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Feedback loop promotes sucrose accumulation in cotyledons to facilitate sugar-ethylene signaling-mediated, etiolated-seedling greening

Highlights

- Sucrose feedback signaling promotes its accumulation in cotyledons under darkness
- Accumulated sucrose in cotyledons stimulates ethylene biosynthesis to enable greening

In brief

Mu et al. describe a sucrose-signaling feedback loop (Suc-ACS7-EIN3-SUC2-Suc) that promotes sucrose accumulation of cotyledons under darkness. This promotes ethylene biosynthesis, facilitates de-etiolation, and enables seedlings to green and grow out of soil when seedlings are exposed to light from darkness.

Authors

Xin-Rong Mu, Chen Tong, Xing-Tang Fang, ..., Xiao-Ying Cao, Ji-Hong Jiang, Lai-Sheng Meng

Correspondence

menglsh@jsnu.edu.cn (L.-S.M.), jhjiang@jsnu.edu.cn (J.-H.J.), cxy4868@jsnu.edu.cn (X.-Y.C.)
Feedback loop promotes sucrose accumulation in cotyledons to facilitate sugar-ethylene signaling-mediated, etiolated-seedling greening

Xin-Rong Mu,1 Chen Tong,1 Xing-Tang Fang,2 Qin-Xin Bao,1 Li-Na Xue,1 Wei-Ying Meng,1 Chang-Yue Liu,1 Gary J. Loake,3,4 Xiao-Ying Cao,1,* Ji-Hong Jiang,1,* and Lai-Sheng Meng1,5,*

1The Key Laboratory of Biotechnology for Medicinal Plants of Jiangsu Province, Jiangsu Normal University, Xuzhou, Jiangsu 221116, People’s Republic of China
2School of Life Science, Jiangsu Normal University, Xuzhou, Jiangsu 221116, People’s Republic of China
3Jiangsu Normal University, Edinburgh University, Centre for Transformative Biotechnology of Medicinal and Food Plants, Jiangsu Normal University, 101 Shanghai Road, Xuzhou, Jiangsu 221116, People’s Republic of China
4Institute of Molecular Plant Sciences, School of Biological Sciences, Edinburgh University, King’s Buildings, Mayfield Road, Edinburgh EH9 3JR, UK
5Lead contact
*Correspondence: menglsh@jsnu.edu.cn (L.-S.M.), jhjiang@jsnu.edu.cn (J.-H.J.), cxy4868@jsnu.edu.cn (X.-Y.C.)
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SUMMARY

De-etiolation is indispensable for seedling survival and development. However, how sugars regulate de-etiolation and how sugars induce ethylene (ET) for seedlings to grow out of soil remain elusive. Here, we reveal how a sucrose (Suc) feedback loop promotes de-etiolation by inducing ET biosynthesis. Under darkness, Suc in germinating seeds preferentially induces 1-amino-cyclopropane-1-carboxylate synthase (ACS7; encoding a key ET biosynthesis enzyme) and associated ET biosynthesis, thereby activating ET core component ETHYLENE-INSENSITIVE3 (EIN3). Activated EIN3 directly inhibits the function of Suc transporter 2 (SUC2; a major Suc transporter) to block Suc export from cotyledons and thereby elevate Suc accumulation of cotyledons to induce ET. Under light, ET-activated EIN3 directly inhibits the function of phytochrome A (phyA; a de-etiolation inhibitor) to promote de-etiolation. We therefore propose that under darkness, the Suc feedback loop (Suc-ACS7-EIN3-SUC2-Suc) promotes Suc accumulation in cotyledons to guarantee ET biosynthesis, facilitate de-etiolation, and enable seedlings to grow out of soil.

INTRODUCTION

The light-dependent switch from skotomorphogenesis to photomorphogenesis is essential for seedling survival. In the life cycle of a plant, the first step is seedling establishment after seed germination under darkness. During this process, seedlings develop elongated hypocotyls and suppressed cotyledons. These dark-grown seedlings are capable of utilizing sucrose (Suc) as their main energy source. Suc is mainly derived from oil bodystored neutral lipids, triacyclglycerols (TAGs), which are considered a main energy source for postgerminative growth (Mazzella et al., 2014). Glyoxysomes are present in cotyledons of etiolated seedlings, and they are specialized peroxisomes engaged in the degradation of TAGs by both the glyoxylate cycle and β-oxidation (Graham, 2008). In Arabidopsis, the loss of glyoxylate-cycle enzymes (glyoxysome mutants), for example, isocitrate lyase (ICL) and malate synthase (MLS), shows a distinctive characteristic of sugar starvation after germination due inability to convert stored lipids into Suc (Eastmond et al., 2000; Cornah et al., 2004).

The second step is that germinating seedlings undergo photomorphogenesis upon light irradiation, including hypocotyl shortening, and cotyledon opening and greening (i.e., chlorophyll biosynthesis), allowing seedlings to turn photosynthetically autotrophic and competent (Zhong et al., 2009, 2014). Specifically, during this process, seedlings exhibit a decreased hypocotyl growth rate, the cotyledons begin to unfold, the apical hook starts to open, and, significantly, the initiation of chlorophyll biosynthesis drives cotyledon greening. Therefore, white light is the major factor triggering the cotyledon-greening process of etiolated seedlings (Mazzella et al., 2014).

The E3 ubiquitin ligase constitutively photomorphogenic 1 (COP1) is a key suppressor of photomorphogenesis and has been reported to be indispensable in regulating cotyledon greening (Moon et al., 2008; Zhong et al., 2009). In addition, phytochrome-interacting factors (PIFs) are also thought to play a fundamental role in regulating the switch from skoto- to photomorphogenesis (Zhong et al., 2009; Duek and Fankhauser, 2005). For example, PIF1 and PIF3 facilitate cotyledon greening partly via suppressing protochlorophyllide accumulation under darkness (Duek and Fankhauser, 2005; Stephenson et al., 2009).

Ethylene (ET), a gaseous hormone, is also thought to be integral to the regulation of cotyledon greening. In this context, ET
and PIF1 redundantly regulate the skoto-to-photomorphogenesis transition by facilitating cotyledon greening upon light irradiation (Zhong et al., 2009). Activation of a key ET signaling component, ETHYLENE-INSENSITIVE3 (EIN3), a plant-specific transcription factor, with an N-terminal DNA-binding domain and a unique fold structure, is both necessary and sufficient for seedling greening (Zhong et al., 2009). Under darkness, ET facilitates the greening of etiolated seedlings by EIN3 directly binding to the promoters of protochlorophyllide oxidoreductase A/B (PORA/B) (Zhong et al., 2009). Further, the mechanical stress of the soil activates ET production to promote cotyledon greening by a key ET component, EIN3, activating a PIF3-ERF1 (ET response factor 1) transcriptional circuitry to synchronize tissue-specific developments (Zhong et al., 2014). However, whereas the mechanical stress of the soil activates ET production and, in turn, promotes cotyledon greening by EIN3 controlling a few phytochrome and associated factors, how the mechanical stress of the soil induces ET production remains unclear.

Whereas white light is the major factor triggering the cotyledon-greenving process of etiolated seedlings, far-red light inhibits this phenomenon by decreasing the level of PORA/B, which catalyzes the light-dependent reduction of protochlorophyllide A to chlorophyllide A, which is subsequently converted to chlorophyll (Barnes et al., 1996; Spoerling et al., 1997). Interestingly, continuous far-red irradiation suppresses cotyledon greening in Arabidopsis, which can be restored by exogenously applied Suc (Barnes et al., 1996), suggesting that sugars may promote etiolated-seedling greening.

Sugars affect many aspects of growth and development throughout the plant life cycle, including germination, flowering, and senescence. Suc is a major soluble sugar in seed embryos and is also the predominant transported sugar in Arabidopsis, with phloem loading essential for plant growth, development, and reproduction (Chen et al., 2012). In Arabidopsis, phloem-specific sucrose transporter 2 (SUC2) is specifically expressed in companion cells (Stadler and Sauer, 1996) and has a central role in the phloem loading of Suc, which is essential for high-performance Suc transport from source to sink tissues (Stadler and Sauer, 1996; Gottwald et al., 2000; Srivastava et al., 2008). suc2 mutants exhibit stunted growth, delayed development, and sterility. Further, this line accumulates starch and Suc in leaf blades and is blocked in the transport of sugars to sink organs, including the shoot apex, inflorescences, and roots (Gottwald et al., 2000).

