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The low-oxygen response is triggered by an ATP-dependent shift in oleoyl-CoA in *Arabidopsis*

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Plant response to environmental stimuli involves integration of multiple signals. Upon low-oxygen stress, plants initiate a set of adaptive responses to circumvent an energy crisis. Here, we reveal how these stress responses are induced by combining (a) energy dependent changes in the composition of the acyl-CoA pool and (b) the cellular oxygen concentration. A hypoxia-induced decline of cellular ATP levels reduces LONG-CHAIN ACYL-COA SYNTHETASE (LACS) activity, which leads to a shift in the composition of the acyl-CoA pool. Subsequently, we show that different acyl-CoAs induce unique molecular responses. Altogether, our data disclose a role for acyl-CoAs acting in a cellular signaling pathway in plants. Upon hypoxia, high oleoyl-CoA levels provide the initial trigger to release the transcription factor RAP2.12 from its interaction partner ACYL-COA BINDING PROTEIN (ACBP) at the plasma membrane. Subsequently, according to the N-end rule for proteasomal degradation, oxygen-concentration dependent stabilization of the subgroup VII ETHYLENE-RESPONSE FACTOR (ERFVII) transcription factor RAP2.12 determines the level of hypoxia-specific gene expression. This research unveils a specific mechanism activating low-oxygen stress responses only when a decrease in the oxygen concentration coincides with a drop in energy.

low oxygen stress | integrative signalling | acyl-CoA | ERFVII | ACBP

Flooding contributes almost 60% to the worldwide cost and damage to crops provoked by natural disasters [1]. Due to heavy precipitation and concomitant waterlogging or flooding events in large areas of the world, climate change will cause plants to be even more frequently exposed to oxygen limiting conditions (hypoxia) in the near future [2].

In plants, subgroup VII ETHYLENE-RESPONSE FACTOR (ERFVII) transcription factors act as key regulators of hypoxic gene expression [3,4,5,6]. During non-stress conditions, the ERFVII protein RELATED TO APETALA 2.12 (RAP2.12) is sequestered to the plasma membrane via direct interaction with ACYL-CoA BINDING PROTEIN (ACBP) [3,7,8,9]. Upon hypoxia, RAP2.12 is released from the plasma membrane and subsequently accumulates in the nucleus [3,7,9]. Further, the stability of ERFVII proteins is tightly controlled in an oxygen-dependent manner employing the Cys-branch of the N-end rule [3,4]. That is, ERFVII protein degradation is prevented under hypoxic conditions when N-end rule-assisted degradation is impaired due to oxygen limitation [10]. Although the homeostatic regulation of adaptive responses to low oxygen stress in plants is well-investigated [3,4,11], the identity of the initial trigger to release the ERFVII transcription factors from its membrane docking protein ACBP remains unknown and the existence of multiple signal queues that are integrated into low-oxygen specific responses is likely [12].

ACBPs represent an evolutionary conserved protein family found in *Escherichia coli*, yeast, animals and plants [13,14] and participate in the regulation of unbound acyl-CoA levels by sequestration and transportation of acyl-CoAs [15,16]. The interaction between members of a protein family capable of reversibly binding acyl-CoAs with the ERFVII proteins RAP2.12 [3,7] and RAP2.3 [8,9] provided a first indication that acyl-CoAs can be involved in the release of ERFVII transcription factor protein during hypoxia. We could elaborate this mode of action with experiments on RAP2.12 as a representative member of ERFVII transcription factors.

Acyl-CoAs are intermediates in both lipid catabolism and anabolism. In the catabolic pathway, fatty acids are activated in the cytosol by ACYL-CoA SYNTHETASES prior to their transport into the mitochondria or peroxisomes where β-oxidation occurs. In plants, lipid anabolism occurs through two pathways: *de novo* fatty acid synthesis takes place in plastids and the generated fatty acids can be incorporated into complex lipids within the plastid by the so-called prokaryotic pathway. Alternatively, the fatty acid may be exported from the plastid to the cytosol to become substrate for the eukaryotic lipid biosynthesis pathway in the endoplasmic reticulum (ER). Transport of fatty acids from the plastid, through the cytosol into the ER is mainly mediated via palmitoyl-CoA (C16:0-CoA) and oleoyl-CoA (C18:1-CoA) that are produced from palmitic and oleic acid by the enzyme LONG-CHAIN ACYL-CoA SYNTHETASES (LACS) at the outer plastid membrane in root and shoot tissues [17,18,19,20]. In addition to their involvement in lipid metabolism, acyl-CoAs are also known to modulate the activity of numerous enzymes, ion-channels and transcription factors in animals and microorganisms [15]. Examples of acyl-CoAs directly affecting transcription factor activity by functioning as ligands have been reported for humans (HNF-4α [21]) and *E. coli* (FadR [22]). For plants, direct involvement of acyl-CoAs controlling transcription factor activity was not

Significance

To control adaptive responses to the ever-changing environment that plants are continuously exposed to, plant cells must integrate multiple information to make optimal decisions. Here, we reveal how plants can link information about the cellular energy status with the actual oxygen concentration of the cell in order to trigger a response reaction to low-oxygen stress. We reveal that oleoyl-CoA has a moonlighting function in an energy (ATP) dependent signal transduction pathway in plants, and we provide a model that explains how diminishing oxygen availability can initiate adaptive responses when it coincides with a decreased energy status of the cell.

