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ABA-insensitive (ABI) 4 and ABI5 synergistically regulate *DGAT1* expression in *Arabidopsis* seedlings under stress

Yinfei Kong, Songbin Chen, Yang Yang, Chengcai An *

State Key Laboratory of Protein and Plant Gene Research, College of Life Sciences, Peking University, Beijing 100871, People's Republic of China

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ABSTRACT

Triacylglycerol (TAG) accumulation is essential for seed maturation in plants. Diacylglycerol acyltransferase 1 (DGAT1) is the rate-limiting enzyme in TAG biosynthesis. In this study, we show that TAG accumulation in *Arabidopsis* seedlings is correlated with environmental stress, and both ABI4 and ABI5 play important roles in regulating *DGAT1* expression. Tobacco transient assays revealed the synergistic effect of ABI4 with ABI5 in regulating *DGAT1* expression. Taken together, our findings indicate ABI5 is an important accessory factor with ABI4 in the activation of *DGAT1* in *Arabidopsis* seedlings under stress.

1. Introduction

TAG is an important component of seeds and is essential for seed development during the plant life cycle. If the TAG synthesis pathway is blocked, embryonic development will be disrupted [1]. Although TAG is typically synthesised during seed development in *Arabidopsis thaliana*, there is a close relationship between stress and TAG accumulation in other vegetative tissues. For instance, low-nitrogen stress and the stress hormone abscisic acid (ABA) stimulate TAG accumulation in *Arabidopsis* seedlings [2], and senescence, drought stress, and oxidative stress will trigger TAG accumulation in plants [3–6]. Photo-oxidative stress and other adverse environmental conditions also stimulate TAG production in microalgae [7].

Diacylglycerol acyltransferase 1 (DGAT1) is the rate-limiting enzyme in TAG biosynthesis, and it catalyses the conversion of *sn*-1,2-diacylglycerol to TAG during seed development [8]. dgat1 Null mutant seeds have significantly decreased TAG content [1,9,10], whereas *DGAT1* overexpression leads to increased seed oil contents [11–13]. DGAT1 is elevated not only in seeds but also in other vegetative tissues under stress. *DGAT1* transcript levels increased enormously in *Arabidopsis* leaves under stress-induced senescence[6] and in *Arabidopsis* seedlings under low-nitrogen stress or ABA treatment [2,14].

Controlling DGAT1 availability appears to be the mechanism by which plant seedlings regulate TAG accumulation under stress. The transcription factor *ABSCISIC ACID INSENSITIVE* 4 (*ABI4*) binds directly to the *DGAT1* promoter and regulates TAG accumulation under low-nitrogen stress and ABA treatment. The transcription factor ABI5 also participates in *DGAT1* transcriptional regulation under low-nitrogen stress [2,15]. However, the molecular mechanism by which ABI5 regulates TAG accumulation is still unclear.

Despite extensive study of the TAG accumulation phenomenon, no systematic analysis of different stressors effects on TAG accumulation in *Arabidopsis* has been made. In this study, we systematically analysed the relationship between TAG accumulation and several stressors, namely ABA, jasmonic acid (JA), salicylic acid (SA), high salt, and hyperosmosis; the relationship between *DGAT1* expression patterns and these different stressors; and the roles of ABI4 and ABI5 in regulating TAG accumulation under stress. To our knowledge, this is the first report to describe an ABI4 and ABI5 synergistic effect on *DGAT1* expression in plant seedlings under stress.

2. Materials and methods

2.1. Plant materials and growth conditions

A. thaliana seeds with the Col-0 and Ws genotypes were sterilised and plated on Murashige and Skoog (MS) medium as described [16]. The seeds were kept at 4 °C for 72 h in the dark.

* Corresponding author. Fax: +86 10 62759070.
E-mail address: chcaian@pku.edu.cn (C. An).

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Arabidopsis seedlings were cultivated in a growth chamber under 16 h of light at 22 °C and 8 h of dark at 20 °C. The *dgat1* mutant (CS3861) was purchased from TAIR. An *abi4-1* mutant line in a Col-0 background and an *abi5-1* mutant line in a Ws background were obtained from Dr. Dapeng Zhang. We generated the double mutant (*abi4abi5*) by crossing the single mutants.

