Running title: Retrograde coordinates light and hormone signals

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Retrograde induction of phyB orchestrates ethylene-auxin hierarchy to regulate growth

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One-sentence summary: The plastidial retrograde metabolite MEcPP orchestrates coordination of light and hormonal signaling cascade by inducing phytochrome B abundance and modulating auxin and ethylene levels.

Author Contributions

J.J. and K.D. designed the study, J.J., Y.X., H.C. W.H., U.D., H.K., and F.D. performed the experiments, L.Z performed the bioinformatics analyses, J.M. and K.P. provided experimental tools and K.D. wrote the manuscript.

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Abstract

Exquisitely regulated plastid-to-nucleus communication by retrograde signaling pathways is essential for fine-tuning of responses to the prevailing environmental conditions. The plastidial retrograde signaling metabolite methylerythritol cyclodiphosphate (MEcPP) has emerged as a stress signal transduced into a diverse ensemble of response outputs. Here we demonstrate enhanced phytochrome B protein abundance in red light–grown MEcPP-accumulating ceh1 mutant plants relative to wild-type seedlings. We further establish MEcPP-mediated coordination of phytochrome B with auxin and ethylene signaling pathways and uncover differential hypocotyl growth of red light–grown seedlings in response to these phytohormones. Genetic and pharmacological interference with ethylene and auxin pathways outline the hierarchy of responses, placing ethylene epistatic to the auxin signaling pathway. Collectively, our findings establish a key role of a plastidial retrograde metabolite in orchestrating the transduction of a repertoire of signaling cascades. This work positions plastids at the zenith of relaying information coordinating external signals and internal regulatory circuitry to secure organismal integrity.
Introduction

Dynamic alignment of internal and external cues through activation of corresponding signal transduction pathways is a defining characteristic of organisms essential for fitness and the balancing act of metabolic investment in growth versus adaptive responses. The integrity of these responses is achieved through finely controlled communication circuitry, notably retrograde (organelle-to-nucleus) signaling cascades. Despite the central role of retrograde signaling in regulation and coordination of numerous adaptive processes, the nature and the operational mode of action of retrograde signals have remained poorly understood.

Through a forward-genetic screen, we identified a bifunctional plastid-produced metabolite methylerythritol cyclodiphosphate (MEcPP) that serves as a precursor of isoprenoids produced by the plastidial methylerythritol phosphate (MEP) pathway and functions as a stress-specific retrograde signaling metabolite (Xiao et al., 2012). We further demonstrated that stress-induced MEcPP accumulation leads to growth retardation and induction of selected nuclear-encoded, stress-response genes (Xiao et al., 2012; Walley et al., 2015; Lemos et al., 2016; Wang et al., 2017a). We specifically established that regulation of growth is in part via MEcPP-mediated modulation of levels and distribution patterns of auxin (IAA) through dual transcriptional and post-translational regulatory inputs (Jiang et al., 2018).

Auxin functions as a key hormone regulating a repertoire of plant development processes including hypocotyl growth (J. Jensen et al., 1998; De Grauwe et al., 2005). The auxin biosynthesis pathway that converts tryptophan (Trp) to IAA in plants is established to be through conversion of Trp to indole-3-pyruvate (IPA) by the TAA family of amino transferases and subsequent production of IAA from IPA by the YUC family, a family of flavin monooxygenases (Zhao, 2012). Subsequently, establishment of auxin gradient is achieved by transporters such as the auxin-efflux carrier PIN-FORMED1 (PIN1) (Galweiler et al., 1998; Geldner et al., 2001). Interestingly, IAA biosynthesis, transport, and signaling during light-mediated hypocotyl growth
are in turn regulated by ethylene (Liang et al., 2012), and conversely ethylene is regulated by auxin (Vandenbussche et al., 2003; Ruzicka et al., 2007; Stepanova et al., 2007; Swarup et al., 2007; Negi et al., 2010). As such, auxin-ethylene crosstalk inserts an additional layer of complexity to the already intricate and multifaceted growth regulatory mechanisms.

Ethylene in plants is derived from conversion of S-adenosyl-L-methionine (AdoMet) to 1-aminocyclopropane-1-carboxylate (ACC) by ACC synthase (ACS) (Yang and Hoffman, 1984), followed by conversion of ACC to ethylene catalyzed by ACC oxidase (L.-C. Wang et al., 2002). Ethylene stimulates hypocotyl growth in the light but inhibits it in the dark (Smalle et al., 1997; J. Jensen et al., 1998; Vandenbussche et al., 2012).

Light signaling is a common environmental stimulus controlling developmental processes through hormonal modulation, such as regulation of auxin biosynthesis and signaling genes by phytochrome B (phyB) (Morelli and Ruberti, 2002; Tanaka et al., 2002b; Tian et al., 2002; Nozue et al., 2011; Hornitschek et al., 2012a; de Wit et al., 2014; Leivar and Monte, 2014). PhyB is the main photoreceptor mediating red-light photomorphogenesis; phyB is activated by red light and imported into the nucleus where it forms phyB-containing nuclear bodies (phyB-NBs) (Nagy and Schafer, 2002; Quail, 2002). Formation of phyB-NBs depends on binding to and sequestration of the basic helix-loop-helix (bHLH) transcription factors, Phytochrome Interacting Factor 1 (PIF1), PIF3, PIF4, PIF5, and PIF7 (Rausenberger et al., 2010; Leivar and Quail, 2011). The prominent role of phyB in auxin regulation is best displayed by simulation of shade avoidance responses (SAR) through exogenous application of auxin or via genetic manipulation of auxin (Tanaka et al., 2002a; Hornitschek et al., 2012b). In addition, PIFs, specifically PIF4, PIF5, and PIF7, play a major role in regulating auxin by targeting promoter elements of multiple auxin biosynthetic and signal transduction genes (Franklin et al., 2011; Nozue et al., 2011; Leivar et al., 2012; Sellaro et al., 2012; Leivar and Monte, 2014). Moreover, the ethylene–promoted hypocotyl elongation in light is regulated by the PIF3-dependent, growth-promoting pathway.
activated transcriptionally by EIN3, whereas under dark conditions, ethylene inhibits
growth by destabilizing the ethylene response factor 1 (ERF1) (Zhong et al., 2012).
Here, we identify MEcPP as a retrograde signaling metabolite that coordinates
internal and external cues, and we further delineate light and hormonal signaling
cascades that elicit adaptive responses to ultimately drive growth-regulating processes
tailored to the prevailing environment.
Results

