Salicylic Acid-Mediated Diacylglycerol/Triacylglycerol Conversion Affects the Freezing Tolerance of Arabidopsis

Le Xu (✉ 597582012@qq.com)

Yangtze University

Jun Wu

Yangtze University

Yancui Zhao

Yangtze University

Huaqiong Liu

Yangtze University

Wenying Zhang

Yangtze University

Yanhao Xu

Yangtze University

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Abstract

Diacylglycerol (DAG) is likely converted to triacylglycerol (TAG) by the enzyme diacylglycerol acyltransferase (DGAT), and this conversion is important in freezing tolerance of *Arabidopsis*. The phytohormone salicylic acid (SA) plays important roles in the chilling and freezing tolerance of plants. In our study, we analysed the chilling phenotype, proline and sugar accumulation, phytohormone measurement, and lipid profiling of *dgat1* mutants during chilling or freezing stress. We found that *dgat1-1* mutants exhibited higher sensitivity to long exposure to cold stress and showed lower proline and sugar accumulation under cold acclimation conditions. The freezing-sensitive phenotype of *dgat1* mutants can be ameliorated by mutations of key salicylic acid (SA) signalling components SAG101, EDS1, and PAD4 through phenotyping analysis of double mutants. *Dgat1* mutants accumulated more SA, ABA, JA-Ile (jasmonate isoleucine) and OPDA (12- oxyphytodienoic acid) during freezing stress and after recovery. In addition, the DAG/TAG content in the SA-deficient mutant *sid2* was lower than that in the wild type, while the SA-excessive accumulated mutant *siz1* showed the opposite trend. In summary, SA could mediate the freezing tolerance of *Arabidopsis* by regulating the ratio of DAG and TAG, which influences the integrity of the membrane.

Introduction

Cold stress can be divided into chilling (temperature above 0°C) and freezing stress (temperature lower than 0°C), which are major environmental factors that severely restrict plant growth, development, productivity, and geographical distribution (Thomashow, 1999). Most temperate plants have evolved complex strategies, such as the accumulation of cryoprotective polypeptides and osmolytes (e.g., soluble sugars and proline), transcriptome reprogramming, and remodelling of biochemical membranes to cope with low temperatures (Thomashow 1999). Prior exposure to nonfreezing temperatures allows plants to survive freezing stress and involves the activation of several physiological processes to enhance the plant tolerance to freezing temperatures (Thomashow, 1999) and this process is called cold acclimation.

Recent work has shown that the defence hormones salicylic acid (SA), ethylene (Shi et al., 2012), and jasmonic acid (JA) modulate cold stress responses in *Arabidopsis*. JAs function as critical upstream signals in the ICE-CBF/DREB1 pathway and they positively regulate *Arabidopsis* freezing tolerance through the JASMONATE ZIM-DOMAIN (JAZ) proteins JAZ1 and JAZ4 (Hu et al. 2013). Unlike the immediate accumulation of JAs in *Arabidopsis* seedlings after exposure to cold stress, SA levels increased only after a week of being kept at cold temperatures (Scott et al. 2004; Shi et al., 2012; Hu et al. 2013; Kim et al. 2013). Nonetheless, cold stress induces an increase in endogenous SA, and the existing evidence suggests that there is a strong negative correlation between the growth rate under cold conditions and the levels or perception of SA (Scott et al. 2004). The wild type suffers more stagnant growth than the SA-deficient mutant *NahG* and *eds5* (inhibited SA transport from the chloroplast) mutants under cold stress, and the partially SA-insensitive *npr1* mutant (a nonexpresser of SA-inducible PR genes) displays growth intermediate between that of *NahG* and wild type (Scott et al. 2004, Kim et al. 2013). In addition, the SA-deficient mutants *eds5* and *sid2* (chloroplastic SA synthesis deficient) had

higher survival rates during recovery after freezing stress (Kim et al. 2013; Majláth et al. 2011). In contrast, the *cpr1* mutant (constitutive expresser of PR genes) that accumulated very high levels of SA at 5°C showed more inhibited growth than the wild type. In addition, the accumulation of SA in *siz1* and *acd6* mutants confers hypersensitivity to freezing stress (Miura and Ohta, 2010).

