

BRIEF COMMUNICATION

Lipid profiling and tolerance to low-temperature stress in *Thellungiella salsuginea* in comparison with *Arabidopsis thaliana*

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Abstract

Changes in membrane lipid composition is a fundamental strategy for plants to resist low-temperature stress. We compared members of 11 membrane glycerolipid classes in *Thellungiella salsuginea* and its close relative *Arabidopsis thaliana* at normal growth temperature and during cold acclimation (CA), freezing (FR), and post-freezing recovery (PFR). The results showed several properties of *T. salsuginea* distinct from that in *A. thaliana* which included: 1) low relative content of phosphatidic acid (PA) and a rapid increase and decrease of PA during FR and PFR, respectively; 2) insensitivity of lyso-phospholipids to freezing; and 3) high ratio of phosphatidylcholine to phosphatidylethanolamine. All these properties were in favour of maintaining membrane integrity and stability and therefore enable *T. salsuginea* to be more tolerant to freezing than *A. thaliana*.

Additional key words: cold acclimation, freezing, membrane glycerolipids, post-freezing recovery.

Low-temperature stress, in particular freezing, is a major environmental limitation for plant growth and agricultural production and causes substantial economic losses every year (Boyer 1982). In nature, the response of overwintering plants to low temperature can be divided into three distinct phases: cold acclimation (CA), freezing (FR), and post-freezing recovery (PFR). The composition of membrane glycerolipids, the major constituents of cellular membranes, undergo distinct changes during these three phases (Uemura and Steponkus 1999, Li *et al.* 2008). Membranes are the primary sites of injuries induced by low temperature (Levitt 1980). The tolerance of plants to low-temperature stress depends mainly on the capacity of their membranes to avoid injury and maintain their integrity. For example, the formation of the

hexagonal II (H_{II}) phase - a non-lamellar phase - is a typical membrane injury during freezing. Overexpression of the cold-regulated gene *COR15a* can reduce the formation H_{II} phase and increase freezing tolerance in *Arabidopsis* protoplasts (Steponkus *et al.* 1998). Plants employ several strategies to maintain membrane integrity during low-temperature stress. Accumulation of cellular osmolytes can reduce the risk of intracellular ice formation (Levitt 1980, Atici *et al.* 2003). An increased proportion of unsaturated lipid chains confers greater membrane fluidity which helps to maintain membrane function during exposure to low temperature (Upchurch 2008, Zhang *et al.* 2010). Changes in lipid composition, such as enhancing the ratio of phosphatidylcholine (PC) to phosphatidylethanolamine (PE), can reduce formation

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Abbreviations: CA - cold acclimation; CBF - C-repeat binding factor; DGDG - digalactosyldiacylglycerol; FR - freezing; LPC - lysophosphatidylcholine; LPE - lysophosphatidylethanolamine; LPG - lysophosphatidylglycerol; LPLs - lysophospholipids; MGDG - monogalactosyldiacylglycerol; PA - phosphatidic acid; PC - phosphatidylcholine; PE - phosphatidylethanolamine; PFR - post-freezing recovery; PG - phosphatidylglycerol; PI - phosphatidylinositol; PS - phosphatidylserine.

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of the non-bilayer phase (Uemura *et al.* 1995, Harwood 1998). Suppression of phosphatidic acid (PA) can decrease ion leakage induced by freezing because formation of the H_{II} phase is decreased (Verkleij *et al.* 1982, Welti *et al.* 2002, Li *et al.* 2008). Therefore, profiling lipid changes during low-temperature stress is an important approach to gain insight into plant tolerance to such stress.

Previously, we have studied the changes in 120 lipid molecular species from 10 classes of membrane glycerolipid under normal temperature, CA, FR, and PFR in *Arabidopsis thaliana* by electrospray ionization tandem mass spectrometry (ESI-MS/MS)-based lipidomics (Welti *et al.* 2002, Li *et al.* 2004, 2008). We found several characteristic changes in lipid composition in response to low temperature such as a marked increase of PA and lysophospholipids (LPLs) upon FR; degradation of the chloroplastic lipid monogalactosyl-diacylglycerol (MGDG) into PA under FR; and differential degradation of extraplastidic and plastidic lipids during FR and PFR (Li *et al.* 2008). To understand the mechanisms involved in responses to environmental stresses, wild plant species from specific or extreme habitats are usually chosen and compared with model plants. In the current study, we studied the changes in lipid composition in *Thellungiella salsuginea* under the same stress conditions.

