ABI4 Activates *DGAT1* Expression in Arabidopsis Seedlings during Nitrogen Deficiency[1[C][W][OA]]

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Triacylglycerol (TAG) is the major seed storage lipid and is important for biofuel and other renewable chemical uses. Acyl-coenzyme A:diacylglycerol acyltransferase1 (DGAT1) is the rate-limiting enzyme in the TAG biosynthesis pathway, but the mechanism of its regulation is unknown. Here, we show that TAG accumulation in Arabidopsis (*Arabidopsis thaliana*) seedlings increased significantly during nitrogen deprivation (0.1 mM nitrogen) with concomitant induction of genes involved in TAG biosynthesis and accumulation, such as *DGAT1* and *OLEOSIN1*. Nitrogen-deficient seedlings were used to determine the key factors contributing to ectopic TAG accumulation in vegetative tissues. Under low-nitrogen conditions, the phytohormone abscisic acid plays a crucial role in promoting TAG accumulation in Arabidopsis seedlings. Yeast one-hybrid and electrophoretic mobility shift assays demonstrated that ABSCISIC ACID INSENSITIVE4 (ABI4), an important transcriptional factor in the abscisic acid signaling pathway, bound directly to the CE1-like elements (CACCG) present in *DGAT1* promoters. Genetic studies also revealed that TAG accumulation and *DGAT1* expression were reduced in the *abi4* mutant. Taken together, our results indicate that abscisic acid signaling is part of the regulatory machinery governing TAG ectopic accumulation and that ABI4 is essential for the activation of *DGAT1* in Arabidopsis seedlings during nitrogen deficiency.

Triacylglycerol (TAG) is an important renewable resource for biofuel production. It is the major form of carbon (C) reserves that accumulate mainly in plant seeds. When the seed germinates, TAG is degraded and converted to sugars that support early seedling growth immediately after germination. Although TAG is typically synthesized during seed maturation, a considerable amount of TAG may also accumulate in leaves or other vegetative tissues in response to certain abiotic stresses. For instance, galactolipids and phospholipids are converted to TAG in plant leaves during senescence, drought, or oxidative stress (Sakaki et al., 1990a, 1990b, 1990c; Kaup et al., 2002). Moreover, nutrient conditions also influence TAG content in plant leaves. Nitrogen (N) and C are important nutrients and signals for plants. CN availability affects post-germination growth, chloroplast lipid metabolism, and TAG content in Arabidopsis (*Arabidopsis thaliana*) seedlings, demonstrating that nutrient supply is important for the regulation of lipid composition and turnover in plant leaves (Martin et al., 2002; Gaude et al., 2007).

There is a close connection between the "stress hormone" abscisic acid (ABA) and TAG metabolism. During seed maturation, ABA promotes the accumulation of stored reserves and inhibits lipid breakdown in the embryo (Phillips et al., 1997; Brocard-Gifford et al., 2003). ABSCISIC ACID INSENSITIVE (ABI) transcription factors are essential for TAG metabolism. *ABI4* encodes an Activator Protein-2/Ethylene-Responsive Factor transcription factor that binds the CE1-like element (CACCG) present in many ABA- and sugar-responsive promoters (Finkelstein et al., 1998; Niu et al., 2002; Acevedo-Hernández et al., 2005). ABI4 is a crucial determinant of ABA sensitivity during TAG breakdown in the embryo (Penfield et al., 2006). *ABI3* and *ABI5*, which encode B3 and basic-region Leu zipper-type transcription factors, respectively, participate in the repression of TAG degradation by close association with the N-end rule components PRT6 and ATE (Holman et al., 2009). In addition to the inhibitory effect on TAG degradation in plant seeds, ABA is also important for TAG accumulation. In response to ABA treatment, ABI3 induces oleosin expression, which is essential for TAG accumulation and oil body stability (Zou et al., 1995; Crowe et al., 2000). In Arabidopsis seedlings, Glc and ABA actively regulate the transcription of *ACYL-COENZYME A:DIACYLGLYCEROL ACYLTRANSFERASE1* (*DGAT1*), which is crucial for TAG biosynthesis (Lu et al., 2003), although the exact molecular mechanism has not been determined.

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DGAT1, which catalyzes the final acylation step of *sn*-1,2-diacylglycerol to TAG, is thought to be the rate-limiting enzyme of TAG biosynthesis (Ichihara et al., 1988). Arabidopsis *dgat1* mutant seeds only accumulate about 55% to 75% of TAG (Routaboul et al., 1999; Zou et al., 1999; Kaup et al., 2002), whereas seed-specific overexpression of *DGAT1* increases oil content from 11% to 28% (Jako et al., 2001; Taylor et al., 2009; Andrianov et al., 2010). *DGAT1* is also important for TAG accumulation in leaves (Slocombe et al., 2009). For instance, *DGAT1* is up-regulated in senescing leaves, correlating with the plastid fatty acid partition into TAG (Kaup et al., 2002). Leaf-specific expression of *DGAT1* in transgenic tobacco (*Nicotiana tabacum*) resulted in a 20-fold increase in TAG accumulation in leaves, and the total fatty acids also increased 2-fold up to 5.8% dry weight (Andrianov et al., 2010). In addition to DGAT1, PHOSPHOLIPID:DIACYLGLYCEROL ACYLTRANSFERASE1 (PDAT1), which catalyzes the acyl-CoA-independent synthesis of TAG, also contributes to seed oil biosynthesis in Arabidopsis (Zhang et al., 2009). Although neither the T-DNA insertion nor the overexpression of *PDAT1* in Arabidopsis alters the seed oil content (Ståhl et al., 2004; Mhaske et al., 2005), RNA interference silencing of *PDAT1* in the Arabidopsis *dgat1* background results in a 63% decrease in oil content compared with the *dgat1* control (Zhang et al., 2009), which indicates that *PDAT1* is the gene responsible for most of the TAG synthesis in the *dgat1* mutant.

Despite extensive reports mentioning the accumulation of storage oil in leaves, none of these studies have addressed the mechanisms or the factors that regulate the expression of key genes in TAG metabolism. In this study, we systemically analyzed the storage oil content and the expression levels of TAG biosynthesis genes in Arabidopsis seedlings grown under different N and C treatments and established an N limitation medium to highly induce storage oil accumulation in Arabidopsis leaves. Our results show that seedling TAG content was highest on Murashige and Skoog (MS) medium containing 0.1 mM N and 50 mM Suc. To our knowledge, this is the first report showing that high CN medium significantly induced genes involved in TAG biosynthesis, such as *DGAT1* and *OLEOSIN1*, in 7-d-old Arabidopsis seedlings. Furthermore, phytohormone ABA played a crucial role in mediating the inhibitory effect of N on TAG biosynthesis in Arabidopsis seedlings. Additionally, ABI4 regulated *DGAT1* transcription under low-N conditions by directly binding CE1-like elements located near the transcription start site. Our study demonstrates a regulation mechanism of *DGAT1* in Arabidopsis seedlings.

