Arabidopsis acyl-CoA-binding protein ACBP3 participates in plant response to hypoxia by modulating very-long-chain fatty acid metabolism

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SUMMARY

In Arabidopsis thaliana, acyl-CoA-binding proteins (ACBPs) are encoded by a family of six genes (ACBP1 to ACBP6), and are essential for diverse cellular activities. Recent investigations suggest that the membrane-anchored ACBPs are involved in oxygen sensing by sequestration of group VII ethylene-responsive factors under normoxia. Here, we demonstrate the involvement of Arabidopsis ACBP3 in hypoxic tolerance. ACBP3 transcription was remarkably induced following submergence under both dark (DS) and light (LS) conditions. ACBP3-overexpressors (ACBP3-OEs) showed hypersensitivity to DS, LS and ethanolic stresses, with reduced transcription of hypoxia-responsive genes as well as accumulation of hydrogen peroxide in the rosettes. In contrast, suppression of ACBP3 in ACBP3-KOs enhanced plant tolerance to DS, LS and ethanol treatments. By analyses of double combinations of OE-1 with npr1-5, coi1-2, ein3-1 as well as ctr1-1 mutants, we observed that the attenuated hypoxic tolerance in ACBP3-OEs was dependent on NPR1- and CTR1-mediated signaling pathways. Lipid profiling revealed that both the total amounts and very-long-chain species of phosphatidylserine (C42:2- and C42:3-PS) and glucosylinositolphosphorylceramides (C22:0-, C22:1-, C24:0-, C24:1-, and C26:1-GIPC) were significantly lower in ACBP3-OEs but increased in ACBP3-KOs upon LS exposure. By microscale thermophoresis analysis, the recombinant ACBP3 protein bound VLC acyl-CoA esters with high affinities in vitro. Further, a knockout mutant of MYB30, a master regulator of very-long-chain fatty acid (VLCFA) biosynthesis, exhibited enhanced sensitivities to LS and ethanolic stresses, phenotypes that were ameliorated by ACBP3-RNAi. Taken together, these findings suggest that Arabidopsis ACBP3 participates in plant response to hypoxia by modulating VLCFA metabolism.

Keywords: acyl-CoA-binding proteins, Arabidopsis thaliana, hypoxic tolerance, sphingolipids, very-long-chain fatty acids.

INTRODUCTION

Flooding is one of the most important abiotic stresses of worldwide concern that determines crop productivity, geographic distribution of plant species and abundance of natural ecosystems (Perata and Voesenek, 2007). Flooding events including root waterlogging and submergence markedly affect diffusion of gases into plant cells, which eventually leads to hypoxia, and carbohydrate shortages in terrestrial plants (Geigenberger, 2003; Bailey-Serres and Voesenek, 2008, 2010; Voesenek and Bailey-Serres, 2013). In response to flooding, plants develop aerenchyma and adventitious roots to facilitate gas exchange, or shift from aerobic to anaerobic metabolism to sustain survival (Bailey-Serres and Voesenek, 2008). With increasing anaerobic respiration, the accumulation of toxic metabolites such as lactic acid, acetaldehyde and ethanol cause damage to plant cells. Moreover, the accumulation of reactive oxygen species (ROS) during re-oxygenation upon subsiding of floodwaters can cause further injury to plant tissues (Bailey-Serres and Voesenek, 2008; Voesenek and Bailey-Serres, 2013).

In recent years, considerable progress has been made in understanding how plants sense and respond to hypoxic stress. For example, many hypoxia-responsive genes and related microRNAs have been identified in Arabidopsis...
and Zea mays through genomic technologies (Chang et al., 2000; Klok et al., 2002; Licausi et al., 2011b; Mithran et al., 2013). Phenotypic analyses of attenuated or enhanced hypoxic tolerant mutants and transgenic lines demonstrated that four members of the group VII ethylene response transcription factors (ERFs), including ERF3/HE1, ERF71/HE2, RAP2.2, and RAP2.12, are positive regulators of the hypoxia response in Arabidopsis (Hinz et al., 2010; Licausi et al., 2010, 2011b; Yang et al., 2011). Moreover, homeostatic hypoxia sensing is tightly regulated by RAP2.12, via the N-end rule proteolysis pathway (Gibbs et al., 2011; Licausi et al., 2011a). In this model, under normoxic conditions, the RAP2.12 protein is subcellularly associated with the plasma membrane by interacting with two membrane-anchored acyl-CoA-binding proteins ACBP1 and ACBP2 (Licausi et al., 2011a; Bailey-Serres et al., 2012). Upon hypoxia, RAP2.12 is released and translocates to the nucleus to activate the transcription of hypoxia-responsive genes. During re-oxygenation following hypoxia, the RAP2.12 protein is rapidly ubiquitinated and proteolytically degraded thereby terminating hypoxia signaling (Licausi et al., 2011a; Bailey-Serres et al., 2012). Besides, ACBP2 and cytosolic ACBP4 proteins were found to interact with another member of group VII ERFs, RAP2.3/AtEBP (Li and Chye, 2004; Li et al., 2008), whose biological significance in hypoxia response is yet to be determined. It is therefore conceivable that ACBPs or ACB-associated lipids may play crucial roles in hypoxia signaling in plants.