Elevated phyB abundance suppresses hypocotyl growth in ceh1

Given the stunted hypocotyl phenotype of the high MEcPP–accumulating mutant ceh1, we explored the nature of the photoreceptors involved by examining hypocotyl length of seedlings grown in the dark and under various monochromatic light conditions. The analyses showed comparable hypocotyl lengths of dark-grown ceh1 and control seedlings (WT) (Fig. 1A). However, under continuous red light (Rc; 15 μE m⁻² sec⁻¹), ceh1 seedlings displayed notably shorter hypocotyls than those of WT plants (Fig. 1A). This data led us to question the role of phyB, the prominent red-light photoreceptor, in regulating ceh1 hypocotyl growth. To answer this question, we generated a ceh1/phyB-9 double-mutant line, and subsequently compared seedling hypocotyl length with WT, ceh1, and phyB-9 seedlings grown under continuous dark and Rc conditions (Fig. 1A & B). The data clearly demonstrated phyB-dependent suppression of hypocotyl growth in ceh1 under Rc, as evidenced by the recovery of ceh1 retarded hypocotyl growth in ceh1/phyB-9 to lengths comparable to those of phyB-9 seedlings.

Hypocotyl growth of the aforementioned four genotypes was also examined under continuous blue (Bc) and far-red (FRc) light conditions. The reduced hypocotyl growth of the ceh1 mutant grown under Bc, albeit not as severe as those grown under Rc, further implicate blue light–receptor cytochromes (Yu et al., 2010) in regulating growth of these seedlings (Fig. S1A & B). Additionally, ceh1 and ceh1/phyB-9 seedlings grown under Bc exhibited equally shortened hypocotyls, and under FRc light hypocotyl growth was almost similarly retarded in all genotypes (Fig. S1A & B). Collectively, these results support the involvement of cryptochromes as well as phyB in ceh1 hypocotyl growth, albeit to different degrees. However, the more drastic effect of phyB in regulating hypocotyl growth of Rc-grown, high MEcPP–accumulating seedlings, in conjunction with the supporting evidence from earlier data using white light–grown ceh1 seedlings (Jiang et al., 2019), led us to primarily focus on the role of phyB.

Next, we measured MEcPP levels in the four genotypes grown in the dark and in
various monochromatic wavelengths to examine a potential correlation between

Fig. 1. *Ceh1* hypocotyl growth in red light is phyB-dependent
(A) Representative images of 7-day-old WT, *ceh1*, *ceh1/phyB-9* and *phyB-9* seedlings grown in the dark and continuous red light (Rc: 15 μEm²sec⁻¹). (B) Quantification of hypocotyl lengths from aforementioned genotypes shown in Fig.1A. (C) MEcPP levels of samples from Fig.1A. The break indicates a change of scale on the y axis. Statistical analyses were performed using Tukey’s HSD method (n ≥), different letters indicate significant difference (P < 0.05). Scale bars: 1cm.
growth phenotypes and altered levels of the retrograde signaling metabolite (Fig. 1C & S1C). The analyses showed almost undetectable MEcPP levels in dark-grown plants of all the genotypes, and low levels of the metabolite in Rc-grown WT and phyB-9 seedlings. By contrast, ceh1 seedlings grown in Rc accumulated high MEcPP levels, a phenotype that was partially (~10-fold) suppressed in ceh1/phyB-9 seedlings. This reduction was not unexpected since phyB-controlled PIF regulates the expression of DXS, the first MEP-pathway gene encoding the flux determinant enzyme (Chenge-Espinosa et al., 2018). It is noteworthy that despite this significant reduction, the MEcPP content of ceh1/phyB-9 seedlings remained ~100-fold above those of WT or phyB-9 plants grown simultaneously and under the same conditions. This reduction of MEcPP in ceh1/phyB-9 also occurred in seedlings grown in Bc (Fig. S1C), likely because of the direct interaction between PIFs and blue light–receptor cryptochromes (Pedmale et al., 2016). However, in spite of reduced MEcPP levels in Rc- or Bc-grown ceh1/phyB-9 seedlings, the hypocotyl growth recovery is exclusive to mutant seedlings grown in Rc (Fig. 1A-C & S1A-C), leading to the conclusion that there is also a blue light–dependent pathway that regulates ceh1 hypocotyl growth in Bc. Moreover, hypocotyls of all genotypes, regardless of their MEcPP levels, remained stunted in FRc, a light condition known to inactivate phyB. Collectively, the results further verify the function of phyB in altering the observed growth phenotype of ceh1 mutant seedlings.

To examine the correlation between accumulation of MEcPP and alteration of growth in response to red-light treatment, we further examined the hypocotyl length of Col-0 ecotype, Col-0 transformed with HPL:LUC construct (WT), ceh1, and complemented ce1 (CP) seedlings (Fig. 2A-B). In contrast to the stunted hypocotyl growth of ceh1, these data clearly showed recovery of hypocotyl growth in CP to lengths comparable to Col-0 and WT seedlings (Fig. 2A-B).

Next, we questioned whether phyB transcript and/or protein levels are altered in ceh1 mutants grown in Rc. The expression data analyses revealed similar PHYB transcript levels in ceh1 and WT seedlings (Fig. S1D). To determine the hyB protein levels, we performed immunoblot analyses using proteins isolated from the aforementioned
The data showed higher PhyB levels in ceh1 versus other genotypes specifically as compared with the complemented line (CP), supporting conclusion that MEcPP mediates enhanced abundance of phyB.