2.2. Transgenic plant generation

The ABI4-1345 to 0 and ABI5-1097 to 0 promoter sequences were amplified by PCR. The products were cloned into the *Hin*dIII–*Bam*HI-digested pBI121 vector. To generate the 35S:ABI4 and 35S:ABI5 plant expression vector, the full-length coding region of ABI4 and ABI5 was amplified by PCR. The product was then inserted into the pCAMBIA 1381-Xa plasmid containing the 35S promoter, and Col-0 seedlings were transformed with the constructs by *Agrobacterium tumefaciens*-mediated method [17]. The transient T3 homozygote from the pBI121 construct was screened with kanamycin and rifampin and the 1381-Xa construct was screened with hygromycin and rifampin. Plant samples were immersed in GUS staining buffer (0.5 mM 5-bromo-4-chloro-3-indolyl-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) for 16 h at 37 °C and were then immersed in 95% (v/v) ethanol at 37 °C to remove the chlorophyll. The plasmid construction primers are shown in Supplementary Table S2.

2.3. Microscopic and lipid analyses

Arabidopsis seedlings (7-d-old) were stained with 0.1% (w/v) Nile Red (Molecular Probes) in acetone for 10 min at room temperature [18]. After a brief distilled water rinse, the neutral lipids were observed with a Leica TCS SP2 confocal laser-scanning microscope. For TLC analysis, the total lipids were extracted from 7-d-old seedlings with equal fresh weights in chloroform/methanol/formic acid (10:10:1, v/v/v) as described [19] and then separated by TLC in hexane/diethyl ether/acetic acid (80:20:1, v/v/v) on pre-coated silica gel 60 plates (Merck). Lipids were visualised by exposing the plates to iodine vapour.

2.4. Quantitative real-time reverse transcription–PCR (qRT-PCR) analysis

The total RNA from 7-d-old seedlings was isolated with Trizol reagent (Invitrogen). Total RNA (2 μg) was used to synthesise cDNA with a First-Strand cDNA Synthesis Kit (Fermentas). The relative mRNA levels were determined by qRT-PCR using a SYBR Green real-time PCR master mix (ToYoBo) with ACTIN1 mRNA as an internal control. 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. A $2^{-\Delta\Delta CT}$ calculation was used to determine the relative mRNA levels normalised to ACTIN1. Supplementary Table S1 lists the primers used for PCR.

2.5. Transient expression assay

35S-ABI4, 35S-ABI5, and pD1000:GUS (DGAT1 −1000 to +226 promoter sequences were cloned into the *Hin*dIII–*Bam*HI-digested pBI121 vector) were transformed into *Agrobacterium* strain EHA105, and *Agrobacterium* cells were cultured overnight at 28 °C. Cells were collected and resuspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES, pH 5.7, and 150 mM acetosyringone) and used to infiltrate the tobacco leaves [20]. 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 [21], with 4-methylumbelliferyl-*b*-D-glucuronide (Sigma) as a substrate. Histochemical staining for GUS was performed as described [22].

2.6. Luciferase assay

To generate the mammalian expression vectors pcmv:*ABI4* and pcmv:*ABI5*, the full-length coding region of *ABI4* and *ABI5* were amplified by PCR and then inserted into the pcmv-3xHA plasmid. To generate the *DGAT1*-luciferase reporter vector, the *DGAT1* promoter sequence (−1000 to + 226 bp) was amplified by PCR and inserted into the plasmid pGL3-luciferase. HEK293 cells were transiently transfected with 100 ng of pcmv-3xHA-*ABI4* and/or 100 ng of pcmv-3xHA-*ABI5*, and with 50 ng of the *DGAT1*-luciferase reporter. A luciferase assay was performed after 24 h with a Dual-Lucy Assay Kit (Vigorous). The plasmid construction primers are shown inSupplementary Table S3.

