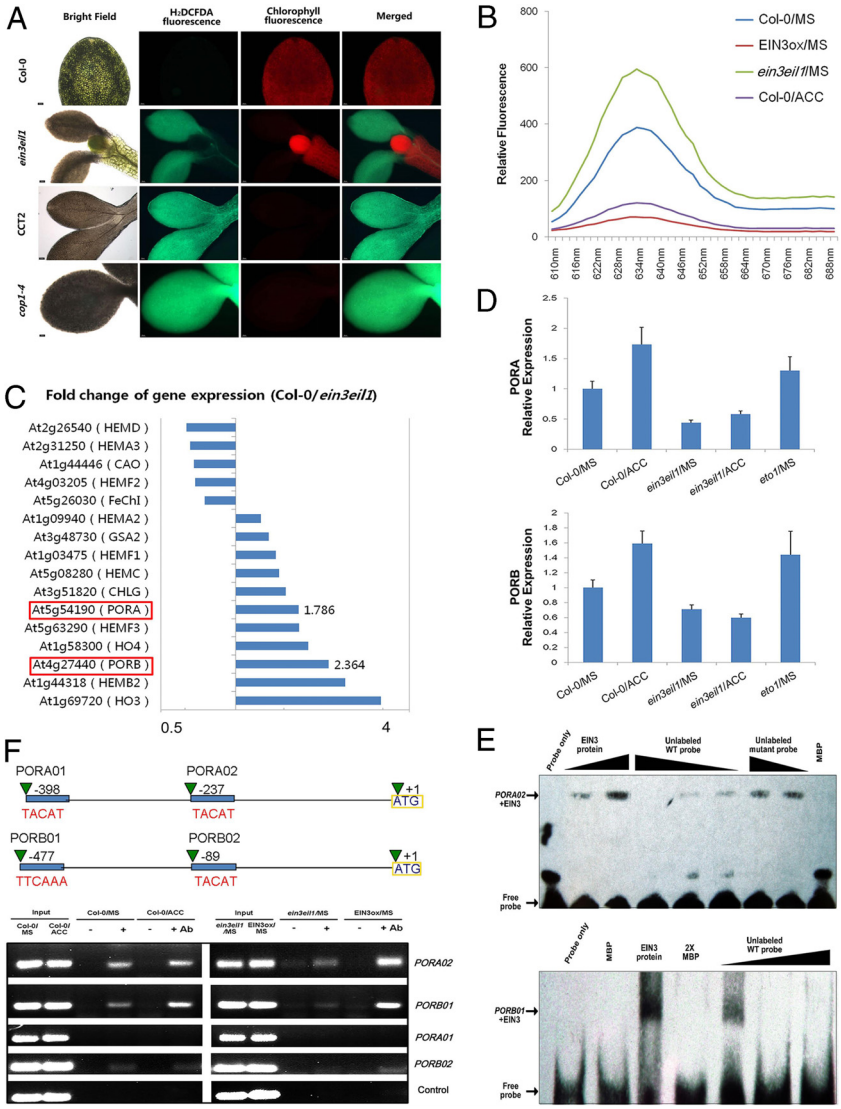

Fig. 2. EIN3/EIL1 are required for protecting seedlings from photooxidation by directly inducing the gene expression of *PORA/B*. (*A*) Fluorescence microscopy images of ROS (indicated by H_2DCFDA fluorescence) and chlorophyll fluorescence in the cotyledons of 4-day-old etiolated seedlings followed by 2 days of WL. (*B*) Relative fluorescence of protochlorophyllide in 3-day-old etiolated seedlings grown on MS medium with or without 10 μM ACC. (*C*) Relative expression levels of selected chlorophyll biosynthetic genes that are induced or repressed by EIN3/EIL1 based on microarray data. (*D*) Analysis of *PORA/B* gene expression by quantitative real-time RT-PCR of 4-day-old etiolated seedlings. (*E*) EMSA assays with recombinant EIN3 protein. Competition for the labeled promoter sequences was performed by adding 100-/200-/400-fold excess of unlabeled wild-type (WT) or mutant probes. (*F*) ChIP-PCR assays using 4-day-old etiolated seedlings. +/- Ab indicates chromatin immunoprecipitation with or without anti-EIN3 antibody. Two distinctive EIN3-binding sites were predicted in the promoter region of the *PORA* or *PORB* gene (PORA01/02, PORB01/02). Primers used for ChIP-PCR were specific to the promoter regions containing PORA01/02 or PORB01/02. Primers specific to the coding region of *PORA* were used as negative control. Input indicates samples before immunoprecipitation.