To further examine the potential role of MEcPP in ceh1 in reducing growth and altering phyB levels, we employed a pharmacological approach using fosmidomycin (FSM), a MEP-pathway inhibitor (Fig. 2D-F). This inhibitor interferes with and highly reduces the flux through the pathway and abolishes MEcPP-mediated actions such as formation of otherwise stress–induced subcellular structures known as ER.
bodies or furthering the reduced auxin levels in *ceh1* mutant plants (Gonzalez-Cabanelas et al., 2015; Wang et al., 2017b; Jiang et al., 2018). We examined hypocotyl growth of red light–grown 7-day-old seedlings that were treated with FSM for 3 days. These data showed enhanced hypocotyl growth of FSM-treated *ceh1* compared to non-treated seedlings (Fig. 2D–E). It is of note that the length of FSM-treated *ceh1* hypocotyls did not recover to that of the WT seedlings, suggesting an inefficiency of FSM treatment and/or the presence of other regulatory factors. In addition, immunoblot analysis showed a very slight reduction in phyB abundance in FSM-treated *ceh1* compared to non-treated seedling (Fig. 2F). There may be two reasons for not detecting an overall stronger response to FSM treatment. One is the very high MEcPP levels in the *ceh1* mutant, and the other the degree of FSM penetration. However, the clearly higher PhyB levels in the *ceh1* mutant compared to CP, WT, and Col lines supports the notion of MEcPP-mediated increase of phyB abundance, verifying the earlier report using white light–grown seedlings (Jiang et al., 2019).

In addition to MEcPP, the *ceh1* mutant accumulates substantial amounts of the defense hormone salicylic acid (SA) (Xiao et al., 2012; Bjornson et al., 2017). The reported involvement of phyB in SA accumulation and signaling (Chai et al., 2015; Nozue et al., 2018) prompted us to examine the potential role of this defense hormone in regulating *ceh1* hypocotyl growth. For these experiments, we employed the previously generated SA-deficient double-mutant line *ceh1/eds16* (Xiao et al., 2012). All four genotypes (WT, *ceh1*, *ceh1/eds16*, and *eds16*) displayed similar hypocotyl length when grown in the dark, whereas in Rc both *ceh1* and *ceh1/eds16* seedlings displayed equally reduced hypocotyl lengths as compared to their respective control backgrounds (Fig. S1G). These results illustrate SA-independent regulation of *ceh1* hypocotyl growth in Rc.

Given the well-established role of PIFs in transduction of phyB signals, we examined PIFs expression levels and found significantly reduced *PIF4* and *-5* transcripts in Re-grown *ceh1* compared to WT seedlings (Fig. S2). These data led us to genetically investigate the potential role of PIFs in regulating hypocotyl length of Re-grown *ceh1*
Fig. 3. Overexpression of *PIF4* and *PIF5* recover stunted hypocotyl growth of *ceh1*

(A) Quantification of hypocotyl lengths of 7-day-old WT, *ceh1*, *ceh1/pifq*, and *pifq* grown in Rc (15 μEm⁻²sec⁻¹).

(B) Quantification of hypocotyl lengths from 7-day-old WT, *ceh1*, *ceh1/PIF4-OX*, *PIF4-OX*, *ceh1/PIF5-OX* and *PIF5-OX* grown in Rc (15 μEm⁻²sec⁻¹). Data are presented with n≥20 for the *pif* mutant backgrounds and n≥30 for the experiments containing *PIF-OX* seedlings. The break indicates a change of scale on the y axis. Statistical analyses were carried out using Tukey’s HSD method, different letters indicate significant difference.
and -5) alone and in lines introgressed into the ceh1 mutant background. The results revealed similarly dwarf hypocotyls in ceh1/pifq and pifq backgrounds, which were slightly but significantly shorter than that of ceh1 seedlings (Fig. 3A). Furthermore, equally reduced hypocotyl growth in ceh1/pifq and pifq suggest that PIFs are the predominant growth regulators in ceh1 under the experimental conditions employed. The role of PIFs in determining hypocotyl growth was further tested by examining ceh1 seedlings overexpressing PIF4 and -5 grown in Rc (Fig. 3B). The data showed the expected enhanced hypocotyl growth of PIF overexpressors compared to WT seedlings and recovery of the retarded growth observed in ceh1 in ceh1/PIF4 and -5 overexpression lines.

Collectively, these data illustrate growth regulatory function of PIFs, and identify MEcPP–mediated transcriptional regulation of PIF4 and -5 as an integral regulatory circuit controlling ceh1 hypocotyl growth.

Reduced expression of auxin biosynthesis and response genes in ceh1

To identify the downstream components of the MEcPP–mediated phyB signaling cascade, we performed RNAseq profiling of WT and ceh1 seedlings grown in the dark and in Rc. A multi-dimensional scaling (MDS) plot revealed significant overlap between expression profiles of WT and ceh1 seedlings grown in the dark, in contrast to their distinct expression profiles when grown in Rc (Fig. S3). GO-term analyses identified over-representations of auxin signaling and response genes amongst the significantly (≥2-fold) altered transcripts (Fig. S4). Confirmation of the data through RT-qPCR identified auxin biosynthesis (YUC3 and -8) and response genes (IAA6 and -19) as the most significantly differentially expressed genes under Rc conditions (Fig. 4A-B). We further quantified the IAA content in plants and found similar auxin levels in dark-grown plans of all genotypes in contrast to significantly reduced auxin levels (50%) in Rc-grown ceh1 versus WT plants (Fig. 4C). We validated this finding by testing Rc-grown WT and ceh1 lines expressing the auxin signaling reporter DR5-GFP (Jiang et al., 2018). The reduced GFP signal in ceh1 was on par with lower IAA levels in the mutant compared to the WT seedling (Fig. 4D).