RESULTS

Storage Oil Accumulates in Arabidopsis Seedlings during N Deprivation

To investigate the influence of N on TAG accumulation in Arabidopsis seedlings, wild-type genotype Columbia (Col-0) seeds were grown for 7 d on MS medium containing 0, 0.01, 0.1, 1, 3, 6, 30, or 60 mM total N without sugar. Total lipid was extracted and analyzed by thin-layer chromatography (TLC). Storage oil was only detected for 0.1 mM or less, with the highest TAG content at 0.1 mM N (Fig. 1A). To further examine the role of N and C on TAG accumulation, 50 and 100 mM Suc were added to the MS medium containing 0.1 or 60 mM N. All the seedlings in 60 mM N showed cotyledon expansion and greening within 7 d after sowing. However, the provision of Suc to 0.1 mM N medium caused a stunted growth phenotype (Fig. 1B). The 7-d-old seedlings were stained with Nile Red and observed by confocal microscopy (Fig. 1C). All seedlings in 60 mM N had no oil droplets, whereas seedlings in 0.1 mM N showed various increases in TAG levels, with the highest level at 50 mM Suc (i.e. 0.1–50 medium). We further confirmed the TAG contents by TLC of total lipid extracted from the seedlings (Fig. 1D). Taken together, N deficiency caused an increase in TAG content in Arabidopsis seedlings, and the addition of Suc led to further enrichment of storage oil TAG.

N Deficiency Induces the Expression of TAG Biosynthesis Genes in Arabidopsis Seedlings

To determine whether the increased TAG level in N-limited medium resulted from incomplete storage oil

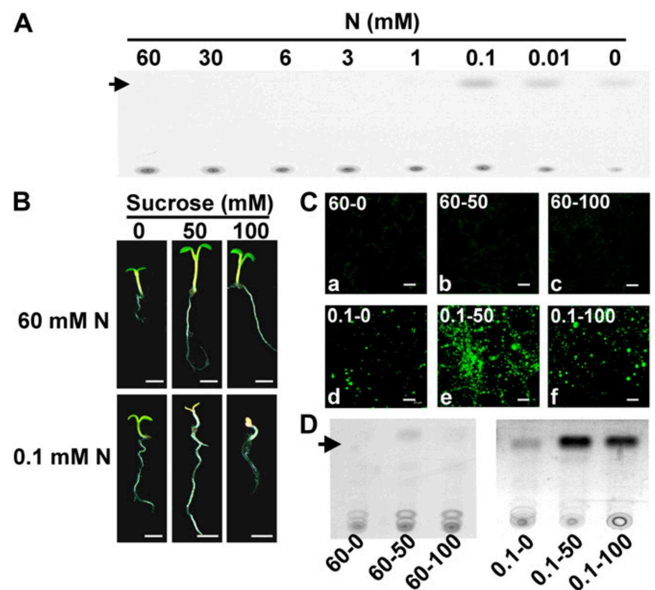

Figure 1. Accumulation of storage oil in seedlings for different CN treatments. A, TAG content of 7-d-old Arabidopsis seedlings in Suc-free medium containing different N concentrations. Arabidopsis seedlings were grown in a growth chamber under a cycle of 16 h of light at 22°C and 8 h of dark at 20°C. Total lipids were extracted and separated by TLC. The arrow indicates the TAG bands. B, Phenotypes of 7-d-old Arabidopsis seedlings in different CN medium. Bars = 2 mm. C, Oil bodies in Arabidopsis cotyledons of seedlings grown in different CN medium. Bars = 20 μm. D, Total lipid was extracted from 7-d-old seedlings grown on different CN medium to determine the TAG content (arrow) by TLC.

degradation or induction of TAG biosynthesis, we isolated RNA from 7-d-old seedlings grown on different CN medium and used quantitative real-time reverse transcription (RT)-PCR to examine the transcription levels of genes involved in TAG metabolism, with *ACTIN1* as an internal control (Fig. 2A). N deficiency led to the induction of TAG biosynthesis genes, such as *DGAT1*, *DGAT2*, and *PDAT1*, in Arabidopsis seedlings. At every Suc concentration, the transcription levels of these genes in 0.1 mM N seedlings were much higher than that in 60 mM N. Additionally, we also found that Suc actively regulates TAG biosynthesis gene expression in an N-dependent way. On the 60 mM N medium, different Suc concentrations had little effect on the transcription profile of *DGAT1*, *DGAT2*, and *PDAT1*. In contrast, under 0.1 mM conditions, all of these genes were induced by Suc, with maximum induction at 50 mM Suc. *DGAT1* expression was enhanced nearly 20 fold in 0.1–50 medium compared with the 60–0 medium, whereas the expression of *DGAT2* and *PDAT1*, which also catalyze the final step of TAG biosynthesis, increased 2.5- and 5-fold, respectively. Genes involved in the production of seed-specific oil bodies, such as *OLEOSIN1*, also showed a strong response to Suc under low-N conditions. In addition to the TAG biosynthetic pathway, we also analyzed the expression of genes involved in storage lipid degradation. *Sugar-Dependent1* (*SDP1*) encodes a TAG lipase in Arabidopsis that catalyzes the initial step in oil breakdown (Eastmond, 2006). Fatty acyl-CoA oxidase (*ACX*) catalyzes the first step of β-oxidation, which subsequently breaks down the free fatty acids released from TAG (Adham et al., 2005; Pinfield-Wells et al., 2005). All of these genes were also induced by low N (Fig. 2A), indicating the accelerated TAG breakdown during N deprivation. Thus, the oil bodies in seedlings grown on 0.1 mM N medium were likely caused by the induction of TAG biosynthesis and accumulation.

To further test the influence of N concentration on ectopic biosynthesis of TAG, seedlings were grown on MS medium containing 60 mM N for 7 d and then transferred to 0.1 mM N for another 7 d, at which time Nile Red was used to visualize the seedling oil bodies. Seedlings grown in both 60–50 and 60–100 medium for 7 d had no oil droplets in cotyledons (Fig. 2B, a and b). However, after transferring to low N for another 7 d, storage oil bodies appeared in cotyledons (Fig. 2B, c and d). All these results indicated that the oil bodies observed under 0.1–50 or 0.1–100 medium were formed by ectopic synthesis of TAG in seedlings rather than slow degradation. Thus, the 0.1–50 medium provides a suitable growth system for inducing TAG accumulation in Arabidopsis seedlings.