sequences of *PORA* or *PORB*, we conducted a chromatin immunoprecipitation (ChIP) assay using anti-EIN3 antibody. In wild-type seedlings, ACC treatment evidently enhanced the binding of EIN3 to the *PORB01* element and, to a lesser extent, to the *PORA02* element (Fig. 2*F*). Consistently, EIN3ox seedlings showed greatly increased binding of EIN3 to the *PORB01* or *PORA02* element compared with the minimal association in *ein3eil1* mutant seedlings. We also detected a weak binding of EIN3 to the *PORB02* element but not to the *PORA01* element. Combining the results of in vitro EMSA and in vivo ChIP assay, we conclude that *PORA* and *PORB* are direct target genes of EIN3.

EIN3/EIL1 Cooperate with PIF1 in Promoting Seedling Greening. PIF1 was recently shown to be a positive regulator of cotyledon greening by inducing *PORC* gene expression as well as inhibiting protochlorophyllide accumulation (18). As our data indicate that EIN3/EIL1 play a role similar to PIF1, we then assessed the genetic interaction between PIF1 and EIN3/EIL1. Upon light irradiation, the dark-grown *pif1ein3eil1* triple mutant displayed a severe greening defect phenotype manifested by completely bleached cotyledons, exhibiting an additive effect between the *pif1* and *ein3eil1* mutant (Fig. 3*A*). Quantitative data indicated that the *pif1ein3eil1* triple mutant showed a considerably lower greening rate than either *pif1* or *ein3eil1* mutant after 4 days of dark growth (Fig. 3*B*). Accordingly, the level of protochlorophyllide in *pif1ein3eil1* seedlings was extremely elevated when compared with either mutant (Fig. 3*E*). We also found that ACC treatment notably enhanced the greening rate of 5-day dark-grown *pif1* seedlings (Fig. 3 *C* and *D*). Consistently, the excess level of protochlorophyllide and ROS accumulation in dark-grown *pif1* seedlings was suppressed by ACC treatment (Fig. 3 *E* and *F*). Moreover, activation of ethylene signaling by either *ctr1* mutation or EIN3 overexpression remarkably salvaged the greening defect of the *pif1* mutant (Fig. 3 *C–E* and Fig. S3). Taken together, we conclude that EIN3/EIL1 and PIF1 act cooperatively in promoting cotyledon greening of etiolated seedlings, and that activation of EIN3 compensates for the loss of PIF1 function.

COP1 Positively Regulates EIN3 Protein Accumulation. Because the greening defect of *cop*-like CCT2 as well as a weak *cop1* allele (*cop1–6*) was noticeably rescued by ACC treatment (Fig. 1 *A* and *B*), we speculated that EIN3/EIL1 might function downstream of COP1 in regulating seedling de-etiolation. In support of this hypothesis, we found that overexpression of EIN3 in *cop1* (EIN3ox/*cop1*) resulted in light green cotyledons, which was in contrast with the white bleached cotyledons seen in *cop1* seedlings after 3 days of dark growth (Fig. 4*A*). The greening rate of EIN3ox/*cop1* was dramatically increased compared with that of *cop1* seedlings (Fig. 4*B*). Accordingly, ACC treatment or EIN3 overexpression reduced

