Phytohormone ethylene-responsive Arabidopsis organ growth under light is in the fine regulation of Photosystem II deficiency-inducible AKIN10 expression

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For photoautotrophic plants, light-dependent photosynthesis plays an important role in organismal growth and development. Under light, Arabidopsis hypocotyl growth is promoted by the phytohormone ethylene. Despite well-characterized ethylene signaling pathways, the functions of light in the hormone-inducible growth response still remain elusive. Our cell-based functional and plant-system-based genetic analyses with biophysical and chemical tools showed that a chemical blockade of photosystem (PS) II activity affects ethylene-induced hypocotyl response under light. Interestingly, ethylene responsiveness modulates PSII activity in retrospect. The lack of ethylene responsiveness-inducible PSII inefficiency correlates with the induction of AKIN10 expression. Consistently, overexpression of AKIN10 in transgenic plants suppresses ethylene-inducible hypocotyl growth promotion under illumination as in other ethylene-insensitive mutants. Our findings provide information on how ethylene responsiveness-dependent photosynthetic activity controls evolutionarily conserved energy sensor AKIN10 that fine-tunes EIN3-mediated ethylene signaling responses in organ growth under light.

A photosynthetic organism plant converts solar energy into organic molecules and self-supplies cellular energy under light. Intuitively, plants largely determine their cell fates in response to light information. For example, the plant hormone ethylene-inducible organ growth is differentially regulated by light conditions. More specifically, Arabidopsis hypocotyl growth is inhibited and promoted in response to ethylene in the absence and presence of light, respectively. The light-dependent ethylene-inducible hypocotyl growth promotion results mainly from cellular enlargement rather than proliferation; hence cell division cycle regulation may be excluded in their controlling mechanisms.

Ethylene is perceived by ETHYLENE RESPONSE1 (ETR1) and four other closely related gene products that act as the hormone receptor in Arabidopsis. The high-molecular-weight ethylene receptor complexes consist of the hormone receptors, CONSTITUTIVE TRIPLE RESPONSE1 (CTR1), and other unknown component(s). In the absence of ethylene, CTR1 directly interacts with and phosphorylates another membrane protein ETHYLENE INSENSITIVE2 (EIN2). In the presence of ethylene, the inverse agonistic receptor complexes block the CTR1 function on EIN2, enabling an unknown protease to cleave the C-terminal half of nonphosphorylated EIN2 (EIN2C). EIN2C moves into the nucleus, stabilizes key transcription factors, EIN3 and EIN3-LIKE1 (EIL1), and triggers ethylene-responsive gene expression.

For ethylene-inducible Arabidopsis hypocotyl growth in light, EIN3 specifically binds to the PHOTOCYROME-INTERACTING FACTOR3 (PIF3) promoter and induces the gene expression. Two other
homologs of PIF3 seem to play key roles in modulation of the organ growth as well. Despite well-characterized ethylene-dependent regulatory pathways, how light information is involved in the hormone–inducible growth promotion remains largely elusive. For instance, how light-labile PIF3 and its homologs can contribute to the light-dependent organ growth is still puzzling.

ETRI is thought to have originated from the chloroplast genome, but its cellular functions have not been widely studied in terms of plastid activity. Recently, ethylene insensitivity has been implicated in decreasing photosynthetic outputs through the transcriptional down-regulation of the most abundant enzyme involved in the first commitment step of carbon fixation RUBISCO expression. Therefore, ethylene insensitivity, resulting in low photosynthetic activity, must lead to low energy status in plant cells along the light and dark cycle of the day.

Intracellular energy deprivation is known to activate the two isoforms of the evolutionarily conserved energy sensor Snf1-related protein kinase (SnRK1), namely, ARABIDOPSIS KINASE (AKIN)10 and AKIN11 in Arabidopsis thaliana, that can turn catabolic and anabolic pathways on and off, respectively, in order to sustain cellular viability. The chemical blockade of the photosystem (PS) II pathway with 3-(3,4-dichlorophenyl)-1,1-dimethyleurea (DCMU), as well as the intervention condition of mitochondrial aerobic respiration by hypoxia, potently induces energy depletion and readily activates AKIN10 activity in Arabidopsis. However, ethylene sensitivity has never been examined as a potential factor of cellular energy status by means of its regulation of chloroplast activity.