ABA Regulates N-Dependent TAG Accumulation in Arabidopsis Seedlings

To investigate the mechanism of ectopic TAG biosynthesis in Arabidopsis seedlings, we used the 0.1–50

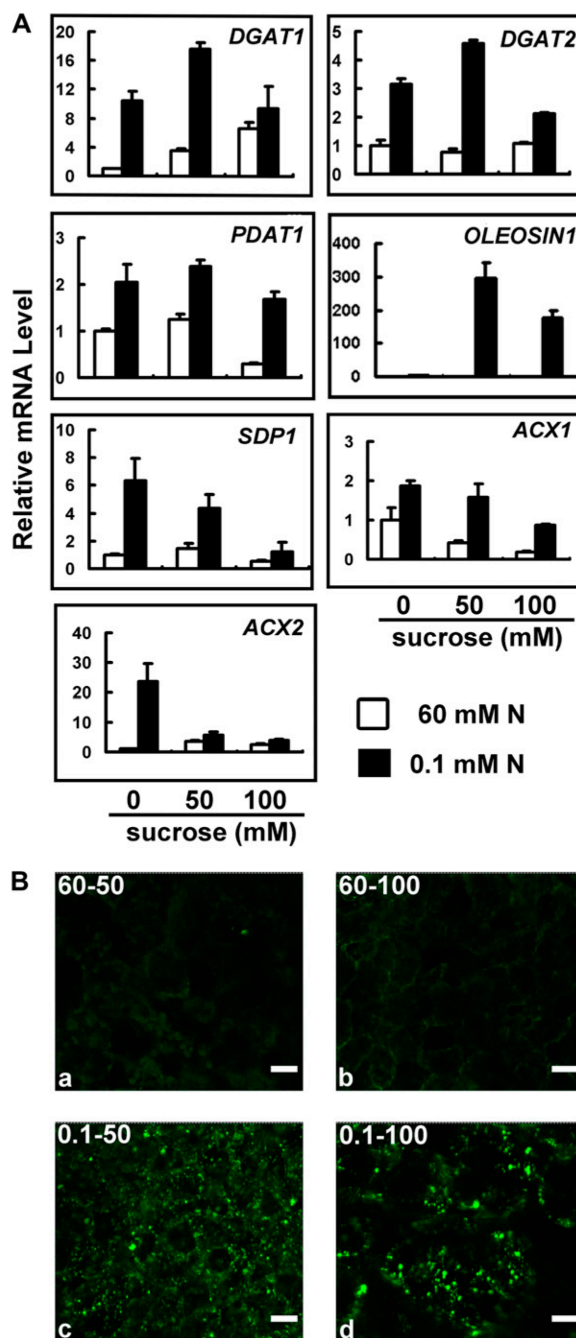

Figure 2. TAG biosynthesis is induced with 0.1 mM N. A, Total RNA was isolated from 7-d-old seedlings grown on medium with 0.1 and 60 mM N and different Suc concentrations. Relative mRNA levels of key genes in storage oil metabolism were determined by quantitative real-time RT-PCR using *ACTIN1* as an internal control. Data represent three independent experiments, and the error bars represent SD. *DGAT1*, *DGAT2*, and *PDAT1* are genes in the TAG biosynthesis pathway. *OLEOSIN1* is essential for the production of seed-specific oil bodies. *SDP1*, *ACX1*, and *ACX2* are genes involved in TAG degradation. B, Oil bodies in seedlings grown on 60 mM N for 7 d and then transferred to 0.1 mM N medium for another 7 d (c and d). Plants grown for 7 d on the 60–50 medium were used as controls (a and b). Seedlings were stained with Nile Red. Bars = 20 μm. [See online article for color version of this figure.]

medium to induce the highest TAG content in seedlings, with the 60–50 medium as a control. Seeds were sown directly on the 0.1–50 or 60–50 medium, and the 7-d-old seedlings were used to examine the connection between hormone signaling and TAG biosynthesis. Seedlings were grown on the 60–50 medium containing 10 μM cytokinin, GA_3, auxin, ethylene, or ABA for 7 d. Only ABA induced yellow and smaller cotyledons, which resembled those resulting from N limitation, whereas other hormone-treated seedlings were green (Fig. 3A). In addition, we used TLC to analyze the TAG content of 7-d-old seedlings grown on 60–50 or 0.1–50 medium with different hormone treatments. Only ABA triggered additional TAG accumulation in seedlings on both 0.1 and 60 mM N medium (Fig. 3B; Supplemental Fig. S1), whereas the TAG content in other hormone-treated seedlings was not significantly increased. Hence, N regulation of early seedling development and ectopic biosynthesis of storage oil were closely associated with ABA.

We further examined the transcription levels of key genes involved in ABA biosynthesis and signaling under low-N conditions (Fig. 3C). Compared with seedlings grown on 60–50 medium, genes in both the ABA biosynthesis and signaling pathways were significantly induced in the 0.1–50 medium. *NCED3* (*9-cis-epoxycarotenoid dioxygenase3*) and *ABI3*, which are involved in ABA biosynthesis and signaling, respectively, were enhanced nearly 25-fold, whereas *ABI4* and *ABI5*, which are also important for ABA signaling, increased more than 100-fold. Because ABA has inhibitory effects on seed germination and early seedling development (Lopez-Molina et al., 2001, 2002), we observed the growth arrest phenotype in 0.1 mM N medium by examining cotyledon expansion and greening. Under sugar-free conditions, all seedlings had green cotyledons within 7 d after sowing. Following the addition of Suc, however, some of the seedling cotyledons became yellow and the true leaves rarely grew. The postgermination growth rate in 0.1 mM N was much lower than that on 60 mM N seedlings. Only less than 40% of seedlings turned green with the 0.1–50 medium, and no seedlings turned green on the 0.1–200 medium (Fig. 3D). In contrast, all the seedlings on the 60–200 medium grew normally. We also analyzed the seed germination rate on different CN medium. After 24 h of growth in light, seed germination was determined as radicle emergence from the seed coated. The germination rate did not differ significantly between 0.1 and 60 mM N at every Suc concentration (Fig. 3E).