Ice formed during freezing stress could cause substantial damage to the cell membrane. Thus, membrane integrity is a vital factor influencing the freezing tolerance of plants, and ion leakage is a reliable indicator of membrane damage (Chen and Thelen, 2013). Lipids are important components of the membrane, and the species, ratio, and remodelling of different lipids could determine the freezing tolerance of plants. Increasing evidence suggests that both plastidic and extraplastidic membrane lipid alterations affect membrane fluidity and integrity in plants exposed to cold stress. (Uemura et al., 1995; Welte et al., 2002; Li et al., 2004; Moellering et al., 2010; Chen and Thelen, 2013). For example, in *Arabidopsis* rosettes, freezing induces significant decreases in phosphatidylcholine (PC) and phosphatidylethanolamine (PE), as well as plastidic monogalactosyldiacylglycerol (MGDG) and phosphatidylglycerol (PG), while phosphatidic acid (PA) and lysophospholipids increase dramatically (Welte et al., 2002). MGDGs have a small head group, and PA lacks a head group; they are inclined to form the inverted hexagonal II (HII)-type phase, and these nonlamellar lipid structures can drive the disruption of cell membrane integrity (Verleij et al., 1982; Moellering et al., 2010; Steponkus, 1984; Uemura et al., 1995; Thomashow, 1999; Welte et al., 2002).

In *Arabidopsis*, disruption of SFR2 (sensitive to freezing tolerance), which encodes a galactolipid:galactolipid galactosyltransferase (GGGT) that converts MGDG to oligogalactolipids (such as diacylglycerol, DAG), results in severe injury during freezing stress (Thorlby et al., 2004; Moellering et al., 2010; Fourrier et al., 2008). Furthermore, DAG produced by GGGT can be subsequently converted to triacylglycerol (TAG) by acyl-coenzyme A diacylglycerol acyltransferase (DGAT), likely resulting in the removal of excess adverse membrane lipids after freezing (Moellering et al., 2010; Moellering and Benning, 2011). Phosphorylation of DAG by DAG kinases (DGKs) can produce PA (Wang, 2004), and DGKs transcripts are induced rapidly in response to chilling stress (Go'mez-Merino et al., 2004; Arisz et al., 2013).

The conversion of DAG to TAG by DGAT1 is critical for plant freezing tolerance by balancing TAG and PA production in *Arabidopsis*. The *dgat1* mutants are more sensitive to freezing stress than the wild type. The blockage of DAG to TAG conversion in the *dgat1* mutant activates the DAG to PA conversion pathway through the function of DGKs. During freezing stress, the accumulation of PA in *dgat1* plants stimulated NADPH oxidase activity and enhanced RbohD-dependent hydrogen peroxide production compared with the wild type. Consistent with this observation, *dgk2*, *dgk3*, and *dgk5* knockout mutants showed attenuated PA production and improved tolerance in response to freezing temperatures (Tan et al., 2018). The *sag101*, *eds1*, and *pad4* mutants had much lower SA and DAG contents than the wild type, and exogenous application of SA and DAG compromised the freezing tolerance of the mutants. Furthermore, SA suppressed the cold-induced expression of DGATs and DGKs in wild-type leaves. These

findings indicate that SAG101, EDS1, and PAD4 are involved in the freezing response in *Arabidopsis*, at least in part, by modulating the homeostasis of SA and DAG (Chen et al., 2015).

However, it is still unknown how the enzymes downstream of DAG contribute to the balance of TAG and PA production under freezing conditions, and the precise cellular functions of *Arabidopsis* DGKs remain elusive. Based on previous reports and references, the purpose of this work was to elucidate how the phytohormone SA regulates DAG-TAG conversion and the cold tolerance of *Arabidopsis*.

Materials And Methods

The *Arabidopsis* ethyl methanesulfonate-generated mutant *dgat1-1* (CS3861, a gift from Shi Lei of oil crops research institute, Chinese Academy of Agricultural Science) and T-DNA insertional mutant *dgat1-2* (SALK_039456) were the same as the seeds used by Tan et al (2018).

