Docking of acetyl-CoA carboxylase to the plastid envelope membrane attenuates fatty acid production in plants

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In plants, light-dependent activation of de novo fatty acid synthesis (FAS) is partially mediated by acetyl-CoA carboxylase (ACCase), the first committed step for this pathway. However, it is not fully understood how plants control light-dependent FAS regulation to meet the cellular demand for acyl chains. We report here the identification of a gene family encoding for three small plastidial proteins of the envelope membrane that interact with the \( \alpha \)-carboxyltransferase (\( \alpha \)-CT) subunit of ACCase and participate in an original mechanism restraining FAS in the light. Light enhances the interaction between carboxyltransferase interactors (CTIs) and \( \alpha \)-CT, which in turn attenuates carbon flux into FAS. Knockouts for CTI exhibit higher rates of FAS and marked increase in absolute triacylglycerol levels in leaves, more than 4-fold higher than in wild-type plants. Furthermore, WRINKLED1, a master transcriptional regulator of FAS, positively regulates CTI1 expression by direct binding to its promoter. This study reveals that in addition to light-dependent activation, “envelope docking” of ACCase permits fine-tuning of fatty acid supply during the plant life cycle.
Vegetable oils are an important source of food, renewable energy, and industrial feedstocks. As demand for this commodity steadily increases, development of improved oil crops has become an agronomic priority. Oil biosynthesis begins with the de novo fatty acid synthesis (FAS) pathway in the plastids. The acyl chains thus obtained are eventually esterified to glycerol to produce triacylglycerols (TAGs), the major form of storage lipid in plants. Like many biosynthetic processes in plastids, the overall rate of FAS oscillates with light/dark cycles, increasing in the light and decreasing in the dark. Upon light illumination, stomatal pH, Mg2+, ATP/ADP ratio, and redox potential increase in the chloroplasts, promoting carbon fixation and FAS. Light-dependent control of FAS is partially achieved by regulation of acetyl-coenzyme A carboxylase (ACCase; EC.6.4.1.2) which catalyzes the first committed step in FAS, the ATP-dependent formation of malonyl-CoA from acetyl-CoA and bicarbonate. In the plastids of dicots and non-graminaceous monocots, the predominant form of ACCase is a multisubunit, heteromeric complex (htACCase) comprised of four distinct subunits named biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP), and α- and β-carboxylytransferases (CTIs). In vitro, increasing pH, Mg2+, ATP/ADP ratio, as well as reducing conditions activates ACCase. Additionally, ACCase is subject to feedback inhibition by downstream metabolites, and effector proteins like the PII protein, a signal integrator that interacts with BCCP subunits to reduce the Vmax of the enzyme. Additionally, a set of noncatalytic biotin/lipoyl attachment domain-containing (BADC) proteins are involved in the regulation of ACCase activity, including light-dark modulation.

Here, we report the isolation and characterization of a gene family, CARBOXYLTRANSFERASE INTERACTORS (CTIs), encoding three plastid membrane proteins, which are involved in the regulation of de novo FAS. Using the noncatalytic domain of α-CT as bait in a yeast two-hybrid screen, we identified CTI1, an uncharacterized gene. The cti mutants display higher rates of FAS and higher TAG content in leaves. Expression of CTI1 is activated by the WRINKLED1 transcription factor. Aside from its overall activating role in FAS, light can simultaneously attenuate FAS by promoting interactions between α-CT and CTIs, which in-turn inhibit htACCase activity and subsequently fatty acid synthesis. Our results provide important insights into how plants control light-dependent FAS to meet the cellular demand for acyl chains.

Results
The noncatalytic domain of α-CT directly interacts with CTIs. In seed plants, the α-CT subunit of htACCase contains a large, noncatalytic C-terminus predicted to have coiled-coil structure, a structural motif that typically mediates protein-protein interactions. This observation prompted us to identify potential interactors with the putative coiled-coil domain of α-CT. Using the C-terminus (amino acid 420–769) of Arabidopsis thaliana α-CT as bait, a yeast two-hybrid (Y2H) screen was performed. Among 16 candidates, the screen identified the C-terminus of At1g42960 from two different clones. This candidate was named CARBOXYLTRANSFERASE INTERACTOR1 (CTI1). The sequence of the CTI1 protein contains a chloroplast transit peptide, a single transmembrane domain, followed by a coiled-coil domain (Supplementary Fig. 1a–d and Supplementary Table 1). By surveying the Arabidopsis genome, we found two CTI1 homologs, CTI2 (At3g02990) and CTI3 (At5g16660), and the three CTIs share similar predicted protein domains (Supplementary Fig. 1a–d and Supplementary Table 1). Based on phylogenetic analysis (Supplementary Fig. 2, and Supplementary Table 2), the CTI family appears to be of cyanobacterial origin, with the C-terminal domain conserved between plants and cyanobacteria. While green algae, bryophytes, and gymnosperms generally possess a single CTI, an apparent duplication event in an ancestral angiosperm gave rise to two divergent angiosperm CTI subfamilies, one of which includes Arabidopsis CTI1, while the other includes the closely-related Arabidopsis CTI2/3 genes. Interestingly, the CTI1 subfamily is apparently absent from grasses that lack the heteromeric form of ACCase. In contrast, the CTI2/3 subfamily is conserved across major angiosperm groups.