3. Results

3.1. TAG accumulation in Arabidopsis seedlings under stress

We previously found that TAG levels increase in *Arabidopsis* seedlings during ABA treatment and during low-nitrogen stress [2]. To explore the influence of additional stressors on TAG accumulation in *Arabidopsis* seedlings, wild-type Columbia (Col-0) seeds were cultivated for 7 d on medium containing increasing concentrations of ABA, JA, SA, NaCl, or sorbitol (which leads to osmotic stress). *Arabidopsis* post-germination growth was markedly inhibited in the presence of all compounds, and the inhibition rate was concentration-dependent (Fig. 1B). The TAG level in seedlings grown in the presence of these stressors has a close relationship to the strength of the stress (Fig. 1A). Seedlings were grown on untreated medium and medium containing 10 μM ABA, 100 μM JA, 200 μM SA, 100 mM NaCl, or 100 mM sorbitol, which were then stained with Nile Red and observed by confocal microscopy. Seedlings grown in the presence of the stressors showed striking increases in the proportion of oil droplets in comparison to the control (Fig. 1C). Thus, medium containing 10 μM ABA, 100 μM JA, 200 μM SA, 100 mM NaCl, or 100 mM sorbitol provided suitable conditions for finding the relationship between TAG accumulation and stress in *Arabidopsis* seedlings.

3.2. Expression of DGAT1 in Arabidopsis seedlings increased under stress

DGAT1 promoter activity was observed by using a *DGAT1-β-glucuronidase* (*GUS*) reporter vector. GUS expression was observed in the cotyledons and hypocotyls of 7-d-old seedlings. The presence of stressors increased the GUS activity (Fig. 2A). To better characterise the relationship between *DGAT1* expression and stress, *DGAT1* expression patterns in 7-d-old seedlings cultivated on different media were analysed. *DGAT1* transcript levels dramatically increased in response to the different stressors in comparison with seedlings cultivated on medium alone, and *DGAT1* expression was enhanced by approximately 10-fold by 10 μM ABA, 7-fold by 100 μM JA, 6-fold by 200 μM SA, 9-fold by 100 mM NaCl, and 3-fold by 100 mM sorbitol (Fig. 2B). These results demonstrated that seedling *DGAT1* expression is closely associated with stress.

3.3. ABI4 is essential to stress-induced TAG accumulation

ABI4 plays an important role in TAG accumulation under low-nitrogen stress [2]. To explore the relationship between ABI4 and different stressors, *ABI4* promoter activity was examined by using an *ABI4-GUS* reporter vector. As in *DGAT1*, the addition of 10 μM ABA, 100 μM JA, 200 μM SA, 100 mM NaCl, or 100 mM sorbitol to

