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). ABA promotes 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.
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 dgd1 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 (Stähl et al., 2004; Mhaske et al., 2005), RNA interference silencing of PDAT1 seed oil content (Stål et al., 2004; Mhaske et al., 2005), the overexpression of PDAT1 et al., 2009). Although neither the T-DNA insertion nor the overexpression of PDAT1 in Arabidopsis alters the seed oil content (Stähl et al., 2004; Mhaske et al., 2005), RNA interference silencing of PDAT1 in the Arabidopsis dgd1 background results in a 63% decrease in oil content compared with the dgd1 control (Zhang et al., 2009), which indicates that PDAT1 is the gene responsible for most of the TAG synthesis in the dgd1 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 systematically 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 synthesis, the expression levels of key TAG biosynthesis genes in seedlings were analyzed by qRT-PCR and compared with the expression levels in the Col-0 wild type grown under N-sufficient conditions. As shown in Figure 1A, the expression levels of the gene encoding DGAT1 were significantly induced under low-N conditions, with the highest level at 0.1 mM N (Fig. 1A). In addition, the expression levels of the genes encoding PHOSPHOLIPID:DIACYLGLYCEROL ACYLTRANSFERASE1 (PDAT1), PHOSPHOLIPID:DIACYLGLYCEROL ACYLTRANSFERASE2 (PDAT2), and OLEOSIN1 were significantly induced under low-N conditions, with the highest levels at 0.1 mM N (Fig. 1A). The expression levels of the genes encoding PHOSPHOLIPID:DIACYLGLYCEROL ACYLTRANSFERASE3 (PDAT3), PHOSPHOLIPID:DIACYLGLYCEROL ACYLTRANSFERASE4 (PDAT4), and OLEOSIN2 were also significantly induced under low-N conditions, with the highest levels at 0.1 mM N (Fig. 1A). These results suggest that low-N conditions significantly induced the expression of key genes involved in TAG biosynthesis, such as DGAT1 and OLEOSIN1, in 7-d-old Arabidopsis seedlings.
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 involved in TAG 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 SE. 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, GA₃, 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 coat. The germination rate did not differ significantly between 0.1 and 60 mM 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), GA₃, 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.

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 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 Arabobacterium 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 so. (See online article for color version of this figure.)](image-url)
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. DGA1 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 DGA1 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 DGA1 in response to ABA and low-N conditions. The transcription profile of DGA1 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 DGA1 transcription levels in abi3 and abi5 seedlings in different CN medium containing 10 μM ABA. We found that DGA1 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 DGA1 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 5. Interaction between DGA1 CE1-like elements and ABI4. A, Interaction of ABI4 and the DGA1 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 DGA1 CE1-like elements. The sequence of the 165-bp DGA1 fragment is shown at the top. Labeled CE1-CE1 DGA1 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 DGA1 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.]
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 seedling 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 phosphoenolpyruvate 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 cta1, 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 Gutierrez, 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 NO3− 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 μM 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
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 KNO₃ to NH₄NO₃ was maintained in each medium as 8:1 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. Daipeng Zhang. The abi4-1 mutant line is in the Col-0 background. The abi5-1 mutant line is in the Landsberg erecta background. All these mutant lines were also obtained from Dr. Daipeng 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 (10:1:1, 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–ΔΔCt 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 HindIII-Not-digested pBII21 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 MgCl₂, 10 mM MES, pH 5.7, and 150 μM ascorbyliron) 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 (Sköldberg et al., 1993). Plant samples were immersed in GUS staining buffer (0.5 mM 5-bromo-4-chloro-3-indoly-β-D-glucuronide, 0.5 mM NaH₂PO₄ 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 +112 promoter sequence was amplified by PCR. The products were cloned into the plasmids pLaZi and pHIS-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-amino-triazole (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-Cloning kit (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 Guil廷nan 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 fragment from Guiltinan and Miller (1994), with some modifications. GST-ABI4 was purified from Escherichia coli using the MagneGST glutathione particles purification system (Promega). Competitor DNA fragments were also digested with EcoRI and XhoI and filled with deoxyribonucleotide triphosphates. The binding reaction used 24 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% (v/v) glycerol, and 500 ng of poly(dIdc). The primers used for plasmid construction and electrophoretic mobility shift assay were amplified by PCR and digested with EcoRI and XhoI. The 5’ ends of the PCR products were filled with [α-32P]dATP and unlabeled dCTP, dGTP, and dTTP by Klenow DNA polymerase (Promega). Competitor DNA fragments were also digested with EcoRI and XhoI 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% (v/v) glycerol, and 500 ng of poly(dIdc). The primers used for plasmid construction are shown in Supplemental Table S2.

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 CEE-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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