To confirm the interaction between α-CT and CTI1, we cloned the coiled-coil-containing C-terminus of CTI1 (Supplementary Fig. 1a) and full-length α-CT for targeted Y2H assays. The results confirmed an interaction of α-CT with CTI1, as well as with the CTI2 and 3 proteins (Fig. 1a). To control for assay specificity, the interactions between CTIs and β-CT, TRIGALACTOSYLDIACYLGLYCEROLO2 (TGD2) or the coil-coiled-containing PROTEIN TARGETING TO STARCH2 (PTST2) with α-CT were tested; the results showed no interactions between either of these pairs (Fig. 1a). Next, we conducted bimolecular fluorescence complementation (BiFC) assays using a split YFP experimental system in Arabidopsis protoplasts. When CTI1-nYFP and α-CT-cYFP were coexpressed in protoplasts, a YFP signal was detectable that colocalized with chlorophyll, confirming α-CT/CTI1 interaction in planta (Fig. 1b). In contrast, no interaction could be detected between CTI1 and BCCP2 or PTST2. Additionally, both CTI2 and 3 interacted with α-CT in BiFC assays (Supplementary Fig. 3a, b). Binding affinity of the CTIs for α-CT was then quantified by microscale thermophoresis (MST) using the C-terminal sequence of both CTIs and α-CT. CTI1 exhibited a 10- and 100-fold higher affinity for α-CT compared to CTI3 and 2, respectively, based upon Kd values (Fig. 1c). We thus identified a family of proteins that associate with htACCase through direct interaction with α-CT.

CTI proteins are localized in the chloroplast inner envelope membrane. Consistent with in silico predictions, CTIs were previously detected in chloroplast envelope membranes. Transient expression of constructs encoding for CTI-YFP fusion proteins in Arabidopsis protoplasts revealed discrete foci at the periphery of chloroplasts similar to that observed with α-CT-YFP (Fig. 2a) and TGD2-GFP fusions. This pattern of localization also mirrored the distribution of two other ACCase interactors, i.e., PII and BADC1 proteins, in plastids. It is not clear, however, whether or not these foci denote subdomains of the membrane where htACCase units are preferentially clustered in a wild-type context, considering that protein overexpression produced similar patterns. To specifically confirm colocalization of CTI1 with TGD2, a well-characterized protein of the plastid inner envelope membrane, or with the CT subcomplex of htACCase previously shown to be associated with the plastid inner envelope membrane, CTI1-GFP was transiently coexpressed with α-CT-RFP (Fig. 2b) or with TGD2-RFP (Fig. 2c) in leaves of Nicotiana benthamiana. Colocalization of GFP and RFP signals suggested that CTI1 is indeed a component of the chloroplast inner envelope membrane. Furthermore, co-immunolocalization experiments were carried out between CTI1 and E37, a methyltransferase involved in tocopherol and plastoquinone synthesis localized to the plastid inner envelope membrane. In Arabidopsis protoplasts transfected with the Pro35S:CTI1:HA construct and in embryonic cells of Arabidopsis transgenic lines stably transformed with the Pro35S:CTI1:HA construct, the CTI1:HA fusion protein appeared as a punctate pattern colocalized with a subset of the wider distribution of E37, confirming targeting of CTI1 to the chloroplast inner envelope membrane. Finally, the membrane topology of CTI1 was determined by dual-protease digestion assay. Intact chloroplasts were
isolated from Arabidopsis leaf tissues and treated with thermolysin (a protease unable to penetrate the outer envelope membrane) or trypsin (a protease able to penetrate the outer envelope but not the inner envelope membrane). CTI1, like α-CT, was resistant to both proteases, suggesting that the C-terminus of the CTI1 protein faces the stromal side of the inner envelope membrane (Fig. 2c).

CTIs negatively regulate fatty acid synthesis through htACCase. To determine the function of CTIs in Arabidopsis, we used CRISPR/Cas9 to knockout the CTI genes individually. We recovered CRISPR/Cas9-induced homozygous frameshift mutants for each CTI gene (Supplementary Fig. 4a). The frameshifts induced premature termination codons preceding the coiled-coil domain for all three CTIs (Supplementary Fig. 4b). The efficiency of the cti1 knockout mutant was confirmed by immunoblotting using anti-CTI1 antibody (Supplementary Fig. 4c). Double and triple mutants were then generated by crosses to examine genetic interactions among the CTIs. When grown under long-day conditions, the cti123 triple mutant displayed a growth defect with smaller rosette leaves and decreased aerial biomass compared to wild-type (WT) plants, indicating a growth penalty in the triple mutant (Fig. 3a, b).

We then assayed ACCase activity in leaves of the different genotypes. Soluble protein fractions from crude chloroplasts prepared from wild-type leaves were assayed in the presence of two different ACCase inhibitors; avidin, a general inhibitor of ACCase activity, and the herbicide haloxyp, which specifically targets homomorphic ACCase.24 Whereas addition of avidin strongly reduced the measured ACCase activity, haloxyp did not cause a significant reduction in specific activity (Supplementary Fig. 5), demonstrating that htACCase activity predominates in the plant material prepared. Then, supernatants and pellets from clarified (13,000 × g) chloroplast lysates from 4-week-old leaves of the different cti mutants were assayed for soluble and membrane-bound ACCase activity, respectively. In the supernatants, the cti1 mutation yielded a 3.5-fold increase in ACCase activity with respect to the WT control. ACCase activity was further enhanced in cti13 and cti123 mutants (Fig. 3c). Conversely, in the pellets, the cti1 mutation yielded lower ACCase activity, and further reduction was observed in the cti123 triple mutant (Fig. 3c). To estimate the rate of FAS in cti mutants, we performed in vivo 14C-acetate labeling experiments using 4-week-old leaf strips. After an incubation of 40 min, the rate of 14C-acetate incorporation was around 3.5-fold higher in cti1 than in WT, while a slight increase was observed in cti3 mutant. The double and triple mutants exhibited enhanced rates of incorporation with respect to the single mutants (Fig. 3d), reflecting the variations observed in soluble ACCase activity (Fig. 3c). Incorporation of 14C-malate into nascent fatty acids was not higher in cti mutants (Supplementary Fig. 6). Next, we quantified htACCase subunit levels in the cti mutants by western blotting. The results indicated that higher FAS rates were not the consequence of increased levels of htACCase subunits (Supplementary Fig. 7).
As a first approach to study the impact of higher soluble hTACCcase activity in cti mutants, we determined the total fatty acid content in leaves of plants grown under long-day (LD) photoperiod. Consistent with increased FAS rate, leaf fatty acid content in the cti1 mutant was significantly higher than WT (a 22% increase). An additive effect of the cti mutations could be observed in double and triple mutants (Fig. 3e). The cti mutants also showed altered leaf fatty composition, with a decrease in the relative proportion of 18:3 polyunsaturated fatty acids and a concomitant increase in 18:0, 18:1, and 18:2 yielding a reduced desaturation ratio (Fig. 3f). Analysis of leaf lipid extracts by thin-layer chromatography (TLC) revealed accumulation of neutral lipids comigrating with TAGs in cti mutants (Fig. 3g).