This gap in our knowledge results partly because further examination of ETRI function using the dominant ethylene-insensitive Arabidopsis etr1-1 has been hampered because it encloses an unexpected abnormal chloroplast structure, which may or may not interfere with photosynthetic activity. The rigorously studied etr1-1 turned out to be recessive with a second site mutation for ACCUMULATION AND REPPLICATION3 (ARC3), which is involved in the positioning of plastid division sites. This chloroplast abnormality has, thus, disallowed the unambiguous understanding of the ETRI-dependent ethylene function in the modulation of chloroplast/photo-synthetic activity, which results in cellular energy modulation.

Spectroscopic analysis of chlorophyll fluorescence emission is a well-established tool to measure the photosynthetic efficiency of PSII in chloroplasts of higher plants. Pulsed amplitude-modulated (PAM) fluorimetry has been the technique of choice for such chlorophyll fluorescence analysis in leaves. This analysis provides information about the quantum yield of PSII, the relative size of the reduced quinone pool, and the extent of nonphotochemical quenching. More recently, chlorophyll fluorescence lifetime (CFL) analysis has also been applied to measure the photosynthetic kinetics. Time-correlated chlorophyll fluorescence analysis of individual chloroplasts was monitored for Arabidopsis and Alocasia leaves under a two-photon excitation fluorescence lifetime imaging microscope (FLIM) with femtosecond pulses. Although FLIM-based data correlated well with ensemble-based data in Synsechocystis sp PCC 6803, the FLIM-based CFL analysis has not been applied to live single cells of multicellular higher plants so far.

Arabidopsis leaf mesophyll protoplast is a versatile cellular system that is acquired after enzymatic removal of cell walls. Leaf mesophyll protoplasts have robust physiological cellular responses to extracellular signals, and their genetic constituents are readily manipulated by transient expression of genes of interest. Microscopic images taken from mesophyll protoplasts also provide useful information about the organization and development of subcellular organelles.

Here, we showed that PSI activity is involved in regulation of ethylene-inducible Arabidopsis hypocotyl growth in light. Then, we took advantage of the protoplast system to reconstitute ethylene insensitivity in live single cells and demonstrated that the lack of ethylene responsiveness causes PSI inefficiency, leading to cellular energy deprivation, which activates AKIN10 expression. Consistently, AKIN10 activation suppresses ethylene-inducible hypocotyl growth in light. Our findings revealed a functional link between ethylene responsiveness–dependent PSI efficiency and the PSI efficiency–dependent cellular energy sensor AKIK10 expression for the control of ethylene-inducible hypocotyl growth promotion under illumination.

Results and Discussion

Ethylene promotes hypocotyl growth through PSI regulation in light. In our previous work an abnormal chloroplast structure was found in etr1-1 resulting from a second site mutation on arc3-3. Since etr1-7 is an intragenic suppressor of etr1-1, etr1-7 also encoded abnormal chloroplasts in mesophyll cells similar to those of etr1-1 (Fig. 1a). To separate the etr1-1 and etr1-7 alleles from the second mutation arc3-3, etr1-1 and etr1-7 were crossed with the wild type (WT; Col-0). Individual allele-specific mutants were isolated without arc3-3 and named as etr1-1sg and etr1-7sg, respectively. Each genetic background was confirmed by using derived cleaved amplified polymorphic sequences (dCAPS) analysis and DNA sequencing (Fig. 1b). Unlike WT, several large chloroplasts with an abnormal structure were observed in protoplasts of ethylene insensitive etr1-1 and ethylene hypersensitive etr1-7 (Fig. 1a). Without the arc3-3 allele, many small chloroplasts were observed in etr1-1sg and etr1-7sg as in WT, confirming that arc3-3 caused the chloroplast abnormality. In WT, etr1-1sg, etr1-7sg, and arc3-3, the accumulation of two representative photosynthetic proteins, PSI D1 (PsbA) and Rubisco large subunit (RbcL) were detected at a similar level by protein blot analysis using specific antibodies (Fig. 1b). Thus, both the PSI protein complex and the downstream metabolic–output machinery in photosynthesis seem to be normal in etr1-1sg and etr1-7sg.