Suc is a major soluble sugar in seed embryos and is also the predominant transported sugar in Arabidopsis, and continuous far-red irradiation suppresses cotyledon greening in Arabidopsis, which can be restored by the exogenous application of Suc (Barnes et al., 1996). This suggests that Suc may promote etiolated-seedling greening. Therefore, it should be investigated if Suc promotes cotyledon greening by regulating the phloem loading of Suc.

Here, we identify a Suc feedback loop and demonstrate that, under darkness, this Suc feedback loop following seed germination promotes Suc accumulation in cotyledons. When cotyledons are exposed to light from darkness, accumulated Suc in cotyledons stimulates ET biosynthesis to promote sugar-ET signaling-mediated, etiolated-seedling greening. Specifically, under darkness, ACS7 expression and associated ET biosynthesis are induced preferentially by accumulated Suc in germinating seeds. Induced ET enhances EIN3 activity, which in turn directly suppresses the function of the key Suc transporter SUC2, thereby inhibiting Suc phloem loading in cotyledons. Consequently, the exporting Suc from cotyledons is inhibited. Therefore, Suc accumulation is enhanced by the Suc feedback loop. On seedling emergence and concomitant exposure to white light irradiation, accumulated Suc stimulates ET biosynthesis. ET-activated EIN3 negatively regulates phytochrome A (phyA), an inhibitor of etiolated-seedling greening, promoting this key developmental process. Collectively, this enables the possibility of specifically manipulating both Suc accumulation and ET production and associated signaling in mesophyll cells to enable coordination of both Suc phloem loading and ET signaling in cotyledons with a few phytochrome and associated factors (including phyA) without disturbing the whole plant body plan.

RESULTS

The cotyledon greening of etiolated seedlings was promoted by endogenous sugars

Cytosolic invertase 1/2 (CINV1/2) irreversibly catalyzes Suc into glucose (Glc) and fructose (Frc) (Barratt et al., 2009). Here, we utilized the loss-of-function mutant of CINV1/2, cinv1/2 (sugar signaling/metabolism mutant [Barratt et al., 2009; Meng et al., 2021]), to obtain the endogenous sugars of genetic manipulation.

The percentage of seedlings exhibiting cotyledon greening (greening rate) of the cinv1/2 mutant (Figures S1A and S1C) substantially decreased as endogenous Glc content decreased (Figure S1D). These etiolated seedlings were grown under high white light (230 μmol quanta PAR m⁻² s⁻¹) and no sugars. High white light conditions can provide a large amount of photosynthetic product (such as endogenous Suc). Further, the decrease in greening-seedling rate can be restored by the exogenous application of Glc but not Suc and Man (Figures S1B and S1C), probably because Suc and Man cannot be directly utilized. Suc is the substrate of CINV1/2, but Glc is the product of CINV1/2 (Barratt et al., 2009; Meng et al., 2020). Moreover, whereas Glc content declined in cinv1/2 mutant seedlings under our conditions (Figure S1D), it was elevated in this mutant under other conditions (Lou et al., 2007; Barnes and Anderson, 2018). These findings suggested that decreasing Glc content in the cinv1/2 mutant interrupts signaling, which leads to blocked etiolated-seedling greening. Together, endogenous Glc may promote etiolated-seedling greening by signaling, but not metabolism.

To examine whether osmotic stress factors cause etiolated-seedling greening, we used the loss of vacuolar invertase 2 (VIN2) (vin2v, a sugar osmotic stress mutant; Sergeeva et al., 2006) to obtain the endogenous sugars of genetic manipulation. This mutant line exhibits considerably shorter roots and ~50% reduction in vacuolar invertase (Vac-Inv) activity compared with parental lines (Sergeeva et al., 2006). We examined etiolated-seedling greening in the vin2v mutant under high light and without sugars. Our findings indicated that whereas Glc content of the vin2v mutant evidently declined (Figure S1D), the
greening-seeding rate in this mutant was not different with wild-type seedlings (Figures S1A and S1C). These different Invs (cystolic invertase [Cinv-Inv] or Vac-Inv) caused the differential genetic phenotypes, indicating the importance of subcellular localization of invertases in signaling (Leskow et al., 2016). The Vac-Inv quantitative trait locus (QTL) influences both Arabidopsis carbon partitioning and biomass accumulation by a different regulation of Vac-Inv inhibitors at the mRNA level (Leskow et al., 2016). Therefore, that the loss of VINV2 lowered Glc content may block osmotic stress but not interrupt Glc signaling. Together, these findings confirmed that Glc may promote etiolated-seedling greening, but not metabolism, by signaling.

Taken together, the cotyledon greening of etiolated seedlings was promoted by endogenous Glc.

The cotyledon greening of etiolated seedlings was promoted preferentially by the exogenous application of Suc

While the endogenous sugar promoted etiolated-seedling greening (Figure S1), we have not provided evidence to identify whether exogenous sugars promote this process. To confirm and extend these observations, we performed the following experiments. With the increase of retention time under darkness, the capability of etiolated-seedling greening will gradually reduce (Zhong et al., 2009). We found that 8-day-old wild-type (Col-0) etiolated seedlings subjected to white light irradiation for 2 days was a desired time point to determine which of a few kinds of soluble sugars preferentially promote the cotyledon greening of etiolated seedlings.

We observed 8-day-old wild-type (Col-0) etiolated seedlings followed by 2 days of white light irradiation on solid MS medium with either 1% or 3% of sucrose (Suc), glucose (Glc), fructose (Frc), glucose-6-phosphate (G6P), and mannose (Man) or without sugar. Bar, 3 mm.

(P) Bar graph illustrating the greening rate of the given etiolated seedlings in (A)–(O).
(Q) Bar graph illustrating chlorophyll concentration of the given etiolated seedlings in (A)–(O).

Error bars represent SD (n = 46 in P; n = 3 in Q). Student’s t test (**p < 0.01; ***p < 0.001). See also Figure S1.

Suc preferentially induces ACS7 expression and associated ET biosynthesis, which in turn promotes etiolated-seedling greening

Both ET production (Jeong et al., 2010) and etiolated-seedling greening (Figure 1) were preferentially induced by Suc relative to other sugars. ET promotes etiolated-seedling greening (Zhong
Quantification was normalized to the expression of \textit{Yoo et al., 2008}). ACS2, ACS4, ACS6, and ACS7 are major factors of ET biosynthesis (\textit{Liu and Zhang, 2004; Yoo et al., 2008}).

Consistent with these genetic data, molecular experiments presented that exogenous Suc, but not Glc and Man, preferentially induce \textit{ACS7} expression (\textit{Zhong et al., 2009}). Very recently, \textit{Xu et al. (2021)} reported that \textit{ACS7}, a key enzyme in ACC biosynthesis, has dual activities of ACC synthase, further suggesting that \textit{ACS7} may be a distinctive ACC synthase enzyme that is involved in the regulation of etiolated-seedling greening.

Figure 2. Suc promotes cotyledon greening by preferentially inducing \textit{ACS7} expression and associated ethylene production

(A–L) Images illustrating 8-day-old wild-type (Col-0), \textit{acs2}, \textit{acs4}, \textit{acs6}, and \textit{acs7}, and 35S:\textit{ACS7} etiolated seedlings followed by 2 days of median-level white light irradiation on solid MS medium with 2% Suc or without sugars. Bar, 3 mm.

(M) Bar graph illustrating the greening rate of the given etiolated seedlings in (A)–(L).

(N) Bar graph illustrating chlorophyll concentration of the given etiolated seedlings in (A)–(L).

(O) Bar graph illustrating \textit{ACS7} expression in different exogenous Suc, Glc and Man. Eight-day-old wild-type etiolated seedlings were followed by 2 days of median-level white light irradiation. Subsequently, these seedlings were exposed to 0%, 2%, 4%, or 6% Suc, Glc, and Man for 30 min. The \textit{ACS7} expression following exposure to 0% Suc, Glc, and Man is set as 1.

(P) Bar graph illustrating \textit{ACS2}, \textit{ACS4}, \textit{ACS6}, and \textit{ACS7} expression in the wild-type etiolated seedlings exposed to 3% Suc or no sugars for 1 h. Eight-day-old wild-type etiolated seedlings were followed by 2 days of median-level white light irradiation were used in this experiment. Quantification of the \textit{ACS2}, \textit{ACS4}, \textit{ACS6}, and \textit{ACS7} expression at no sugars is set as 1.