Next, we examined possible modulation of other phytohormones such as abscisic acid
Fig. 4. Auxin is reduced in ceh1
Expression levels of YUC3, 8 (A) and IAA6, 19 (B) in WT and ceh1 seedlings. RNAs were extracted from 7-day-old WT and ceh1 seedlings grown in the dark and Rc (15 μEm⁻²sec⁻¹). Transcript levels of target genes were normalized to the levels of At4g26410 (M3E9). Data are presented with three biological replicates and three technical replicates. Statistical analyses were determined by a two-tailed Student's t tests with a significance of $P < 0.05$, $P < 0.01$ (C) IAA levels in 7-day-old WT and ceh1 seedlings grown in the dark and Rc (15 μEm⁻²sec⁻¹). Data are presented with three biological replicates. The break indicates a change of scale on the y axis. Statistical analyses were carried out by a two-tailed Student's t tests with a significance of $P < 0.05$. (D) Representative images of DR5-GFP signal intensity in 7-day hypocotyls of Rc (15 μEm⁻²sec⁻¹) grown WT and ceh1 seedlings. DR5-GFP (green), chloroplast fluorescence (red) and merged images.
(Fig. S5). Similar ABA and JA levels found in WT and ceh1 plants grown in the dark and in Rc strongly support the specificity of MEcPP-mediated regulation of auxin.

Enhanced tolerance of ceh1 to auxin and auxinole

Reduced IAA levels in ceh1 led us to examine whether external application of this hormone could rescue the retarded hypocotyl growth in ceh1 seedlings. The analyses showed longer hypocotyls in ceh1 seedlings treated with IAA at concentrations (10 and 100 μM) that inhibited growth in WT seedlings (Fig. 5A-B). Interestingly, ceh1 and WT hypocotyls displayed similar lengths when treated with the highest IAA concentration used here (100 μM), albeit through two opposing responses, namely growth suppression in WT and induction in ceh1.

This finding led to the hypothesis that the enhanced tolerance of ceh1 to auxin treatment is not solely the result of reduced auxin levels in the mutant, but also a consequence of modified auxin signaling in the mutant. To address this possibility, we treated WT and ceh1 seedlings with auxinole, an auxin signaling inhibitor that functions as an auxin antagonist for TIR1/AFB receptors (Hayashi et al., 2008; Hayashi et al., 2012). The analyses showed clear dose-dependent suppression of hypocotyl growth of WT seedlings in response to auxinole treatment, in contrast to the unresponsiveness of ceh1 seedlings at all concentrations examined (Fig. 5C-D). Collectively, the data indicated enhanced tolerance of ceh1 to otherwise inhibitory concentrations of auxin and auxinole, likely stemming from reduced auxin levels and compromised signaling in the mutant line.

Altered auxin transport in ceh1

We have previously established that MEcPP-mediated modulation of levels and distribution patterns of auxin (IAA) is via dual transcriptional and post-translational regulatory inputs (Jiang et al., 2018). We specifically demonstrated reduced transcript and protein levels of auxin efflux transporter PIN-FORMED 1 (PIN1) in ceh1 seedlings grown in white light. Here, we extended these analyses to Rc-grown seedlings, initially by expression analyses of PIN1 in WT and ceh1. The analyses showed similar PIN1 transcript levels in ceh1 and the WT seedlings (Fig. 6A). By contrast, the combined approaches of immunoblot and immunolocalization analyses
confirmed a significant reduction in PIN\textsuperscript{16} protein levels in ceh\textsuperscript{1} compared to WT.
seedlings (Fig. 6B-C). Specifically, immunolocalization clearly showed reduced PIN1
protein abundance in plasma membranes of xylem parenchyma cells (along tracheids), most notably in the meristems of ceh1 compared to WT seedlings, albeit with an unchanged polarity (Fig. 6C). These data support the earlier finding establishing the role of MEcPP in modulating PIN1 protein abundance both in Rc- and white light–grown seedlings (Jiang et al., 2018).

The reduced levels of the major auxin transporter PIN1 led us to examine the impact of varying concentrations of a general auxin polar transport inhibitor, specifically 1-naphthylphthalamic acid (NPA) (Scanlon, 2003), on the hypocotyl growth of WT and ceh1 seedlings grown in Rc (Fig. 6D-E). As expected, NPA application reduced WT hypocotyl growth in a dose-dependent manner, which contrasts the lack of detectable response in ceh1, thereby confirming compromised auxin transport in the mutant.

*Ethylene regulates hypocotyl growth in ceh1*

Comparative transcriptomic profiling of WT and ceh1 seedlings grown in Rc revealed reduced levels of ethylene biosynthesis genes, ACSs (Table S1), in the mutant. This observation, in conjunction with the established crosstalk between ethylene and auxin (Yu et al., 2013; Sun et al., 2015; Das et al., 2016), prompted us to further investigate the potential function of ethylene in regulating ceh1 hypocotyl growth. Initially, we performed RT-qPCR analyses on ethylene biosynthesis genes to validate the original transcriptomic profile data (Table S1, and Fig. S4). The data showed that compared to WT seedlings, there is a prominent reduction in the transcript levels of ACS4 in dark- and Rc-grown ceh1 seedlings (≥2-fold and ~60-fold, respectively), as well as a notable (3–10-fold depending on the gene) reduced expression of ACS5, -6, and -8, albeit solely in Rc-grown ceh1 (Fig. 7A).

Measurements of ethylene in these seedlings confirmed reduced levels (~80%) of the hormone in Rc-grown ceh1 compared to WT seedlings (Fig. 7B). This led us to examine hypocotyl growth of seedlings grown in the presence of varying concentrations of ethylene precursor, ACC (Fig. 7C-D). The data show suppression of WT hypocotyl growth at all concentrations examined, as opposed to equally enhanced hypocotyl growth in ceh1 at both ACC concentrations (10 and 20 μM), an indication
Fig. 7. Ethylene regulates hypocotyl growth in ceh1
(A) Expression levels of ACS4, 5, 6 and 8 in 7-day-old WT and ceh1 seedlings grown in the dark and Rc (15 μEm−2sec−1). Experiment was performed as described in Fig. 4A. Data are presented with three biological replicates and three technical replicates. Statistical analyses were determined by a two-tailed Student’s t tests with a significance of P < 0.05 *, P < 0.01 **. (B) Ethylene levels in samples used in panel (A). (C) Representative images of 7-day-old WT and ceh1 seedlings grown in the absence (0) and presence of ACC in the Rc (15 μEm−2sec−1). Scale bar: 1 cm. (D) Quantification of hypocotyl length of seedlings from panel (C). Data are presented with 45 seedlings. The break indicates a change of scale on the y axis. Statistical analyses were carried out using Tukey’s HSD method, different letters indicate significant difference (P < 0.05).
coordination of red-light signaling cascades with ethylene levels and ethylene regulation of hypocotyl growth.