T. salsuginea is a halophytic plant and can tolerate extreme environmental conditions, which include salinity (Taji *et al.* 2004), low temperature (Griffith *et al.* 2007), drought (Rahman *et al.* 2010), ozone (Li *et al.* 2006), and nitrogen limitation (Kant *et al.* 2008). *T. salsuginea* is closely related to *A. thaliana*, and genes from these two species share approximately 90 % identity (Zhu 2001). This similarity could facilitate research on *T. salsuginea* because the complete genome sequence of *A. thaliana* is known. *T. salsuginea* was selected as a model plant for studies of abiotic stress tolerance (Zhu 2001, Griffith *et al.* 2007). Its tolerance mechanisms have been compared with *A. thaliana* at the transcriptome and proteome levels (Gong *et al.* 2005, Du *et al.* 2008, Gao *et al.* 2008, 2009). However, whether and how membrane lipids contribute to the tolerance of *T. salsuginea* to cold stress is unknown. Herein, we report several characteristic changes in membrane composition in *T. salsuginea* in response to low-temperature stress and make comparisons with *A. thaliana*.

The Shandong ecotype of *Thellungiella salsuginea* (C.A. Meyer) O.E. Schulz (kindly provided by Prof. Hui Zhang and Prof. Zhiyi Cao, Shandong Normal University, China) was grown in a Hoagland nutrient solution with minor modification (Tocquin *et al.* 2003). Seeds were stratified at 4 °C for 7 d after surface sterilization and sown in seed-holders that contained 1/2 MS medium. Seedlings with 2 to 4 leaves were transferred to containers with 1/4 Hoagland solution for further growth at temperature of 22 °C, irradiance of

120 µmol m⁻² s⁻¹ (fluorescent tubes), 12-h photoperiod, and relative humidity of 60 %.

Low-temperature treatment of *T. salsuginea* was carried out as described previously for *A. thaliana* (Welti *et al.* 2002, Li *et al.* 2008). For CA, 8-week-old *T. salsuginea* plants were grown at 4 °C for 3 d under irradiance of 30 µmol m⁻² s⁻¹ and a 12-h photoperiod. For FR, cold-acclimated plants were subjected to programmed lowering the temperature from 4 to -2 °C at 3 °C h⁻¹, and then incubated with ice chips for ice nucleation. Subsequently, the temperature was lowered to -8 °C at 1 °C h⁻¹ and held at -8 °C for 2 h before sampling. For PFR, the temperature was raised from -8 to 4 °C at 1 °C h⁻¹ and held at 4 °C for 12 h before sampling.

Lipid extraction, sample analysis, and data processing were performed as described previously with minor modifications (Welti *et al.* 2002, Li *et al.* 2008). Briefly, the rosettes of a plant were harvested at sampling time and were transferred immediately into 2 cm³ of isopropanol with 0.01 % butylated hydroxytoluene at 75 °C. The tissue was extracted with chloroform/methanol (2:1) three times with 2 h of agitation each time. The remaining plant tissue was heated overnight at 105 °C and weighed. Lipid samples were analyzed on a triple quadrupole MS/MS equipped for ESI. The lipids in each class were quantified by comparison with two internal standards for the class. Data processing was performed as previously described (Welti *et al.* 2002). Five replicates from each sampling time were analyzed. A Q test was performed on the total amount of lipid in each class of head group, and data from discordant samples were removed (Welti *et al.* 2002). The data were subjected to one-way analysis of variance (ANOVA) with SPSS 13.0. Statistical significance was tested by Fisher's least significant difference (LSD) method.