Lipids are essential cellular constituents that not only provide the structural basis for cell membranes and energy for metabolic processes, but also serve as signals in plant responses to environmental cues (Wang, 2004). ACBP proteins are a family of lipid-binding proteins conserved in eukaryotic and prokaryotic organisms, which show ability to bind acyl-CoA esters and phospholipids via the acyl-CoA-binding (ACB) domain (Burton et al., 2005; Xiao and Chye, 2011a). In addition to the conventional 10-kDa cytosolic ACBPs, recent investigations have identified several larger proteins with the ACB domain from both mammals and plants (Xiao and Chye, 2009, 2011a; Fan et al., 2010; Yurchenko and Wesselake, 2011). In Arabidopsis, there are six genes encoding ACBP proteins with different subcellular localizations. Among them, the small 10-kDa ACBP is involved in phospholipid metabolism by interacting with phosphatidylcholine (PC), and its overexpression confers freezing tolerance in Arabidopsis (Chen et al., 2008). The five larger forms of ACBP (designated ACBP1 to ACBP5) range in size from 37.5 to 73.1 kDa and show distinct binding affinities to acyl-CoA esters and phospholipids (Xiao and Chye, 2009, 2011a; Du and Chye, 2013). More recently, the functions of these ACBP proteins have been intensely explored by reverse genetics and biochemical approaches (Xiao and Chye, 2011a). In particular, the existence of ankyrin repeats in ACBP1 and ACBP2, and a kelch motif present in ACBP4 and ACBP5, enables these proteins to interact with various protein partners (Du and Chye, 2013). To date, the identified targets for ACBP1 and ACBP2 include the ethylene-responsive factors RAP2.3/AtEBP and RAP2.12 (Li and Chye, 2004; Licausi et al., 2011a), heavy-metal-binding protein AtFP6 (Gao et al., 2009), lysophospholipase LysoPL2 (Gao et al., 2010), phospholipase PLD1 (Du et al., 2013) and ABA-responsive-element-binding protein1 AREB1 (Du and Chye, 2013). It is interesting to note that all these ACBP1 and/or ACBP2 interactors are stress-responsive proteins or transcription factors, which indicates that ACBPs are involved in stress tolerance by either binding to differential acyl-CoAs/lipids or combining with other protein interactors.

Arabidopsis ACBP3 is a unique protein that possesses an ACB domain and an overlapping N-terminal transmembrane domain with extracellular-targeting signal peptides, ensuring ACBP3 is directed to the extracellular region and within intracellular membranes (Xiao et al., 2010). Overexpression of ACBP3 accelerates age-dependent and starvation-triggered leaf senescence through binding to phosphatidylethanolamine (PE), an interaction that is confirmed to interfere with the PE lipidation of the autophagy-related protein ATG8 (Xiao et al., 2010). Moreover, ACBP3 overexpression constitutively activates the expression of pathogenesis-related (PR) genes, induces cell death and leads to accumulation of hydrogen peroxide (H2O2) and salicylic acid (SA) in the rosettes (Xiao and Chye, 2011b). Observations that ACBP3-overexpressors (ACBP3-OEs) display enhanced resistance to the hemi-biotrophic pathogens, but greater susceptibility to the necrotrophic pathogens (Xiao and Chye, 2011b), support a role for ACBP3 in SA-dependent plant defense signaling.

In this study, ACBP3 gene expression was remarkably induced by submergence under both dark (DS) and light (LS) conditions. ACBP3-OEs attenuated plant resistance, whereas down-regulation of ACBP3 (ACBP3-KOs) enhanced plant tolerance to DS, LS and ethanolic applications. Moreover, the phenotypes of ACBP3-OEs and ACBP3-KOs were correlated with dynamic changes of VLCFA-containing phospholipids and sphingolipids. In addition, recombinant ACBP3 (rACBP3) protein was shown to bind VLC acyl-CoA esters with high affinities in vitro. These findings suggest that ACBP3 is involved in plant response to hypoxic stress by modulating VLCFA metabolism.

RESULTS

The expression patterns of Arabidopsis ACBPs upon hypoxia exposure

To determine the involvement of Arabidopsis ACBPs in hypoxia response, 4-week-old plants were subjected to DS or darkness alone as a control (Dark), and the transcript
profiles of six ACBPs were determined by real-time quantitative reverse transcription PCR (qRT-PCR) analysis. The expression of ACBP3 was significantly induced but the levels of ACBP4 and ACBP5 were significantly repressed by exposing Arabidopsis plants to the extended darkness (Figure 1a), which is consistent with previous observations (Xiao and Chye, 2009; Xiao et al., 2010). Upon DS exposure, ACBP3 transcription was significantly elevated, with levels increasing 24.3- to 94.5-fold from 3 to 24 h (Figure 1a). Expression of ACBP6 was also slightly inducible by darkness and was further up-regulated at 6, 9 and 12 h after DS treatment. Both ACBP2 and ACBP5 mRNA levels were elevated at 6 h under DS. Whereas the level of ACBP2 had further increased, the level of ACBP5 had decreased at 12 h after treatment (Figure 1a). In contrast, the expression of ACBP1 and ACBP4 was not responsive to DS.