Hierarchy of ethylene and auxin signaling pathways

The partial recovery of ceh1 hypocotyl growth by external application of auxin and ethylene, albeit to varying degrees, prompted us to genetically explore their potential interdependency and hierarchy of their respective growth regulatory actions in Rc-grown seedlings. To address this, we applied ACC and IAA independently to mutant lines ceh1; ceh1 introgressed into auxin receptor mutant tir1-1 (ceh1/tir1-1); and ceh1 introgressed into single ethylene-signaling mutants ein3 and eil1 (ceh1/ein3, ceh1/eil1) and the double mutant ein3 eil1 (ceh1/ein3 eil1).

Analyses of hypocotyl lengths of Rc-grown WT, ceh1, ceh1/tir1-1, and tir1-1 seedlings in the absence and presence of ACC demonstrated TIR1-dependent growth-promoting action of ACC in tir1-1 and ceh1/tir1-1 (Fig. 8A-B). We furthered these studies by applying ACC alone or together with NPA (Fig. 8C-D). Consistent with the earlier data, ACC treatment promoted ceh1 hypocotyl growth, but less effectively when combined with the auxin polar transport inhibitor NPA (Fig. 8C-D).

In parallel, we examined hypocotyl growth of Rc-grown WT, ceh1, ein3, ceh1/ein3, eil1, ceh1/eil1, ein3/eil1, and ceh1/ein3eil1 seedlings in the presence and absence of externally applied IAA (Fig. 8E-F). Enhanced growth of ceh1 hypocotyls in the presence of IAA irrespective of mutant backgrounds (single or double ein3/eil1) reaffirmed the growth-promoting function of auxin even in lines perturbed in ethylene signaling.

This finding establishes the dependency of ethylene function on auxin signaling, delineating the hierarchy of responses and positioning ethylene as epistatic to the auxin signaling pathway.
Fig. 8. Ethylene is epistatic to auxin
(A) Representative images of 7-day-old WT, oeh1, oeh1/WT-1 and
in/1-1 seedlings grown in the Rc (15 μM/sec) in the absence (a)
and presence (e) of ACC. (B) Representative images of 7-day-old
WT and oeh1 seedlings grown in the Rc (15 μM/sec) in the
absence (a) and presence (e) of ACC/HN alone or in combination.
(D) Representative images of 7-day-old WT, oeh1, eeh1, eeh1/WT-1,
eeh1/oeh1, eeh1/oeh1/WT-1 seedlings grown in the Rc
(15 μM/sec) in the absence (a) and presence (e) of IAA.
(6) & (6) (F) Quantification of hypocotyl length of seedlings from
panel (A) & (C) & (E), respectively. Data are presented with 45
seedlings. The error bar indicates a change of scale on the y-axis.
Statistical analyses were carried out using Tukey's HSD method,
different letters indicate significant difference (P < 0.05). Scale bars:
1 cm.

Discussion
An inherent feature of plant growth and development is the capacity to coordinate and integrate external cues with endogenous regulatory pathways through tightly regulated signaling cascades. Recent studies have identified retrograde signaling as a quintessential mode of cellular communication required for optimal organismal response to prevailing conditions. Here, we provide a coherent picture of how the stress-specific plastidial retrograde signaling metabolite (MEcPP) coordinates light and hormonal signaling circuitries to adjust growth to the most prevalent environmental cue, light conditions.

Our simplified schematic model (Fig. 9) depicts MEcPP as the upstream signal coordinating and modulating drivers of growth, specifically through enhancing phyB protein abundance and the consequential reduction of auxin levels and distribution in conjunction with diminished ethylene content. The degradation of phyB is established to be through intermolecular transaction of this photoreceptor with PIF transcription factors (Ni et al., 2013), thereby supporting the prospect of significantly reduced PIF4 and -5 transcript levels as the likely cause of enhanced phyB protein abundance in ceh1 seedlings grown in Rc. Furthermore, reversion of ceh1 stunted hypocotyls in ceh1/phyB-9 confirms the key role of enhanced phyB protein abundance in growth retardation of the mutant, confirming the earlier finding using white light–grown seedlings (Jiang et al., 2019).

The role of phyB in regulating growth is reported to be through repressing auxin-response genes (Devlin et al., 2003; Halliday et al., 2009). The red light–mediated reduction of auxin biosynthesis and signaling together with decreased hormone levels in ceh1 supports phyB function in auxin regulation. In addition, reduced levels of PIN1 protein abundance as evidenced by immunoblot and immunolocalization assays suggest the regulatory role of phyB in controlling auxin transport via modulation of PIN1 protein levels. This notion is supported by the ineffectiveness of auxin transport inhibitor in modulating hypocotyl growth of Re-grown ceh1 seedlings.

Similar to auxin, reduction of ethylene levels, partly due to decreased transcript levels of the respective biosynthesis genes in Rc-grown ceh1, strongly supports the
regulatory role of MEcPP-mediated induction of phyB in the process. Partial and
differential recovery of ceh1 hypocotyl growth under Rc in the presence of external
auxin or ACC identifies auxin as the key growth-regulating hormone under these
experimental conditions. Moreover, measurement of hypocotyl growth of ceh1
seedlings introgressed into auxin and ethylene signaling receptor mutants, places
ethylene epistatic to auxin, and supports a one-directional control mechanism of
ethylene-auxin interaction under Rc conditions.