Comprehensive, quantitative lipidomic analyses confirmed specific accumulation of TAGs in cti1 and cti3 single mutants as well as an additive effect in double mutants (Fig. 3h). In contrast, TLC and quantitative lipidomic analyses of polar lipids showed that these classes of lipids were not affected by the cti mutations (Supplementary Fig. 8a, b). In mutant seeds, the total seed oil content was unmodified but a reduced desaturation ratio was observed as in leaves (Supplementary Fig. 9). To confirm the CRISPR mutant phenotypes, we then characterized a T-DNA insertion mutant corresponding to a second null allele of CTI1 (Supplementary Fig. 10a). This T-DNA allele (cti1-2) phenocopied the cti1 CRISPR/Cas9 allele, including increased soluble ACCase activity, FAS rate, total fatty acid content, and TAG accumulation.
accumulation in leaves (Supplementary Fig. 10b–f), as well as modified fatty acid composition of leaves and mature dry seeds (Supplementary Fig. 10g). This last phenotype was complemented by introgression of the ProCTI1:CTI1-HA or ProAT2S2:CTI1 construct in the cti1-2 mutant background (Supplementary Fig. 10g). A partial complementation of the cti1-2 seed oil phenotype could also be obtained by introgression of CTI2 and CTI3 cDNAs placed under the control of the seed-specific ProAT2S2 promoter29, but the efficiency of these phenotypic reversions was lower (Supplementary Fig. 10g). Taken together, these results demonstrate that CTIs attenuate soluble hAACCCase activity, so that cti mutations enhance carbon flux into fatty acid synthesis, resulting in TAG over accumulation in leaves. If the three CTIs have partially redundant functions, CTI1 appears to be the predominant effector of hAACCCase activity. The phenotypic impact of the CTI1 isoform is consistent with both higher affinity for α-CT and phylogeny data showing that CTI1 coevolved with hAACCase.

**WRINKLED1 activates CTI1 expression through direct binding to CTI1 promoter.** Expression patterns of the CTI genes were investigated using qRT-PCR (Supplementary Fig. 11a) and translational ProCTI1:CTI1:uidA fusions assayed in transgenic plants (Supplementary Fig. 11b–d). The three CTIs appeared to be ubiquitously expressed, and CTI1 mRNAs were more abundant than CTI2 or CTI3 mRNAs in all tissues analyzed, providing another possible explanation for the predominant regulatory role of CTI1. Furthermore, a peak of CTI1 transcript accumulation was detected at the onset of seed maturation and this pattern was not observed for CTI2 and CTI3. The expression pattern of CTI1 in seeds was similar to the profiles observed for several genes encoding FAS enzymes known to be transcriptionally activated by WRI1 on the pattern of CTI1 promoter activity, the ProCTI1:uidA construct was stably expressed in transgenic Arabidopsis lines. Unlike ProCTI1:CTI1:uidA transgenic lines, ProCTI1:uidA lines displayed GUS staining in reproductive organs but not in vegetative parts of the plants (Supplementary Fig. 10, 11), indicating that important regulatory elements exist downstream of the translational start codon of CTI1. The construct was then introduced into Pro35S:WRRII lines by crosses, and a strong induction of ProCTI1 activity was detected in rosette leaves (Fig. 4d). In contrast to the observations made in the WT, ProCTI1 activity could not be detected in wri1 embryos (Fig. 4e). In agreement with these observations, CTI1 mRNA levels were significantly decreased in wri1 embryos, whereas higher CTI1 mRNA levels were measured in leaves of WRI1 overexpressors (Fig. 4f). Together, these results showed that WRI1 enhances the activity of the CTI1 promoter, in a similar way to the activities of promoters of genes coding core FA biosynthetic enzymes and regulators of hAACCCase like the BADC subunits and PII16,30.