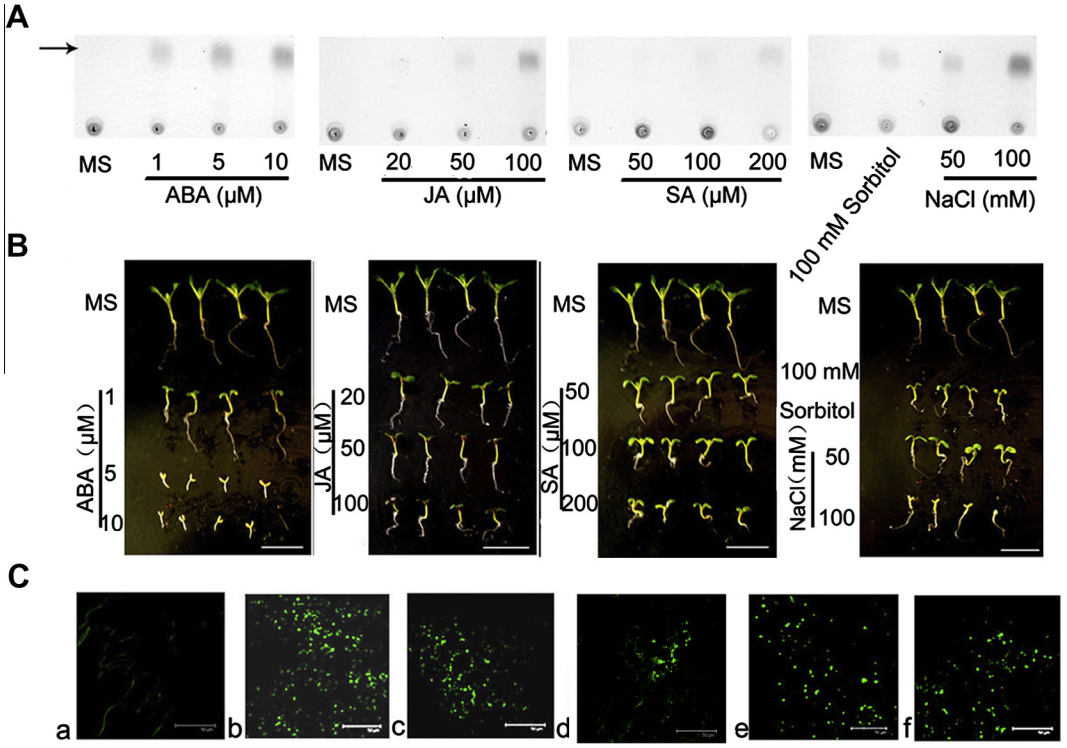

Fig. 1. Stress triggers TAG accumulation in *Arabidopsis* seedlings. (A) TAG content of 7-d-old Col-0 *Arabidopsis* seedlings cultivated on different media (arrow). The total lipids were extracted and separated by thin-layer chromatography (TLC). (B) Growth phenotypes of 7-d-old Col-0 seedlings cultivated on different media. Bars = 5 mm. (C) Nile Red-stained oil droplets (green fluorescence) in Col-0 seedlings cultivated on different media for 7-d. a, MS; b, MS with 10 μM ABA; c, 100 μM JA; d, 200 μM SA; e, 100 mM NaCl; f, 100 mM sorbitol. Bars = 50 μm.

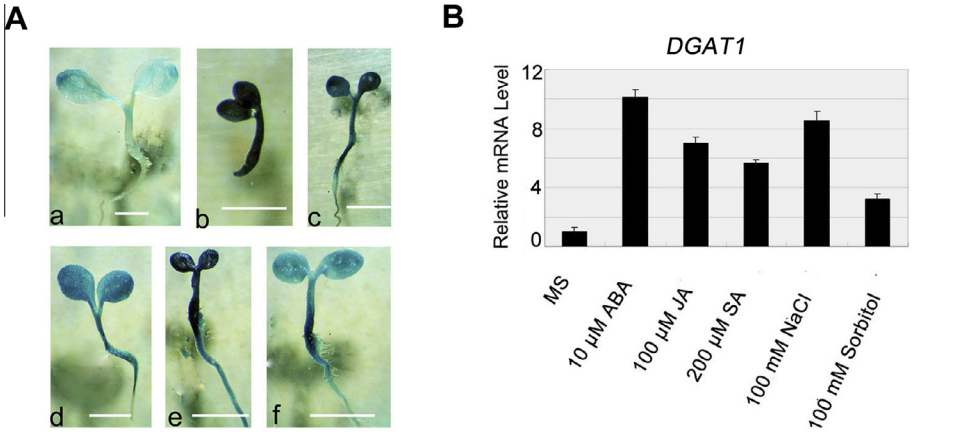

Fig. 2. Stress induces *DGAT1* expression. (A) Expression of the *DGAT1*-GUS reporter in 7-d-old Col-0 seedlings cultivated on different media. a, MS; b, MS with 10 μM ABA; c, 100 μM JA; d, 200 μM SA; e, 100 mM NaCl; f, 100 mM sorbitol. Bars = 2 mm. (B) *DGAT1* expression in 7-d-old Col-0 seedlings cultivated on different media. Relative mRNA levels were determined by qRT-PCR using ACTIN1 as an internal control. Data represent the average of three independent experiments, and the error bars represent the standard deviation.

the medium increased the GUS activity in 7-d-old seedlings (Fig. 3A). ABI4 expression was markedly induced by ABA, JA, SA, NaCl, and sorbitol, with approximately 50-, 30-, 25-, 35-, and 20-fold increases, respectively (Fig. 3B). To confirm ABI4 regulated *DGAT1* expression under stress, the *DGAT1* expression was assayed in 7-d-old *abi4* loss-of-function seedlings cultivated in the presence of the different stressors. The *DGAT1* expression in the *abi4* mutant did not obviously increase in the presence of the stressors (Fig. 3C), and very little TAG accumulation was detected (Fig. 3D). When ABI4 was overexpressed in *Arabidopsis* Col-0, we found both *DGAT1* expression and TAG accumulation were induced when the plant was treated in MS medium (Supplementary Fig. 1). All these results strongly suggested that ABI4 positively regulates *DGAT1* expression and TAG accumulation under stress.

