PNAS Nexus, 2023, 2, 1–15
https://doi.org/10.1093/pnasnexus/pgad216
Advance access publication 18 July 2023
Research Report

Ethylene-mediated metabolic priming increases photosynthesis and metabolism to enhance plant growth and stress tolerance

Eric Brenya[a], Esha Dutta[b], Brittani Herron[a], Lauren H. Walden[a], Daniel M. Roberts[a,b] and Brad M. Binder[a,b,*]

[a]Department of Biochemistry & Cellular and Molecular Biology, University of Tennessee, Knoxville, TN 37996, USA
[b]Genome Science and Technology Program, University of Tennessee, Knoxville, TN 37996, USA
*To whom correspondence should be addressed: Email: bbinder@utk.edu
Edited By: Edward Bayer

Abstract

Enhancing crop yields is a major challenge because of an increasing human population, climate change, and reduction in arable land. Here, we demonstrate that long-lasting growth enhancement and increased stress tolerance occur by pretreatment of dark grown *Arabidopsis* seedlings with ethylene before transitioning into light. Plants treated this way had longer primary roots, more and longer lateral roots, and larger aerial tissue and were more tolerant to high temperature, salt, and recovery from hypoxia stress. We attributed the increase in plant growth and stress tolerance to ethylene-induced photosynthetic-derived sugars because ethylene pretreatment caused a 23% increase in carbon assimilation and increased the levels of glucose (266%), sucrose/trehalose (446%), and starch (87%). Metabolomic and transcriptomic analyses several days posttreatment showed a significant increase in metabolic processes and gene transcripts implicated in cell division, photosynthesis, and carbohydrate metabolism. Because of this large effect on metabolism, we term this "ethylene-mediated metabolic priming." Reducing photosynthesis with inhibitors or mutants prevented the growth enhancement, but this was partially rescued by exogenous sucrose, implicating sugars in this growth phenomenon. Additionally, ethylene pretreatment increased the levels of *CINV1* and *CINV2* encoding invertases that hydrolyze sucrose, and *cinv1; cinv2* mutants did not respond to ethylene pretreatment with increased growth indicating increased sucrose breakdown is critical for this trait. A model is proposed where ethylene-mediated metabolic priming causes long-term increases in photosynthesis and carbohydrate utilization to increase growth. These responses may be part of the natural development of seedlings as they navigate through the soil to emerge into light.

Keywords: ethylene, photosynthesis, metabolism, growth, stress tolerance

Significance Statement

The ability of seedlings to transition from darkness to light as they emerge from the soil is critical for plant survival. Here, we demonstrate that ethylene is an important factor early in seedling development that has long-lasting effects on plant growth and tolerance to stresses after they transition from darkness into light. Our study illustrates that transient exposure to ethylene in darkness results in long-term increases in photosynthesis and carbohydrates upon transition to light. These changes lead to increased growth and stress tolerance. This response is likely to be widespread in angiosperms since several angiosperm species show growth enhancement under these conditions.

Introduction

Enhancing plant vigor is a major challenge because of an increasing human population and reduction in arable land. Methods to increase growth and stress resistance are key to addressing this challenge. However, the success of these approaches is challenging, since improvement in growth often leads to compensation through a decrease in stress tolerance and vice versa. This trade-off can have profound implications on strategies to enhance both plant growth and stress tolerance.

Plant growth is regulated by a variety of hormones including ethylene, which is a gaseous hormone that has wide-ranging effects on plants that impact growth, development, and responses to various stresses that reduce crop yield and postharvest storage (1, 2). Because of its complex and widespread signaling role in growth and stress responses, ethylene biosynthesis and signaling are often targeted for genetic or chemical control to improve agricultural outcomes and postharvest storage (3). The current model for ethylene signaling posits that ethylene receptors signal

Competing Interest: The authors declare no competing interest.
Received: January 23, 2023. **Revised:** June 13, 2023. **Accepted:** June 20, 2023

to constitutive triple response 1 (CTR1), which functions as a negative regulator of the pathway (4–6). Downstream of CTR1 is ethylene-insensitive 2 (EIN2) which is a central regulator of ethylene signaling (7). When bound to ethylene, the receptors are inhibited leading to a reduction in CTR1 activity resulting in EIN2 dephosphorylation and cleavage to release the C-terminal portion of EIN2 (8). This leads to stabilization of EIN3 and EIN3-like1 (EIL1) transcription factors that bind to target gene promoters causing changes in other ethylene-responsive genes, including other transcription factors, leading to ethylene responses (9–14). Light affects some responses to ethylene and alters the networks of transcription factors regulated by ethylene downstream of EIN3 and EIL1 (15–18). Ethylene also promotes greening of seedlings during photomorphogenesis (19). These changes have been linked to seedling survival as they emerge from under the soil into the light.

Many of the core elements of the ethylene signaling pathway were discovered using the "triple response" assay of dark-grown *Arabidopsis thaliana* seedlings characterized by a shorter root and hypocotyl, a thicker hypocotyl, and an exaggerated apical hook (4, 20). These responses help the seedlings to grow out of the soil (18). While conducting a "triple response" screen on dark-grown *Arabidopsis* seedlings, we surprisingly observed that after removal of ethylene and transfer to light, seedlings pretreated with ethylene grew larger and were more stress tolerant than control seedlings. This long-lasting increase in growth included longer primary and lateral roots, a higher density of lateral roots, and an increase in aerial tissue fresh weight. We show that these changes in response to ethylene in the dark are caused by an increase in photosynthesis and carbohydrate levels after transfer to light and removal of ethylene. The growth enhancement response occurs in multiple angiosperm species suggesting that this is a general feature of ethylene signaling in angiosperms transitioning from darkness (underground) to light during germination. The mechanisms for these responses are likely to be different from the effects of continuous ethylene exposure, which can also affect photosynthesis and growth (2, 21).

Results

Seedlings grow larger after transient treatment with ethylene

Ethylene typically inhibits the growth of dark-grown eudicot seedlings (1). As expected, *Arabidopsis* seedlings treated for 3 days in the dark with ethylene displayed a typical "triple response" with shorter hypocotyls and roots, exaggerated apical hooks, and thicker hypocotyls compared with controls (Fig. S1). Unexpectedly, when ethylene was removed and the seedlings were grown under a 16-h photoperiod, the seedlings that had been pretreated with ethylene developed longer primary and lateral roots, a higher number of lateral roots, and more aerial tissue fresh weight than control seedlings that were not exposed to ethylene (Fig. 1A–D). Hypocotyls remained shorter than air controls days after ethylene removal (Fig. S2). Using end-point analysis, the faster primary root growth became evident 3 days after transfer to light and persisted throughout the observation period (Fig. 1B). The effects of ethylene were long-lasting and resulted in an increase in rosette and plant height after transfer to light compared with controls (Fig. 1E and F).

To determine how rapidly plants respond with increased primary root growth, we used time-lapse imaging to examine the rate of root growth for the first 22 hours after transfer to light (Fig. 1G). We estimated the latent time for an effect of ethylene pretreatment with two methods to provide a range of times where ethylene pretreatment may begin to affect growth. The time at which there was first a statistical difference (Students *t* test, $P \leq 0.05$) in the mean value was 2.25 hours, whereas a value of 3.75 hours was obtained using cumulative sum control chart analysis to determine when the trends diverge.

The enhancement of growth is not limited to the Columbia (Col) ecotype (Fig. S3), and other flowering plant species also exhibit the ethylene-induced enhanced growth response (Fig. 2). Ethylene-pretreated tomato (*Solanum lycopersicum*, cultivar Floridade) plants sown in soil were taller and had more leaf area than controls, whereas ethylene-pretreated cucumber (*Cucumis sativus*, cultivar Beit Alpha Burpless) plants sown in soil had an accelerated production of leaves and wheat (*Triticum aestivum*) seedlings responded with faster primary root growth. Thus, the ethylene-induced growth stimulation signaling pathway may be widespread in the angiosperms.

One possible explanation for enhanced growth is that pretreatment with ethylene might lead to a negative feedback loop to reduce ethylene biosynthesis or responsiveness. However, this does not appear to be the case since there was no statistical difference in ethylene production of the control and ethylene-pretreated *Arabidopsis* seedlings after transfer to ethylene-free conditions (Fig. S4A). Furthermore, the mRNA levels of several ethylene-inducible genes, which were upregulated immediately after treatment with ethylene for 3 days in the dark, returned to basal expression levels 5 days after removal of ethylene and transfer to light. This is consistent with no long-term changes in ethylene responses (Fig. S4B).

The ethylene-induced growth response was not simply a delayed response to ethylene since seedlings that were administered continuous ethylene treatment after transfer to light lacked the growth enhancement phenotype and showed shorter roots (Fig. S5) in agreement with the earliest studies on *Arabidopsis* (4, 20). Additionally, the hypocotyls were taller in the seedlings kept in continuous ethylene upon transfer to light, which is consistent with prior reports documenting that ethylene stimulates the growth of hypocotyls in light-grown *Arabidopsis* seedlings (22). Thus, the removal of ethylene is required for growth enhancement.

To determine if ethylene pretreatment in darkness is required, we conducted experiments in continuous light. This showed that ethylene treatment in darkness is required for growth stimulation since ethylene pretreatment of seedlings in light (rather than darkness) followed by ethylene-free conditions did not cause stimulation of growth (Fig. S6). These results indicate that there is a critical period during seedling development during which transient ethylene exposure in darkness causes lasting growth stimulation upon subsequent exposure to light.

Ethylene signaling via EIN2 and EIN3/EIL1 mediates ethylene-induced growth enhancement

The above experiments indicate that ethylene pretreatment in darkness is having profound and long-lasting effects on plants after removal of ethylene and transfer to light. To determine if the primary ethylene signaling pathway is involved, we examined *ein2-5* and *ein3-1;eil1-1* ethylene-insensitive *Arabidopsis* seedlings. Neither mutant responded to the ethylene pretreatment with longer primary roots (Fig. 1H) and *ein2-5* mutants did not respond with increased leaf growth (Fig. 1D). These data demonstrate that the observed growth phenotypes require the primary ethylene signaling pathway.