**CTI/α-CT module regulates fatty acid synthesis in a light-dependent manner.** It was proposed that light-dependent 14C-acetate incorporation into fatty acids is due to the regulation of hAACCCase31–33. To determine if the CTIs are involved, rates of FAS in 4-week-old leaves were monitored using 14C-acetate labeling. Under a classical LD regime, incorporation rates were higher at the end of the day than at the end of the dark period for most genotypes. Increased 14C-acetate incorporation observed in cti mutants was moderated at the end of the dark period (Fig. 5a). The enhancement of 14C-acetate incorporation measured in cti mutants was abolished by an extended dark period (24 h) but recovered after a 16-h-light period (Fig. 5b). To exclude the possibility that the variations in incorporation rates measured were reflecting differences in unlabeled acetate in leaves, a competition assay with unlabeled acetate was performed. Before adding 14C-acetate, leaf tissues were incubated in the presence of unlabeled acetate for 10 min. No competition effect could be detected in the WT nor in the cti1 mutant (Supplementary Fig. 13). However, considering that acetate is not the principal carbon source for FAS in planta34,35, we next quantified the total leaf fatty acid content from Arabidopsis plants grown under various photoperiods. No significant modifications of the leaf fatty acid content in the cti mutants could be observed under short-day conditions. In contrast, increased fatty acid content was measured in cti leaves under long-day conditions and under constant light (Fig. 5c). Like the 14C-acetate labeling assay, fatty
acid quantification suggested that CTIs inhibit FAS in the light. We finally tested whether a light-regulated CTI/α-CT interaction was responsible for the modulation of FAS. A split-luciferase assay was implemented in leaves of *N. benthamiana*. In leaves of plants grown 4 d under continuous light after agroinfiltration, a strong split-luciferase (LUC) signal could be detected (Fig. 5d). When agroinfiltrated leaves were kept 2 d in the light then 2 d in the dark, interactions between α-CT and the CTIs appeared significantly weaker. However, a further 24 h recovery period in the light was sufficient to restore equivalent levels of α-CT/CTI interactions.
interactions in all the leaves, regardless of the light/dark regime previously imposed (Fig. 5d, e and Supplementary Fig 14). Light-dependent variations in CTI1/α-CT interactions detected in split-luciferase assays were not due to differential protein accumulation (Supplementary Fig. 14a). To further demonstrate the light-dependence of CTI1/α-CT interaction, co-immunoprecipitation experiments were carried out using leaf samples harvested after different light/dark treatments. In agreement with split-luciferase assays, an extended (24 h) dark period reduced the interaction between α-CT and CTI (Fig. 5f). A strong interaction between the proteins could be restored by an extended 16 h light illumination. These observations suggest that light-dependent CTI/α-CT interactions play an important role in the regulation of htACCase and FAS. In summary, we identified a membrane regulatory subunit to the plant htACCase, and light appears to trigger both positive and negative regulatory mechanisms targeting FAS. This is evident from light-induced CTI/α-CT interactions attenuating htACCase activity and limiting carbon flux into de novo FAS (Fig. 5g). We suggest this permits the plant cell to finely regulate the allocation of products of photosynthesis, thus optimizing carbon and energy homeostasis in response to changing environmental and developmental parameters.

Discussion

De novo fatty acid synthesis in higher plants occurs mainly in plastids, and is catalyzed by a series of enzymes and acyl-carrier proteins. For thermodynamic efficiency, it was proposed that the enzymes, substrates and cofactors for FAS may be compartmentalized to form a metabolic channel. However, we lack a clear picture of such a FAS metabolon. Another dilemma regards how does de novo FAS in the plastids ‘communicate’ with the cytosol to meet the acyl chain demand of the entire cell? One possibility would be that FAS enzymes sense cellular demand across the envelope by directly associating with this membrane conduit. The carboxylation of acetyl-CoA to malonyl-CoA by ACCase is the first committed step of FAS, and is considered rate-limiting for FAS. ACCase was detectable on the inner envelope membrane of pea plastids. Subsequently, a protein of 96 kDa (IEP96) associated with the chloroplast inner envelope membrane was characterized, which shares high similarity with the 35 kDa α-carboxytransferase subunit of ACCase from Escherichia coli, proved to be the α-CT subunit of plant htACCase. The CTI subcomplex of htACCase is tightly membrane associated whereas BC/BCCP subcomplex can be partially dissociated from chloroplast membranes. Because of the strong association of α-CT with the chloroplast envelope and the lack of a predicted transmembrane domain in α-CT, it was proposed that a chloroplast membrane protein may mediate such membrane association of α-CT. In this study, we used the noncatalytic domain of α-CT as bait in a yeast two-hybrid screen, and we identified an uncharacterized gene, that we termed CTI1. A survey of the Arabidopsis genome revealed two genes related to CTI1, CTI2, and CTI3. All three CTI proteins localize to the plastid envelope membrane and interact specifically with the noncatalytic domain of α-CT. The thorough characterization of ctI mutants generated by CRISPR/Cas9 technology showed that CTI1 is the predominant isoform with respect to the molecular function of the CTIs. This could be attributed to (i) a higher expression level of CTI1 combined with (ii) a higher affinity of CTI1 to α-CT. In agreement with these observations, CTI1 appears to have coevolved with htACCase. The occurrence of CTI2/CTI3 in graminaceous monocots, which lack htACCase, then raises the unresolved question of the function of these homologs in such species.

While FAS in leaves is essential to support the production of membrane lipids and waxes, overproduction of FAs can lead to membrane damage so that excess FAs are detoxified via TAG biosynthesis and accumulation, or via degradation through beta-oxidation pathway. TAGs function as a buffer for cytotoxic FAs and other lipid intermediates, thereby playing a key role to maintain intracellular lipid homeostasis. Leaf TAG production is energetically wasteful though. Under normal circumstances, FAS is highly regulated in vegetative tissues to minimize TAG production. The α-CT/CTI module clearly participates in this regulatory mechanism: illumination promotes α-CT/CTI interactions, which in-turn attenuates htACCase activity, thus reducing carbon flux into FAS. Decreased C18 fatty acid desaturation ratio was observed in both in leaves and seeds of ctI mutants. This phenotype probably originates from the disconnection between the FAS module in the plastid and FA modification/elongation modules in the endoplasmic reticulum, the location of production of polyunsaturated fatty acids (PUFAs) and very long chain fatty acids (VLCFAs). FA modifications seem to function at the same rate regardless of de novo FA production capacity. In seeds of Arabidopsis pfi mutants and transgenic rapeseed lines overexpressing ACC1 in plastids, increased ACCase activity was associated with decreased proportion of polyunsaturated FAs, just like in ctI mutants. Reciprocally, in wr1 mutant seeds, FA production is decreased, and the relative proportions of PUFAs and VLCFAs are higher. Light exposure upregulates FAS by activating htACCase. Illumination first creates optimal physiological conditions for htACCase by raising pH and concentrations of Mg2+, ATP, and reductants. Light-regulated post-translational modifications of catalytic subunits of htACCase have also been described.

