Depletion of the Membrane-Associated Acyl-Coenzyme A-Binding Protein ACBP1 Enhances the Ability of Cold Acclimation in Arabidopsis[1][OA]

Zhi-Yan Du, Shi Xiao, Qin-Fang Chen, and Mee-Len Chye*

School of Biological Sciences, The University of Hong Kong, Pokfulam, Hong Kong, China

In Arabidopsis (*Arabidopsis thaliana*), a family of six genes encodes acyl-coenzyme A-binding proteins (ACBPs). A member of this family, ACBP1, contains an amino-terminal transmembrane domain that targets it to the plasma membrane and the endoplasmic reticulum. To investigate ACBP1 function, ACBP1-overexpressing transgenic Arabidopsis plants were characterized using lipid analysis. ACBP1 overexpressors showed reduction in several species of diunsaturated phosphatidylcholine (PC), prompting us to investigate if they were altered in response to freezing stress. ACBP1 overexpressors demonstrated increased freezing sensitivity accompanied by a decrease in PC and an increase in phosphatidic acid (PA), while *acbp1* mutant plants showed enhanced freezing tolerance associated with PC accumulation and PA reduction. We also showed binding of a recombinant eukaryotic ACBP (ACBP1) to PA, indicative of the possibility of enhanced PA interaction in ACBP1 overexpressors. Since phospholipase Dα1 (PLDα1) is a major enzyme promoting the hydrolysis of PC to PA, *PLDα1* expression was examined and was observed to be higher in ACBP1 overexpressors than in *acbp1* mutant plants. In contrast, the expression of PLDδ, which plays a positive role in freezing tolerance, declined in the ACBP1 overexpressors but increased in *acbp1* mutant plants. Given that ACBP1 is localized to the endoplasmic reticulum and plasma membrane, it may regulate the expression of PLDα1 and PLDδ by maintaining a membrane-associated PA pool through its ability to bind PA. Moreover, both genotypes showed no alterations in proline and soluble sugar content or in cold-regulated (*COR6.6* and *COR47*) gene expression, suggesting that the ACBP1-mediated response is PLD associated and is independent of osmolyte accumulation.

Arabidopsis (*Arabidopsis thaliana*) acyl-CoA-binding proteins (ACBPs) are conserved at the acyl-CoA-binding domain and range in size from 10.4 to 73.1 kD (Leung et al., 2004). They include membrane-associated ankyrin repeat-containing ACBP1 and ACBP2, extracellularly targeted ACBP3, Kelch motif-containing ACBP4 and ACBP5, and cytosolic 10-kD ACBP6 (for review, see Xiao and Chye, 2009). These proteins are localized in various subcellular compartments, and their recombinant derivatives have been shown to bind different acyl-CoA esters (Engeseth et al., 1996; Chye, 1998; Chye et al., 1999, 2000; Leung et al., 2004, 2006; Gao et al., 2008; Li et al., 2008a; Xiao et al., 2008b, 2009). Previous studies have indicated that ACBP6 overexpression in transgenic Arabidopsis enhances freezing tolerance accompanied by an up-regulation of *phospholipase Dδ* (*PLDδ*) expression (Chen et al., 2008). These findings prompted us to investigate whether other ACBPs are involved in cold stress.

Low temperature is a major environmental factor that restricts the geographical distribution and productivity of plants. Under low-temperature stress, complex processes including changes in gene regulation occur, culminating in cold acclimation and the acquirement of cold and freezing tolerance in some plants (Thomashow, 1999). Since the ability to manipulate cold tolerance leads to implications in agriculture and food production, intensive investigations in recent years have led to the identification and characterization of genes that confer cold and freezing tolerance.

Initial studies on cold-regulated gene expression in Arabidopsis have revealed that the family of CBF (also known as DREB1) transcription factors regulates many cold-inducible genes (Thomashow, 1999; Yamaguchi-Shinozaki and Shinozaki, 2006; Maruyama et al., 2009). The overexpression of CBFs (CBF1, CBF2, or CBF3) in Arabidopsis up-regulated cold-regulated (*COR*) gene expression and concomitantly improved freezing tolerance (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999; Gilmour et al., 2004). Constitutive

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* Corresponding author; e-mail mlchye@hkucc.hku.hk.

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expression of the *COR* genes also improves freezing tolerance (Artus et al., 1996; Steponkus et al., 1998), while their disruption dramatically decreases tolerance (Knight et al., 1999, 2009; Boyce et al., 2003). Some studies have shown that CBF expression is regulated by other transcription factors, such as ICE1, which elevates the expression of *CBF3* and *COR* to enhance freezing tolerance (Chinnusamy et al., 2003, 2007; Miura et al., 2007). Other independent pathways known to affect freezing tolerance are related to the accumulation of osmolytes such as Pro and soluble sugars (Xin and Browse, 1998; Gilmour et al., 2000; Rajashekar et al., 2006).

PLDα1 and PLDδ have been implicated in mediating freezing tolerance. Previous studies have demonstrated that *PLDα1*-deficient Arabidopsis plants are more tolerant to freezing and show decreased membrane lipid hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA) and an accumulation of osmolytes in comparison with the wild type (Welti et al., 2002; Rajashekar et al., 2006). PLDδ enhances freezing tolerance (Li et al., 2004) by binding microtubules and interaction with the cytoskeleton, thereby stabilizing the plasma membrane (Gardiner et al., 2001; Li et al., 2004, 2008b).

In Arabidopsis, the overexpression of ACBP6 was observed to enhance freezing tolerance (Chen et al., 2008). While ACBP6 is a 10-kD cytosolic protein, the larger ankyrin repeat-containing ACBP1 has been subcellularly localized to the plasma membrane and endoplasmic reticulum (ER; Chye, 1998; Chye et al., 1999; Li and Chye, 2003). In this report, we show that the *acbp1* mutant is tolerant to freezing stress. We suggest that reduction in *PLDα1* expression and decrease in the hydrolysis of PC to PA likely enhances membrane stability in the *acbp1* mutant plants, resulting in enhanced cold acclimation and freezing tolerance. Moreover, increased expression of *PLDδ* in the *acbp1* mutant plants may enhance tolerance by stabilization of the membranes. We also demonstrate here that a recombinant eukaryotic ACBP (ACBP1) can bind PA and that ACBP1 can possibly regulate the expression of PLDα1 and PLDδ through its interaction with membrane-associated PA.

RESULTS

ACBP1 Overexpressors Show Alterations in PC Molecular Species

ACBP1-overexpressing transgenic lines (ox-1 and ox-2) expressing the full-length *ACBP1* cDNA from the cauliflower mosaic virus (CaMV) *35S* promoter were generated, tested, and confirmed to accumulate the 37-kD ACBP1 protein by western-blot analysis in an earlier study (Xiao et al., 2008a). When these lines grown at 23°C were subjected to lipid analysis (Tables I and II), we observed that two species of diunsaturated PC, 36:5 PC and 38:6 PC, were significantly lower ($P < 0.05$) in the ACBP1 overexpressors (ox-1 and ox-2) than in the wild type. This result indicates that ACBP1 may play a role in the freezing response, since diunsaturated PCs are known to reduce the formation of the hexagonal II (H_{II}) phase and enhance freezing tolerance in plants (Uemura and Steponkus, 1994). Hence, we next investigated if the ACBP1 overexpressors and *acbp1* mutant plants would perform differently from the wild type under freezing stress.

The *acbp1* Knockout Mutant Shows Enhanced Freezing Tolerance, While ACBP1-Complemented Plants Are Freezing Sensitive

A T-DNA knockout mutant of *ACBP1* (designated *acbp1*) from Syngenta (SAIL_653_B06) that had been previously characterized (Xiao et al., 2008a) was used to investigate the role of ACBP1 in freezing stress. This mutant was complemented by a construct expressing *ACBP1* from the CaMV *35S* promoter in *Agrobacterium tumefaciens*-mediated transformation as reported by Xiao et al. (2008a). An *ACBP1*-complemented transgenic line (*cACBP1-2*) that was confirmed to accumulate the 37-kD ACBP1 protein by western-blot analysis (Xiao et al., 2008a) was subsequently used in the freezing treatment.

To investigate the role of ACBP1 in freezing tolerance, nonacclimated (NA) and cold-acclimated (CA) 11-d-old seedlings of *acbp1* mutant and ACBP1-complemented seedlings grown on Murashige and Skoog (MS) medium were treated at −12°C for 1 h using the wild type as a control. Exposure at −12°C killed all NA seedlings (Fig. 1A). With CA, ACBP1-complemented seedlings showed enhanced tolerance over NA; however, the survival rate of complemented seedlings (30%) was significantly ($P < 0.01$) lower than in the wild type (82%; Fig. 1, A and B). In contrast, all *acbp1* mutant seedlings survived in comparison with only 82% ($P < 0.05$) of the wild type (Fig. 1, B and C).

Five-week-old mature plants were also subjected to freezing treatment. NA *acbp1* mutant plants tolerated temperatures of −6°C and −8°C better than NA wild-type or ACBP1-complemented plants (Fig. 2A). As shown in Figure 2B, more CA *acbp1* mutant plants survived at −6°C, −8°C, and −10°C than either CA wild-type or ACBP1-complemented plants. Both NA and CA ACBP1-complemented plants displayed greater freezing sensitivity than the wild type (Fig. 2, A and B).