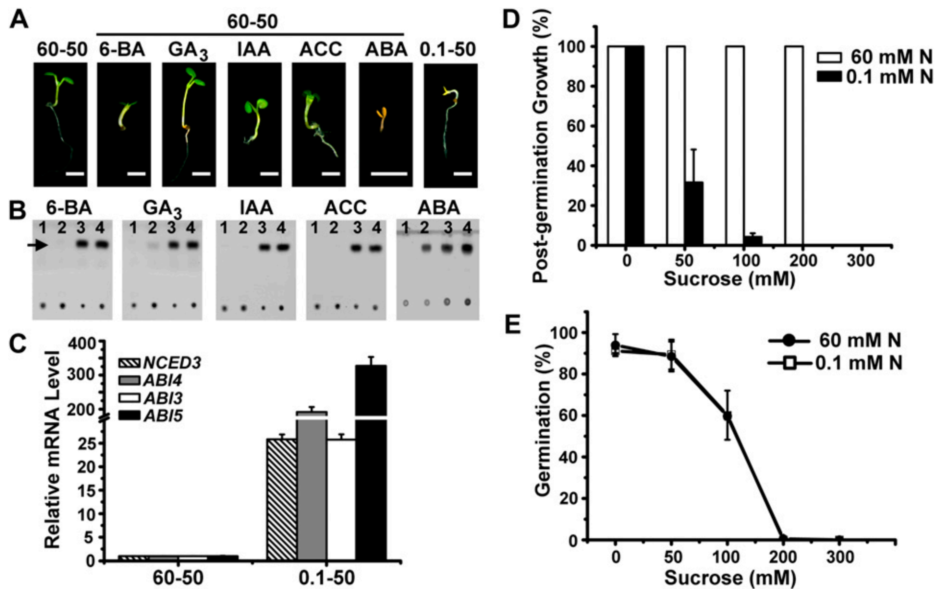

Figure 3. Influence of ABA on ectopic accumulation of storage oil during N deprivation. A, Phenotypes of 7-d-old seedlings on 60–50 medium without or with 10 μM final concentrations of different plant hormones. The 7-d-old seedling grown on 0.1–50 medium was chosen as a control. Bars = 2 mm. B, TAG content (arrow) of 7-d-old Arabidopsis seedlings grown on 60–50 (lanes 1 and 2) or 0.1–50 (lanes 3 and 4) medium in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 10 μM final concentrations of 10 μM cytokinin (6-BA), GA_3, auxin (IAA), ethylene (ACC), or ABA. Total lipid was extracted and separated by TLC. C, Expression levels of key genes involved in ABA biosynthesis and signaling in 7-d-old seedlings grown on 60–50 or 0.1–50 medium. Relative mRNA levels were determined by quantitative real-time RT-PCR using *ACTIN1* as an internal control. Data represent three independent experiments, and the error bars represent SD. D, Percentage of postgermination growth, defined as cotyledon expansion and greening, in 7-d-old seedlings grown on different CN medium. About 100 seeds were used in each experiment. Data represent three independent experiments, and average values are shown with SD. E, Germination rate of Arabidopsis seeds sown on MS medium with different CN concentrations. The percentage of germination (radicle emergence) was determined after 24 h of growth in light. About 100 seeds were used in each experiment. Data represent three independent experiments, and the error bars represent SD. [See online article for color version of this figure.]

ABI4 Is Essential for the Activation of *DGAT1* Expression in Tobacco

Many studies have shown that Arabidopsis *DGAT1* is important for TAG biosynthesis, whereas *DGAT2* is not a major determining factor (Shockey et al., 2006). In addition, although *PDAT1* and *DGAT1* have overlapping functions for TAG synthesis in Arabidopsis (Zhang et al., 2009), the transcription profile of *PDAT1* changed less than that of *DGAT1* when N concentration was reduced. We thus focused our further study on the key factors that regulate *DGAT1* expression under low-N conditions. By analyzing the 1-kb *DGAT1* promoter sequence upstream of the ATG start codon, two putative CE1-like elements (CACCG) that function as the core ABI4-binding site were found at –55 and +88 from the transcription start site (Fig. 4A). We predicted that the induction of *DGAT1* transcription in 0.1–50 medium is dependent on ABI4. A tobacco transient expression assay was used to investigate the interaction between ABI4 and the *DGAT1* promoter. The reporter plasmids pD1000:GUS and pD117:GUS and the effector plasmid 35S:ABI4 were constructed (Fig. 4B). When *Agrobacterium tumefaciens* cells carrying the reporter plasmids were injected separately into tobacco leaves, no GUS expression was detected. When these constructs were injected together with 35S:ABI4, GUS induction was detected within 48 h after injection (Fig. 4C). Coexpression with ABI4 showed about 25-fold activation of pD1000:GUS and pD117:GUS reporters (Fig. 4D). These results suggested that ABI4 actively regulated the expression of *DGAT1*, and the –117 to +230 promoter region of *DGAT1* was sufficient to trigger the ABI4-induced activation of GUS in tobacco cells.

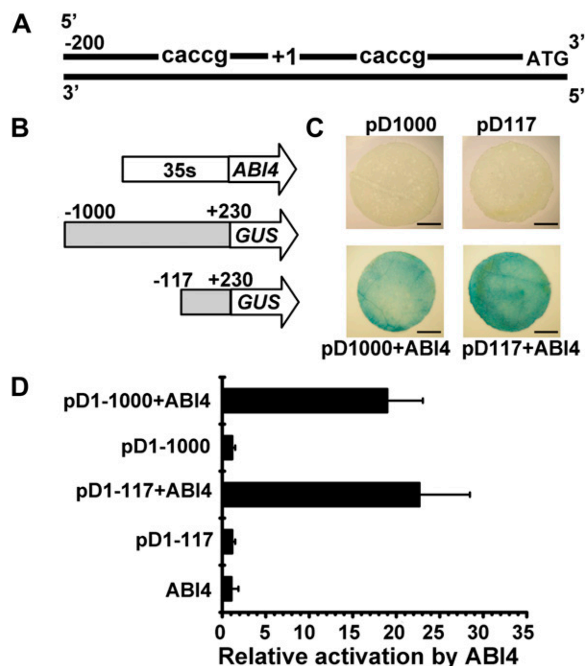

Figure 4. Tobacco transient assay for the interaction between ABI4 and the *DGAT1* promoter. A, Analysis of the Arabidopsis *DGAT1* promoter sequence, showing the core sequence of the CE1-like element. B, Schematic of the ABI4 plant expression constructs and the 5′ deletion of the *DGAT1* promoter used in the tobacco transient assay. The effector plasmid contained the cauliflower mosaic virus 35S promoter fused to *ABI4* cDNA. The reporter plasmid contained the *DGAT1* −1,000 to +226 or −117 to +226 promoter region fused to the *GUS* gene. C, Histochemical GUS assays of *Agrobacterium* infiltrated with different constructs. Bars = 5 mm. D, Relative GUS activity directed by pD1000 and pD117 alone or together with 35S:ABI4. Data represent three independent experiments, and the error bars represent SD. [See online article for color version of this figure.]

ABI4 Binds to the CE1-Like Elements in the *DGAT1* Promoter

To determine the interaction between the *DGAT1* promoter and ABI4 in yeast, the –63 to +102 *DGAT1* promoter region containing two CE1-like elements was used as bait in the yeast one-hybrid system. Yeast transformed with both the *DGAT1* promoter and *ABI4* had markedly higher β-galactosidase activity (Fig. 5A). Furthermore, the leaky His$^+$ phenotype of yeast transformants with the *DGAT1* promoter region was suppressed by increasing the concentration of the His synthase inhibitor, 3-aminotriazole, in the His$^-$ synthetic dextrose (SD) medium. When ABI4 was transformed together with the *DGAT1* promoter into yeast, they grew well in the 45 mM 3-aminotriazole His$^-$ SD medium (Fig. 5B). These results indicated that ABI4 bound to the promoter region of *DGAT1* and activated its expression in yeast cells. An electrophoretic mobility shift assay was used to examine the direct interaction between ABI4 and the *DGAT1* promoter. The –63 to +102 promoter region of *DGAT1* containing the CE1-like elements was used as the probe (Fig. 5C). Recombinant ABI4 fused with glutathione *S*-transferase (GST) was purified from *Escherichia coli*. The probe was incubated with and without the purified ABI4 protein and separated on a native polyacrylamide gel. Recombinant ABI4 bound to the 165-bp *DGAT1* promoter region, and the protein-DNA binding was dependent on the presence and the concentration of ABI4 (Fig. 5D). A 50-fold molar excess of unlabeled *DGAT1* promoter fragment was sufficient to compete for ABI4 binding. To further examine the binding specificity of the CE1-like elements, the CE1-like elements were mutated (mCE1), and the 50-fold molar excess of unlabeled mutant fragment acted as an efficient competitor (Fig. 5D). Our results demonstrated that ABI4 bound to the two CE1-like elements located upstream of *DGAT1*.