Seeds of *Arabidopsis* wild type (ecotype Columbia-0) and mutants were surface sterilized with 20% bleach (v/v) for 15 min and then washed three times with sterilized water followed by sowing on MS medium supplemented with 1% sucrose. Seeds were dark treated at 4 °C for 3 d and subsequently transferred to plant growth room under a 16-h light (23 °C)/8-h dark (21 °C) cycle. After germination for 8 days, the seedlings were transplanted to soil in the plant growth room until treatment. Chilling and freezing treatments were carried out as described previously (Chen et al. 2015) with minor modifications. For cold acclimation and chilling treatment, 4-week-old plants were transferred from the growth room to a cold growth chamber (4 °C) for 3 d of acclimation or several weeks under a normal light/dark cycle, respectively. For the freezing treatment, all 4-week-old NA (non-acclimated) or CA (cold-acclimated) soil-grown plants or 11-d-old seedlings grown on MS medium plates were transferred to a growth chamber (Blue Pard LRH- LRH-500CA) with temperatures reduced steadily from 4 °C to −2 °C (2 °C per hour). The temperature remained at −2 °C for 2 h and continued to decrease until reaching the final temperatures. After staying at the final temperatures for 1 hour, the plants were thawed overnight at 4 °C. Plants were photographed after a 5-d recovery under normal growth conditions.

Lipid Profiling

Leaf samples in chloroform/methanol/water (2/1/1, v/v/v) solution were vortexed for 1 min, and centrifuged at 3000 rpm for 10 min. Organic phase was collected and transferred to a new tube and lyophilized using nitrogen. Dried metabolites were reconstituted in 400 ul isopropanol/methanol (1/1, v/v) solution, vortexed, centrifuged at 12000 rpm for 10 min at 4 °C and supernatant was analyzed using LC-MS. A Kinetex C18 column (100 × 2.1 mm, 1.9 μm) and the following gradient: 0–2 min 30% mobile phase B; 2–20 min 100% B; 20–40 min 100% B; 40–40.01 min 30% B; 40.01–45 min 30% B, was applied for the experiment. Mobile phase A was acetonitrile/ water (3:2, v/v), 10mM ammonium formate. Mobile phase B was acetonitrile/ isopropanol (1:9, v/v), 10mM ammonium formate and 0.1% formic acid. The flow rate was 0.4 ml/min, the column was at 45 °C. Parameters used for mass spectrometry were described as follows: positive ion mode, Heater Temp 300 °C, Sheath Gas Flow rate, 45arb, Aux Gas Flow Rate, 15 arb, Sweep Gas Flow Rate, 1arb, spray voltage, 3.0KV, Capillary Temp, 350

°C, S-Lens RF Level, 30%. Scan ranges:200–1500; negative ion mode, Heater Temp300 °C, Sheath Gas Flow rate, 45arb, Aux Gas Flow Rate, 15arb, Sweep Gas Flow Rate, 1arb, spray voltage, 2.5KV, Capillary Temp, 350 °C, SLens RF Level, 60%. Scan ranges: 200–1500. Lipidomics assays was performed in Biocluster Inc.

Extraction and Measurement of Phytohormone Levels

The levels of ABA, SA, JA, and OPDA were determined by Zoonbio Biotechnology Co., Ltd., and the methods were modified from those described by Pan et al (2002). Approximately 0.5 g leaves were ground in a precooled mortar that contained mL extraction buffer comprised of isopropanol/hydrochloric acid. The extract was shaken at 4 °C for 30 min. Then, 10 ml dichloromethane was added, and the sample was shaken at 4 °C for 30 min and centrifuged at 13,000 rpm for 5 min at the same temperature. We then extracted the lower, organic phase. The organic phase was dried under N₂ and dissolved in 150 µL methanol (0.1% methane acid) and filtered with a 0.22-µm filter membrane. The purified product was then subjected to high-performance liquid chromatography-tandem mass spectrometry (HPLC–MS/MS) analysis. HPLC analysis was performed using a ZORBAX SB-C18 (Agilent Technologies) column (2.1 mm X 150 mm; 3.5 mm). Mobile phase A solvents consisted of methanol/0.1% methanoic acid, and mobile phase B solvents consisted of ultrapure water/0.1% methanoic acid. The injection volume was 2 µL. The MS conditions were as follows: the spray voltage was 4500 V; the pressure of the air curtain, nebulizer, and aux gas was 15, 65, and 70 psi, respectively; and the atomizing temperature was 400 °C.