**Conclusions**

Here, we revealed MEcPP-mediated enhanced abundance of PhyB, in part via
suppression of PIF4 and -5 expression levels, and the resulting reduced hypocotyl
growth. We further established MEcPP-mediated coordination of phytochrome B with auxin and ethylene signaling pathways, and the function of the collective signaling circuitries in the regulation of hypocotyl growth of red light–grown seedlings. In addition, hormonal applications and pharmacological treatments support hierarchical functions of auxin and ethylene in regulating growth, with ethylene being epistatic to auxin.

In summary, this finding illustrates MEcPP-mediated coordination of light and hormonal signaling cascades to ultimately reprogram plant growth in responses to the light environment and further provides information on the functional hierarchy of these growth regulatory inputs. As such, this finding identifies plastids as the control hub of growth plasticity in response to environmental cues.

**MATERIALS AND METHODS**

**Plant materials**

The wild-type seedlings used here are the earlier-reported Col-0 ecotype transformed with *HPL:LUC* constructs and used as the parent (WT) for isolation of the *ceh1* mutant (Xiao et al., 2012). All experiments were performed with 7-day-old seedlings grown in 15 μE m⁻² sec⁻¹ continuous monochromatic light at 22°C, unless specified otherwise. The *ein3/eil1* double mutant is provided by Hongwei Guo (Southern University of Science and Technology); *DR5-GFP* is a gift from Mark Estelle (University of California, San Diego); and *tir1-1* (CS3798) was ordered from ABRC.

**Light treatment**

Surface-sterilized seeds were planted on half-strength Murashige and Skoog medium (1/2 MS: 2.2 g/L Murashige and Skoog salts, 1 g/L MES (2-(N-morpholino)ethanesulfonic acid, pH 5.7, and 8 g/L agar), stratified at 4°C for 5 days, grown in 15 μE m⁻² sec⁻¹ of monochromatic red, far-red, and blue LEDs (Quantum Devices Snap-Lite) in a custom chamber at ~22°C for 7 days prior to hypocotyl measurement.

Dark control experiments were performed by exposing seedlings to white light for 3 hours after stratification, and then wrapping the plates with 3 layers of aluminum foil, and growing seedlings for 7 days before quantification of hypocotyl length. Each treatment was performed on three biological replicates, each replicate with 15
Hypocotyl length measurement

Seven-day-old seedlings were scanned with an Epson flatbed scanner, hypocotyl length was measured using Image J.

RNA isolation and RNA-Seq library construction

Total RNA was isolated using TRIzol (Life Technologies) from 7-day-old seedlings grown in the dark and in Rc. The RNA quality and quantity were assessed by Nanodrop ND 1000 (Nanodrop technologies), 4 µg of qualified total RNA was used for RNA-Seq library preparation using Illumina’s TruSeq v1 RNA sample Preparation kit (RS-930-2002) with a low-throughput protocol following manufacturer’s instructions with modifications as described (Devisetty et al., 2014). Illumina’s 12 indices were used during adaptor ligation and library construction. The constructed libraries were size-selected using 1:1 volume of AMPure XP beads (Beckman Coulter, Brea CA). Size and quality of libraries were examined using Bioanalyzer 2100 (Agilent, Santa Clara, CA). The 12 libraries were quantified using Quant-iT™ PicoGreen® ds DNA Assay Kit (Invitrogen) and equally pooled in 1 lane of single-end 50-bp sequencing in HiSeq 2000 machine (Illumina, San Diego, CA) at the QB3 facility at UC Berkeley.

Quality filtering and alignment of RNA-Seq data

To ensure good read quality for downstream analysis, raw reads were pre-processed using FastX-tool kit software (http://hannonlab.cshl.edu/fastx_toolkit/) and custom Perl scripts. First, the de-multiplexed raw reads were filtered with fastq_quality_filter, parameters (−q 20, minimum quality score to keep: 20; −p 95, minimum percent of bases that must satisfy the quality score cut-off: 95). Next, reads with custom adapters were removed using a custom script. Quality of reads was examined before and after quality control with FastQC quality assessment software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Then Reads (1x50 bp) were mapped against the Arabidopsis representative_gene_model (TAIR10) using BWA v0.6.1-r104 (Li and Durbin, 2009) with parameters (-l 20) and SAMtools (Li et al., 2009). The resulting BAM files were used to calculate the read counts using a
custom R script, and then the counts were used for differential gene expression analysis.

**Differential expression analysis of RNA-Seq data**

The EdgeR Bioconductor package implemented in R was used to generate the pseudo-normalized counts for visualization and to carry out differential gene expression analysis (Robinson et al., 2010). Genes were kept for further analysis if read counts were greater than 1 count per million (cpm) in at least 3 of the 12 libraries. The EdgeR Generalized linear models (GLM) framework with explanatory variables of genotype and treatment allowed us to specify a design matrix estimating the effect of run number (batch) as a nuisance parameter. After fitting the model for our experiment, we defined contrasts between parent lines (WT) and mutant (*ceh1*) in red light and tested for significant expression differences using a likelihood ratio test (`glmLRT`). *P*-values for the remaining genes were adjusted using Benjamini-Hochberg method for false discovery correction. Genes with an FDR-adjusted *P*-value less than or equal to 0.01 were identified as differentially expressed.

**Multi-dimensional scaling (MDS) plot**

A multi-dimensional scaling (MDS) plot was generated in edgeR to analyze relationship between samples. Distance between each pair of RNA-seq profiles corresponded to the average (root-mean-square) of absolute logFC between each pair of samples.

**GO Term enrichment**

Goseq package in R (Young et al., 2010) was used to identify enriched Gene Ontology (GO) terms (mainly biochemical process) in the differentially expressed gene list.