(Q and R) Bar graph illustrating Suc (Q) and ethylene (R) levels in 3-, 5-, 7-, and 9-day-old wild-type (Col-0) and \textit{acs7-1} etiolated seedlings. The 2-, 4-, 6-, and 8-day-old wild-type (Col-0) and \textit{acs7-1} etiolated seedlings were followed by 1 day of median-level white light irradiation.

Quantification was normalized to the expression of \textit{UBQ5} in (Q) and (P). Error bars represent SD (\(n = 44\) in M; \(n = 3\) in N–R). Student’s t test (***\(p < 0.001\); **\(p < 0.01\); *\(p < 0.05\)). See also Figure S2.

et al., 2009). We therefore speculated whether Suc promotes etiolated-seedling greening by inducing the expression of genes encoding enzymes involved in ET biosynthesis. In this experiment, we employed a few loss-of-function mutants in \textit{1-amino-cyclopropane-1-carboxylate synthase} (\textit{ACSs}), which encode key enzymes involved in ET biosynthesis (\textit{Liu and Zhang, 2004; Yoo et al., 2008}). \textit{ACS2}, \textit{ACS4}, \textit{ACS6}, and \textit{ACS7} are major factors of ET biosynthesis (\textit{Liu and Zhang, 2004; Yoo et al., 2008}).

Genetic experiments revealed that the loss-of-function mutants of \textit{ACS2}, \textit{ACS4}, and \textit{ACS6}, \textit{acs2}, \textit{acs4}, and \textit{acs6}, were not significantly different from wild-type in the greening rate of etiolated seedling (Figures 2A–2H, 2M, and 2N). In contrast, whereas the loss-of-function of the \textit{ACS7} mutant \textit{acs7} (Figures S2A and S2B) reduced the greening rate, \textit{ACS7} overexpression (35S:\textit{ACS7}) (Figures S2A and S2B) elevated the greening rate relative to wild-type etiolated seedlings (Figures 2A, 2B, 2L, and 2N). This may result from a decline or an increase of 1-amino-cyclopropane-1-carboxylic acid (ACC) or ET production in \textit{acs7-1} or 35S:\textit{ACS7} seedlings, respectively, as ACC/ET promotes cotyledon greening (\textit{Zhong et al., 2009}). Very recently, \textit{Xu et al. (2021)} reported that \textit{ACS7}, a key enzyme in ACC biosynthesis, has dual activities of ACC synthase, further suggesting that \textit{ACS7} may be a distinctive ACC synthase enzyme that is involved in the regulation of etiolated-seedling greening.

Figure 2. Suc promotes cotyledon greening by preferentially inducing \textit{ACS7} expression and associated ethylene production

(A–L) Images illustrating 8-day-old wild-type (Col-0), \textit{acs2}, \textit{acs4}, \textit{acs6}, and \textit{acs7}, and 35S:\textit{ACS7} etiolated seedlings followed by 2 days of median-level white light irradiation on solid MS medium with 2% Suc or without sugars. Bar, 3 mm.

(M) Bar graph illustrating the greening rate of the given etiolated seedlings in (A)–(L).

(N) Bar graph illustrating chlorophyll concentration of the given etiolated seedlings in (A)–(L).

(O) Bar graph illustrating \textit{ACS7} expression in different exogenous Suc, Glc and Man. Eight-day-old wild-type etiolated seedlings were followed by 2 days of median-level white light irradiation. Subsequently, these seedlings were exposed to 0%, 2%, 4%, or 6% Suc, Glc, and Man for 30 min. The \textit{ACS7} expression following exposure to 0% Suc, Glc, and Man is set as 1.

(P) Bar graph illustrating \textit{ACS2}, \textit{ACS4}, \textit{ACS6}, and \textit{ACS7} expression in the wild-type etiolated seedlings exposed to 3% Suc or no sugars for 1 h. Eight-day-old wild-type etiolated seedlings were followed by 2 days of median-level white light irradiation were used in this experiment. Quantification of the \textit{ACS2}, \textit{ACS4}, \textit{ACS6}, and \textit{ACS7} expression at no sugars is set as 1.

(Q and R) Bar graph illustrating Suc (Q) and ethylene (R) levels in 3-, 5-, 7-, and 9-day-old wild-type (Col-0) and \textit{acs7-1} etiolated seedlings. The 2-, 4-, 6-, and 8-day-old wild-type (Col-0) and \textit{acs7-1} etiolated seedlings were followed by 1 day of median-level white light irradiation.

Quantification was normalized to the expression of \textit{UBQ5} in (Q) and (P). Error bars represent SD (\(n = 44\) in M; \(n = 3\) in N–R). Student’s t test (***\(p < 0.001\); **\(p < 0.01\); *\(p < 0.05\)). See also Figure S2.

et al., 2009). We therefore speculated whether Suc promotes etiolated-seedling greening by inducing the expression of genes encoding enzymes involved in ET biosynthesis. In this experiment, we employed a few loss-of-function mutants in \textit{1-amino-cyclopropane-1-carboxylate synthase} (\textit{ACSs}), which encode key enzymes involved in ET biosynthesis (\textit{Liu and Zhang, 2004; Yoo et al., 2008}). \textit{ACS2}, \textit{ACS4}, \textit{ACS6}, and \textit{ACS7} are major factors of ET biosynthesis (\textit{Liu and Zhang, 2004; Yoo et al., 2008}).

Consistent with these genetic data, molecular experiments presented that exogenous Suc, but not Glc and Man, preferentially induce \textit{ACS7} expression (\textit{Figure 2O}). Moreover, \textit{ACS7} expression strikingly increased relative to \textit{ACS2}, \textit{ACS4}, and \textit{ACS6} upon 3% Suc treatment (\textit{Figure 2P}). Further, following exposure to white light for 1 day, 2-, 4-, 6-, and 8-day-old wild-type etiolated seedlings accumulated Suc and ET, with the accrual of these molecules increasing with seedling age (\textit{Figures 2Q} and \textit{2R}). In contrast, the accumulation of Suc and ET in the \textit{acs7-1} mutant had no significant difference during this time stage (\textit{Figures 2Q} and \textit{2R}). These findings indicate that Suc/ET signaling-mediated cotyledon greening is partially dependent of \textit{ACS7} function.
In aggregate, our data reveal that Suc preferentially induces ACS7 expression and associated ET biosynthesis, which in turn promotes etiolated-seedling greening.

Suc/ET signaling-mediated cotyledon greening is partially dependent of EIN3/EIL1 acting downstream of ACS7

Suc drives etiolated-seedling greening by preferentially inducing ACS7 expression and associated ET biosynthesis (Figure 2), and induced ET activates a key ET component, EIN3, by its canonical signal transduction pathway (Cho et al., 2006; Binder et al., 2007) to promote ET-mediated etiolated-seedling greening (Zhong et al., 2009). Therefore, we determined if EIN3 might be a component integral to the developmental process of Suc/ET signaling-mediated etiolated-seedling greening. The close paralogs EIN3 and EIN3-like 1 (EIL1) have redundant functions (Cho et al., 2006; Binder et al., 2007). Thus, we determined whether EIN3/EIL1 participates in the regulation of Suc/ET signaling-mediated etiolated-seedling greening.

The greening rate of 8-day-old wild-type (Col-0) etiolated seedlings subjected to white light irradiation for 2 days on MS medium without or with 1%, 2%, or 3% Suc was significantly elevated with the increase of exogenous Suc concentrations. By contrast, under the same conditions, ein3/eil1 seedlings exhibited no obvious difference in greening rate in response to different Suc concentrations (Figures 3A–3J). These findings indicate that Suc/ET signaling-mediated etiolated-seedling greening is dependent of EIN3/EIL1 function.

To extend these data, we determined the consequence of EIN3 overexpression on Suc/ET signaling-mediated etiolated-seedling greening. A 35S:EIN3 in the wild-type background showed increased greening seedling rate relative to wild-type plants (Figures 3K, 3L, 3S, and 3T). This observation contrasted with that of the acs7-1 mutant defective in ET biosynthesis, which exhibited reduced greening-seedling rate (Figures 3K, 3M, 3S, and 3T). Further, we crossed the 35S:EIN3 transgene to the acs7-1 mutant and determined the rate of seedling greening in the resulting acs7-1/35S:EIN3 line (Figure S2B). This line produced a greening rate similar to the 35S:EIN3 line.