To uncouple the effects of darkness on the DS-affected expression of ACBPs, Arabidopsis plants were submerged under LS and the expression levels of ACBPs were examined. Consistently, ACBP3 was induced at 3, 6, 9, 12 and 24 h after LS treatment, with the highest expression

![Figure 1. Expression profiles of Arabidopsis ACBPs under submergence.](image)

Total RNA was extracted from 4-week-old soil-grown seedlings upon dark and DS (a), or LS (b) treatment. The samples were harvested at 0, 3, 6, 9, 12 or 24 h after treatment, and the relative expression levels of Arabidopsis ACBPs (ACBP1 to ACBP6) were determined by qRT-PCR. Expression levels relative to 0 h for each time point were normalized to that of ACTIN2. Data are means ± SD of three independent replicates. Asterisks indicate significant difference from untreated control (0 h); *P < 0.05; **P < 0.01 by Student’s t-test. The experiment was repeated with similar results.

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The Plant Journal published by Society for Experimental Biology and John Wiley & Sons Ltd., The Plant Journal, (2015), 81, 53–67
occurring at 3 h (Figure 1b). However, transcription of ACBP1, ACBP2, ACBP4, ACBP5 and ACBP6 was not significantly induced by LS (Figure 1b).

The DS- and LS-inducible expression of ACBP3 was further confirmed by subjecting ACBP3pro::GUS transgenic lines to dark, DS and LS treatments. Histochemical staining showed that GUS expression in ACBP3pro::GUS lines was clearly activated by DS and LS (Figure S1).

**Altered expression of ACBP3 changed plant tolerance to submergence**

To investigate the role of ACBP3 in hypoxia response, wild-type, ACBP3-OEs and ACBP3-KOs plants were used to determine their tolerance to hypoxic stress. The growth and development of 4-week-old ACBP3-OEs and ACBP3-KOs lines was indistinguishable from that of wild type (Figure 2a). When the plant lines were DS-treated for 2 days or LS-treated for 5 days plus a 3-day recovery period, two ACBP3-OEs (OE-1 and OE-4) showed increased sensitivity to submergence in comparison with wild type (Figure 2a, d). In contrast, when wild type and ACBP3-KOs (acbp3 and ACBP3-RNAi) were treated for 3 days under DS or for 7 days under LS, most ACBP3-KOs survived, whereas most wild-type plants died (Figure 2a, d), indicating that depletion of ACBP3 in ACBP3-KOs improved plant tolerance to submergence. The attenuated and enhanced hypoxic tolerance of ACBP3-OEs and ACBP3-KOs, respectively, was further confirmed by analyses of the survival rates (Figure 2b, e) and dry weights (Figure 2c,f) of DS- or LS-treated plants followed by a 3-day recovery. As shown in Figure 2, both survival rates and dry weights of ACBP3-OEs were significantly lower than wild type upon treatment with either 2-day DS or 5-day LS. In contrast, data of both ACBP3-KOs were significantly higher than wild type under the extended treatment with 3-day DS or 7-day LS (Figure 2).

Previously, we observed that ACBP3-overexpressors showed up-regulation of several defense genes and conferred enhanced resistance to bacterial pathogens (Xiao and Chye, 2011b). To explore whether the differential sensitivities of ACBP3-OEs and ACBP3-KOs are associated with the expression levels of hypoxia-responsive marker genes, the transcripts of ADH1, PDC1 and SUS1 were further determined by qRT-PCR in wild-type, OE-1 and acbp3 rosettes. As shown in Figure 3, all three transcripts were all significantly induced in wild-type seedlings by DS and LS exposure. Of note, the expression of ADH1 and SUS1 at 0, 1 and 3 h under DS were significantly down-regulated in OE-1 in comparison with...
wild type (Figure 3a). Similarly, in OE-1 plants, the expression of all three genes was up-regulated by 3-h LS but down-regulated by 6- and 24-h LS treatments, while the level of PDC1 was unchanged under 24-h LS (Figure 3b). In contrast, the expression of ADH1, PDC1 and SUS1 in the acbp3 mutant showed significant up-regulation following DS and LS treatments at the specific stages, i.e. ADH1 at 3- and 6-h LS, PDC1 at 3-h LS and DS, as well as SUS1 at 6- and 24-h LS (Figure 3). The reduced expression of hypoxia-responsive genes in the OE-1 line is seemingly correlated with an increased sensitivity to DS and LS stresses.

Accumulation of H$_2$O$_2$ in ACBP3-OEs under hypoxia

Given ACBP3-OEs accumulate high levels of H$_2$O$_2$ in rosettes after pathogen infection (Xiao and Chye, 2011b), we tested H$_2$O$_2$ levels in ACBP3-OEs and ACBP3-KOs by diaminobenzidine (DAB) staining upon LS or DS exposure. As shown in Figure 4, both LS (1-, 2- and 3-day) and DS (1-day) treatments triggered the production of H$_2$O$_2$, indicated by brown coloration, in wild-type rosettes; however, much higher levels of H$_2$O$_2$ were detected in the rosettes of LS- and DS-treated ACBP3-OEs. In contrast, the H$_2$O$_2$ signals in the rosettes of ACBP3-KOs were weaker than wild type at day 2 and day 3 after LS treatment (Figure 4).

