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# Transgenic Arabidopsis Flowers Overexpressing Acyl-CoA-Binding Protein ACBP6 are Freezing Tolerant

Pan Liao[1], Qin-Fang Chen[1,2] and Mee-Len Chye[1,*]

[1]School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong, China
[2]Present address: State Key Laboratory of Biocontrol and Guangdong Key Laboratory of Plant Resources, School of Life Sciences, Sun Yat-sen University, Guangzhou 510275, China.
*Corresponding author: E-mail, mlchye@hkucc.hku.hk; Fax, +852-28583477.
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**Low temperature stress adversely affects plant growth. It has been shown that the overexpression of ACYL-COENZYME A-BINDING PROTEIN6 (ACBP6) resulted in enhanced freezing tolerance in seedlings and rosettes accompanied by a decrease in phosphatidylcholine (PC), an increase in phosphatidic acid (PA) and an up-regulation of *PHOSPHOLIPASE Dδ*(*PLDδ*) in the absence of *COLD-RESPONSIVE* (*COR*)-related gene induction. Unlike rosettes, ACBP6-overexpressor (OE) flowers showed elevations in PC and monogalactosyldiacylglycerol (MGDG) accompanied by a decline in PA. The increase in PC species corresponded to a decline in specific PAs. To better understand such differences, the expression of PC-, MGDG-, proline-, ABA- and *COR*-related genes, and their transcription factors [C-repeat binding factors (CBFs), INDUCER OF CBF EXPRESSION1 (ICE1) and MYB15] was analyzed by quantitative real-time PCR (qRT-PCR). ACBP6-conferred freezing-tolerant flowers showed induction of *COR*-related genes, *CBF* genes and *ICE1*, PC-related genes (*PLDδ*, *CK*, *CK-LIKE1*, *CK-LIKE2*, *CCT1*, *CCT2*, *LPCAT1*, *PLA2α*, *PAT-PLA-IIβ*, *PAT-PLA-IIIα*, *PAT-PLA-IIIδ* and *PLDζ2*), MGDG-related genes (*MGD* genes and *SFR2*) and ABA-responsive genes. In contrast, ACBP6-conferred freezing-tolerant rosettes were down-regulated in *COR*-related genes, *CBF1*, PC-related genes (*PEAMT1*, *PEAMT2*, *PEAMT3*, *CK1*, *CCT1*, *CCT2*, *PLA2α*, *PAT-PLA-IIIδ* and *PLDζ2*), MGDG-related genes (*MGD2*, *MGD3* and *SFR2*) and some ABA-responsive genes including *KIN1* and *KIN2*. These results suggest that the mechanism in ACBP6-conferred freezing tolerance varies in different organs.**

**Keywords:** COR • C-repeat binding factors • Flowers • Monogalactosyldiacylglycerol • Phosphatidylcholine • Proline.

**Abbreviations:** ABI5, ABA-INSENSITIVE5; ACBP, acyl-CoA-binding protein; AtPOX, *Arabidopsis thaliana* proline oxidase; CA, cold-acclimated; CBF, C-repeat binding factor; CCT, phosphorylcholine cytidylyltransferase; CK, choline kinase; COR, COLD-RESPONSIVE; CRT, C-repeat element; DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; DRE, dehydration-responsive; ER, endoplasmic reticulum; F, freeze-treated; GGGT, galactolipid galactosyltransferase; ICE1, INDUCER OF CBF EXPRESSION1; LPCAT, lysophosphatidylcholine acyl-transferase; LysoPC, lysophosphatidylcholine; MGDG, monogalactosyldiacylglycerol; NA, non-acclimated; NCED3, 9-CIS-EPOXYCAROTENOID DIOXYGENASE3; OE, overexpressor; PA, phosphatidic acid; PC, phosphatidylcholine; P5CS, $\Delta^1$-pyrroline-5-carboxylate synthase; PE, phosphatidylethanolamine; PEAMT, phosphoethanolamine *N*-methyltransferase; PG, phosphatidylglycerol; PI, phosphatidylinositol; PLA, phospholipase A; PLD, phospholipase D; PS, phosphatidylserine; qRT-PCR, quantitative real-time PCR; R, recovery; SFR2, SENSITIVE TO FREEZING2; WT, wild type.

## Introduction

Low temperature stress including chilling (<20°C) and freezing (<0°C) adversely affects plant growth, development and agricultural productivity (Li et al. 2004, Chinnusamy et al. 2007). Plants including Arabidopsis adapt to survive freezing when pre-exposed to low temperature, i.e. during cold acclimation (Moellering et al. 2010). Changes in gene expression, metabolism and remodeling of tissue and cell structures occur as the plant responds (Chinnusamy et al. 2007, Moellering et al. 2010).

The identification of genes and transcription factors that enhance cold and freezing tolerance provides a first step towards developing potential applications in agriculture (Thomashow 1999, Chinnusamy et al. 2007, Zhou et al. 2011, Theocharis et al. 2012). Manipulation of the C-repeat binding factor (CBF)-dependent signaling pathway allowed plants to attain freezing tolerance (Gilmour et al. 1998, Gilmour et al. 2000). Transcription factors CBF1, CBF2 and CBF3 up-regulate many *COLD-RESPONSIVE* (*COR*) genes such as *COR6.6/KIN2*, *COR15a*, *COR47* and *LTI78* by binding to the C-repeat (CRT)/dehydration-responsive (DRE) element (Gilmour et al. 1998, Thomashow 1999, Gilmour et al. 2000, Thomashow 2010), and enhanced freezing tolerance was conferred on transgenic

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Arabidopsis overexpressing CBF1, CBF2 or CBF3 (Jaglo-Ottosen et al. 1998, Liu et al. 1998, Kasuga et al. 1999, Gilmour et al. 2004).

In contrast, CBF4 was not responsive to cold but was induced by ABA, a phytohormone which plays an important role in adaptation to drought (Haake et al. 2002, Knight et al. 2004). CBF1, CBF2 and CBF3 were also up-regulated by ABA but less so than by cold (Knight et al. 2004). Hence, an ABA-inducible signaling pathway triggered by cold-inducible CBFs may still activate CRTs (Gilmour and Thomashow, 1991, Knight et al. 2004). The ectopic expression of ABA-INSENSITIVE3 (ABI3) resulted in freezing tolerance in Arabidopsis (Tamminen et al. 2001). The MYC-type basic helix–loop–helix transcription factor INDUCER OF CBF EXPRESSION1 (ICE1) positively regulates *CBF3*, and transgenic Arabidopsis overexpressing ICE1 were freezing tolerant (Chinnusamy et al. 2003).

Plants accumulate osmolytes including soluble sugars and proline during cold acclimation to protect membranes against freezing injury (Strauss and Hauser 1986, Anchordoguy et al. 1987, Li et al. 2004). Genes related to lipid metabolism, particularly those associated with stabilizing membranes, can protect against low temperature (Li et al. 2004, Devaiah et al. 2006, Chen et al. 2008, Du et al. 2010, Moellering et al. 2010). Upon freezing, phospholipase Dα (PLDα)-deficient Arabidopsis showed an increase in phosphatidylcholine (PC) and reduction in phosphatidic acid (PA), stabilizing the membrane bilayer structure and resulting in freezing tolerance (Welti et al. 2002). On the other hand, transgenic Arabidopsis overexpressing plasma membrane-associated PLDδ accumulated PA on freezing treatment and achieved tolerance, while the PLDδ knockout mutant became more sensitive. Thus, PLDδ-regulated PA production at the plasma membrane altered the freezing response but PLDδ overexpression did not affect *COR* (*COR47* and *LTI78*) gene expression or soluble sugar and proline contents (Li et al. 2004). Unlike *COR47*, *SENSITIVE TO FREEZING2* (*SFR2*) is constitutively expressed and is not cold inducible (Thorlby et al. 2004). SFR2 stabilizes the outer chloroplast membrane, enhancing freezing tolerance (Moellering et al. 2010). SFR2 encodes a galactolipid galactosyltransferase (GGGT), which catalyzes the transgalactosidation of monogalactosyldiacylglycerol (MGDG) to di-, tri- and tetragalactosyldiacylglycerol (DGDG, TGDG and TeDG, respectively), concomitantly generating diacylglycerol (DAG) (Benning and Ohta 2005). Upon freezing, DAG is further converted to triacylglycerol (TAG), a non-polar lipid not normally present in membranes (Moellering et al. 2010).

Six isogenes encode acyl-CoA-binding proteins (ACBPs) in Arabidopsis (Xiao and Chye 2009, Xiao and Chye 2011). ACBPs are involved in lipid metabolism, plant development and stress responses to heavy metals, oxidation and drought (Xiao and Chye, 2009, Xiao and Chye 2011, Du et al. 2013a, Du et al. 2013b). ACBP1 and ACBP6 have been shown to be associated with freezing through different mechanisms (Chen et al. 2008, Du et al. 2010). The *acbp1* mutant displayed enhanced freezing tolerance accompanied by an increase in PC and a decrease in PA, while seedlings and rosettes of ACBP1-overexpressors (OEs) showed greater freezing sensitivity accompanied by a reduction in PC and a gain in PA (Du et al. 2010). Loss of ACBP1 function was related to down-regulation of *PLDα1* and up-regulation of *PLDδ*, but the expression of *COR6.6* and *COR47*, and soluble sugar and proline contents were not affected (Du et al. 2010). In contrast, the *acbp6* mutant was freezing sensitive, Arabidopsis ACBP6-OE seedlings and rosettes were tolerant and the expression of *PLDδ*, but not that of the *COR* genes, was induced in ACBP6-OEs (Chen et al. 2008). Lipid profiling of ACBP6-OE rosettes post-freezing revealed a decline in PC, while PA increased in comparison to the wild type (WT) (Chen et al. 2008).

Many studies on freezing tolerance utilized Arabidopsis seedlings and rosettes which represent the vegetative stages, but flowers comprising the reproductive stage have seldom been tested. An understanding of freezing tolerance in flowers will enable us to better direct strategies in floral protection in floriculture. Previous studies have revealed that the up-regulation of many *COR* genes in Arabidopsis leaf tissues after cold stress is not conserved in pollen (Lee and Lee 2003). Also, 86% of the genes that show cold induction are not conserved between Arabidopsis leaves and roots (Kreps et al. 2002). These results indicate that the transcriptional networks in cold regulation differ amongst various organs (Chinnusamy et al. 2007). As *ACBP6* is expressed in both leaves and flowers (Chen et al. 2008), we addressed herein ACBP6 function during freezing stress in these organs of ACBP6-OE plants, and report observations on their differential gene regulation, and changes in lipid and proline.

## Results

### Flowers of ACBP6-OEs are freezing tolerant

Flowers of ACBP6-OEs (OE-3 and OE-5) and the WT were subjected to freezing treatment in this study as shown in **Fig. 1**. Non-acclimated (NA) flowers were harvested from 5-week-old Arabidopsis plants grown at 23°C. NA flowers were treated at 4°C for 3 d to obtain cold-acclimated (CA) flowers. CA flowers were subjected to freezing treatment (−7°C for 1 h) to obtain freeze-treated (F) flowers. F flowers were left to recover at 4°C for 12 h, to yield flowers after recovery (R).

There were no differences in phenotype between NA and CA WT *Arabidopsis thaliana* (Col-0) and ACBP6-OE flowers. After freezing (−7°C) treatment followed by a 7 d recovery, OE-3 and OE-5 flowers showed better tolerance and the percentage of intact OE flowers after freezing was significantly ($P < 0.01$) higher (82.6% and 89.6%, respectively) than the WT (54.5%) (**Fig. 2**). The results revealed that the overexpression of ACBP6 enhanced freezing tolerance of transgenic Arabidopsis flowers.

### Changes in lipid molecular species of ACBP6-OE flowers after freezing treatment

Except for a significant ($P < 0.05$) increase in PA in the ACBP6-OEs (OE-3 and OE-5) compared with the WT (**Table 1**), lipid

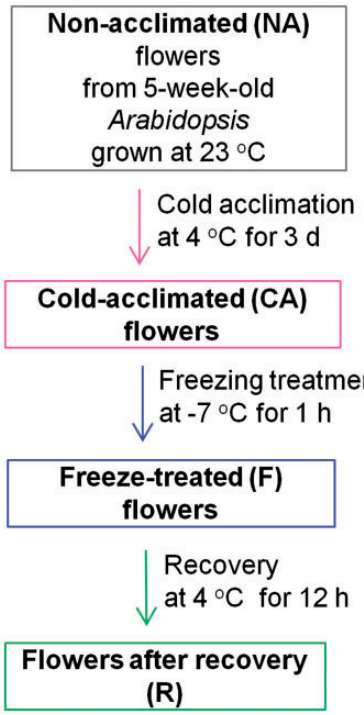


**Fig. 1** Flowchart to illustrate the treatments carried out on flowers used in this study. Non-acclimated (NA) flowers were harvested from 5-week-old Arabidopsis plants grown at 23°C. These flowers were treated at 4°C for 3 d to obtain cold-acclimated (CA) flowers. CA flowers were subjected to freezing treatment (−7°C for 1 h) to obtain freeze-treated (F) flowers. F flowers were left to recover at 4°C for 12 h, to yield flowers after recovery (R). Color codes used: NA (gray), CA (pink), F (blue) and R (green). NA, CA, F and R samples were used for lipid analysis (**Table 1**; **Supplementary Tables S1**, **S2**, **S3**; **Fig. 3**), qRT-PCR (**Figs. 4–8**, **10**; **Supplementary Figs. S1–S7**), measurement of soluble sugar and proline contents (**Figs. 9**, **10**) and Northern blot analysis (**Fig. 9**).