Figure 3. EIN3 acts downstream and upstream of ACS7 and SUC2 in the regulation of Suc signaling-mediated cotyledon greening of etiolated seedlings, respectively

(A–H) Images illustrating 8-day-old wild-type (Col-0) and ein3/eil1 etiolated seedlings followed by 2 days of median-level white light irradiation on solid MS medium with 1%, 2%, or 3% of Suc or without sugar. Bars, 4 mm.

(I) Bar graph illustrating the greening rate of the given etiolated seedlings in (A)–(H).

(K–R) Images illustrating 8-day-old wild-type (Col-0) (K), 35S:EIN3 (L), acs7-1 (M), acs7-1/35S:EIN3 (N), ein3/eil1 (O), 35S:SUC2 (P), suc2-5/+ (Q), and ein3/eil1/suc2-5/+ (R) etiolated seedlings followed by 2 days of median-level white light irradiation on solid MS medium with 2% Suc. Bar, 1.0 mm.

(S) Bar graph illustrating the greening rate of the given etiolated seedlings in (K)–(R).

(T) Bar graph illustrating chlorophyll concentration of the given etiolated seedlings in (K)–(R).

Error bars represent SD (n = 43 in I and S; n = 3 in J and T). Student’s t test (**p < 0.001; ***p < 0.01; *p < 0.05). See also Figure S2.
EIN3 represses SUC2 expression

Chromatin immunoprecipitation sequence (ChIP-seq) analysis of EIN3 (Chang et al., 2013) indicated that EIN3 may directly regulate SUC4 expression. Further, phloem transport is promoted in response to light cues, and sugar export from sinks is directly linked to SUC activity (Xu et al., 2018). In this context, SUC2 is a major transporter of Suc during phloem loading in Arabidopsis (Kühn et al., 1997; Srivastava et al., 2009). As we identified the transcriptional regulator EIN3 as a key component in Suc/ET-signaling etiolated-seedling greening (Figure 3), we therefore explored if transcription factor EIN3 could directly interact with SUC2 promoter sequences to regulate SUC2 expression and, by extension, Suc transport.

While it is well established that EIN3 protein levels are enhanced with increasing seedling age (Li et al., 2013), our data revealed that SUC2 transcripts declined with age and were of greater abundance in ein3/eil1 seedlings relative to wild-type ones (Figure S3C). This result was further supported by the upregulation of SUC2 expression in ein3/eil1 seedlings (Jeong et al., 2010). Further, transient expression experiments in Nicotiana benthamiana showed that coexpression of EIN3 decreased the expression of a SUC2 promoter-reporter gene, ProSUC2::LUCIFERASE (LUC) (Figures S3A and S3B). Thus, these findings suggest that EIN3 inhibits SUC2 expression.

ET suppression of SUC2 expression may be partially dependent of EIN3 function

To determine whether SUC2 expression is sensitive to transiently applied ET, we monitored SUC2 expression over time in response to the exogenously supplied ET precursor ACC. Informatively, SUC2 expression decreased after ACC application. Further, SUC2 transcripts remained suppressed 60 min after ACC treatment of wild-type plants (Figure S3D). Conversely, there was no obvious suppression of SUC2 expression in ein3/eil1 seedlings (Figure S3D). We also monitored SUC2 expression over time in response to either an exogenously supplied ET synthesis inhibitor, aminooxyacyclidine (AVG), or an ET-binding inhibitor, silver (Jeong et al., 2010). SUC2 expression was significantly elevated after application of either AVG or silver (Figures S3E and S3F). Moreover, SUC2 transcripts remained strikingly elevated at 60 min post treatment in wild-type plants, but this elevation was not detected in ein3/eil1 seedlings (Figures S3E and S3F).

In sum, these data indicate that ET suppression of SUC2 expression may be partially dependent on EIN3 function.

EIN3 directly binds to the SUC2 promoter

Examination of DNA sequences comprising the SUC2 promoter revealed putative EIN3 binding sites (EBSs) (Li et al., 2013) (Figure 4A). To explore whether EIN3 can interact with any of these EBSs, we employed a transgenic Arabidopsis line possessing an EIN3pro:EIN3-GFP line (Figure S2C). We thus performed ChIP analysis to determine whether EIN3 bound directly to SUC2 promoter sequences. Indeed, EIN3 strongly bound to one SUC2 promoter sequence (S2), which contained a single EBS, but not to three other SUC2 promoter sequences (S1, S3, or S4) (Figure 4B). Moreover, ET treatment enhanced EIN3 binding to S2 of the SUC2 promoter (Figure 4C), likely because ET is known to enhance EIN3 stability or activity (Yanagisawa et al., 2003). In addition, EIN3 association with sequence S2 in the SUC2 promoter increased over time in EIN3pro:EIN3-GFP seedlings (Figure 4D), possibly because EIN3 stability or activity was enhanced with increasing seedling age (Li et al., 2013). This association was strongly correlated with a decline in SUC2 expression with increasing age (Figure S3A), which may be because with increasing seedling age, the increase of EIN3 association with sequence S2 (Figure 4D) inhibits SUC2 expression.

Further, a SUC2 promoter construct containing a mutated EBS (mEBS), SUC2(mEBS)pro::SUC2, was transformed into a suc2-5 line expressing 35S:EIN3-GFP [35S:EIN3-GFP/SUC2 (mEBS)pro::SUC2/suc2-5]. The absence of an intact EBS within the S2 sequence of the SUC2 promoter construct decreased EIN3 binding (Figure 4E). Significantly, the seedling-greening rate of the 35S:EIN3-GFP/SUC2(mEBS)pro::SUC2/suc2-5 seedlings was reduced compared with the 35S:EIN3-GFP/ein3/eil1 seedlings (Figures 4F and 4G), suggesting that EIN3 binding to the SUC2 promoter is required to suppress SUC2 function and that its impairment blocks etiolated-seedling greening. Taken together, our findings indicate that EIN3 directly binds to the promoter of the SUC2 gene in vivo to inhibit SUC2 expression.

To determine if EIN3 can bind directly to the S2 EBS within the SUC2 promoter in vitro, we performed an electrophoretic mobility shift assay (EMSA). EIN3 bound exclusively to the S2 EBS element [mEBS] pro::SUC2/suc2-5 line expressing 35S:EIN3-GFP/SUC2 pro::SUC2/suc2-5] within the 3T promoter-sequence of the S2 sequence of the SUC2 promoter in vivo. (Figure 4H). Excess unlabeled competitor DNA effectively abolished this binding ability in a dose-dependent manner, thereby establishing the specificity of this binding (Figure 4H). We confirmed and extended these findings by determining that mutant S2 DNA sequences cannot abolish EIN3 binding to the labeled S2 EBS element in vitro (Figure 4I).

Together, these data indicate that EIN3 directly binds to the SUC2 promoter to suppress SUC2 expression in vivo and in vitro.

SUC2 acts genetically downstream of EIN3, repressing etiolated-seedling greening

The homozygous SUC2 loss-of-function suc2-5/− mutant is a null allele (Figure S2E); it is well established that these seedlings exhibit severely retarded growth, so seedlings of this line were not viable (Lei et al., 2011). We therefore utilized a heterozygote suc2-5/+ mutant to explore cotyledon greening. Eight-day-old etiolated suc2-5/+ seedlings (Figure S2E) grown on solid MS medium with 2% Suc subjected to white light irradiation for 2 days promoted etiolated-seedling greening (Figures 3K, 3Q, 3S, and 3T). In contrast, a 35S:SUC2 transgenic line (Figure S2D) failed to show etiolated-seedling greening (Figures 3K, 3P, 3S, and 3T). Thus, SUC2 negatively regulates the cotyledon greening of etiolated seedlings.

Subsequently, we analyzed the genetic interactions between EIN3 and SUC2. The generated ein3/eil1/suc2-5/+ line (Figure S2F) exhibited accelerated seedling greening compared
with the wild-type line. This result was similar to that obtained with the suc2-5/+ line (Figures 3K and 3O–3T). Thus, SUC2 acts genetically downstream of EIN3/EIL1 to block etiolated-seedling greening.

The ACS7–EIN3–SUC2 module is located on phloems of cotyledons

In Arabidopsis, SUC2 encodes a proton/Suc symporter localized throughout collection and transport phloem; thus, SUC2 is...
essential for the effective transport of Suc from source to sink tissues (Stadler et al., 1995; Srivastava et al., 2009). Building on our findings, we therefore determined if the ACS7–EIN3–SUC2 module might be located on phloems of cotyledons.