The responses of ACBP3-OEs and ACBP3-KOs to ethanolic treatment

Our preliminary data suggested that exogenous application of ethanol, the end product of anaerobic respiration in plant cells (Bailey-Serres and Voesenek, 2008), could be used to mimic hypoxic stress under certain conditions. To further assess the role of ACBP3 in hypoxia response, wild type, ACBP3-OEs and ACBP3-KOs were subjected to two types of ethanolic stress. When 4-week-old plants were sprayed with 0.5% ethanol or water (control) and analyzed after 5 days, ACBP3-OEs showed greater sensitivity while ACBP3-KOs had increased tolerance to ethanol than wild type, as indicated by the extent of yellowing in the rosettes (Figure 5a). Chlorophyll measurements in wild type, ACBP3-OEs and ACBP3-KOs at 0 and 5 days after ethanol application, confirmed that relative chlorophyll contents had declined significantly in ACBP3-OEs, but had increased significantly in ACBP3-KOs, compared with wild type after 5 days (Figure 5b). When the seeds of wild type, ACBP3-OEs and ACBP3-KOs were germinated on Murashige and Skoog (MS) medium supplemented with 0, 50 or 75 mM ethanol for 2 weeks, the ACBP3-OEs and ACBP3-KOs seedlings displayed less or more ethanol tolerance, respectively, compared with wild type (Figure 5c). Data illustrated that the percentage of ACBP3-OEs with true leaves and...
green cotyledons was significantly lower than wild type on MS medium containing 50 mM ethanol (Figure 5d, upper graph), while that of ACBP3-KOs was significantly higher than wild type on MS medium supplemented with 75 mM ethanol (Figure 5d, lower graph).

Hypoxia hypersensitivity in OE-1 is dependent on NPR1 and CTR1

To investigate whether the hypoxia hypersensitive phenotype in ACBP3-OE lines relies on SA or jasmonate (JA)/ethylene (ET) responses, we generated OE-1 coi1-2, OE-1 npr1-5, and OE-1 ein3-1 double combinations, which suppressed the SA, JA and ET signaling pathways, respectively. From the phenotypic analyses of single mutants, npr1-5 plants displayed more tolerance, while the coi1-2 mutant showed increased sensitivity to both DS and LS, in comparison with wild type (Figure 6a). However, the ein3-1 mutant was not significantly different to wild type under either DS or LS (Figure 6b), possibly due to the functional redundancy of the EIL1 gene in Arabidopsis. Interestingly, the hypoxia-sensitive phenotype of OE-1 was significantly rescued by the npr1-5 mutation in the OE-1 npr1-5 double combination (Figure 6a). In contrast, DS and LS treatments revealed that the OE-1 coi1-2 and OE-1 ein3-1 double combinations were hypoxia sensitive, resembling the OE-1 phenotype (Figure 6a,b).

The OE-1 line was also crossed with the constitutive triple-response mutant ctr1-1(Kieber et al., 1993), to generate the OE-1 ctr1 double combination. Phenotypic analysis showed that upon either DS or LS exposure, the increased hypoxia sensitivity of OE-1 was partially restored in a ctr1-1 mutant background (Figure 6c). Together, these results suggest that the hypoxia-sensitive phenotype of OE-1 is dependent on NPR1 and CTR1, associated with SA and ET signaling, respectively.

Lipid profiling of wild type, ACBP3-OEs and ACBP3-KOs upon LS exposure

To investigate the potential role of ACBP3 in regulating lipid metabolism during hypoxia, lipid profiles of Arabidopsis rosettes following LS exposure were analyzed. Under normal growth conditions, few differences were detected between wild type and ACBP3-OEs or ACBP3-KOs except that the levels of phosphatidylserine (PS) increased significantly in ACBP3-OEs(Figure 7a). When specific lipid species were analyzed, the levels of C34:3-, C34:2-, C34:1-, C36:5-, C38:3-, C40:3- and C42:3-PS were significantly higher in both OE-1 and OE-4 lines compared with wild type (Figure 7b, upper graph). After a 4-day LS treatment, total phosphatidylinositol (PI), PS and phosphatidic acid (PA) levels were significantly elevated in wild-type rosettes, while the levels of other lipids remained unchanged (Figure 7a). In contrast, galactolipids, including digalactosyl-diacylglycerol (DGDG) and monogalactosyldiacylglycerol (MGDG), as well as phospholipids such as phosphatidylglycerol (PG), PC, PE and PI, declined in wild-type rosettes.
but PA levels showed significant elevation compared to the untreated control (Figure 7a). In particular, more significant changes were observed in the membrane lipid content between wild-type and ACBP3-OE rosettes after LS treatment for 4 days, and between wild-type and ACBP3-KOs rosettes after LS treatment for 6 days (Figure 7a). Specifically, total levels of MGDG, DGDG, PG, PC, PI, PS and PA were significantly lower in the rosettes of LS-treated wild type and untreated control compared to those of wild type under 4-day LS stress, while most of these lipid molecules were remarkably higher in the rosettes of ACBP3-OEs than that of 6-day LS-treated wild type (Figure 7a). These data are consistent with increased hypoxia sensitivity in ACBP3-OEs but increased tolerance in ACBP3-KOs.