In the ethylene response, etiolated Arabidopsis WT seedlings grown on a full-strength Murashige and Skoog (MS) medium containing 1% (w/v) sucrose manifested a triple response of shortened hypocotyls and roots and exaggerated apical hook formation in the presence of an ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC, Fig. 1c). The etr1-1sg exhibited ethylene insensitivity with its elongated hypocotyls in the same manner as etr1-1. Furthermore, etr1-7sg exhibited the same ethylene hypersensitive phenotype as etr1-7.

All results
Figure 1. etr1 and arc3-3 alleles, respectively, control ethylene sensitivity and chloroplast morphology in the etr1-1 background. (a) Chloroplast morphology in mesophyll protoplasts isolated from the leaves of WT, etr1-1, etr1-7, arc3-3, etr1-1sg, and etr1-7sg. etr1-1 and etr1-7 uncoupled from arc3-3 are denoted as etr1-1sg and etr1-7sg, respectively. (b) Rubisco large subunit (RbcL) and PSII protein D1 (PsbA) protein levels are shown as representative photosynthetic enzymes. Protein blot analysis was performed with commercially available specific antibodies. (c) Triple response assay for WT, etr1-1, etr1-7, arc3-2, etr1-1sg, etr1-7sg, and arc3-3. Scale bar, 10 mm. (d,e) The light-controlled growth response of Arabidopsis hypocotyls observed (d) and measured (e) in the absence and presence of ACC with and without DCMU. Scale bar, 1 mm. n > 20. (f) Marker gene expression measured in WT and ethylene-insensitive etr1-1sg using qRT-PCR. All experiments conducted in triplicate with consistent results; the means of triplicate measurements are shown with standard error bars. ***p < 0.001, **p < 0.01, and *p < 0.05.
indicated that the second site mutation on ARC3 within etr1-1 only affects the chloroplast phenotype, and the mutation on ETR1 is critical for determining ethylene sensitivity in etr1-1 and etr1-7.

In the light, Arabidopsis WT hypocotyl growth promotion was pronounced on an MS-deficient growth medium in the presence of 50 µM ACC43, but not those of ethylene-insensitive etr1-1, etr1-1sg, and another well-characterized strong ethylene insensitive mutant ein2-1 (Fig. 1d,e). On the other hand, the hypocotyl growth promotion of two arc3 alleles, arc3-2 and arc3-3, was again pronounced in the presence of ACC and was similar to that of WT. To further investigate photosynthesis functions uncoupled from other effects of light on organ growth promotion, Arabidopsis hypocotyl growth was quantitatively analyzed in the presence and absence of DCMU, which blocks the PSII pathway40. Surprisingly, ethylene dependent hypocotyl growth promotion in light was largely suppressed in the presence of DCMU (Fig. 1d,e). Since DCMU is a sensitive blocker that interrupts the electron-transport chains in photosynthesis, its loss-of-photosynthetic activity may modulate cellular energy status and ethylene-dependent hypocotyl elongation under light.

To understand the molecular basis of light-grown Arabidopsis seedling responses to ethylene/ACC in terms of PSII regulation, the expression pattern of marker genes including skotomorphogenesis-related PIF3, photosynthetic/sucrose-responsive SUCROSE TRANSPORTER1 (SUC1) and SUC449 were examined using quantitative reverse transcriptase-dependent real-time PCR (RT-qPCR) with total RNA extracted from the shoots of 5-day-old WT and etr1-1sg seedlings grown in light. The expression of PIF3, SUC1 and SUC4 was induced by ACC in WT, but not in ethylene insensitive etr1-1sg (Fig. 1f). Moreover, the ACC-inducible marker gene expression was largely suppressed in WT by DCMU. Because EIN3-induced PIF3 plays main regulatory role in hypocotyl growth elongation, regulation of EIN3 level would result in the modulation of ACC/ethylene dependent hypocotyl growth promotion under light. All results suggest that the receptor-dependent ethylene signaling controls EIN3-dependent gene expression leading to hypocotyl growth promotion and perhaps PSII activity is involved in the ethylene-inducible organ growth regulation.