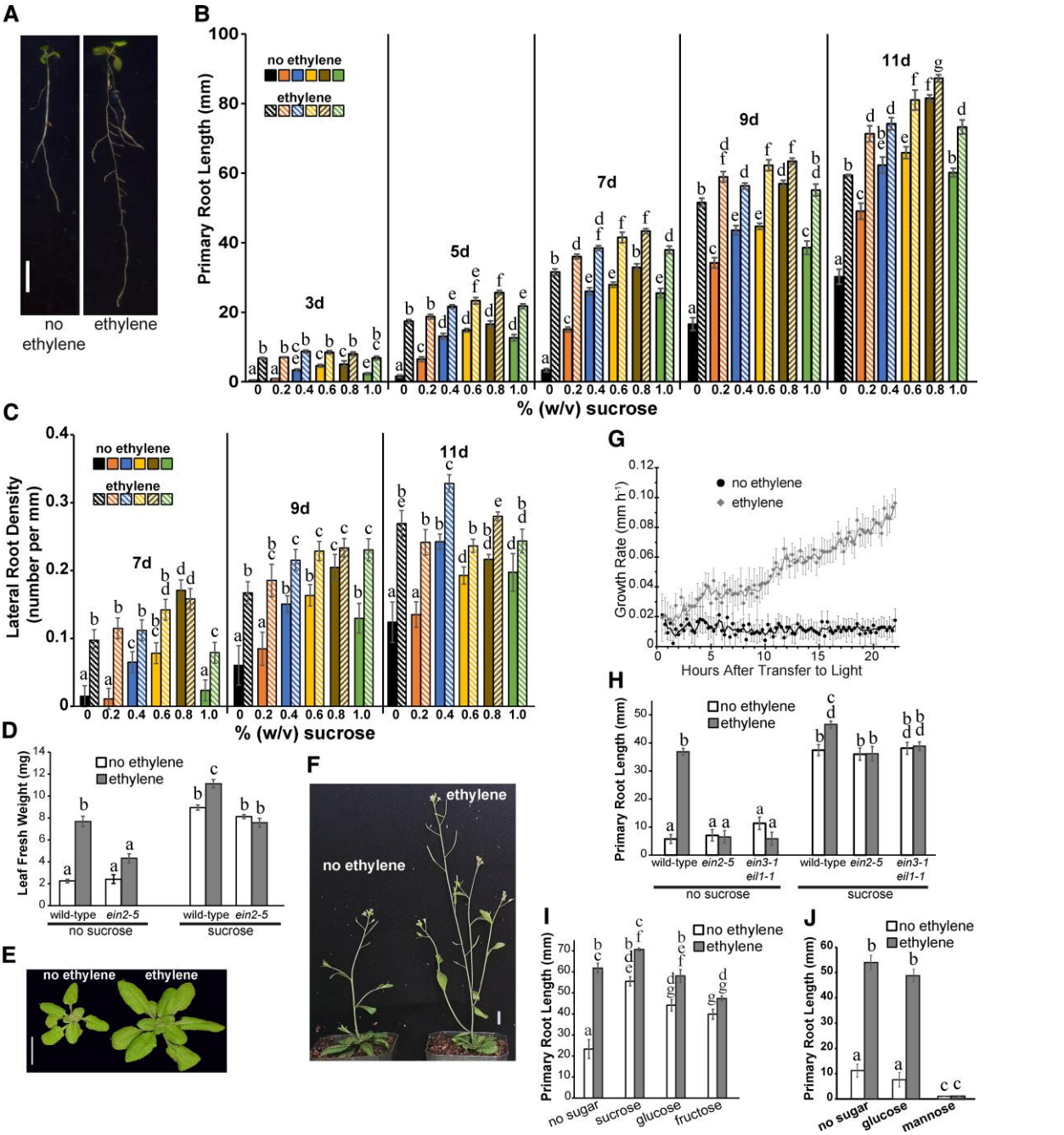

Fig. 1. Phenotypes of plants after exposure to ethylene in the dark in the presence and absence of added sugar. Germinating *Arabidopsis* seeds were exposed to 0.7 ppm ethylene or ethylene-free air in the dark. At this time (day 0), they were transferred to ethylene-free conditions and grown under a 16-h photoperiod. In some cases, exogenous sugar was included as indicated. A) Images of wild-type (Col) seedlings were acquired 10 days after transfer to light. Scale bar = 5 mm. B, C) The effects of ethylene pretreatment at the indicated concentrations of sucrose on wild-type B) new primary root growth and C) lateral root density at the indicated times after transfer to light and ethylene-free conditions. D) The effects of ethylene pretreatment on leaf fresh weight of wild-type and *ein2-5* seedlings in the presence or absence of 0.8% (*w/v*) sucrose at 12 days after transfer to light and ethylene-free conditions. E, F) Images of plants grown in soil for D) 22 days or E) 33 days after transfer to light and ethylene-free conditions. Scale bars = 1 cm. G) Images of growing roots were captured every 15 min for the first 22 h after transfer to light. The average growth rate ± SEM for each time interval is plotted ($n \geq 10$). H) The effects of ethylene pretreatment on new root growth of wild-type, *ein2-5*, and *ein3-1;eil1-1* seeds in the presence or absence of 0.8% (*w/v*) sucrose at 9 days after transfer to light and ethylene-free conditions. I) The effects of ethylene pretreatment on new primary root growth in wild-type seedlings in the presence or absence of 0.8% (*w/v*) sucrose, glucose, or fructose at 9 days after transfer to light and ethylene-free conditions. J) The effects of ethylene pretreatment on new primary root growth in wild-type seedlings in the presence or absence of 0.02% (*w/v*) glucose or mannose at 9 days after transfer to light and ethylene-free conditions. Data in B–D) and H–J) represent the mean ± SEM ($n \geq 15$), the data were analyzed by ANOVA, and the different letters indicate significant difference ($P < 0.05$).

Signaling downstream of EIN3/EIL1 varies depending on light conditions and involves the phytochrome-interacting factors (PIFs) which are important for the transition from darkness to light, and, depending on lighting conditions, they can determine the levels of and binding sites for EIN3 to regulate which genes are regulated (15–19, 23–27). PIF1, PIF3, PIF4, and PIF5 have overlapping gene targets with EIN3 (28). Because of this, and our observation that a transition from darkness to light is important for the

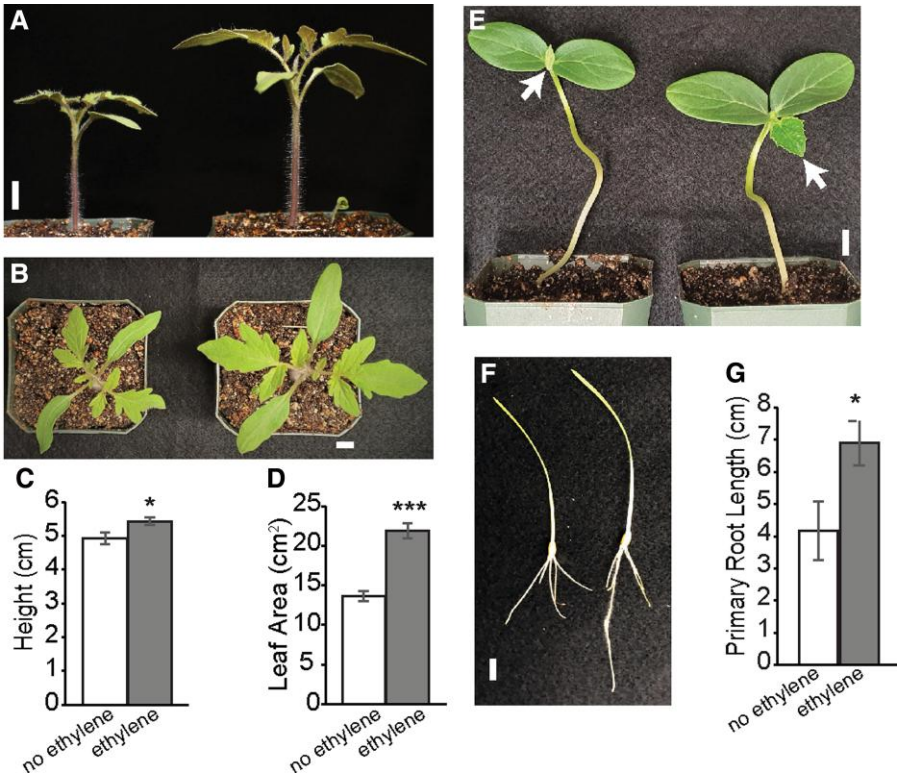

Fig. 2. Ethylene enhances the growth of several plant species. Germinating tomato (*S. lycopersicum*, cultivar Floridade) A–D) and cucumber (*C. sativus*, cultivar Beit Alpha Burpless) E) seeds sown in soil were treated with ethylene or ethylene-free air in the dark for 4 days and wheat (*T. aestivum*) seeds grown on agar F, G) for 3.5 days. At this time, the seedlings were transferred to light and ethylene-free conditions. Photos show representative plants A) 7 days, B) 9 days, E) 11 days, and F) 1.5 days after transfer to light. E) Arrows point to first true leaves. Seedlings on the left in each panel are ethylene-free controls and on the right pretreated with 0.7 ppm ethylene. Scale bars = 1 cm. C, D) Quantification of tomato height and leaf area of tomato seedlings 9 days after transfer to light. G) Quantification of wheat primary root length 1.5 days after transfer to light. Data in C), D), and G) are the mean ± SEM ($n \geq 6$) and statistical differences from the untreated controls determined with Student's *t* test (*$P < 0.05$; ***$P < 0.001$).

ethylene-induced growth stimulation, we examined the effects of ethylene on *pif1-1;pif3-7;pif4-2;pif5-3* (*pifq*) seedlings. The *pifq Arabidopsis* seedlings still responded to ethylene pretreatment with enhanced growth comparable to wild-type (Fig. S7) indicating these transcription factors are not involved in this response.

Sucrose, glucose, and fructose phenocopy ethylene pretreatment

The basipetal flow of auxin and photosynthesis-derived sugars are important for root development (29, 30). Therefore, we explored the potential roles for each of these in ethylene-induced stimulation of growth. Application of *N*-1-naphthylphthalamic acid (NPA) to *Arabidopsis* seedlings, which blocks auxin transport, reduced primary root growth by ~40%, but did not block stimulation of primary root growth or leaf growth by ethylene (Fig. S8A and B). In contrast, NPA prevented changes in lateral root density (Fig. S8C) suggesting that auxin transport is required for lateral root formation, but not the other two traits. Auxin transport is known to be critical for lateral root initiation and emergence (30). We also monitored the expression of DR5::GUS in control and experimental seedlings 5 days after transfer to light. The DR5 promoter is sensitive to auxin and provides a measure of auxin responsiveness (31). Ethylene pretreatment had little or no measurable effect on GUS expression in either leaves or root tips indicating auxin responses are not measurably altered by ethylene pretreatment under these conditions (Fig. S8D and E).

We typically carry out "triple response" assays in the absence of added sugar. To explore if the enhanced growth is controlled by sugar, we conducted ethylene treatment experiments in the presence of different concentrations of sucrose. A typical ethylene-induced growth inhibition response of hypocotyls and roots (3 days ethylene in darkness) was observed for all seedlings regardless of sucrose concentration (Fig. S1B and C). After subsequent transfer to light, the length of the primary roots of control seedlings increased as a function of sucrose concentration, with maximum growth observed with 0.8% (*w*/*v*) sucrose (Fig. 1B). Ethylene pretreatment followed by transfer to light led to increased root growth compared with the controls at all concentrations of exogenous sucrose tested.

Sucrose also regulates lateral root formation (32). Increasing sucrose concentrations caused an increase in lateral root density in the air controls and ethylene led to a further increase at all sucrose concentrations, although it took longer for a statistical difference to occur at 0.8% (*w*/*v*) sucrose (Fig. 1C). Exogenous sucrose also led to higher leaf fresh weight (Fig. 1D). Unlike stimulation of growth by ethylene, ethylene signaling is not required for increased root or leaf growth in response to exogenous sucrose (Fig. 1D and H).

Since sucrose is broken down to fructose and glucose, we next tested whether or not these monosaccharides also phenocopy the growth enhancement phenotype caused by ethylene pretreatment, or whether the effect is specific for sucrose. Addition of 0.8% (*w*/*v*) glucose or fructose led to increased primary root growth similar to the effect of sucrose (Fig. 1I). Pretreatment

with ethylene led to a further enhancement of growth. Thus, like sucrose, the application of either glucose or fructose phenocopies the effects of ethylene pretreatment suggesting that the enhanced growth in ethylene-pretreated plants might be mediated by an increase in photosynthesis-derived sugars.