Given the significant accumulation of PS in the rosettes of LS-treated wild type and untreated ACBP3-OEs, we further analyzed the lipid compositions of different PS species. As shown in Figure 7(b), the levels of unsaturated species such as C36:5-, C36:4-, C36:2-, C38:6-, C38:4-, C38:3-, C40:2-, C42:2- and C44:2-PS declined in both OE-1 and OE-4 lines, while those of C34:3-, C34:2-, C36:3-, C36:2-, C38:6-, C38:5-, C38:3-, C38:2-, C40:2-, C42:2-, C42:3-, C42:4- and C44:3- and C44:2-PS increased significantly in ACBP3-KOs. Among them, the levels of PS containing VLC species 42:3- and 42:2-PS were the most significantly affected species under LS treatment, which respectively decreased or increased more than two-fold in the rosettes of ACBP3-OEs and ACBP3-KOs (Figure 7b).

**Sphingolipid profile in rosettes of wild type, ACBP3-OEs and ACBP3-KOs upon hypoxia**

Given the significant changes of PS in ACBP3-OEs and ACBP3-KOs plants exposed to hypoxia, we measured the profiles of the two most abundant sphingolipid species, glucosylinositolphosphorylceramides (GIPCs) and glycosylceramides (GlcCers), as well as the simple ceramides (Cers), in wild type, ACBP3-OEs and ACBP3-KOs upon LS treatment. Under normal growth conditions, the levels of C22:0-GIPC, C18:0-, C20:0-, C22:0-GlcCer and C16:0-Cer were significantly higher in the rosettes of ACBP3-OEs compared with wild type and ACBP3-KOs compared with wild type, but few differences were observed between wild type and ACBP3-KOs (Figure 8a–c). Upon 4-day LS exposure, remarkable increases in the levels of most species of GIPCs, GlcCers and Cers were detected in the wild-type rosettes (Figure 8). Compared with the wild-type control, a significant decline in the levels of GIPC classes containing VLCFAs, such as C22:1-, C22:0-, C24:1-, C24:0-, C26:1- and C26:0-GIPC, occurred in ACBP3-OEs (Figure 8a). The levels were correspondingly increased in ACBP3-KOs after a 6-day LS treatment compared with wild type (Figure 8b).
No significant changes in the compositions of GlcCer were detected in any of the three genotypes upon LS exposure. In contrast, significant decreases of C22:1- and C24:1-Cer were detected in LS-treated OE-1 (Figure 8c). However, the levels of C16:0-Cer varied significantly, increasing 4.9-fold in the OE-1 and declining 2.3-fold in the acbp3 mutant in comparison with wild type after LS treatment (Figure 8c).

**ACBP3 recombinant protein binds VLC-acyl-CoAs in vitro**

The binding of rACBP3 to 20:4-CoA esters (Leung et al., 2006) may represent its ability to target VLC acyl-CoAs. To address this possibility, the rACBP3 protein was expressed and purified from Escherichia coli and its binding affinities to different VLC acyl-CoAs were determined by microscale thermophoresis (MST) analysis, which is a sensitive approach to measure protein-ligand interactions in vitro. Data presented in Figure 9 show that the dissociation constant ($K_d$) of rACBP3-18:2-CoA interaction was 0.788 μM in MST measurement, which is comparable with that of Lipidex assay (Xiao et al., 2010). Moreover, we found that rACBP3 bound VLC acyl-CoAs including 20:0-, 22:0- and 24:0-CoA with higher affinities than observed for 18:2-CoA, as reflected by the $K_d$ values at nmol levels (Figure 9). These results indicate that ACBP3 preferentially binds VLC acyl-CoAs in vitro.

**The VLCFA-mediated hypoxia response is controlled by MYB30**

The transcription factor MYB30 serves as a key regulator of VLCFA metabolism by regulating various genes encoding subunits of the acyl-CoA elongase complex (Raffaele et al., 2008). To evaluate the role of MYB30 in the potential regulation of hypoxia-induced VLCFA metabolism, the MYB30 knockout mutant myb30-1 (Zheng et al., 2012b) was obtained and the ACBP3-RNAi myb30 double combination was generated. As shown in Figure 10, the myb30-1 single mutant displayed hypersensitivity to both LS (Figure 10a) and ethanol (Figure 10b) treatments, indicating that MYB30 plays an essential role in moderating the hypoxia response. In contrast, the ACBP3-RNAi myb30 plants showed enhanced tolerance to both LS and ethanolic stresses, similar to that of the ACBP3-RNAi phenotype (Figure 10). These results, together with lipid profiling data showing increased accumulation of VLCFA-containing PS and GIPC in ACBP3-RNAi, suggest that ACBP3 acts downstream of MYB30 in VLCFA-mediated hypoxia signaling.

**DISCUSSION**

We have previously demonstrated that Arabidopsis ACBP3 is crucial for the promotion of leaf senescence and plant resistance to hemi-biotrophic pathogens (Xiao et al., 2010; Xiao and Chye, 2011b). Here, we present several lines of evidence to support a role for ACBP3 in the modulation of hypoxic tolerance, which is associated with the cellular homeostasis of VLCFA metabolism. First, rACBP3 protein bound to VLC acyl-CoAs esters with high affinities. Second, LS triggered a significant accumulation of VLC-enriched PS and GIPC compounds in rosettes, whose levels remarkably declined or increased in ACBP3-OEs and ACBP3-KOs, respectively, upon LS exposure. Third, deletion of Arabidopsis MYB30, the master regulator of VLCFA biosynthesis.
in response to pathogen attack (Raffaele et al., 2008), attenuated plant sensitivity to LS and ethanolic stresses. Moreover, ACBP3-RNAi ameliorated the myb30-1 hypoxia-sensitive phenotype. Therefore, our findings demonstrate a mechanism for the involvement of VLCFAs or VLCFA-derivatives in plant response to hypoxia.