Sugars, the products of photosynthesis, function not only as energy resources, but also as signaling molecules that modulate the expression of genes such as SUC1 and SUC449. To examine whether sugar signaling plays a role in ethylene-inducible hypocotyl growth promotion under light, hypocotyl growth of gin2-1, the plant glucose sensor HEXOKINASE1-null mutant, was monitored under light. The hypocotyls of gin2 grew more or less like those of WT (Ler) when grown in combinations of ACC and DCMU under light (Fig. S2); thus, glucose/sugar as a nutrient resource, but not as an intracellular signal that antagonizes ethylene signaling, apparently has a major role in connecting PSII activity in chloroplasts to ethylene-inducible hypocotyl growth promotion under light. In line with this notion, exogenous 1% (w/v) sucrose could induce ACC-dependent and -independent hypocotyl growth promotion in Col-0, but not in ein2-1 and ein3eil1 block EIN3-dependent ethylene signaling. This is consistent with our observation that EIN3-dependent PIF3 is necessary in Arabidopsis hypocotyl elongation in the presence of ACC/ethylene under light. Moreover, PSII-dependent photosynthetic activity may modulate EIN3 and its downstream regulator PIF3 in ethylene-inducible hypocotyl elongation under light (Fig. S3).

FLIM-based CFL measures PSII activity at single cell resolution. Next we investigated whether ethylene responsiveness could affect PSII activity. First, we measured chlorophyll fluorescence using traditional pulse-amplitude modulation (PAM) fluorimetry and expressed these values as photochemical yields (F/Fm). Because each leaf of a plant ages differently, only the third and fourth leaves from 3- to 5-week-old soil-grown plants were used for analyses. F/Fm was always higher in WT than in etr1-1 at 3 to 5 weeks after germination (Fig. 2a,b).

We then measured PSII activity in the chloroplasts at single-cell resolution. To do so, an FLIM technique was applied to obtain CFLs in mesophyll protoplasts freshly isolated from mature leaves of ethylene sensitive WT and ethylene insensitive etr1-1. When color-coded CFLs were superimposed over leaf cell images, many small chloroplasts were largely shown to be bluish-green in WT protoplasts, but several large chloroplasts were yellowish-red in etr1-1 protoplasts (Fig. 2c,d). These results indicated that chlorophyll fluorescence decays rather quickly in the chloroplasts of WT than in those of etr1-1. The convoluted intensity-weighted average CFLs of WT and etr1-1 were estimated to be ca.797 ps and ca.1144 ps, respectively (Fig. 2e, Table 1). The CFL value obtained from WT protoplasts was much shorter than the values obtained from isolated antenna complexes, which were around 2 to 4 ns41. This may imply that photo energy transfer to the reaction center occurs rather quickly and efficiently in the cellular environment than in isolated light-harvesting complexes.

CFL reflects the efficiency of PSII activity in the experiment. PSII fluorescence is predominant at 700(+/-20) nm and nearly 100 times greater than that of PSI at room temperature. Furthermore, the plastoquinone pool is fully oxidized by dark incubation before CFL analysis41. Under such conditions, CFL values most likely echo the quenching efficiency of the PSII pathway by energy transfer to the reaction center, i.e., photochemical quenching42,43. Therefore, when PSII efficiency is low, photochemical quenching is delayed/slow and CFL becomes longer in etr1-1 than in WT (Fig. 2c-e). The differences in CFLs between WT and etr1-1 were consistent throughout different developmental stages from 3 (mature) to 5 weeks (just before bolting), indicating that ethylene-sensitivity/responsiveness modulates PSII activity throughout plant leaf development (Fig. 2g,h). In summary, both FLIM- and PAM fluorimetry-based chlorophyll fluorescence analyses consistently suggest that PSII photosynthetic activity in abnormal chloroplasts of ethylene-insensitive etr1-1 plants is less efficient than in normal chloroplasts in ethylene-sensitive WT plants.