Electrolyte leakage was measured with freezing-treated leaves from NA and CA wild-type, *acbp1* mutant, and ACBP1-complemented plants. Results showed that the ionic leakage at −4°C, −6°C, and −8°C of NA and CA *acbp1* mutant plants was significantly lower ($P < 0.05$) than in the wild type (Fig. 2C). For NA Arabidopsis, ionic leakage of ACBP1-complemented plants was significantly greater than in the wild type following treatment at −6°C and −8°C ($P < 0.05$; Fig. 2C). For CA plants, ionic leakage of ACBP1-complemented lines was significantly greater than in

Table I. *Changes in PC species of wild-type (Col-0) and ACBP1-overexpressing (ox-1 and ox-2) plants grown at 23°C or CA followed by freezing treatment*

The values are means ± SD (nmol g^{-1} dry weight; $n = 3$). Significant differences ($P < 0.05$) from the wild type in the same experiment are indicated in boldface.

PC Species	23°C			−8°C		
	Wild Type	ox-1	ox-2	Wild Type	ox-1	ox-2
32:0	6.5 ± 1.5	5.2 ± 1.0	4.6 ± 1.4	6.5 ± 1.8	4.7 ± 1.3	5.3 ± 0.4
34:4	134.1 ± 9.0	117.1 ± 15.4	126.2 ± 4.8	132.7 ± 21.5	**76.7 ± 19.0[a]**	**89.9 ± 13.3[a]**
34:3	3,690.4 ± 180.7	3,768.6 ± 629.4	**4,176.2 ± 69.2[b]**	3,568.3 ± 487.3	3,030.0 ± 552.7	3,035.0 ± 594.3
34:2	3,123.3 ± 275.4	3,344.1 ± 414.6	3,288.5 ± 300.1	2,614.0 ± 440.2	2,139.7 ± 477.3	2,206.6 ± 227.9
34:1	299.8 ± 57.2	279.1 ± 42.3	307.1 ± 63.8	145.2 ± 37.0	110.2 ± 15.3	131.9 ± 15.9
36:6	1,886.2 ± 184.7	1,651.7 ± 179.7	1,892.0 ± 97.2	1,786.0 ± 204.7	**1,330.9 ± 217.4[a]**	1,347.7 ± 312.3
36:5	4,934.5 ± 294.0	**4,151.1 ± 470.8[a]**	**4,271.8 ± 471.2[a]**	3,620.0 ± 590.8	**2,439.9 ± 689.8[a]**	**2,696.8 ± 382.7[a]**
36:4	2,424.8 ± 165.5	2,137.7 ± 205.5	2,183.0 ± 232.2	1,753.6 ± 332.3	**1,104.6 ± 265.6[a]**	**1,160.8 ± 205.5[a]**
36:3	1,020.3 ± 156.3	983.4 ± 184.3	1,022.4 ± 117.2	527.3 ± 79.6	**387.9 ± 61.4[a]**	411.1 ± 66.8
36:2	365.4 ± 39.7	**485.7 ± 70.5[b]**	436.2 ± 101.4	225.4 ± 46.0	220.6 ± 40.9	226.9 ± 16.3
36:1	0	0	0	0	0	0
38:6	15.4 ± 2.2	**11.2 ± 2.0[a]**	**11.1 ± 1.7[a]**	12.0 ± 3.4	8.7 ± 1.9	8.7 ± 2.1
38:5	46.9 ± 2.0	39.9 ± 6.5	**34.3 ± 4.2[a]**	34.0 ± 6.8	24.2 ± 5.7	**21.3 ± 4.8[a]**
38:4	48.6 ± 10.5	48.7 ± 7.6	42.6 ± 8.7	35.0 ± 5.5	**22.8 ± 4.8[a]**	**23.1 ± 2.2[a]**
38:3	44.4 ± 6.2	34.7 ± 9.5	33.1 ± 7.4	22.3 ± 4.5	16.5 ± 5.5	17.0 ± 3.7
38:2	25.9 ± 6.9	24.1 ± 2.3	**14.8 ± 4.2[a]**	18.9 ± 1.3	15.5 ± 4.0	**14.8 ± 3.1[a]**
40:5	1.5 ± 1.6	2.5 ± 1.4	1.2 ± 0.9	5.7 ± 1.3	**3.3 ± 1.3[a]**	3.6 ± 1.8
40:4	4.9 ± 1.8	10.4 ± 4.9	5.4 ± 5.0	10.6 ± 1.6	**5.9 ± 1.4[a]**	**5.4 ± 1.4[a]**
40:3	3.7 ± 1.7	8.0 ± 5.4	4.1 ± 2.5	10.1 ± 1.5	**5.0 ± 1.2[a]**	**3.3 ± 0.9[a]**
40:2	2.2 ± 2.4	2.5 ± 2.2	4.5 ± 0.9	4.4 ± 1.2	2.4 ± 1.7	3.9 ± 1.4

[a]Value lower than the wild type in the same experiment ($P < 0.05$). [b]Value higher than the wild type in the same experiment ($P < 0.05$).

the wild type following treatment at −4°C, −6°C, and −8°C ($P < 0.05$; Fig. 2C).

ACBP1 Overexpressors Are More Sensitive to Freezing Stress

NA and CA 11-d-old seedlings of the wild type and ACBP1 overexpressors (ox-1 and ox-2) were grown on MS medium and treated at −12°C for 1 h to investigate the effects of freezing treatment on seedling development. For NA seedlings, −12°C was lethal to both the wild type and ACBP1 overexpressors (Fig. 3A). After CA at 4°C for 3 d, the survival rate of ACBP1-overexpressing seedlings increased to 30% (ox-1) and 23% (ox-2) but were still significantly ($P < 0.01$) lower than in the wild type (78%; Fig. 3, B and C).

To test the effect of freezing treatment on mature plants, 5-week-old wild-type and ACBP1-overexpressing plants from NA and CA sets were examined. When the temperature reached −6°C, NA wild-type

Table II. *Total amount of each head group class in rosettes of wild-type (Col-0) and ACBP1-overexpressing (ox-1 and ox-2) plants grown at 23°C or CA followed by freezing treatment*

The values are means ± SD (nmol mg^{-1} dry weight; $n = 3$). Significant differences ($P < 0.05$) from the wild type in the same experiment are indicated in boldface. DGDG, Digalactosyldiacylglycerol.

Lipid Class	23°C			−8°C		
	Wild Type	ox-1	ox-2	Wild Type	ox-1	ox-2
PC	18.1 ± 1.01	17.1 ± 2.13	17.9 ± 1.02	14.5 ± 2.12	11.0 ± 2.20	**11.4 ± 1.49[a]**
PA	0.2 ± 0.03	0.2 ± 0.02	0.2 ± 0.02	5.5 ± 1.74	8.7 ± 1.89	**9.8 ± 2.33[b]**
DGDG	37.5 ± 1.93	36.7 ± 2.19	38.5 ± 1.23	41.6 ± 2.22	44.9 ± 1.02	43.9 ± 2.50
MGDG	184.0 ± 8.32	184.3 ± 12.12	177.8 ± 7.64	133.5 ± 10.86	143.6 ± 11.18	137.6 ± 10.35
PG	12.3 ± 0.36	12.6 ± 1.55	13.5 ± 0.81	16.5 ± 0.86	16.0 ± 0.45	15.8 ± 0.88
PE	6.2 ± 0.80	6.4 ± 0.26	5.6 ± 0.29	7.6 ± 1.25	7.4 ± 1.43	8.2 ± 1.23
PI	6.7 ± 0.29	6.2 ± 0.52	6.3 ± 0.34	6.8 ± 0.42	6.3 ± 0.13	6.4 ± 0.35
PS	1.1 ± 0.08	1.0 ± 0.11	1.1 ± 0.04	0.8 ± 0.22	0.6 ± 0.13	0.8 ± 0.11
LysoPG	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.01	0.02 ± 0.01
LysoPC	0.02 ± 0.00	**0.04 ± 0.00[b]**	**0.03 ± 0.00[b]**	0.17 ± 0.01	0.30 ± 0.10	0.36 ± 0.15
LysoPE	0.07 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.31 ± 0.09	0.34 ± 0.06	0.40 ± 0.07

[a]Value lower than the wild type in the same experiment ($P < 0.05$). [b]Value higher than the wild type in the same experiment ($P < 0.05$).

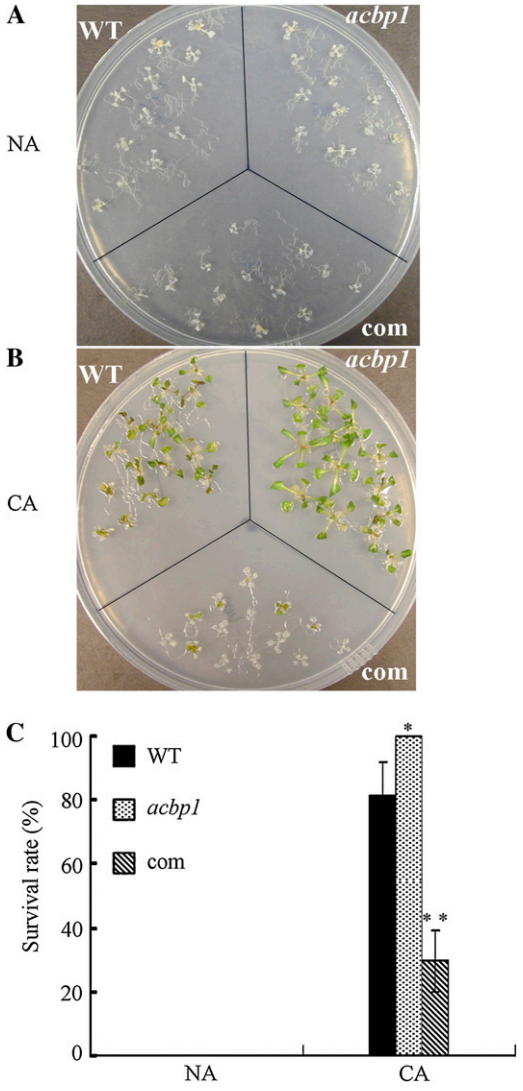

Figure 1. The *acbp1* mutant seedlings display enhanced freezing tolerance, while ACBP1-complemented plants show increased freezing sensitivity. A and B, NA and CA 11-d-old wild-type (WT), *acbp1* mutant, and ACBP1-complemented (com) seedlings after treatment at −12°C for 1 h. After thawing overnight at 4°C, the plates were transferred to a growth chamber (16-h-light [23°C]/8-h-dark [21°C] photoperiods) for a 7-d recovery before photography. C, Survival rate of NA and CA wild-type, *acbp1* mutant, and ACBP1-complemented seedlings shown in A and B. Asterisks denote significant differences from the wild type (** $P < 0.01$, * $P < 0.05$). Values are means ± SD ($n = 3$).