Proline and Soluble Sugar Contents Measurement.

Soluble sugar measurements were carried out according to a previous report (Li et al. 2004). The rosette leaves were harvested, weighed, ground into fine powder in liquid nitrogen and then incubated in 75% ethanol overnight with gentle shaking. After centrifugation at 20,000 g, 20 µL of each extract was incubated with 1000 µL of anthrone reagents [0.15% (w/v) anthrone, 72% (v/v) H₂SO₄, and 28% (v/v) water] at 100 °C for 1 h. The soluble sugar value was measured at 630 nm.

Proline measurement was carried out according to a previously described method (Bates et al. 1973). The rosette leaves were weighed and subjected to extraction using 3% sulfosalicylic acid. After filtration, 2 mL of each filtrate was incubated with 2 mL of glacial acetic acid and 2 mL of acid ninhydrin reagent [2.5% (w/v) ninhydrin, 60% (v/v) glacial acetic acid, and 40% (v/v) 6 M phosphoric acid] at 100 °C for 1 h. The reaction was terminated in an ice bath. Toluene (4 mL) was added to the extraction followed by vigorous shaking for 20 s. After incubation at 23 °C for 24 h, the proline values were measured at 520 nm.

Results

***Dgat1-1* mutant exhibit enhanced chilling sensitivity**

When 3-week-old plants were exposed to 4 °C for several weeks, the rosettes of *dgat1-1* mutants became yellowish when it was exposed to chilling stress for 4 weeks compared with the wild-type rosettes under the same conditions (Figure 1). This result is consistent with previous reports of Tan et al (2018) and *dgat1* mutants exhibit enhanced sensitivity to freezing stress (data not shown).

Contents of Proline and Sugars were Decreased in *DGAT1* Mutants under Cold Conditions

In *Arabidopsis*, osmolytes such as proline and soluble sugars accumulate in response to cold stress, which may help reduce cellular damage from dehydration during freezing (Xin and Browse 1998). In this study, the contents of proline, glucose and fructose in wild-type, *dgat1-1*, and *dgat1-2* mutants after 3 days of cold accumulation and normal conditions were measured (Figure 2). The proline content in the CA group was generally higher than that in normal (non-cold acclimation, NA) plants. The contents of glucose and proline in both *dgat1* mutants were lower than those in the wild type under normal conditions and under cold acclimation. The content of fructose in the *dgat1-2* mutant was lower than that in the wild type under normal growth conditions, but there was no significant difference after cold acclimation. Lower accumulation of proline and carbohydrates during cold acclimation may contribute to the sensitive phenotype of the *dgat1* mutants.

Phenotypic analysis of *DGAT1/SAG101* double mutants.

Previous studies found that *dgat1* mutants are sensitive to freezing and chilling stress, while SA-deficient mutants *sag101-3* (senescence-associated gene101), *eds1-22* (enhanced disease susceptibility1), and *pad4-1* (phytoalexin deficient4) exhibit enhanced tolerance to freezing stress (Chen et al., 2015; Tan et al., 2018). We obtained similar results (data not shown). In this study, *dgat1-1* and *sag101-3*, *eds1-22* and *pad4-1* double mutants were constructed, and homozygous double mutants were used for freezing tolerance analysis. No significant differences were found between the wild type, single mutant, and double mutant under normal growth conditions. After freezing treatment and 7 days after recovery, the *dgat1-1* single mutant was more sensitive than the wild type, the freezing tolerance of the double mutant was between those of the two single mutants, and the dry weight and survival ratio also support this conclusion (Figure 3). This indicates that *SAG101-3*, *EDS1-22* and *PAD4-1* mutations can recover the freezing tolerance of *dgat1* mutants at least partially. We can speculate that the DAG to TAG conversion in these mutants was an important factor affecting freezing resistance and are possibly mediated by SA.