In higher plants, most of the plastidial C16:0- and C18:0-fatty acyl-CoAs are exported to the endoplasmic reticulum (ER) membrane for either biosynthesis of membrane lipids by ER-resident enzymes, or assembly of VLCFAs by the fatty acid elongase complex (Ohlrogge and Browse, 1995; Li-Beisson et al., 2013). By their binding ability to different acyl-CoA esters, ACBPs are primarily deemed to maintain acyl-CoA pools and modulate lipid biosynthesis in vivo (Fan et al., 2010; Xiao and Chye, 2011a). Besides, ACBPs can also function in the delivery of acyl-CoAs to enzymes such as lysophosphatidic acid acyltransferase and glycerol-3-phosphate acyltransferase, both of which require acyl-CoAs as substrates for lipid biogenesis (Xiao and Chye, 2011a).

In this study, MST analysis showed that rACBP3 binds not only LC acyl-CoA esters (C18-CoA), which is consistent with previous findings (Leung et al., 2006; Xiao et al., 2010), but also to VLC acyl-CoA esters (C20:0-, C22:0- and C24:0-CoAs) with higher affinities (Figure 9). This suggests that rACBP3 is able to interact with C18-CoA and VLC...
acyl-CoA esters, and deliver them as potential substrates for lipid metabolism. In agreement with this result, a study using isothermal titration calorimetry (ITC) revealed that rACBP1 could also bind VLC acyl-CoA in vitro (Xue et al., 2014). Furthermore, investigations using T-DNA insertion mutants by two independent groups have recently demonstrated that depletion of either ACBP1 or ACBP3 impaired cuticle development in Arabidopsis stems and leaves, respectively (Xia et al., 2012; Xue et al., 2014). Thus, it is conceivable that Arabidopsis ACBP1 and ACBP3 play crucial roles in cuticle formation, possibly by shuttling VLC acyl-CoAs to elongases, and thereby influencing the subsequent cuticular lipid biosynthesis.

In addition to cuticular lipids, VLCFAs are known to be precursors for synthesis of sphingolipids, which originate in the ER by condensation of serine and palmitoyl-CoA to produce 3-ketosphinganine (Lynch and Dunn, 2004; Chen et al., 2009; Li-Beisson et al., 2013). Ceramides are assembled by acylating sphinganine to an acyl-CoA molecule and, in turn, serve as direct substrates for the generation of complex sphingolipids such as GlcCers and GIPCs, which are enriched in C16-fatty acids and VLCFAs.
respectively (Lynch and Dunn, 2004; Chen et al., 2009; Li-Beisson et al., 2013). Our lipidomic data further extend the significance of ACBP3 in modulating the biosynthesis of sphingolipids, particularly the classes of VLCFA-enriched GIPCs in Arabidopsis. The effect of ACBP3 in modulating the composition of sphingolipids resembles that of yeast ACB1, whose depletion in *Saccharomyces cerevisiae* led to dramatic reductions of C26:0-VLCFA and sphingolipids by 50–70% (Gaigg et al., 2001). However, evidence that yeast ACB1 binds to C16:0-CoA, but not VLC acyl-CoAs, suggests that ACB1 may function in yeast sphingolipid biosynthesis by shuttling and incorporating C16:0-CoA into d18:0 sphinganine for the production of LC-ceramides (Gaigg et al., 2001). Given that rACBP3 did not bind to C16:0-CoA (Leung et al., 2006), it may therefore function in the sphingolipid pathway in plants through a different way to that of yeast. Instead, its high affinity to VLC acyl-CoAs may contribute to controlling the incorporation of VLC acyl-CoAs into t18:0 sphinganine for production of VLC-ceramides as well as GIPCs. In agreement with this proposal, we observed that under normal growth conditions, the levels of C22:0-GIPC and C20:0- and C22:0-GlcCers were significantly increased in the ACBP3-OEs in comparison with wild type (Figure 8). In contrast, upon LS exposure, a significant decline in VLC-GIPCs (C22:0, C22:1, C24:0, C24:1, C26:0 and C26:1) was detected in the ACBP3-OEs, and vice versa in ACBP3-KOs (Figure 8), indicating that VLC-GIPCs are essential for activation of hypoxia response and for protecting Arabidopsis from hypoxic stress. Interestingly, we also observed that the levels of C16:0-Cer showed contrasting accumulation patterns in LS-treated ACBP3-OEs and ACBP3-KOs, with high accumulation occurring in ACBP3-OEs but a decline occurring in

Figure 9. rACBP3 protein binds VLC acyl-CoA esters in vitro. The interactions between rACBP3 and 18:2-, 20:0-, 22:0- and 24:0-acyl-CoA esters were determined by MST analyses. The dissociation constant (K_d) calculated for each binding assay is shown.