plants were more freezing tolerant than NA ACBP1 overexpressors (Fig. 4A). However, when the temperature was lowered to −8°C and −10°C, most wild-type plants and ACBP1 overexpressors were killed (Fig. 4A). After CA at 4°C for 3 d, the wild type and ACBP1 overexpressors were better protected against freezing stress (Fig. 4B). Most (93%) CA wild-type plants survived at −6°C, while the survival rates of ACBP1 overexpressors (ox-1 and ox-2) were 62% ($P < 0.01$) and 67% ($P < 0.05$), respectively. Few (4%) CA ACBP1 overexpressors survived at −8°C, while 56% of the wild type remained viable (Fig. 4B). These results suggested that ACBP1 overexpressors are more sensitive to freezing stress at −6°C and −8°C and that CA enhanced freezing tolerance in the wild type. Both NA and CA ACBP1-overexpressing plants displayed a phenotype similar to ACBP1-complemented plants under freezing stress (Figs. 2, A and B, and 4, A and B), confirming that the accumulation of ACBP1 led to greater freezing sensitivity.

Subsequently, freezing injury was evaluated by taking electrolyte leakage measurements of NA and CA freezing-treated leaves from wild-type and ACBP1-overexpressing plants (Fig. 4C). For NA Arabidopsis,

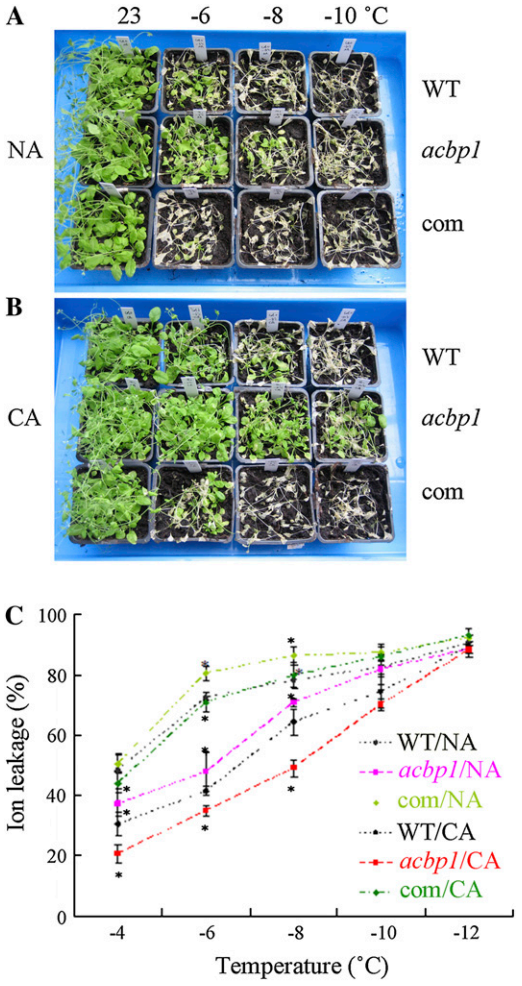

Figure 2. The *acbp1* mutant plants are more tolerant to freezing stress, while ACBP1-complemented plants show increased freezing sensitivity. A and B, NA and CA 5-week-old wild-type (WT), *acbp1* mutant, and ACBP1-complemented (com) plants after freezing treatment at the indicated temperatures. The plants were photographed after thawing overnight at 4°C and recovery in a growth chamber (16-h-light [23°C]/8-h-dark [21°C] photoperiods) for 7 d. C, Electrolyte leakage measurement of NA and CA wild-type, *acbp1* mutant, and ACBP1-complemented plants after freezing treatment at the indicated temperatures lasting 1 h followed by thawing at 4°C overnight. Asterisks denote significant differences from the wild type (* $P < 0.05$). Values are means ± SD ($n = 3$).

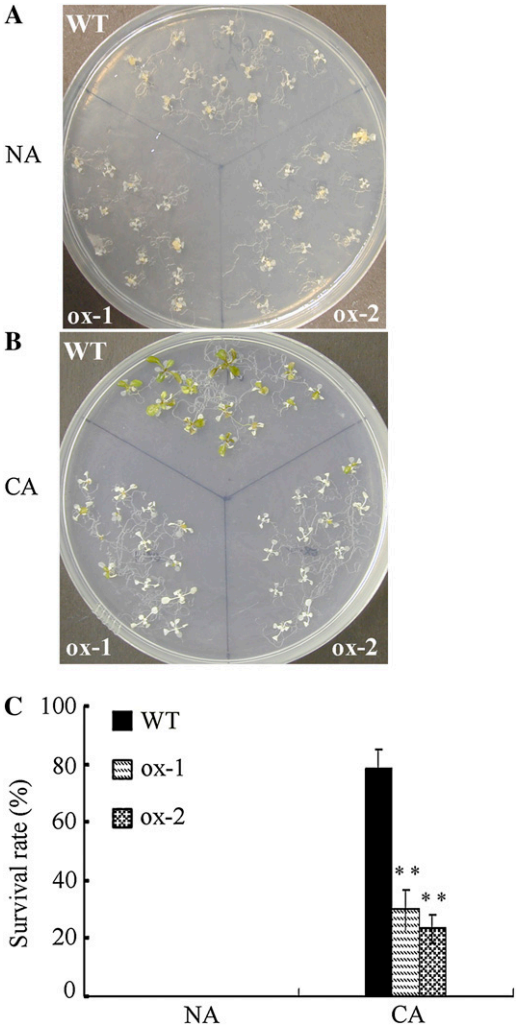

Figure 3. ACBP1-overexpressing seedlings show increased sensitivity to freezing stress in comparison with the wild type. A and B, NA and CA 11-d-old wild-type (WT) and ACBP1-overexpressing (ox-1 and ox-2) seedlings after treatment at −12°C for 1 h. Plates were thawed overnight at 4°C following a 7-d recovery in a growth chamber (16-h-light [23°C]/8-h-dark [21°C] photoperiods) before photography. C, Survival rate of NA and CA wild-type and ACBP1-overexpressing seedlings after freezing displayed in A and B. Asterisks denote significant differences from the wild type (** $P < 0.01$). Values are means ± SD ($n = 3$).

ionic leakage was greater in ACBP1 overexpressors than in the wild type following treatment at −6°C and −8°C ($P < 0.05$; Fig. 4C). For CA plants, ionic leakage was greater in ACBP1 overexpressors than in the wild type following treatment at −4°C, −6°C, and −8°C ($P < 0.05$; Fig. 4C).

The Expression of *ACBP1* Is Not Induced by Cold Treatment

The expression profiles of *ACBP1* during cold acclimation were examined by northern-blot analysis. Total RNA was extracted from 5-week-old wild-type Arabidopsis exposed to 4°C for 0, 6, 12, 24, and 48 h. As shown in Figure 5A, the expression of *ACBP1* was first down-regulated after 6 h of cold treatment and then increased to the untreated level 12 and 24 h after treatment. At 48 h, the expression of *ACBP1* was slightly weaker than at 0 h (Fig. 5A) but rose to a similar level after cold acclimation for 3 d (Fig. 5B). Similar expression profiles were observed in microarray data (http://bar.utoronto.ca/). These results indicate that the expression of *ACBP1* is not cold inducible.