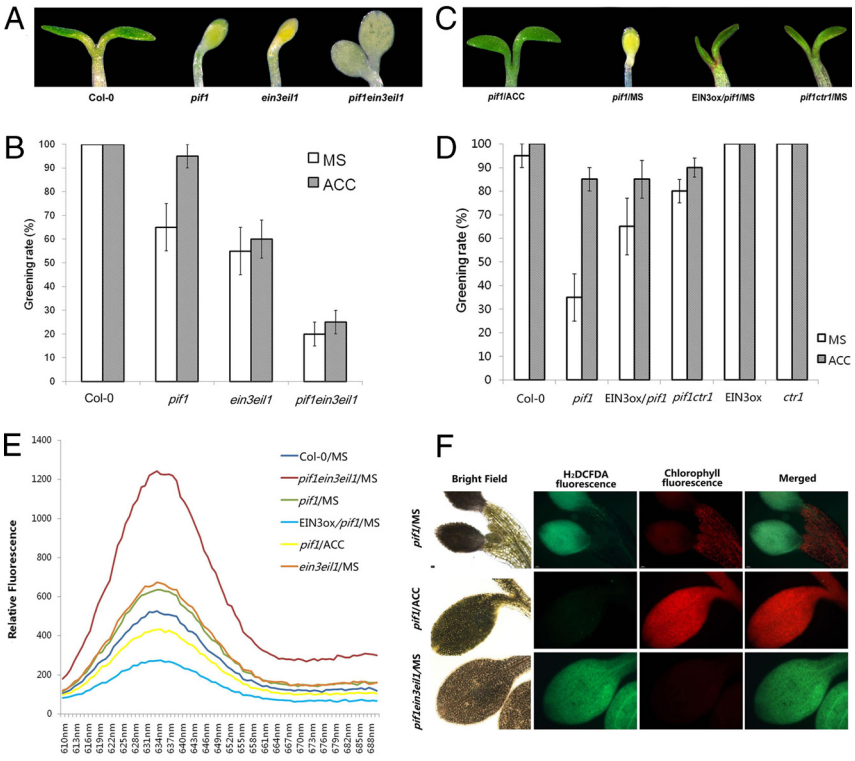

Fig. 3. EIN3/EIL1 act in cooperation with PIF1 in promoting cotyledon greening. (*A-D*) Cotyledon phenotypes and greening rates of 4-day-old (*A* and *B*) or 5-day-old (*C* and *D*) etiolated seedlings followed by 2 days of WL. Error bars represent standard error of at least three independent experiments. (*E*) Relative fluorescence of protochlorophyllide in 4-day-old etiolated seedlings. (*F*) Fluorescence microscopy images of ROS (indicated by H_2DCFDA fluorescence) and chlorophyll fluorescence in the cotyledons of 4-day-old etiolated seedlings followed by 2 days of WL.

the accumulation of protochlorophyllide in *cop1* seedlings (Fig. 4*C*). To further understand how EIN3 is regulated by COP1, we measured the levels of EIN3 protein in the *cop1* mutant as well as in the COP1-overexpression line (COP1ox), which showed an enhanced cotyledon greening phenotype (Fig. S1). Compared with the wild type, EIN3 protein accumulation decreased in *cop1* and CCT2 but slightly increased in COP1ox (Fig. 4*D*). Moreover, the level of EIN3 protein in EIN3ox markedly decreased when COP1 function was eliminated (Fig. 4*D*). Given that COP1 is repressed by light, we were not surprised to find that EIN3 protein level gradually diminished upon exposure to light while remaining constant in the dark (Fig. 4*E*). Taken together, our data show that EIN3 is a downstream effecter of COP1, which stimulates the cotyledon greening process at least partly by increasing EIN3 level.

Activation of EIN3 Rescues FR-Blocked Cotyledon Greening. It is well documented that FR light blocks cotyledon greening in a phyA-dependent manner. The FR-induced blockage is caused by the repression of PORA/B accumulation (8, 9). As our studies revealed that activation of EIN3 by ethylene was able to induce *PORA* and *PORB* gene expression and to partially rescue *cop1* defect, we investigated whether EIN3 activation could also reverse the FR-evoked suppression of cotyledon greening. We found that ACC application significantly improved the greening rate of wild-type seedlings after exposure to FR light for 2.5 days. Overexpression of EIN3 was sufficient to stimulate the greening process blocked by FR light, whereas the *ein2* or *ein3eil1* mutant showed hypersensitivity to FR (Fig. 4 *F* and *G*). These data indicate that activation of EIN3 initiates the cotyledon greening process that is blocked by FR- and phyA-mediated signaling.

Discussion

Ethylene Is an Essential Phytohormone for the Proper Establishment of Green Seedlings. In this study, we discovered that ethylene plays a pivotal role in protecting etiolated seedlings from photo-oxidative damage, thus enhancing cotyledon greening and seedling survival. Several lines of evidence support this finding. (*i*) Ethylene or ACC application remarkably increased the greening rate of wild-type seedlings after prolonged dark treatment. Consistently, the level of protochlorophyllide accumulation and ROS production was repressed by ACC treatment. (*ii*) Ethylene-insensitive mutants (*ein2* and *ein3eil1*) were defective in cotyledon greening, whereas mutant or transgenic plants in which the ethylene signaling pathway is constitutively activated (*ctr1*, *eto1*, EIN3ox) showed enhanced cotyledon greening of etiolated seedlings. Accordingly, protochlorophyllide accumulation was elevated in *ein3eil1* seedlings but reduced in EIN3ox seedlings. (*iii*) ACC treatment or EIN3 overexpression rescued the greening defect of *cop*-like mutants. (*iv*) ACC treatment or EIN3 overexpression rescued the greening defect of the *pif1* mutant. (*v*) ACC treatment or EIN3 overexpression reversed FR-induced suppression of cotyledon greening. (*vi*) EIN3 activated the expression of the *PORA* and *PORB* genes by directly binding to its cognate EBS sites in their promoter regions. Taken together, our data demonstrate that ethylene is a critical hormonal signal to promote cotyledon greening and to avert ROS-induced photobleaching by means of activating *PORA*/*B* gene expression and repressing protochlorophyllide accumulation.