After treatment at 4 °C for 3 d, *T. salsuginea* looked normal by visual inspection. This treatment might not make *T. salsuginea* fully cold-acclimated but its time course was comparable with the previous experiment on *Arabidopsis* (Welti *et al.* 2002). There were no significant changes in the lipid composition of the membrane, except for digalactosyldiacylglycerol (DGDG) and PA which showed significant increases (Table 1). The results indicate that the effects of nonfreezing low temperature on membrane lipid composition is much smaller in *T. salsuginea* than that in *A. thaliana* (Welti *et al.* 2002).

During FR at -8 °C and PFR at 4 °C for 12 h, *T. salsuginea* showed no severe damage. This means that -8 °C is a sublethal freezing temperature for *T. salsuginea*. However, obvious changes in the composition of membrane lipids took place. PA content increased about eight-fold (Table 1). During PFR, the PA content declined to almost the background level during CA (Table 1). In contrast, PA increased in *A. thaliana* approximately seven-fold during FR and remained at the same level during PFR (Li *et al.* 2008). FR induced high

Table 1. Molar percentage of lipids in each head group class during growth in *T. salsuginea* at normal temperature (C), or during cold acclimation (CA), freezing (FR) and post-freezing recovery (PFR). Values in the same row with different letters are significantly different ($P < 0.05$). Values are means $\pm$ SD ($n = 4$ or 5).

Lipids	C	CA	FR	PFR
Digalactosyldiacylglycerol (DGDG)	15.14 $\pm$ 0.66 ^c	16.13 $\pm$ 0.55 ^b	17.25 $\pm$ 0.78 ^a	16.21 $\pm$ 0.84 ^b
Monogalactosyldiacylglycerol (MGDG)	65.12 $\pm$ 1.57 ^a	63.47 $\pm$ 2.52 ^a	58.96 $\pm$ 2.05 ^b	63.74 $\pm$ 2.78 ^a
Phosphatidylglycerol (PG)	5.611 $\pm$ 0.486 ^b	6.371 $\pm$ 0.608 ^{ab}	7.157 $\pm$ 0.839 ^a	5.783 $\pm$ 0.758 ^{ab}
Phosphatidylcholine (PC)	8.639 $\pm$ 0.328 ^a	7.827 $\pm$ 1.181 ^a	8.095 $\pm$ 0.687 ^a	8.536 $\pm$ 1.563 ^a
Phosphatidylethanolamine (PE)	3.768 $\pm$ 0.020 ^b	3.891 $\pm$ 0.494 ^b	3.748 $\pm$ 0.123 ^b	4.370 $\pm$ 0.409 ^a
Phosphatidylinositol (PI)	1.627 $\pm$ 0.146 ^c	1.759 $\pm$ 0.171 ^{bc}	2.330 $\pm$ 0.148 ^a	2.037 $\pm$ 0.348 ^{ab}
Phosphatidylserine (PS)	0.120 $\pm$ 0.016 ^a	0.108 $\pm$ 0.024 ^a	0.106 $\pm$ 0.015 ^a	0.127 $\pm$ 0.015 ^a
Phosphatidic acid (PA)	0.030 $\pm$ 0.010 ^c	0.271 $\pm$ 0.101 ^b	2.213 $\pm$ 0.803 ^a	0.447 $\pm$ 0.138 ^b
Lysophosphatidylglycerol (LPG)	0.009 $\pm$ 0.006 ^b	0.007 $\pm$ 0.003 ^b	0.020 $\pm$ 0.010 ^a	0.012 $\pm$ 0.006 ^{ab}
Lysophosphatidylethanolamine (LPE)	0.041 $\pm$ 0.004 ^b	0.046 $\pm$ 0.005 ^b	0.072 $\pm$ 0.020 ^a	0.061 $\pm$ 0.010 ^a
Lysophosphatidylcholine (LPC)	0.004 $\pm$ 0.003 ^c	0.010 $\pm$ 0.007 ^{bc}	0.029 $\pm$ 0.014 ^a	0.018 $\pm$ 0.006 ^{ab}

content of PA and this was associated with ionic leakage resulting from membrane damage (Welti *et al.* 2002). Thus, the PA content in *A. thaliana* was much higher during FR and PFR (Welti *et al.* 2002, Li *et al.* 2008) than that in *T. salsuginea* (Table 1). These results indicated that the increase in PA in *T. salsuginea* was a response of membrane lipids to FR. They also suggested that membrane composition was more able to recover to pre-freezing levels and PA-related membrane injuries are predicted to occur less frequently in *T. salsuginea* than in *A. thaliana*.

