Salicylic acid-mediated diacylglycerol/triacylglycerol conversion affects the freezing tolerance of *Arabidopsis*

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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 the freezing tolerance of *Arabidopsis*. The phytohormone salicylic acid (SA) and DAG/TAG ratio are known to play important roles in the chilling and freezing tolerance of *Arabidopsis* while the interaction or connection was not clear. In our study, we analysed the chilling phenotype, proline and sugar accumulation, phytohormone content, and lipid profiling of *dgat1* mutants during chilling or freezing stress. We found that *dgat1-1* mutants exhibited higher sensitivity to long time 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 SA signalling components SAG101, EDS1, and PAD4 through phenotyping analysis of double mutants. *Dgat1* mutants accumulated more SA, ABA (abscisic acid), 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 negatively mediate the freezing tolerance of *Arabidopsis* by regulating the ratio of DAG and TAG, which influences the integrity of the membrane and it provide a novel research direction.

**Keywords** Salicylic acid · Diacylglycerol · Triacylglycerol · Cold stress · *Arabidopsis*

**Abbreviations**

ABA  Abscisic acid  
*acd6* Accelerated cell death 6  
CA Cold-acclimated  
*cpr1* Constitutive expresser of PR genes  
DAG Diacylglycerol  
DGAT Diacylglycerol acyltransferase  
DGKs Diacylglycerol kinases  
EDS1 Enhanced disease susceptibility 1  
eds5 Enhanced disease susceptibility 5  
GGGT Galactolipid galactosyltransferase  
HPLC–MS/MS High-performance liquid chromatography–tandem mass spectrometry  
JA Jasmonic acid  
JA-Ile Jasmonate isoleucine  
JAZ Jasmonate zim-domain  
MGDG Monogalactosyl diacylglycerol  
NA Non-acclimated  
NADPH Nicotinamide adenine dinucleotide phosphate  
OPDA 12-Oxyphytodienoic acid  
PA Phosphatidic acid  
PAD4 Phytoalexin deficient4  
PC Phosphatidyleholine  
PE Phosphatidylethanolamine  
PG Phosphatidylglycerol  
PI Phosphoinositides  
PI-PLC Phosphoinositide-specific phospholipase C  
PLD Phospholipase D  
SA Salicylic acid

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SAG101  Senescence-associated gene101
SFR2   Sensitive to freezing 2
sid2   SA-deficient mutant
siz1   SA-excessive accumulated mutant
TAG   Triacylglycerol
WT   Wild type

**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 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*. Jasmonates (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; Wang et al. 2018). 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; Xia et al. 2009; Kim et al. 2013). Interestingly, at low temperature, *NahG* plants was correlated with enhanced transcription of the CK-regulated D-type cyclin CYCD3:1 that promotes the G1/S phase transition in *Arabidopsis* (Xia et al. 2009) and it suggested SA could affect cell cycle progression in plants and this may contribute to SA-induced growth inhibition in the cold (Wolters and Jürgens 2009).

In addition, the SA-deficient mutants *eds5* and *sid2* (chlo- roplastic SA synthesis deficient) had higher survival rates during recovery after freezing stress (Kim et al. 2013; Majlath 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 extraplastic membrane lipid alterations affect membrane fluidity and integrity in plants exposed to cold stress (Uemura et al. 1995; Welti 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 monogalactosyl diacylglycerol (MGDG) and phosphatidylglycerol (PG), while phosphatidic acid (PA) and lysophospholipids increase dramatically (Welti et al. 2002). MGDGs have a small head group, and PAs lack 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 (Verkleij et al. 1982; Moellering et al. 2010; Steponkus 1984; Uemura et al. 1995; Thomashow 1999; Welti et al. 2002).