To further compare FLIM-based CFL and PAM fluorescence analysis, we tried to obtain photochemical yields with FLIM-based fluorescence analysis. When the chlorophyll fluorescence of PSII was measured in mesophyll protoplasts, parts of reaction centers must be closed by laser excitation. In the results, fluorescence values can be attributed to two different states of the reaction centers, open and closed. In WT and etr1-1, these two peaks were found under optimized fitting conditions (Fig. 2i). By referring to previous studies on the CFL of leaves and isolated chloroplasts, shorter (~500 ps) and longer (~1000 ps) peaks in WT were identified for
Figure 2. Differences in chlorophyll fluorescence emission between ethylene-sensitive WT and ethylene-insensitive etr1-1 in Arabidopsis. (a,b) Photochemical yields (Fv/Fm) were measured using PAM fluorimetry in WT (a) and etr1-1 (b) leaves 3 to 5 weeks after germination. (c,d) Chlorophyll fluorescence lifetimes (CFLs) -color-coded cellular images from leaf mesophyll protoplasts of WT (c) and etr1-1 (d) were obtained using a time-correlated and space-resolved single photon-counting FLIM technique. Image sizes are 200μm × 200μm. (e) Chlorophyll fluorescence decay of etr1-1 (black) and WT (red) are shown with instrumental response function (IRF, blue). CFL values were obtained using FLIM and then analyzed using the Symphotime Program. (f) Working model of photo-energy dissipation. (g,h) CFLs were measured using FLIM in WT (g) and etr1-1 (h) mesophyll protoplasts isolated from leaves at 3 to 5 weeks after germination. (i) Normalized amplitude of CFL components from mesophyll protoplasts of WT and etr1-1. (j) Amplitudes of CFL components from WT protoplasts in the presence of DCMU.
reaction centers at open (τ_{open}) and partially closed states, respectively (Fig. 2i) 44–47. Interestingly, fluorescence values at both peaks were always relatively longer in etr1–1 than in WT. When DCMU that chemically closes PSII pathway was applied, a new fluorescence component of 2700 ps was identified for reaction centers at completely closed state (τ_{closed}) in WT (Fig. 2j). Then, photochemical yields was calculated as following F_{v}/F_{m} \approx (\tau_{open}^{-1} - \tau_{closed}^{-1})/\tau_{open}^{-1} 48. This F_{v}/F_{m} value calculated from CFL values was close to the values obtained by PAM fluorimetry (Fig. 2a), supporting its fidelity in analyzing PSII activity. The advancement of cellular imaging microscopy now allows specific observations of cellular structures and their functions in vivo. Its application to the measurement of chlorophyll fluorescence from chloroplasts in a single cell enables the accurate and dynamic detection of PSII photochemical efficiency at high resolution in live single cells of a higher plant, Arabidopsis.

Ethylene responsiveness modulates the chlorophyll fluorescence lifetime in PSII. Next we examined whether the chloroplast abnormality observed in etr1-1 may affect the chlorophyll fluorescence of PSII. Unlike etr1-1, the CFLs of etr1-7 and arc3-2 were similar to those of WT (Fig. 3a,b). The arc3-2 allele produces defective chloroplast division, resulting in development of giant chloroplasts as in etr1-721, 49. We then examined whether ethylene responsiveness affects the photochemical efficiency of PSII. A FLIM device was fabricated in house and used to monitor the CFLs of other strong ethylene insensitive ein2-1 protoplasts, in addition to ethylene sensitive WT, ethylene insensitive etr1-1, and ethylene hypersensitive etr1-7. CFLs were again longer in etr1-1 and ein2-1 than in WT and etr1-7 (Fig. 3c,d) indicating that the CFL of PSII is under the control of ethylene responsiveness.

|          | A_{1}^* (a.u.) | τ_{1} (ns) | A_{2}^* (a.u.) | τ_{2} (ns) | τ_{int. avg} (ns) |
|----------|---------------|-----------|---------------|-----------|------------------|
| WT       | 63.59         | 0.55      | 35.41         | 1.24      | 0.797            |
| etr1-1   | 51.23         | 0.78      | 48.77         | 1.53      | 1.144            |

Table 1. Average lifetimes of chlorophyll fluorescence in Arabidopsis WT and etr1-1. A_{i} = \sum C_{i} \tau_{i}, where C_{i} is the amplitude at time zero. ^{a}Intensity-weighted average lifetime, \tau_{int. avg} = \sum A_{i} \tau_{i}. ^{a}A biexponential decay function for deconvolution was used to calculate the amplitude of lifetime components and the average lifetime. Biexponential decay function I(t) = C_{1}e^{-t/\tau_{1}} + C_{2}e^{-t/\tau_{2}}. Deconvolution function I(t) = \int_{-\infty}^{t} IRF(\tau')(C_{1}e^{-\tau'/\tau_{1}} + C_{2}e^{-\tau'/\tau_{2}})d\tau'.