3.4. ABI5 is important for TAG accumulation during stress

ABI5 is induced by low-nitrogen stress [2], but the function of ABI5 with respect to TAG accumulation under stress is still unknown. To further investigate the relationship between ABI5 and the stress response, *ABI5* expression was observed in 7-d-old seedlings cultivated in the presence of different stressors. An assessment of *ABI5* promoter activity with an *ABI5-GUS* reporter vector showed that GUS expression was barely detectible in 7-d-old

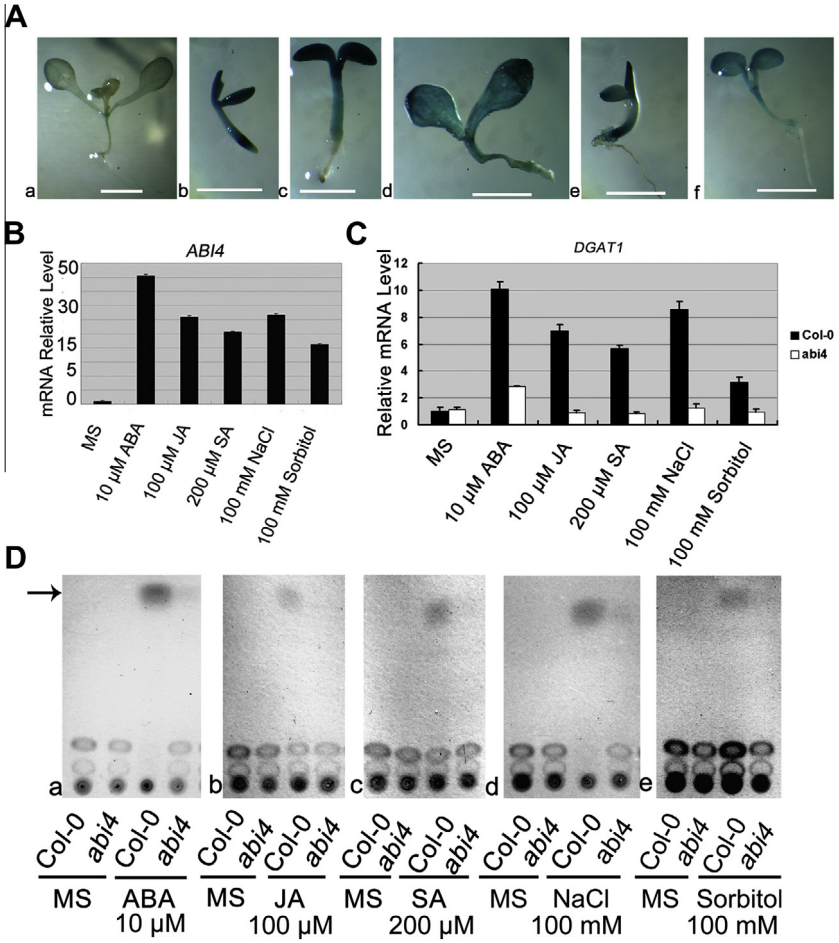

Fig. 3. Influence of ABI4 on TAG accumulation under stress. (A) Expression of the ABI4-GUS reporter in 7-d-old Col-0 seedlings cultivated on different media. a, MS; b, MS with 10 μM ABA; c, 100 μM JA; d, 200 μM SA; e, 100 mM NaCl; f, 100 mM Sorbitol. Bars = 2 mm. (B) Expression levels of ABI4 in 7-d-old Col-0 seedlings grown on different media. Relative mRNA levels were determined by qRT-PCR using ACTIN1 as an internal control. Data represents the average of three independent experiments, and the error bars represent the standard deviations. (C) Relative *DGAT1* mRNA levels in 7-d-old abi4 seedlings grown on different media. (D) TAG content of 7-d-old Col-0 and abi4 seedlings cultivated on indicated media (arrow). Total lipids were extracted and separated by TLC.

seedlings cultivated on medium alone, but the addition of ABA, JA, SA, NaCl or sorbitol markedly activated GUS activity (Fig. 4A). *ABI5* transcriptional activity markedly increased in the presence of stressors in comparison to the activity of seedlings cultivated on medium alone. We observed ~350-, 150-, 50-, 150-, and 60-fold increases in the ABA-, JA-, SA-, NaCl-, and sorbitol-treated seedlings, respectively (Fig. 4B). This regulatory function of ABI5 in TAG accumulation was further confirmed by using an *abi5* loss-of-function mutant. We measured the *DGAT1* transcript levels and TAG levels in 7-d-old *abi5* seedlings cultivated on media containing the different stressors. The *DGAT1* expression in the *abi5* mutant was only slightly induced by stressors (Fig. 4C), and TAG accumulation markedly decreased in the *abi5* mutant in the presence of stressors (Fig. 4D). When ABI5 was overexpressed in *Arabidopsis* Col-0, we found both *DGAT1* expression and TAG accumulation were induced when the plant was treated in MS medium, as in ABI4 (Supplementary Fig. 2). Hence, ABI5 also positively regulated *DGAT1* expression and TAG accumulation under stress.