Reserved for Publication Footnotes

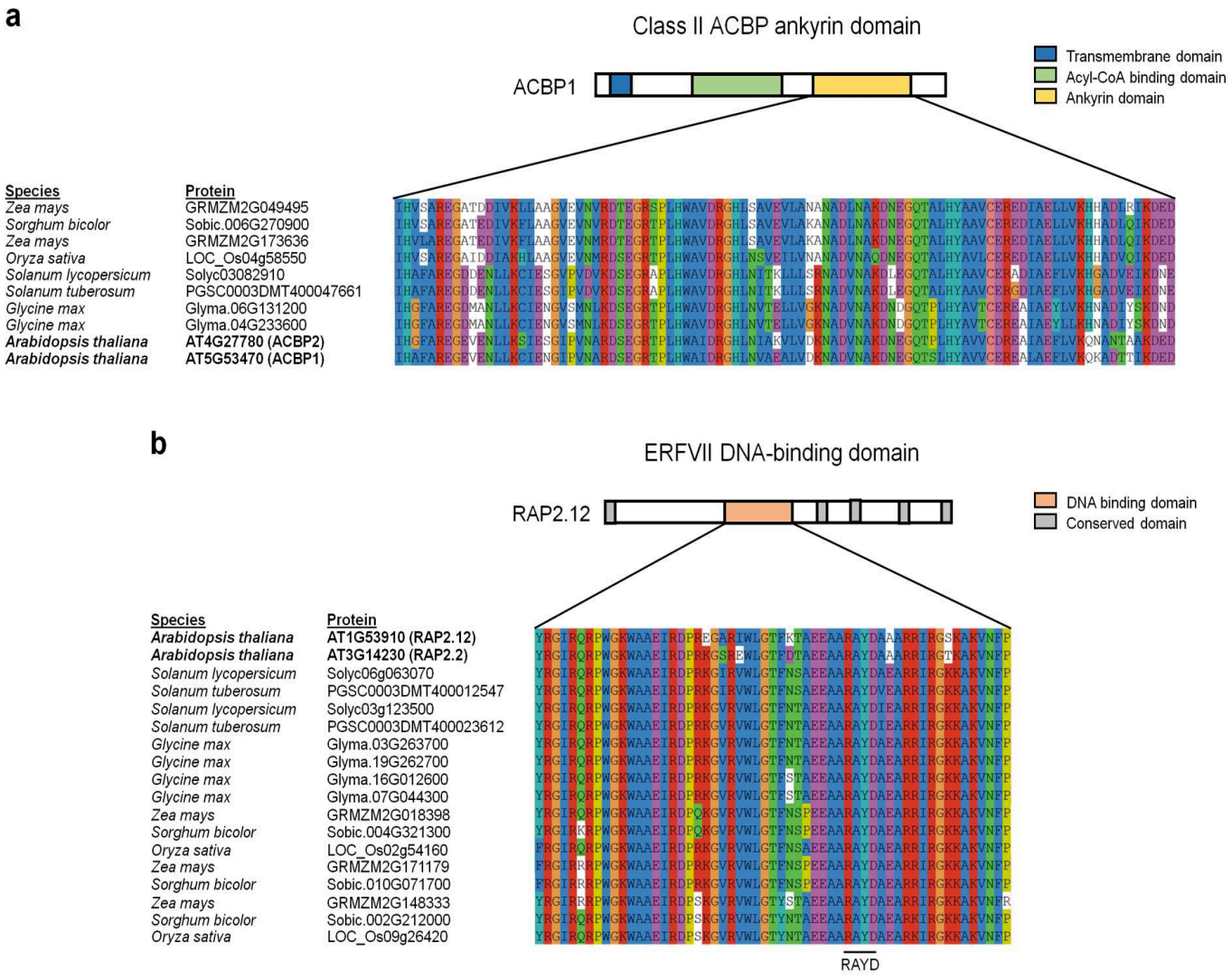

Fig. 1. ACBP is an important element of the low-oxygen stress response pathway in plants a, Multiple sequence alignment of Class II ACBP ankyrin domain as compared to ACBP1 and ACBP2 ankyrin domains in *Arabidopsis thaliana* [27]. **b**, Multiple sequence alignment of ERFVII DNA-binding domain as compared to RAP2.12 and RAP2.2 in *Arabidopsis thaliana* [26]. Sequences of homologous proteins were obtained from Phytozome v12.0 and aligned with Clustal X. **c**, Eleven-day-old seedlings of wildtype and *acbp1* after 9 h of anoxia and 3 d recovery. Scale bar, 1.0 cm. **d**, Survival scores for wildtype and *acbp1* after 9 h anoxia and 3 d recovery. Data are mean values ± s.d.; * $p < 0.05$, n = 4 (15 seedlings per replicate). **e**, Effect of 2 hours of hypoxia treatment (1% O_2) on the localization of GFP-tagged ACBP1 in epidermal cells. Representative pictures are shown. Bar = 10 µm.

demonstrated yet, although an indirect regulatory role has been suggested [23].

Hypoxia has detrimental effects on the plant's cellular homeostasis, in the first place because oxidative phosphorylation in mi-

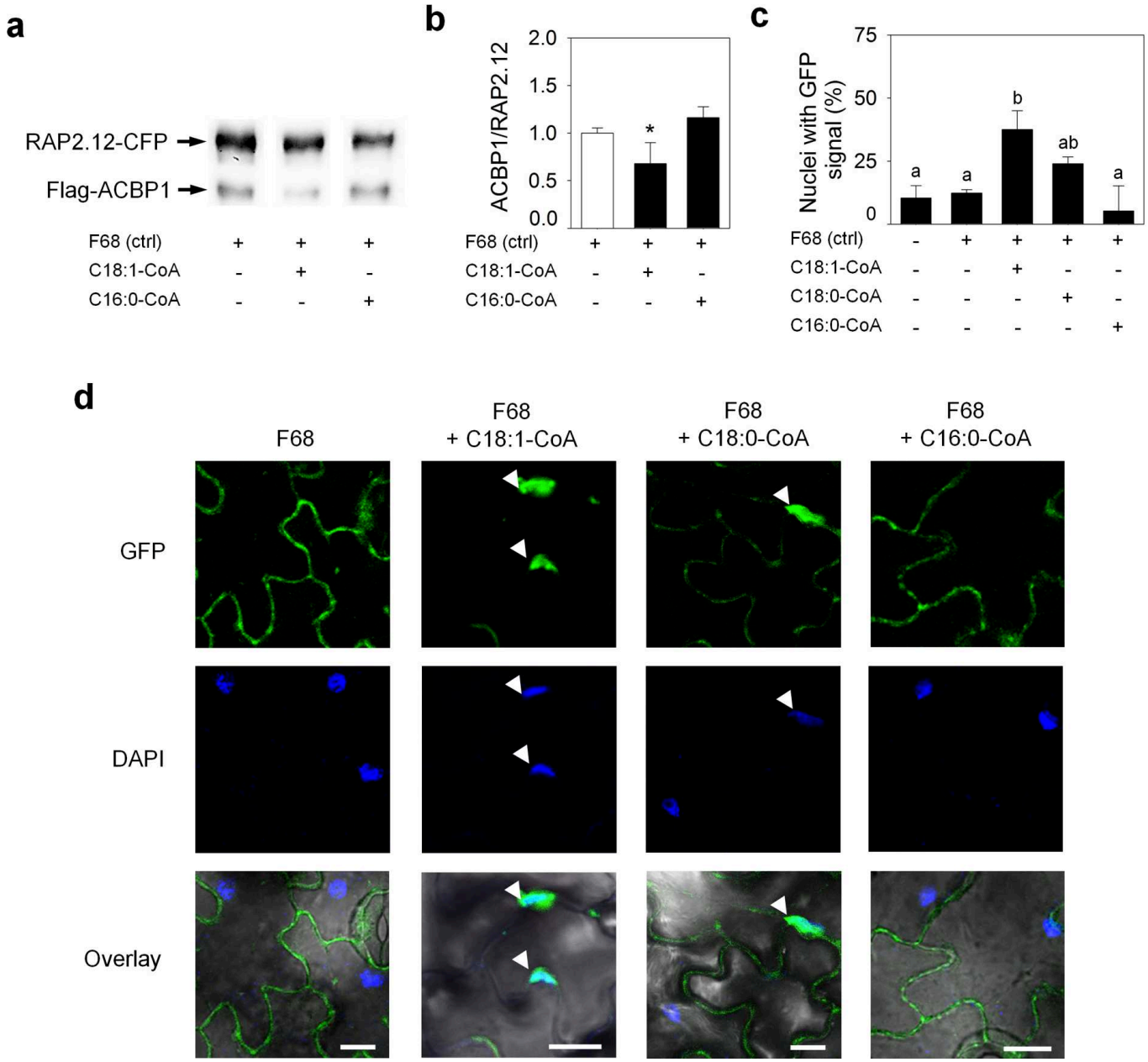

Fig. 2. Application of C18:1-CoA induces RAP2.12 relocalization into the nucleus. a, Representative Western-blot showing *in vitro* ACBP1-RAP2.12 complex stability after treatment with C18:1-CoA or C16:0-CoA. Pluronic F68 treatment served as control. **b**, Quantification of ACBP1-to-RAP2.12-ratio as shown in panel a. Data are mean values ± s.d. *p < 0.05, n = 8. **c**, Percentage of epidermal cells with nuclear localization of RAP2.12-GFP after treatment with different acyl-CoAs. Data are mean values ± s.d.; *p < 0.05. **d**, Localisation of RAP2.12-GFP in detached leaves incubated with 0.1 % C18:1-CoA, C18:0-CoA or C16:0-CoA dissolved in 0.01 % Pluronic F68 under normoxic conditions for 3 hours. Treatment with Pluronic F68 only served as negative control. DAPI staining was used to identify nuclei. Arrows indicate nuclei with GFP signal. Bar = 10 µm.

tochondria is reduced which ultimately leads to a decrease of the cellular energy charge. This results in ATP-consuming metabolic processes being attenuated, including fatty acid synthesis and processing [24]. Consequently, the export of newly synthesized fatty acids from the plastid into the cytosol is affected, since the activation to acyl-CoAs is ATP dependent [18, 25]. Therefore, an energy-related impact of hypoxia on acyl-CoA levels in the cytosol is expected and investigated here.