Sugars are acting as a carbon source to affect growth after ethylene pretreatment

Our observations suggest that the growth enhancement phenotype is due to higher levels of sugars. Sugars can affect growth both as signaling molecules and as carbon sources. To first determine whether or not changes in sugar sensing are important, we examined the effects of ethylene pretreatment on a double mutant in *Arabidopsis* lacking two proteins involved in sugar sensing, hexokinase1 (HXK1) and regulator of G protein signaling 1 (RGS1) (Fig. S9A). The *hxk1-3;rgs1-2* double mutant still responded to ethylene with more growth. Consistent with HXK1 not being involved, the transcript abundance of *HXK1* was not altered 5 days after ethylene treatment (Fig. S9B). To explore this more, we compared the effects of glucose with mannose. Mannose and glucose are epimers that both signal via HXK1; however, unlike glucose, mannose is poorly metabolized via glycolysis (33–35). If sugar signaling has a major role in the growth enhancement from ethylene pretreatment, then mannose and glucose should have similar effects. However, unlike glucose, mannose inhibited root growth enhancement caused by ethylene (Fig. 1J). These data indicate that the HXK1 sugar sensing pathway is not essential for ethylene-induced growth enhancement. Rather, sugars act as carbon sources to enhance growth after ethylene treatment.

Ethylene pretreatment increases photosynthesis

In the absence of added sucrose, primary growth inhibition, rather than growth enhancement, was observed in seedlings kept in darkness after removal of ethylene (Fig. S10A). This combined with the above results with sucrose, glucose, fructose, and mannose suggests that photosynthesis-derived sugars are important for the growth stimulation. We therefore examined which wavelengths of light cause growth enhancement since transition to light is important for the growth phenotype. *Arabidopsis* seedlings transferred to monochromatic blue, green, or red light had enhanced growth after ethylene pretreatment compared with controls suggesting each contributes to the growth response. However, blue light resulted in growth enhancement similar to white light, whereas less growth enhancement occurred with green and red light. Transfer to far-red light led to no growth enhancement. When these experiments were conducted in the presence of 0.2% (*w/v*) sucrose, growth enhancement by ethylene pretreatment was observed under all wavelengths of light tested and a small, but statistically significant, increase in darkness also occurred (Fig. S10B).

To elucidate the role of photoreceptors in the growth enhancement phenotype, we examined mutants lacking various photoreceptors including mutants that lack the phototropin (Phot) 1 or 2 blue light photoreceptors, a mutant lacking both Phot1 and 2 (*phot1;phot2*), and a quadruple mutant (*phyA,B/cry1,2*) lacking the phytochrome (Phy) A and B photoreceptors for red/far-red light and the cryptochrome (Cry) 1 and 2 photoreceptors for blue light. All of these mutants responded to ethylene pretreatment with growth enhancement (Fig. S11) indicating these receptors are not critical for this trait under these conditions.

Based on these results, we hypothesized that photosynthesis-derived sugars are increased by ethylene pretreatment and are required for this trait. To test this, we evaluated *Arabidopsis* seedling growth in the presence of norfluorazone or lovastatin to block chlorophyll biosynthesis, or in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) which blocks photosynthesis by disrupting electron transfer in photosystem II (36–39). None of these chemicals blocked root growth inhibition caused by application of ethylene for 3 days in darkness (Fig. S12A and B), but, in the absence of added sugar, all three chemicals prevented stimulation of growth by ethylene pretreatment (Figs. 3A and S12C). However, these chemicals also greatly reduced root growth in the control plants making it difficult to determine if they are blocking the effects of ethylene or are having toxic effects on the seedlings. To address this, we examined mutants that affect photosynthesis. Constitutive photomorphogenic 1 (COP1) affects photomorphogenesis, and *cop1* mutants are pale in the light (40). *Arabidopsis* root growth of *cop1-4* seedlings was inhibited by a 3-day treatment with ethylene in darkness (Fig. S12D) showing that this mutant responds to ethylene. However, ethylene pretreatment in the absence of added sucrose failed to stimulate growth of the *cop1-4* roots (Fig. 3B). Ethylene pretreatment did not affect *COP1* transcript abundance 5 days after transfer to light (Fig. S12E). We also examined the effects of several higher order mutants of members of the family of small subunits of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) which have been shown to greatly reduce carbon fixation and growth (41). Neither *rbcs1a2b* nor *rbcs1a3b* double mutants affected responses to ethylene pretreatment (Fig. 3C). However, in the absence of exogenous sucrose, the *rbcs1a2b3b* triple mutants, which fix much less carbon than the double mutants (41), failed to have growth stimulation after ethylene pretreatment. These results show that interfering with either the light reactions of photosynthesis or carboxylation reactions of the Calvin cycle blocks the effects of ethylene on growth. Addition of exogenous sucrose to *cop1-4*, *rbcs1a2b3b*, or DCMU-treated wild-type seedlings partially rescued the enhancement of growth from ethylene pretreatment (Fig. 3A–C). Thus, sucrose is necessary and when sucrose is available, ethylene pretreatment enhances growth, perhaps by enhancing sucrose breakdown.

Since photosynthesis is the source of sugars in plants, we examined various aspects of photosynthesis in more detail to determine whether or not ethylene pretreatment affects photosynthesis. The cotyledons of seedlings treated with ethylene were greener than control seedlings for several hours after transfer to light. This combined with our results with sucrose and the fact that photosynthesis is most rapid in blue light where we see the largest growth enhancement led us to question whether or not increased chlorophyll content and photosynthesis underlie the increased growth we observe. Both the optimum quantum efficiency (F_v/F_m) (Fig. 3D) and chlorophyll content (Fig. 3E) of ethylene-pretreated seedlings were higher for several hours after transfer to light and ethylene-free conditions. However, these differences were not long-lasting. Thus, it seems unlikely that these early, transient changes cause the enduring growth changes, but may be involved in the early stages of growth enhancement after transfer to light.

We hypothesized that genes involved in the growth enhancement would be altered several days after removal of ethylene. Therefore, we analyzed the transcript levels of several genes that encode proteins related to photosynthesis. Of the six genes examined, ethylene pretreatment caused an increase in the transcript abundance of four genes 5 days after transfer to light (Fig. 3F). This included *chlorophyll a/b binding protein 2* (*CAB2*), which encodes a protein that is part of the light-harvesting

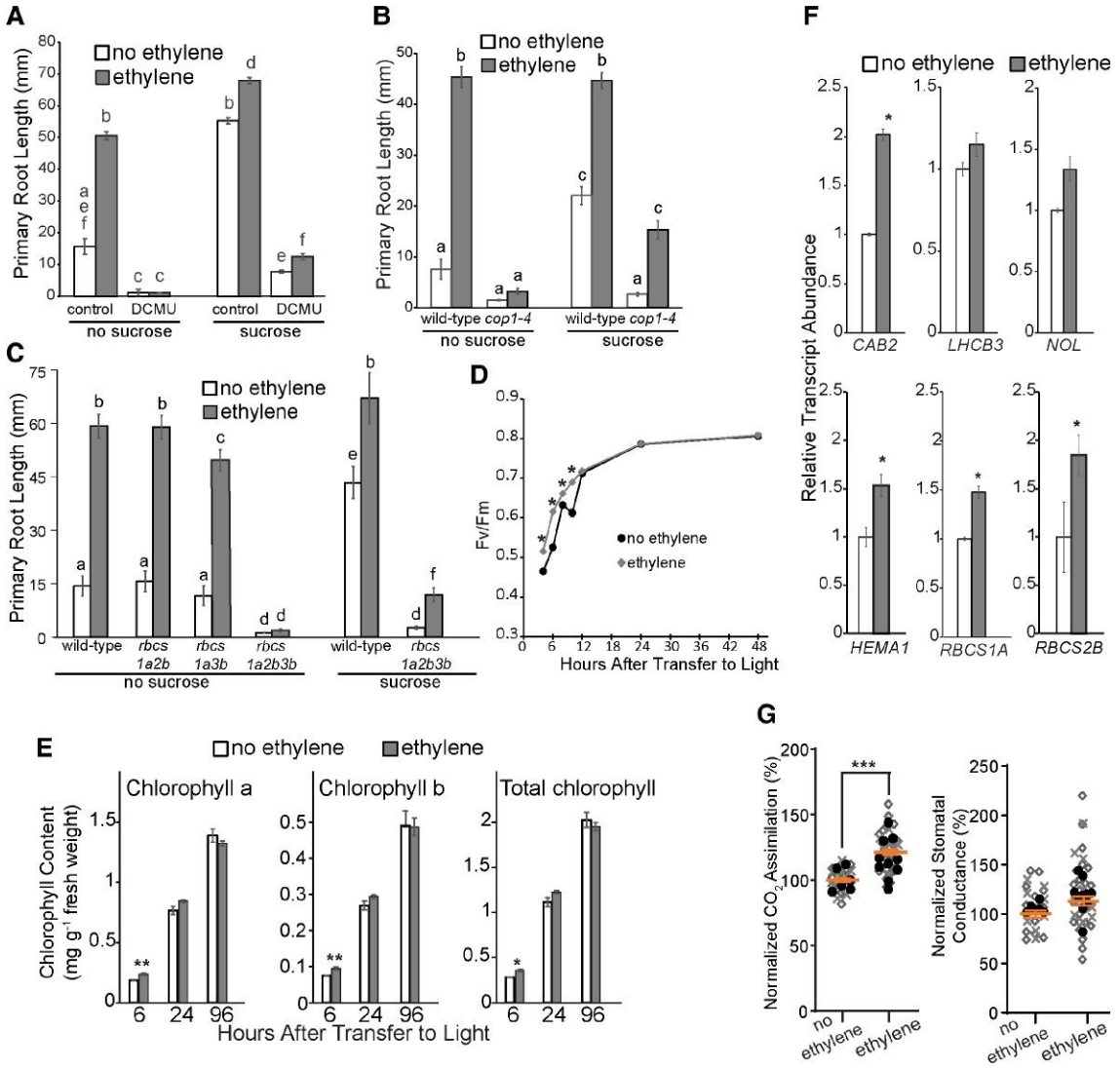

Fig. 3. Ethylene pretreatment increases carbon assimilation. Germinating *Arabidopsis* seeds were treated with 0.7 ppm ethylene or ethylene-free air for 3 days in the dark and then transferred to ethylene-free air and light. Unless otherwise indicated, no exogenous sugar was added. A) Wild-type seeds were sown in the presence or absence of 0.2% sucrose in the presence of 2.5 μM DCMU to block electron transport. Solvent-treated samples are included as controls. B, C) Wild-type and *cop1-4* B) or *rbcs* C) mutants were sown in the absence or presence of 0.2% (*w/v*) sucrose. A–C) New root primary root growth was measured 9 days after transfer to light. Data are the average ± SEM of at least 15 seedlings. Different letters denote statistical difference ($P < 0.05$) using ANOVA. D) The F_v/F_m was determined in tissue of wild-type seedlings at the indicated times after transfer to white light. Data represent the mean ± SEM ($n \geq 9$). E) Chlorophyll was extracted from excised cotyledons of wild-type seedlings and quantified at different times after transfer to white light as indicated. Data were normalized to tissue fresh weight and represent the mean ± SEM ($n \geq 6$). D, E) Data were analyzed by Student's *t* test and found to be statistically different from seedlings not treated with ethylene with a $^*P < 0.05$ and $^{**}P < 0.005$. F) Transcript levels of selected genes that encode proteins involved in photosynthesis and chlorophyll metabolism were evaluated by qPCR as described in the Materials and methods at 5 days after transfer to light. Each gene was normalized to its levels in the control condition and to housekeeping genes. G) Measurements of carbon assimilation and stomatal conductance in individual leaves 3 weeks after transfer to light were made in three separate experiments and normalized to the amount in control samples. Different symbols represent individual data points from the different experiments. The mean ± SEM is plotted ($n \geq 29$). In D), F), and G), data were analyzed by Student's *t* test and found to be statistically different from seedlings not treated with ethylene with a $^*P < 0.05$ or $^{***}P < 0.001$.

complex associated with photosystem II, *glutamyl-tRNA reductase* (*HEMA1*) involved in chlorophyll biosynthesis, and *RBCS1A* and *RBCS2B*. Correlating with these increases in *RBCS1a* and *RBCS2B*, ethylene pretreatment led to a 23% increase in CO_2 assimilation 3 weeks after transfer to light (Fig. 3G) showing that photosynthetic capacity is increased long-term. In contrast, stomatal conductance was not significantly affected. Together, these results indicate that ethylene pretreatment leads to long-lasting increases in expression of select transcripts encoding proteins related to photosynthesis which are likely involved in the long-term increase in carbon fixation.