3.5. Synergistic effects of ABI4 and ABI5 on stress-induced TAG accumulation

Because both ABI5 and ABI4 are involved in the regulation of stress-induced TAG accumulation in *Arabidopsis*, we next investigated the relationship between these two transcription factors by comparing *DGAT1* expression in the *abi4*, *abi5*, and *abi4abi5* double-mutant strains. *DGAT1* expression in *abi4abi5* is similar to *abi4*, and it is lower than that of *abi5* (Fig. 5A). A tobacco transient assay showed that *ABI4* activated the expression of *DGAT1* by ~14-fold, whereas *ABI5* did not activate expression. When tobacco was transfected with the *ABI4* and *ABI5* together, there was a 30-fold increase in GUS activity in comparison to the empty-vector control (Fig. 5B). TAG accumulation was not detected in the *abi4* or *abi4abi5* mutants in the presence of stressors, and TAG accumulation, although present in the *abi5* mutant, was strikingly lower than that of the wild type strains (Fig. 5C). These results suggest that ABI4 is essential for *DGAT1* expression and TAG accumulation under stress, and ABI5 is able to enhance ABI4 in activating *DGAT1* expression in heterologous system. Thus, we believe ABI4 and ABI5 act synergistically in regulating *DGAT1* expression in response to stress.

4. Discussion

Some secondary metabolites play important roles in plants that respond to different stresses. For example, the plant signalling hormone JA is activated by necrotrophic pathogens and herbivorous insects, SA by biotrophic pathogens [23], and ABA by drought and high salinity [24,25]. Some lipids are also involved in responding to hyperosmotic stress [26–28]. In this study, we found that