ABI4 Actively Regulates *DGAT1* Transcription in Arabidopsis Seedlings in Response to N Deprivation and ABA

To further study the interaction between ABI4 and the *DGAT1* promoter, the TAG contents in *abi4* and Col-0 were tested by TLC. Seedlings were grown for 7 d on 60–50 and 0.1–50 medium in the presence or

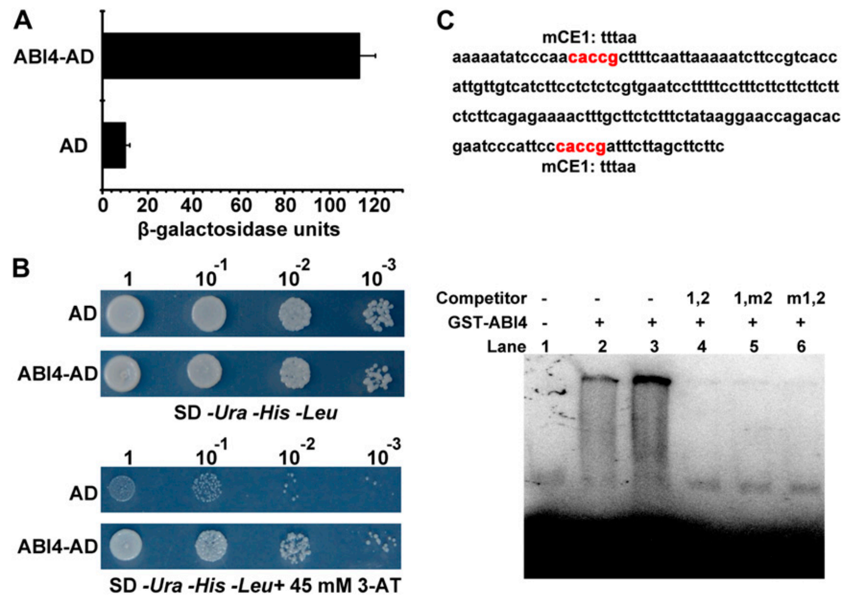

Figure 5. Interaction between *DGAT1* CE1-like elements and ABI4. A, Interaction of ABI4 and the *DGAT1* promoter in yeast cells. The β-galactosidase activity indicates the LacZ expression level. Data represent three independent experiments, and the error bars represent SD. B, Growth of yeast cells on 45 mM 3-aminotriazole (3-AT) His$^-$ SD medium. Cells were grown in liquid medium to an optical density at 600 nm of 1.0. The numbers at the top indicate the dilutions. C, Electrophoretic mobility shift analysis of interactions between ABI4 and *DGAT1* CE1-like elements. The sequence of the 165-bp *DGAT1* fragment is shown at the top. Labeled CE1-CE1 *DGAT1* promoter sequence was incubated with 20 ng (lane 2) or 100 ng (lane 3) of purified ABI4 protein, and the DNA probe incubated with GST served as the negative control (lane 1). Nonlabeled *DGAT1* promoter sequences CE1-CE1 (lane 4), mCE1-CE1 (lane 5), and CE1-mCE1 (lane 6) were used at a 50-fold molar excess as competitors. The numerals 1 and 2 indicate nonmutated sequences, whereas m1 and m2 indicate mutated sequences. [See online article for color version of this figure.]

absence of 10 μM ABA. Both Col-0 and *abi4* showed greening on 60–50 medium (Fig. 6A, a and e) and lost most TAG content in 7 d (Fig. 6B, a and e). In the 0.1–50 medium, vegetative growth of both Col-0 and *abi4* was inhibited, and the cotyledons did not turn green (Fig. 6A, c and g). Although the mutant seedlings exhibited a similar phenotype to wild-type seedlings grown in low-N medium, the TAG content in the *abi4* mutant was much lower (Fig. 6B, c and g). When 10 μM ABA was added to either 60–50 or 0.1–50 medium, the growth of Col-0 seedlings was arrested (Fig. 6A, b and d) and the accumulation of TAG was increased obviously (Fig. 6B, b and d), whereas the *abi4* seedlings showed a nonarrest phenotype (Fig. 6A, f and h) and the TAG content in the mutants was reduced remarkably (Fig. 6B, f and h). Hence, ABI4 plays an important role in mediating ABA-dependent activation of TAG accumulation during N deprivation. *DGAT1* promoter activity was examined by transforming the reporter construct pD1000:GUS into both Col-0 and *abi4* (Fig. 6C). GUS was expressed in the cotyledons, hypocotyls, and root tips of 60–50 7-d-old Col-0 seedlings, in accordance with the expression pattern of ABI4 (Bossi et al., 2009). On 0.1 mM N, GUS expression levels increased in cotyledons and hypocotyls. The addition of 10 μM ABA to either 60–50 or 0.1–50 medium significantly increased GUS activity. These observations confirmed that *DGAT1* expression was induced in response to low-N and ABA treatments. In *abi4*, cotyledons and hypocotyls had very low GUS expression for most treatments. On the 60–50 medium, GUS expression was only slightly reduced. On the 60–50 medium containing 10 μM ABA, GUS expression was confined to the hypocotyl and root region. On 0.1 mM N, GUS expression was very low in the presence or absence of ABA. These results demonstrated that ABI4 participated in the activation of *DGAT1* in response to ABA and low-N conditions. The transcription profile of *DGAT1* coincided with the changes in GUS activity for all growth conditions (Fig. 6D).

Because the expression of *ABI3* and *ABI5* increased significantly in 0.1–50 seedlings, we also analyzed the *DGAT1* transcription levels in *abi3* and *abi5* seedlings in different CN medium containing 10 μM ABA. We found that *DGAT1* mRNA levels were slightly reduced in both *abi3* and *abi5* seedlings grown on 0.1–50 medium (Fig. 6E). Hence, ABI3 and ABI5 may also participate in the transcriptional regulation of *DGAT1* during N deficiency. In addition, previous studies have shown that ABI3 is required for the regulation of *OLEOSIN1* in response to ABA (Crowe et al., 2000). To further determine the roles of ABI transcription factors in TAG accumulation at low N, we examined *OLEOSIN1* expression levels in *abi3*, *abi4*, and *abi5* mutant seedlings. On the 0.1–50 medium, the transcription level of *OLEOSIN1* in *abi3* mutants was reduced compared