When buried seeds germinate in the subterranean darkness, large amounts of ethylene gas are usually produced because of mechanical compression or blockage of the elongating seedlings. With the production of ethylene, an etiolated seedling normally develops exaggerated bending cotyledons to form a compact hook, a feature of the "triple response." This ethylene-evoked hook structure is thought to protect apical meristematic tissues residing between the two cotyledons when the seedling emerges from the soil (32). Our study revealed another feat of ethylene for germinating etiolated seedlings whereby ethylene protects cotyledons from photobleaching and facilitates chlorophyll biosynthesis when young seedlings are initially exposed to light irradiation. Therefore, ethylene is a plant hormone that is indispensable in the establishment of a green seedling during plant's transition from the etiolated state to photomorphogenic growth.

EIN3/EIL1 Work in Parallel with PIF1 to Induce *POR* Gene Expression and Repress Protochlorophyllide Accumulation. Recent studies have demonstrated that PIF1, a bHLH transcription factor, is a critical

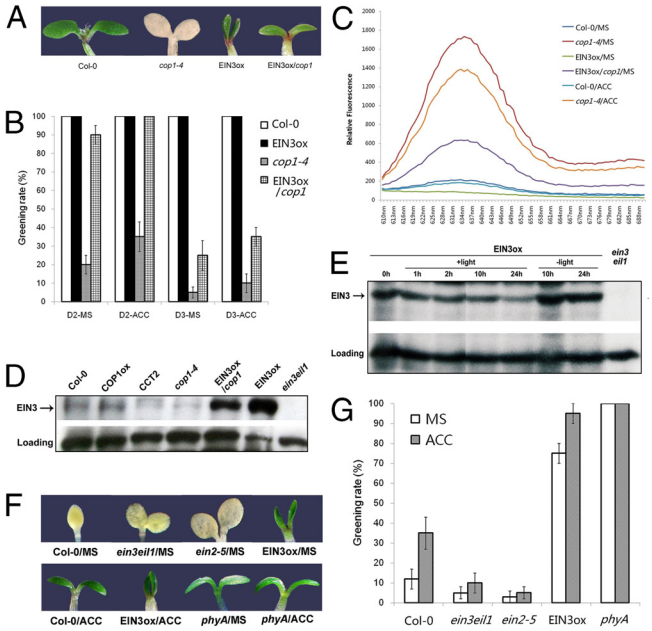

Fig. 4. EIN3 protein accumulation is positively regulated by COP1 but negatively regulated by light. (*A*) Cotyledon phenotype of 3-day-old etiolated seedlings followed by 2 days of WL. (*B*) Greening rate of 2- or 3-day-old etiolated seedlings followed by 2 days of WL. (*C*) Relative fluorescence of protochlorophyllide in 2-day-old etiolated seedlings. (*D*) Immunoblot assays of EIN3 protein in 4-day-old etiolated seedlings grown on MS medium. (*E*) Immunoblot assays of EIN3 protein in 4-day-old etiolated seedlings grown on MS medium with indicated hours of light or dark treatment. Cotyledon phenotype (*F*) and greening rate (*G*) of seedlings that were grown in far red light (FR) for 60 h followed by 36 h of WL. Error bars represent standard error of at least three independent experiments.