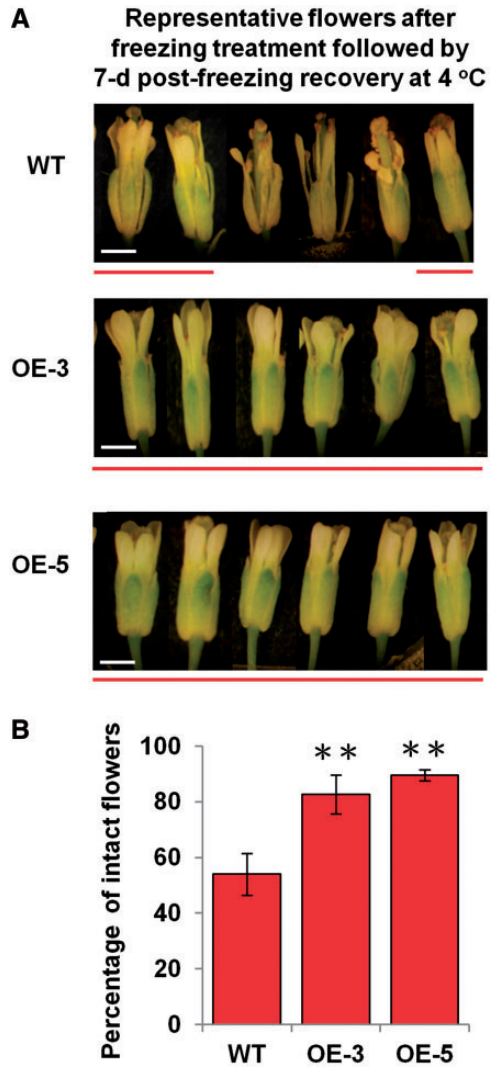


**Fig. 2** Flowers of ACBP6-OEs are freezing tolerant. (A) Representative flowers after freezing treatment followed by a 7 d post-freezing recovery at 4°C. Representative intact flowers are marked with a red line. Scale bar = 0.1 cm. (B) Percentage of intact flowers after freezing treatment followed by a 7 d post-freezing recovery at 4°C. The red column represents the percentage of intact flowers. Asterisks indicate significant differences from the WT (**$P < 0.01$). Values are means ± SD, $n = 3$ (30 flowers per group for each line were tested and the experiment was repeated twice).

profiling showed no other significant changes in the total amounts of PC, digalactosyldiacylglycerol (DGDG), MGDG, phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) between the NA ACBP6-OE and WT flowers. However, freeze-treated (F) ACBP6-OE flowers accumulated less PA than the WT (**Table 1**). Although this difference in PA content was not significant, the total PA content increased 2.7-fold in the WT in contrast to 1.6-fold in OE-3 and 2.0-fold in OE-5 (**Table 1**). Therefore, flowers of OE-3 and OE-5 accumulated 29% and 18% less PA than the WT, respectively (**Table 1**). In particular, 34:6 PA and 36:5 PA in the ACBP6-OEs were significantly ($P < 0.05$) lower than the WT (**Supplementary Table S1**). Also, 34:4 PA, 34:3 PA, 34:2 PA, 36:6 PA and 36:4 PA in the ACBP6-OEs were lower than the WT (**Supplementary Table S1**).

In contrast, PC levels decreased by 36% in OE-3, 42% in OE-5 and 45% in WT flowers after freezing treatment (**Table 1**). OE-3 and OE-5 flowers accumulated 34% and 25% more PC than the WT, respectively ($P < 0.05$) (**Table 1**). The contents of several PC species including 34:4 PC, 34:3 PC, 34:2 PC, 36:6 PC, 36:5 PC, 36:4 PC, 36:3 PC, 36:2 PC, 38:5 PC and 38:4 PC were significantly ($P < 0.05$) higher than the WT (**Supplementary Table S2**).

Furthermore, MGDG was reduced in OE-3, OE-5 and WT flowers after freezing treatment. The total MGDG content declined by 55% in the WT, and by 40% and 44%, respectively, in OE-3 and OE-5 (**Table 1**). Thus, OE-3 and OE-5 flowers accumulated 34% and 21% more MGDG than the WT ($P < 0.05$) (**Table 1**). In particular, contents of 34:6 MGDG, 34:5 MGDG, 34:3 MGDG, 36:6 MGDG, 36:5 MGDG and 36:4 MGDG were significantly ($P < 0.05$) higher than the WT (**Supplementary Table S3**).

**Table 1** Total amount of lipid in each head group class in flowers of non-acclimated and freeze-treated ACBP6-OEs and the WT

| Lipid class | NA | | | F | | |
|---|---|---|---|---|---|---|
| | WT | OE-3 | OE-5 | WT | OE-3 | OE-5 |
| PC | 32.6 ± 2.29 | 37.6 ± 1.45 | 39.1 ± 3.78 | 17.6 ± 1.28 | **23.6 ± 1.40**[b] | **22.0 ± 1.32**[b] |
| PA | 0.86 ± 0.14 | **1.61 ± 0.18**[b] | **1.19 ± 0.14**[b] | 3.18 ± 0.74 | 2.25 ± 0.94 | 2.61 ± 0.26 |
| DGDG | 16.2 ± 0.56 | 17.1 ± 0.94 | 14.8 ± 1.62 | 11.2 ± 0.41 | **12.1 ± 0.57**[b] | 11.9 ± 1.09 |
| MGDG | 66.2 ± 2.61 | 66.0 ± 4.48 | 64.6 ± 4.69 | 29.6 ± 1.33 | **39.6 ± 0.53**[b] | **36.0 ± 2.95**[b] |
| PG | 5.54 ± 0.22 | 6.00 ± 0.38 | 5.55 ± 0.52 | 3.19 ± 0.48 | **3.86 ± 0.23**[b] | **3.79 ± 0.17**[b] |
| PE | 13.7 ± 2.35 | 13.9 ± 0.56 | 12.0 ± 0.97 | 8.21 ± 0.54 | **10.3 ± 0.28**[b] | 8.59 ± 0.34 |
| PI | 6.72 ± 0.46 | 7.90 ± 0.90 | 6.52 ± 0.79 | 4.95 ± 0.03 | **5.71 ± 0.45**[b] | **5.60 ± 0.50**[b] |
| PS | 0.84 ± 0.13 | 0.89 ± 0.26 | 0.78 ± 0.12 | 0.51 ± 0.08 | 0.44 ± 0.07 | **0.30 ± 0.03**[a] |
| LysoPG | 0.06 ± 0.00 | 0.06 ± 0.02 | 0.09 ± 0.03 | 0.15 ± 0.06 | 0.10 ± 0.03 | 0.11 ± 0.02 |
| LysoPC | 0.21 ± 0.01 | 0.25 ± 0.02 | 0.26 ± 0.05 | 0.51 ± 0.03 | **0.32 ± 0.11**[a] | **0.38 ± 0.06**[a] |
| LysoPE | 0.21 ± 0.04 | 0.26 ± 0.03 | 0.26 ± 0.04 | 0.39 ± 0.04 | 0.33 ± 0.05 | **0.32 ± 0.01**[a] |

Non-acclimated (NA) and freeze-treated (F) samples were obtained as shown in Fig. 1.
Values are means ± SD (nmol $mg^{-1}$ DW; $n = 4$); significant differences ($P < 0.05$) from the WT in the same experiment are shown in bold.
[a] Value lower than the WT in the same experiment ($P < 0.05$).
[b] Value higher than the WT in the same experiment ($P < 0.05$).

A comparison of the total lipid content of flowers was made between the ACBP6-OEs and the WT before and after freezing treatment. Before freezing treatment, there were no significant differences between them (Fig. 3A). After freezing treatment, the total lipid content of ACBP6-OE and WT flowers declined by 37% (OE-3), 37% (OE-5) and 46% (WT) ($P < 0.05$) (Fig. 3A). Accordingly, the total lipids of OE-3 and OE-5 flowers were 20% and 15% higher than the WT ($P < 0.05$) (Fig. 3A).

Decreases in the ratio of PA to PC have been reported to enhance membrane stability and improve freezing tolerance (Welti et al. 2002, Li et al. 2008, Du et al. 2010). Freezing-tolerant PLDα-deficient plants and ACBP1-deficient plants demonstrated a decrease in the ratio of PA to PC (Welti et al. 2002, Du et al. 2010). Hence, when the ratio of PA to PC was compared after freezing treatment, WT flowers showed a 5.8-fold increase, while OE-3 and OE-5 increased 2.1- and 3.1-fold, respectively, in comparison with the NA WT (Fig. 3B). The ratio of PA to PC in freeze-treated OE-3 and OE-5 was 45% and 37% lower, respectively, than the WT ($P < 0.05$) (Fig. 3B).

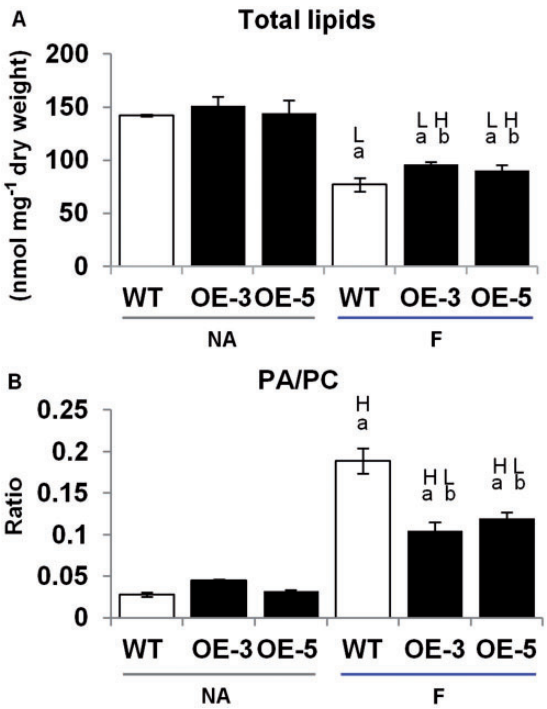


**Fig. 3** Freezing-induced changes in floral lipids of Arabidopsis WT and ACBP6-OEs. Non-acclimated (NA) and freeze-treated (F) samples were obtained as shown in Fig. 1. The white bar represents the WT, and the black bars represent OE-3 and OE-5. a indicates a significant difference in comparison with the NA WT; b indicates a significant difference in comparison with the F WT ($P < 0.05$). H, value higher than the WT ($P < 0.05$); L, value lower than the WT ($P < 0.05$). Values of total lipids are means ± SD ($n = 4$).

## The expression of PC-related genes in ACBP6-OE flowers

To investigate the molecular mechanism of PC changes in NA, CA, 'freeze-treated (F)' and 'after recovery (R)' ACBP6-OE flowers, the expression of PC biosynthesis-related genes encoding phosphoethanolamine *N*-methyltransferase (PEAMT), choline kinase (CK), phosphorylcholine cytidylyltransferase (CCT) and lysophosphatidylcholine acyl-transferase (LPCAT) which reacylates lysophosphatidylcholine (LysoPC) to regenerate PC or deacylates PC to produce LysoPC (Lands, 1960), as well as PC degradation-related genes encoding phospholipase A (PLA) and PLD families were analyzed by quantitative real-time PCR (qRT-PCR). The results showed that the expression of *PEAMT2* and *PEAMT3* in ACBP6-OEs significantly ($P < 0.05$) decreased in comparison with the NA WT, the expression of *PEAMT1*, *PEAMT2* and *PEAMT3* in both the WT and ACBP6-OEs decreased after cold acclimation, freezing and recovery, while there were no differences in *PEAMT1*, *PEAMT2* and *PEAMT3* expression between ACBP6-OEs and the WT after cold

acclimation, freezing and recovery (Fig. 4), suggesting that PC accumulation in ACBP6-OE flowers after freezing did not arise from up-regulation of *PEAMT* expression. *CK* encodes a choline kinase, catalyzing the first committed step in the choline pathway (Tasseva et al. 2004) in PC biosynthesis. The expression of *CK1* and *CK-LIKE2* in ACBP6-OE flowers was significantly ($P < 0.05$) higher than the WT after cold acclimation, freezing and recovery (Fig. 4). *CK-LIKE1* expression was also significantly ($P < 0.05$) higher than the corresponding WT after freezing and recovery (Fig. 4). *CCT1* and *CCT2* are considered to affect PC biosynthesis substantially (Wang et al. 2012) and the expression of *CCT1* was significantly ($P < 0.05$) higher than the WT after cold acclimation and recovery, respectively, and *CCT2* expression in ACBP6-OE flowers was significantly ($P < 0.05$) higher than the WT after cold acclimation, freezing and recovery, respectively (Fig. 4). The expression of *LPCAT1* in ACBP6-OE flowers was significantly ($P < 0.05$) higher than the WT after cold acclimation and recovery (Fig. 4). These results imply that ACBP6-conferred freezing tolerance in flowers is related to an increased expression of *CK*, *CCT* and *LPCAT*, but not *PEAMT*.