![Figure 9](image_url)

| Acyl-CoA Concentrations (μM) | Thermophoresis with jump |
|-----------------------------|-------------------------|
| 10^-1                      | 10^-2                  |
| 10^-3                      | 10^-4                  |
| 10^-5                      | 10^-6                  |

Figure 10. Regulation of ACBP3-mediated hypoxia response by MYB30.
(a) Phenotypes of 4-week-old WT, ACBP3-RNAi, RNAi myb30, and myb30-1 plants before treatment (day 0) and after 5-day LS treatment followed by 3 days’ recovery.
(b) Seeds of WT, ACBP3-RNAi, RNAi myb30, and myb30-1 were germinated on MS medium supplemented with 0 (MS) or 75 mM ethanol. Images were taken 2 weeks after germination.
ACBP3-KOs. The corresponding cell death phenotypes (Xiao and Chye, 2011b) and C16:0-Cer accumulation in ACBP3-OEs resembled the knockout mutant phenotype corresponding to LOH1, a ceramide synthase responsible for synthesis of VLC-Cers in Arabidopsis (Markham et al., 2011; Ternes et al., 2011), further confirming the potential function of ACBP3 in VLC-sphingolipid biogenesis.

The transcription factor MYB30 is a positive regulator in the activation of hypersensitive cell death (Vailleau et al., 2002) and abscisic acid response (Zheng et al., 2012b). MYB30 regulates genes encoding key components of the acyl-CoA elongase complex, and changes to MYB30 expression greatly influence accumulation patterns of VLCFA as well as VLCFA-derived metabolites including leaf wax and sphingolipids (Raffaele et al., 2008). This suggests that MYB30 is an upstream master regulator for the stress-triggered production of VLCFAs. Consistent with this proposal, our phenotypic data obtained from analyzing the responses of myb30-1 to submergence and ethanolic treatments, indicate that MYB30 is required for hypoxia response (Figure 10), and provides validation of our biochemical data. Many reports on plant defense signaling have revealed that VLCFAs and their derivatives GIPCs are essential components for mediating the SA response (Raffaele et al., 2008; Wang et al., 2008; Mortimer et al., 2013). For example, two Arabidopsis mutants eer1 and gosn1 that lack the inositol-phosphorylceramide synthase and the GDP-D-mannose transporter, respectively, have severely reduced GIPCs as well as SA-mediated programmed cell death in rosettes (Wang et al., 2008; Mortimer et al., 2013). Similarly, the SA dependence of ACBP3 in both plant defense and hypoxia responses has been established previously (Xiao and Chye, 2011b) and confirmed in the present study (Figure 6) by phenotypic analyses of the OE-1 npr1-5 double combination. Nonetheless, observations that defense signaling mutants such as npr1-5, coi1-2, ein3-1 and ctr1-1 of the SA and JA/ET pathways showed altered tolerance to hypoxia (Figure 6) suggest a link between the defense and hypoxia signaling pathways. Moreover, a recent investigation demonstrated that submergence strongly induces the transcription of a large number of defense genes in Arabidopsis (Hsu et al., 2013). By targeting genes involved in plant innate immunity, the transcription factor WRKY22 was shown to be a key regulator in protecting plants against pathogen infection during or after submergence (Hsu et al., 2013). Further investigations that determine how defense hormones control the plant response to hypoxic stress are required in order to deepen our understanding of the interaction between hypoxia and defense signaling.

Finally, we would like to propose the potential mechanism of ACBP3, VLCFAs and their derivatives in the plant response to hypoxia. Our results illustrate that the sensitivities of ACBP3-OEs and ACBP3-KOs to hypoxic stress were negatively correlated with the rosette H2O2 levels (Figure 4). The hypoxia-induced accumulation of H2O2 in ACBP3-OEs is consistent with the compositional alterations of Cer species in these lines (Figures 7 and 8), whose abnormal changes severely affect the SA-dependent H2O2 production in plant cells (Raffaele et al., 2008; Mortimer et al., 2013). In general, low levels of H2O2 act as an essential second messenger to transmit initial signals to activate plant stress responses. However, excessive accumulation of H2O2 can result in irreversible oxidative damages to cellular components and eventually lead to cell death (Apel and Hirt, 2004). Recent studies have also highlighted a direct link between hypoxia/anoxia responses and ROS signaling (Baxter-Burrell et al., 2002; Pucciariello et al., 2012; Yang, 2014). Particularly, the ROP-triggered elevation of H2O2 levels under anoxia was accompanied with an increase in the activity of ADH1 and enhanced plant tolerance to oxygen deprivation (Baxter-Burrell et al., 2002). Thus, it is most likely that the hypoxia-activation of ACBP3, VLCFAs as well as VLCFA-derivatives may enhance plant survival during hypoxic stress by modulating the cellular homeostasis of ROS. However, the over-production of H2O2 in the rosettes of ACBP3-OEs may contribute to the hypersensitivity to hypoxia observed here. Whereas in the ACBP3-KO lines, the moderate levels of H2O2 appear to improve plant tolerance to submergence.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Wild-type Arabidopsis used in this study was Columbia ecotype (Col-0). Characterizations of ACBP3-OEs (OE-1 and OE-4) and ACBP3-KOs (acbp3 and ACBP3-RNAi) and ACBP3pro:GUS lines have been previously described (Xiao et al., 2010; Zheng et al., 2012a). The double combinations of OE-1 coi1 and OE-1 npr1 were generated following Xiao et al. (2010). OE-1 ein3 and OE-1 ctr1 combinations were generated by crossing OE-1 with ein3-1 (Chao et al., 1997) and ctr1-1 (Kieber et al., 1993). ACBP3-RNAi myb30 was generated by crossing ACBP3-RNAi with myb30-1 (SALK_122884; Zheng et al., 2012b). Seedlings displaying insensitivity to ethylene (Chao et al., 1997) and constitutive triple-response phenotypes (Kieber et al., 1993) were deemed to be homozygous for ein3-1 and ctr1-1, respectively. The T-DNA insertion in the myb30-1 line was identified by PCR (Zheng et al., 2012b).