Freezing Tolerance of the *acbp1* Mutant and Sensitivity of ACBP1 Overexpressors Are Independent of *COR* Gene Expression

To obtain further insight into *ACBP1* function in freezing stress, the expression of *ACBP1* and two *COR*

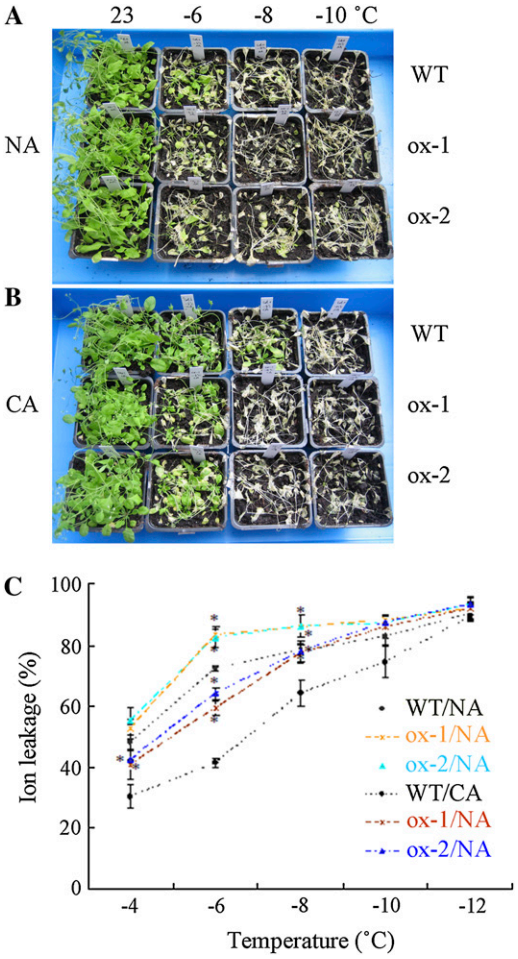

Figure 4. ACBP1-overexpressing plants are more sensitive to freezing stress in comparison with the wild type. A and B, NA and CA 5-week-old wild-type (WT) and ACBP1-overexpressing (ox-1 and ox-2) plants after freezing treatment at the indicated temperatures. Plants were photographed after a 7-d recovery in a growth chamber (16-h-light [23°C]/8-h-dark [21°C] photoperiods). C, Electrolyte leakage of NA and CA wild-type and ACBP1-overexpressing plants after freezing treatment at the indicated temperatures lasting 1 h followed by thawing at 4°C overnight. Asterisks denote significant differences from the wild type (* $P < 0.05$). Values are means ± SD ($n = 3$).

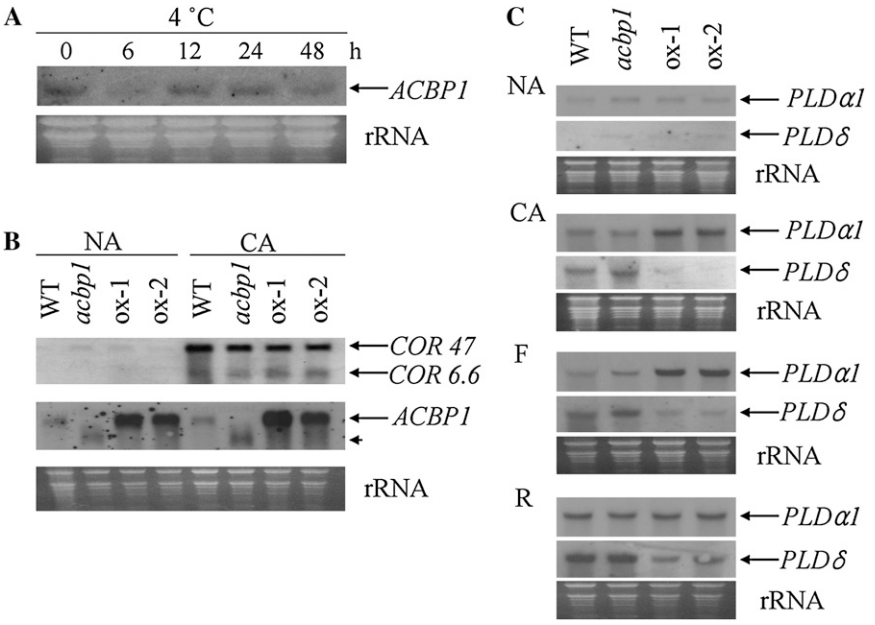

Figure 5. The expression of *ACBP1*, *COR* genes, *PLDα1*, and *PLDδ* in wild-type (WT), *acbp1* mutant, and ACBP1-overexpressing (ox-1 and ox-2) plants. A, Cold induction of *ACBP1* expression. Total RNA (20 μg lane^{-1}) was isolated from wild-type Arabidopsis rosettes at the indicated hours (h) after treatment. B, Total RNA (20 μg lane^{-1}) was prepared from rosettes of wild-type, *acbp1* mutant, and ACBP1-overexpressing plants before (NA) or after (CA) 3 d of cold acclimation at 4°C. The blots were hybridized with digoxigenin-labeled probes for *COR6.6*, *COR47*, and *ACBP1*. C, The transcript levels of *PLDα1* and *PLDδ* in the wild type, *acbp1*, and ACBP1 overexpressors. Total RNA (20 μg lane^{-1}) was prepared from wild-type, *acbp1*, ox-1, and ox-2 rosettes harvested before (NA) or after (CA) 3 d of cold acclimation, followed by freezing at −8°C for 1 h (F) and recovery (R; the temperature was raised to 4°C at 1°C h^{-1} and held at 4°C for 12 h before sampling). All bottom panels show ethidium bromide-stained rRNA, indicating the relative amounts of total RNA loaded per lane.

genes (*COR6.6* and *COR47*) was measured in 5-week-old Arabidopsis plants by northern-bolt analysis. After cold acclimation (4°C for 3 d), *ACBP1* was not induced in the wild type (Fig. 5B), in contrast to the cold-induced *ACBP6* (Chen et al., 2008). Without CA, the *COR6.6* and *COR47* transcripts were barely detectable in the wild type, the *acbp1* mutant, and ACBP1 overexpressors (Fig. 5B). After cold acclimation for 3 d, the mRNA levels of *COR6.6* and *COR47* increased in all three genotypes. Although cold acclimation enhanced freezing tolerance of *acbp1* mutant plants, the expression levels of *COR6.6* and *COR47* were similar among wild-type, *acbp1* mutant, and ACBP1-overexpressing plants (Fig. 5B), suggesting that tolerance of the *acbp1* mutant and sensitivity of ACBP1 overexpressors are not dependent on cold-induced *COR* gene expression.

Tolerance of the *acbp1* Mutant and Sensitivity of ACBP1 Overexpressors Are Correlated to the Expression of *PLDα1* and *PLDδ*

PLDα1 and *PLDδ* are important in mediating freezing tolerance in Arabidopsis. Previous studies have demonstrated that *PLDα1*-deficient Arabidopsis plants are more tolerant to freezing (Welti et al., 2002; Rajashekar et al., 2006). In contrast, knockout of *PLDδ* decreased and its overexpression increased freezing tolerance (Li et al., 2004). We have shown that the overexpression of the 10-kD ACBP6 resulted in enhanced freezing tolerance that was correlated to elevated *PLDδ* expression (Chen et al., 2008).

To determine whether tolerance in the *acbp1* mutant and sensitivity in ACBP1 overexpressors are associated with *PLD* expression, the expression of *PLDα1* and *PLDδ* in the wild type, *acbp1* mutant, and ACBP1 overexpressors (ox-1 and ox-2) was investigated by northern-blot analysis using PCR-generated digoxigenin-labeled cDNA probes. As shown in Figure 5C, ACBP1 overexpressors (ox-1 and ox-2) showed higher levels of *PLDα1* mRNA than the wild type at CA and freezing stages, while the expression of *PLDα1* was lower in the *acbp1* mutant than in the wild type. In contrast, the mRNA levels of *PLDδ* were lower in ox-1 and ox-2 plants than in the wild type at CA, freezing, and postfreezing recovery stages, while its expression in the *acbp1* mutant was higher than in the wild type at CA and freezing stages (Fig. 5C). However, the expression of *PLDα1* and *PLDδ* was similar in all three genotypes when nonacclimated (Fig. 5C).

Changes in Lipid Molecular Species after Freezing Treatment of CA Wild-Type, *acbp1* Mutant, ACBP1-Overexpressing, and ACBP1-Complemented Plants

Lipid profiling of Arabidopsis plants was carried out by electrospray ionization tandem mass spectrom-

Table III. *Total amount of each head group class in rosettes of wild-type (Col-0), acbp1 mutant, and ACBP1-complemented (com) plants grown at 23°C or CA followed by freezing treatment*

The values are means ± SD (nmol mg^{-1} dry weight; $n = 3$). Significant differences ($P < 0.05$) from the wild type in the same experiment are indicated in boldface. DGDG, Digalactosyldiacylglycerol.

Lipid Class	23°C			−8°C		
	Wild Type	*acbp1*	com	Wild Type	*acbp1*	com
PC	18.1 ± 1.01	17.5 ± 2.03	**16.2 ± 0.22**[a]	14.5 ± 2.12	**18.4 ± 2.07**[b]	**8.9 ± 1.41**[a]
PA	0.2 ± 0.03	0.2 ± 0.07	0.3 ± 0.14	5.5 ± 1.74	**2.8 ± 0.35**[a]	**9.6 ± 0.69**[b]
DGDG	37.5 ± 1.93	34.2 ± 2.74	36.7 ± 0.80	41.6 ± 2.22	41.0 ± 1.31	44.3 ± 1.94
MGDG	184.0 ± 8.32	168.6 ± 11.44	**169.1 ± 5.27**[a]	133.5 ± 10.86	139.9 ± 7.72	135.9 ± 9.93
PG	12.3 ± 0.36	11.1 ± 1.72	12.0 ± 0.18	16.5 ± 0.86	16.7 ± 1.02	**13.6 ± 1.65**[a]
PE	6.2 ± 0.80	5.7 ± 0.60	5.5 ± 0.12	7.6 ± 1.25	8.9 ± 1.12	6.3 ± 1.40
PI	6.7 ± 0.29	6.5 ± 0.48	5.8 ± 0.12	6.8 ± 0.42	7.1 ± 0.42	6.4 ± 0.30
PS	1.1 ± 0.08	1.1 ± 0.10	1.0 ± 0.08	0.8 ± 0.22	1.1 ± 0.16	0.7 ± 0.07
LysoPG	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.01
LysoPC	0.02 ± 0.00	**0.03 ± 0.00**[b]	**0.03 ± 0.01**[b]	0.17 ± 0.01	0.13 ± 0.06	0.38 ± 0.27
LysoPE	0.07 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.31 ± 0.09	0.23 ± 0.04	**0.44 ± 0.03**[b]