With respect to PC degradation genes, *PLA* family members including *PLA2α* and the patatin-like *PLA* (*PAT-PLA-IIβ*, *PAT-PLA-IIIα* and *PAT-PLA-IIIδ*), and *PLD* family members such as *PLDδ*, *PLDζ2* and *PLDα1* were selected (Wang et al. 2012). *PLA2α* expression in ACBP6-OE flowers was lower than the NA WT, but higher than the corresponding WT after cold acclimation and recovery (Fig. 5). The expression of *PAT-PLA-IIβ* in ACBP6-OE flowers was lower than the NA WT, but higher than the corresponding WT after cold acclimation and recovery. *PAT-PLA-IIIα* was more highly expressed in ACBP6-OEs than the WT after freezing (Fig. 5). The expression of *PAT-PLA-IIIδ* in ACBP6-OE flowers was lower than the NA WT, but higher than the WT after freezing (Fig. 5). The expression of *PLDδ* in ACBP6-OE flowers was significantly ($P < 0.05$) higher than the corresponding WT after cold acclimation and recovery (Fig. 5). *PLDζ2* showed up-regulation only after cold acclimation in ACBP6-OEs (Fig. 5). The expression of *PLDα1* in OE-3 and OE-5 was lower than the NA WT, but was higher than the WT after cold acclimation and freezing (Fig. 5). These results indicate that not only *PLDδ*, but also other PC degradation-related genes such as *PLA2α*, *PAT-PLA-IIβ*, *PAT-PLA-IIIα*, *PAT-PLA-IIIδ*, *PLDζ2* and *PLDα1* were up-regulated in ACBP6-OE flowers after cold acclimation and freezing.

## The expression of MGDG-related genes in ACBP6-OE flowers

As significant changes in floral MGDG were also observed between the ACBP6-OEs and the WT after freezing treatment, the expression of MGDG-related genes encoding monogalactosyldiacylglycerol synthase1 (MGD1), MGD2, MGD3 and SFR2 was investigated. The results revealed that the expression of *MGD1* and *SFR2* was higher in the WT after cold acclimation, freezing and recovery than the NA WT, while the expression of MGD2 and MGD3 was lower in the WT after cold acclimation, freezing and/or recovery than the NA WT (Fig. 6). *MGD1* mRNA in OE-3 and OE-5 flowers was significantly ($P < 0.05$) higher than the WT after cold acclimation, freezing and recovery, while *MGD2* showed a slight increase in OE-3 and OE-5 after freezing, but was down-regulated after recovery (Fig. 6). *MGD3* showed a significant ($P < 0.05$) increase in OE-3 and OE-5 after cold acclimation and freezing, and *SFR2* displayed higher expression in OE-3 and OE-5 after cold acclimation and recovery (Fig. 6). Therefore, ACBP6-conferred freezing tolerance in flowers appeared to be associated with expression of MGDG-related genes.

## ACBP6-conferred freezing tolerance in flowers is related to *COR* gene expression

In ACBP6-OE rosettes, the expression of *COR* genes was no higher than the WT after cold acclimation, implying that ACBP6-conferred freezing tolerance in rosettes is not related to the induction of *COR* expression (Chen et al. 2008). To explore whether the expression of *COR* genes in OE-3 and OE-5 flowers behaves similarly, *COR* (*COR6.6*, *COR47*, *LTI78* and *COR15a*) expression was analyzed by qRT-PCR. The results showed that *COR6.6*, *COR47*, *LTI78* and *COR15a* mRNAs in OE-3 and OE-5 flowers were significantly lower than the NA WT, but increased significantly after cold acclimation, freezing or recovery (Fig. 7).

To address the participation of the *COR* pathway in ACBP6-conferred freezing tolerance in flowers, the expression of transcription factors (CBF1, CBF2 and CBF3) and the positive regulator of *CBF3*, *ICE1*, was analyzed. Results of qRT-PCR showed that the expression of *CBF1*, *CBF2* and *CBF3* in ACBP6-OEs was significantly higher than the WT after cold acclimation and freezing (Fig. 8). Correspondingly, the *ICE1* mRNA was higher in OE-3 and OE-5 than the WT after cold acclimation, freezing and recovery (Fig. 8). These results confirm that ACBP6-conferred freezing tolerance in flowers is related to the *COR* pathway.

When the expression of mRNA encoding MYB15, a nuclear factor that negatively regulates the expression of *CBF* genes (Chinnusamy et al. 2007), was measured, the results indicated that its expression in OE-3 and OE-5 flowers was lower than the NA WT, while there were no significant differences between ACBP6-OEs and WT after cold acclimation and freezing. *MYB15* mRNA in OE-3 and OE-5 flowers was significantly higher than the WT after recovery (Fig. 8). These results suggested that ACBP6-conferred freezing tolerance in flowers is not associated with *MYB15*.

## Expression of ABA-related genes in flowers before and after freezing

In contrast to *CBF1*, *CBF2* and *CBF3*, which are up-regulated by cold and ABA, CBF4 is induced only by ABA. Furthermore, many genes including *KIN1*, *KIN2* and *RESPONSE TO DESICCATION29A* (*RD29A*) are up-regulated by both cold and ABA (Wang et al. 1995, Xiong et al. 2002). Given the significant induction of COR6.6/*KIN2* and *RD29A* in ACBP6-OE flowers after cold acclimation and freezing (Fig. 7), the role of ABA

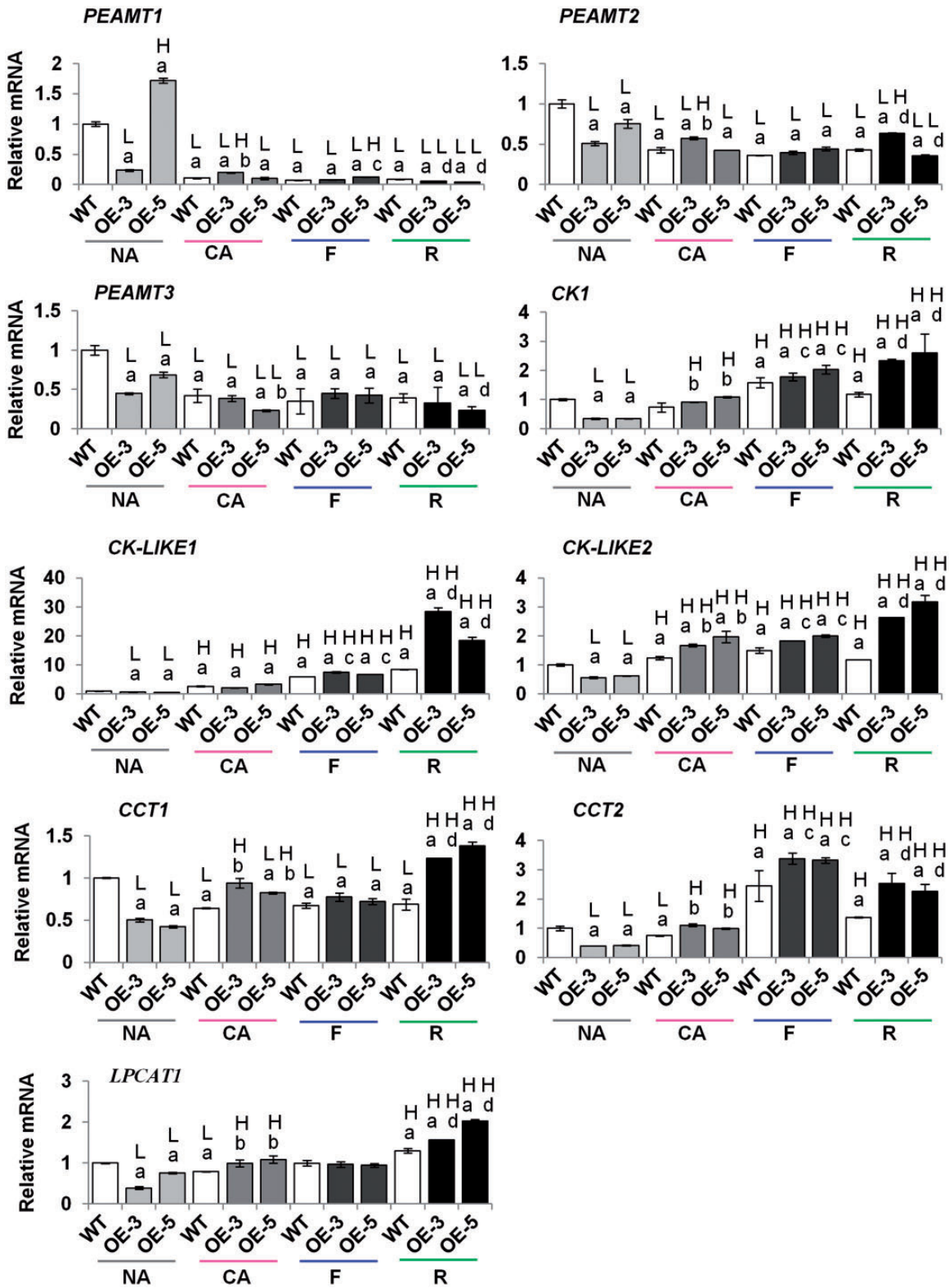


**Fig. 4** Expression of PC biosynthesis-related genes in flowers before and after freezing treatment. Total RNA was extracted from non-acclimated (NA), cold-acclimated (CA), freeze-treated (F) and after recovery (R) flowers of WT, OE-3 and OE-5. NA, CA, F and R samples were obtained as shown in **Fig. 1**. a indicates a significant difference in comparison with the NA WT; b indicates a significant difference in comparison with the CA WT; c indicates a significant difference in comparison with the F WT; d indicates a significant difference in comparison with the R WT. H, value higher than the control ($P < 0.05$); L, value lower than the control ($P < 0.05$). Values are means ± SD ($n = 3$).