All Arabidopsis seeds were surface-sterilized with 20% bleach containing 0.1% Tween-20 for 20 min, and then washed five times with sterile water. Seeds were sown on MS medium (Murashige and Skoog, 1962), followed by cold treatment for 3 days. After germination for 7 days, seedlings were transplanted to soil and grown in a plant growth room under 16-h light/8-h dark cycle at 22°C.

Hypoxic treatments

Hypoxic treatments were carried out following Licausi et al. (2011a). Briefly, 4-week-old plants were submerged under LS and DS or darkness as a control. Rosettes were submerged 10 cm
below the water surface. Plant samples were collected or photographed at the indicated times. Survival rates and dry weights were scored after treatment following recovery for 3 days. Data presented in this study are means ± standard deviation (SD) (n = 3) of three independent experiments (for one experiment, >15 plants were scored for each genotype).

For ethanolic spraying assays, 4-week-old plants were sprayed with 0.5% (v/v) ethanol and covered to maintain high humidity for 5 days. Samples were collected after treatment and chlorophyll contents were measured according to Xiao et al. (2010). For germination assays, surface-sterilized seeds were sown on MS solid medium with 0, 50 or 75 mM ethanol, followed by cold treatment for 2 days, and grown under normal growth conditions for 2 weeks. The experiment was repeated with similar results.

**RNA extraction and qRT-PCR analysis**

RNA extraction and qRT-PCR analysis were performed as described previously (Xiao and Chye, 2011b). The isolated RNA was reverse transcribed using the PrimeScript™ RT reagent Kit (TaKaRa, http://www.takara-bio.com/) following the manufacturer’s instructions. qRT-PCR was analyzed using SYBR Green (TaKaRa, http://www.takara-bio.com/) following the manufacturer’s instructions for 2 weeks. The experiment was repeated with similar results.

**DAB staining**

DAB staining was performed as described previously (Xiao and Chye, 2011b). Rosettes were excised and placed in 1 mg ml⁻¹ DAB staining solution (pH 3.8) for 3 h at room temperature under darkness and subsequently cleared in 96% boiling ethanol for 10 min. After cooling, the leaves were retained in ethanol and photographed.

**Measurements of polar membrane lipids and sphingolipids**

For membrane lipid analysis, the total plant lipids were extracted following Welti et al. (2002). The profiles of membrane lipids were determined by electrospray ionization-tandem mass spectrometry (ESI-MS/MS) as previously described (Devaiah et al., 2006; Xiao et al., 2010). Data showed in this study are means ± SD of four independent samples and each sample was pooled from the rosettes of three plants.

Extraction of sphingolipids was carried out according to Markham et al. (2006) using 100 mg tissue samples. Briefly, an 8-ml isopropanol/n-hexane/water solvent (50/20/25; v/v/v) was added to the freeze-dried samples, and the mixture was fully homogenized using a glass homogenizer. The extract was transferred to a glass tube, and C17 LCB, C12 Ceramide, C12 glucosylceramide (Avanti, http://www.avantilipids.com/) were added as internal standards, using a glass homogenizer. The extract was transferred to a fresh tube and the pellet was extracted twice with 0.5% (v/v) ethanol and covered to maintain high humidity for 10 min. After cooling, the leaves were retained in ethanol and photographed.

**ACKNOWLEDGEMENTS**

We thank the ABRC for provision of ein3-1, ctr1-1 and npr1-5 mutant seeds, Y. Guo (Chinese Agricultural University) for myb30-1 seeds, D.X. Xie (Tsinghua University) for the coil1-2 seeds and M. L. Chye (University of Hong Kong) for the ACPB3-OEs, ACPB3-KOs and ACPB3pro::GUS lines. We also thank M. Roth and R. Welti (Kansas Lipidomics Research Center, USA) for membrane lipid profiling. This work was supported by the National Natural Science Foundation of China (Projects 31461143001, 31370298 and 31202020), Program for New Century Excellent Talents in University (Project NCET-13-0614), the Key Project of Department of Education of Guangdong Province (Project 2012KZD0003), the Fundamental Research Funds for the Central Universities (Project 121gdz02) and Sun Yat-sen University (Start-up fund to S.X.).

**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** Expression patterns of ACPB3pro::GUS upon DS and LS treatments.

**Table S1.** Sequence of gene-specific primers used for qRT-PCR.
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