More
Recently, pH modulation of BADC/BCCP association within the biotin carboxylase subcomplex was identified and proposed to contribute to light-dependent regulation of the activity of htACCase. BADC subunits were shown to inhibit ACCase in the dark through dark-favored interactions with BC, which correlates with lower htACCase activity and FAS in the dark. To study the effects of light on CTI-mediated FAS regulation, we carried out 14C-acetate labeling assays to quantify the FAS rates. 14C-acetate labeling in FAS is light-dependent, and has been proposed to be achieved by light-dependent carboxylation of acetyl-CoA by htACCase. Like other studies, we found that 14C-acetate labeling is a very practical approach to study htACCase in vivo. It revealed that CTIs attenuate the rate of FAS in the light. In addition, 14C-acetate labeling suggests that both the accumulation of fatty acids in leaves of ctI mutants and CTI/α-CT interactions are regulated by light (Fig. 5). Altogether, these data demonstrate that interactions of CTI isoforms with the α-CT subunits regulate FAS in a light-dependent manner.

Phosphorylation of α-CT was previously described and two phosphorylation sites (Ser741 and Ser744 on Arabidopsis ortholog) were identified by phosphoproteomic studies. Both of the identified α-CT phosphorylation sites are near the

**Fig. 4 WRINKLED1 enhances CTI1 expression through direct binding to the CTI1 promoter.** a AW cis-regulatory element present in the CTI1 promoter. Conserved nucleotides denoting the presence of an AW box are in red. The numbers between brackets indicate the position of the element relative to the start codon. b WR1 directly activates the CTI1 promoter in yeast one-hybrid assay. Yeast strains containing the HIS3 reporter gene under the control of either the BANYULS (BAN, negative control), BCCP2 (positive control), or CTI1 promoter were transformed with either the empty expression vector or with versions of the vectors allowing expression of WR1 or WR12 (negative control) before being plated on synthetic dropout (SD-WU or SD-WUH) media. The experiment was repeated five times (with independent clones) with similar results. c WR1 activates the promoter of CTI1 in transactivation assay in leaves of Nicotiana benthamiana. Pro:uidA reporter constructs (ProBCCP2 was used as positive control, ProODD as negative control) alone or in combination with a vector allowing the expression of WR1 or MYB118 (negative control) were coinfected in young leaves of N. benthamiana. The experiment was repeated twice with similar results. For each experiment and for each condition tested, six independent infiltrations were realized. Representative pictures are displayed. Bars = 2 mm. d Activity of the ProCTI1(994 bp):uidA cassette in rosette leaves of Pro35Sdual:WR1 over-expressing (OE) lines (WR1 OE T81 and T63) and in a wild-type (WT) background. The experiment was repeated twice (using independent ProCTI1(994 bp):uidA transformants) with similar results. For each experiment and for each genotype, six different leaves were observed. Representative pictures are displayed. Bars = 5 mm. e Activity of the ProCTI1(994 bp):uidA cassette in wr1 mutant embryos and in a WT embryo. The experiment was repeated twice (using independent ProCTI1(994 bp):uidA transformants) with similar results. For each experiment and for each genotype, 10 different embryos were observed. Representative pictures are displayed. Bars = 150 μm. f Accumulation levels of CTI1 transcripts in seeds of wr1 and in leaves of two independent WR11 overexpressing lines (WR1 OE T81 and T63) analyzed by qRT-PCR. The results obtained are standardized to the constitutive EFlαA4 gene expression level. Data are means ± SEM, n = 3 biologically independent plants. Asterisks indicate significant difference from the wild-type control as determined by a two-tailed Student’s t-test at **P < 0.01 and * P < 0.05, respectively.
C-terminus, within the noncatalytic domain of the protein, which is responsible for its interaction with CTI. Additionally, a different phosphoproteomic study identified phosphorylation sites on the C-terminus of CTI1 (Ser160, Thr161) and CTI3 (Thr151, Ser152, and Ser153). The identified phosphorylation sites are conserved in all three CTIs. Protein phosphorylation can be used to modulate the nature and strength of protein-protein interactions thereby regulating protein binding. Considering the occurrence of phosphorylation sites in the protein binding regions (coiled-coil domains) of both α-CT and CTIs, it would be interesting to further study whether such protein modifications affect the dynamics of CTI/α-CT binding.