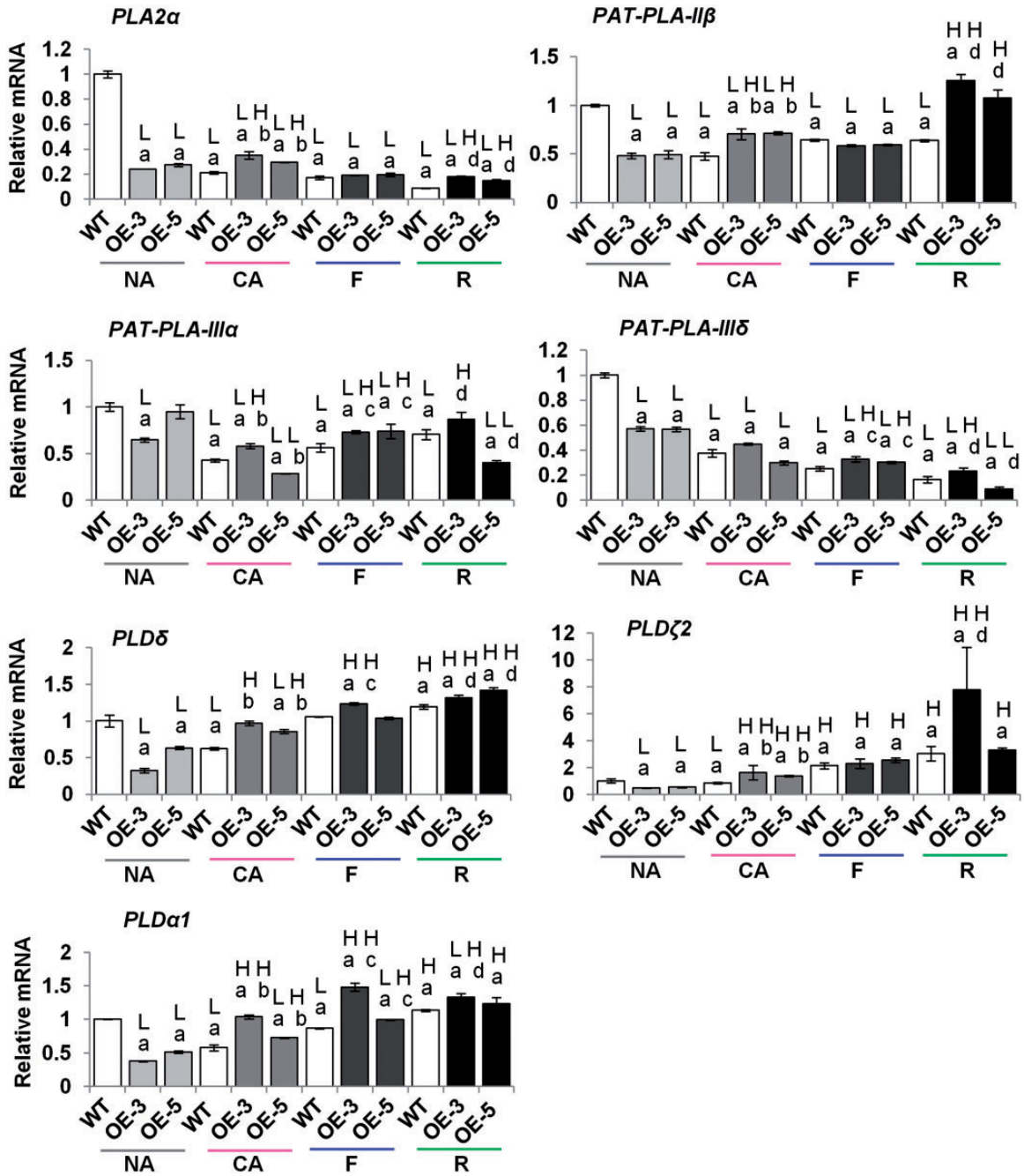


**Fig. 5** Expression of PC degradation-related genes in flowers before and after freezing treatment. Total RNA was extracted from non-acclimated (NA), cold-acclimated (CA), freeze-treated (F) and after recovery (R) flowers of WT, OE-3 and OE-5. NA, CA, F and R samples were obtained as shown in **Fig. 1**. a indicates a significant difference in comparison with the NA WT; b indicates a significant difference in comparison with the CA WT; c indicates a significant difference in comparison with the F WT; d indicates a significant difference in comparison with the R WT. H, value higher than the control ($P < 0.05$); L, value lower than the control ($P < 0.05$). Values are means ± SD ($n = 3$).

was subsequently investigated. Significant up-regulation of *KIN1*, *CBF4* and *ABA-INSENSITIVE 5* (*ABI5*) was also seen in ACBP6-OEs after cold acclimation and freezing, and *9-CIS-EPOXYCAROTENOID DIOXYGENASE3* (*NCED3*) showed higher expression in ACBP6-OE flowers after cold acclimation (**Figs. 7**, **8**), suggesting that ACBP6-conferred freezing tolerance in flowers is associated with the ABA signaling pathway.

## Measurement of soluble sugar and proline contents and the expression of proline-related genes in NA and CA rosettes

As the accumulation of soluble sugars and proline at low temperatures enhances freezing tolerance in Arabidopsis (Xin and Browse 1998, Rajashekar et al. 2006), the soluble sugar and

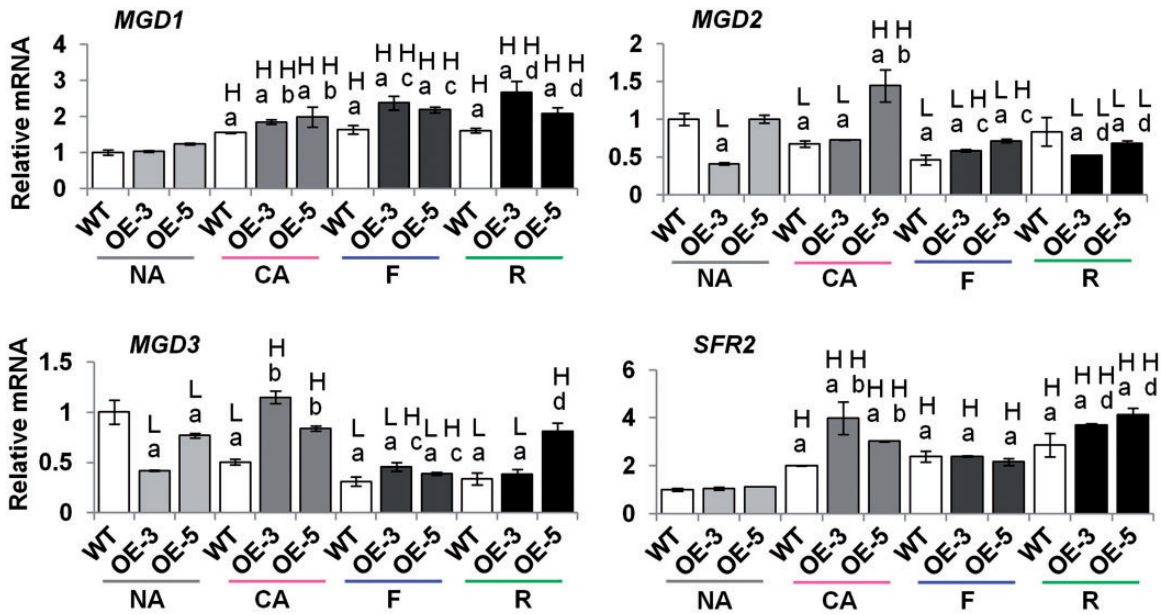


**Fig. 6** Expression of MGDG-related genes in flowers before and after freezing treatment. Total RNA was extracted from non-acclimated (NA), cold-acclimated (CA), freeze-treated (F) and after recovery (R) flowers of WT, OE-3 and OE-5. NA, CA, F and R samples were obtained as shown in **Fig. 1**. a indicates a significant difference in comparison with the NA WT; b indicates a significant difference in comparison with the CA WT; c indicates a significant difference in comparison with the F WT; d indicates a significant difference in comparison with the R WT. H, value higher than the control ($P < 0.05$); L, value lower than the control ($P < 0.05$). Values are means ± SD ($n = 3$).

proline contents of 5-week-old NA and CA WT and ACBP6-OE rosettes were compared. Both NA and CA ACBP6-OE rosettes showed significantly ($P < 0.05$) higher soluble sugar levels than the WT (**Fig. 9A**). Although lower values were observed in the *acbp6* mutant, the Student's *t*-test revealed that this result was not significant. After cold acclimation, the soluble sugar content in ACBP6-OE rosettes reached approximately 2-fold the WT level (**Fig. 9A**).

When the proline content of 5-week-old NA and CA WT and ACBP6 rosettes of OE-3 and OE-5 were compared, both NA and CA ACBP6-OEs showed significantly ($P < 0.05$) higher amounts than the WT (**Fig. 9B**). The proline level in rosettes of OE-3 and OE-5 following cold acclimation was approximately 2-fold the WT level (**Fig. 9B**). Although the proline level in the *acbp6* mutant was below that of the WT (**Fig. 9B**), the change was not deemed significant.

To further understand the mechanism of ACBP6-conferred freezing tolerance related to proline in rosettes, Northern blot analyses were carried out to detect the mRNAs encoding $\Delta^1$-pyrroline-5-carboxylate synthase (P5CS) in proline biosynthesis and the mRNA encoding a proline oxidase (AtPOX) in proline degradation. Consistent with greater accumulation of free proline in ACBP6-OE rosettes, *P5CS* mRNA was higher than the WT. In contrast, *AtPOX* mRNA was lower in ACBP6-OEs and the knockout mutant than the CA WT rosettes (**Fig. 9C**). These observations indicated that the accumulation of soluble sugars and proline may enhance freezing tolerance in the ACBP6-OE rosettes, resembling other freeze-tolerant plants.

## Expression of proline-related genes and metabolite changes of soluble sugars and proline during cold acclimation in ACBP6-OE flowers

In order to address whether proline in ACBP6-OE flowers after cold acclimation was affected, qRT-PCR was subsequently conducted to analyze *P5CS* and *AtPOX* expression. In ACBP6-OE flowers, P5CS mRNA was lower than NA WT, but higher than CA WT (**Fig. 10A**). In contrast, *AtPOX* expression in OEs was lower than both NA and CA WT flowers (**Fig. 10B**). However, no differences in *P5CS* and *AtPOX* expression were evident between OEs and the WT after freezing and recovery (**Fig. 10A**, **B**).

The content of soluble sugars and proline in ACBP6-OE flowers was further analyzed at NA and CA stages to test whether solutes play similar roles in ACBP6-OE rosettes and flowers during cold acclimation. Similar to rosettes (**Fig. 9A**), NA ACBP6-OE flowers showed significantly ($P < 0.05$) higher soluble sugars than the NA WT (**Fig. 10C**). In contrast, there was no significant difference in soluble sugars between the CA WT and the CA ACBP6-OE flowers (**Fig. 10C**). When the proline contents of the NA and the CA WT and ACBP6-OE flowers were compared, both the NA and CA ACBP6-OEs showed significantly ($P < 0.05$) lower amounts than their corresponding WT (**Fig. 10D**). The proline level in the CA flowers of OE-3 and OE-5 (following cold acclimation) was approximately 50% of the CA WT level (**Fig. 10D**). The decrease in proline in the NA flowers of OE-3 and OE-5 was consistent with their lower expression of *P5CS* (**Fig. 10A**, **D**). However, the decrease in proline

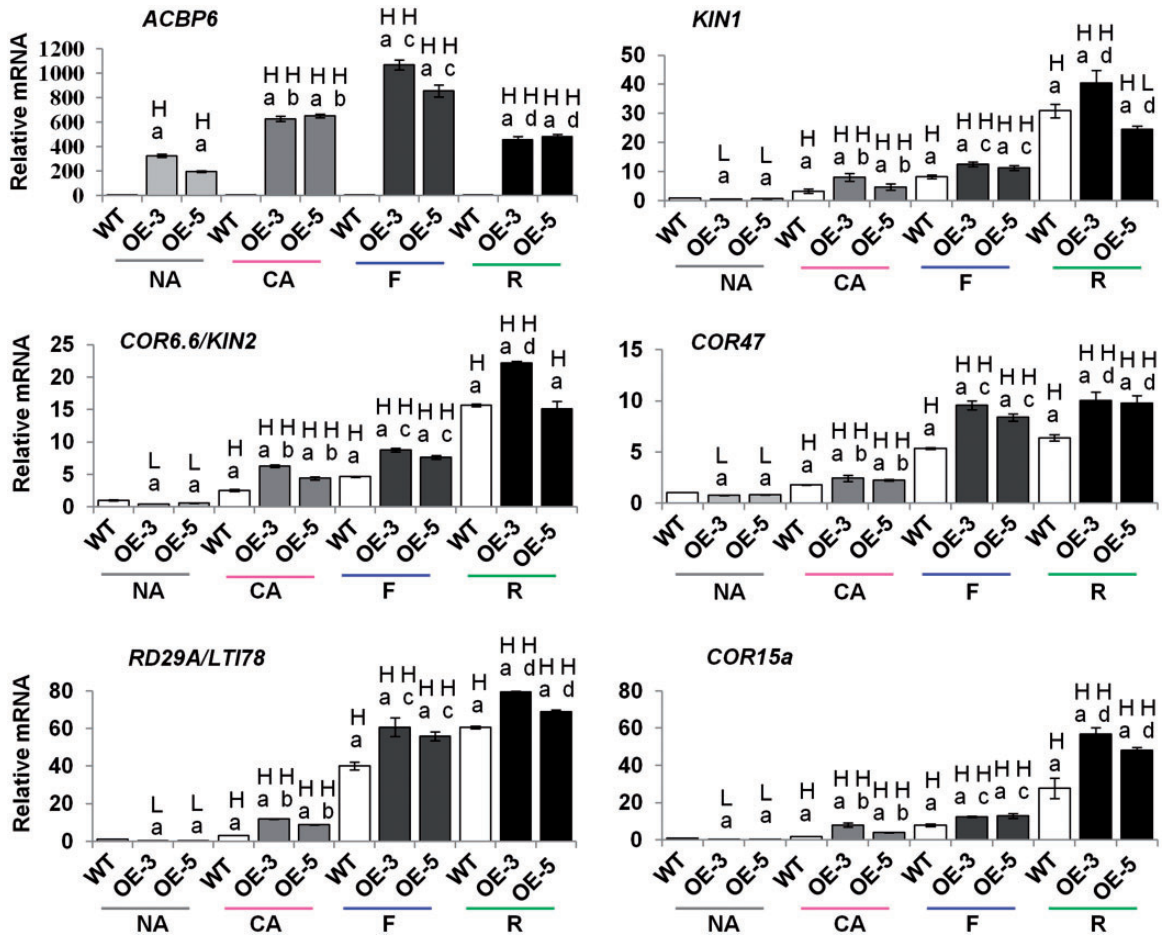


**Fig. 7** Expression of *ACBP6* and cold-related genes in flowers before and after freezing treatment. Total RNA was extracted from non-acclimated (NA), cold-acclimated (CA), freeze-treated (F) and after recovery (R) flowers of WT, OE-3 and OE-5. NA, CA, F and R samples were obtained as shown in **Fig. 1**. a indicates a significant difference in comparison with the NA WT; b indicates a significant difference in comparison with the CA WT; c indicates a significant difference in comparison with the F WT; d indicates a significant difference in comparison with the R WT. H, value higher than the control ($P < 0.05$); L, value lower than the control ($P < 0.05$). Values are means ± SD ($n = 3$).

in the CA flowers of OE-3 and OE-5 appears inconsistent with their higher expression of *P5CS* and lower expression of *AtPOX* (**Fig. 10A**, **D**).