In this study, we reveal a combinatory signaling network by which the reduced energy level under low-oxygen stress is integrated into the ERFVII-dependent hypoxic signaling cascade. We show that dynamic responses of C18:1-CoA and C16:0-CoA levels to hypoxia constitute an early molecular trigger leading to dissociation of the ACBP:RAP2.12 complex thereby activating the molecular low-oxygen response cascade. We describe an integrative signaling mechanism in which adaptive gene expression upon low-oxygen stress results from the specific combination of (1) low-energy triggered release of the transcription factor RAP2.12 from ACBP as mediated by an acyl-CoA signal; and (2) low-oxygen dependent stabilization of the RAP2.12 protein according to the N-end rule of protein degradation.

Results

C18:1-CoA promotes dissociation of the ACBP:RAP2.12 complex *in vitro* and *in vivo*

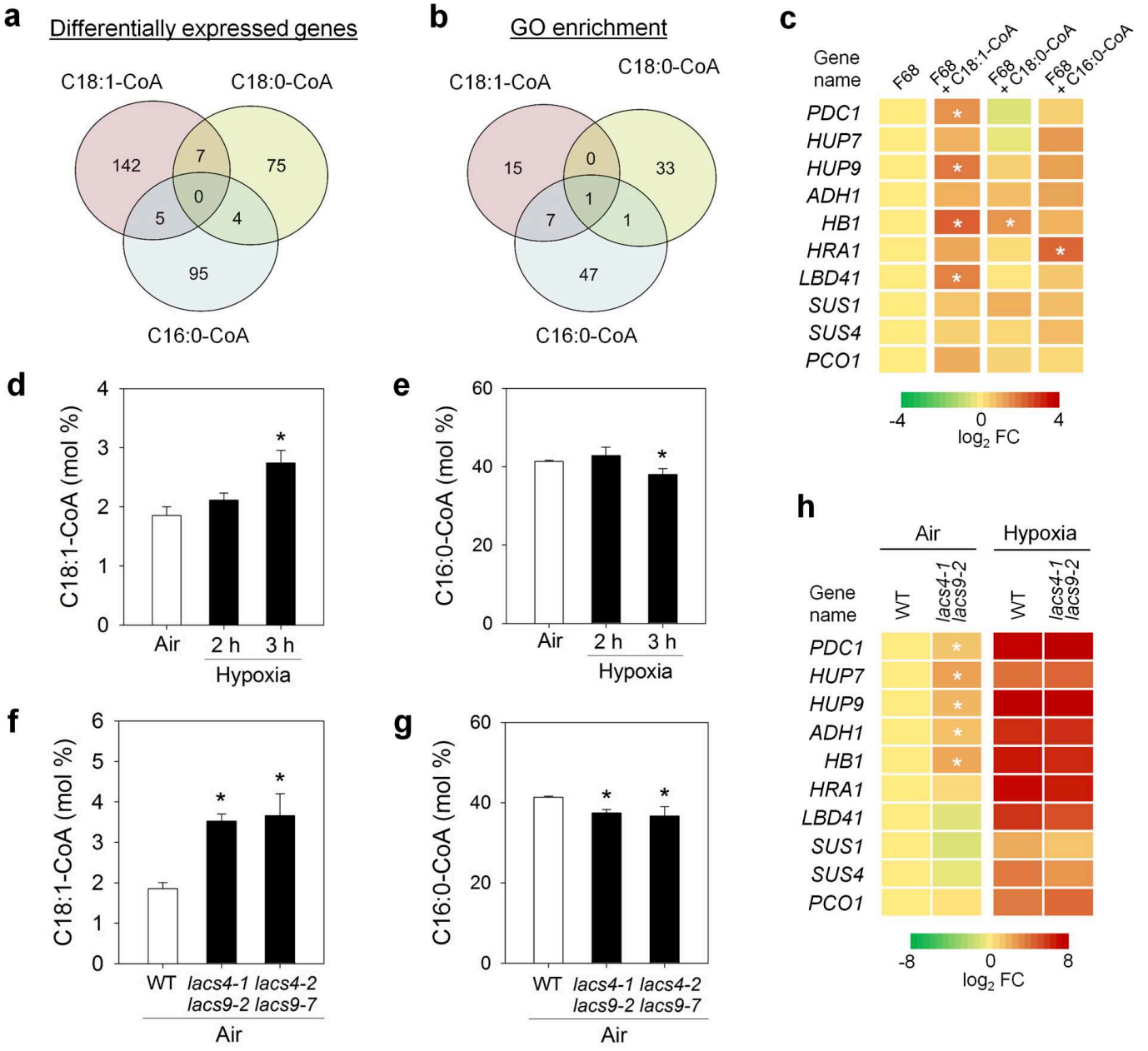

Fig. 3. Changing oleoyl-CoA levels induces low-oxygen responsive gene expression. **a**, Number of significantly differentially expressed genes in leaves after 1.5 hours treatment with different acyl-CoAs as determined by RNA-Seq (FDR-adjusted p-value < 0.05). **b**, Number of GO classes in which differentially expressed genes are significantly over-represented under acyl-CoA treatments as shown in a. **c**, qPCR analysis of differential expression of hypoxia-responsive genes after acyl-CoA treatment in air (reference: F68 only). Data are presented as mean values * $p < 0.05$, n = 5. **d**, C18:1-CoA levels increase upon hypoxia in wildtype. Data are mean values ± s.d.; * $p < 0.05$, n = 3. **e**, C16:0-CoA levels decrease upon hypoxia in wildtype. Data are mean values ± s.d.; * $p < 0.05$, n = 3. **f**, C18:1-CoA levels are increased in *lacs4lacs9* double mutants grown in air. Data are mean values ± s.d.; * $p < 0.05$, n = 3. **g**, C16:0-CoA levels are lowered in *lacs4lacs9* double mutants. Data are mean values ± s.d.; * $p < 0.05$, n = 3. **h**, Expression data for hypoxia-responsive genes comparing wildtype and *lacs4-1 lacs9-2* in air or hypoxia (2 h 1% O_2; mean values, * $p < 0.05$, n = 4).