Ethylene pretreatment leads to increased levels of starch and glucose

To explore further the link between ethylene pretreatment, enhanced growth, and photosynthesis-derived sugars, we grew *Arabidopsis* seedlings in the absence of added sugars and compared the levels of starch and glucose in ethylene-pretreated seedlings compared with controls 5 days after transfer to light. As seen in Fig. 4A and B, ethylene pretreatment led to an increase in starch levels in cotyledons, leaves, and roots after transfer to ethylene-free conditions. Consistent with this increase in starch, the transcript abundance of *starch synthase* 3 (*SS3*) that encodes

a starch biosynthesis enzyme was increased in the seedlings pretreated with ethylene (Fig. 4C). The *sucrose phosphate synthase 3F* (*SPS3F*) gene involved in sucrose biosynthesis and the *glucuronic acid substitution of xylan 2* (*GUX2*) gene involved in cell wall biosynthesis were also up-regulated by ethylene pretreatment.

Ethylene pretreatment also results in 266% elevation of seedling glucose levels compared with controls (Fig. 4D). We also observed a concomitant increase in the transcript abundance of *cytosolic invertase 1* (*CINV1*) and *CINV2* which encode enzymes that degrade sucrose to glucose and fructose and are critical for normal growth (42) (Fig. 4E). Furthermore, mutant seedlings with the loss of both genes (*cinv1;cinv2*) did not respond to ethylene pretreatment with enhanced root growth in either the presence or absence of sucrose (Fig. 4F). Root and hypocotyl growth of *cinv1;cinv2* seedlings were inhibited by a 3-day treatment with ethylene in darkness (Fig. S13) showing that this mutant responds to ethylene. These results are consistent with a model where ethylene pretreatment is leading to an increase in the degradation of sucrose by these invertases to form glucose and fructose which leads to more growth.

The above results show that transient ethylene treatment of dark-grown seedlings grown in the absence of added sugar results in sustained changes in the accumulation of carbohydrates upon subsequent growth under light conditions. To determine how widespread the effects of ethylene pretreatment are on cellular metabolism, we carried out an untargeted metabolomic analysis on seedlings grown in the absence of added sugar 6 days after removal of ethylene and transfer to light. Partial least squares discriminant analysis of the data shows a clear separation of control versus ethylene-pretreated samples (Fig. S14A). Based on variable importance in the projection (VIP) scores, 15 metabolites, including trehalose/sucrose, are contributing the most to this separation (Fig. S14B). Of the 131 metabolites detected, 13 were significantly reduced and 97 were significantly increased ($P \leq 0.05$, *t* test) by pretreatment with ethylene (Table S1 and Fig. S14C). Pathway analysis shows that carbon fixation was up-regulated supporting our observations above for an increase in carbon assimilation (Fig. S15 and Table S2). Additionally, amino acid metabolism, purine and pyrimidine metabolism, glyoxylate/dicarboxylate metabolism, glutathione metabolism, and nicotinate/nicotinamide metabolism were affected by ethylene pretreatment. The map of altered metabolism illustrates the interconnectedness of the pathways involved and the widespread up-regulation of metabolism. These data show that ethylene pretreatment results in prolonged changes in many metabolites in multiple metabolic pathways. We refer to this as ethylene-mediated metabolic priming.

To further explore processes affected by ethylene-mediated metabolic priming, we conducted RNA sequencing analyses on seedlings 5 days after transfer to light. Under these conditions, ethylene pretreatment resulted in 1,938 gene transcripts being altered 2x or more with an adjusted $P \leq 0.05$. Of these, 1,102 were up-regulated and 836 were down-regulated (Fig. S16 and Spreadsheet S1). We examined these genes for enrichments in Gene Ontology (GO) biological processes (43). This revealed that processes associated with up-regulated genes include development, differentiation, morphogenesis, cell cycle, cell division, and cell wall organization (Table S3). In contrast, almost all GO biological processes associated with down-regulated genes have to do with responses to different environmental signals and stress (Table S4). Thus, there appears to be a shift toward growth-related processes and away from defense-related processes. Many of the altered gene transcripts mapped onto the same metabolic pathways as the altered metabolites including carbohydrate metabolism, carbon fixation, amino acid metabolism, and purine and pyrimidine metabolism (Fig. S17). However, additional processes altered at the transcript level by ethylene pretreatment became apparent from the RNA-seq results including fatty acid metabolism, plastoquinone cycling, and the biosynthesis of tocopherol, isoprenoids, monolignol, flavanol, and flavone. These data indicate that transient treatment of plants with ethylene results in up-regulation of processes involved in plant growth and development.

Seedlings are more stress tolerant after transient treatment with ethylene

Ethylene is a critical hormone that coordinates many stress responses in plants (1). Ethylene pretreatment is known to "prime" plants to survive better upon subsequent challenge with various stresses (44–54). Therefore, we asked whether or not ethylene pretreatment under the conditions reported here also affects tolerance to various abiotic stresses. Since exogenous sugar can increase stress tolerance (55, 56), unless otherwise noted, no sugar was added to the media for these stress experiments. *Arabidopsis* plants were exposed to each stress 5 days after transfer to light and removal of ethylene. In the absence of ethylene pretreatment, most seedlings exposed to 22 min of high temperature (43°C) or 7 days of 150 mM NaCl, or during recovery from 12 h of hypoxia in darkness, became bleached and died (Figs. 5 and S18). Ethylene priming led to more seedlings surviving exposure to the stress. In seedlings exposed to either high temperature or salt stress, nearly 100% of seedlings survived the stress after exposure to ethylene. By 2 days after hypoxia, the ethylene-pretreated seedlings showed a clear and statistically significant higher survival rate compared with the control seedlings. This trend continued to day 3 of recovery from hypoxia. In all three stress conditions, ethylene pretreatment had no measurable effect on the survival of seedlings not challenged with the stress. Loss of both CINV1 and CINV2 resulted in seedlings that did not respond to ethylene pretreatment with enhanced heat tolerance (Fig. 5A) and application of sucrose phenocopied ethylene with increased heat tolerance (Fig. 5D) suggesting that carbohydrate levels are important for the effects of ethylene priming on stress tolerance.

Discussion

Enhancing plant growth and vigor is an important challenge that needs to be met to feed a growing human population. Methods to increase growth and stress resistance are key to addressing this challenge. However, there is often an inverse relationship between growth and stress tolerance (57). This trade-off can have profound implications on bioengineering strategies to enhance plant yield. Despite these challenges, bioengineering has led to plants with 25% or more photosynthetic efficiency, growth, and yield (58–60). Here, we show that treating dark-grown *Arabidopsis* seedlings with ethylene for several days followed by transfer to ethylene-free conditions and light results in robust increases in the growth of leaves and roots and increased tolerance to abiotic stresses. Correlating with these results, seedlings pretreated with ethylene have almost a 25% increase in carbon fixation and large increases in carbohydrate levels. Although auxin is often involved in growth-related phenotypes, our results indicate that ethylene pretreatment is not altering auxin responses to stimulate leaf and primary root growth. NPA blocks the enhanced formation of lateral roots by ethylene pretreatment. More experiments will be needed to determine if this simply

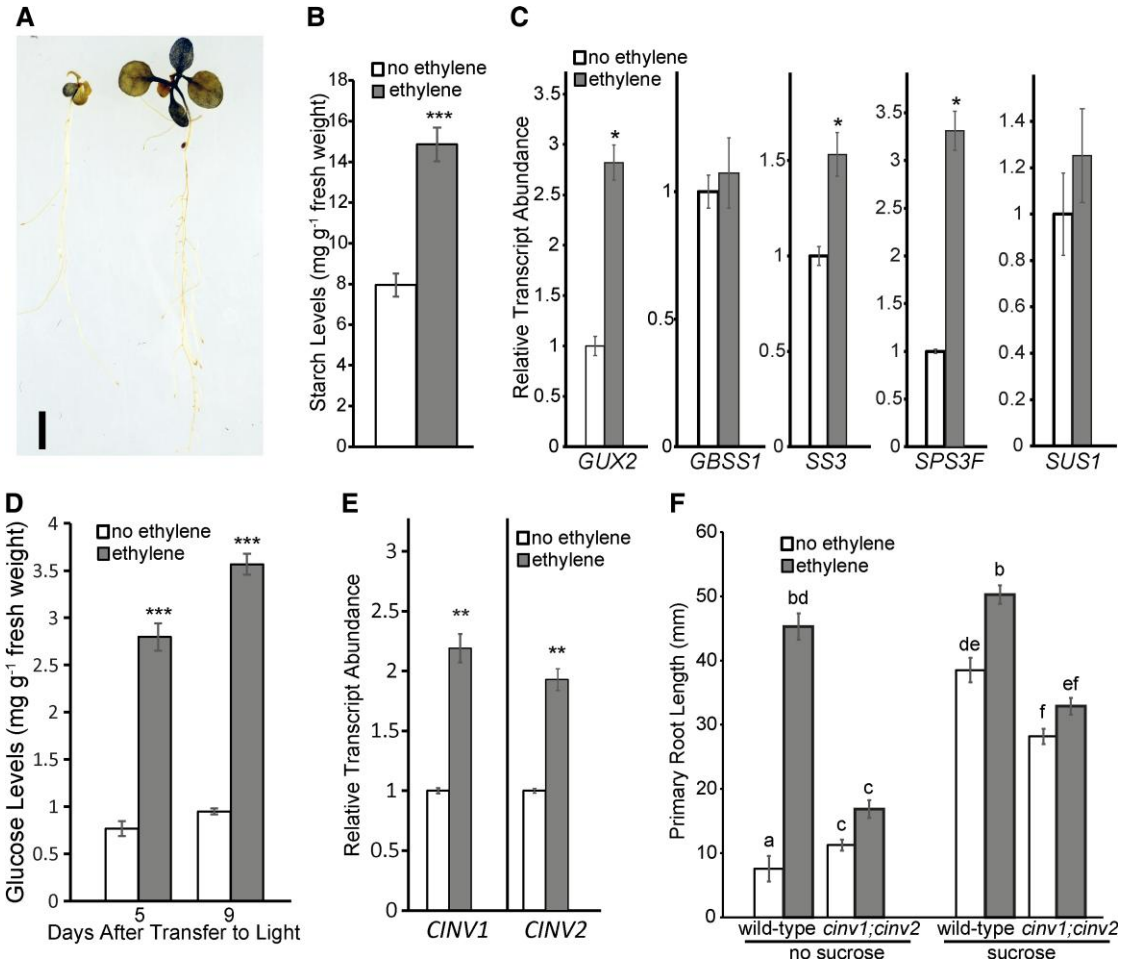

Fig. 4. Ethylene pretreatment increases starch and glucose levels. Germinating *Arabidopsis* seeds in the absence of added sugar were treated with 0.7 ppm ethylene or ethylene-free air for 3 days in the dark and then transferred to ethylene-free air and white light with a 16-h photoperiod. A) Control (left) and ethylene-treated (right) seedlings stained for starch as described in the Materials and methods 9 days after transfer to light. Scale bar is 3 mm. B) Quantification of starch levels normalized to fresh weight of the tissue at 9 days after transfer to light ($n = 6$). C) Transcript levels of genes that encode enzymes involved in starch and sucrose biosynthesis were evaluated 5 days after transfer to ethylene-free air and light by qPCR as described in the Materials and methods. Each gene was normalized to its levels in the control condition and to housekeeping genes. D) Quantification of glucose levels normalized to fresh weight of the tissue at the indicated days after transfer to light ($n = 5$). E) Transcript levels of *CINV1* and *CINV2* were evaluated 5 days after transfer to ethylene-free air and light by qPCR as described in the Materials and methods. Each gene was normalized to its levels in the control condition and to housekeeping genes. In B–E), Student's *t* test was used to determine statistically significant change from the control samples (*$P < 0.05$; **$P < 0.005$; ***$P < 0.001$). F) The amount of new primary root growth was compared in Col (wild-type) and *cinv1;cinv2* double mutants 9 days after transfer to light in the presence or absence of 0.8% (*w/v*) sucrose. Different letters denote statistically significant differences ($P < 0.05$) as determined by ANOVA. Data in B–F) are the mean ± SEM ($n \geq 15$).

reflects a requirement for auxin transport or if ethylene pretreatment is altering auxin responses for this trait. In contrast, our studies reveal that transient ethylene treatment results in large and long-term increases in both photosynthesis and carbohydrate abundance resulting in metabolic priming to impact growth and stress tolerance that is comparable to existing bioengineering strategies.