To observe the location of the ACS7–EIN3–SUC2 module on phloems of cotyledons, we firstly employed a transgenic line containing the β-glucuronidase (GUS) reporter gene downstream of five tandem repeats of an EBS (5×EBS:GUS), which was utilized to monitor EIN3 binding activity (Li et al., 2013). EIN3 activity was observed in the veins of cotyledons, true leaves, and hypocotyls either under white light or in darkness (Figures 5A–5E). EIN3 activity was also detected in sieve tubes and companion cells in phloems (Figures 5H–5J). EIN3 binding activity revealed by GUS staining was elevated with increasing ACC (Figures 5I and 5J), suggesting an increase in EIN3 binding to its cognate EBS and, by extension, a localization of ET accumulation during etiolated-seedling greening. Further, when Suc levels were increased, EIN3 binding activity was enhanced together with the cotyledon greening of the 5×EBS-GUS line (Figures 5L–5N), suggesting that Suc-signaling-induced ET promotes cotyledon greening by the ACS7–EIN3–SUC2 module.

Employing the EIN3pro:EIN3-GFP line to report EIN3 abundance and localization, EIN3 was located primarily within the phloem sieve tube elements and companion cells (Figures 5F and 5G), congruent with SUC2 (Stadler et al., 1995; Kühn et al., 1997; Srivastava et al., 2008, 2009). Further, previous findings showed that ACS7, integral to ET biosynthesis, promotes vascular cambium development in Arabidopsis (Yang et al., 2020), suggesting that ACS7 function may regulate the development of phloem sieve tube elements and associated companion cells. In agreement with this posit, our data indicate that an ACS7 promoter driving expression of a GUS reporter gene (ACS7pro-GUS) generated strong GUS activity within phloem sieve tube elements and associated companion cells (Figure 5K).

Taken together, these findings indicate that the ACS7–EIN3–SUC2 module is located on phloems of cotyledons.
The ACS7–EIN3–SUC2 module mediates the phloem loading of Suc in cotyledons

To further elucidate the mechanism by which the ACS7–EIN3–SUC2 module mediates Suc phloem loading, a [14C] Suc phloem loading assay was performed. Our findings revealed that the rate of Suc phloem loading in the cotyledons of wild-type, acs7-1, ein3/eil1, suc2-5/+; 3SS:SUC2, 35S:EIN3, and 35S:ACS7 seedlings was remarkably different (Figure S4A). The phloem-loading rate was significantly increased in acs7-1, ein3/eil1, and 35S:SUC2 seedlings. In contrast, this rate decreased in suc2-5/+ 35S:EIN3, and 35S:ACS7 lines (Figure S4A). These findings thus indicate that the ACS7–EIN3–SUC2 module suppresses Suc transport in the phloem cell of cotyledons.

Further, we assayed the Suc contents of cotyledons (source tissues) and shoot apexes (sink tissues) in 9-, 11-, and 13-day-old wild-type, acs7-1, suc2-5/+; and ein3/eil1 seedlings exposed to white light for 5 and 10 h, respectively. In all seedlings, Suc accumulation increased in both source and sink tissues with increasing seedling age (Figures S4B and S4C), suggesting that phloem loading of Suc results in typical Suc distributions in both source and sink tissues. Moreover, after white light exposure for 5 h, Suc accumulation decreased over time in acs7-1 and ein3/eil1 cotyledons but increased in suc2-5/+ cotyledons compared with that of wild-type (Figure S4B). Consistent with this, the phloem-loading rate of Suc in cotyledons increased over time in acs7-1 and ein3/eil1 cotyledons but decreased in suc2-5/+ cotyledons compared with that of wild-type (Figure S4A). As a result, after light exposure for 10 h, Suc accumulation increased over time in the shoot apex of acs7-1 and ein3/eil1 plants but decreased in the shoot apex of the suc2-5/+ line compared with that of wild-type plants (Figure S4C). These data were strongly correlated with a decline in Suc2 expression with increasing age (Figure S4A), and with increasing seedling age, the increase of EIN3 association with sequence S2 (Figure 4D) inhibits SUC2 expression. Although Suc contents in sink leaves were substantially altered in acs7-1, suc2-5/+; and ein3/eil1 seedlings compared with those of wild-type, Glc contents were not evidently different between wild-type and mutant sink leaves (Figure S4D), suggesting that the ACS7–EIN3–SUC2 module only affects Suc accumulation. Furthermore, in potato plants, Suc content in sink leaves (such as shoot apical meristems) was examined at different developmental stages. Whereas the levels of Fru and Glc in sink leaves were not obviously altered between transgenic and wild-type plants, the contents of Suc differ conspicuously (Chincinska et al., 2008).

In sum, these findings indicated that under darkness, the ACS7–EIN3–SUC2 module inhibits Suc export from cotyledons and thus accumulates Suc in cotyledons (source tissues) by suppressing the phloem loading of Suc in cotyledons.

**EIN3 inhibits phyA expression**

Far-red light has been shown to block etiolated-seeding greening by a phyA-dependent pathway, which can be restored by the application of exogenous Suc (Barnes et al., 1996). ChIP-seq analysis of EIN3 (Chang et al., 2013) has previously revealed that EIN3 might directly regulate phyA expression. Therefore, we determined whether phyA might be a downstream target gene of EIN3.

Results from a time course qPCR analysis revealed that phyA transcripts decreased with age and, over time, were higher in ein3/eil1 than in wild-type seedlings (Figure S5A). However, EIN3 accumulated with increasing age (Li et al., 2013). This suggests that EIN3 might inhibit phyA expression. Further, we employed transient expression analysis in *N. benthamiana*. Coexpression of EIN3 together with a phyA promoter driving the expression of a LUC reporter gene established that EIN3 suppressed expression from the phyA promoter (Figures 6A and 6B). These findings indicate that EIN3 inhibits phyA expression and that phyA may be a downstream target gene of EIN3.

ET suppression of phyA expression may be partially dependent of EIN3 function

To explore whether phyA expression is sensitive to transiently applied ET, we monitored phyA expression over time in response to the exogenously supplied ET precursor ACC. phyA expression decreased 60 min after treatment of the wild-type line, but this was not the case in ein3/eil1 double mutant seedlings (Figure S5B). Furthermore, we monitored phyA expression over time in response to the exogenously supplied ET synthesis inhibitors AVG and silver, phyA expression increased after application of either AVG or silver and remained highly elevated 60 min after the treatment of wild-type but not ein3/eil1 seedlings (Figures S5C and S5D). This suggests that ET, induced by accumulated Suc in germinating seeds under darkness, appears to promote etiolated-seeding greening, likely by EIN3 transcriptional suppression of phyA, a negative regulator of etiolated-seeding greening, following white light illumination.

Together, ET suppression of phyA expression may be partially dependent on EIN3 function.

**EIN3 directly binds to phyA promoter**

Our observations prompted us to determine whether EIN3 directly regulates phyA transcription. Examination of the phyA promoter revealed the presence of a putative EBS (TTCAA) [or its complementary TTTGAA] (Li et al., 2013). For subsequently analysis, we designated phyA promoter sequences as P1 or P2 (Figure 6C). The DNA sequences within the phyA promoter were utilized for ChIP analysis together with *EIN3* promoter containing mutations within the P1 region of the phyA promoter (Figure 6C) produced transcripts of the greatest abundance (Figure 6D). Furthermore, EIN3 association with the P1 sequence in the phyA promoter increased over time in *EIN3* promoter *phyA* seedlings (Figure 6E). In contrast, a phyA promoter containing mutations within the EBS (phyA*pro::phyA*pro::phyA*) displayed reduced EIN3 binding within the P1 region of the phyA promoter (Figure 6F).

Subsequently, EMSA was performed to determine whether EIN3 can directly bind to the phyA promoter P1 sequence in vitro. Indeed, the recombinant EIN3 protein bound to the labeled P1 element in vitro (Figure 6G). Excessive amounts of the unlabeled competitor P1 DNA sequence effectively abolished this binding in a dose-dependent manner (Figure 6G). Further, excessive amounts of the mutated competitor mP1 DNA did not induce effective binding in a dose-dependent manner (Figure 6H). Our data indicates that EIN3 directly binds to the phyA promoter.
to suppress phyA expression, further indicating that phyA is the downstream target gene of EIN3.

Taken together, these findings reveal that EIN3 directly binds to the phyA promoter to inhibit phyA expression in vivo and in vitro.