Figure 3. ETR1-dependent ethylene insensitivity modulates the CFL of PSII. (a,b) CFL-color-coded cellular images (a) and values (b) from leaf mesophyll protoplasts of WT, etr1-1, etr1-7, and arc3-2 were obtained using a commercial FLIM device. (c,d) CFL-color-coded cellular images (c) and values (d) of leaf mesophyll protoplasts of WT, etr1-1, etr1-7, and ein2-1 plants were obtained using an in-house fabricated FLIM device. All experiments were repeated three times with consistent results. The means of at least three measurements are shown with standard error bars.
To further verify our original observation that ethylene responsiveness modulates the chlorophyll fluorescence of PSII (Fig. 2), FLIM- and PAM fluorimetry-based analyses were conducted with mesophyll protoplasts isolated from the leaves of WT, etr1-1sg, etr1-7sg, and arc3-3 plants. As shown in etr1-1 (Fig. 3a,b), CFL was also longer in etr1-1sg than in WT, etr1-7sg, and arc3-3 (Fig. 4a). Consistently, the quantum yields of PSII were relatively lower in etr1-1sg, but higher in etr1-7sg, than those in ethylene sensitive WT (Fig. 4c). The photochemical efficiency of arc3-3 that is segregated from etr1-1 was similar to that of the WT (Fig. 4a-c), confirming that abnormal chloroplast structure does not affect PSII activity. As in ctrl-1 (Fig. 3e), the quantum yields of NPQ in etr1-1sg were relatively higher than those in WT (Fig. 4c). Both FLIM and PAM fluorimetry data reliably demonstrated that ethylene responsiveness, but not chloroplast structure, modulates the photochemical efficiency of PSII in the chloroplasts.

To substantiate that the lack of ethylene responsiveness leads to photochemical inefficiency of PSII, changes in CFL were measured from WT protoplasts concomitantly with etr1-1 expression. Protoplasts were co-transfected with a construct encoding a nuclear marker-GFP that harbored DOF1a, allowing for the selection of...
transfection-positive ones. CFL was relatively longer in WT protoplasts expressing etr1-1 than in those expressing the N-terminal end of split-YFP as a control (Fig. 4d), indicating that the reconstitution of lack of ethylene responsiveness by etr1-1 expression results in a longer CFL of PSII, i.e., low photochemical efficiency.

**Photochemical activity affects ethylene-inducible hypocotyl elongation under light through the cellular energy sensor SnRK1/AKIN10.** Since ethylene responsiveness controls PSII efficiency (Figs 2–4) and has a regulatory role in photosynthesis outputs15, 16, we reasoned that inefficient PSII activity caused by the lack of ethylene responsiveness might affect the cellular energy supply. To test this possibility, the cellular energy stress sensor AKIN10 expression was monitored in WT protoplasts transfected with an etr1-1 construct using a semi-quantitative RT-PCR. Notably, AKIN10 expression was clearly increased by the lack of ethylene responsiveness imposed by etr1-1 expression (Fig. 5a). More specifically mature AKIN10 mRNA expression increased (Fig. 5b), but its pre-mRNA that retains the first intron decreased (Fig. 5c) in our RT-qPCR analysis.

To further examine the notion of ethylene responsiveness-dependent AKIN10 expression, we monitored AKIN10 expression in genetically predisposed ethylene insensitive etr1-1sg together with ethylene-sensitive WT. Mature AKIN10 mRNA transcripts accumulated to a much higher level in etr1-1sg than in WT (Fig. 5d). Pre-mRNA transcripts of AKIN10, however, accumulated to a relatively lower level in etr1-1sg than in WT (Fig. 5e). These consistent but conditional results indicated that post-transcriptional regulation seemingly plays a role in AKIN10 induction under the lack of ethylene responsiveness in a condition sensitized to their differential photosynthetic capacities. The mature AKIN10 mRNA accumulated again more in both ethylene-insensitive ein2-1 and ein2-5 than in WT (Fig. 5f), supporting that ethylene responsiveness modulates AKIN10 expression, perhaps through photosynthetic activity regulation. In line with this, mature mRNA of AKIN10 accumulated highly in the presence of DCMU (Fig. 5a).