## Gene expression in ACBP6-OE rosettes before and after freezing

Differences in lipid changes after freezing were addressed using qRT–PCR to check the expression of PC-, MGDG-, proline-, ABA- and *COR*-related genes in rosettes subjected to non-acclimation, cold acclimation, freezing and recovery.

For PC biosynthesis-related genes, *PEAMT1* showed lower expression in rosettes of ACBP6-OEs after cold acclimation, in comparison with the CA WT; *PEAMT2* displayed lower expression after cold acclimation, freezing and recovery, and *PEAMT3* showed lower expression after cold acclimation and freezing (**Supplementary Fig. S1**). No differences in the expression of *PEAMT1*, *PEAMT2* and *PEAMT3* were observed between ACBP6-OE and WT flowers during cold acclimation, freezing and recovery (**Fig. 4**), indicating that *PEAMT1*, *PEAMT2* and *PEAMT3* maintain the PC content in ACBP6-OE flowers after freezing, while decreased expression of *PEAMT1*, *PEAMT2* and *PEAMT3* may be attributed to a decrease in the PC content in the ACBP6-OE rosettes after freezing. Similarly, the PC biosynthesis-related genes (*CK1*, *CCT1* and *CCT2*) showed decreased expression in ACBP6-OE rosettes after cold acclimation, but *CK1*, *CK-LIKE1* and *CCT1* displayed increased expression after freezing (**Supplementary Fig. S1**). No significant differences in the expression of *CK-LIKE2* and *LPCAT1* were observed between ACBP6-OE and WT rosettes at any stage (**Supplementary Fig. S1**). These results suggested that the decrease in PC content in ACBP6-OE rosettes after freezing was related to the down-regulation of *PEAMT1*, *PEAMT2*, *PEAMT3*, *CK1*, *CCT1* and *CCT2*.

For PC degradation-related genes, the expression of *PLA2α* and *PAT-PLA-IIIδ* in ACBP6-OE rosettes was lower than the WT after cold acclimation and freezing (**Supplementary Fig. S2**). *PLDζ2* displayed lower expression in ACBP6-OE rosettes after cold acclimation, but higher expression after

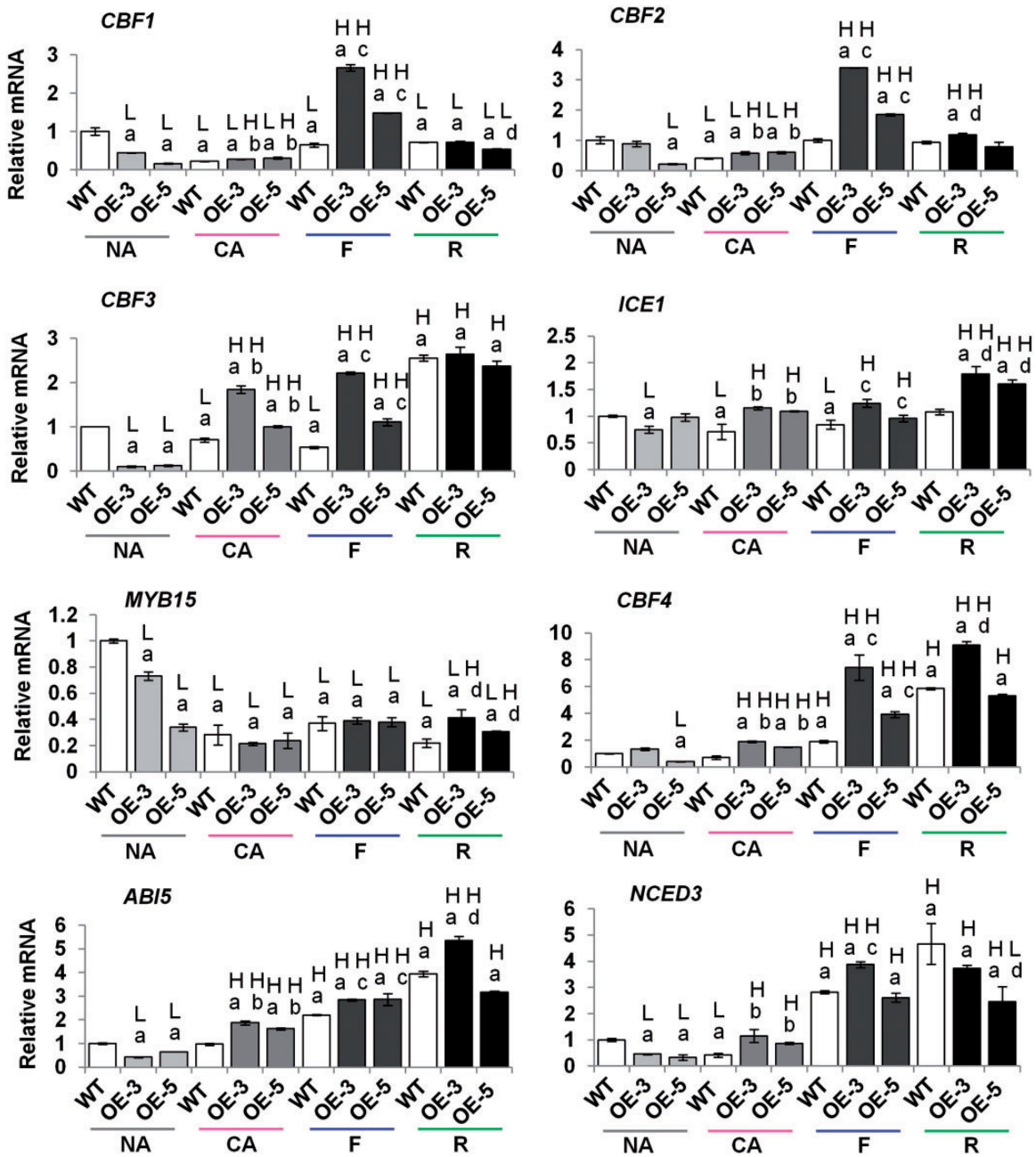


**Fig. 8** Expression of *CBF* genes, *ICE1*, *MYB15* and ABA-related genes in flowers before and after freezing treatment. Total RNA was extracted from non-acclimated (NA), cold-acclimated (CA), freeze-treated (F) and after recovery (R) flowers of WT, OE-3 and OE-5. NA, CA, F and R samples were obtained as shown in **Fig. 1**. a indicates a significant difference in comparison with the NA WT; b indicates a significant difference in comparison with the CA WT; c indicates a significant difference in comparison with the F WT; d indicates a significant difference in comparison with the R WT. H, value higher than the control ($P < 0.05$); L, value lower than the control ($P < 0.05$). Values are means ± SD ($n = 3$).

recovery (**Supplementary Fig. S2**). Other genes including *PAT-PLA-IIβ* and *PAT-PLA-IIIα* did not show any significant differences between ACBP6-OE and WT rosettes at any stage (**Supplementary Fig. S2**). These results implied that the change in PC content in ACBP6-OE rosettes after freezing is related to the down-regulation of *PLA2α*, *PAT-PLA-IIIδ* and *PLDζ2*. In contrast to the up-regulation of several MGDG-related genes in ACBP6-OE flowers after cold acclimation and freezing (**Fig. 6**), *MGD2*, *MGD3* and *SFR2* expression declined in ACBP6-OE rosettes after cold acclimation and/or freezing (**Supplementary Fig. S3**).

*COR*-related genes (*COR6.6*, *COR47*, *LTI78* and *COR15a*) showed higher expression in ACBP6-OE flowers than the WT after cold acclimation, freezing and recovery (**Fig. 7**). However,

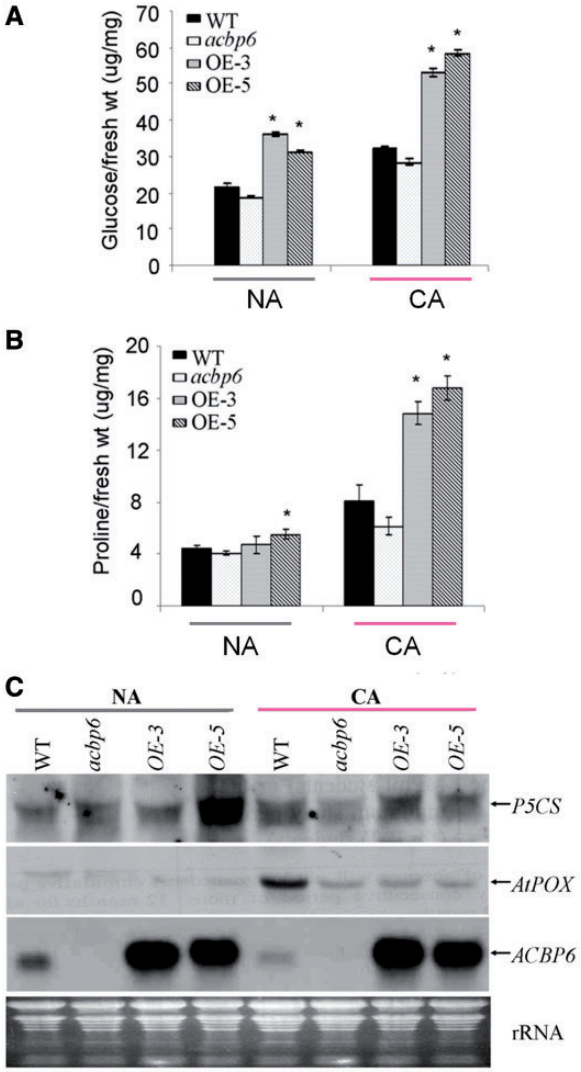


**Fig. 9** Changes in leaf soluble sugar and proline contents and expression of proline-related genes from non-acclimated (NA) and cold-acclimated (CA) 5-week-old plants. (A) Changes in soluble sugar content in NA and CA rosettes of the WT, the *acbp6* mutant and ACBP6-OEs (OE-3 and OE-5). NA and CA leaf samples were obtained as shown in **Fig. 1**. Asterisks indicate a significant difference from the WT ($P < 0.05$). Values are means ± SD ($n = 3$) calculated from three independent experiments. (B) Proline content in NA and CA rosettes of the WT, the *acbp6* mutant and ACBP6-OEs (OE-3 and OE-5). Asterisks indicate a significant difference from the WT ($P < 0.05$). Values are means ± SD ($n = 3$) from three independent experiments. (C) Northern blot analysis using digoxgenin-labeled *P5CS*, *AtPOX* and *ACBP6* cDNA probes. Total RNA was isolated from NA and CA rosettes of the WT, *acbp6* and ACBP6-OEs. Bottom panel: total RNA (30 μg per lane) stained with ethidium bromide.

the expression of *COR6.6*, *COR47*, *LTI78* and *COR15a* in ACBP6-OE rosettes was significantly lower than the WT after cold acclimation and/or freezing (**Supplementary Fig. S4**), consistent with the results of Northern blot analysis (Chen et al. 2008). Similarly, the expression of *CBF1* and *CBF2* in ACBP6-OE flowers was higher than the WT after cold acclimation and freezing (**Fig. 8**), while the expression of *CBF1* and *CBF2* in ACBP6-OE rosettes was lower than the WT after cold acclimation and recovery (**Supplementary Fig. S5**). Correspondingly, the expression of the negative regulator of CBFs (*MYB15*) in ACBP6-OE rosettes was higher than the WT after freezing and recovery (**Supplementary Fig. S5**). *CBF3* and *ICE1* showed increased expression after cold acclimation and recovery but showed no significant differences after freezing in ACBP6-OE rosettes (**Supplementary Fig. S5**). These results confirmed our previous finding that unlike flowers, ACBP6-conferred freezing tolerance in rosettes is independent of *COR* induction, suggesting that ACBP6-conferred freezing tolerance varied in different organs.