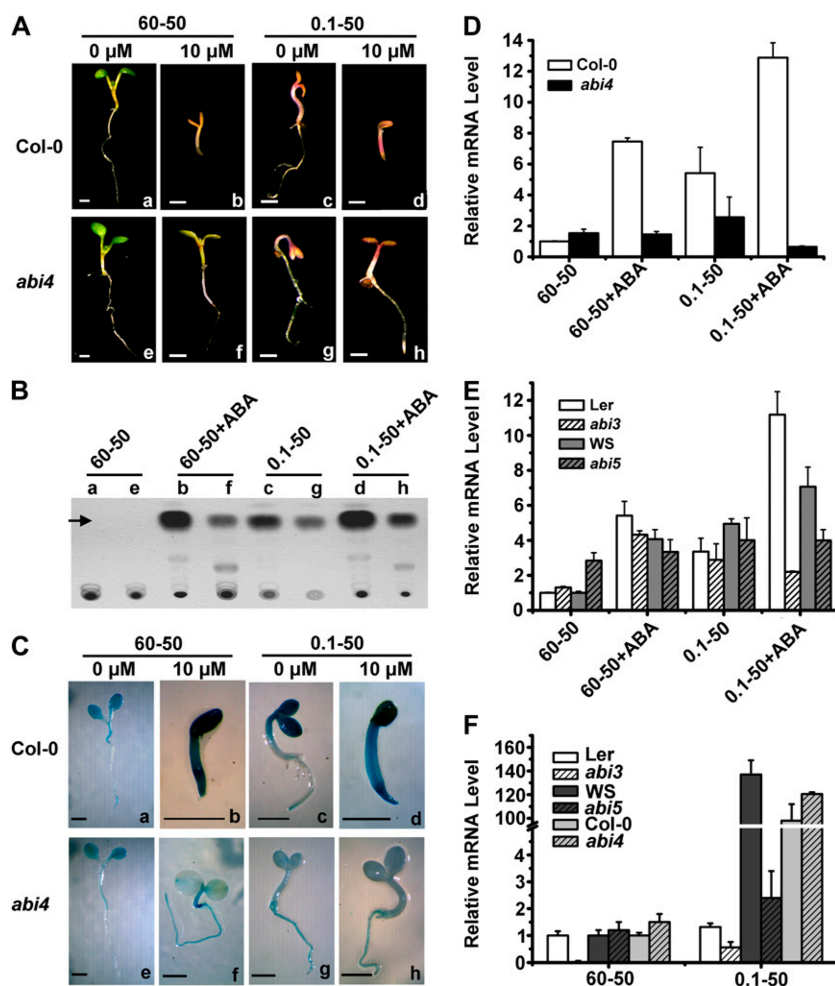

Figure 6. Analysis of the TAG content and the *DGAT1* expression levels in *abi* mutants. A to C, The 7-d-old Col-0 (a−d) and *abi4* (e−h) seedlings grown on 60–50 (a, b, e, and f) or 0.1–50 (c, d, g, and h) medium in the absence (a, c, e, and g) or presence (b, d, f, and h) of 10 μM ABA. Bars = 1 mm. A, The phenotypes of the seedlings. B, Total lipid was isolated from seedlings and analyzed by TLC to show TAG content (arrow). C, GUS expression of the *DGAT1* promoter in 7-d-old seedlings. D, The transcription level of *DGAT1* in 7-d-old Col-0 and *abi4* seedlings grown on 60–50 or 0.1–50 medium. E, Levels of *DGAT1* mRNA in 7-d-old *abi3*, *abi5*, and their wild-type (Landsberg *erecta* [Ler] and Ws) seedlings grown on 0.1–50 or 60–50 medium with or without 10 μm ABA. F, Levels of *OLEOSIN1* mRNA in 7-d-old *abi3*, *abi4*, *abi5*, and wild-type (Landsberg *erecta*, Col-0, and Ws) seedlings grown on 60–50 or 0.1–50 medium. Relative mRNA levels were determined by quantitative real-time RT-PCR using *ACTIN1* as an internal control. Data represent three independent experiments, and the error bars represent SD.

with wild-type seedlings. Additionally, we found that the *OLEOSIN1* transcription level in *abi5* was also reduced, but it did not change significantly in *abi4* (Fig. 6F). Therefore, ABI3 and ABI5 participated in the positive regulation of TAG accumulation by activating both TAG biosynthesis and accumulation pathways in N-deprived seedlings.

Taken together, ABA signaling is part of the regulatory machinery governing TAG ectopic accumulation under low-N conditions. The transcription of *ABI3*, *ABI4*, and *ABI5*, key genes involved in ABA signaling, was highly induced in the 0.1–50 medium. ABI4 positively regulates *DGAT1* transcription by binding the CE1-like promoter element during N deprivation. ABI3 and ABI5 also participate in TAG accumulation under low-N conditions by actively regulating *OLEOSIN1*, and they may also participate in TAG biosynthesis by positively regulating *DGAT1* expression.

DISCUSSION

N and C Are Tightly Coordinated during Plant Development and Oil Accumulation

N and C are essential nutrients and signals for plant growth. Their metabolism and signaling are tightly coordinated, which enables plants to grow in different environmental conditions. C is the photosynthetic product that provides energy and C skeletons for amino acid biosynthesis, and N is important for carbohydrates to be utilized for photosynthesis, protein synthesis, and plant growth (Stitt, 1999; Fritz et al., 2006). In our study, we found that N deprivation led to a hypersensitive response to sugar for seedling establishment. High sugar concentrations above the physiology range can arrest early seedling development, which is characterized by the absence of cotyledon greening and leaf formation (Dekkers et al., 2008). Under 60 mM N conditions, the growth-arrested phenotype was only detected in 300 mM Suc medium (Fig. 3D). In contrast, under 0.1 mM N conditions, 50 mM Suc was enough to block the early seedling development (Figs. 1B and 3D).

On the other hand, we also found that there is an antagonistic interaction between N and C in the regulation of TAG biosynthesis in Arabidopsis seedlings. The activation effect of low N was significantly enhanced by C. Similarly, the addition of Suc to N starvation medium also resulted in high oil production in *Brassica napus* seedlings (data not shown) and the microalga *Chlorella protothecoides* (Xu et al., 2006).

These results suggest that controlling TAG metabolism based on CN status is universal in both microalgae and higher plants.

There Is a Homeostasis between TAG Biosynthesis and Degradation in Plant Seedlings

Storage oil breakdown takes place immediately after seed germination and provides the C skeletons and energy that support seeding growth following germination (Quettier and Eastmond, 2009). Most of the oil bodies in Arabidopsis seedlings disappeared within 6 d after germination on 60 mM N (Siloto et al., 2006). Oil drops in seedlings often correlate with the inhibition of stored lipid breakdown. A previous study showed that simultaneously decreasing N concentration and adding exogenous sugar resulted in 80% of eicosenoic acid, a 20:1 fatty acid that is specific to seed oil, being maintained 6 d after germination, suggesting delayed degradation of storage oil (Martin et al., 2002). However, we found that genes involved in TAG biosynthesis and accumulation were increased when the N concentration was reduced from 60 to 0.1 mM. Our results suggest that TAG synthesis was induced during vegetative development under low-N conditions, which is in accordance with the observation that TAG and free fatty acids were increased when seedlings were grown on 65 mM N medium for 2 weeks and then transferred to 0.65 mM N for another 10 d (Gaude et al., 2007). Unexpectedly, the expression of lipases and key genes in β-oxidation was also increased with reduced N, indicating accelerated TAG and fatty acid breakdown in seedlings. Hence, storage oil in N-limited seedlings is caused by the induction of TAG biosynthesis rather than the inhibition of degradation.