[a]Value lower than the wild type in the same experiment ($P < 0.05$). [b]Value higher than the wild type in the same experiment ($P < 0.05$).

etry. In the absence of freezing treatment (23°C), the ACBP1 overexpressors, *acbp1* mutant, and ACBP1-complemented plants all showed significantly higher LysoPC content than the wild type (Tables II and III). However, they showed no significant differences in LysoPC when compared with the wild type after CA and freezing treatment at −8°C (Tables II and III). Results indicate that after CA and freezing treatment, the total amount of PA significantly increased in the ACBP1 overexpressor ox-2 (Table II) and the ACBP1-complemented line (Table III) when compared with the wild type. Although the difference in PA content between the wild type and the ACBP1 overexpressor ox-1 was not significant, total PA increased 43.5-fold in ox-1 compared with 27.5-fold in the wild type after CA and freezing treatment (Table II). Thus, ox-1 accumulated 58% more PA than the wild type, while ox-2 and complemented plants accumulated 78% and 75% more PA, respectively (Tables II and III). Particularly, several PA species were significantly higher in ox-1, ox-2, and the ACBP1-complemented line than in the wild type, including 34:6 PA, 34:3 PA, 34:2 PA, 36:6 PA, and 36:2 PA (Tables IV and V). After CA and freezing treatment, the *acbp1* mutant accumulated significantly less (51%) PA than the wild type (Table III). Specifically, 32:0 PA, 34:4 PA, 34:3 PA, 36:6 PA, and 36:5 PA were reduced in the *acbp1* mutant (Table V).

In contrast, PC levels declined 20% in the wild type after CA and freezing, while the decrease was 36%, 36%, and 45% in ox-1, ox-2, and ACBP1-complemented lines, respectively (Tables II and III). In this case, ox-1, ox-2, and the ACBP1-complemented line accumulated 24%, 21%, and 39% less PC than the wild type, respectively (Tables II and III). In particular, 34:4 PC, 36:5 PC, 36:4 PC, 38:4 PC, 40:4 PC, and 40:3 PC in ACBP1-overexpressing (ox-1 and ox-2) and ACBP1-

Table IV. *Changes in PA species of wild-type (Col-0) and ACBP1-overexpressing (ox-1 and ox-2) plants grown at 23°C or CA followed by freezing treatment*

The values are means ± SD (nmol g^{-1} dry weight; $n = 3$). Significant differences ($P < 0.05$) from the wild type in the same experiment are indicated in boldface.

PA Species	23°C			−8°C		
	Wild Type	ox-1	ox-2	Wild Type	ox-1	ox-2
32:0	0	0	0	2.26 ± 1.2	3.9 ± 2.0	4.6 ± 3.2
34:6	0.2 ± 0.4	0.1 ± 0.2	0 ± 0.1	395.7 ± 132.9	**786.0 ± 224.3**[a]	**835.9 ± 59.0**[a]
34:5	0	0 ± 0.2	0	12.4 ± 5.0	11.7 ± 14.6	20.7 ± 11.6
34:4	0.3 ± 0.5	0.6 ± 0.5	1.7 ± 1.3	169.3 ± 77.6	275.9 ± 53.7	233.6 ± 97.9
34:3	48.9 ± 3.4	**63.6 ± 3.7**[a]	**67.0 ± 7.6**[a]	1,121.7 ± 334.7	**1,904.6 ± 355.2**[a]	**2,083.4 ± 375.9**[a]
34:2	55.7 ± 7.2	62.1 ± 10.8	59.9 ± 10.3	1,034.7 ± 334.7	**1,759.2 ± 410.1**[a]	**1,894.8 ± 582.9**[a]
34:1	1.1 ± 2.3	1.1 ± 1.4	2.2 ± 1.2	61.5 ± 28.2	83.3 ± 24.7	112.6 ± 35.8
36:6	6.7 ± 4.7	8.3 ± 3.8	**13.8 ± 3.5**[a]	524.9 ± 145.4	**842.0 ± 179.1**[a]	**955.8 ± 99.9**[a]
36:5	32.8 ± 7.6	32.0 ± 3.1	32.4 ± 5.3	1,127.6 ± 354.1	1,626.7 ± 357.2	**1,885.3 ± 495.5**[a]
36:4	22.3 ± 5.6	17.6 ± 5.3	25.5 ± 5.7	799.5 ± 269.4	1,071.2 ± 240.4	1,309.6 ± 497.9
36:3	4.9 ± 3.1	8.3 ± 5.3	6.8 ± 2.1	149.6 ± 59.3	230.6 ± 53.3	**287.4 ± 85.7**[a]
36:2	2.7 ± 2.6	3.6 ± 2.5	2.9 ± 3.4	73.1 ± 24.8	**136.7 ± 36.4**[a]	**146.1 ± 44.3**[a]

[a]Value higher than the wild type in the same experiment ($P < 0.05$).

Table V. *Changes in PA species of wild-type (Col-0), acbp1 mutant, and ACBP1-complemented (com) plants grown at 23°C or CA followed by freezing treatment*

The values are means ± SD (nmol g^{-1} dry weight; $n = 3$). Significant differences ($P < 0.05$) from the wild type in the same experiment are indicated in boldface.

PA Species	23°C			−8°C		
	Wild Type	*acbp1*	com	Wild Type	*acbp1*	com
32:0	0	0	0.1 ± 0.3	2.26 ± 1.2	**0.3 ± 0.1**[a]	3.7 ± 0.7
34:6	0.2 ± 0.4	0	0.4 ± 0.6	395.7 ± 132.9	269.3 ± 26.6	**751.8 ± 60.0**[b]
34:5	0	0	0	12.4 ± 5.0	8.1 ± 4.5	24.3 ± 17.4
34:4	0.3 ± 0.5	1.5 ± 1.6	1.8 ± 2.3	169.3 ± 77.6	**52.0 ± 16.8**[a]	195.5 ± 42.2
34:3	48.9 ± 3.4	51.1 ± 12.5	99.3 ± 52.5	1,121.7 ± 334.7	**516.9 ± 75.1**[a]	**1,985.0 ± 134.1**[b]
34:2	55.7 ± 7.2	54.6 ± 19.2	89.8 ± 28.9	1,034.7 ± 334.7	563.7 ± 90.0	**1,838.8 ± 191.0**[b]
34:1	1.1 ± 2.3	1.3 ± 1.1	3.8 ± 5.5	61.5 ± 28.2	22.4 ± 13.1	82.2 ± 3.5
36:6	6.7 ± 4.7	9.9 ± 6.0	21.2 ± 15.8	524.9 ± 145.4	**257.1 ± 3.8**[a]	**946.3 ± 65.4**[b]
36:5	32.8 ± 7.6	37.5 ± 13.1	50.5 ± 18.7	1,127.6 ± 354.1	**561.5 ± 89.5**[a]	**2,014.7 ± 139.6**[b]
36:4	22.3 ± 5.6	31.8 ± 20.8	**38.5 ± 9.3**[b]	799.5 ± 269.4	406.0 ± 82.5	**1,305.3 ± 90.9**[b]
36:3	4.9 ± 3.1	8.5 ± 3.1	11.9 ± 8.0	149.6 ± 59.3	76.0 ± 12.9	**283.1 ± 29.2**[b]
36:2	2.7 ± 2.6	2.0 ± 2.1	5.3 ± 3.6	73.1 ± 24.8	43.1 ± 8.9	**140.0 ± 15.9**[b]

[a]Value lower than the wild type in the same experiment ($P < 0.05$). [b]Value higher than the wild type in the same experiment ($P < 0.05$).

complemented plants were significantly lower ($P < 0.05$) than in the wild type (Table VI). Among these PC species, 34:4 PC (16:1–18:3), 36:5 PC (18:3–18:2), 36:4 PC (18:2–18:2 > 18:1–18:3), and 38:4 PC (20:1–18:3 > 20:2–18:2) are diunsaturated species (Devaiah et al., 2006). After CA and freezing treatment, *acbp1* mutant plants accumulated 27% more PC than the wild type. Several species of PC were significantly higher ($P < 0.05$) in the *acbp1* mutant than in the wild type (Table III). These include 34:4 PC, 34:2 PC, 36:5 PC, 36:4 PC, 36:2 PC, 38:5 PC, 38:4 PC, 38:3 PC, and 40:4 PC (Table VI). In addition, 34:4 PC (16:1–18:3), 36:5 PC (18:3–18:2), 36:4 PC (18:2–18:2 > 18:1–18:3), 38:5 PC (20:3–18:3), 38:4 PC (20:1–18:3 > 20:2–18:2), and 38:3 PC (20:1–18:2 > 20:0–18:3) are diunsaturated species (Devaiah et al., 2006). Prior to CA and freezing treatment, LysoPC was significantly higher ($P < 0.05$) in the ACBP1 overexpressors (ox-1 and ox-2), *acbp1* mutant, and the ACBP1-complemented line than in the wild type (Tables II and III). Following CA and freezing, no

Table VI. *Changes in PC species of wild-type (Col-0), acbp1 mutant, and ACBP1-complemented (com) plants grown at 23°C or CA followed by freezing treatment*

The values are means ± SD (nmol g^{-1} dry weight; $n = 3$). Significant differences ($P < 0.05$) from the wild type in the same experiment are indicated in boldface.