For ABA-responsive genes, inconsistent up-regulation of the expression of *KIN1*, *KIN2*, *CBF4*, *ABI5* and *NCED3* was observed in ACBP6-OE rosettes at different stages (**Supplementary Fig. S4** for *KIN1* and *KIN2* and **Supplementary Fig. S5** for *CBF4*, *ABI5* and *NCED3*). *KIN1* and *KIN2* showed decreased expression in ACBP6-OE rosettes after cold acclimation and freezing (**Supplementary Fig. S4**), *CBF4* displayed increased expression after cold acclimation and decreased expression after freezing, while *ABI5* showed decreased expression after cold acclimation and increased expression after freezing (**Supplementary Fig. S5**). However, *NCED3* displayed higher expression in ACBP6-OE rosettes after cold acclimation and recovery (**Supplementary Fig. S5**). These inconsistencies in up-regulation of ABA-responsive genes between rosettes and flowers suggested that ACBP6-conferred freezing tolerance may differ between various plant organs.

## Discussion

### Phenotypic differences between ACBP6-OE and WT flowers arise after freezing treatment

In this study, no phenotypic differences in seedlings, rosettes and flowers were observed under normal growth conditions amongst ACBP6-OEs, the *acbp6* mutant and the WT. Earlier, Chen et al. (2008) had shown that there were no differences in phenotype or in lipid content between the *acbp6* mutant and WT rosettes under normal conditions and that the expression of *ACBP6* in WT plants was only induced by cold treatment but not by any other treatments such as high salt and a fungal elicitor (arachidonic acid) (Chen et al. 2008). Lack of induction with high salt deduced from Northern blot analysis (Chen et al. 2008) was consistent with microarray data analysis (www.weigelworld.org/resources/microarray) on *ACBP6* (At1g31812) expresssion. Taken together, these observations suggest that the phenotypic differences between ACBP6-OE and WT flowers we observed herein arose from freezing treatment.

### Differences between ACBP6-OE rosettes and flowers in freezing stress

We report that ACBP6-OEs showed enhanced freezing tolerance in flowers coinciding with PC and MGDG accumulation

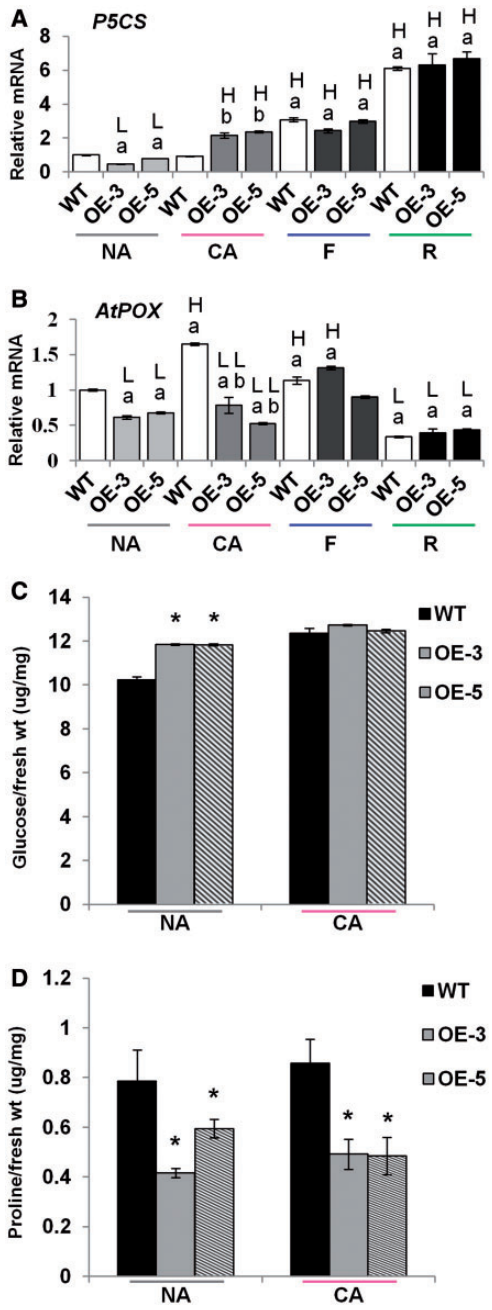


**Fig. 10** Expression of proline-related genes in flowers before and after freezing treatment and changes in soluble sugar and proline contents from non-acclimated and cold-acclimated flowers. (A and B) Expression of *P5CS* and *AtPOX* in flowers by qRT-PCR. Total RNA was extracted from non-acclimated (NA), cold-acclimated (CA), freeze-treated (F) and after recovery (R) flowers of WT, OE-3 and OE-5. NA, CA, F and R samples were obtained as shown in **Fig. 1**. a indicates a significant difference in comparison with the NA WT; b indicates a significant difference in comparison with the CA WT. H, value higher than the control ($P < 0.05$); L, value lower than the control ($P < 0.05$). Values are means ± SD ($n = 3$). (C) Changes in soluble sugar content in NA and CA flowers of WT and ACBP6-OEs (OE-3 and OE-5). Asterisks indicate a significant difference from the WT ($P < 0.05$). Values are means ± SD ($n = 3$) calculated from three independent experiments. (D) Differences in proline content in NA and CA flowers of WT and ACBP6-OEs (OE-3 and OE-5). Asterisks indicate a significant difference from the WT ($P < 0.05$). Values are means ± SD ($n = 3$) from three independent experiments.

and PA reduction (**Table 1**). ACBP6-mediated freezing tolerance in rosettes was correlated with an increase in *PLDδ*, but not *COR* gene expression (Chen et al. 2008), while ACBP6-conferred freezing tolerance in flowers was related to the induction of *COR*-related genes, *CBF* genes and *ICE1*, but not *MYB15* (**Figs. 7**, **8**). Furthermore, ACBP6-mediated freezing tolerance in flowers was associated not only with elevated *PLDδ* expression, but also with that of other PC-related genes including *CK*, *CK-LIKE1*, *CK-LIKE2*, *CCT1*, *CCT2*, *LPCAT1*, *PLA2α*, *PAT-PLA-IIβ*, *PAT-PLA-IIIα*, *PAT-PLA-IIIδ* and *PLDζ2* (**Figs. 4**, **5**). Additionally, ACBP6-conferred freezing tolerance in flowers was affiliated with the up-regulation of ABA-related genes and MGDG-related genes such as *MGD1*, *MGD3* and *SFR2* (**Fig. 6**). *P5CS* and *AtPOX* expression in CA ACBP6-OE flowers showed a similar trend to that in CA rosettes (**Figs. 9**, **10**). In contrast to CA OE rosettes that showed an increase in proline, the proline content declined in the CA OE flowers, which appears inconsistent with the higher expression of *P5CS* and lower expression of *AtPOX* (**Fig. 10A**, **D**). Such a disparity between the proline content and *P5CS* mRNA level has also been previously observed in an Arabidopsis mutant lacking *HISTIDINE KINASE1* and this was attributed to post-transcriptional regulation of proline accumulation by P5CS (Kumar et al. 2013). These results imply that ACBP6-conferred freezing tolerance in flowers may not necessarily be dependent on proline accumulation, and proline seems to play a different role in CA ACBP6-OE rosettes and flowers, although proline-related genes did show similar changes in both organs (**Figs. 9**, **10**).

## Comparison of the lipid content and gene expression between WT flowers and rosettes after freezing

A comparison of the lipid content and gene expression between WT flowers and rosettes was carried out after freezing to address any variation between them. The results showed similar changes in the content of most lipids in WT flowers and rosettes. The PC, DGDG, MGDG, PE and PS content in flowers (**Table 1**) and rosettes (Chen et al. 2008) decreased after cold acclimation and freezing, while PA, LysoPG, LysoPC and LysoPE increased after freezing, consistent with the results of Welti et al. (2002). When the expression of PC-, PA-, MGDG- and *COR*-related genes at different stages (NA, CA, F and R) in WT flowers and rosettes was tested, the results indicated that *ACBP6* expression was up-regulated after cold acclimation and recovery in flowers (**Supplementary Fig. S6A**) and after cold acclimation in rosettes (**Supplementary Fig. S6B**). Correspondingly, the expression of some PC-, PA-, MGDG-,

**Fig. 10** Continued

proline- and *COR*-related genes including *PLDζ2*, *CCT2*, *CK1*, *COR6.6*, *COR47*, *LTI78*, *COR15a*, *KIN1* and *NCED3* was up-regulated in both flowers and rosettes after cold acclimation and/or freezing and/or recovery (**Figs. 4–8**; **Supplementary Figs. S1–S5**).

However, differences in expression of some genes were evident between WT flowers and rosettes at various stages. For example, *MGD1* expression slightly increased in flowers after cold acclimation, freezing and recovery, but greatly increased in rosettes after freezing and recovery (**Supplementary Fig. S6A**, **B**). *SFR2* expression increased 2- to 3-fold in flowers after cold acclimation, freezing and recovery, while its expression was only slightly up-regulated in rosettes after cold acclimation but decreased after freezing and recovery (**Supplementary Fig. S6A**, **B**). Furthermore, the expression of cold-related TFs (*CBF1*, *CBF2*, *CBF3*, *ICE1* and *CBF4*) dramatically increased in rosettes after cold acclimation and/or freezing and/or recovery (**Supplementary Fig. S6B**), but the expression of *CBF1*, *CBF2*, *CBF3* and *ICE1* decreased or showed no change in flowers after cold acclimation and freezing (**Supplementary Fig. S6A**). Slight increases in *CBF3* expression in flowers after recovery, and in *CBF4* expression after freezing and recovery were noted (**Supplementary Fig. S6A**). Differences for several PC biosynthesis-related genes were also observed. For instance, *PEAMT1* expression decreased in flowers after cold acclimation, freezing and recovery, but its expression significantly increased in rosettes after cold acclimation and decreased after freezing and recovery (**Supplementary Fig. S7A**, **B**). *CK-LIKE1* expression increased in flowers after cold acclimation, freezing and recovery but decreased in rosettes after cold acclimation, freezing and recovery (**Supplementary Fig. S7A**, **B**). Although *CCT2* showed up-regulation in both flowers and rosettes after freezing and recovery, it displayed down-regulation in flowers after cold acclimation but showed the highest increase in rosettes after cold acclimation (**Supplementary Fig. S7A**, **B**). Furthermore, four PC degradation-related genes showed variation between WT flowers and rosettes after cold acclimation, freezing and recovery; *PAT-PLA-IIβ*, *PAT-PLA-IIIα*, *PAT-PLA-IIIδ* and *PLA2α* expression increased in WT rosettes (**Supplementary Fig. S7B**), but decreased in WT flowers during cold acclimation and/or freezing and/or recovery (**Supplementary Fig. S7A**). These results revealed that WT flowers and rosettes showed some similar responses in gene expression during cold acclimation, freezing and recovery and in lipid content in freeze-treated flowers, but differential gene expression between flowers and rosettes reinforces the finding that variation in the transcriptional networks for cold regulation occurs between these organs (Lee and Lee 2003, Chinnusamy et al. 2007).

## Role of PC and PA in ACBP6-mediated freezing tolerance in flowers

Accumulation of diunsaturated PC species may cause a decrease in propensity for formation of the freeze-induced non-lamellar phase, thus promoting membrane stabilization (Thomashow 1999, Uemura et al. 2006). The yeast 10-kDa ACBP is known to regulate the expression of genes involved in stress responses and phospholipid and fatty acid synthesis (Feddersen et al. 2007). Rather similarly, Arabidopsis ACBP6-OE rosettes showed enhanced freezing tolerance by the up-regulation of *PLDδ* (Chen et al. 2008).

We also reported that ACBP6 binds PC but not PA or LysoPC (Chen et al. 2008), suggesting a close relationship between ACBP6 and PC. Consistently, our results herein revealed that ACBP6 overexpression in flowers enhanced freezing tolerance by up-regulating PC biosynthesis-related genes, resulting in significant accumulation of diunsaturated PC (34:4 PC, 34:3 PC, 34:2 PC, 36:6 PC, 36:5 PC, 36:4 PC, 36:3 PC, 36:2 PC, 38:5 PC and 38:4 PC) after freezing (**Figs. 3**, **4**; **Table 1**; **Supplementary Table S2**), resembling changes in PC content of freezing-tolerant PLDα-deficient Arabidopsis (Welti et al. 2002) and *acbp1* knockout mutant rosettes (Du et al. 2010). ACBP6 overexpression may allow greater PC binding and accumulation in ACBP6-OE flowers. The PA content in ACBP6-OE flowers was significantly higher than the WT before treatment, but was lower after freezing, albeit without significant variation (**Table 1**). As accumulation of freeze-induced PA is detrimental to cell membranes by promoting the formation of the non-lamellar phase (Li et al. 2008), PA reduction in ACBP6-OE flowers probably enhanced membrane stability and freezing tolerance.