Photosynthetically derived sugars control root growth early in seedling development (61), and hexoses and sucrose can affect *Arabidopsis* root growth and architecture (32, 62, 63). Previous work showed that ethylene affects chloroplast development and chlorophyll levels and photosynthesis in diverse ways depending on factors such as plant species, age of the plant, lighting conditions, and stress conditions (2, 21), and treating etiolated *Arabidopsis* seedlings with ethylene promotes greening when they are transferred to light (18, 19). Here, we show an interesting effect of ethylene on photosynthesis that is induced in darkness, but is sustained long-term after removal of ethylene and growth in the light. Similar to a prior study on cucumber (64), an increase in chlorophyll levels and optimum quantum efficiency occurred in etiolated *Arabidopsis* seedlings pretreated with ethylene compared with controls. However, these differences disappeared by 12 h after transfer to light making it unlikely that these changes are important for the long-lasting changes in growth. However, the increase in root growth occurs within several hours after transfer to light and these early, transient changes in photosynthesis may have a role in the early stages of growth stimulation. Long-lasting increases in *RBCS1a*, *RBCS2b*, *CINV1*, and *CINV2* occurred after ethylene treatment which correlates with the higher carbon assimilation and carbohydrate levels we measured and increased growth.

Our results with monochromatic light indicate that blue light is important for growth enhancement after priming. Although photosynthesis is stimulated by blue light and photosynthesis is important for this trait, plants also respond to blue light via photoreceptors such as the Cry and Phot (65, 66). Higher order *phot1;*

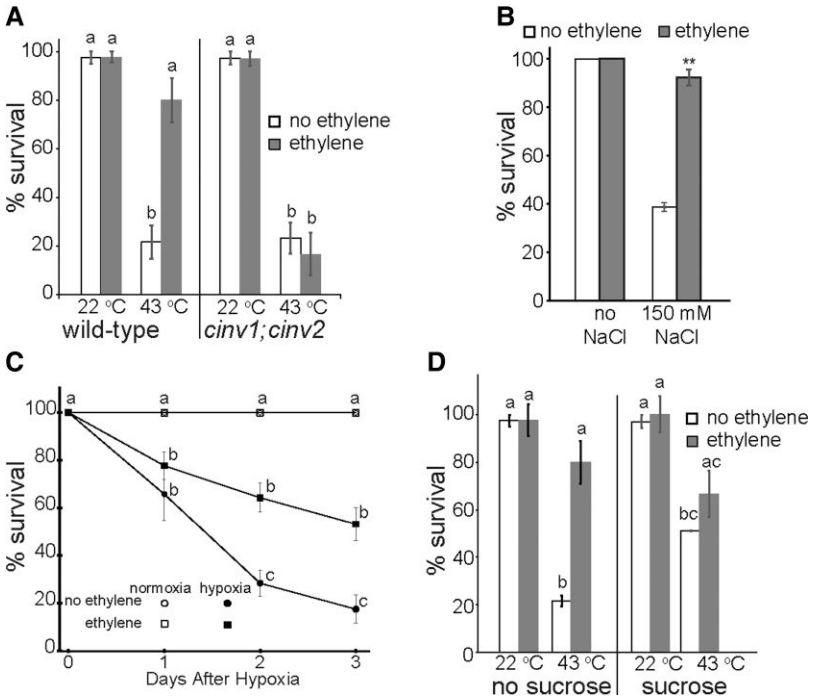

Fig. 5. Ethylene pretreatment increases tolerance to abiotic stresses. Germinating *Arabidopsis* seeds in the absence of added sugar were treated with 0.7 ppm ethylene or ethylene-free air for 3 days in the dark and then transferred to ethylene-free air and white light with a 16-h photoperiod. A) Five days after transfer to light, Col (wild-type) and *cinv1;cinv2* seedlings were exposed to high temperature (43°C) for 22 min. Survival rates 2 days after exposure to high temperature are shown compared with seedlings not stressed with high temperature (22°C). B) Five days after transfer to light, seedlings were exposed to 150 mM NaCl stress and survival assessed 1 week later compared with no stress controls. $^{**}P < 0.01$ indicates statistical difference from the no ethylene condition using Student's t test. C) Five days after transfer to light, the seedlings were exposed to hypoxia stress for 12 h in darkness and then transferred back to white light and normoxia and allowed to recover. Normoxia controls are shown for comparison. Survival was assessed each day for 3 days. Different letters denote statistically significant differences ($P < 0.05$) on each day as determined by ANOVA. D) Wild-type seedlings were treated as in A) in the presence or absence 0.2% (*w/v*) sucrose. In A) and D), different letters denote statistically significant differences ($P < 0.05$) as determined by ANOVA.

phot2 and *phya,b;cry1,2* mutants still respond to ethylene priming with enhanced growth. However, both mutant combinations slightly reduce the growth enhancement response. One interpretation of this is that these photoreceptors are involved, but not required, for this response. For instance, *phot* mutants affect chloroplast movement which could alter photosynthesis (67) to reduce the effects of priming. Alternatively, these photoreceptors may be required but have overlapping functions that will only become evident with higher order mutant combinations that eliminate both sets of blue light receptors. It is also possible that other blue light receptors not tested in this study are required (68, 69).

The growth enhancement from ethylene pretreatment was blocked in *cop1* and *rbcs1a2b3b* mutants and by chemical inhibitors of photosynthesis and chlorophyll synthesis. Thus, in the absence of exogenous sucrose, proper chloroplast development and photosynthesis are required to observe growth stimulation after treatment with ethylene. Addition of sucrose to *cop1*, *rbcs1a2b3b*, and DCMU-treated wild-type seedlings partially rescues the effects of ethylene pretreatment. These observations coupled with the fact that ethylene pretreatment causes a long-lasting up-regulation of *RBCS1a*, *RBCS2b*, and carbon fixation are consistent with the idea that increases in photosynthetically derived sugars are involved in the growth enhancement phenotype. The fact that such enhancement is not observed in *rbcs1a2b3b* seedlings shows that the increases in *RBCS* transcripts are required for the effects of ethylene pretreatment.

Addition of sucrose to seedlings maintained in darkness after ethylene treatment results in ethylene-mediated root growth stimulation supporting the idea that ethylene is leading to changes in sugar utilization independently of photosynthesis. Ethylene pretreatment leads to large increases in sucrose, glucose, and starch which correlate with increases in several genes related to carbohydrate metabolism including *CINV1* and *CINV2* that encode invertases that break down sucrose and are critical for carbon allocation for cellulose biosynthesis and normal plant growth (42, 70, 71). We show that CINV1 and CINV2 are required for the growth enhancement caused by ethylene pretreatment, and addition of sucrose does not rescue this trait in *cinv1;cinv2* seedlings. Thus, these invertases are regulated by ethylene and are required for the long-term growth effects caused by ethylene-mediated metabolic priming. However, our data do not distinguish between direct regulation of CINV1 and CINV2 by ethylene and indirect regulation via other factors such as increased sucrose levels from higher photosynthesis. Taken together, our results support a model where transient treatment of dark-grown seedlings with ethylene results in a factor or factors that, upon transfer to light and ethylene-free conditions, result in the long-lasting up-regulation of genes that lead to increased photosynthesis and carbon assimilation that lead to higher levels of glucose, sucrose, and starch and other metabolites to enhance growth (Fig. 6). Whether or not any of these effects are directly due to ethylene signaling or are secondary effects caused by other changes has yet to be determined. Although the metabolomic data indicate many metabolites are up-regulated by ethylene, the results with *cinv1;cinv2* suggest that it is the breakdown of sucrose that is critical for growth enhancement. Our experiments demonstrated that the

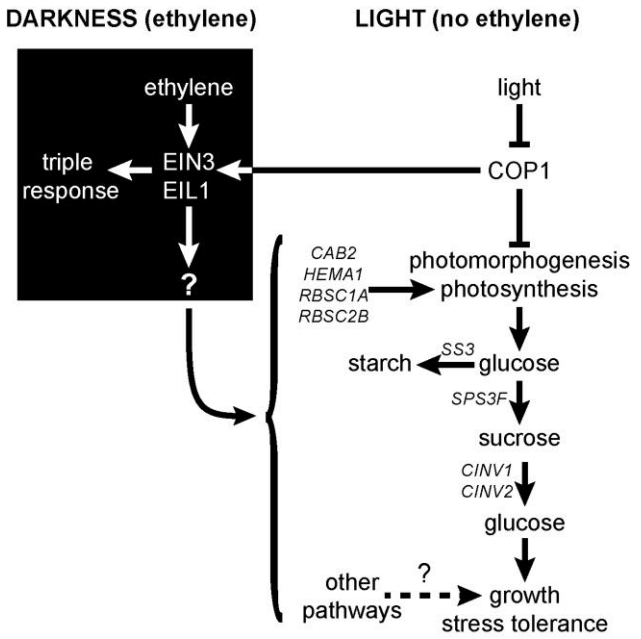

Fig. 6. Model for enhanced plant vigor from ethylene pretreatment in darkness. In this model, exposure to ethylene while seeds are germinating in darkness activates ethylene signaling resulting in higher EIN3/EIL1 activity. In the dark, this results in the "triple response." Also, an unknown factor or factors downstream of EIN3 and EIL1 lead to long-lasting changes that, upon illumination and removal of ethylene, result in increased photosynthesis and sugar metabolism resulting in more stress tolerance and growth. It is currently unclear if ethylene is directly affecting all of these pathways or if some effects are secondary to a primary response. In either case, increased photosynthesis leads to higher glucose levels in the leaves to increase starch accumulation. The synthesis of sucrose is also increased which is transported from source to sink tissues where it is broken down to glucose (and fructose). Metabolomic data indicate other metabolic pathways are also affected. Although COP1 is known to affect EIN3 levels in the dark, our data suggest that COP1 affects priming via its role in photomorphogenesis. The changes in photosynthesis and carbohydrate metabolism might occur naturally when seeds germinate underground in darkness and ethylene levels are high due to mechanostimulation from the soil. Upon emergence into light aboveground, the seedlings are exposed to less ethylene because of less mechanostimulation and diffusion away from the aboveground parts of the plant leading to long-lasting developmental changes.

growth stimulation phenotype is probably due to increased carbon availability, rather than sugar signaling via HXK1. However, we cannot rule out that sugar signaling via other pathways that control growth and stress responses, such as via the target of rapamycin (TOR) and sucrose non-fermenting 1-related protein kinase 1 (SnRK1) pathways (72), are important for this response.