Vegetative tissues in cti1 mutants display measurable TAG accumulation, resulting in at least a four-fold increase in 14C acetate incorporation (DPM/mg fresh weight) compared to wild-type (WT) plants. The increase is more pronounced under 16-h light + 8-h dark conditions than under 24-h dark conditions. The relative fatty acid content (% WT) also shows an increase in cti1 mutants compared to WT plants, with a significant difference observed in the 16-h light condition. The input controls (IgG) and α-CT IP show specific bands for α-CT and CTI1, indicating specific interactions. The experiment was repeated for 4-d light and 3-d light conditions, with statistical significance assessed using ANOVA and Tukey’s test. The results show a significant difference (P < 0.001) in the relative luminance units between the WT and cti1 mutants. The reaction mechanisms include the conversion of Acetyl-CoA to Malonyl-CoA and the regeneration of HCO3⁻ + ATP to ADP + Pi, indicating the importance of these reactions in TAG synthesis. The figure illustrates the intermembrane space with BC, pH/Mg²⁺, HDH, SH, Stroma, and Intermembrane space.
extractable leaf oil compared with wild-type Arabidopsis leaves (Fig. 3h). From a biotechnological point of view, CTIs may thus represent promising targets for the engineering of vegetable oil crops. While oilseed-based fuel production can meet only a small proportion of world transportation needs before impinging on the global food supply, the overproduction of oil in non-seed tissues such as leaves and stems may be exploited as part of an overall strategy to maximize the recovery of renewable resources from agricultural products39,48.

Methods

Plant material and growth conditions. Arabidopsis thaliana accession Col-0 was used as wild-type control in this study. The T-DNA mutant line (cti-2; N599125) was ordered from ABRC (Arabidopsis Biological Resource Center). Characterization of the wrt-3 insertions and wrt-1 T-DNA insertion lines and construction of the Pro35Sdual:WRT lines (T63 and T81) were previously reported28. All plants were grown in growth chamber at 22 °C. Light intensity was 82–115 (98 on average) μmol m⁻² s⁻¹ and humidity was 50%. The long-day (LD) photoperiod corresponded to an alternate 12 h light/12 h dark regime. The short-day (SD) photoperiod corresponded to an alternate 12 h light/12 h dark regime.

Constructions preparation and stable transformation of Arabidopsis. The primers used for construct preparation are listed in Supplementary Table 3. We designed single-guide RNAs (sgRNAs) for CRISPR/Cas9 using the online CRISPR-P 2.0 software50. We annealed the oligo pairs to generate double-stranded DNA and cloned the sgRNAs into the BbsI site of the gateway compatible entry vector pU6-sgRNA. We then introduced the pU6-sgRNA cassette into a modified pCambia1300 binary vector, in which hspCas9 expression is driven by the YAO promoter32.

To construct ProCTI1:CTI1:uidA, ProCTI2:CTI2:uidA, and ProCTI3:CTI3:uidA transgenes, gene fragments extending from up to 2 kb upstream of the ATG of the gene to the last coding codon were amplified with the Pfu Ultra DNA polymerase (Stratagene) from Col-0 genomic DNA. The PCR products were introduced by BP recombination into the pDONR207 entry vector (Invitrogen) and transferred into the destination vector pB101-R1R2-GUS31 by LR recombination. The resulting binary vector was electroporated into Agrobacterium tumefaciens C58C strain and transformed to the C58C strain and used for transformation1. Depending on the construct considered, between 17 and 19 independent transgenic lines were analyzed. To construct the ProCTI1:uidA transgenic line, region -994 to -1 bp relative to the CTI1 translational start codon was introduced by BP recombination into the pDONR207 entry vector as described above, and transferred into the destination vector pB101-R1R2-GUS by LR recombination. Construction of the ProBCCP2:uidA transgene29, just like that of the ProODD:uidA transgene29 followed a similar procedure.

To construct ProAT52S:CTI transgene, CTI CDSs were amplified from a mixture of seed DNA (Col-0 accession), cloned into the pDONR207 as described above, and finally transferred to the binary vector ProAT52S-R1R2-HYGRO53 by LR recombination. The corresponding binary vectors were used for agroinfiltration of cti-1-2 flower buds. T2 seeds were subjected to segregation analyses for hygromycin resistance and lines segregating 3:1 were selected (heterozygous lines, one insertion locus). T2 lines were then grown in a greenhouse and their progeny (T3 seeds) was subjected to segregation analyses. Lines producing 100% resistant plantslets were selected (homozygous lines, single insertion locus) and used for further characterization.

To construct Pro35S:CTI1:YFP, Pro35S:CTI2:YFP, Pro35S:CTI3:YFP, and Pro35S:α-CT:YFPtransgenes for protransient transformation, CTI and α-CT coding sequences (CDSs) without STOP codons were amplified from a mixture of cDNA (Col-0 accession), cloned into the pENETr-D/TOPO, and transferred into the pAM-PAT-35SS:YFP/GW vector54 by LR recombination. To construct Pro35SS:CTI1:GFP, Pro35SS:CTI2:RFP, and Pro35SS:CTI2:RFP transgenes, CDSs were cloned into the pENETr-D/TOPO vector as above, before being transferred into destination vectors pGW6605 (GFP) and pGW6654 (RFP) by LR recombination55.

To construct BiFC and split-luciferase vectors, CDSs previously cloned into the pENTR-D/TOPO were transferred into pAM-PAT-35SS::YFP/nYFP/GW, and pEarley-nLUC/cLUC vectors by LR recombination. To construct BiFC and split-luciferase vectors, CDSs previously cloned into the pDONR207 (see above) was transferred into the pDONR207 (see above) was transferred into the pDONR207 (see above) was transferred into the pDONR207 (see above) was transferred into the pDONR207 (see above) was transferred into the pDONR207 to destination vectors pGWB6654 (GFP) and pGW6654 (RFP) by LR recombination56.

To construct BiFC and split-luciferase vectors, CDSs previously cloned into the pDONR207 (see above) was transferred into the pDONR207 (see above) was transferred into the pDONR207 (see above) was transferred into the pDONR207 to destination vectors pGWB6654 (GFP) and pGW6654 (RFP) by LR recombination56.