PC Species	23°C			−8°C		
	Wild Type	*acbp1*	com	Wild Type	*acbp1*	com
32:0	6.5 ± 1.5	7.4 ± 1.4	5.0 ± 2.3	6.5 ± 1.8	5.0 ± 4.8	4.1 ± 3.1
34:4	134.1 ± 9.0	129.8 ± 8.7	**108.0 ± 2.6**[a]	132.7 ± 21.5	**162.6 ± 8.7**[b]	**52.2 ± 36.9**[a]
34:3	3,690.4 ± 180.7	3,376.6 ± 235.5	3,711.3 ± 172.9	3,568.3 ± 487.3	4,027.9 ± 426.4	**1,730.1 ± 1,180.1**[a]
34:2	3,123.3 ± 275.4	3,443.0 ± 142.9	2,822.0 ± 126.7	2,614.0 ± 440.2	**3,401.0 ± 402.0**[b]	**1,289.1 ± 892.9**[a]
34:1	299.8 ± 57.2	379.6 ± 59.7	288.7 ± 55.7	145.2 ± 37.0	162.2 ± 6.0	77.8 ± 52.5
36:6	1,886.2 ± 184.7	1,796.2 ± 206.5	1,823.2 ± 188.7	1,786.0 ± 204.7	2,084.9 ± 386.7	**728.9 ± 495.0**[a]
36:5	4,934.5 ± 294.0	4,713.7 ± 665.5	**3,944.8 ± 211.5**[a]	3,620.0 ± 590.8	**5,032.5 ± 597.8**[b]	**1,595.2 ± 1,113.4**[a]
36:4	2,424.8 ± 165.5	2,741.6 ± 182.6	**2,018.4 ± 222.0**[a]	1,753.6 ± 332.3	**2,364.6 ± 317.8**[b]	**727.7 ± 514.9**[a]
36:3	1,020.3 ± 156.3	1,092.8 ± 110.7	920.0 ± 38.2	527.3 ± 79.6	597.9 ± 35.9	**269.1 ± 181.6**[a]
36:2	365.4 ± 39.7	411.7 ± 48.3	390.1 ± 57.4	225.4 ± 46.0	**310.2 ± 39.7**[b]	129.3 ± 87.6
36:1	0	0	0	0	0	0
38:6	15.4 ± 2.2	14.8 ± 2.1	14.0 ± 5.7	12.0 ± 3.4	14.2 ± 2.3	5.3 ± 3.5
38:5	46.9 ± 2.0	46.0 ± 4.7	**36.3 ± 1.8**[a]	34.0 ± 6.8	**47.2 ± 7.3**[b]	**14.1 ± 9.8**[a]
38:4	48.6 ± 10.5	57.6 ± 16.7	38.7 ± 10.0	35.0 ± 5.5	**50.9 ± 3.0**[b]	**16.2 ± 11.8**[a]
38:3	44.4 ± 6.2	38.4 ± 11.9	**31.4 ± 4.5**[a]	22.3 ± 4.5	**30.1 ± 3.1**[b]	**11.4 ± 7.7**[a]
38:2	25.9 ± 6.9	24.4 ± 4.9	**15.0 ± 4.5**[a]	18.9 ± 1.3	24.6 ± 4.7	9.0 ± 8.0
40:5	1.5 ± 1.6	3.5 ± 0.5	3.0 ± 2.6	5.7 ± 1.3	7.8 ± 1.7	**1.1 ± 1.5**[a]
40:4	4.9 ± 1.8	8.1 ± 3.9	**8.6 ± 1.5**[a]	10.6 ± 1.6	**16.1 ± 2.6**[b]	**2.7 ± 2.8**[a]
40:3	3.7 ± 1.7	**9.6 ± 3.0**[b]	7.0 ± 2.4	10.1 ± 1.5	11.0 ± 4.1	**2.5 ± 1.7**[a]
40:2	2.2 ± 2.4	1.9 ± 1.6	2.0 ± 1.6	4.4 ± 1.2	4.5 ± 2.0	**1.1 ± 1.0**[a]

[a]Value lower than the wild type in the same experiment ($P < 0.05$). [b]Value higher than the wild type in the same experiment ($P < 0.05$).

significant differences in LysoPC were evident among all phenotypes (Tables II and III). More significant changes occurred in the ACBP1-complemented line than in the wild type (Table III). Before CA and freezing, total PC and monogalactosyldiacylglycerol (MGDG) were significantly lower in the ACBP1-complemented line than in the wild type, while phosphatidylglycerol (PG) and lysophosphatidylethanolamine (LysoPE) in the ACBP1-complemented line were significantly lower and higher than in the wild type after CA and freezing, respectively (Table III). Nevertheless, these changes were not observed in the ACBP1 overexpressors (ox-1 and ox-2; Table II).

No Change in Soluble Sugar and Pro Accumulation during Cold Acclimation in the *acbp1* Mutant

The accumulation of soluble sugar and Pro is associated with enhanced freezing tolerance in Arabidopsis and tobacco (*Nicotiana tabacum*; Xin and Browse, 1998; Gilmour et al., 2000; Rajashekar et al., 2006; Zhao et al., 2009a). In the absence of cold acclimation, similar levels of soluble sugar were observed in wild-type, *acbp1* mutant, ACBP1-overexpressing, and ACBP1-complemented plants (Fig. 6A). After 3 d of cold acclimation at 4°C, soluble sugar content in all genotypes increased by 2-fold (Fig. 6A). Pro content increased by 1.8-fold in the wild type after cold acclimation (Fig. 6B). Similar increases occurred in the other genotypes, but there were no significant differences between them (Fig. 6B).

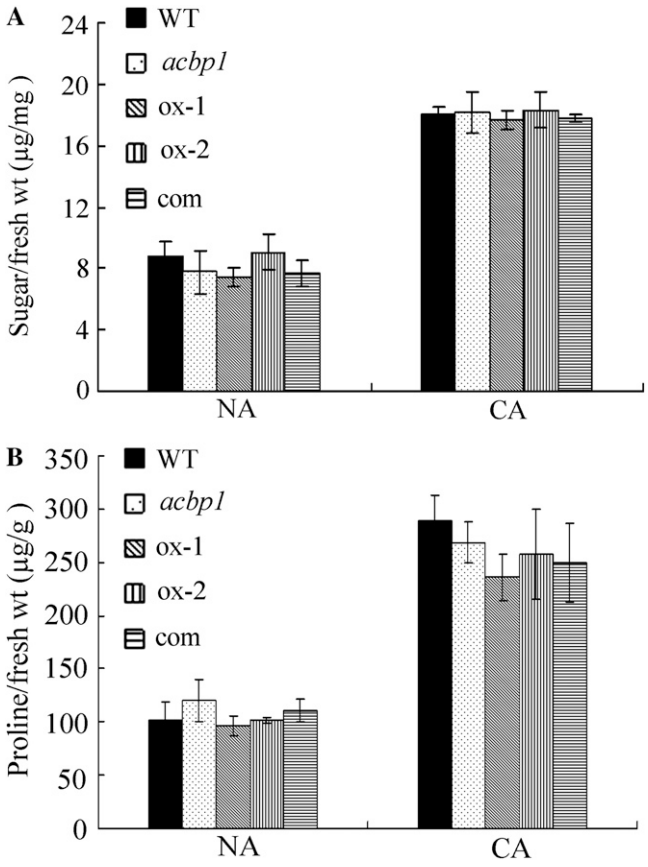

Figure 6. Changes in soluble sugar (A) and Pro (B) after cold acclimation of wild-type (WT), *acbp1* mutant, ACBP1-complemented (com), and ACBP1-overexpressing (ox-1 and ox-2) plants. Values are means ± SD ($n = 3$).

His_6-ACBP1 Binds PA in Vitro

To explore the interaction between ACBP1 and the phospholipids (PLs) PA and PC, which influence freezing tolerance, we tested the binding of purified recombinant ACBP1 (Chye, 1998) to various lipids. We have previously shown using similar filter-binding assays that recombinant ACBP6 binds PC in vitro (Chen et al., 2008). When we used a protein-lipid overlay assay of commercially available membrane lipid strips, recombinant ACBP1 (rACBP1) was observed to specifically bind PA but not other lipids, including diacylglycerol, phosphatidylserine (PS), PE, PC, PG, phosphatidylinositol (PI), phosphatidylinositol 4-phosphate, phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol 3,4,5-trisphosphate, and 3-sulfogalactosylceramide, which were simultaneously tested (Fig. 7, A and B).

To examine the significance of the acyl-CoA-binding domain of ACBP1 in mediating PA binding, a 20-kD His-tagged fusion protein lacking the ACBP domain (rACBP1ΔACB) was expressed, purified from *Escherichia coli* (data not shown), and tested using the protein-lipid binding assay. As shown in Figure 7B, deletion of the acyl-CoA-binding domain in ACBP1 abolished PA binding. Furthermore, results from blots containing serial dilutions of PA (16:0) indicated that rACBP1 binds PA in a dose-dependent manner (Fig. 7C). As the PL species used in Figure 7, A and B, were 16:0, the binding of other fatty acid species of PA to rACBP1 was subsequently tested. Results indicated that rACBP1 binds all species of PA (16:0-PA, 18:0-PA, and 18:1-PA) tested.