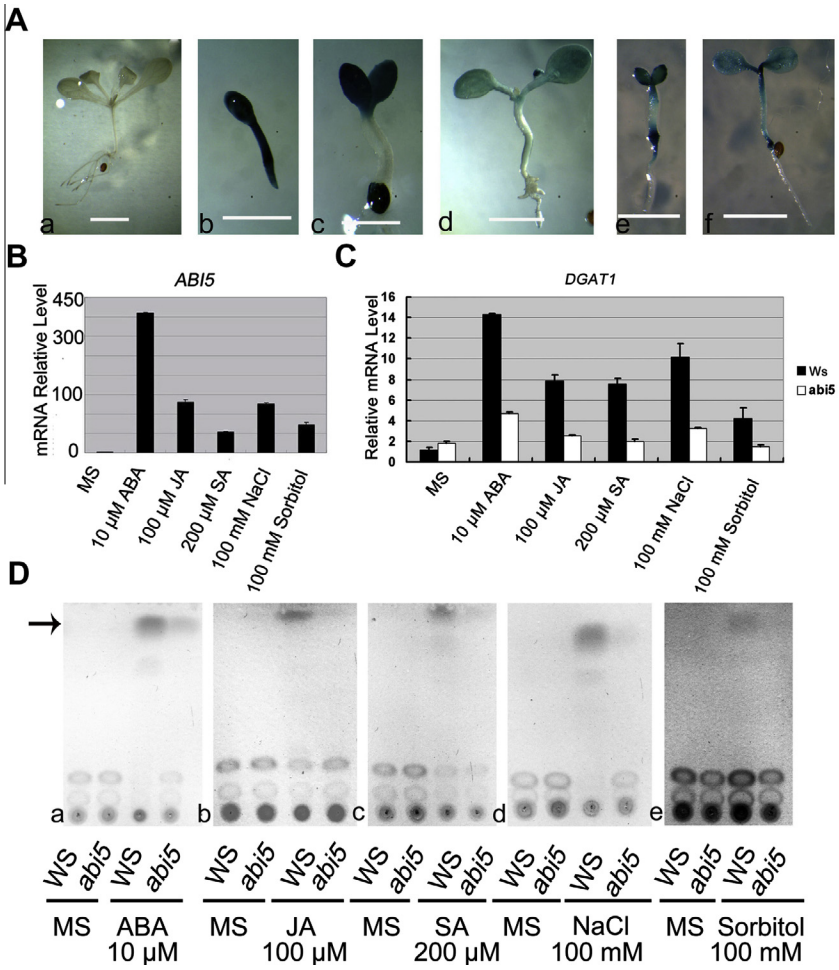

Fig. 4. Influence of ABI5 on TAG accumulation under stress. (A) Expression of the ABI5-GUS reporter in 7-d-old Col-0 seedlings cultivated on different media. a, MS; b, MS with 10 μM ABA; c, 100 μM JA; d, 200 μM SA; e, 100 mM NaCl; f, 100 mM sorbitol. Bars = 2 mm. (B) Expression levels of ABI5 in 7-d-old Col-0 seedlings cultivated on different media. The relative mRNA levels were determined by qRT-PCR using ACTIN1 as an internal control. Data represents the average of three independent experiments, and the error bars represent the standard deviations. (C) Relative *DGAT1* mRNA levels in 7-d-old abi5 seedlings cultivated on different media. (D) TAG content of 7-d-old Wassilewskija (Ws) and abi5 seedlings cultivated on indicated media (arrow). Total lipids were extracted and separated by TLC.

TAG accumulation in *Arabidopsis* seedlings is closely associated with the concentration of ABA, JA, SA, salt, and sorbitol stressors (Fig. 1A and B). We observed a similar phenomenon in *Brassica napus* and *Eichhornia crassipes* seedlings (data not show). We also observed that TAG accumulation occurred in biotrophic pathogen-infected *B. napus* seedlings (data not show); in contrast, signalling molecules that promote plant development, such as 6-BA, IAA, and ACC, do not induce TAG accumulation [2]. There is still on doubt about that TAG accumulation under stress resulted from incomplete storage oil degradation or TAG biosynthesis. Our study showed that the expression of *DGAT1*, which is the key gene that catalyses TAG biosynthesis, is significantly induced in *Arabidopsis* seedlings by ABA, JA, SA, salt, and hyperosmosis (Fig. 2A and B), and the expression of SDP1, which catalyses TAG degradation, was also induced by stressors (Supplementary Fig. 3). These results indicate that biosynthesis is the primary source of TAG accumulation as induced by stress, and there is a homeostasis between TAG biosynthesis and degradation in plant seedlings under stress, and there is still a question as to what the function of TAG biosynthesis is under stress. *dgat1* mutants have no visible defects during seed development, but they are more sensitive to ABA and osmotic stress during germination than wild-type plants [1,29]. Our study found the germination rate of *dgat1* was much lower than the wild-type when treated by JA, SA, and NaCl (Supplementary Fig. 4). These results suggest that TAG accumulation in *Arabidopsis* seedlings is an important stress response.

The seed oil bodies production accumulated in *Arabidopsis* including TAG and storage protein biosynthesis. Both ABI4 and ABI5 are very important in the TAG biosynthesis pathway because they regulate *DGAT1* expression during low-nitrogen conditions [2], our study showed both ABI4 and ABI5 are important for TAG biosynthesis under stress (Figs. 3 and 4). Furthermore, both ABI4 and ABI5 can trigger TAG accumulation and *DGAT1* expression in both 35S:*ABI4*/*Col-0* and 35S:ABI5/Col-0 plants (Supplementary Figs. 1 and 2), and interestingly, the expression of *ABI4* in *35S:ABI5/Col-0* also increased (Supplementary Fig. 5). Tobacco and mammalian cell assays showed that ABI4 alone can activate *DGAT1* expression but not ABI5, and ABI4 and ABI5 synergistically regulate *DGAT1* expression (Fig. 5B and Supplementary Fig. 6). Interestingly, the synergistic action of ABI4 with ABI5 also regulates Oleosin1 expression [30]. Our study found that both ABI4 and ABI5 are positive regulators of Oleosin1 expression under stress (Supplementary Fig. 7), and as in Oleosin1, there is no previously characterised ABI5-binding motif in the *DGAT1* promoter sequence [2,30]. These results suggest that maybe the synergistic effect of ABI4 together with ABI5 is an important mechanism for controlling the seed oil bodies production accumulated in *Arabidopsis* under stress, while theoretically possible that ABI5 cannot