## Role of ACBP6 in LPCAT1-mediated exchange between intracellular acyl-CoA and PC pools in flowers

Arabidopsis ACBP6 binds acyl-CoA esters in vitro, protects acyl-CoAs from degradation by microsomal acyl-hydrolases (Engeseth et al. 1996) and also binds PC (Chen et al. 2008). Yurchenko et al. (2009) reported that the recombinant 10-kDa ACBP from *Brassica napus* (BnACBP) binds acyl-CoA esters in vitro and enhances acyl exchange between acyl-CoA and PC catalyzed by LPCAT by promoting the incorporation of 18:1-CoA (Yurchenko and Weselake, 2011). The up-regulation of *LPCAT1* in ACBP6-OE flowers after cold acclimation and recovery (**Fig. 4**) and accumulation of PC in ACBP6-OE flowers after freezing (**Table 1**) again implied a role for ACBP6 in *LPCAT1*-mediated maintenance of intracellular acyl-CoA and PC pools.

It was observed that PC degradation-related genes (PLAs and PLDs) were up-regulated in the flowers of ACBP-OEs after cold acclimation and freezing (**Fig. 5**), but the PC content had increased or was maintained at higher levels than the WT at the same stage (**Table 1**). Several possible reasons may account for this observation. First, up-regulation of PC degradation-related genes in ACBP6-OE flowers may cause a higher production of PA derived from PC, but the resultant PA could be metabolized to other lipid compounds which can be recycled to PC. This explanation is supported by the observation that PA content was lower in ACBP6-OE flowers than the WT after freezing treatment (**Table 1**). Secondly, LPCAT

mediates the interchange between PC and LysoPC (Wang et al. 2012), and the up-regulation of *LPCAT1* in ACBP6-OE flowers after cold acclimation and recovery (**Fig. 4**) may have increased PC by remolding the intracellular acyl-CoA and PC pools or, more precisely, by reacylation of LysoPC to regenerate PC (Wang et al. 2012) in ACBP6-OE flowers after freezing treatment. This is supported by the observation that the level of LysoPC in ACBP6-OE flowers was lower than the WT after freezing treatment (**Table 1**). Thirdly, as ACBP6 binds PC but not PA or LysoPC in vitro (Chen et al. 2008), ACBP6 overexpression may allow greater PC binding and accumulation in ACBP6-OE flowers after freezing treatment.

## Role of MGDG and MGDG-related enzymes in ACBP6-mediated freezing tolerance in flowers

MGDG is the most abundant galactolipid which is exclusively localized at plastids in plants (Douce and Joyard 1980, Härtel et al. 2000, Nakamura 2013). MGDG is synthesized by MGDG synthases (Nakamura et al. 2010). Arabidopsis has three members in the MGDG synthase family (Awai et al. 2001). Type A MGDG synthase (MGD1), localized at the inner envelope of the chloroplast and up-regulated by light and cytokinins, is needed for photosynthetic growth (Jarvis et al. 2000, Kobayashi et al. 2007). Type B MGDG synthases (MGD2 and MGD3) reside at the outer envelope of chloroplast, are suppressed by cytokinins and are primarily expressed in non-photosynthetic organs (flowers for MGD2 and roots for MGD3) (Awai et al. 2001, Kobayashi et al. 2009). While previous studies have demonstrated an essential role for galactolipids in photosynthesis (Jarvis et al. 2000, Kobayashi et al. 2007), recent studies showed that galactolipids are abundant in flowers, and type B MGDs participate more actively than type A in floral MGDG synthesis (Awai et al. 2001, Kobayashi et al. 2009, Nakamura, 2013). Our results herein revealed a function for MGDs in flowers upon freezing treatment. Floral MGDG content did not significantly differ between the WT and ACBP6-OEs before freezing, but decreased in both the WT and ACBP6-OEs after freezing treatment (**Table 1**). However, the MGDG content in OE-3 and OE-5 flowers was significantly higher than the WT after freezing treatment (**Table 1**), suggesting a positive role for MGDG in enhancing floral freezing tolerance. Consistently, elevation in OE-3 and OE-5 flowers compared with the WT of *MGD1* after cold acclimation, freezing and recovery, of *MGD2* after freezing, and of *MGD3* after cold acclimation and freezing suggested that both type A and type B are important in ACBP6-mediated freezing tolerance in flowers (**Fig. 6**).

Recently, SFR2, a GGGT that shuttles galactose groups from MGDG, was reported to confer freezing tolerance in Arabidopsis and indicated that GGGT remodels galactoglycerolipid biosynthesis at the outer chloroplast membrane and stabilizes membranes during freezing (Moellering et al. 2010). Interestingly, the expression of *SFR2* in WT flowers increased after cold acclimation, freezing and recovery and its expression in OE-3 and OE-5 flowers was higher than the WT after cold acclimation and recovery (**Fig. 6**), suggesting that remodeling of galactoglycerolipid at the outer chloroplast membrane can occur in ACBP6-OE flowers upon freezing treatment and *SFR2* may contribute in enhancing freezing tolerance in ACBP6-OE flowers. However, it should be noted that *MGD2*, *MGD3* and *SFR2* expression declined in ACBP6-OE rosettes after cold acclimation and/or freezing (**Supplementary Fig. S3**), implying that the roles of *MGD2*, *MGD3* and *SFR2* can vary in flowers and rosettes under freezing stress.

## Function of ACBP6 as a transporter for lipids in flowers

De novo fatty acid biosynthesis predominantly occurs in the chloroplasts of higher plants (Ohlrogge and Browse 1995). Subsequently, most plastid-synthesized fatty acids are exported as oleoyl-CoA (18:1-CoA) and palmitoyl-CoA (16:0-CoA) to the endoplasmic reticulum (ER) for biosynthesis of non-plastidial membrane lipids and triacylglycerols (Moreau et al. 1998, Ohlrogge and Browse 1995). Extensive exchange of acyl-CoAs and their derivatives occurs between the plastids and the ER through the cytosol (Ohlrogge and Browse 1995). As AtACBP6 has been shown to bind oleoyl-CoA (Engeseth et al. 1996) and is localized at the cytosol (Chen et al. 2008), it may be a candidate for the transfer of acyl-CoAs from the plastids to the ER. Interestingly, significant changes in plastid-derived MGDG and the regulation of MGDG-related genes (**Table 1**) were observed between the WT and ACBP6-OE flowers after freezing (**Fig. 6**). ACBP6 may possibly transfer acyl-CoAs and their derivatives between the plastids and the ER in flowers exposed to freezing stress. Interestingly, the cytosolic 10-kDa *Brassica* ACBP affected the balance between acyl-CoA and phospholipid pools in fatty acids and triacylglycerol, and BnACBP overexpression resulted in the accumulation of fatty acids for oil biosynthesis in transgenic Arabidopsis seeds (Yurchenko et al. 2009), suggesting its role in enhancing acyl-CoA transfer (Yurchenko and Weselake 2011). In conclusion, our work using electrospray ionization tandem mass spectrometry (ESI MS/MS) and qRT-PCR analyses demonstrated the differences in ACBP6-mediated freezing tolerance in flowers and rosettes, and the potential of ACBP6 in floral protection from freezing stress in floriculture.

# Materials and Methods

## Plant materials and growth conditions

Transgenic ACBP6-OE homozygous lines (OE-3 and OE-5) transformed with the *35S::ACBP6* construct were generated by *Agrobacterium*-mediated transformation of Arabidopsis in a previous study (Chen et al. 2008). $T_3$ Arabidopsis seeds were surface-sterilized and germinated on MS medium (Murashige and Skoog 1962) supplemented with 2% sucrose. The plates were incubated at 4°C for 2 d and then transferred to a growth chamber under 16 h light (23°C)/8 h dark (21°C) cycles. Ten-day-old seedlings were potted in soil for further growth under the same light/dark regime.

## Freezing treatment

Freezing treatment was performed following Chen et al. (2008) and is summarized in **Fig. 1**. NA WT and ACBP6-OEs (OE-3 and OE-5) were grown in a growth chamber under 16 h light (23°C)/8 h dark (21°C) cycles before treatment. Detached flowers from 5-week-old plants were subjected to 4°C (cold room) for 3 d to obtain CA flowers. For freezing treatment, CA flowers were transferred to a growth chamber (Watlow series 942) and the temperature was reduced from 4°C to −7°C at 2°C $h^{-1}$. After 1 h at −7°C, the flowers were thawed overnight (12 h) at 4°C. F flowers were then subjected to a 7 d recovery at 4°C under white light before photography. NA, CA, F and R flowers were collected in liquid nitrogen and stored at −80°C for lipid profiling, qRT-PCR, measurement of sugar and proline contents and Northern blot analysis.

## Lipid profiling

Lipid extraction was conducted according to the protocol provided by the Kansas Lipidomics Research Center (www.K-state.edu/lipid/lipidomics). Detached Arabidopsis flowers were placed on water-moistened Whatman No. 1 filter paper in Petri plates. After cold acclimation for 3 d at 4°C, detached flowers in Petri plates were frozen at −7°C for 1 h, and then collected. NA flowers were also harvested. All the harvested samples were transferred to 3 ml of isopropanol 0.01% butylated hydroxytoluene at 75°C for 15 min, and subsequently 1.5 ml of chloroform and 0.6 ml of water were added. Tubes were shaken for 1 h and the extract was transferred to a new glass tube for lipid analysis. The remaining tissue was re-extracted 4–5 times using chloroform : methanol (2 : 1) 0.01% butylated hydroxytoluene with 30 min agitation at each extraction until all the samples became white. The remaining flower tissue was dried overnight at 105°C and weighed to obtain the dry weight. The combined extracts were washed using 1 ml of 1 M KCl followed by 2 ml of water. Finally, the solvent was evaporated under nitrogen. Samples were then sent to the Kansas Lipidomics Research Center for lipid profiling by ESI MS/MS (Devaiah et al. 2006).

## qRT-PCR

Total RNA (5 μg) from NA, CA, F and R flowers and rosettes harvested from 5-week-old plants was extracted using the RNeasy Plant Mini Kit (Qiagen) and was reverse-transcribed into first-strand cDNA using the SuperScript First-Strand Synthesis System (Invitrogen), followed by qRT-PCR with a StepOne Plus Real-Time PCR System (Applied Biosystems) and FastStart Universal SYBR Green Master (Roche). The conditions for qRT-PCR were as follows: denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Three experimental replicates for each reaction were carried out using gene-specific primers, and Arabidopsis *ACTIN2* as an internal control. The relative changes in gene expression were analyzed (Schmittgen and Livak 2008) using data from three independent experiments. Primers for qRT-PCR are listed in **Supplementary Table S4**.

## Measurement of sugar and proline contents

Soluble sugar content was measured according to Li et al. (2004). NA or CA rosettes and flowers from 5-week-old plants were collected, weighed and ground into powder, then 1 ml of 75% ethanol was added before overnight incubation at 4°C with shaking. Subsequently, the mixtures were centrifuged at 20,000 × *g* and the soluble sugars were harvested. Sugar content was measured by incubating a 20 μl aliquot in 1 ml of anthrone reagent [0.15% anthrone (w/v), 72% $H_2SO_4$ (v/v) and 28% $H_2O$ (v/v)] at 100°C for 1 h. Absorbance was measured at 625 nm and the sugar value expressed as glucose equivalents. For proline measurements (Li et al. 2004), a 100 μl aliquot was incubated with 900 μl of ninhydrin reagent [1% ninhydrin (w/v), 60% glacial acetic acid (v/v) and 40% $H_2O$] at 100°C for 1 h. Subsequently, 3 ml of toluene was added, vortexed and incubated at 23°C for 24 h. Absorbance was read at 520 nm.

## Northern blot analysis

Total RNA from 5-week-old NA and CA rosettes was extracted by TRIzol reagent (Invitrogen) according to the manufacturer's instruction. A 30 μg aliquot of RNA per well was separated on a 1.5% agarose gel containing 6% formaldehyde and transferred to Hybond N membrane (Amersham) for Northern blot analysis (Xiao et al. 2008). Probes were generated using the PCR Digoxigenin Probe Synthesis Kit (Roche), and Northern blot analysis was carried out using the Digoxigenin Nucleic Acid Detection Kit (Roche). The gene-specific primers used are listed in **Supplementary Table S4**.

## Accession numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under the numbers listed in **Supplementary Table S4**.

## Supplementary data

**Supplementary data** are available at PCP online.

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## Disclosures

The authors have no conflicts of interest to declare.

## References


Anchordoguy, T.J., Rudolph, A.S., Carpenter, J.F. and Crowe, J.H. (1987) Modes of interaction of cryoprotectants with membrane phospholipids during freezing. *Cryobiology* 24: 324–331.

Awai, K., Maréchal, E., Block, M.A., Brun, D., Masuda, T., Shimada, H. et al. (2001) Two types of MGDG synthase genes, found widely in both 16:3 and 18:3 plants, differentially mediate galactolipid syntheses in photosynthetic and nonphotosynthetic tissues in *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA* 98: 10960–10965.

Benning, C. and Ohta, H. (2005) Three enzyme systems for galactoglycerolipid biosynthesis are coordinately regulated in plants. *J. Biol. Chem.* 280: 2397–2400.