In Arabidopsis, the ERFVII transcription factor RAP2.12 is constitutively expressed but sequestered at the plasma membrane by binding to ACBP1 during normoxic conditions, while it accumulates in the nucleus under oxygen concentrations below 10% (v/v) [3,7]. The interaction domains of ACBP1 and RAP2.12 were previously identified in Arabidopsis [3,26,27] and appear to be well conserved among plant species (**Fig. 1a-b**), indicating that complex formation of both proteins is a general feature in plants. Importantly, changing the expression of *ACBPs* affects tolerance to low oxygen [28] (**Fig. 1c-d**) similar to what was shown previously for *RAP2.12* [3]. During hypoxia GFP-tagged ACBP1 remains at the plasma membrane (**Fig. 1e**), while GFP-tagged RAP2.12 was shown to accumulate in the nucleus upon hypoxia [3,7]. This indicates that RAP2.12 dissociates from ACBP1 prior to its relocation to the nucleus. Consequently, the release of RAP2.12 from ACBP1 is considered as a trigger that activates adaptive gene expression in response to hypoxia.

To investigate if acyl-CoAs interfere with the interaction between ACBP1 and RAP2.12, we performed an *in vitro* affinity assay (**SI Appendix, Fig. S1**) in the presence of either oleoyl-CoA (C18:1-CoA), which is a preferred substrate for ACBP1, or palmitoyl-CoA (C16:0-CoA) that is not a strongly interacting

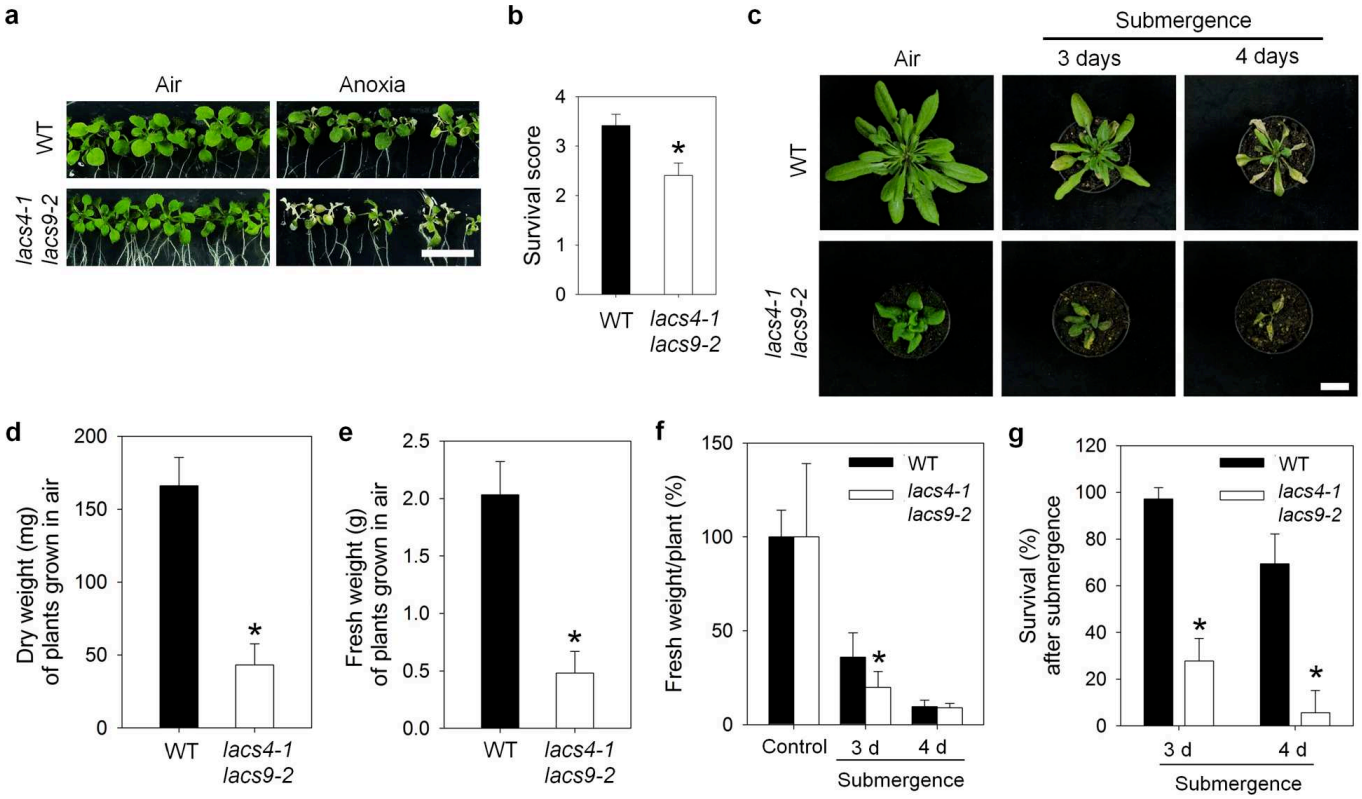

Fig. 4. Decreased tolerance of *lacs4 lacs9* knock-out lines to anoxia and submergence. **a**, Eleven-day old seedlings of wildtype and *lacs4-1 lacs9-2* after 9 h of anoxia and 3 d recovery. Scale bar, 1.0 cm. **b**, Survival scores for wildtype and *lacs4-1 lacs9-2* after 9 h anoxia and 3 d recovery. Data are mean values ± s.d.; * $p < 0.05$, n = 4 (15 seedlings per replicate). **c**, Phenotype of wildtype and *lacs4 lacs9* mutant grown in air (control), or after 3 or 4 days submergence-induced hypoxic treatment. Scale bar = 2 cm. Photographs were taken 4 days after the submergence treatment. **d**, Absolute dry weight of wild-type and *lacs4-1 lacs9-2* plants grown in air. Data represent mean ± s.d. (3 replicate experiments with each 12 plants per genotype). Asterisk indicates significant differences after one-way ANOVA ($P < 0.05$). **e**, Absolute fresh weight of wildtype and *lacs4-1 lacs9-2* plants grown in air. Data represent mean ± s.d. (3 replicate experiments with each 12 plants per genotype). Asterisk indicates significant differences after one-way ANOVA ($P < 0.05$). **f**, Relative fresh weight of wildtype and *lacs4-1 lacs9-2* plants grown in air, or after 3 or 4 days of submergence followed by 4 days of recovery. Data represent mean ± s.d. (3 replicate experiments with each 12 plants per genotype). Asterisk indicates significant differences after one-way ANOVA ($P < 0.05$). **g**, Percentage of plants that survived the 3 or 4 days of flooding-induced hypoxia, respectively (mean values ± s.d., 3 replicate experiments with each 12 plants per genotype). * $P < 0.05$ according to one-way ANOVA.

agent [29, 30]. *In vitro* exposure of an ACBP1-RAP2.12 protein complex to C18:1-CoA, but not C16:0-CoA, significantly decreased the binding affinity between the two proteins as indicated by the reduced ratio of Flag-tagged ACBP1 and CFP-tagged RAP2.12 protein (**Fig. 2a-b**). Apparently, interaction between ACBP1 and C18:1-CoA reduces the binding capacity of ACBP1 for the transcription factor RAP2.12. To confirm that C18:1-CoA-induced dissociation of RAP2.12 from ACBP1 also occurs *in vivo*, we exposed detached leaves of plants expressing *35S:RAP2.12-GFP* to various acyl-CoAs under aerobic conditions. Application of C18:1-CoA, but not C18:0-CoA or C16:0-CoA, significantly induced nuclear accumulation of RAP2.12-GFP, indicating that RAP2.12 was released from ACBP1 *in vivo* after the application of C18:1-CoA, but not by C16:0-CoA (**Fig. 2c-d**). Translocation of RAP2.12 to the nucleus was previously described to occur during hypoxic conditions [3, 7]. Therefore, we tested if application of acyl-CoA to leaves might have induced hypoxia due to increased beta-oxidation or mitochondrial respiration. However, no increase of the oxygen consumption rate by leaf tissue after treatment with acyl-CoAs was observed, indicating that our experimental treatment did not affect the oxygen concentration of the tissue in this experiment (**SI Appendix Fig. S2**). We concluded, remobilization of the transcription factor RAP2.12 from the plasma membrane into the nucleus can be triggered *in vivo* by increasing the level of C18:1-CoA.