**Hormones and chemical treatments**

Surface-sterilized seeds were planted on 1/2 MS, stratified at 4°C for 3 days, germinated under continuous red light at 15 μmol m$^{-2}$ sec$^{-1}$ for 2 days and subsequently transformed to 1/2 MS medium with 1 g/L MES (2-(N-morpholino)ethanesulfonic acid) in combination with hormones or chemicals. These plates were
vertically placed in continuous red light for 5 extra days before hypocotyl measurements. IAA, ACC, auxinole, and NPA were dissolved in ethanol, water, DMSO, and DMSO, respectively. The corresponding solvents were used as control treatment (mock) for the respective experiments.

**MEcPP and hormone measurements**

Quantification of SA, JA, ABA, and IAA was carried out by gas chromatography-mass spectrometry (GC-MS), using dihydro-JA, deuterated SA, ABA, and IAA as internal standard, respectively, as previously described (Savchenko et al., 2010). MEcPP extraction and quantification was performed as previously described (Jiang et al., 2019).

**Microscopy**

Confocal fluorescence imaging was performed using Zeiss LSM 710. GFP signal was examined in 7-day-old DR5-GFP and ceh1/DR5-GFP seedlings grown on 1/2 MS in Rc (15 μE m⁻² sec⁻¹).

**Immunolocalization of PIN1**

Immunolocalization of PIN1 was performed using anti-PIN1 monoclonal primary antibody and FITC anti mouse secondary antibody as previously described (Jiang et al., 2018).

**Reverse Transcription Quantitative PCR**

Total RNA was isolated from 7-day-old seedlings grown in the Rc using TRIzol (Life Technologies) and treated with DNase to eliminate DNA contamination. 1 μg total RNA was reverse transcribed into cDNA using SuperScript III (Invitrogen). *At4g26410* was used to normalize target gene expressions. Gene-specific primers were designed using QuantPrime qPCR primer design tool (http://www.quantprime.de/) and are listed (Supplemental Table S2). Each experiment was performed with three biological replicates and three technical replicates.

**Protein extraction and immunoblot analyses**

For protein extraction 7-day-old seedlings were collected, ground with liquid nitrogen, homogenized in extraction buffer (10 mM Heps, pH 7.6, 1 M Sucrose, 5 mM KCl, 5 mM MgCl₂, 5 mM EDTA, 14 mM 2-ME, 0.4% (W/V) Triton X-100, 0.4 mM PMSF,
20 μM MG132, 20 μM MG115, and Proteinase Inhibitor), centrifuged at 1X10^5G
for 10 min at 4°C, after which supernatants were transferred to new tubes as total
proteins. Then the proteins were separated on 7.5% SDS-PAGE gel and transferred to
PVDF membranes. Blots were probed with B1+B7 (1:500) primary antibodies
obtained from Peter Quail lab. The secondary was anti-mouse horseradish peroxidase
(HRP) (KPL, catalog no. 074-1806) (1:10000). Immunoblots for PIN1 protein were
performed as previously described (Jiang et al., 2018) using anti PIN1 monoclonal
antibody (1:100) primary antibody and secondary anti-mouse Horseradish peroxidase
(HRP) (1:3000). Chemiluminescent reactions were performed using the Pierce ECL
Western Blotting Substrate following the manufacturer’s instructions. Excess
substrate was removed from membranes before placing them between two plastic
sheets to develop with X-ray, and subsequently scanned with Epson Perfection V600
Photo Scanner.

Statistical analyses
All experiments were performed with at least three biological replicates. Data are
mean ± standard deviation (SD). The statistical analyses were performed using library
agricolae, Tukey’s HSD test method in R with a significance of P < 0.05 (Bunn, 2008).
We have specified the method we used for statistical analysis in all figure legends.
The names and accession numbers of all genes named in the paper are presented in
Table S1.

Accession Numbers
Sequence data from this article can be found in the GenBank/EMBL data libraries
under accession numbers PRJNA601482.

Supplemental Data
Supplemental Figure S1. Hypocotyl growth of ceh1 in continuous blue and far-red
light is phyB-independent.
Supplemental Figure S2. Expression levels of PIF1, -3, -4, and -5 in WT and ceh1
seedlings grown in the dark and Rc (15 μEm^-2sec^-1).
Supplemental Figure S3. MDS plot of sequencing data from 7-day-old WT and ceh1
seedlings grown in the dark and Rc (15 μEm-2sec-1).
Supplemental Figure S4. Heatmap of Top 50 significantly enriched Go terms of down-regulated genes in ceh1/WT under continuous Rc (15 μEm-2sec-1).

Supplemental Figure S5. Similar ABA and JA levels in WT and ceh1 seedlings grown in the dark and in Rc (15 μEm-2sec-1).

Supplemental Table S1. List of differentially expressed genes.

Supplemental Table S2. List of primers used in RT-qPCR analyses.

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Figure legends
Fig. 1. Ceh1 hypocotyl growth in red light is phyB-dependent
(A) Representative images of 7-day-old WT, ceh1, ceh1/phyB-9 and phyB-9 seedlings grown in the dark and continuous red light (Rc: 15 μEm-2sec-1). (B) Quantification of hypocotyl lengths from aforementioned genotypes shown in Fig.1A. (C) MEcPP levels of samples from Fig.1A. The break indicates a change of scale on the y axis. Statistical analyses were performed using Tukey’s HSD method (n ≥ 45), different letters indicate significant difference (P < 0.05). Scale bars: 1cm.
Fig. 2. MECPP induction of phyB results in stunted ceh1 hypocotyl growth

(A) Representative images of 7-day-old Col-0, WT, ceh1 and complementation line (CP) seedlings grown in Rc15 (15 μE m⁻²sec⁻¹). Scale bars: 1 cm. (B) & (E) Quantification of hypocotyl length of seedlings from panel (A) and (D), respectively. Data are presented with 45 seedlings. Statistical analyses were carried out using Tukey’s HSD method, different letters indicate significant difference (P < 0.05). (C) & (F) Immunoblots of phyB protein abundance, using RPN6 antibody a loading control. (D) Representative images of 7-day-old WT and ceh1 seedlings grown in Rc15 (15 μE m⁻²sec⁻¹) in the absence (-) and presence (+) of fosmidomycin (20 μM).