Chen, Q.F., Xiao, S. and Chye, M.L. (2008) Overexpression of the *Arabidopsis* 10-kilodalton acyl-coenzyme A-binding protein ACBP6 enhances freezing tolerance. *Plant Physiol.* 148: 304–315.

Chinnusamy, V., Ohta, M., Kanrar, S., Lee, B.H., Hong, X., Agarwal, M. et al. (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. *Genes Dev.* 17: 1043–1054.

Chinnusamy, V., Zhu, J.H. and Zhu, J.K. (2007) Cold stress regulation of gene expression in plants. *Trends Plant Sci.* 12: 444–451.

Devaiah, S.P., Roth, M.R., Baughman, E., Li, M., Tamura, P., Jeannotte, R. et al. (2006) Quantitative profiling of polar glycerolipid species from organs of wild-type *Arabidopsis* and a *PHOSPHOLIPASE Dα1* knockout mutant. *Phytochemistry* 67: 1907–1924.

Douce, R. and Joyard, J. (1980) Lipids: structure and function. *In* The Biochemistry of Plants. Edited by Stumpf, P.K. Vol. 4. pp. 321–362. Academic Press, New York.

Du, Z.Y., Chen, M.X., Chen, Q.F., Xiao, S. and Chye, M.L. (2013a) Arabidopsis Acyl-CoA-Binding Protein ACBP1 participates in the regulation of seed germination and seedling development. *Plant J.* 74: 294–309.

Du, Z.Y., Chen, M.X., Chen, Q.F., Xiao, S. and Chye, M.L. (2013b) Overexpression of Arabidopsis Acyl-CoA-Binding Protein ACBP2 enhances drought tolerance. *Plant Cell Environ.* 36: 300–314.

Du, Z.Y., Xiao, S., Chen, Q.F. and Chye, M.L. (2010) Depletion of the membrane-associated acyl-coenzyme A-binding protein ACBP1 enhances the ability of cold acclimation in *Arabidopsis*. *Plant Physiol.* 152: 1585–1597.

Engeseth, N.J., Pacovsky, R.S., Newman, T. and Ohlrogge, J.B. (1996) Characterization of an acyl-CoA-binding protein from *Arabidopsis thaliana*. *Arch. Biochem. Biophys.* 331: 55–62.

Feddersen, S., Neergaard, T.B., Knudsen, J. and Faergeman, N.J. (2007) Transcriptional regulation of phospholipid biosynthesis is linked to fatty acid metabolism by an acyl-CoA-binding-protein-dependent mechanism in *Saccharomyces cerevisiae*. *Biochem. J.* 407: 219–230.

Gilmour, S.J., Fowler, S.G. and Thomashow, M.F. (2004) *Arabidopsis* transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. *Plant Mol. Biol.* 54: 767–781.

Gilmour, S.J., Sebolt, A.M., Salazar, M.P., Everard, J.D. and Thomashow, M.F. (2000) Overexpression of the *Arabidopsis CBF3* transcriptional activator mimics multiple biochemical changes associated with cold acclimation. *Plant Physiol.* 124: 1854–1865.

Gilmour, S.J. and Thomashow, M.F. (1991) Cold acclimation and cold-regulated gene expression in ABA mutants of *Arabidopsis thaliana*. *Plant Mol. Biol.* 17: 1233–1240.

Gilmour, S.J., Zarka, D.G., Stockinger, E.J., Salazar, M.P., Houghton, J.M. and Thomashow, M.F. (1998) Low temperature regulation of the *Arabidopsis* CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression. *Plant J.* 16: 433–442.

Haake, V., Cook, D., Riechmann, J.L., Pineda, O., Thomashow, M.F. and Zhang, J.Z. (2002) Transcription factor CBF4 is a regulator of drought adaptation in *Arabidopsis*. *Plant Physiol.* 130: 639–648.

Härtel, H., Dörmann, P. and Benning, C. (2000) DGD1-independent biosynthesis of extraplastidic galactolipids after phosphate deprivation in *Arabidopsis*. *Proc. Natl Acad. Sci. USA* 97: 10649–10654.

Jaglo-Ottosen, K.R., Gilmour, S.J., Zarka, D.G., Schabenberger, O. and Thomashow, M.F. (1998) *Arabidopsis CBF1* overexpression induces *COR* genes and enhances freezing tolerance. *Science* 280: 104–106.

Jarvis, P., Dörmann, P., Peto, C.A., Lutes, J., Benning, C. and Chory, J. (2000) Galactolipid deficiency and abnormal chloroplast development in the *Arabidopsis* MGD synthase 1 mutant. *Proc. Natl Acad. Sci. USA* 97: 8175–8179.

Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat. Biotechnol.* 17: 287–291.

Knight, H., Zarka, D.G., Okamoto, H., Thomashow, M.F. and Knight, M.R. (2004) Abscisic acid induces *CBF* gene transcription and subsequent induction of cold-regulated genes via the CRT promoter element. *Plant Physiol.* 135: 1710–1717.

Kobayashi, K., Awai, K., Nakamura, M., Nagatani, A., Masuda, T. and Ohta, H. (2009) Type-B monogalactosyldiacylglycerol synthases are involved in phosphate starvation-induced lipid remodeling, and are crucial for low-phosphate adaptation. *Plant J.* 57: 322–331.

Kobayashi, K., Kondo, M., Fukuda, H., Nishimura, M. and Ohta, H. (2007) Galactolipid synthesis in chloroplast inner envelope is essential for proper thylakoid biogenesis, photosynthesis, and embryogenesis. *Proc. Natl Acad. Sci. USA* 104: 17216–17221.

Kreps, J.A., Wu, Y., Chang, H.S., Zhu, T., Wang, X. and Harper, J.F. (2002) Transcriptome changes for *Arabidopsis* in response to salt, osmotic, and cold stress. *Plant Physiol.* 130: 2129–2141.

Kumar, M.N., Jane, W.N. and Verslues, P.E. (2013) Role of the putative osmosensor Arabidopsis *histidine kinase1* in dehydration avoidance and low-water-potential response. *Plant Physiol.* 161: 942–953.

Lands, W.E.M. (1960) Metabolism of glycerolipids. 2. The enzymatic acylation of lysolecthin. *J. Biol. Chem.* 235: 2233–2237.

Lee, J.Y. and Lee, D.H. (2003) Use of serial analysis of gene expression technology to reveal changes in gene expression in Arabidopsis pollen undergoing cold stress. *Plant Physiol.* 132: 517–529.

Li, W., Li, M., Zhang, W., Welti, R. and Wang, X. (2004) The plasma membrane-bound phospholipase Dδ enhances freezing tolerance in *Arabidopsis thaliana*. *Nat. Biotechnol.* 22: 427–433.

Li, W., Wang, R., Li, M., Li, L., Wang, C., Welti, R. et al. (2008) Differential degradation of extraplastidic and plastidic lipids during freezing and post-freezing recovery in *Arabidopsis thaliana*. *J. Biol. Chem.* 283: 461–468.

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K. et al. (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and

low-temperature-responsive gene expression, respectively, in Arabidopsis. *Plant Cell* 10: 1391–1406.

Moellering, E.R., Muthan, B. and Benning, C. (2010) Freezing tolerance in plants requires lipid remodeling at the outer chloroplast membrane. *Science* 330: 226–228.

Moreau, P., Bessoule, J.J., Mongrand, S., Testet, E., Vincent, P. and Cassagne, C. (1998) Lipid trafficking in plant cells. *Prog. Lipid Res.* 37: 371–391.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.* 15: 473–497.

Nakamura, Y. (2013) Galactolipid biosynthesis in flowers. *Bot. Stud.* 54: 29.

Nakamura, Y., Kobayashi, K., Shimojima, M. and Ohta, H. (2010) Biosynthesis and function of monogalactosyldiacylglycerol (MGDG), the signature lipid of chloroplasts. *In* The Chloroplast: Basics and Applications. Edited by Rebeiz, A.C., Benning, C., Bohnert, H.J., Lichtenthaler, H.K., Portis, A.R. and Tripathy, B.C. pp. 185–202. Springer Press, Dordrecht.

Ohlrogge, J. and Browse, J. (1995) Lipid biosynthesis. *Plant Cell* 7: 957–970.

Rajashekar, C.B., Zhou, H.E., Zhang, Y., Li, W. and Wang, X. (2006) Suppression of phospholipase Dα 1 induces freezing tolerance in *Arabidopsis*: response of cold-responsive genes and osmolyte accumulation. *J. Plant Physiol.* 163: 916–926.

Schmittgen, T.D. and Livak, K.J. (2008) Analyzing real-time PCR data by the comparative $C_T$ method. *Nat. Protoc.* 3: 1101–1108.

Strauss, G. and Hauser, H. (1986) Stabilization of lipid bilayer vesicles by sucrose during freezing. *Proc. Natl Acad. Sci. USA* 83: 2422–2426.

Tamminen, I., Mäkelä, P., Heino, P. and Palva, E.T. (2001) Ectopic expression of ABI3 gene enhances freezing tolerance in response to abscisic acid and low temperature in *Arabidopsis thaliana*. *Plant J.* 25: 1–8.

Tasseva, G., Richard, L. and Zachowski, A. (2004) Regulation of phosphatidylcholine biosynthesis under salt stress involves choline kinases in *Arabidopsis thaliana*. *FEBS Lett.* 566: 115–120.

Theocharis, A., Clément, C. and Barka, E.A. (2012) Physiological and molecular changes in plants grown at low temperatures. *Planta* 235: 1091–1105.

Thomashow, M.F. (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50: 571–599.

Thomashow, M.F. (2010) Molecular basis of plant cold acclimation: insights gained from studying the CBF cold response pathway. *Plant Physiol.* 154: 571–577.

Thorlby, G., Fourrier, N. and Warren, G. (2004) The *SENSITIVE TO FREEZING2* gene, required for freezing tolerance in *Arabidopsis thaliana*, encodes a β-glucosidase. *Plant Cell* 16: 2192–2203.

Uemura, M., Tominaga, Y., Nakagawara, C., Shigematsu, S., Minami, A. and Kawamura, Y. (2006) Responses of the plasma membrane to low temperatures. *Physiol. Plant.* 126: 81–89.

Wang, H., Datla, R., Georges, F., Loewen, M. and Cutler, A.J. (1995) Promoters from kin1 and cor6.6, two homologous *Arabidopsis* thaliana genes: transcriptional regulation and gene expression induced by low temperature, ABA, osmoticum and dehydration. *Plant Mol. Biol.* 28: 605–617.

Wang, L., Shen, W., Kazachkov, M., Chen, G., Chen, Q., Carlsson, A.S. et al. (2012) Metabolic interactions between the Lands cycle and the Kennedy pathway of glycerolipid synthesis in Arabidopsis developing seeds. *Plant Cell* 24: 4652–4669.

Welti, R., Li, W., Li, M., Sang, Y., Biesiada, H., Zhou, H.E. et al. (2002) Profiling membrane lipids in plant stress responses. Role of phospholipase D alpha in freezing-induced lipid changes in *Arabidopsis*. *J. Biol. Chem.* 277: 31994–32002.

Xiao, S. and Chye, M.L. (2009) An *Arabidopsis* family of six acyl-CoA-binding proteins has three cytosolic members. *Plant Physiol. Biochem.* 47: 479–484.

Xiao, S. and Chye, M.L. (2011) New roles for acyl-CoA-binding proteins (ACBPs) in plant development, stress responses and lipid metabolism. *Prog. Lipid Res.* 50: 141–151.

Xiao, S., Gao, W., Chen, Q.F., Ramalingam, S. and Chye, M.L. (2008) Overexpression of membrane-associated acyl-CoA-binding protein ACBP1 enhances lead tolerance in Arabidopsis. *Plant J.* 54: 141–151.

Xin, Z. and Browse, J. (1998) *eskimo1* mutants of *Arabidopsis* are constitutively freezing-tolerant. *Proc. Natl Acad. Sci. USA* 95: 7799–7804.

Xiong, L., Schumaker, K.S. and Zhu, J.K. (2002) Cell signaling during cold, drought, and salt stress. *Plant Cell* 14: S165–S183.

Yurchenko, O.P., Nykiforuk, C.L., Moloney, M.M., Ståhl., U., Banaś, A., Stymne, S. et al. (2009) A 10-kDa acyl-CoA-binding protein (ACBP) from *Brassica napus* enhances acyl exchange between acyl-CoA and phosphatidylcholine. *Plant Biotechnol. J.* 7: 602–610.

Yurchenko, O.P. and Weselake, R.J. (2011) Involvement of low molecular mass soluble acyl-CoA-binding protein in seed oil biosynthesis. *New Biotechnol.* 28: 97–109.

Zhou, M.Q., Shen, C., Wu, L.H., Tang, K.X. and Lin, J. (2011) CBF-dependent signaling pathway: a key responder to low temperature stress in plants. *Crit. Rev. Biotechnol.* 31: 186–192.