Acyl-CoAs provoke distinct transcript responses

To determine if specific transcriptional responses are provoked by the application of different acyl-CoAs, RNA-Seq transcriptome analysis was performed on wild-type seedlings exposed to either 1 mM C18:1-CoA, C18:0-CoA or C16:0-CoA. Uptake of these externally applied acyl-CoAs is mediated by ABCD-transporters that first cleave the CoA-group from the acyl-chain, allowing the resulting fatty acid to cross lipid membranes. Once inside the cell, CoA is immediately reattached, which traps the acyl-CoA in the cellular compartment in which it has been imported [31]. This analysis revealed that each of these acyl-CoAs modulates distinct sets of genes (**Fig. 3a**, **SI Appendix, Table S1**). The high specificity of induced changes in gene expression underlines the eligibility of acyl-CoAs as signaling molecules in plants. To investigate the biological function of the differentially regulated genes, a Gene Ontology enrichment analysis [32] was carried out. While application of C18:0-CoA or C16:0-CoA mainly affected the expression of genes related to reproductive development and hormone signaling, C18:1-CoA mainly modulated the expression of genes associated with hypoxia and low oxygen responses (**Fig. 3b**, **SI Appendix, Table S2**). This result was confirmed by qPCR-assisted expression profiling executed on wild-type plants incubated with C18:1-CoA. Indeed, RAP2.12-regulated hypoxia-response genes were induced by C18:1-CoA treatment, while C18:0-CoA and C16:0-CoA had only minor effects on the expression of these genes (**Fig. 3c**, **SI Appendix Table S1**). This observation is readily explained by our earlier

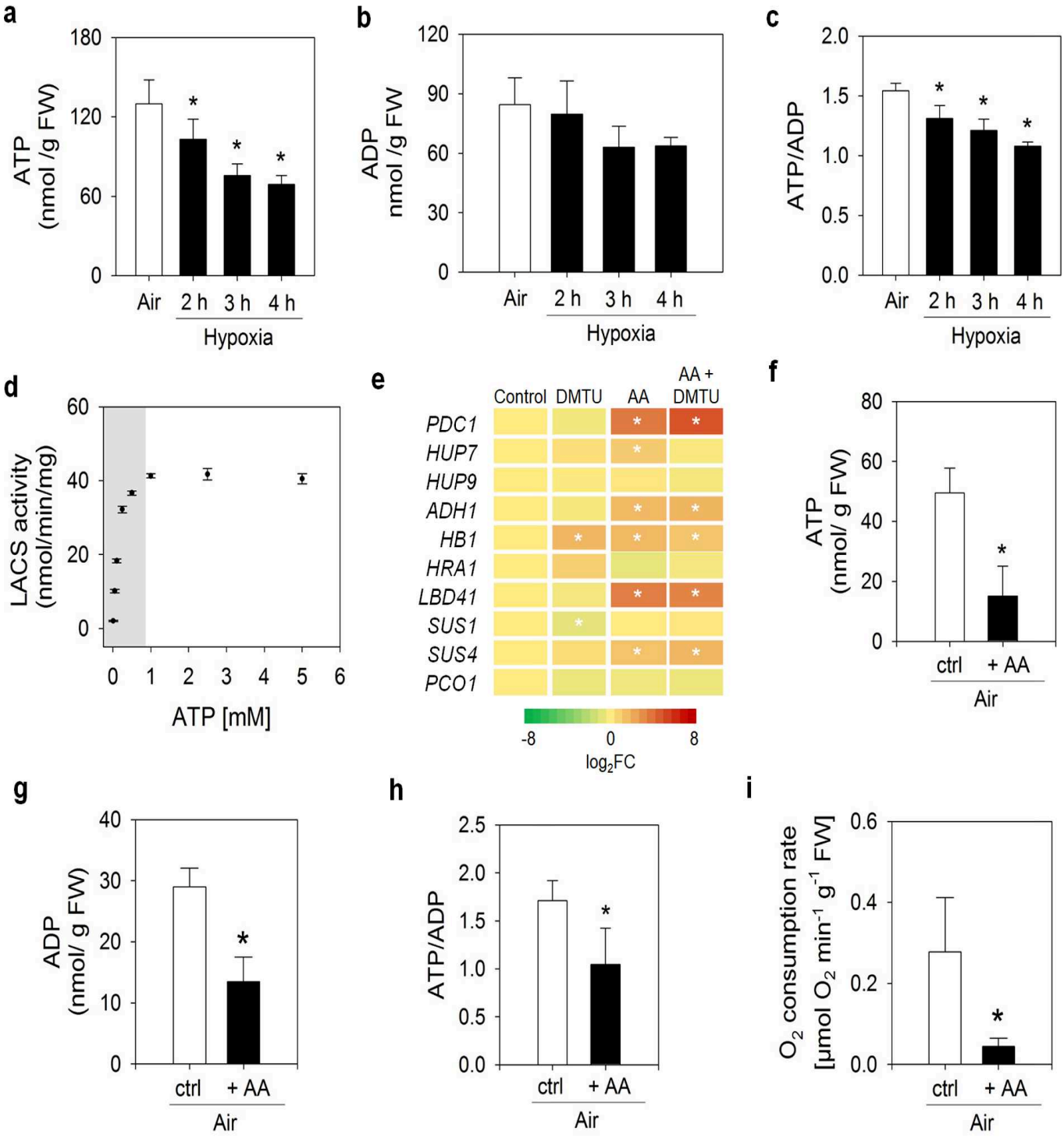

Fig. 5. Decreasing the cellular ATP level constitutes limiting conditions for LACS activity and induces the expression of low-oxygen responsive genes. a, ATP levels under hypoxia (mean ± s.d, * $p < 0.05$, n = 5). **b**, Concentration of ADP in wild-type seedlings grown under long-day conditions and exposed to hypoxia. Data shown are given in nmol per gram fresh weight and represent the mean ± s.d. of independent replicates (n = 5). **c**, ATP-to-ADP-ratio under hypoxia (mean ± s.d, * $p < 0.05$, n = 5). **d**, *In vitro* LACS activity depends on ATP concentration (mean ± s.d, * $p < 0.05$, n = 5). The grey area marks the ATP-concentration range usually determined in plant cells. **e**, Differential expression of hypoxia-responsive genes after 3 hours of 1 mM dimethylthiourea (DMTU) and/or 50 µM antimycin-A (AA) treatment under aerobic conditions (reference: mock-treated control). Data are presented as mean ± s.d.* $p < 0.05$, n = 5. **f**, ATP levels after 3 hours of 50 µM antimycin-A treatment (AA) (mean ± s.d, * $p < 0.05$, n = 5). **g**, Concentration of ADP in wild-type seedlings exposed to 3 hours of 50 µM antimycin-A (AA) treatment. Data represent mean ± s.d. (n = 5). Asterisk indicates significant differences after one-way ANOVA ($p < 0.05$). **h**, ATP–to-ADP-ratio after 3 hours of 50 µM antimycin-A (AA) treatment (mean ± s.d, * $p < 0.05$, n = 5).. **i**, Oxygen consumption rate in wild-type leaves upon 3 hours of 50 µM antimycin-A treatment. Data represent mean ± s.d. (n = 7). Asterisk indicates significant difference after Student's t-test ($p < 0.05$).