DISCUSSION

Upon freezing, the plasma membrane of the plant cell is most susceptible to injury (Steponkus, 1984; Uemura et al., 2006). Its destabilization and disruption is believed to be the primary cause of freezing injury in plants (Steponkus, 1984). Hence, stabilization of plant membranes against cellular dehydration would alleviate such injury. Plants have developed an innate ability to combat freezing after exposure to low non-freezing temperature, and this is defined as cold acclimation (Thomashow, 1999). During cold acclimation in rye (*Secale cereale*), the plasma membrane increases cryostability by reducing the formation of nonlamellar phase (like H_{II} phase) lipids, which prevent expansion-induced lysis (Uemura and Steponkus, 1994). This is accompanied by changes in lipid

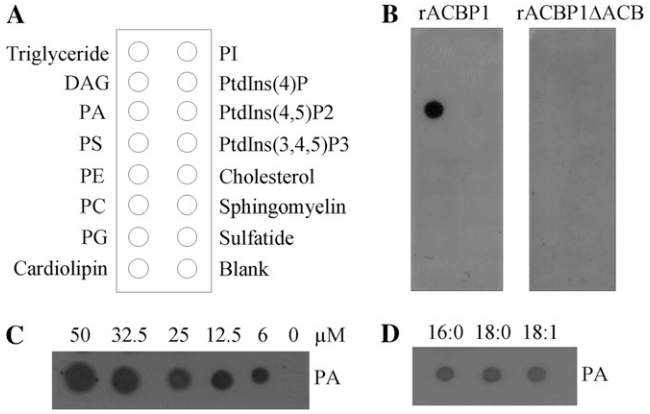

Figure 7. Interaction of recombinant ACBP1 with PA. A, Diagram of lipid species on membrane lipid strips (Echelon Biosciences). B, Binding of full-length (rACBP1) and deletion mutant (rACBP1ΔACB) ACBP1 to lipids on membrane lipid strips in A. The strips were incubated with 1 μg mL^{-1} purified rACBP1 or rACBP1ΔACB protein, and their binding was detected by immunoblotting with HRP-conjugated anti-penta-His antibodies. C, rACBP1/PA binding on filters. Serial concentrations (0, 6, 12.5, 25.0, 32.5, and 50 μM) of 16:0-PA were spotted onto nitrocellulose and incubated with 1 μg mL^{-1} purified rACBP1 protein. The rACBP1/PA binding was detected by immunoblotting with HRP-conjugated anti-penta-His antibodies. D, Effect of PA acyl species on rACBP1/PA binding. Twenty micromolar lipids (16:0-PA, 18:0-PA, and18:1-PA) spotted onto nitrocellulose were incubated with 1 μg mL^{-1} purified rACBP1 protein. The rACBP1/PA binding was detected by immunoblotting with HRP-conjugated anti-penta-His antibodies. DAG, Diacylglycerol; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PtdIns (3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; sulfatide, 3-sulfogalactosylceramide.

composition that modulate membrane stabilization (Thomashow, 1999). Increases in the proportion of PL in the plasma membrane during cold acclimation are commonly observed in various herbaceous and woody species, such as rye (Uemura and Steponkus, 1994), oat (*Avena sativa*; Uemura and Steponkus, 1994), seashore paspalum (*Paspalum vaginatum*; Cyril et al., 2002), Arabidopsis (Uemura et al., 1995; Welti et al., 2002), and mulberry (*Morus bombycis*; Yoshida, 1984). Under low-temperature stress, an increase in the ratio of unsaturated PL is a crucial factor in maintaining the biological functions of membranes (Nishida and Murata, 1996). Some studies have shown that an increase in unsaturated PG enhanced cold tolerance in transgenic tobacco (Ishizaki-Nishizawa et al., 1996; Sakamoto et al., 2003) and rice (*Oryza sativa*; Ariizumi et al., 2002), while a decrease in unsaturated PG impaired the recovery of photosynthesis after low-temperature photoinhibition in transgenic tobacco (Moon et al., 1995; Maréchal et al., 1997). Other than PL increases during cold acclimation, an accumulation of diunsaturated PC species may lead to a decreased propensity for freeze-induced formation of the nonlamellar phase and minimize the incidence of expansion-induced lysis (Uemura and Steponkus, 1994).

In this study, lipid analysis revealed that the freezing-tolerant *acbp1* mutant accumulated more diunsaturated PC (34:4 PC, 36:5 PC, 36:4 PC, 38:5 PC, 38:4 PC, and 38:3 PC) following freezing treatment, while ACBP1-overexpressing and ACBP1-complemented plants displayed reductions in diunsaturated PC (34:4 PC, 36:5 PC, 36:4 PC, and 38:4 PC). Such differences in diunsaturated PC content may provide a plausible explanation for the varied response to freezing treatment in the *acbp1* mutant, ACBP1 overexpressors, and ACBP1-complemented plants. In addition, after treatment, varying levels of diunsaturated PC in these three genotypes corresponded to distinct differences in the transcription of *PLDα1*. PLDα1 is the most abundant *PLD* gene product in plants and accounts for approximately half of the hydrolysis of PC to PA and more than half of the PA generated under freezing stress (Welti et al., 2002; Li et al., 2008b). PC is believed to be a major substrate for PLDα1 during freezing stress, and in vitro experiments have confirmed that PC is preferentially hydrolyzed in comparison with PE (Pappan et al., 1998; Welti et al., 2002). Northern-blot analysis revealed that the expression of *PLDα1* was down-regulated in the *acbp1* mutant at CA and freezing stages, while ACBP1 overexpressors showed enhanced *PLDα1* expression at the same stages. Correspondingly, after cold acclimation followed by freezing treatment, *acbp1* mutant plants accumulated more PC and less PA than wild-type plants in comparison with reduced PC and elevated PA in ACBP1 overexpressors (ox-1 and ox-2). Interestingly, previous studies of *PLDα1*-deficient Arabidopsis suggest that the suppression of *PLDα1* resulted in PC increase and PA decrease, similar to what was observed in the *acbp1* mutant plants (Welti et al., 2002; Li et al., 2008b). Under freezing stress, *PLDα1*-deficient plants were more tolerant due to a reduced ratio of PA to PC (Welti et al., 2002; Li et al., 2008b). This low ratio of PA to PC likely enhances membrane stability and improves freezing tolerance (Welti et al., 2002; Li et al., 2008b). Similarly, a low ratio of PA to PC in *acbp1* mutant plants may possibly be attributed to enhanced freezing tolerance. In contrast, ACBP1-overexpressing and ACBP1-complemented plants with a high ratio of PA to PC were observed to be more sensitive to freezing.

Besides PLDα1, PLDδ is another phospholipase involved in mediating the plant freezing response, as demonstrated in the *PLDδ* knockout mutant and *PLDδ* overexpressors in Arabidopsis (Li et al., 2004). In our earlier study, ACBP6-overexpressing Arabidopsis attained freezing tolerance through the increased expression of *PLDδ*, while the *acbp6* mutant was freezing sensitive due to a decrease in *PLDδ* expression (Chen et al., 2008). Other reports suggest that PLDδ contributes to approximately 20% of total PA following freezing, which would promote nonlamellar phase membrane lipids and inhibit the function of phospholipase A (Li et al., 2004, 2008b). In addition, the activation of PLD may cause the reorganization of microtubules (Dhonukshe et al., 2003). Thus, PLDδ

may contribute to membrane stabilization through its ability to bind tubulin (Gardiner et al., 2001; Li et al., 2004, 2008b). Results from this study support a role for PLDδ in mediating freezing tolerance in the *acbp1* mutant and in freezing sensitivity in ACBP1 overexpressors.

Furthermore, in vitro filter-binding assays on the binding of His-tagged ACBP1 to PL showed that it binds PA. ACBP1 is localized at the plasma membrane and ER (Chye, 1998; Chye et al., 1999; Li and Chye, 2003), while PLDα1 is also associated with the plasma and intracellular membranes (Fan et al., 1999). Similarity in their membrane localization suggests that ACBP1 could maintain a membrane-associated PA pool resulting from the PLDα1-promoted hydrolysis of PC to PA. Previous studies have shown that PA is a considerable negative curvature and fusigenic lipid (Kooijman et al., 2005). It has also been suggested that freeze-induced PA species may harm cell membranes by promoting the formation of H_{II} phase due to the cone-like molecular shape of PA (Li et al., 2008b). Interaction of ACBP1 with membrane-associated PA may possibly promote the formation of the nonlamellar phase, which could result in a reduction in membrane stability and freezing sensitivity.

It has been reported that the yeast 10-kD ACBP can regulate the expression of genes in stress responses involving catalase and heat shock proteins as well as those related to lipid metabolism, such as genes encoding OLE1 (stearoyl-CoA desaturase), INO1 (myoinositol-3-phosphate synthase), PSD1 (PS decarboxylase 1), OPI3 (methylene-fatty acyl-phospholipid synthase), and CHO2 (PE *N*-methyltransferase; Feddersen et al., 2007). It has been suggested that the yeast ACBP-acyl-CoA ester complex modulates gene expression directly or indirectly by the donation of acyl-CoA esters (Feddersen et al., 2007). Thus, ACBP1 could possibly resemble the yeast 10-kD ACBP in regulating gene expression. Furthermore, we have previously suggested that ACBP1 may be able to transfer acyl-CoA esters from the ER to the plasma membrane to form a membrane-associated acyl-CoA pool (Chye, 1998; Chye et al., 1999; Li and Chye, 2003). Possibly, together with the PA-binding ability of ACBP1, the expression levels of *PLDα1* and *PLDδ* could be regulated by the sequestering action of PA or acyl-CoAs maintained by ACBP1.