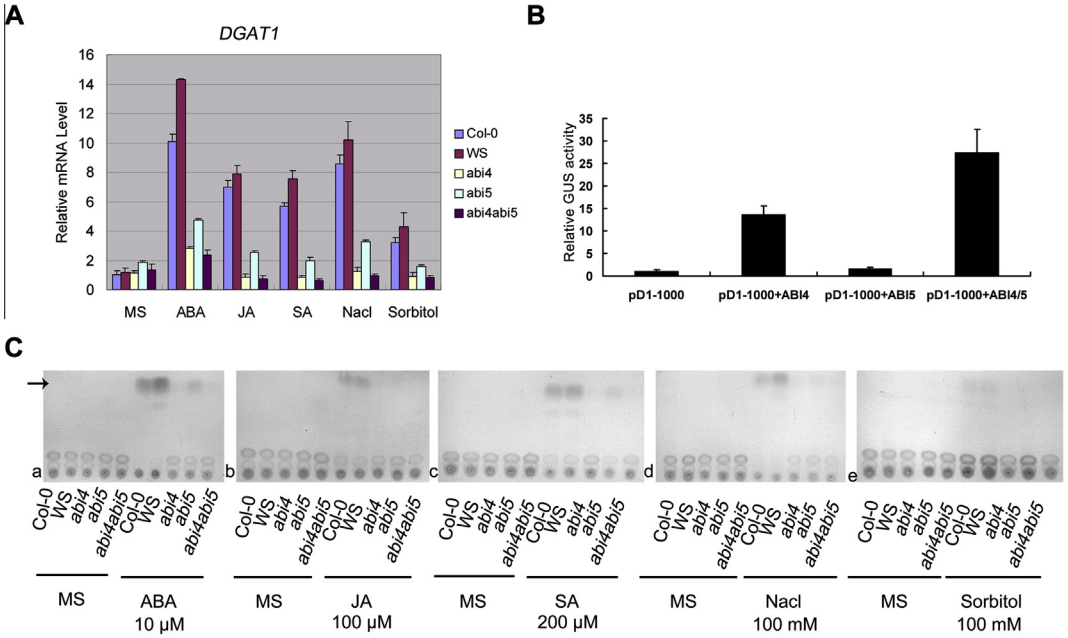

Fig. 5. Combined influence of ABI4 and ABI5 on TAG accumulation under stress. (A) Expression of *DGAT1* in 7-d-old Col-0, Ws, abi4, abi5, and abi4abi5 seedlings cultivated on different media (MS, MS with 10 μM ABA, 100 μM JA, 200 μM SA, 100 mM NaCl, 100 mM Sorbitol). Relative mRNA levels were determined by qRT-PCR using ACTIN1 as an internal control. Data represent the average of three independent experiments, and the error bars represent the standard deviation. (B) Tobacco was transfected with 35S:ABI4 or 35S:ABI5 or 35S:ABI4 with 35S:ABI5 along with a *DGAT1*−1000–+226 promotor reporter construct. Data represent the average ± the standard deviations from independent replicates. (C) TAG level of 7-d-old Col-0, Ws, abi4, abi5, and abi4abi5 seedlings grown on the indicated media (arrow). Total lipids were extracted and separated by TLC.

recognize *DGAT1* promoter sequence, it would seem likely that it influences the promoter complex assembled at *DGAT1* in some other, possibly indirect manner.

ABA, JA, and SA all play very important roles in plant development and environmental stress resistance, and crosstalk between these hormones is important in determining either stress responses or developmental processes [31]. ABA can interfere with the signal transduction pathway of JA and SA [32–36], and both SA and JA can regulate the biosynthesis and signal transduction pathway of ABA [37,38]. Our study found both ABI4 and ABI5 are induced by different stressors (Fig. 3A and B and Fig. 4A and B). Furthermore, ABI4 was essential for *ABI5* expression under JA and SA treatments, but not in the presence of ABA (Supplementary Fig. 8). These results suggest there may be different regulatory mechanisms pertaining to *ABI5* expression among hormones; we believe *ABI4* plays an important role in crosstalk among JA, SA, and ABA in regulating TAG accumulation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.07.045.

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