observation that RAP2.12 relocalizes to the nucleus upon C18:1-CoA application (**Fig. 2c-d**). Therefore, it is concluded that C18:1-CoA provides a specific cellular signal that is substantially

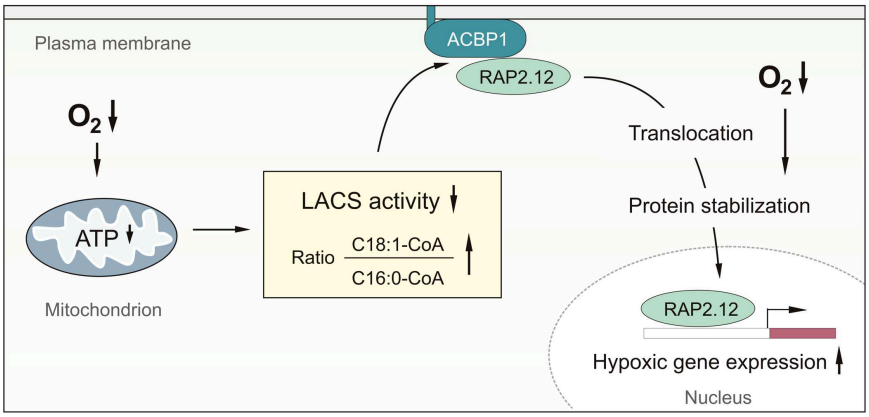

Fig. 6. Triggering low-oxygen responses in plants integrates the cellular energy and oxygen status via modulation of oleoyl-CoA levels. Oxygen limitation reduces cellular ATP levels, which results in increased C18:1-CoA levels. Dissociation of ERFVII protein (as shown here for RAP2.12) bound to ACBP1 at the plasma membrane is promoted by C18:1-CoA. Free ERFVII protein is stable under low-oxygen conditions and relocalizes into the nucleus to activate hypoxic responses.

involved in the control of gene expression by releasing RAP2.12 from ACBP1.

Increase of C18:1-CoA to C16:0-CoA ratio induces hypoxic gene expression *in vivo*

In a physiological context, C18:1-CoA-mediated dissociation of RAP2.12 from ACBP1 only makes sense when the endogenous acyl-CoA pool responds to hypoxia. Indeed, HPLC-assisted quantification of acyl-CoAs revealed a shift to elevated C18:1-CoA (**Fig. 3d**) and C20:0-CoA (**SI Appendix, Fig. S3**) levels with less C16:0-CoA (**Fig. 3e**) after 3 hours of hypoxia, while no significant changes of the total pool of acyl-CoAs included in our analyses were observed (**SI Appendix, Fig. S3**). These dynamic responses of specific acyl-CoAs to changing environmental conditions as exemplified here for hypoxia are in line with the suggestion that acyl-CoAs in plants can play a role in stress signaling.

A similar shift of the acyl-CoA pool as observed during low-oxygen conditions was observed in transgenic plants in which two LACS genes, *LACS4* and *-9*, were knocked out[33] (**Fig. 3f-g**, **SI Appendix, Fig. S4**). Under aerobic conditions, *lacs4lacs9* double knock-out plants have elevated C18:1-CoA and reduced C16:0-CoA levels. To provide further proof that endogenous changes of the C18:1-CoA or C16:0-CoA level can have an effect on low-oxygen responses of plants, we tested the induction of hypoxic gene expression in *lacs4lacs9* plants in air. Indeed, many RAP2.12-dependent hypoxia-response genes were activated under aerobic conditions in *lacs4lacs9* (**Fig. 3h**, **SI Appendix, Table S2**). In addition, also tolerance to hypoxia was altered. Performance of *lacs4lacs9* was impaired under both anoxia and submergence conditions as compared to wild-type plants (**Fig. 4**). Similar observations were described for plants with constitutive activation of the hypoxia stress response pathway [34], although it should be mentioned that the impact of constitutive activation of the ERFVII signaling pathway on plant performance under stress conditions also appears to depend on the experimental growth conditions and the recovery treatment [4,35]. Altogether, our data give rise to a model describing that the control of endogenous acyl-CoA levels through LACS activity is a prerequisite to properly induce hypoxia tolerance responses in plants.

LACS activity is reduced by depletion of ATP

To explain the mechanism by which LACS activity in plants is affected by hypoxic conditions, we analyzed LACS activity in dependence of its substrate adenosine-tri-phosphate (ATP). First, we monitored the cellular energy status in wild-type plants grown under hypoxia. Already after 2 hours, the ATP level as well as the ATP-to-ADP ratio were significantly reduced as compared to the air-treated control (**Fig. 5a-c**). To examine at which ATP concentration LACS activity becomes substrate limited, an *in vitro* LACS activity assay was performed. This experiment showed that the activity of LACS4 was ATP dependent at ATP concentrations below 0.8 mM (**Fig. 5d**), which is close to the estimated cytosolic ATP concentration under aerobic conditions *in planta* [36,37,38]. Since ATP concentrations in plant cells decrease during hypoxia [24], LACS activity is expected to diminish concomitantly. However, it should be mentioned that variations of the ATP concentration between tissues or even within a cell (between organelles) might lead to different local ATP-limiting conditions for LACS during hypoxic stress. Moreover, hypoxia-induced low-energy status will also influence other processes that are not covered by the analyses that we describe here.

To verify that a drop in ATP can trigger hypoxia responses of plants in air, we chemically inhibited mitochondrial respiration using antimycin-A. Indeed, after three hours of 50 µM antimycin-A treatment of rosettes in air, several hypoxia responsive genes were significantly induced (**Fig. 5e**, **SI Appendix Table S3**), while ATP levels and the ATP-to-ADP ratio were reduced (**Fig. 5f-h**). Since application of antimycin-A strongly reduced the oxygen consumption rate of the tissue (**Fig. 5i**), it is unlikely that the induction of hypoxia responsive genes is the consequence of low-oxygen concentrations here. Also an antimycin-A induced ROS burst appeared unlikely to be responsible for the gene induction, since the induced genes responded similarly when in addition to antimycin-A also 1 mM of the hydrogen peroxide scavenger dimethylthiourea (DMTU) [39,39] was supplied (**Fig. 5e**). Although it cannot be concluded that reduced ATP levels are exclusively responsible for triggering hypoxia responses in plants without performing dose response analyses of individual and combined compounds, the data provide evidence that the cellular energy status is involved in the regulation of hypoxic gene expression.

Discussion

In this study, we demonstrate that acyl-CoAs provoke distinct transcriptional responses in plants, suggesting that they are involved in different signaling pathways. Specifically, binding of C18:1-CoA to ACBP triggers dissociation of the ACBP:RAP2.12 complex upon an hypoxia-induced energy crisis, resulting in mobilization of the transcription factor RAP2.12 into the nucleus. Consequently, RAP2.12-mediated gene expression is induced. Therewith we reveal the trigger of the ERFVII-mediated signaling cascade to activate cellular hypoxia-tolerance responses in plants (**Fig. 6**).