**phyA acts genetically downstream of EIN3 to inhibit etiolated-seedling greening**

We further analyzed the genetic interactions between EIN3 and phyA. Despite the absence of phyA function, etiolated-seedling greening was promoted in the phyA mutant line, whereas greening of ein3/eil1 seedlings was considerably reduced compared with that of wild-type seedlings (Figure S6). Moreover, in an ein3/eil1/phyA triple mutant line, etiolated-seedling greening was similar to that of the phyA line (Figure S6).

Taken together, therefore, phyA acts genetically downstream of EIN3/EIL1 to negatively regulate etiolated-seedling greening.
DISCUSSION

The switch from skotomorphogenesis to photomorphogenesis is indispensable for seedling survival and development. Integral to this process is etiolated-seedling greening, where the onset of illumination initiates an etioplast-to-chloroplast transition. While sugars have long been shown to affect etiolated-seedling greening, the underpinning molecular mechanisms have remained elusive. Further, whereas the mechanical stress of the soil has been reported to activate ET production to promote cotyledon greening by a key ET component, EIN3, controlling a few phytochrome and associated factors (Zhong et al., 2009). These reports suggest that Suc might induce ET production to promote etiolated-seedling greening. Further, during etiolated-seedling greening, a large amount of Suc is consumed, dependent on phytochrome (Kozuka et al., 2020). Therefore, it is probable that under darkness, Suc export from cotyledons must be blocked, or Suc must be accumulated in cotyledons to continually stimulate ET biosynthesis to promote etiolated-seedling greening, when exposed to light. However, the relative regulatory pathway is unknown.

In the present study, both ACS7 expression (Figure 2O) and ET production (Jeong et al., 2010) were preferentially induced by the exogenous application of Suc. Further, both endogenous Suc accumulation and ET production increased gradually with age in wild-type, but not acs7-1, seedlings (Figures 2Q and 2R). Moreover, ET biosynthesis did not occur in suc2-5/+ mutant seedlings (Figure S7). These findings indicate that both ACS7 and SUC2 are essential for Suc-induced ET production. We also demonstrated that Suc/ET signaling-mediated cotyledon greening is dependent on the EIN3/EIL1 component (Figure 3). Therefore, ACS7, EIN3, and SUC2 are essential for Suc/ET signaling-mediated cotyledon greening. Further, the direct suppression of SUC2 activity via an ACS7-EIN3 module (Figures 4 and S3) led to the suppression of Suc phloem loading in cotyledons (Figure S4), resulting in Suc accumulation in cotyledons (Figure S4). Therefore, these components form a Suc feedback loop (Suc-ACS7-ET-EIN3-ACS2-Suc) to promote Suc accumulation in cotyledons.

Further, EIN3 is elevated, and SUC2 transcripts were reduced under darkness (Zhong et al., 2009; Kühn et al., 1997). Darkness is known to suppresses SUC2 activity (Wright et al., 2003). These reports further suggested that Suc is constantly accumulated under subterranean darkness, possibly due to the decline in the Suc-feedback-loop-mediated Suc phloem loading. Therefore, when buried seeds germinate under subterranean darkness, the accumulating Suc enables ET production by preferential inducting ACS7 expression via the Suc feedback loop (Figure 7). Greater mechanical stress of the soil will lead to the exudation of less ET production from the soil. As a result, more ET production in the soil accelerates cotyledon greening and enables seedlings to grow out of soil.

Collectively, under darkness, these components form a Suc feedback loop (Suc-ACS7-ET-EIN3-ACS2-Suc) to constantly promote Suc accumulation, ACS7 expression, and associated ET biosynthesis in cotyledons, and to promote Suc/ET signaling-mediated cotyledon greening, following white light illumination (Figure 7).

The cooperation of the Suc feedback loop and the ET-EIN3-PORA/B module promotes Suc/ET signaling-mediated etiolated-seedling greening

Under darkness, Suc accumulation, ACS7 expression, and associated ET biosynthesis in cotyledons are promoted by the Suc feedback loop (Figure 7). Following white light illumination, generated ET activates EIN3 to facilitate etiolated-seedling greening by EIN3 directly binding to the promoters of PORA/B, which encode key enzymes in the chlorophyll synthesis pathway.

The Suc feedback loop (Suc-ACS7-ET-EIN3-ACS2-Suc) is essential for Suc accumulation and associated ET biosynthesis

ET is a gaseous hormone that has key functions in plant growth, development, and stress responses. It has been previously reported that the mechanical stress of the soil stimulates ET production to promote etiolated-seedling greening (Zhong et al., 2014). However, the molecular mechanisms of ET production under this condition have remained elusive.

Compared with other soluble exogenous sugars, Suc preferentially stimulates ET production in the leaf blades of Arabidopsis (Jeong et al., 2010), rice (Kobayashi and Saka, 2000), and tobacco (Philosoph-Hadas et al., 1985) in a dose-dependent manner. Further, ET production promotes etiolated-seedling greening by a key ET component, EIN3, controlling a few phytochrome and associated factors (Zhong et al., 2009). These reports suggest that Suc might induce ET production to promote etiolated-seedling greening. Further, during etiolated-seedling greening, a large amount of Suc is consumed, dependent on phytochrome (Kozuka et al., 2020). Therefore, it is probable that under darkness, Suc export from cotyledons must be blocked, or Suc must be accumulated in cotyledons to continually stimulate ET biosynthesis to promote etiolated-seedling greening, when exposed to light. However, the relative regulatory pathway is unknown.

In the present study, both ACS7 expression (Figure 2O) and ET production (Jeong et al., 2010) were preferentially induced by the exogenous application of Suc. Further, both endogenous Suc accumulation and ET production increased gradually with age in wild-type, but not acs7-1, seedlings (Figures 2Q and 2R). Moreover, ET biosynthesis did not occur in suc2-5/+ mutant seedlings (Figure S7). These findings indicate that both ACS7 and SUC2 are essential for Suc-induced ET production. We also demonstrated that Suc/ET signaling-mediated cotyledon greening is dependent on the EIN3/EIL1 component (Figure 3). Therefore, ACS7, EIN3, and SUC2 are essential for Suc/ET signaling-mediated cotyledon greening. Further, the direct suppression of SUC2 activity via an ACS7-EIN3 module (Figures 4 and S3) led to the suppression of Suc phloem loading in cotyledons (Figure S4), resulting in Suc accumulation in cotyledons (Figure S4). Therefore, these components form a Suc feedback loop (Suc-ACS7-ET-EIN3-ACS2-Suc) to promote Suc accumulation in cotyledons.

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Collectively, under darkness, these components form a Suc feedback loop (Suc-ACS7-ET-EIN3-ACS2-Suc) to constantly promote Suc accumulation, ACS7 expression, and associated ET biosynthesis in cotyledons, and to promote Suc/ET signaling-mediated cotyledon greening, following white light illumination (Figure 7).
(Zhong et al., 2009). Therefore, etiolated-seedling greening is promoted by the ET-EIN3-PORA/B module. This module may be another regulatory pathway of Suc/ET signaling-mediated etiolated-seedling greening (Figure 7). Therefore, the Suc feedback loop and the ET-EIN3-PORA/B module synergistically facilitate Suc/ET signaling-mediated etiolated-seedling greening.

The Suc-ACS7-ET-EIN3-ERF1-PIF3 module promotes Suc/ET signaling-mediated etiolated seedlings and enables seedlings to grow out of soil

The EIN3/EIL1 proteins activate an ET signaling-mediated PIF3-ERF1 transcriptional circuitry to synchronously promote etiolated-seedling greening by tissue-specific patterns (Zhong et al., 2014). That is, the EIN3/EIL1-ERF1 gene cascade modulates the cell elongation of hypocotyls. As a result, cotyledons of the etiolated seedlings are able to penetrate the soil cover. By contrast, the EIN3/EIL1-PIF3 gene cascade synchronously regulates the preassembly of photosynthetic machinery in cotyledons (Zhong et al., 2014). The cooperation of these two processes allows etiolated seedlings to gain photosynthetic capacity but avoid being damaged through photooxidation from dark to light. However, under darkness, the Suc-feedback-loop-induced Suc accumulation is a prerequisite for ET production to activate EIN3 and thus promote sugar-ET signaling-mediated etiolated-seedling greening (Figure 7). Therefore, following white light illumination, accumulated Suc under darkness are essential for sugar-ET signaling-mediated etiolated-seedling greening by a key ET component, EIN3, controlling both ERF1 and PIF3 gene expression (Figure 7). Together, under subterranean darkness, ET production is promoted preferentially by Suc relative to other sugars by the Suc feedback loop to promote Suc-ET signaling-mediated etiolated-seedling greening and to enable these seedlings to grow out of the soil by the Suc-ACS7-ET-EIN3-ERF1-PIF3 module in the light (Figure 7).