In *Arabidopsis*, disruption of SFR2 (sensitive to freezing 2), which encodes a galactolipid:galactolipid galactosyltransferase (GGGT) that converts MGDG to oligogalactolipids (such as diacylglycerol, DAG), the results in severe injury during freezing stress (Thorby 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 DGK transcripts are induced rapidly in response to chilling stress (Gómez-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 dga1 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 dga1-1 (CS3861, a gift from Shi Lei of Oil Crops Research Institute, Chinese Academy of Agricultural Science) and T-DNA insertion mutant dga1-2 (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 days and subsequently transferred to a 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 or several weeks under a normal light/dark cycle, chilling treatment, 4-week-old plants were transferred from the growth room to a cold growth chamber (4 °C) for 3 days of acclimation or several weeks under a normal light/dark cycle. For the freezing treatment, all 4-week-old NA (non-acclimated) or CA (cold-acclimated) soil-grown plants or 11-day-old seedlings grown on MS medium plates were transferred to a growth chamber (Blue Pard LRH- LRH-500CA) with temperatures reduced steadily from 4 to −2 °C (2 °C/h). 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 h, the plants were thawed overnight at 4 °C. Plants were photographed after a 7-day 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 μL isopropanol/methanol (1/1, v/v) solution, vortexed, and centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatant was analysed using LC–MS. A Kinetex C18 column (100×2.1 mm, 1.9 μm) and the following gradient were applied for the experiment: 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. Mobile phase A was acetonitrile/water (3:2, v/v) and 10 mM ammonium formate. Mobile phase B was acetonitrile/isopropanol (1:9, v/v), 10 mM ammonium formate and 0.1% formic acid. The flow rate was 0.4 mL/min, and the column was at 45 °C. Parameters used for mass spectrometry were described as follows: positive ion mode, heater temperature 300 °C, sheath gas flow rate 45 arb, aux gas flow rate 15 arb, sweep gas flow rate 1 arb, spray voltage 3.0 kV, capillary temperature 350 °C, S-Lens RF level, 30%. scan ranges 200–1500; negative ion mode, heater temperature 300 °C, sheath gas flow rate 45 arb, aux gas flow rate 15 arb, sweep gas flow rate 1 arb, spray voltage 2.5 kV, capillary temperature 350 °C, S-Lens RF level, 60%. scan ranges 200–1500. Lipidomics assays were 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 (2010). 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, dissolved in 150 μL methanol (0.1% methanoic 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×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 pressures of the air curtain, nebulizer, and aux gas were 15, 65, and 70 psi, respectively; and the atomizing temperature was 400 °C.

**Proline and soluble sugar content measurements**

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**

**The dgat1-1 mutant exhibits 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 they were exposed to chilling stress for 4 weeks compared with the wild-type rosettes under the same conditions (Fig. 1). This result is consistent with previous reports by Tan et al. (2018), and dgat1 mutants exhibited enhanced sensitivity to freezing stress (data not shown).

**Proline and sugar contents 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 (Fig. 2). The proline...
content in the CA (cold-acclimated) group was generally higher than that in normal (noncold 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 (EDS1 and PAD4) 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 (Fig. 3). This indicates that SAG101-3, EDS1-22 and PAD4-1 mutations can at least partially recover the freezing tolerance of dgat1 mutants. We can speculate that the DAG to TAG conversion in these mutants was an important factor affecting freezing resistance and is possibly mediated by SA.

Freezing-induced phytohormone changes in dgat1 mutants

The phytohormones SA, jasmonates (including 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 Fig. 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.

![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 ± SD calculated from three technical replicates. Asterisks indicate significant differences from the wild type (*P<0.05; **P<0.01 by Student’s t test)](image-url)
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 sizl 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 (Fig. 5, detailed 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 (Fig. 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 a higher SA content could inhibit the DAG to TAG conversion.

**Discussion**

Previous studies indicate that the cold-inducible transcripts of DKG2, DKG3, and DKG5 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).

The phytohormone SA could regulate the level of phospholipids. For example, previous research shows that SA induces an increase in phosphoinositides (PIs) 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). Besides, recent study suggested that SA could mediate plasmodesmal closure and trigger Remorin-dependent membrane lipid nanodomain reorganization, leading to enhancement of the liquid-ordered phase (Huang et al. 2019).

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. 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, 2000; Horváth et al. 2002). Preliminary SA treatment could increase antioxidant enzymes, reduce lipid peroxidation through the inhibition of lipoygenase activity and decrease the H2O2 content, leading to the maintenance of cellular membrane integrity under cold stress conditions (Janda et al. 1999; Lapenna et al. 2009; Wang et al. 2020a, b). In addition, exogenous SA caused an increase in ice nucleation activity under cold and control conditions.
Phytohormone salicylic acid could negatively mediate the freezing tolerance of *Arabidopsis* by weakening the transformation of DAG to TAG, which influences the integrity of the membrane.

**Author contributions** LX conceived and designed this paper. JW, YZ, and HL conducted the experiments. LX and YX revised the manuscript.

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**Conflict of interest** The authors declare that they have no conflicts of interest.

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**Conclusions**

**Fig. 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 triacylglycerol (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 the wild type (*P<0.05; **P<0.01 by Student’s t test)
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