To genetically examine whether AKIN10 modulates ethylene-inducible hypocotyl growth under light, hypocotyl growth of transgenic plants expressed with AKIN10 (ref. 19) was observed in the presence of ACC under light together with WT, ethylene-insensitive etr1-1sg, and ethylene-constitutive responsive ctrl-1. The WT hypocotyl growth was again promoted in the presence of ACC/ethylene under light, but not in AKIN10-expressing transgenic plants much as for etr1-1sg (Fig. 5g,h). Consistently, the organ growth was increased in ctrl-1 in the absence and presence ACC under light14, thereby reflecting the constitutive ethylene responsive nature of the genetic background. Interestingly, both ACC-dependent and independent growth promotion of ctrl-1 was repressed by DCMU as in WT (Fig. 5g,h). Taken together, these results strongly support that lack of ethylene responsiveness affects PSII activity and modulates hypocotyl growth promotion by regulating AKIN10 expression under light conditions.

In this study we took advantage of a time-correlated and space-resolved fluorescence microscopic technique to characterize CFLs inversely reflecting PSII activity in vivo utilizing uniform and abundant mesophyll protoplasts isolated from mature Arabidopsis leaves. The CFL-based PSII activity in live single cells correlates well with chlorophyll fluorescence analysis using conventional PAM fluorimetry analysis at the tissue level. Here, we have described that Arabidopsis hypocotyl growth is under the regulation of ethylene signaling pathway (Fig. 5i). Ethylene responsiveness modulates PSII-dependent photosynthesis activity, controlling cellular energy sensor AKIN10 expression. Cellular AKIN10 activity could involve in negative modulation of EIN3-mediated ethylene responsive gene expression. In turn ethylene responsiveness could secure its signaling responses by assisting full activation of PSII efficiency. Further studies on the functional mechanisms of ethylene signaling underlying the regulation of photosystem II efficiency is needed to yield novel strategies for more efficient solar-energy harvesting in higher plants.

**Materials and Methods**

**Plant materials and genetic analysis.** Plants were grown in soil at 24°C for 20 to 22 days under 60 μmol/m²/s light intensity with a 12-h photoperiod. Arabidopsis Columbia-0 served as the wild-type (WT) and etr1-1, etr1-7, arc3-2 (SALK_057144), ein2-1, and ein2-5 mutant plants were used for experiments. The arc3-3 allele was segregated from the etr1-1 allele (etr1-1sg) or etr1-7 allele (etr1-7sg). The genetic backgrounds of the segregated mutants were confirmed using dCAPS markers and DNA sequencing. Homozygous lines were identified by PCR with dCAPS primers (S5′-TTGGTGGTGTATCGGCRGAT-3′ and S5′-TCCGCTTCTTCTCCTAGTCTC-3′ for etr1-1, digested using NdeI; 5′-TGGAGCAACTCATCTTAATTACG-3′ and S5′-TCCGTTTTCTCCTACGATCC-3′ for etr1-7, digested using HpaII; and 5′-ATGGTCCTCGAATATCGCTTAA-3′ and S5′-AAATACCTCTCTTCTAGACGCTC-3′ for arc3-3, digested using HpaII).

The ethylene response assay of etiolated seedlings was performed in complete darkness for 3 days with or without ACC. After 4 days of stratification, seeds were germinated on MS agar plates supplemented with 1% sucrose to ensure uniform germination.

For hypocotyl elongation assays, seedlings were grown on water-agar plates (without added salts or sugars) containing either no ACC or 50 μM ACC and/or 5 μM DCMU for 5 days under photon irradiance of 80 μmol·m⁻²·s⁻¹. Experiments were repeated at least three times with 50 seedlings each time.