Freezing-Induced Phytohormone Changes in *dgat1* Mutants

The phytohormones SA, jasmonates (jasmonic acid, JA-Ile, OPDA), and ABA play important roles in regulating plant responses to chilling and freezing temperatures in *Arabidopsis* (Scott et al. 2004; Miura and Ohta, 2010; Shi et al., 2012; Hu et al. 2013; Kim et al. 2013; Shinkawa et al., 2013). We measured the endogenous levels of free SA and jasmonates in the rosettes of wild-type, *dgat1-1*, and *dgat1-2* plants under normal growth conditions (NA) upon freezing treatment and recovery. As shown in Figure 4, the levels of free SA increased slightly after freezing exposure, and the *dgat1* mutants showed enhanced accumulation of SA under NA and freezing conditions, whereas no significant changes in ABA, JA, JA-Ile,

or OPDA were observed between NA and the freezing plants. After recovery, the contents of ABA, SA, and jasmonates increased remarkably, and the levels of SA, JA, and OPDA were higher in the *dgat1* mutants than in the wild type, while the contents of ABA and JA-Ile were comparable between the wild type and mutants.

Changes in Lipid Species in Wild-Type and *DGAT1* Mutants Following Freezing Treatment

To further confirm that SA might affect freezing tolerance by regulating DAG and TAG in the *DGAT1* mutants, we measured DAG and TAG content in SA-deficient *sid2* and SA-overexpressing *siz1* mutants (Miura and Ohta, 2010) under normal growth conditions (22 °C) and freezing treatment (-8 °C). At 22 °C, we observed little difference in either DAG or TAG levels between the wild type and mutants (Figure 5, detail species of DAG and TAG are not shown). In contrast, after freezing treatment, the DAG and TAG levels were dramatically elevated in all plants compared to the untreated control (Figure 5). Interestingly, after freezing treatment, the accumulation of DAG was lower in the *sid2* mutant than in the wild type, and *siz1* showed a more remarkable increase in DAG; accordingly, the trend of TAG accumulation was in contrast to DAG in the *sid2* and *siz1* mutants compared to the wild type. We can speculate that higher SA content could inhibit the DAG to TAG conversion.

Discussion

Previous studies indicate that the cold-inducible transcripts of *DGK2*, *DGK3*, and *DGK5* were significantly more upregulated and thus the PA content was higher in *dgat1* mutants than in the wild type during cold stress. Moreover, *dgat1* mutants showed stimulated NADPH oxidase activity and enhanced RbohD-dependent hydrogen peroxide production compared with the wild type. These findings demonstrate that the conversion of DAG to TAG by *DGAT1* is critical for plant freezing tolerance, and plants tend to choose the DAG to PA pathway when the DAG to TAG conversion pathway is blocked (Tan et al., 2018).

Phytohormone SA could regulate the level of phospholipids. For example, previous research shows that SA induces an increase in phosphoinositides (PI) with no increase in PA (the phosphorylated derivative of DAG, a phospholipase C product), which likely reflects an in vivo inhibition of PI-PLC by SA. Ruelland et al. proposed a model in which SA inhibits PI-PLC activity and alters the levels of PI-PLC products and substrates, thereby divergently regulating gene expression (2014). In addition, previous studies have shown that SA activates PLD in *Arabidopsis* cell suspension cultures (Krinke et al., 2009).

SA has a contradictory role in cold stress tolerance. High-dose SA treatment causes stress, while exogenous SA treatment can protect plants against chilling stress at optimal doses. SA and other phenol derivatives are known to improve the cold tolerance of plants such as maize, banana, cucumber, rice, and pomegranate fruit (Janda et al. 1997, 1999; Kang et al. 2007; Kang and Saltveit 2002; Sayyari et al. 2009). Other related compounds, such as BA, aspirin or coumaric acid, may also have a protective role in young maize plants against cold stress (Janda et al. 1998, Janda, Szalai et al. 2000, Horváth et al. 2002). Preliminary SA treatment could increase antioxidant enzymes, reduce lipid peroxidation through the

inhibition of lipoxygenase activity and decrease the H₂O₂ content, leading to the maintenance of cellular membrane integrity under cold stress conditions (Janda et al. 1997, 1999; Lapenna et al. 2009; Wang et al. 2006; Sasheva et al. 2010). In addition, exogenous SA caused an increase in ice nucleation activity under cold and control conditions, similar to cold acclimation in winter wheat leaves (Taşgn et al. 2003). The SA analogue 4-hydroxybenzoic acid ameliorated the freezing tolerance of the spring wheat strain Chinese Spring that is due to reinforcement of the cell wall (Horvth et al. 2007).