Lysophospholipids (LPLs), which include lysophosphatidylcholine (LPC), lysophosphatidylglycerol (LPG), and lysophosphatidylethanolamine (LPE), are derived by hydrolysis of phospholipids at the sn-1 or sn-2 positions of the glycerol backbone. At normal temperature, the content of LPLs in *T. salsuginea* (Table 1) and *A. thaliana* (Li *et al.* 2008) was $< 0.1\%$ which was a very small proportion of the total membrane lipid. During FR and PFR, LPLs changed subtly in *T. salsuginea* (Table 1). This is remarkable different to that in *A. thaliana*. FR led to a 7- to 10-fold increase in LPLs in *A. thaliana* (Welti *et al.* 2002, Li *et al.* 2008). The results indicated that LPLs in *T. salsuginea* were not as sensitive to FR as those in *Arabidopsis*, though it might also have a role in freezing process. This suggested that the membrane lipid composition in *T. salsuginea* was relatively stable during freezing.

PC is a bilayer-forming lipid in extraplastidic membranes. Accumulation of PC is thought to be helpful for membrane stabilization and a high PC/PE ratio is beneficial for plants to survive at low temperature (Uemura *et al.* 1995, Harwood 1998). In *A. thaliana*, PC was degraded and PC/PE ratios were 1.68, 1.48, and 1.28 during CA, FR, and PFR, respectively (Table 2). In *T. salsuginea*, PC/PE ratios were 1.97, 2.16, and 1.93 during CA, FR, and PFR, respectively. Thus, the ratios in *T. salsuginea* were significantly higher than those in

A. thaliana and they were maintained at higher levels throughout the low-temperature treatment (Table 2). These results showed that the extraplastidic membrane was stable during low-temperature stress which might enhance tolerance to freezing in *T. salsuginea*.

Table 2. PC/PE ratio during cold acclimation (CA), freezing (FR), and post-freezing recovery (PFR) in *T. salsuginea* and *A. thaliana*. Values in the same row with different lowercase letters are significantly different ($P < 0.05$). Values in the same column with different capital letters are significantly different ($P < 0.05$). *Values were calculated on the basis of previously published data. Values are means $\pm$ SD ($n = 5$).

Species	CA	FR	PFR
<i>T. salsuginea</i>	1.97 $\pm$ 0.33 ^{aA}	2.16 $\pm$ 0.26 ^{aA}	1.93 $\pm$ 0.13 ^{aA}
<i>A. thaliana</i> *	1.68 $\pm$ 0.01 ^{aA}	1.48 $\pm$ 0.11 ^{bB}	1.28 $\pm$ 0.29 ^{bB}

T. salsuginea has attracted much attention in recent years due to its tolerance to salinity and close genetic relationship to *A. thaliana* (Inan *et al.* 2004, Gong *et al.* 2005, Stepien and Johnson 2009). *T. salsuginea* also possesses strong tolerance to freezing. For example, its LT₅₀ (the temperature at which 50 % of the plants die from freezing) is as low as -13 °C for non-acclimated plants and -19 °C after acclimation, which is much greater than *A. thaliana* (Griffith *et al.* 2007). Although the C-repeat binding factor (CBF) regulation pathway has been reported to be involved in tolerance to freezing (Jaglo-Ottosen *et al.* 1998, Griffith *et al.* 2007), further investigation of the mechanisms of tolerance to freezing is still required. The membrane lipids did indeed exhibit characteristic properties in *T. salsuginea*. Basically, the composition of membrane glycerolipids changed less and recovered quicker in *T. salsuginea* than those in *A. thaliana*. We speculate that these properties enable *T. salsuginea* to be more tolerant to freezing than