Our previous study indicated that the overexpression of cytosolic ACBP6 enhances freezing tolerance in Arabidopsis by the up-regulation of *PLDδ* (Chen et al., 2008) together with an increased accumulation of Glc and Pro (Q.-F. Chen and M.-L. Chye, unpublished data). In this study, we further investigated the role of an Arabidopsis membrane-associated ACBP, ACBP1, in the freezing response. Our data from ion leakage experiments suggest that the key point in acquiring freezing tolerance in the *acbp1* mutant is possibly related to its enhanced membrane stability. In contrast to ACBP6, a mutation in ACBP1 suppressed *PLDα1* expression and the hydrolysis of PC to PA, possibly protecting the plasma membrane. In the *acbp1* mutant, *PLDδ* expression is up-regulated, which may then enhance freezing tolerance by its positive role in stabilizing membranes under freezing stress. The differences in the regulation of *PLDα1* and *PLDδ* between ACBP1 and ACBP6 may be due to their differences in lipid binding and subcellular localization. Additionally, the ACBP1-mediated freezing response is independent of osmolyte accumulation, in contrast to ACBP6. Furthermore, PA, to which recombinant ACBP1 binds, as shown in this study, is known to be an important stress-signaling lipid in plants (Testerink and Munnik, 2005). Also, membrane-associated ACBP1 possesses C-terminal ankyrin repeats that may interact with protein partners, as has been demonstrated with ACBP2 (Li and Chye, 2004; Gao et al., 2008), which is highly conserved to ACBP1. Hence, ACBP1 could be involved in other plant stress responses besides freezing stress (as has been shown herein) and heavy metal tolerance (as has been demonstrated previously; Xiao et al., 2008a).

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Treatments

The *acbp1* knockout mutant, ACBP1 overexpressors, and ACBP1-complemented plants were generated from Arabidopsis (*Arabidopsis thaliana* ecotype Columbia [Col-0]) in an earlier study (Xiao et al., 2008a). The loss, and the overexpression and complementation, of ACBP1 were confirmed by northern- and western-blot analyses as reported by Xiao et al. (2008a). As described previously (Xiao et al., 2008a), ACBP1-overexpressing transgenic lines were generated by *Agrobacterium tumefaciens*-mediated transformation. Three independent T2 transgenic lines were observed to overexpress *ACBP1* mRNA. Two lines (ox-1 and ox-2) in the T2 population showed an approximately 3:1 (resistant:sensitive) segregation ratio on selective medium, indicating the presence of a single copy of the *35S::ACBP1* transgene. These two lines were subsequently confirmed to accumulate the 37-kD ACBP1 protein by western-blot analysis (Xiao et al., 2008a). T4 transgenic plants from these two lines were used in this study. Northern-blot analysis revealed that these two T4 lines were stable after several generations (Fig. 5B). Arabidopsis seeds were surface sterilized and planted on MS medium (Murashige and Skoog, 1962) containing 2% Suc. 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. Plants were also grown in soil under the same conditions.

NA plants or seedlings were grown in the growth chamber under 16-h-light (23°C)/8-h-dark (21°C) cycles until treatment. For cold acclimation, 5-week-old plants in soil pots and 11-d-old seedlings on agar plates were transferred from the growth chamber to a 4°C cold room under white light for 3 d prior to treatment or harvest.

For freezing treatment, NA and CA plants or seedlings were transferred to a growth chamber (Watlow series 942) and subjected to a temperature drop from 4°C to −2°C at 2°C h^{-1}. The temperature was held at −2°C for 2 h, and ice crystals were placed on the soil or plates to induce crystallization and prevent supercooling. Subsequently, the temperature was lowered to −12°C at 2°C h^{-1}. After 1 h at the final temperature, the plants or seedlings were thawed at 4°C overnight. The plants were photographed after recovery for 7 d under the normal growth conditions described above.

Lipid Profiling

Lipid extraction was performed following the protocol provided by the Kansas Lipidomics Research Center. After CA treatment for 3 d at 4°C, 5-week-old plants were frozen at −8°C for 2 h, and rosettes from three plants were harvested. NA plants were incubated in a growth chamber at 23°C until rosettes were harvested and transferred to 3 mL of isopropanol containing 0.01% butylated hydroxytoluene at 75°C for 15 min, after which 1.5 mL of

chloroform and 0.6 mL of water were added. Tubes were then shaken for 1 h, and the extract was moved for lipid analysis. The remaining tissue was subject to reextraction with chloroform:methanol (2:1) with 0.01% butylated hydroxytoluene four to five times, each with 30 min of agitation, until the tissue became completely white. The remaining plant tissue was incubated overnight at 105°C and subsequently weighed to obtain the dry weight. Finally, the combined extracts were washed with 1 mL of 1 M KCl followed by 2 mL of water. The solvent was evaporated under nitrogen, and samples were mailed by courier for lipid profiling at the Kansas Lipidomics Research Center.

Electrolyte Leakage

To measure ionic leakage, Arabidopsis rosettes from NA and CA plants were collected after freezing at the indicated temperature for 1 h and then incubated at 4°C for 24 h. Deionized water was added and gently agitated at 23°C for 1 h. Ionic leakage of the solution was measured using a conductivity meter (YSI model 55). Total ionic strength was determined after heating the solution in a 100°C water bath for 10 min and cooling to 23°C as described previously (Welti et al., 2002).

RNA Gel-Blot Analysis

Total RNA was prepared from rosettes of NA or CA 5-week-old plants collected in liquid nitrogen at the indicated times following treatment using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Twenty micrograms of total RNA per sample was separated on a 1.5% agarose gel containing 6% formaldehyde. RNA was transferred to Hybond-N membranes (Amersham) by capillary action. To generate probes, the PCR Digoxigenin Probe Synthesis Kit was used following the manufacturer's instructions (Roche).

The gene-specific primers used were ML179 (5′-AATCTTTGGTTTGATCTTCGC-3′) and ML759 (5′-GTCTACAATTGGAATCCTTCTTCTC-3′) for *ACBP1*, ML882 (5′-CAGAGACCAACAAGAATGCC-3′) and ML883 (5′-CGTAGTACATCTAAAGGGAG-3′) for *COR6.6*, ML884 (5′-CAAGATTACTCTGCTAGAGGAGC-3′) and ML885 (5′-GTATACGATGAGTGTTATGGG-3′) for *COR47*, ML921 (5′-TATGCGACGATTGATCTGCA-3′) and ML922 (5′-CTGAGAGCCTGAATCACATC-3′) for *PLDα1*, and ML923 (5′-AGCGACTCTAGCTCGAACAC-3′) and ML924 (5′-CAAGCATAAGAAGAACCCAG-3′) for *PLDδ*. Northern-blot analysis was performed using the Digoxigenin Nucleic Acid Detection Kit (Roche) according to standard procedures.

Sugar and Pro Measurements

Sugar measurements were carried out according to Li et al. (2004). Rosettes from NA or CA 5-week-old plants were harvested, weighed, and ground to powder in liquid nitrogen and then incubated in 75% ethanol overnight with gentle shaking. After centrifugation at 20,000*g*, 20 μL of each extract was incubated with 1,000 μL of anthrone reagents (0.15% [w/v] anthrone, 72% [v/v] H_2SO_4, and 28% [v/v] water) at 100°C for 1 h. The soluble sugar value was expressed as Glc equivalents and measured at 625 nm. Pro measurements were carried out according to a previously described method (Bates et al., 1973; Zhao et al., 2009b). Harvested rosettes from 5-week-old plants were weighed and subjected to extraction using 3% sulfosalicylic acid. After filtration, 2 mL of each filtrate was incubated with 2 mL of glacial acetic acid and 2 mL of acid ninhydrin reagent (2.5% [w/v] ninhydrin, 60% [v/v] glacial acetic acid, and 40% [v/v] 6 M phosphoric acid) at 100°C for 1 h. The reaction was terminated in an ice bath. Toluene (4 mL) was added into the extractions followed by vigorous shaking for 20 s. After incubation at 23°C for 24 h, A_{520} was measured to obtain values of Pro.

Purification of Recombinant Proteins rACBP1 and rACBP1ΔACB for Protein-Lipid Binding Assays

Expression and purification of His-tagged ACBP1 recombinant protein rACBP1 were performed according to Chye (1998). To construct the plasmid for rACBP1ΔACB expression, a 4.5-kb *Pst*I-*Pst*I fragment from pAT61 (*ACBP1* coding sequence without the transmembrane domain in pRSET B vector; Chye, 1998) was recovered and self-ligated to generate plasmid pAT473. Purification of rACBP1ΔACB was carried out under native conditions as described (Chye, 1998). The protein-lipid overlay assay for rACBP1 and rACBP1ΔACB proteins on membrane lipid strips (Echelon Biosciences; catalog no. P-6002) was performed according to the manufacturer's instructions. His_6-ACBP1 was used to test the binding of various lipids on filters following Zhang et al. (2004) with minor modifications. Various concentrations of lipids were spotted on nitrocellulose membranes followed by incubation at room temperature for 1 h in the dark. The lipid-bound filter was blocked in Tris-buffered saline (TBS) with 1% nonfat milk for 1 h and incubated with 1 μg mL^{-1} purified His_6-ACBP1 protein in blocking buffer for 2 h. The filter was then gently washed three times, each for 10 min, with TTBS (TBS plus 0.1% Tween 20). After incubation with the horseradish peroxidase (HRP)-conjugated anti-His_6 antibodies (1:2,000; Qiagen) for 1 h at room temperature, the filter was again washed three times with TTBS, each for 10 min, and subjected to detection using the ECL Western-Blotting Detection Kit (Amersham) following the manufacturer's protocols.

Sequence data from this article are available in the GenBank/EMBL data libraries under accession numbers NM_124726 (*ACBP1*), NM_121602 (*COR6.6*), NM_101894 (*COR47*), NM_112443 (*PLDα1*), and NM_119745 (*PLDδ*).

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