SA treatment may alter endogenous SA metabolism, either by inducing de novo synthesis (Szalai et al. 2011) or by decreasing and disturbing it (Rakhmankulova, et al. 2010). *DGAT1* mutants showed significantly lower levels of DAG and PA and higher levels of TAG than the wild type. The application of SA suppressed the cold-induced expression of *DGATs* and *DGKs* in wild-type leaves (Chen et al. 2015). It is conceivable that DAG may act as a common substrate for PA and TAG production upon cold exposure, the homeostasis of which may determine the plant tolerance to such stresses. However, the molecular mechanism of how SA regulates lipid remodelling and responses to chilling and freezing remains to be investigated.

Declarations

Author Contributions: LX, WZ, YX conceived and designed this paper. JW, YZ, and HL conduct the experiments. YX, LX revised the manuscript.

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Conflicts of Interest: The authors declare that no conflict of interest.

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Figures

Figure 1

***DGAT1* Knockout Mutants Show Increased Sensitivity to Chilling Stress.**

Images of three-week-old WT, *dgat1-1*, and *dgat1-2* plants grown at 22 °C before (CK, top panel) and after cold treatment (4 °C, bottom panels) for 1 to 4 weeks.

Figure 2

Accumulation of Osmolytes in Wild Type and *dgat1* mutants.

Contents of proline and soluble sugars in WT, *dgat1-1*, and *dgat1-2* plants under NA and CA conditions. WT, *dgat1-1*, and *dgat1-2* plants were untreated (NA) or cold-treated for 3 days and rosettes were collected for metabolite extraction. Ribitol was added as an internal quantitative standard for GC-MS analysis. The experiments were repeated (biological replicates) four times, with similar results, and the representative data from one replicate are shown. Data are means $\pm$ SD calculated from three technical replicates. Asterisks indicate significant differences from wild type (* $P < 0.05$; ** $P < 0.01$ by Student's *t*-test).

Figure 3

Phenotype of *DGAT1/SAG101* (*EDS1*, and *PAD4*) double mutants

Images of three-week-old WT, *dgat1-1/sag101*, *dgat1-1/eds1* and *dgat1-1/pad4* plants grown at 22 °C before (CK, top panel) and after freezing treatment (4 °C, bottom panels) for 1 to 4 weeks.

Figure 4

Levels of Free SA, ABA and Jasmonates in the Rosettes of Wild-Type (WT), and *dgat1* Plants.

Free SA and SA glucosides were extracted from rosettes of 4-week-old WT, *dgat1-1*, and *dgat1-2* plants harvested after NA, freezing treatment (-8 °C) for 1 h (Freezing) and recovery for 12 h (Recovery), and the extracts were analyzed by LC-MS/MS. The experiments were repeated (biological replicates) three times, with similar results, and the representative data from one replicate are shown. Data are means ± SD calculated from three technical replicates. Asterisks with “a” indicate significant differences from that of NA wild type and with “b” indicate significant differences from wild type (*P < 0.05; **P < 0.01 by Student’s *t*-test).

Figure 5

DAG and TAG Profiles of 4-Week-Old Wild-Type (WT), *dgat1-1*, and *dgat1-2* Rosettes Before (22 °C) and After CA Followed by Freezing Treatment (-8 °C).

Total amounts (signal/mg dry weight) of diacylglycerol (DAG; left column) and tri-

acylglycerol (TAG; right column) in WT, *dgat1-1*, and *dgat1-2* rosettes. Data are means ± SD calculated from four technical replicates. Asterisks indicate significant differences from wild type (*P < 0.05; **P < 0.01 by Student’s *t*-test).