To construct the Pro35SS:CTI1:α-CT transgene29, the α-CT/CTI interactions, possibly on a different timescale (4). Additionally, BADC proteins modulate hypothiaction activity by competing with BCPB subunits for access to BC.

Fig. 5 CTIs interacting with α-CT regulate fatty acid synthesis in a light-dependent manner. a, b, 14C-acetat incorporation experiments carried out on rosette leaves aged 4 weeks and subjected to different light/dark regimes. Data are means ± SEM, n = 3 biologically independent plants. c, leaf total fatty acid content of rosette leaves from plants grown under different light regimes as determined by gas chromatography. Plants were grown in short-day (SD), long-day (LD) or constant light (CL) conditions for 4 weeks before analyses. Data are means ± SEM, n = 4 biologically independent plants. Asterisks indicate significant difference from the 2-d light/2d-dark treatment (before recovery) as determined by a two-tailed Student’s t-test at **P < 0.01. f-Co-immunoprecipitation assays. IgG was used as a negative control. Experiments were repeated three times with similar results. g, Model for the regulation of hACCase activity by light. Light upregulates FAS by increasing the ATP/ADP ratio (1), optimizing Mg²⁺ concentration and pH (2), and reduct potential in the plastid (3). Light also attenuates hACCase activity by enhancing α-CT/CTI interactions, possibly on a different timescale (4). Additionally, BADC proteins modulate hACCase activity by competing with BCPB subunits for access to BC.
obtained were digested with NcoI and integrated into the yeast strain YM4271 at the URA3 locus. Yeast cells presenting the HIS3 reporter gene under the control of the different promoters studied were transformed with pDEST23 using LiAc/SSDNA/PEG method. Yeast cells were grown in YPD medium and harvested by centrifugation at 3000 × g. For each transformation, around 10^6 cells were mixed with T Mix (240 μL PEG 3350 50% (w/v), 36 μL 1 M lithium acetate, 50 μL bovine SS-Carrier DNA (2 mg/mL), 34 μL plasmid DNA (0.1–1 μg)). After vigorous mix by vortexing when OD600 reached 0.6, pellets were obtained by centrifugation, washed with sterile water, resuspended in 200 μL sterile water before being plated on selective medium and grown for 30°C for 3 d.

**Yeast two-hybrid assays.** The cDNA library used was prepared by introducing Arabidopsis cDNA prepared from seedlings into the pGADT7-GW vector. The screening was performed according to yeast two-hybrid yeast-hunter system. Briefly, the sequence encoding the coiled-coil domain of α-CT (amino acid 420–769) was cloned into the pGBKKT7 vector. This vector was used to transform the AH109 yeast strain. Yeast transformants were selected on synthetic dropout (SD) medium lacking tryptophan. Then, 100 μg of cDNA library were used to transform AH109 cells containing the pGBKKT7 vector expressing the coiled-coil domain of α-CT. Cells plated on SD medium lacking leucine, tryptophan, histidine, adenine, were incubated at 30°C for 4 d and cDNAs of positive clones were amplified by PCR and sequenced using T7 and 3′AD primers (Supplementary Table 6).

For oriented interaction assays, the sequences coding for the coiled-domain of CTI1, CTI2, CTI3 and α-CT were cloned into the pGADT7 vector as described above. Then, the AH109 strain was cotransformed with the pGADT7 vector expressing CTI coiled-coil domains and the pGBKKT7 vector expressing the α-CT coiled-coil domain, before being plated on SD medium lacking leucine and tryptophan. After 3 days at 30°C, positive clones were transferred to SD medium lacking leucine, histidine, and tryptophan, in the presence of 1 mM 3AT for another 4 d at 30°C.

**Transformation of Arabidopsis protoplasts.** Arabidopsis protoplasts were prepared from 4-week-old Col-0 plants57. About 40 leaves were cut into strips with a sharp razor blade and then transferred into 20 mL enzyme solution (20 mM MES pH 5.7, 1.5% (w/v) cellulase R10, 0.4% (w/v) macerozyme R10, 0.4 M mannitol, 20 mM KCl and 0.1% BSA) for 3 h at room temperature. After 3 h, the enzyme solution was filtered through 75-μm nylon mesh that was then washed with 200 mL W5 solution (2 mM MES pH 5.7, 154 mM NaCl, 125 mM CaCl2, 5 mM KCl). The flow-through was centrifuged at 200 × g for 2 min. After removing as much supernatant as possible, the protoplast pellet was resuspended in 1 mL W5 solution and kept on ice for 30 min. The protoplasts were pelleted at 200 × g for 2 min and resuspended in 500 μL MCMG solution (4 mM MES pH 5.7, 0.4 M mannitol, 15 mM MgCl2). Then, 10 μL purified plasmid (10–20 μg) were mixed with 100 μL protoplasts before 110 μL PEG solution, namely 40% (w/v) PEG4000 in ddH2O conditioned for 5 min. After mixing by gently tapping the tube and incubated at room temperature for 10 min, the mixture was diluted in 500 μL W5 solution, mixed by vortexing, incubation period was implemented. The mixture was finally spun down and washed three times with washing buffer (50 mM MES pH 7.5, 150 mM NaCl). After washing, the resin was incubated with 100 μL of 1× SDS-PAGE loading buffer, and then heated at 80°C for 10 min. Proteins were separated by SDS-PAGE and then transferred to PVDF membrane. After transfer, the proteins were detected by immunoblotting with anti-CTI1 or anti-α-CT antibody. A horseradish peroxidase-conjugated secondary antibody was used and the horseradish peroxidase activity was detected by enhanced chemiluminescence (ECL) western blotting substrate (ThermoFisher; Cat. No. 32106). Ponceau S Staining was used as a loading control.