When the oxygen availability to cells diminishes, mitochondria produce less ATP due to a reduced activity of oxidative phosphorylation [41]. Indeed, our plants showed a rapid decrease of their energy status upon hypoxia. Already within two hours, the ATP-to-ADP ratio dropped significantly and remained decreasing throughout the rest of the experiment (**Fig. 5c**). Consequently, the activity of ATP-mediated reactions within the cell is expected to

reduce too [24]. Here, we show that ATP concentration-dependent LACS activity reaches its maximum at an ATP concentration of 1 mM (**Fig. 5d**) which resembles the concentration in a non-stressed plant cell [36, 37, 38]. This means that any decrease of the cellular energy status is translated into a reduction of LACS activity. The enzyme LACS is among others located in the plastidial outer envelope where it is provided with C16:0 and C18:1 fatty acids from the stroma by thioesterases that are located in the inner envelope [42, 43]. Using CoA and ATP as co-substrates, LACS activates the fatty acids and releases them as C16:0-CoA and C18:1-CoA in the cytosol [18]. When the level of ATP drops and LACS activity decreases, the export rate of fatty acids will be reduced. As the elongation and rapid desaturation reactions in the plastidial stroma from C16:0 to C18:1 commence, the ratio C18:1 compared to C16:0 that is provided to LACS from the stroma is likely to increase through time under these conditions. As a consequence, the ratio of C18:1-CoA to C16:0-CoA that is released by LACS into the cytosol will increase and would readily explain why we observe an increased level of C18:1-CoA as compared to C16:0-CoA in plants that were exposed to low oxygen (**Fig. 3d,e**) as well as in *lacs4lacs9* double knockout lines in air (**Fig. 3f,g**).

Acyl-CoA fatty acid esters bind to ACBP proteins. Here, we show that specifically the interaction between C18:1-CoA with ACBP1 results in release of RAP2.12 from the ACBP1:RAP2.12 complex while C16:0-CoA does not affect the interaction between RAP2.12 and ACBP1 (**Fig. 2**). A shift of the ratio between C18:1-CoA and C16:0-CoA as described above, will therefore lead to the release of RAP2.12 from ACBP. Indeed, application of C18:1-CoA, but not C16:0-CoA, increased the number of nuclei in which GFP-tagged RAP2.12 accumulated (**Fig. 2c, d**). Consequently, also upregulation of hypoxia responsive marker genes was observed (**Fig. 3a-c**).

Similar to this external application of acyl-CoAs, also an endogenous shift of the C18:1-CoA to C16:0-CoA ratio as observed in *lacs4lacs9* mutant lines resulted in the upregulation of hypoxia responsive genes already in aerobic conditions (**Fig. 3h**). Altogether, these data describe how the ultimate trigger for release of RAP2.12 from ACBP is constituted by an energy-crisis provoked response of the acyl-CoA pool under hypoxia (**Fig. 6**).

Changes of the cellular energy status happen all the time as most biotic and abiotic stress conditions affect energy metabolism in one way or another [44]. It would be most detrimental for plant fitness, when each fluctuation of the ATP-level immediately led to the activation of hypoxic gene expression [34]. Therefore, the lifetime of ERFVII proteins depends on the actual cellular oxygen concentration via the Cys-branch of the N-end rule for proteasomal protein degradation [3,4,11]. Only when an energy crisis is provoked by low-oxygen conditions, the stabilization of RAP2.12 enables the protein to accumulate in the nucleus in a sufficient amount to activate hypoxic gene expression. However, when RAP2.12 is released from ACBP due to an energy deficit that is not related to low-oxygen stress, the protein will be rapidly degraded due to proteasomal activity. Therefore, we propose that the ACBP:RAP2.12 complex forms the initial hub capable of integrating signal inputs related to the cellular energy charge with oxygen-concentration dependent determination of the life-time of RAP2.12 protein (**Fig. 6**).Subsequently, RAP2.12 protein that is newly synthesized after the onset of hypoxia does still undergo N-end-rule-assisted stabilization but may not be linked directly to the energy status of the cell.

Constitutive activation of the molecular stress response to low-oxygen in the *lacs4lacs9* mutant background lead to reduced tolerance to low-oxygen as well as to flooding stress (**Fig. 4**). This observation is congruent with earlier observations that constitutive activation of the hypoxia stress response in plants *via* overexpression of a stable version of RAP2.12 protein reduced the tolerance of the plants to hypoxia [34] although other studies indicate that the latter phenotype is likely conditional to growth conditions and recovery treatment too [4,34]. This underlines the importance of a timely control of stress responses that are optimally adjusted to the actual environmental conditions. The integration of (a) energy-dependent changes in C18:1-CoA levels as cellular trigger signal with (b) the homeostatic control of the lifetime of RAP2.12 in an oxygen-concentration dependent manner provides a highly specific control mechanism to initiate hypoxic responses. The mechanism guarantees that a full low-oxygen response is activated only when hypoxia is detrimental for the plant's energy status.

The ATP dependence of oleoyl-CoA synthesis by LACS in combination with the impact of oleoyl-CoA on the interaction between RAP2.12 and ACBP exposes mitochondrial activity as an early trigger for hypoxia signaling. Consistently, manipulating mitochondrial ATP synthesis using inhibitors of specific respiratory complexes like antimycin-A induced RAP2.12-controlled hypoxic gene expression even under aerobic conditions [45] (**Fig 5e**). It is striking that the induction of hypoxic gene expression in air by acyl-CoAs (**Fig. 3c,h**) or antimycin-A (**Fig. 5e**) was lower as compared to the induction of these genes by hypoxic conditions (**Fig. 3h**). This observation stresses the impact of additional oxygen-dependent regulatory mechanisms, such as the N-end rule-mediated reduction of RAP2.12 life-time in air. In this context, it is worth mentioning that the control of low-oxygen stress responses is not only linked to the oxygen and energy status of the cell, but it is also known to be influenced by other cellular factors such as nitric oxide [46,47], hydrogen peroxide [48], calcium [49,50], and potassium [51]. In the near future, it will be intriguing to expand the mechanistic explanation on how the energy and oxygen status of a cell are integrated upon low-oxygen stress with these additional signaling components [12].

ACBPs are found in all kingdoms of life, while ERFVIIs are highly conserved among higher plants. Moreover, the interaction domains of both protein families are highly conserved in plants (**Fig 1**). Therefore, the ACBP:ERFVII signaling hub as presented here may represent a universal mechanism in plants to initiate hypoxia-induced stress responses via the integration of multiple cellular signals. Moreover, the specific interaction of ACBPs to various acyl-CoAs on the one hand (**Fig 2**) and the distinct cellular responses provoked by individual acyl-CoAs on the other hand (**Fig 3a,b**) suggest that many additional possibilities may exist of how acyl-CoAs can modulate cellular signaling pathways in plants.

Materials and Methods

Plant materials. Arabidopsis thaliana ecotype Col-0 was used as wildtype for all analyses. 35S:RAP2.12-GFP line and *lacs4-1 lacs9-2* and *lacs4-2 lacs9-7* double knock-out lines were described previously [3,33]. The *acbp1* knock-out line (SAIL_683_C03) was obtained from NASC (**SI Appendix, Fig. S5**).