**Transient expression of Arabidopsis mesophyll protoplasts.** Protoplast isolation and transient expression of genes of interest were carried out using previously described methods12. The etr1-1 construct was generated by inserting cDNA between the 35SCPPD promoter and the NOS terminator in a plant expression vector. The transfected protoplasts were incubated in WS solution for 15 h and the CFL was measured.
Figure 5. Lack of ethylene responsiveness induces AKIN10 expression, which inhibits ethylene-inducible hypocotyl growth promotion under light. (a) AKIN10 expression was monitored in mesophyll protoplasts transfected with and without etr1-1 construct using semi-quantitative RT-PCR. UBQ10 served as a control. Experiments were duplicated with consistent results. A representative result is shown. (b,c) Mature mRNA (b) and pre-mRNA (c) of AKIN10 expression was monitored in mesophyll protoplasts transfected with and without etr1-1 construct using RT-qPCR. (d,e) Mature mRNA (d) and pre-mRNA (e) of AKIN10 expression was monitored in WT and etr1-1sg using RT-qPCR. (f) Mature mRNA of AKIN10 expression was monitored in WT, ein2-1 and ein2-5 using RT-qPCR. (g,h) The light-controlled growth response of hypocotyls of WT, etr1-1sg, AKIN10-expressing transgenic seedlings, and ctrl-1 were observed (g) and measured (h) in the absence and presence of ACC with and without DCMU. Scale bar, 1 mm. n > 20. All experiments conducted in triplicate with consistent results; the means of triplicate measurements are shown with standard error bars. *** p < 0.001, ** p < 0.01, and * p < 0.05. (i) Working model of ethylene-inducible hypocotyl growth promotion under the fine control of ethylene responsiveness-dependent PSII activity in the light.
Photochemical efficiency measurement. MicroTime 200 (PicoQuant, Germany) was used for fluorescence lifetime imaging microscopy (FLIM) analysis. A diode-pulsed laser (470 nm, 40 MHz, 480 mW, LDH-F-2C470-PDL 800-B; PicoQuant) was used to excite the chlorophyll, and emissions from 665 to 685 nm were collected in a 40,000-pixel mode. Images with Chi-squared values of 0.70 to 1.30 were used in the analysis. Protoplasts were placed on an inverted microscope without any actinic light. The lifetime data of living protoplasts were collected according to the manufacturer’s recommendations. The collection time was approximately 1 min for each protoplast image. FLIM data were analyzed and fitted using the Symphotime Program provided by PicoQuant. The photochemical efficiency of PSII was measured using a portable plant efficiency analyzer (Hansatech Instruments, King’s Lynn, Norfolk, UK).

PAM fluorimetry analysis. The quantum yield of PSII photochemistry, nonphotochemical quenching, and intrinsic decay were estimated using equations developed previously. Chlorophyll fluorescence from bulk leaves was measured by FMS2 fluorimetry (Hansatech instruments, Norfolk, UK). The saturating pulse and actinic light intensity were fixed at 5000 µE and 500 µE, respectively.

RNA isolation and transcript accumulation analysis. ACC and/or DCMU-mediated gene regulation was investigated using monitoring marker gene expression in WT and etr1-1gr mutants. For gene expression analysis, total RNA was isolated using RNAiso Plus (Takara Bio, Otsu, Japan) from the shoots of 5-day-old seedlings grown on water-agar plates in the presence and absence of ACC and/or DCMU, and 1 µg of total RNA was used for cDNA synthesis using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). Gene expression was measured using real-time PCR (CFX Connect™ Real-Time System with C1000 Thermal Cycler, Bio-Rad), with seedlings grown on water-agar plates serving as controls. RT-qPCR was carried out using an iQ SYBR Green dye-supplemented PCR mix (Bio-Rad). Either Tubulin4 (TUB4, At1g04820) or Elongation initiation factor4a (EIF4a, At1g13920) transcripts were used as real-time PCR controls with gene-specific primers. Primer sequences are presented in Supplemental Table S1. The production of a single gene product by each primer set in the PCR reactions was validated before each experiment. Experiments were repeated three times (using more than 20 seedlings each time), with consistent results.

Protein extraction and protein blot analysis. Total proteins were extracted from each plant using an extraction buffer containing 150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 5 mM EDTA, 1 mM DTT, 1% (v/v) Triton X-100, and complete protease inhibitor. Protein blot analysis was performed using 40 µg of total protein and protein amounts were quantified using the Bradford method (Bio-Rad). Anti-RbCl-rabbit or anti-PsbA-rabbit antibody (Agrisera) was used for detection.

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Author Contributions

S.Y. initiated and designed experiments; G.K. and Y.C. performed experiments and analyzed data; G.K. and Y.C. were involved in manuscript preparation. S.Y. and Y.C. wrote the manuscript.

Additional Information

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