Suc represents a class of regulators that function in conjunction with ET signaling, Suc transport, and a few phytochromes and EIN3 controlling a few phytochromes and associated factors to optimize the de-etiolation of Arabidopsis seedlings as they emerge from subterranean darkness and are exposed to light (Figure 7).

Summary of main findings in this study

In this study, we demonstrated that sugars promote etiolated-seedling greening. We identified that ACS7, CINV1/2, and SUC2 are endogenous regulators of the Arabidopsis etiolated-seedling greening. Further, Suc, relative to other sugars, preferentially promotes etiolated-seedling greening. We also identified a Suc feedback loop (Suc-ACS7-ET-EIN3-SUC2-Suc). This loop promotes Suc accumulation in cotyledons, and this process is essential for ET biosynthesis, EIN3 activity, and, finally, etiolated-seedling greening mediated by EIN3 controlling a few phytochromes and associated factors. Finally, we found that EIN3 inhibits phyA functions by directly binding to the phyA promoter to promote etiolated-seedling greening.
Limitations of the study
In this study, we have identified a Suc feedback loop (Suc-ACS7-ET-EIN3-SUC2-Suc), which is essential for ET biosynthesis, EIN3 activity, and Suc-ET signaling-mediated etiolated-seedling greening. However, the connections between Suc and ACS7 are unclear. For example, whether Suc promotes ACS7 expression by signaling or metabolism remains unknown. Suc promotes ACS7 expression by an unknown pathway. Moreover, cellular nitrogen and carbon metabolism are tightly coordinated to sustain optimal growth and development for plants and other cellular organisms. We currently know little about the mechanisms if cellular nitrogen regulates etiolated-seedling greening. How mineral nutrient nitrogen regulates etiolated-seedling greening remains unknown. It is also unclear whether phyA, as a key component, is involved in cellular nitrogen-mediated etiolated-seedling greening.

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Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2022.110529.

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AUTHOR CONTRIBUTIONS

DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES


**STAR METHODS**

**KEY RESOURCES TABLE**

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<td>Arabidopsis:5 × EBS-GUS/wild-type</td>
<td>Li et al., 2013</td>
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<td>Arabidopsis:phyA(CS6223)</td>
<td>Kang et al., 2009</td>
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<tr>
<td>Arabidopsis:ein3-1</td>
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<td>An et al., 2010</td>
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**RESOURCE AVAILABILITY**

**Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Lai-Sheng Meng (menglsh@jsnu.edu.cn).
Materials availability
All unique/stable reagents generated in this study are available from the lead contact without restriction.

Data and code availability
This paper does not report original code. “Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.”

EXPERIMENTAL MODEL AND SUBJECT DETAILS
All plants are in the Col-0 background, unless indicated differently. Seedlings grown on agar were maintained in a growth room under a 16/8 h of light/dark cycle with cool white fluorescent light at 21 ± 2 °C. Plants grown in soil were maintained in a controlled environmental growth chamber under a 16/8 h light/dark cycle with white light [60 (low light), 130 (middle light/normal), 230 (high light) μmol quanta PAR m⁻² s⁻¹] conditions at 21 ± 2 °C. Unless otherwise stated, seedlings grown on agar were under middle light conditions at 21 ± 2 °C.

METHOD DETAILS

Plant materials and growth conditions
The 5 × EBS-GUS/wild-type (Li et al., 2013), phyA (CS6223) (Kang et al., 2009), ein3-1 and ein3/eil1 (An et al., 2010) mutants, 3SS:SUC2 and suc2-5 (Lei et al., 2011), 5 × EBS-GUS (Li et al., 2013), FLAG-EIN3 and 35S:EIN3-GFP (An et al., 2010) were all described previously.

The 35S:EIN3-GFP, ein3/eil, and ein3-1 seeds were kindly provided by Prof H. W. Guo (South University of Science and Technology of China, China). pGreen0800-LUC vector and 5 × EBS:GUS seeds were kindly provided by Associate Prof Ziqiang Zhu (Nanjing Normal University, Nanjing, China). The 3SS:SUC2 and suc2-5 (SALK_087046) seeds were kindly provided by Prof D Liu (Tsinghua University, China). The phyA (CS6223) mutant was obtained from the ABRC (Ohio State University). The acs2 (SALK_208272C), acs4 (SALK_068371C), acs6 (SALK_138142C) mutants were requested from AraShare (a non-profit Arabidopsis share center, http://www.arashare.cn). The acs7-1 (CS16570) seeds were kindly provided by Prof N. N Wang (Nankai University, China).

The ein3/eil1/phyA mutant was obtained from F2 plants [ein3/eil1/phyA (CS6223)] that have long hypocotyl and unexpanded cotyledons in far-red light (Barnes et al., 1996) and have elongated hypocotyls on solid MS medium with 6.0 μm ACC (An et al., 2010). The ein3/eil1/suc2-5/+ mutant was obtained from F2 plants (ein3/eil1/suc2-5/+) that had elongated hypocotyls on solid MS medium with 6.0 μm ACC (An et al., 2010) and then were identified through PCR-based genotyping for suc2-5/+.

For simultaneous germination, seeds were treated with jarovization at 4 °C overnight and then sown on solid MS medium supplemented with 1% Suc, pH 5.8 and 0.8% agar (Meng and Yao., 2015). Seedlings grown on agar were maintained in a growth room under a 16/8 h of light/dark cycle with cool white fluorescent light at 21 ± 2 °C (5). Plants grown in soil were maintained in a controlled environmental growth chamber under a 16/8 h light/dark cycle with white light [60 (low light), 130 (middle light/normal), 230 (high light) μmol quanta PAR m⁻² s⁻¹] conditions at 21 ± 2 °C. Unless otherwise stated, seedlings grown on agar were under middle light conditions at 21 ± 2 °C.

Wild-type is Col-0 unless otherwise stated. In all experiments, three biological replicates were performed and error bars represent SD.

GUS assay and histochemical assay of GUS activity
Using a mix buffer (1 mM X-gluc, 60 mM NaPO₄ buffer, 0.4 mM of K₃Fe(CN)₆/K₄Fe(CN)₆ and 0.1% (v/v) Triton X-100), samples were incubated at 37 °C overnight and then stained with 5 × EBS (EIN3 binding site)-GUS, ACS7pro-GUS, FLAG-EIN3-GUS activity or X-gluc was detected by observing and recording fluorescence images with a confocal microscope (Nikon). All conditions were performed in triplicate. The 3SS-3×FLAG-EIN3; ACS7pro-GUS plasmid was digested. (5) Using ClonExpress II One Step Cloning Kit, above four fragments were linked to pCB2004:ACS7-GUS plasmid. Using the same method, we generated pHB:EIN3-GFP/PHYA(mEBS)pro::PHYA plasmid. In detail, (1) used primers were phyApcpF; R-M-EMSA-phyA and M-EMSA-phyA; phyApcR for generating the phyA promoter sequence. (2) Used primers were phyApcpF; phyApcR for generating the phyA promoter sequence. (3) Using Plasmid constructs
For obtaining pCB308R:ACS7-GUS plasmid, used primers can be seen in Table S1. For obtaining EIN3pro::EIN3-GFP plasmid, used primers can be seen in Table S1. For obtaining pCB2004:ACS7-GUS plasmid, used primers can be seen in Table S1.
Quantitative PCR
Total RNA was prepared from tissues indicated in the figures by the TRIZOL reagent (Invitrogen), and 1 μg of RNA from each sample was used for the reverse transcription reaction. Subsequently, 1 μL of the reverse transcription reaction was used as template for PCR amplification. The same RNA samples and primers were used for real-time PCR analysis that was performed and statistically analyzed as described (Meng et al., 2021). Kinetic detection of PCR products using Sybgreen real-time RT-PCR. As an internal control, Tubulin transcripts were used to quantify the relative transcriptional level of each target gene in each tissue type. Primer pairs are listed in Table S2. Unless otherwise specified, the number of PCR cycles is 30. Three repeated biological experiments were carried out. Primer pairs of SUC2 were utilized. Primer pairs of EIN3 have been described by (Meng et al., 2018). Primer pairs of ACS7 were utilized. Used primers can be seen in Table S1.