RNA-seq revealed that ethylene pretreatment leads to the long-term up-regulation of genes involved in development and morphogenesis and down-regulation of genes annotated to be involved in signaling for environmental stresses. This is consistent with the fact that stress tolerance and growth often show an inverse relationship. However, physiologically, we observe an increase in both growth and tolerance to several abiotic stresses. Given the large increases in carbohydrate levels, the increase in both growth and stress tolerance is consistent with prior research showing that exogenous application of sugars can increase growth (61) and stress tolerance (55, 56), and starch stores are often key to survival of plants under stress (73). Furthermore, ethylene pretreatment increases the levels of both *CINV1* and *CINV2* and *cinv1;cinv2* loss-of-function mutants fail to have increased growth or heat stress tolerance indicating that regulation of sucrose breakdown is a key process affected by ethylene pretreatment. Ethylene has previously been shown to prime plants for specific stresses (44, 45, 47–54), but in most cases, this has not been linked to photosynthesis or carbohydrate levels. However, pretreatment of rice seedlings with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid to increase ethylene levels caused enhanced submergence tolerance and led to higher chlorophyll levels (46) supporting the idea that metabolic priming by ethylene is widespread across angiosperm species. Our results showing enhanced growth of tomato, cucumber, and wheat support this. It is likely that other factors are influenced by ethylene to affect stress tolerance. For instance, the metabolomic analysis indicates that several pathways that affect stress tolerance, such as glyoxylate, nicotinate, and glutathione metabolism, are affected by ethylene-mediated metabolic priming.

It is likely that ethylene signaling in darkness is leading to long-term changes that are indispensable for the growth phenotype. Our data do not identify which factor(s) downstream of EIN3/EIL1 mediate the ethylene effects in darkness that are responsible for these long-term changes upon transfer to light. However, distinct light and dark transcriptional networks regulated by EIN3 and EIL1 could provide a clue to the mechanism for this trait. Two such candidates that are involved in the distinct light and dark responses to ethylene are the PIF transcription factors (15, 17–19, 24, 25) and COP1 (21, 64, 70). Our results with *pifq* plants indicate these PIFs are not involved; however, COP1 is required for the growth enhancement phenotype in the absence of exogenous sucrose. COP1 has previously been shown to regulate EIN3 since EIN3 levels are enhanced by COP1 in darkness (19, 74). However, given the importance of COP1 in the development of chloroplasts, it is more likely that COP1 is required for growth enhancement through its effects on photomorphogenesis and photosynthesis. In support of this, exogenous sucrose does not rescue ethylene-mediated growth enhancement of *ein3;eil1*, but does rescue *cop1-4* suggesting that EIN3/EIL1 are not acting downstream of COP1 for this trait.

During seed germination, mechanical stress caused by growing through soil leads to higher ethylene levels, which is important for seedlings to sense mechanical stress and successfully navigate through the soil from darkness into light (18, 74–78). Our studies show that germinating seeds may require this initial stress to increase ethylene levels to affect development for robust growth and tolerance to stresses. This is consistent with the idea that mechanical stress can prepare plants against future stresses by affecting hormone biology (79). The growth stimulation caused by ethylene seems to be a general trait of angiosperms since we see similar ethylene-induced growth enhancement in several angiosperm species. Thus, this represents a possible new approach to increase plant vigor, perhaps in greenhouse applications where plants can be easily pretreated with ethylene.

Materials and methods

Plant materials

Unless otherwise specified, experiments were carried out on *A. thaliana* in the Col background. Col and Wassilewskija seeds are lab stocks. The *ein2-5* seeds were from Anna Stepanova (7), *ein3-1;eil1-1* seeds from Joseph Ecker (12), *cop1-4* mutants from Peter Schopfer (61), *cinv1;cinv2* seeds from Charles Anderson (70), mutants of the small subunits of Rubisco (*rbcs2b3b*, *rbcs1a3b*,

and *rbcs1a2b3b*) were from Alistair McCormick (41), and *hxk1-3; rgs1-2* (80), *pif1-1;pif3-7;pif4-2;pif5-3* (81), and additional ecotypes and the *phot1* and *phot2* seeds were from the Arabidopsis Biological Resource Center. The DR5::GUS lines were from Elena Shpak. All mutants are in the Col background. Seeds of tomato were obtained from Zellajake Farm and Garden, cucumber from Isla's Garden Seeds, and wheat from Palouse Brand.

Preparation of seeds and growth

Arabidopsis seeds were surface sterilized and sown on 0.8% (*w*/*v*) agar containing half-strength Murashige and Skoog basal salt mixture, pH 6.0 with no added sugar. In some experiments, the medium was supplemented with the indicated concentration of other compounds. Prior to growth experiments, seeds were stratified for 2–3 days at 4°C, light-treated for 2 to 4 h under continuous fluorescent lights, and then wrapped in aluminum foil. Seeds were allowed to germinate and grow on vertically orientated plates for 3 days in darkness in chambers through which ethylene-free air or ethylene was passed at a flow rate of 50 ml min^{-1}. Unless otherwise specified, ethylene treatment of seedlings was carried out with methods modified from Binder et al. (82) using a concentration of 0.7 ppm ethylene. At the end of this treatment (designated day 0), images were acquired by scanning on a flat-bed scanner. The position of each root tip was then marked, and the plates were transferred to ethylene-free chambers under a 16-h photoperiod (120 µmol m^{-2} s^{-1}) at 21 to 23°C for up to 14 days. To examine the effects of monochromatic light, seedlings were transferred to ethylene-free conditions under continuous 40 µmol m^{-2} s^{-1} white, 53 µmol m^{-2} s^{-1} blue (λ_{max} = 462 nm), 20 µmol m^{-2} s^{-1} green (λ_{max} = 525 nm), 28 µmol m^{-2} s^{-1} red (λ_{max} = 672 nm), 12 µmol m^{-2} s^{-1} far-red (λ_{max} = 732 nm), or darkness for 9 days. Monochromatic light was delivered using light-emitting diode arrays from Quantum Devices. The amount of new primary root growth and number of lateral roots were determined using ImageJ (ver. 1.52E). Lateral root density was then calculated for each seedling from these parameters. For all analyses, the length of new root growth after transfer to light is measured. In some experiments, leaf biomass was determined 14 days after transfer to light by removing shoot tissue and weighing the tissue fresh weight for each seedling.

To determine the longer-term effects of ethylene pretreatment, *Arabidopsis* seeds were prepared as above and sown in soil comprised of a 2:1 mixture of peat light:perlite. They were then treated in darkness for 4–5 days with either 0.7 ppm ethylene or ethylene-free air followed by ethylene-free conditions in a 16-h photoperiod. Images were taken at the times designated.

Tomato and cucumber seeds were surface sterilized with 5% (*v*/*v*) bleach for 30 min. Tomato seeds were placed under white light for 2–3 h and then planted in soil mixture. Cucumber seeds were soaked in water for 1 h under white light, then excess water removed and light treated for 2 h. They were then transferred to soil. Wheat seeds were surface sterilized with 33% (*v*/*v*) bleach for 15 min, rinsed three times with sterile distilled water, and sown on 0.8% (*w*/*v*) agar plates. For all three species, the seeds were then treated in darkness for 3.5–4 days with either 0.7 ppm ethylene or ethylene-free air. They were transferred to ethylene-free and long-day conditions. At the indicated times, images were taken of the plants. The height and leaf area of tomato were determined using ImageJ. Wheat seedlings were imaged 1.5 days after transfer to light and the length of the primary root determined using ImageJ.

Growth rate measurements of primary roots

Seedlings were treated and grown on vertically orientated plates as described above. After 3 days in the dark in the presence or absence of 0.7 ppm ethylene, seedlings were transferred to continuous white light (75 µmol m^{-2} s^{-1}) and ethylene-free conditions. At this time, high-resolution time-lapse imaging was carried out on the primary roots using imaging setups previously described (83). Imaging was started 15 min after transfer to light and images were acquired every 15 min for 22 h. New growth in each time interval was then determined and the growth rate calculated for each time interval. To estimate the latent period for an effect of ethylene pretreatment, we used two methods. One was to determine the time at which ethylene pretreatment led to a statistically significant change in the mean growth rate between the two conditions using Student's *t* test ($P \leq 0.05$). The other was using cumulative sum control chart analysis to determine the time at which the growth rate trends diverged between the control and experimental seedlings (84).

Photosynthesis and chlorophyll measurements

To determine optimum quantum efficiency (F_v/F_m) and chlorophyll levels, ~50 *Arabidopsis* seeds were plated on agar plates and treated as above. For quantum efficiency measurements, plates of seeds were transferred to continuous white light and at the times indicated placed in a FluoroCam 800MF (Photon Systems Instruments, Czech Republic), dark acclimated for 3–5 min, and the F_v/F_m measured using a preprogramed protocol. To determine chlorophyll a and chlorophyll b levels, cotyledons were excised at the times indicated. Chlorophyll was extracted with acetone and quantified with a spectrophotometer according to the methods of Lichtenthaler (85). Data were normalized to the tissue fresh weight.

To determine photosynthetic capacity and stomatal conductance, *Arabidopsis* plants were grown in soil and treated as above. After 24 days growth in a 16-h photoperiod, leaf 7 was clamped in a cuvette (LI6400XT, LI-COR, Lincoln, NE, USA) with a light emitting diode. Light intensity was 350 µmol m^{-2} s^{-1} (10% blue and 90% red) with a block temperature of 22°C, humidity of 55%, constant CO_2 concentration maintained at 400 µmol mol^{-1}, and a flow rate of 500 µmol s^{-1}. When stomatal conductance and net assimilation were stable, two to three measurements were made to represent a technical replicate. All experiments were repeated three times.

Ethylene measurements

Arabidopsis seeds were prepared as above except they were placed on agar in 130-ml jelly jars covered with aluminum foil. Eight days after treatment with ethylene, the jars were sealed with gas-tight lids fitted with a septum, and 24 h later, the amount of ethylene in the headspace was measured using an ETD-300 ethylene detector (Sensor Sense).

GUS reporter gene assay and microscopy

Transgenic seedlings expressing DR5::GUS were grown and treated with ethylene or ethylene-free air as described above. GUS staining was carried out 5 days after transfer to light. Images of leaves and roots were taken using a dissecting microscope (Olympus SZH10) with a digital camera (Canon Rebel T1i).

Starch and glucose analyses

Arabidopsis seedlings were grown and treated with ethylene and ethylene-free air as described above. For starch staining, seedlings

were harvested at 9 days after transfer to light and then were boiled in 95% ethanol to remove chlorophyll after which seedlings were rinsed twice in distilled water. Leaves were stained for 10 min in Lugol's iodine solution (Volu-Sol) in the dark and then rinsed in water to clear excess background. Images were acquired using Zeiss Stereo microscope Discovery V8 with a Canon camera. For starch quantification, seedlings were collected 9 days after transfer to light and for glucose quantification 5 and 9 days after transfer to light. For both assays, seedlings were frozen in liquid nitrogen after weighing and then ground into fine power in a 2-ml Eppendorf tube containing two steel balls using a homogenizer (Biospec). Starch quantification was performed as described in the protocol of the starch assay kit (SA20, Sigma) and glucose quantification as described in the glucose assay kit (GAHK20, Sigma). For all assays, seedlings were harvested at 10 h into the photoperiod.