Furthermore, we also found that the expression of key enzymes in the glyoxylate cycle and gluconeogenesis, such as malate synthase, isocitrate lyase, and phospho*enol*pyruvate carboxykinase, was highly induced in the N-limitation seedlings (Supplemental Fig. S2). These results showed that the newly synthesized TAG in the N-limited seedlings is broken down and converted to Suc. A previous study has proved that the anabolic and catabolic processes of TAG act in parallel during the yeast cell division cycle. Such TAG homeostasis is important for cell growth (Kohlwein, 2010). Here, we suggest that there is also a homeostasis between TAG biosynthesis and degradation in higher plants. Experimental evidence has indicated that extended darkness treatment of fatty acid breakdown mutants, such as *pxa1*, *cts2*, and *acx1acx2*, led to the ectopic accumulation of TAG in the leaf (Kunz et al., 2009; Slocombe et al., 2009). The TAG content in naturally senescing leaves of fatty acid breakdown mutants was also increased (Slocombe et al., 2009). These data demonstrated that in the wild-type plants, the ectopically accumulated TAG in leaves is broken down rapidly and proved that a TAG homeostasis exists in higher plants.

The Interaction between N and ABA Signaling

Phytohormones are vital to almost every aspect of plant development. N regulates the plant morphological changes by modulating hormone homeostasis and/or signaling (Vidal and Gutiérrez, 2008). Although ABA plays essential roles in plant development, including seed maturation, dormancy, germination, and root growth (De Smet et al., 2006), the interaction between N and ABA signaling remains poorly defined. Limited evidence shows that the inhibitory effect of high NO_3^- on lateral root development requires ABA signaling (Signora et al., 2001). Our study demonstrates that ABA is important for mediating TAG biosynthesis and postgermination arrest in Arabidopsis seedlings during N starvation. Similar to ABA-treated seedlings (Lopez-Molina et al., 2001; Dekkers et al., 2008), the cotyledons of seedlings grown in 0.1 mM N were small and yellow, and storage oil accumulated ectopically. However, ABA-treated and N-starved seedlings have some different phenotypes. In comparison with ABA-arrested seedlings, significant root growth was observed in N-deprived conditions (Fig. 3A), indicating that the N-dependent regulation of plant leaf and root development was probably not mediated by the same mechanism. In addition, unlike the inhibitory effect of ABA on seed germination, N deprivation did not affect the seed germination rate at every concentration of Suc (Fig. 3E). We suppose that the N-dependent regulation of seed germination and early seedling development may be mediated by a different mechanism.

ABI4 Activates *DGAT1* Transcription by Directly Binding the CE1-Like Elements during N Deficiency

Previous studies have shown that Glc and ABA actively regulate *DGAT1* transcription in 3-d-old Arabidopsis seedlings (Lu et al., 2003). However, the molecular mechanisms are unclear. Our study showed that ABI4 directly bound the CE1-like element in the *DGAT1* promoter and actively regulated *DGAT1* expression in the 0.1–50 medium. We also identified CE1-like elements upstream of genes involved in fatty acid synthesis, TAG biosynthesis, and TAG degradation that are listed in the Arabidopsis lipid gene database (Beisson et al., 2003). Twenty-four genes, including *DGAT2*, *OLEOSIN2*, and *OLEOSIN4*, contained more than one CE1-like element in either strand for 1,500 bp upstream of the start codon (Supplemental Fig. S3), which indicated that *ABI4* may play an important role in the TAG metabolic pathway.

In addition to ABI4, ABI3 and ABI5 may also participate in transcriptional regulation of *DGAT1* during N deficiency. Two RY (CATGCA) motifs, which are essential targets of ABI3 or other B3 domain transcription factors (Mönke et al., 2004), are found at –1,799 and –67 of the *DGAT1* promoter in the reverse orientation (Lu et al., 2003). Hence, *ABI3* may regulate *DGAT1* expression by binding the RY motifs. In contrast, the *DGAT1*

promoter sequence has no ABI5-binding site within 1.5 kb upstream of the ATG start codon, indicating that ABI5 may not interact directly with the *DGAT1* promoter. All these results indicate that the regulation mechanism of *DGAT1* in response to low N is likely to be complex.

Control of TAG Accumulation by CN Availability Can Be Used for High-Yield Oil Production

Storage oil can be produced in plant vegetative tissue (Durrett et al., 2008). Because of their high biomass potential, plant leaves have promising potential for biofuel production (Durrett et al., 2008). There are two approaches to accumulate TAG in vegetative tissues. First, many key genes involved in TAG metabolism are used to increase oil content by genetic engineering. Accumulation of TAG in leaves has been observed in certain Arabidopsis mutants, such as the patatin-like lipase *sdp1* (Eastmond, 2006), the ATP-binding cassette transporter *cts* (Footitt et al., 2007), and the plastid lipid permease *tgd1* (Xu et al., 2005), but their effects were limited. Second, ectopic expression of transcription factors responsible for embryo development, such as *LEAFY COTYLEDON1* or *LEAFY COTYLEDON2*, caused more than 100-fold increase of storage lipid in Arabidopsis leaves (Santos Mendoza et al., 2005; Mu et al., 2008). However, some of these transgenic seedlings did not survive (Mu et al., 2008), thus limiting the applicability of this approach. The fact that CN status plays a major part in regulating oil accumulation offers a further opportunity to produce oil in nonseed tissues. C and N concentrations can be easily controlled, resulting in high oil production. Hence, the CN platform is useful for improving oil production in oil crops.

CONCLUSION

In summary, an N limitation medium is established to highly induce storage oil accumulation in Arabidopsis leaves. Seedling TAG content and genes involved in TAG biosynthesis are induced in 0.1 mM N, with the highest level on MS medium containing 0.1 mM N and 50 mM Suc. Furthermore, the phytohormone ABA plays a crucial role in promoting TAG accumulation in N-limited seedlings. Additionally, ABI4 activates *DGAT1* transcription by binding the CE1-like promoter element during N deprivation. Our study describes the regulation mechanism of *DGAT1*. We also suggest that the CN platform represents a promising approach for improving oil production in plants and offers a new opportunity to characterize the regulation mechanisms of genes in TAG biosynthesis and accumulation.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) seeds were sterilized and plated on MS medium with different concentrations of N and Suc as described by Martin et al. (2002). The molar ratio of KNO_3 to NH_4NO_3 was maintained in each medium as MS medium described by Murashige and Skoog (1962). KCl was added to compensate for the lower K^+ concentration in the medium. Seeds were kept at 4°C for 72 h in the dark. Both Arabidopsis and tobacco (*Nicotiana tabacum*) seedlings were grown in a growth chamber under a cycle of 16 h of light at 22°C and 8 h of dark at 20°C. Arabidopsis genotypes Col-0, Wassilewskija (Ws), and Landsberg *erecta* were obtained from Dr. Dapeng Zhang. The *abi4-1* mutant line is in the Col-0 background. The *abi5-1* mutant line is in the Ws background. The *ai3-1* mutant line is in the Landsberg *erecta* background. All these mutant lines were also obtained from Dr. Dapeng Zhang.