A. thaliana.

Among the characteristic properties of the membrane lipid composition of *T. salsuginea*, the pattern of PA changes might be the dominant one in enhancing its tolerance to freezing. Content of PA increased dramatically during FR and remained the same during PFR in *A. thaliana* (Li *et al.* 2008). In contrast, content of PA in *T. salsuginea* clearly increased and decreased as the temperature decreased below zero and then returned above zero, respectively. How does the transient change in PA contribute to tolerance to FR in *T. salsuginea*? PA can have dual roles in the response of plants to stress. One role is to be a secondary messenger, *e.g.* during drought (Sang *et al.* 2001), mechanical wounding (Wang *et al.* 2000), nitrogen signalling (Hong *et al.* 2009), and signalling by reactive oxygen species (Zhang *et al.* 2009). Another role is as a structural factor that favours formation of the H_{II} phase which is a non-lamellar phase that usually occurs at sites of membrane injury under conditions of cellular dehydration (Verkleij *et al.* 1982). The formation of H_{II} phase is influenced by a complex interplay of factors including the hydration properties of membranes and spatial separation between bilayers (Steponkus *et al.* 1993). Membranes with increase of PA and unsaturated PE have a strong propensity to form H_{II} phase (Cullis and Dekruiff 1979, Verkleij *et al.* 1982); whereas increases in PC reduce the propensity of H_{II} phase formation (Uemura *et al.* 1995, Harwood 1998). However, in *T. salsuginea*, the contents of PE and PC were maintained and the content of PA rose rapidly when the temperature dropped below 0 °C. Therefore, PA may play the role of a structural factor. PA increase in the membrane might transduce signals of environmental temperature change into the cells and trigger intracellular reactions. When the temperature increased above 0 °C, the content of PA decreased rapidly to normal levels. This decrease reduced the risk of the H_{II} phase being present when normal metabolism resumed during PFR and increased likelihood of maintaining membrane integrity. Moreover, the content of PA induced by freezing in *T. salsuginea* was much lower than that in *A. thaliana* which also mitigated against the formation of

H_{II}. Therefore, both signalling and structural roles of PA are potentially to be benefit to freezing tolerance in *T. salsuginea*. Our results also suggest that inhibition of PA accumulation is a common and effective strategy for plants to tolerate freezing stress and consequently can be a useful approach to improve freezing tolerance in crops.

Other properties of the membrane lipid composition of *T. salsuginea* also enhance its tolerance to freezing. An increase in LPLs is a sensitive indicator of freezing-induced membrane damage in *A. thaliana* (Li *et al.* 2008) and *Brassica napus* (Li and Li 2010). Stable content of LPLs in *T. salsuginea* supports the conclusion that the membranes had strong resistance to freezing stress at -8 °C. During cold acclimation, five lipid classes (MGDG, DGDG, PA, LysoPE, and LysoPC) expressed as [$\mu\text{mol g}^{-1}(\text{d.m.})$] changed significantly in *A. thaliana* (Welti *et al.* 2002) but only MGDG and PA contents changed in *T. salsuginea*. During freezing, seven lipid classes (PG, PC, PE, PA, LysoPG, LysoPE, and LysoPC) changed in *A. thaliana* (Li *et al.* 2008) but only five lipid classes (PI, PA, LysoPG, LysoPE, and LysoPC) changed in *T. salsuginea*. Therefore, number of lipid classes involved in low temperature responses in *T. salsuginea* is less than that in *A. thaliana*. All these properties indicated that the membranes of *T. salsuginea* have a high capacity to maintain their integrity during freezing stress.

At present, there are two mechanisms by which *T. salsuginea* may tolerate extreme stress. The first, revealed from a study of salinity stress in *T. salsuginea*, showed that stress-inducible signalling pathways are constitutive and active even under normal growth conditions (Taji *et al.* 2004). The second is suggested from a study of drought and states that *T. salsuginea* can precisely coordinate the antagonistic relationship between osmotic and biotic stress responses (Wong *et al.* 2006). Plants usually use common strategies to tolerate salinity, low temperature, and drought by cross talk between signalling pathways. Here we show a third mechanism by which *T. salsuginea* tolerates stress, namely precise control of lipid metabolism to enable the maintenance of membrane integrity before, during and after freezing stress.

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