Untargeted metabolomic analysis

Arabidopsis seedlings were grown and treated as described above. Seedlings were harvested 6 days after transfer to light and at 10 h into the photoperiod. Samples were weighed and quickly frozen in liquid nitrogen before untargeted metabolomic analysis using ultraperformance liquid chromatography coupled with hybrid quadrupole Orbitrap mass spectrometry (UPLC Orbitrap MS/MS) at the Biological and Small Molecule Mass Spectrometry Core Facility in the Department of Chemistry at the University of Tennessee. MetaboAnalyst 5.0 was used for partial least squares discriminant analysis and VIP scores and to conduct pathway analysis (86). Data represent the analysis of five biological replicates for controls and four replicates for ethylene-pretreated samples which were each analyzed with three technical replicates and normalized to tissue fresh weight.

RNA isolation and real-time qRT-PCR

Whole seedlings were collected and quickly frozen in liquid nitrogen. Total RNA was extracted using the PureLink plant RNA reagent (Ambion, TX, USA) and treated with turbo-DNase (Invitrogen). RNA was purified using acid phenol–chloroform (Invitrogen). RNA concentration was determined using a NanoDrop after which samples were normalized to the same concentration for cDNA synthesis. First-strand cDNA synthesis was done using SensiFAST cDNA synthesis kit (Meridian Bioscience). Primers were designed across exon junction using the online Primer3Plus program. Secondary structures were identified to eliminate primer dimers using the IDT UNAFold program, and primer quality and efficiency were determined to be between 90 and 100%. qPCR was performed using Sensifast SYBR No-Rox (Meridian Bioscience) as described in the manual with the following PCR conditions: 28 cycles of 98°C for 30 s, 98°C for 10 s, 60°C for 30 s, and 72°C for 30 s followed by 72°C for 5 min. Gene expression levels relative to the previously validated reference genes *UBQ10* (At4g05320) and *GAPDH* (At1g13440) (87) were used for each sample following the method of Pfaffl (88). Specific primer sequences for these analyses are listed in Table S5.

RNA sequencing

Arabidopsis seeds were germinated in the presence or absence of 0.7 ppm ethylene in darkness for 3 days, followed by transfer to ethylene-free conditions and light for 5 days. At this time, seedlings were harvested and quickly frozen in liquid nitrogen. Total RNA was extracted using TRIzol and sent to Genewiz (Azenta Life Sciences, South Plainfield, NJ) for library preparation and sequencing using an Illumina HiSeq 4000. This produced over 25 million paired-end reads per sample. All data analyses including read trimming, GO analyses, differential gene analysis, and mapping reads to the *A. thaliana* reference genome were conducted by Genewiz. Three biological replicates were used for each condition. The RNA-seq data discussed in this publication are deposited in the NCBI's Gene Expression Omnibus (89) and are accessible through the GEO Series Accession Number GSE218645 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE218645).

Salt stress assays

Seeds were initially sown on sterile nylon filters placed on agar plates and treated as above. At 5 days after removal of ethylene and transfer to light, the seedlings were transferred to plates with either 150 mM NaCl or no added salt and survival rate determined 1 week later by monitoring chlorosis. All experiments were repeated 3 times.

Heat tolerance and hypoxia assays

For both assays, seeds were prepared as described above and stress administered 5 days after transfer to light. Heat tolerance assays were carried out based on published methods (90, 91) at either 22 or 43°C for 22 min. Seedlings were returned to 22°C, and any that turned white and had not grown new leaves by 3 days afterwards were scored as having died.

Hypoxia stress treatments on *Arabidopsis* seedlings were administered by argon gas as previously described (92). Briefly, hypoxia was administered by purging a sealable chamber (9.5-l jar Gas Pak System, BBL) with argon gas (AR UHP300, Airgas) to a final O_2 level <2% measured by using a Traceable Oxygen probe (Fisher Scientific). Plants were kept in hypoxic conditions in darkness for 12 h and then were returned to normal aerobic long-day growth chamber conditions. Plant survival was then assessed over a 3-day period by monitoring the absence of shoot chlorosis (93). Control (normoxic) plants were treated identically except that the argon purging step was omitted.

Statistical analyses

Pairwise comparisons were conducted with Student's *t* tests using Excel (Office 2019). For multiple group comparisons, ANOVA tests were performed using GraphPad Prism ver. 9.3.1. Cumulative sum control chart analysis was conducted using Excel.

Acknowledgments

We thank Jenny Zhang and Grace Phelan for technical assistance, Eric Schaller, Gloria Muday, Barry Bruce, K. Trout, Andreas Nebenführ, Rachel P. McCord, Ricardo Urquidi-Camacho, and Mariano Labrador for helpful conversations and advice, and the Susan Kalisz lab for use of their LI-COR.

Supplementary material

Supplementary material is available at *PNAS Nexus* online.

Funding

This project was supported by the United States National Science Foundation Grant MCB-1716279 and a Hunsicker Research Incentive Award and Dr. Donald L. Akers Jr. Faculty Enrichment Fellowship from the BCMB department to B.M.B.

Author contributions

E.B., D.M.R., and B.M.B. designed the research; E.B., B.M.B., L.H.W., E.D., and B.H. performed the research and analyzed the data; and E.B., D.M.R., and B.M.B. wrote the paper.

Data availability

All data needed to evaluate this study are present in the paper, supplementary materials, or the publicly available RNA-seq data set at GEO Series Accession Number GSE218645 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE218645).

References

1 Abeles F, Morgan P, Saltveit MJ. 1992. *Ethylene in plant biology*. 2nd ed. San Diego, CA: Academic Press. p. 414.
2 Khan NA, Ferrante A, Munné-Bosch S. 2022. *The plant hormone ethylene: stress acclimation and agricultural applications*. Cambridge (MA): Elsevier. p. 248.
3 Mahajan PV, Caleb OJ, Singh Z, Watkins CB, Geyer M. 2014. Postharvest treatments of fresh produce. *Philos Trans A Math Phys Eng Sci*. 372:20130309.
4 Bleecker AB, Estelle MA, Somerville C, Kende H. 1988. Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. *Science* 241:1086–1089.
5 Chang C, Kwok SF, Bleecker AB, Meyerowitz EM. 1993. *Arabidopsis* ethylene-response gene ETR1: similarity of product to two-component regulators. *Science* 262:539–544.
6 Kieber JJ, Rothenberg M, Roman G, Feldman KA, Ecker JR. 1993. *CTR1*, a negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a member of the Raf family of protein kinases. *Cell* 72:427–441.
7 Alonso JM, Hirayama T, Roman G, Nourizadeh S, Ecker JR. 1999. EIN2, a bifunctional transducer of ethylene and stress responses in *Arabidopsis*. *Science* 284:2148–2152.
8 Binder BM. 2020. Ethylene signaling in plants. *J Biol Chem*. 295:7710–7725.
9 Potuschak T, *et al.* 2003. EIN3-dependent regulation of plant ethylene hormone signaling by two *Arabidopsis* F box proteins: EBF1 and EBF2. *Cell* 115:679–689.
10 Guo HW, Ecker JR. 2003. Plant responses to ethylene gas are mediated by SCF (EBF1/EBF2)-dependent proteolysis of EIN3 transcription factor. *Cell* 115:667–677.
11 Gagne JM, *et al.* 2004. *Arabidopsis* EIN3-binding F-box 1 and 2 form ubiquitin-protein ligases that repress ethylene action and promote growth by directing EIN3 degradation. *Proc Natl Acad Sci U S A*. 101:6803–6808.
12 Alonso JM, *et al.* 2003. Five components of the ethylene-response pathway identified in a screen for weak ethylene-insensitive mutants in *Arabidopsis*. *P Natl Acad Sci U S A*. 100:2992–2997.
13 Chao QM, *et al.* 1997. Activation of the ethylene gas response pathway in *Arabidopsis* by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. *Cell* 89:1133–1144.
14 Solano R, Stepanova A, Chao QM, Ecker JR. 1998. Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. *Genes Dev*. 12:3703–3714.
15 Liu X, *et al.* 2017. EIN3 and PIF3 form an interdependent module that represses chloroplast development in buried seedlings. *Plant Cell* 29:3051–3067.
16 Zhang X, *et al.* 2018. Integrated regulation of apical hook development by transcriptional coupling of EIN3/EIL1 and PIFs in *Arabidopsis*. *Plant Cell* 30:1971–1988.
17 Zhong S, *et al.* 2012. A molecular framework of light-controlled phytochrome action in *Arabidopsis*. *Curr Biol*. 22:1530–1535.
18 Zhong S, *et al.* 2014. Ethylene-orchestrated circuitry coordinates a seedling's response to soil cover and etiolated growth. *Proc Natl Acad Sci U S A*. 111:3913–3920.
19 Zhong S, *et al.* 2009. EIN3/EIL1 cooperate with PIF1 to prevent photo-oxidation and to promote greening of *Arabidopsis* seedlings. *Proc Natl Acad Sci U S A*. 106:21431–21436.
20 Guzmán P, Ecker JR. 1990. Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants. *Plant Cell* 2:513–523.
21 Ceusters J, Van de Poel B. 2018. Ethylene exerts species-specific and age-dependent control of photosynthesis. *Plant Physiol*. 176:2601–2612.
22 Smalle J, Haegman M, Kurepa J, Van Montagu M, Straeten DVD. 1997. Ethylene can stimulate *Arabidopsis* hypocotyl elongation in the light. *P Natl Acad Sci U S A*. 94:2756–2761.
23 Zhang Y, Liu Z, Chen Y, He J-X, Bi Y. 2015. PHYTOCHROME-INTERACTING FACTOR 5 (PIF5) positively regulates dark-induced senescence and chlorophyll degradation in *Arabidopsis*. *Plant Sci*. 237:57–68.
24 Ueda H, *et al.* 2020. Genetic interaction among phytochrome, ethylene and abscisic acid signaling during dark-induced senescence in *Arabidopsis thaliana*. *Front Plant Sci*. 11:564.
25 Wu Q, *et al.* 2020. Allosteric deactivation of PIFs and EIN3 by microproteins in light control of plant development. *Proc Natl Acad Sci U S A*. 117:18858–18868.
26 Xie Y, *et al.* 2021. *Arabidopsis FHY3* and *FAR1* function in age gating of leaf senescence. *Front Plant Sci*. 12:770060.
27 Sakuraba Y, *et al.* 2014. Phytochrome-interacting transcription factors PIF4 and PIF5 induce leaf senescence in *Arabidopsis*. *Nat Commun*. 5:4636.
28 Jeong J, *et al.* 2016. Phytochrome and ethylene signaling integration in *Arabidopsis* occurs via the transcriptional regulation of genes co-targeted by PIFs and EIN3. *Front Plant Sci*. 7:1055.
29 van Gelderen K, Kang C, Pierik R. 2018. Light signaling, root development, and plasticity. *Plant Physiol*. 176:1049–1060.
30 Muday G, Rahman A, Binder BM. 2012. Auxin and ethylene: collaborators or competitors? *Trends Plant Sci*. 17:181–195.
31 Ulmasov T, Murfett J, Hagen G, Guilfoyle TJ. 1997. Aux/1AA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *Plant Cell*. 9:1963–1971.
32 Macgregor DR, Deak KI, Ingram PA, Malamy JE. 2008. Root system architecture in Arabidopsis grown in culture is regulated by sucrose uptake in the aerial tissues. *Plant Cell* 20:2643–2660.
33 Salas J, Salas M, Viñuela E, Sols A. 1965. Glucokinase of rabbit liver. *J Biol Chem*. 240:1014–1018.
34 Jang JC, Sheen J. 1994. Sugar sensing in higher plants. *Plant Cell* 6:1665–1679.
35 Jang JC, León P, Zhou L, Sheen J. 1997. Hexokinase as a sugar sensor in higher plants. *Plant Cell* 9:5–19.
36 Jabben M, Deitzer GF. 1979. Effects of the herbicide san 9789 on photomorphogenic responses. *Plant Physiol*. 63:481–485.
37 Kobayashi K, *et al.* 2007. LOVASTATIN INSENSITIVE 1, a novel pentatricopeptide repeat protein, is a potential regulatory factor of isoprenoid biosynthesis in *Arabidopsis*. *Plant Cell Physiol*. 48:322–331.
38 Rodriéguez-Concepcioén M, *et al.* 2004. Distinct light-mediated pathways regulate the biosynthesis and exchange of isoprenoid precursors during Arabidopsis seedling development. *Plant Cell* 16:144–156.