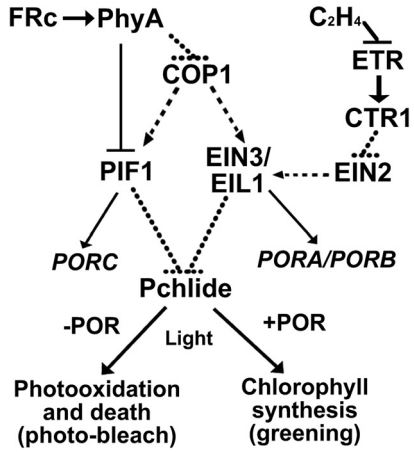

Fig. 5. A model on the action of EIN3/EIL1 and PIF1 in regulating cotyledon greening. EIN3/EIL1 and PIF1 are two classes of transcription factors that redundantly regulate chlorophyll synthesis and alleviate photooxidation of etiolated seedlings. EIN3/EIL1 and PIF1 are able to induce *POR* gene expression by directly binding to their promoters, and regulate a number of tetrapyrrole pathway genes to repress the accumulation of protochlorophyllide, a phototoxic intermediate in chlorophyll synthesis. Ethylene enhances seedling greening by activating EIN3/EIL1 via its canonical signal transduction pathway. Far-red light (FR) blocks seedling greening through PhyA-mediated direct inhibition of PIF1 or indirect repression of COP1, a positive regulator of EIN3/EIL1 and PIF1. Arrows and bars represent positive and negative regulation, respectively. Solid and dotted lines indicate direct and indirect regulation, respectively.

modulator of cotyledon greening of dark-grown seedlings (16). PIF1 promotes seedling greening in two ways: first, it represses the accumulation of protochlorophyllide by regulating the expression of a number of genes involved in the tetrapyrrole pathway; second, PIF1 directly binds to the promoter of *PORC* to activate its transcription, thus promoting the catalysis of protochlorophyllide into chlorophyll (18). In our study, we found that EIN3/EIL1 mediates the ethylene effect in promoting cotyledon greening in a similar manner: EIN3 (and probably EIL1 as well) directly binds to the promoters of both *PORA* and *PORB* (but not *PORC*, as *PORC* is not induced by EIN3/EIL1) genes to activate their expression. Furthermore, EIN3/EIL1 inhibit the accumulation of protochlorophyllide, probably by inducing *HO3*/*HO4* gene expression that helps switch the tetrapyrrole pathway into phytochromobilin synthesis. Interestingly, the greening defect of the *pif1* mutant was largely rescued by either direct ethylene application or activation of the ethylene signaling pathway via a *ctr1* mutation or EIN3 overexpression. Nonetheless, PIF1 was unable to regulate EIN3 protein level (Fig. S4), supporting a parallel function between PIF1 and EIN3. The co-action of PIF1 and EIN3/EIL1 was further reinforced by the additive effect between *pif1* and *ein3eil1* mutants, in which *pif1ein3eil1* triple mutant showed a completely bleached cotyledon phenotype reminiscent of *cop1* seedlings. These results indicate that PIF1 and EIN3/EIL1 are two distinctive families of transcription factors that work cooperatively to protect germinating seedlings from photo-oxidative damage upon light exposure.

COP1 Is a Positive Regulator of EIN3/EIL1 in Control of Seedling Greening. Although the *cop1* mutant displays a constitutive photomorphogenic response (open cotyledons, short hypocotyls and activation of light responsive genes), it is unable to initiate chlorophyll synthesis in cotyledons in the dark (33). Our results revealed that dark-grown *cop1* and *cop*-like seedlings failed to green due to elevated accumulation of protochlorophyllide and ROS formation in cotyledons upon light exposure. As ethylene application or EIN3 activation notably rescued the greening defect of *cop1*, EIN3 is believed to act downstream of COP1 and at least partly mediates its effect on promoting seedling greening. Furthermore, the *ein3eil1pif1* triple mutant showed a photobleached phenotype as severe as *cop1*, so it is conceivable that EIN3/EIL1 and PIF1 are the major transcription factors mediating COP1-regulated POR induction and cotyledon greening. Although COP1 is a RING-type E3 ubiquitin ligase that degrades a number of photomorphogenic transcription factors (e.g., HY5, LAF1, and HFR1) (34), COP1 seems to promote the stability of EIN3 protein. Similarly, COP1 was proposed to positively regulate the accumulation of PIF1 protein although the regulatory mechanism has yet to be identified (15). Therefore, being a negative regulator of photomorphogenesis, COP1 is also required for the normal accumulation of PIF1 and EIN3/EIL1, two distinctive classes of transcription factors essential for cotyledon greening of etiolated seedlings.