**Col-immosi precipitation.** Crude chloroplasts were isolated from 4-week-old Col-0 plants. Around 5 g of fresh leaves was ground in 30 mL ice-cold isolation buffer (50 mM HEPES-KOH pH 7.6, 150 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 1 mM 2-mercaptoethanol, 1% (v/v) Triton X-100, 1X complete protease inhibitor cocktail). The crude chloroplasts were isolated from 5 g of 4-week-old Col-0 leaf tissues. The proteins were isolated by SDS-PAGE and blotted to a PVDF membrane. The BCP, α-CT and β-CT subunits of plastidic HACase were immunologically detected with detected antisera18. A horseradish peroxidase-conjugated secondary antibody was used and the horseradish peroxidase activity was detected by enhanced chemiluminescence (ECL) western blotting substrate (ThermoFisher; Cat. No. 32106). Ponceau S Staining was used as a loading control.
culture hood. To permeabilize protoplasts, a 15 min treatment with 0.5% (v/v) Triton X-100 buffered with PBS-GM was carried out. After three washes with PBS-GM, non-specific binding was blocked with 1% bovine serum albumin in PBS-GM for 1 h. Protoplasts were then incubated overnight at 4 °C with rabbit anti-E3 antibodies19 (1:500) followed by 1 h incubation at room temperature with rat anti- 

HA antibodies (1:2000; Roche). Cells were washed for 20 min in PBS-GM, and incubated with goat anti-rabbit immunoglobulin G-Alexa Fluor 568 (1:1000; Molecular Probes) and with goat anti-mouse immunoglobulin G-Alexa Fluor 488 (1:100; Molecular Probes) or with goat anti-mouse immunoglobulin G-Alexa Fluor 488 (1:500; Molecular Probes) for 1 h. Finally, cells were washed for 20 min in PBS and mounted using Vestashield mounting solution (Vector Laboratories). Cells were observed with an inverted spectral confocal laser microscope (LEICA SP2 AOBS). Preparation of embryos excised from maturing seeds was similar to that of protoplasts except for the permeabilization treatment: cell walls were partially digested by incubating embryos in 2% (w/v) daiselose (Sigma) in PBS-GM for 45 min, then membranes were permeabilized by a 1 h treatment with 10% (v/v) DMSO and 3% (v/v) NP-40 in PBS-GM.

Lipid and fatty acid analyses. To analyze polar lipids and triacylglycerols, 500 mg of leaves were harvested and stored at −80 °C prior to lipid extraction. Leaf samples were ground in 7.2 mL of precooled chloroform/methanol/formic acid (10:10:0.5, v/v/v) and incubated at -20 °C overnight. The mixture was centrifuged at 7500 × g for 10 min at 4 °C, the lower phase containing lipids was collected and evaporated with a stream of N2. Lipids were resuspended in chloroform/methanol (2:1, v/v) and separated on thin-layer chromatography plates developed with hexane/diethylether/acetic acid (35:15:0.1, v/v/v). Polari lipids and triacylglycerols were visualized under UV light by staining with sprayed primuline. Lipid spots were ultimately collected and analyzed by gas chromatography48.

To analyze total fatty acids in leaves or seeds, total lipids were transmethylated into fatty acid methyl esters (FAMEs). FAMEs were analyzed by a Hewlett Packard 6890 gas chromatograph. Leaves from 4-week-old plants were collected and dried by centrifugation SpeedVac overnight. Mature samples were harvested and dried at room temperature for 1 week prior to analysis.

Lipidomics. Lipid extracts were dissolved in chloroform, and appropriate amounts of internal standards were introduced. The lipids were detected by triple quadrupole mass spectrometer (Applied Biosystems API 4000) with an autosampler (LC Mini PAL; CTC Analytics)35. Data processing was performed in a Lipidome DB Data Calculation Environment (http://lipidome.bcf.uu.edu/9000/Lipidomics) with normalization to internal standards.

In vivo acetate labeling. In vivo labeling experiments were performed according to previous report49. Leaves from 4-week-old plants were cut into strips after measuring the fresh weight. The leaf strips were transferred into 2 mL of reaction buffer (20 mM MES pH 5.5, half MS, 0.01% (v/v) Tween 20) in a 6-well plate. The labeling assays were started by the addition of [1-14C]-acetate or 1 μM haloxyfop, and 1 μM 12 N HCl. The reaction solutions were transferred to scintillation vials with a filter paper at the bottom of the vial. Samples were dried at 80 °C for 1 h, after which 1 mL of scintillation cocktail was added and radioactivity counted by liquid scintillation. Controls without acetyl-CoA were included to determine and subtract non-specific background rates.

GUS assay. For histochemical detection of GUS activity, tissues were incubated in 0.1 M phosphate buffer, pH 7.2 containing 2 mM 5-bromo-4-indoly-β-D-glucuronic acid, 0.01% (v/v) Triton X-100, 10 mM Na-EDTA, and 0.2 (Arabidopsis vegetative organs, flowers, and seeds) or 2 mM (Arabidopsis embryos and N. benthamiana leaf disks) each of potassium ferricyanide and potassium ferrocyanide. A vacuum was applied for 1 h before incubating the samples for 4 h (Arabidopsis embryos and N. benthamiana leaf disks) or 15 h (Arabidopsis vegetative organs, flowers, and seeds) at 37 °C in the dark, and chlorophyll was finally removed by room temperature incubation in 70% (v/v) ethanol (vegetative tissues and flowers only).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.