Growth conditions and analysis of oxygen deprivation response. For testing anoxia tolerance of seedlings, seeds were sown on half-strength MS medium containing 0.5% (w/v) sucrose, stratified for 48 hours at 4°C and germinated at 21°C day / 18°C night with a photoperiod of 16 h light (150 µmol m^{-2} s^{-1}) and 8 h dark. At day 11, seedlings were exposed to full anoxia, by placing the culture plates in an environment containing 100% nitrogen, for 9 h in the dark to avoid photosynthetic oxygen production. After 3 days of recovery, the survival score was determined as previously described [4]. For submergence assays, seeds were sown in moist soil, stratified at 4°C in the dark for 48 h and germinated at 21°C day / 18°C night with a photoperiod of 16 h light and 8 h darkness. 4-week-old plants were submerged with water in 40 cm high plastic containers and kept in the dark to avoid photosynthetic oxygen production. Leaves stayed 10 cm under the water surface. After 3 or 4 days, water was removed from the boxes and plants were placed back under photoperiodic conditions (16 h/8 h, light/dark). Submergence tolerance was assayed after 4 days of recovery.

For acyl-CoA pool measurements, plants were grown on horizontal agar plates containing 0.8 % agar in ½ MS medium (pH 5.7) with 15 mM sucrose for two weeks under long-day conditions (16 h in 150 µmol photons m^{-2} s^{-1} at 21°C and 8 h in 0 µmol photons m^{-2} s^{-1} at 19°C). After 2.5 h in light,

they were subjected to hypoxia in the dark by exposition to a stream of air containing 1% (v/v) oxygen, supplemented with nitrogen and 350 ppm carbon dioxide. Plants were harvested by freezing in liquid nitrogen after 2 h, 3 h, and 4 h of hypoxia. For normoxic control, untreated plants were harvested simultaneously with the start of hypoxic treatment.

For expression analysis after acyl-CoA treatment, wild-type seeds were sown in 24-well plates containing half-strength liquid MS-medium, stratified for 48 hours at 4°C and germinated at 21°C day / 18°C night with a photoperiod of 16 h light (150 µmol m^{-2} s^{-1}) and 8 h dark. At day 14, seedlings were treated with different acyl-CoAs at a final concentration of 0.1% in 0.01% Pluronic F68. Pluronic is a non-fatty acid based detergent which means that the detergent properties of pluronic in solubilising fatty acyls are not confounded by fatty acyls derived from the detergent itself [52]

Cloning of constructs. Coding sequences (CDSs) were amplified from a cDNA template using Phusion High Fidelity DNA polymerase (Thermo Scientific). The ACBP1 CDS was cloned into pENTR-D and recombined into pK7FWG2 [51] to fuse it with GFP. For *in vitro* expression, the CDS of ACBP1 was fused N-terminally with a Flag-tag by PCR and cloned into pF3A WG BYDV (Promega), while the CDS of RAP2.12 was fused C-terminally to CFP by PCR and cloned into pF3A WG BYDV (Promega). A complete list of all primers used is provided in **SI Appendix, Table S4**.

Plant transformation. Transgenic ACBP1-GFP plants were generated by transforming wild-type plants with the vector pK7FWG2 [53] containing the ACBP1 CDS fused in frame to GFP at its C-terminus. T0 seeds were screened for kanamycin resistance and the presence of GFP signals by confocal microscopy.

qRT-PCR. RNA extraction, digestion of genomic DNA, cDNA synthesis and qRT-PCR analysis were performed as described previously [54]. For all experiments, 4-5 independent biological replicates were used, as indicated in the figure and table legends. For normalization, UBIQUITIN10 expression was used according to [3] Primers for hypoxia core genes, LACS genes and UBI10 are given in **SI Appendix, Table S4**.

RNA-SEQ analysis. Illumina HiSeq sequencing was performed according to standardized protocols as described in detail in the SI Appendix. Transcriptome analysis was performed by means of CLC Genomics Workbench v.6 using the Arabidopsis thaliana reference sequence (Tair10). Expression values were normalized using quantile normalisation and pair-wise statistical analyses comparing the treatments performed using FDR-corrected p-values based on Baggerly's test [55].

Confocal imaging. For protein localization studies, GFP signals were imaged and analysed with a Leica DM6000 TCS SP8 confocal microscope (Leica Microsystems). Nuclear staining was performed by using DAPI (molecular probes) according to the manufactures instructions. For quantification of nuclear translocation of RAP2.12 after acyl-CoA treatment, twenty DAPI-stained nuclei per plant (5 plants in total) were analyzed per treatment. Leaves of 5-week-old soil-grown 35S:RAP2.12-GFP plants were incubated for 3 h with different acyl-CoAs at a final concentration of 0.1% in 0.01% Pluronic F68 in a 24-well plate under continuous shaking in the light. The experiments were repeated three times.

***In vitro* binding assay.** The method to determine whether acyl-coAs affect the interaction between ACBP1 and RAP2.12 is explained in detail in the SI Appendix, Supplementary Information text and Fig. S1. In brief, both proteins were synthesized using wheat germ extract [54]. The full CDS of RAP2.12 was fused C-terminally with the CDS of CFP, and the CDS of ACBP1 was N-terminally fused with a FLAG-tag. RAP2.12-CFP protein was bound to GFP-Trap-A beads (Chromotek) and incubated with ACBP1 protein overnight. Subsequently, beads were resuspended in wash buffer containing different acyl-CoAs at a final concentration of 0.1% in 0.01% Pluronic F68, or as a mock control only 0.01% F68. After one night of incubation, the buffer was replaced and the composition of the protein complex retained to the beads was analysed on a Western blot.

Analysis of acyl-CoA esters. Acyl-CoAs were extracted, derivatized, and analysed using HPLC as described earlier [56]. Detailed information about the method is provided in the SI Appendix.

LACS4 *in vitro* enzyme assay. The *in vitro* LACS enzyme assay was carried out as described previously [17] using protein that was heterologously expressed in *E. coli* [57]. Details of the method are explained in the SI Appendix.

ATP and ADP quantification ATP and ADP were extracted with 16 % trichloroacetic acid [33] and analysed after derivatisation by HPLC as described previously [58]. Details of the procedure are described in the SI Appendix.

Analysis of oxygen consumption rates. For the determination of oxygen consumption rates four-week-old plants grown on soil under short-day conditions (8 h in 160 µmol photons m^{-2} s^{-1} at 20 °C and 16 h in 0 µmol photons m^{-2} s^{-1} at 16 °C) were used. Starting three hours prior to measurement, the plants were sprayed every hour either with 50 mM MES buffer (pH 6.5) containing 50 µM antimycin-A [59] or with 50 mM MES buffer for control. This treatment was done in the last hour of dark phase and two hours into the light phase, and then measurement of oxygen consumption rates at normoxic conditions was performed as described before [34] using the respective spraying solution. In a similar setup the effect of different acyl-CoAs on the oxygen consumption rate was tested by incubating leaf disks in acyl-CoAs at a final concentration of 0.1% in 0.01% Pluronic F68.

Statistical analysis. Statistical evaluation of significant variation between treatments or genotypes was done by performing Student's t-test or one-way ANOVA where appropriate.

Data availability. The RNAseq gene expression data are available in NCBI's Gene Expression Omnibus (GEO) through GEO Series accession number GSE97186.

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