Taken together, we propose a model depicting the action of EIN3/EIL1 and PIF1 in the regulation of seedling greening (Fig. 5). In this model, EIN3/EIL1 and PIF1 act as key transcription factors that regulate chlorophyll synthesis and prevent photooxidation of etiolated cotyledons. Both EIN3/EIL1 and PIF1 activate *POR* gene expression in which EIN3/EIL1 directly bind to the promoters of *PORA* and *PORB* while PIF1 binds to the promoter of *PORC*. Meanwhile, these transcription factors also regulate a number of tetrapyrrole pathway genes to inhibit the accumulation of protochlorophyllide, a phototoxic intermediate in chlorophyll synthesis. Therefore, when EIN3/EIL1 or PIF1 function is missing, protochlorophyllide accumulation is high whereas POR levels remain low. The mutant seedlings consequently undergo photooxidative damage caused by excessive ROS accumulation upon illumination, culminating in severe photobleaching or seedling death when the functions of both EIN3/EIL1 and PIF1 are absent. Ethylene is thought to promote

seedling greening and survival by activating EIN3/EIL1 via a signal transduction pathway involving CTR1 and EIN2. COP1 is a positive regulator of EIN3/EIL1 and PIF1, probably by enhancing their protein accumulation in the cotyledon greening process. FR light activates phyA to inhibit PIF1 through direct molecular interaction, and at the same time to diminish COP1 activity via a possibly indirect mechanism. As a whole, FR-irradiated seedlings are expected to possess low activity of PIF1 and EIN3/EIL1, thereby accumulating high levels of ROS and triggering photo-oxidative bleaching.

Experimental Procedures

Plant Materials and Growth Conditions. All mutants were in the Columbia background, using Col-0 as control. For C_2H_4 treatment, plants were grown on MS medium supplemented with 10 μM ACC as described (25). For far-red (FR) irradiation, seedlings were grown in the dark at 22 °C for 1 day after germination, followed by FR (flux rate, 6–8 μmol/m^{2}s) irradiation for 2.5 days (8). FR light was provided by a far-red LED light incubator (model LH-55LED-SS, NK system, Japan) with a Plexiglas filter (FRF 700, Westlake Plastics, Lenni, PA). To determine the greening rate, more than 50 seedlings were grown in darkness for indicated number of days after germination and transferred into continuous white light (WL, 80–100 μmol/m^{2}s) to continue growing for 2 days. The seedlings can be divided into two types, normal greening (with dark-green cotyledons) and impaired greening (with white, yellowish, or light-green cotyledons). The greening rate was calculated by the percentage of normal greening seedlings (12, 16).

qRT-PCR, Microarray Experiments, and Western Blot Analysis. Total RNA was isolated from 4-day-old dark-grown seedlings using a TRIZOL Reagent system (Invitrogen). After RNA isolation, reverse transcription was conducted according to the manufacturer's protocol (M-MLV reverse transcription system, Promega), followed by quantitative real-time RT-PCR analysis (SYBR Premix, Takara) to determine the gene expression. Microarray experiments were carried out using Arabidopsis ATH1 arrays by Gene Tech Co. Ltd (Shanghai) with 4-day-old etiolated seedlings grown on MS medium. The expression data were analyzed using GeneSpring version 4.2. Microarray results were confirmed using RT-PCR or real-time RT-PCR. Western blot analysis was performed as described with anti-EIN3 antibody (25).

Protochlorophyllide Determination and Fluorescence Microscopic Imaging of ROS. Pigments in etiolated seedlings were extracted under a dim-green safe light as described (35), except the extraction step was conducted by incubating 10–20 seedlings with ice-cold buffer in the dark for 6 h before centrifugation. To detect Pchlide, the room-temperature fluorescence emission spectra of various samples were acquired with an Infinite M200 (Tecan). The excitation wavelength was 440 nm and the fluorescence emission spectra were recorded between 600 and 700 nm with a bandwidth of 1 nm. After transferring dark-grown etiolated seedlings to WL for 2 days, fluorescence microscopic imaging of ROS was performed as described (36). Fluorescence microscopic images were acquired by a DMI6000 B fluorescence microscope (Leica).

ChIP and DNA Gel-Shift Assays. Chromatin immunoprecipitation (ChIP) was performed as described (37), using 4-day-old dark-grown seedlings on MS medium with or without 10 μM ACC. Anti-EIN3 polyclonal antibody was used for IP. DNA gel-shift assays were done using a Biotin 3′ End DNA Labeling Kit and a Light Shift Chemiluminescent EMSA Kit according to the manufacturer's protocol (Pierce).

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