39 Russell AW, *et al.* 1995. Photosystem II regulation and dynamics of the chloroplast D1 protein in Arabidopsis leaves during photosynthesis and photoinhibition. *Plant Physiol*. 107:943–952.

40 Deng XW, Caspar T, Quail PH. 1991. Cop1: a regulatory locus involved in light-controlled development and gene expression in *Arabidopsis*. *Genes Dev*. 5:1172–1182.

41 Khumsupan P, *et al.* 2020. Generating and characterizing single- and multigene mutants of the Rubisco small subunit family in *Arabidopsis*. *J Exp Bot*. 71:5963–5975.

42 Barratt DHP, *et al.* 2009. Normal growth of *Arabidopsis* requires cytosolic invertase but not sucrose synthase. *Proc Natl Acad Sci U S A*. 106:13124–13129.

43 Ashburner M, *et al.* 2000. Gene Ontology: tool for the unification of biology. *Nat Genet*. 25:25–29.

44 van Veen H, *et al.* 2013. Two Rumex species from contrasting hydrological niches regulate flooding tolerance through distinct mechanisms. *Plant Cell* 25:4691–4707.

45 Vwioko E, Adinkwu O, El-Esawi MA. 2017. Comparative physiological, biochemical, and genetic responses to prolonged waterlogging stress in okra and maize given exogenous ethylene priming. *Front Physiol*. 8:632.

46 Huang Y-C, Yeh T-H, Yang C-Y. 2019. Ethylene signaling involves in seeds germination upon submergence and antioxidant response elicited confers submergence tolerance to rice seedlings. *Rice* 12:23.

47 K?pczyński J. 2021. Gas-priming as a novel simple method of seed treatment with ethylene, hydrogen cyanide or nitric oxide. *Acta Physiologiae Plantarum*. 43:117.

48 Hussain S, *et al.* 2020. Ethylene response of salt stressed rice seedlings following Ethephon and 1-methylcyclopropene seed priming. *Plant Growth Reg*. 92:219–231.

49 Hartman S, *et al.* 2019. Ethylene-mediated nitric oxide depletion pre-adapts plants to hypoxia stress. *Nat Commun*. 10:4020.

50 Peng J, *et al.* 2014. Salt-induced stabilization of EIN3/EIL1 confers salinity tolerance by deterring ROS accumulation in *Arabidopsis*. *PLoS Genet*. 10:e1004664.

51 Tscharntke T, Thiessen S, Dolch R, Boland W. 2001. Herbivory, induced resistance, and interplant signal transfer in *Alnus glutinosa*. *Biochem Syst Ecol*. 29:1025–1047.

52 Bailey BA, Dean JF, Anderson JD. 1990. An ethylene biosynthesis-inducing endoxylanase elicits electrolyte leakage and necrosis in *Nicotiana tabacum* cv Xanthi leaves. *Plant Physiol*. 94:1849–1854.

53 Nascimento WM, Cantliffe DJ, Huber DJ. 2004. Ethylene evolution and endo-β-mannanase activity during lettuce seed germination at high temperature. *Sci Agric*. 61:156–163.

54 Liu Z, *et al.* 2022. Ethylene augments root hypoxia tolerance via growth cessation and reactive oxygen species amelioration. *Plant Physiol*. 190:1365–1383.

55 Wang LH, *et al.* 2019. Effects of exogenous glucose and sucrose on photosynthesis in triticale seedlings under salt stress. *Photosynthetica* 57:286–294.

56 Hernández-Madrigal F, *et al.* 2018. Sucrose protects *Arabidopsis* roots from chromium toxicity influencing the auxin–plethora signaling pathway and improving meristematic cell activity. *J Plant Growth Regul*. 37:530–538.

57 da Silva AC, *et al.* 2020. The Yin and Yang in plant breeding: the trade-off between plant growth yield and tolerance to stresses. *Biotechnol Res Innovation*. 3:73–79.

58 Kromdijk J, *et al.* 2016. Improving photosynthesis and crop productivity by accelerating recovery from photoprotection. *Science* 354:857–861.

59 Wei S, *et al.* 2022. A transcriptional regulator that boosts grain yields and shortens the growth duration of rice. *Science* 377:eabi8455.

60 De Souza AP, *et al.* 2022. Soybean photosynthesis and crop yield are improved by accelerating recovery from photoprotection. *Science* 377:851–854.

61 Kircher S, Schopfer P. 2012. Photosynthetic sucrose acts as cotyledon-derived long-distance signal to control root growth during early seedling development in *Arabidopsis*. *Proc Natl Acad Sci U S A*. 109:11217–11221.

62 Freixes S, Thibaud M-C, Tardieu F, Muller B. 2002. Root elongation and branching is related to local hexose concentration in *Arabidopsis thaliana* seedlings. *Plant Cell Environ*. 25:1357–1366.

63 Mishra BS, Singh M, Aggrawal P, Laxmi A. 2009. Glucose and auxin signaling interaction in controlling *Arabidopsis thaliana* seedlings root growth and development. *PLoS One* 4:e4502.

64 Alscher RG, Castelfranco PA. 1972. Stimulation by ethylene of chlorophyll biosynthesis in dark-grown cucumber cotyledons. *Plant Physiol*. 50:400–403.

65 Hart JE, Gardner KH. 2021. Lighting the way: recent insights into the structure and regulation of phototropin blue light receptors. *J Biol Chem*. 296:100594.

66 Lin C. 2002. Blue light receptors and signal transduction. *Plant Cell* 14:S207–S225.

67 Łabuz J, Sztatelman O, Hermanowicz P. 2022. Molecular insights into the phototropin control of chloroplast movements. *J Exp Bot*. 73:6034–6051.

68 Losi A, Gärtner W. 2012. The evolution of flavin-binding photoreceptors: an ancient chromophore serving trendy blue-light sensors. *Ann Rev Plant Biol*. 63:49–72.

69 Li X, Liang T, Liu H. 2021. How plants coordinate their development in response to light and temperature signals. *Plant Cell* 34: 955–966.

70 Barnes WJ, Anderson CT. 2018. Cytosolic invertases contribute to cellulose biosynthesis and influence carbon partitioning in seedlings of *Arabidopsis thaliana*. *Plant J*. 94:956–974.

71 Pignocchi C, *et al.* 2020. Restriction of cytosolic sucrose hydrolysis profoundly alters development, metabolism, and gene expression in *Arabidopsis* roots. *J Exp Bot*. 72:1850–1863.

72 Margalha L, Confraria A, Baena-González E. 2019. SnRK1 and TOR: modulating growth–defense trade-offs in plant stress responses. *J Exp Bot*. 70:2261–2274.

73 Thalmann M, Santelia D. 2017. Starch as a determinant of plant fitness under abiotic stress. *New Phytol*. 214:943–951.

74 Shi H, *et al.* 2016. Seedlings transduce the depth and mechanical pressure of covering soil using COP1 and ethylene to regulate EBF1/EBF2 for soil emergence. *Curr Biol*. 26:139–149.

75 Pandey BK, *et al.* 2021. Plant roots sense soil compaction through restricted ethylene diffusion. *Science* 371:276–280.

76 Harpham NVJ, *et al.* 1991. The effect of ethylene on the growth and development of wild-type and mutant *Arabidopsis thaliana* (L.) Heynh. *Ann Bot*. 68:55–61.

77 Hussain A, Black C, Taylor I, Roberts JA. 1999. Soil compaction. A role for ethylene in regulating leaf expansion and shoot growth in tomato? *Plant Physiol*. 121:1227–1237.

78 Goeschl JD, Rappaport L, Pratt HK. 1966. Ethylene as a factor regulating the growth of pea epicotyls subjected to physical stress. *Plant Physiol*. 41:877–884.

79 Brenya E, *et al.* 2022. Mechanical stress acclimation in plants: linking hormones and somatic memory to thigmomorphogenesis. *Plant Cell Environ*. 45:989–1010.

80 Huang J-P, Tunc-Ozdemir M, Chang Y, Jones AM. 2015. Cooperative control between AtRGS1 and AtHXK1 in a WD40-repeat protein pathway in *Arabidopsis thaliana*. *Front Plant Sci*. 6:851.

81 Leivar P, *et al.* 2008. Multiple phytochrome-interacting bHLH transcription factors repress premature seedling photomorphogenesis in darkness. *Curr Biol*. 18:1815–1823.
82 Binder BM, Rodriguez FI, Bleecker AB, Patterson SE. 2007. The effects of group 11 transition metals, including gold, on ethylene binding to the ETR1 receptor and growth of *Arabidopsis thaliana*. *FEBS Lett*. 581:5105–5109.
83 Binder BM, *et al.* 2004. Arabidopsis seedling growth response and recovery to ethylene. A kinetic analysis. *Plant Physiol*. 136: 2913–2920.
84 Duncan AJ. 1974. Cumulative sum control charts. *Quality control and industrial statistics*. New York: Wiley. p. 464–482.
85 Lichtenthaler HK. 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. In: Packer L, Douce R, editors. *Methods in enzymology*. Cambridge (MA): Academic Press. p. 350–382.
86 Pang Z, *et al.* 2022. Using MetaboAnalyst 5.0 for LC–HRMS spectra processing, multi-omics integration and covariate adjustment of global metabolomics data. *Nat Protoc*. 17:1735–1761.
87 Jin Y, Liu F, Huang W, Sun Q, Huang X. 2019. Identification of reliable reference genes for qRT-PCR in the ephemeral plant *Arabidopsis pumila* based on full-length transcriptome data. *Sci Rep*. 9:8408.
88 Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 29:e45.
89 Edgar R, Domrachev M, Lash AE. 2002. Gene expression omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res*. 30:207–210.
90 Silva-Correia J, Freitas S, Tavares RM, Lino-Neto T, Azevedo H. 2014. Phenotypic analysis of the *Arabidopsis* heat stress response during germination and early seedling development. *Plant Methods* 10:7.
91 Li S, *et al.* 2014. HEAT-INDUCED TAS1 TARGET1 mediates thermotolerance via HEAT STRESS TRANSCRIPTION FACTOR A1a-directed pathways in Arabidopsis. *Plant Cell* 26:1764–1780.
92 Lokdarshi A, Conner WC, McClintock C, Li T, Roberts DM. 2016. Arabidopsis CML38, a calcium sensor that localizes to ribonucleoprotein complexes under hypoxia stress. *Plant Physiol*. 170: 1046–1059.
93 Beamer ZG, *et al.* 2021. Aquaporin family lactic acid channel NIP2; 1 promotes plant survival under low oxygen stress in Arabidopsis. *Plant Physiol*. 187:2262–2278.