Greening rate
For determining the greening rate, ≥ 40 seedlings were grown on solid MS medium with the indicated sugar concentration under darkness for the indicated number of days after germination and then transferred into white light (60 [low light], 130 [middle light], 230 [high light] μmol quanta PAR m⁻² s⁻¹) to grow for the given time. The greening rate was calculated as the percentage of seedlings exhibiting greening, as described (Zhong et al., 2009).

Seedling observation and photography
Different age seedlings were observed and photographed by using stereo microscope [Leica Microsystems (Switzerland) Ltd. CH-9435 Heerbrugg. Type: DMC4500 (12730517)].

ET assay
Experiments performed on indicated-day-old seedlings using a gas chromatograph equipped with a flame ionization detector [Echrom Technologies (shanghai) Ltd Co]. For ethylene collection, petri dishes containing indicated-day-old seedlings were opened to remove trapped air, and then the original lids were replaced by lids with silicone rubber seals. After a further 24 h of incubation under the same conditions, 1 mL of gas was withdrawn from each plate using a gas-tight syringe. The gas was injected into a gas chromatograph equipped with a flame ionization detector [Echrom Technologies (shanghai) Ltd Co]. The carrier gas (N2) flow rate was 60 mL min⁻¹. The detector response was standardized by injecting known amounts of ethylene prepared by serial dilution. Means and sd values were calculated from three experiments.

Chlorophyll content
Chlorophyll content was extracted from cotyledons of five or six-day-old etiolated seedlings followed by 1 day of white light irradiation by boiling them in 95% ethanol at 80°C. The chlorophyll concentration per fresh weight of cotyledons of the same weight was examined, as has been described by Lichtenthaler (1987).

Assay of sugar metabolites
Plant Suc assay kit (Beijing Solarbio Science & Technology Co., Ltd, Cat#BC2465; http://www.solarbio.com/goods-9298.html) was used for assay of sugar metabolites. 0.1g mature leaf blades were ground into homogenate at 23°C and 0.5 mL extraction buffer added and ground within centrifuge tube, finally kept at 80°C for 10 min and then were centrifuged at 2000 g for 10 min. After cooling these extracts were centrifuged at 4000 g for 10 min, the supernatant was transferred to a fresh tube and 2 mg reagent 5 added to decolorize at 80°C for 30 min. Then 0.5 mL extraction buffer was added, mixed and centrifuged at 4000 g for 10 min. The supernatant was transferred to a fresh tube for visible light analysis. Three centrifuged tubes were per sample were used each with 25 μL of sample and then standard product (reagent 1) and water were added, respectively. Fifteen μL of reagent 2 was added, mixed and boiled at 100°C for 5 min. Subsequently, 175μL of reagent 3 and 50μL of reagent 4 were added, respectively. The resulting solution was boiled in water for 10 min, followed by measurement of the light absorption value at 480 nm after cooling. The Suc content was calculated for each sample.

Phloem loading of Suc
Nine-day-old seedlings grown on solid MS medium with or without sugars were used to assay phloem loading of Suc. Following incubation in pretreatment solution (pH5.7; liquid MS medium) for 40–50 min, the cotyledons were incubated in the phloem loading buffer (pH 5.7; MS medium with 0.1–0.2% Suc) with [¹⁴C] Suc (0.50μCi mL⁻¹) and then were cultivated for 2–3 h. Following three washes in desorption solution (pH 5.7; liquid MS medium and 1% Suc), these seedlings were placed in scintillation vials and 3.0 mL of scintillation cocktail was added. The amount of [¹⁴C] phloem Suc loading was analyzed using a scintillation counter and expressed as cpm mg⁻¹ fresh weight.

Histological analysis
Histological analysis was performed, as was described by Sun et al. (2012). In details, leaf and cotyledon tissues were fixed in FAA (acetic acid 5% and formaldehyde 3.7% in 50% ethanol) for over 2 h, at 4°C. These tissues were dehydrated for 45–60 min in 30, 50, 70, 85, 93, 100% ethanol, followed by 2:1, 1:1, 1:2 ethanol/xylol (v/v), and 100% xylol solutions, followed by infiltration with Paraffin.
(Leica, Germany) at 65°C. And then these materials were embedded in Paraffin. The sections were made by using a Microtome
(Leica, Germany), and then stained with Fast Green FCF and Safranine O. These materials were placed on glass slides, and visualized
by using an optical microscope (Olympus 80i, Olympus Corporation, Japan).

Transactivation assay
To examine if EIN3 activates SUC2 expression by LUC activity assay, we performed the below experiments. To generate proSUC2-
LUC, the promoter was PCR amplified with primers proSUC2-F and proSUC2-R from the gnomic DNA of Arabidopsis and inserted
into the cloning site of the pGreen0800-LUC vector.

The MproSUC2-LUC construct containing mutations in the S2 sequence of the SUC2 promoter was generated using overlap
extension PCR with primers and inserted into pGreen0800-LUC vector. Two fragments of MproSUC2 were combined into a inte-
grated fragments of MproSUC2 by using primers. Similarly, two fragments of Mpro-phyA were combined into an integrated fragment
of Mpro-phyA by using primers. The primers used can be seen in Table S1.

Agrobacteria with the effector or reporter constructs were coinfected for 3 h and then infiltrated into 20-day-old N. Benthamiana
leaf blades. Leaf blades of these seedlings were incubated under light conditions (130 μmol quanta PAR m⁻² s⁻¹) for 2–4 days after
infiltration. The firefly LUC activities were photographed after spraying with 1 mM luciferin. To determine the dual luciferase activities,
firefly lucerase and Renilla lucerase were measured, as has been previously described (Li et al., 2013).

Protein expression and purification
The plasmid pGEX-5X-1 for EIN3 was utilized. The coding sequence of EIN3 was amplified by the primer pair and cloned into the BamH1 and XhoI restriction sites of pGEX-5X-1. Recombinant glutathione S-transferase binding protein (GST)-tagged EIN3 was ex-
tracted from transformed E. coli (Rosetta2) after 10 h of incubation at 16°C following induction with 10 μM isopropyl-B-D-thiogalac-
topyranoside. These recombinant proteins were purified using GST-agarose affinity. The primers used can be seen in Table S1.

ChIP-PCR
The transgenic lines containing EIN3pro::EIN3-GFP were utilized. ChIP was performed with seedlings (Meng et al., 2015, 2018). Leaf
blades were incubated in buffer (1.0 mM PMSF, 0.5 M Suc, 1 mM EDTA, 10–12 mM Tris, pH 8.0, and 1% formaldehyde) under vacuum
for 15–20 min for crosslinking the chromatin. Then, 0.1 M Gly was placed in the mixture, incubating for an additional 5 min for ter-
minalizing the reaction. Leaf blades were placed and ground in liquid nitrogen and re-suspended in lysis buffer (150mM NaCl, 1mM
EDTA, 0.1% SDS, 0.1% deoxycholate, 50 mM HEPES, pH 7.5, 1% Triton X-100, 10 mM Na-butyrate, 1 mM PMSF and 13 complete
protease inhibitor (Roche). Chromatin was sheared to about 200–500 bp fragments via sonication followed by centrifuged. At 4°C,
supernatants were preincubated under protein G agarose beads for 1–1.5 h. Input material (supernatant containing chromatin) was used
for immunoprecipitation with anti-GFP antibody. Anti-GFP antibody bound to EIN3-FLAG or GFP-chromatin complexes was incu-

Electrophoretic mobility shift assay (EMSA)
According to the manufacturer’s instructions, the LightShift chemiluminescence EMSA kit (Beyotime Biotechnology, cat#gs009;
http://www.beyotime.com) was used to detect the electrophoretic mobility shift assay (EMSA). The biotin-labeled SUC2 DNA frag-
ments (5'-caccattatgtttatatccacattaacactctctcataaggtgt-3'; 5'-acacctttagaatatttaaagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagag
the membrane was washed 1 min with 1× washing buffer, and then washed 4 times for 5 min each with 1× washing buffer, and then the membrane was transferred into the detection of equilibrium liquid for 5 min with gentle shaking. The biotin-labeled DNA was detected by chemiluminescence according to the manufacturer’s protocol.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Student’s t test was used to determine the statistical significance between wild-type and mutant lines in measure related to glucose and sucrose levels, invertase activity, qPCR, chlorophyll concentration, luminescence intensity (*p < 0.05; **p < 0.01; ***p < 0.001).