Fig. 3. Overexpression of PIF4 and PIF5 recover stunted hypocotyl growth of ceh1

(A) Quantification of hypocotyl lengths of 7-day-old WT, ceh1, ceh1/pifq, and pifq grown in Rc (15 μE m⁻²sec⁻¹). (B) Quantification of hypocotyl lengths from 7-day-old WT, ceh1, ceh1/PIF4-OX, PIF4-OX, ceh1/PIF5-OX and PIF5-OX grown in Rc (15 μE m⁻²sec⁻¹). Data are presented with n≥20 for the pif mutant backgrounds and n≥30 for the experiments containing PIF-OX seedlings. The break indicates a change of scale on the y axis. Statistical analyses were carried out using Tukey’s HSD method, different letters indicate significant difference.

Fig. 4. Auxin is reduced in ceh1

Expression levels of YUC3, 8 (A) and IAA6, 19 (B) in WT and ceh1 seedlings. RNAs were extracted from 7-day-old WT and ceh1 seedlings grown in the dark and Rc (15 μE m⁻²sec⁻¹). Transcript levels of target genes were normalized to the levels of At4g26410 (M3E9). Data are presented with three biological replicates and three technical replicates. Statistical analyses were determined by a two-tailed Student’s t tests with a significance of P < 0.05 *, P < 0.01 **. (C) IAA levels in 7-day-old WT and ceh1 seedlings grown in the dark and Rc (15 μE m⁻²sec⁻¹). Data are presented with three biological replicates. The break indicates a change of scale on the y axis. Statistical analyses were carried out by a two-tailed Student’s t tests with a significance of P < 0.05. (D) Representative images of DR5-GFP signal intensity in
7-day hypocotyls of Rc (15 μEm-2sec-1) grown WT and ceh1 seedlings. DR5-GFP (green), chloroplast fluorescence (red) and merged images.

**Fig. 5.** Enhanced tolerance of ceh1 to auxin and auxinole

(A) & (C) Representative images of 7-day-old WT and ceh1 seedlings in the absence (0) and presence of IAA and auxinole grown under Rc (15 μEm-2sec-1), respectively.

(B) & (D) Quantification of hypocotyl lengths of seedlings from panel (A) & (C), respectively. Data are presented with 45 seedlings. The break indicates a change of scale on the y axis. Statistical analyses were carried out using Tukey’s HSD method, different letters indicate significant difference ($P < 0.05$). Scale bars: 1cm.

**Fig. 6.** Altered auxin transport in ceh1

(A) PIN1 expression levels in 7-day-old WT and ceh1 seedlings grown in Rc (15 μEm-2sec-1). Experiment was performed as described in Fig. 4A. Data are presented with three biological replicates and three technical replicates. (B) Immunoblots of PIN1 and ATPase as the protein loading control, and signal intensity quantification of the PIN1/ATPase protein abundance in 7-day-old WT and ceh1 seedlings grown under Rc (15 μEm-2sec-1) with two biological replicates. Asterisk denotes significant difference as determined by a two-tailed Student’s $t$ tests. (C) Immunolocalization of PIN1 in the hypocotyls of 7-day-old WT and ceh1 seedlings grown under Rc (15 μEm-2sec-1). Scale bar: 20 μm. (D) Representative images of 7-day-old WT and ceh1 seedlings grown under Rc (15 μEm-2sec-1) in the absence (0) and presence of NPA. Scale bar: 1cm. (E) Quantification of hypocotyl length of seedlings from panel (D). Data are presented with 45 seedlings. Statistical analyses were carried out using Tukey’s HSD method. Data are means ± SD and different letters indicate significant difference ($P < 0.05$).

**Fig. 7.** Ethylene regulates hypocotyl growth in ceh1

(A) Expression levels of ACS4, 5, 6 and 8 in 7-day-old WT and ceh1 seedlings grown in the dark and Rc (15 μEm-2sec-1). Experiment was performed as described in Fig.
Data are presented with three biological replicates and three technical replicates. Statistical analyses were determined by a two-tailed Student’s t tests with a significance of $P < 0.05 \ast$, $P < 0.01 \ast\ast$. 

(B) Ethylene levels in samples used in panel (A). 

(C) Representative images of 7-day-old WT and ceh1 seedlings grown in the absence (0) and presence of ACC in the Rc (15 $\mu$Em-2sec-1). Scale bar: 1cm. 

(D) Quantification of hypocotyl length of seedlings from panel (C). Data are presented with 45 seedlings. The break indicates a change of scale on the y axis. Statistical analyses were carried out using Tukey’s HSD method, different letters indicate significant difference ($P < 0.05$).

Fig. 8. Ethylene is epistatic to auxin 

(A) Representative images of 7-day-old WT, ceh1, ceh1/tir1-1 and tir1-1 seedlings grown in the Rc (15 $\mu$Em-2sec-1) in the absence (-) and presence (+) of ACC. 

(C) Representative images of 7-day-old WT and cehl seedlings grown in the Rc (15 $\mu$Em-2sec-1) in the absence (-) and presence (+) of ACC/NPA alone or in combination. 

(E) Representative images of 7-day-old WT, cehl, ein3, ceh1/ein3, eil1, ceh1/eil1, ein3/eil1, ceh1/ein3eil1 seedlings grown in the Rc (15 $\mu$Em-2sec-1) in the absence (-) and presence (+) of IAA. 

(B) & (D) & (F) Quantification of hypocotyl length of seedlings from panel (A) & (C) & (E), respectively. Data are presented with 45 seedlings. The break indicates a change of scale on the y axis. Statistical analyses were carried out using Tukey’s HSD method, different letters indicate significant difference ($P < 0.05$). Scale bars: 1cm.

Fig.9. Schematic model depicting MEcPP as the integrator of growth regulating pathways. Stress induction of MEcPP accumulation reduces expression of PIF 4 and 5 and enhances abundance of phyB protein and the consequential orchestration of ethylene-auxin hierarchy to regulate growth.