Microscopic and Lipid Analyses

Seven-day-old Arabidopsis seedlings were stained with 0.1% (w/v) Nile Red (Molecular Probes) in acetone for 10 min at room temperature (Greenspan et al., 1985). After brief rinsing with distilled water, neutral lipids were observed using a Leica TCS SP2 confocal laser scanning microscope. For TLC analysis, total lipids were extracted from 7-d-old Arabidopsis seedlings with the same fresh weight in chloroform:methanol:formic acid (10:10:1, v/v/v) as described by Bligh and Dyer (1959) and then separated by TLC in hexane: diethyl ether:acetic acid (80:20:1, v/v/v) on precoated silica gel 60 plates (Merck). Lipids were visualized by exposure of the plates to iodine vapor.

Quantitative Real-Time RT-PCR

Total RNA from 7-d-old Arabidopsis seedlings was isolated using Trizol reagent (Invitrogen). Total RNA (2 μg) was used to synthesize cDNA using the first-strand cDNA synthesis kit (Fermentas). Relative mRNA levels were determined by quantitative real-time RT-PCR using a SYBR Green real-time PCR master mix (Toyobo), with Arabidopsis *ACTIN1* mRNA as an internal control. Supplemental Table S1 lists the primers used. PCR was initiated with denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 20 s, 58°C for 20 s, and 72°C for 20 s, and a final extension at 72°C for 5 min. The $2^{-\Delta\Delta Ct}$ calculation was used to determine relative mRNA levels. The threshold cycle (Ct) is the cycle number at which the amount of amplified target reaches a fixed threshold. ΔCt is the Ct of the target gene subtracted from the Ct of *ACTIN1*. ΔΔCt is the difference in the threshold cycles of the target and *ACTIN1*.

Transient Expression Assay

For the transient expression assay, the *DGAT1* −1,000 to +226 and −117 to +226 promoter sequences were amplified by PCR. The products were cloned into the *Hin*dIII-*Bam*I-digested pBI121 vector. To generate the plant expression vector of 35S:ABI4, the full-length coding region of *ABI4* was amplified by PCR. The product was then inserted into the plasmid pCAMBIA 1381-Xa containing the 35S promoter. *Agrobacterium tumefaciens*-mediated transient transformation was performed as described by Yang et al. (2000). 35S-ABI4, pD1000:GUS, and pD117:GUS were transformed into *Agrobacterium* strain EHA105, and *Agrobacterium* cells were cultured overnight at 28°C. Cells were collected and resuspended with infiltration buffer (10 mM $MgCl_2$, 10 mM MES, pH 5.7, and 150 μM acetosyringone) and infiltrated into tobacco leaves. GUS activity was detected 48 h after infiltration. Total protein was quantified by the Bradford method with a protein assay kit (Bio-Rad). Ten milligrams of protein was used for each GUS activity determination as described by Jefferson et al. (1987), with 4-methylumbelliferyl-β-D-glucuronide (Sigma) as a substrate. Histochemical staining for GUS was performed as described (Stålberg et al., 1993). Plant samples were immersed in GUS staining buffer (0.5 mM 5-bromo-4-chloro-3-indoly-β-D-GlcA, 0.5 M NaH_2PO_4, pH 7.0, 1 mM EDTA, 0.5 mM potassium ferricyanide, and 0.5 mM potassium ferrocyanide). After staining at 37°C for 16 h, the samples were immersed in 95% (v/v) ethanol at 37°C to remove the chlorophyll. The primers used for plasmid construction are shown in Supplemental Table S2.

Yeast One-Hybrid Analysis

For the yeast one-hybrid assay, the *DGAT1* –63 to +102 promoter sequence was amplified by PCR. The products were cloned into the plasmids pLacZi and pHISi-1. *ABI4* was cloned into the plasmid pGAD-T7Rec. All constructs were transformed into yeast strain YM4271; yeast was grown in SD-Ura-Leu-

His medium and then spotted on SD-Ura-Leu-His medium in the presence or absence of 45 mM 3-aminotriazole (Sigma) with different dilutions. The plates were incubated for 3 d at 28°C. The β-galactosidase activity was determined according to the Matchmaker One-Hybrid User Manual (Clontech protocol no. PT1031-1). The primers used for plasmid construction are shown in Supplemental Table S2.

Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays were performed as described by Guiltinan and Miller (1994), with some modifications. GST-ABI4 was purified from *Escherichia coli* using the MagneGST glutathione particles purification system (Promega), and GST was also purified as the negative control. The –63 to +102 *DGAT1* promoter sequence used for the electrophoretic mobility shift assay was amplified by PCR and digested with *Eco*RI and *Xba*I. The 5′ ends of the PCR products were filled with [α-^{32}P]dATP and unlabeled dCTP, dGTP, and dTTP by Klenow DNA polymerase (Promega). Competitor DNA fragments were also digested with *Eco*RI and *Xba*I and filled with deoxyribonucleotide triphosphates. The binding reaction used 20 ng of purified protein and 24 ng of labeled probes incubated for 30 min with or without competitor fragment at room temperature. The binding buffer contained 24 mM Tris, pH 7.9, 24% (v/v) glycerol, 70 mM KCl, 0.14 mM EDTA, 2.15 mM dithiothreitol, 15 mM $MgCl_2$, and 500 ng of poly(dI-dC), as described by Guiltinan and Miller (1994). The protein-DNA complexes were separated on 5% (w/v) polyacrylamide gels in 0.5× Tris-borate-EDTA buffer at 4°C. The gel was dried and autoradiographed. The primers for probe amplification are shown in Supplemental Table S2.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NM_172503 (DGAT1), NM_115011 (DGAT2), NM_121367 (PDAT1), NM_118646 (OLEOSIN1), NM_120486 (SDP1), NM_117778 (ACX1), NM_125910 (ACX2), NM_112304 (NCED3), NM_113376 (ABI3), NM_129580 (ABI4), NM_129185 (ABI5), and NM_129318 (ACTIN1).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Effect of ABA on seedling TAG content.

Supplemental Figure S2. Key genes in the glyoxylate cycle and gluconeogenesis pathways are induced with 0.1 mM N.

Supplemental Figure S3. Many genes involved in fatty acid biosynthesis and TAG metabolism contain CE1-like elements.

Supplemental Table S1. Primers used for real-time RT-PCR.

Supplemental Table S2. Primers for plasmid constructions and electrophoretic mobility shift assays.

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