Overexpression of Arabidopsis ACBP3 Enhances NPR1-Dependent Plant Resistance to Pseudomonas syringe pv tomato DC3000

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ACBP3 is one of six Arabidopsis (Arabidopsis thaliana) genes, designated ACBP1 to ACBP6, that encode acyl-coenzyme A (CoA)-binding proteins (ACBPs). These ACBPs bind long-chain acyl-CoA esters and phospholipids and are involved in diverse cellular functions, including acyl-CoA homeostasis, development, and stress tolerance. Recombinant ACBP binds polyunsaturated acyl-CoA esters and phospholipids in vitro. Here, we show that ACBP3 plays a role in the plant defense response to the bacterial pathogen Pseudomonas syringe pv tomato DC3000. ACBP3 mRNA was up-regulated upon pathogen infection and treatments using pathogen elicitors and defense-related phytohormones. Transgenic Arabidopsis ACBP3 overexpressors (ACBP3-OEs) showed constitutive expression of pathogenesis-related genes (PR1, PR2, and PR5), cell death, and hydrogen peroxide accumulation in leaves. Consequently, ACBP3-OE displayed enhanced resistance to the bacterial pathogen P. syringe pv tomato DC3000. In contrast, the acbp3 T-DNA insertional mutant was more susceptible and exhibited lower PR gene transcript levels upon infection. Using the ACBP3 OE-1 line in combination with nonexpressor of PR genes1 (npr1-5) or coronatine-insensitive1 (coi1-2), we concluded that the enhanced PR gene expression and P. syringe pv tomato DC3000 resistance in the ACBP3-OEs are dependent on the NPR1-mediated, but not the COI1-mediated, signaling pathway. Given that ACBP3-OEs showed greater susceptibility to infection by the necrotrophic fungus Botrytis cinerea while the acbp3 mutant was less susceptible, we suggest that ACBP3 plays a role in the plant defense response against biotrophic pathogens that is distinct from necrotrophic pathogens. ACBP3 function in plant defense was supported further by bioinformatics data showing up-regulation of many biotic and abiotic stress-related genes in ACBP3 OE-1 in comparison with the wild type.

The acyl-CoA-binding proteins (ACBPs) are conserved at the ACB domain (Xiao and Chye, 2009, 2011). They have been identified from various organisms, including plants, yeast, Drosophila, and mammals, and implicated in diverse functions such as acyl-CoA and/or phospholipid metabolism, acyl-CoA trafficking and homeostasis, protection of acyl lipids from cytosolic enzymes, growth, development, and stress tolerance (Knudsen et al., 2000; Faergeman and Knudsen, 2002; Xiao and Chye, 2009, 2011; Yurchenko et al., 2009; Fan et al., 2010; Yurchenko and Weselake, 2011). In addition to the 10-kD ACBPs, which are ubiquitous in eukaryotes, larger forms of ACBPs have been identified from many species (Leung et al., 2004; Xiao and Chye, 2009; Fan et al., 2010; Meng et al., 2011). For example, in the model plant Arabidopsis (Arabidopsis thaliana), in a total of six ACBPs (ACBP1–ACBP6), five are larger than 10 kD (Engeseth et al., 1996; Chye, 1998; Chye et al., 2000; Leung et al., 2004; Chen et al., 2008; Xiao and Chye, 2009), while in rice (Oryza sativa), four of six ACBPs exceed 10 kD (Meng et al., 2011). Functional analyses of the Arabidopsis ACBPs have revealed that despite conservation in the ACB domain, they vary in size from 92 amino acids (10.4 kD) to 668 amino acids (73.1 kD), are located in different cellular compartments, and show distinct in vitro binding affinities to acyl-CoA/phospholipid substrates, suggesting that they may play nonredundant functions in planta (Xiao and Chye, 2009, 2011; Yurchenko and Weselake, 2011). Arabidopsis ACBPs have been demonstrated to serve various roles in lipid metabolism, in early embryo development (Chen et al., 2010) and senescence (Xiao et al., 2010), as well as in conferring tolerance to heavy metals (Xiao et al., 2008a; Gao et al., 2009, 2010) and freezing (Chen et al., 2008; Du et al., 2010).

Among the Arabidopsis ACBPs, three members (ACBP1, ACBP2, and ACBP3) contain N-terminal transmembrane domains and are membrane associated, with ACBP3 also being targeted to the apoplast (Chye, 1998; Chye et al., 1999, 2000; Li and Chye, 2003, 2004; Leung et al., 2006; Xiao et al., 2010). ACBP3 is unique in its domain structure in comparison with other large Arabidopsis ACBPs because it lacks ankyrin repeats or kelch motifs, and it is the only large ACBP...
identified so far that has an ACB domain located at the C terminus (Leung et al., 2004; Xiao and Chye, 2011). Previous studies have revealed that its ACB domain binds to C18- and C20:4-acyl-CoA esters as well as C18-phosphatidylethanolamine (PE) in vitro (Leung et al., 2006; Xiao et al., 2010). Our investigations show correlation between ACBP3 expression and PE position in vivo; for example, PE levels increase in Arabidopsis ACBP3 overexpressors (ACBP3-OEs) and decrease in ACBP3 knockout lines (ACBP3-KOs) comprising the acbp3 T-DNA insertional mutant and RNA interference lines (Xiao et al., 2010). Furthermore, the ACBP3-OE and ACBP3-KO lines exhibited accelerated and delayed leaf senescence phenotypes, respectively. Our observations of disrupted autophagosome formation accompanied by an enhanced degradation of the GFP-ATG8e protein, a translational fusion of GFP to the autophagy protein 8e (ATG8e), in the combined ACBP3 OE-1 GFP-ATG8e line during starvation have linked ACBP3 function to the regulation of ATG8-PE complex formation and autophagy-mediated leaf senescence (Xiao et al., 2010). The autophagy pathway is a highly regulated process essential for bulk degradation and nutrient recycling in many species (Ohsumi, 2001; Levine and Klionsky, 2004). In plants, the autophagy proteins (ATGs) have been reported to be related to salicylic acid (SA) signaling-dependent plant innate immunity-associated programmed cell death (PCD; Liu et al., 2005; Yoshimoto et al., 2009). Hence, it was pertinent to investigate the potential role of ACBP3 in this process, given its abilities to interact with PE and to modulate ATG8 stability.

Here, ACBP3 mRNA expression was observed to be induced following bacterial pathogen infection as well as pathogen elicitor and defense-related phytohormone treatments. Consequently, alterations in ACBP3 expression in ACBP3-OE and acbp3 mutant lines were found to affect responses to bacterial pathogen infection, suggesting a role for ACBP3 in plant defense that was investigated further using SA signaling (nonexpressor of PR genes1 [npr1-1 and npr1-5]) and jasmonic acid (JA) signaling (coronatine-insensitive1 [coi1-2]) mutants.

**RESULTS**

**ACBP3 Expression Is Induced by Phytopathogens, Arachidonic Acid, and Phytohormones**

To examine the potential role of ACBP3 in plant defense, ACBP3 mRNA expression following fungal or bacterial pathogen infection was analyzed in RNA gel-blot analyses. As shown in Figure 1A, the expression of ACBP3 is induced 12, 24, and 48 h post inoculation (hpi) with either *Botrytis cinerea* or *Pseudomonas syringae pv tomato* DC3000. ACBP3 expression was also elevated following treatment with the fungal elicitor arachidonic acid (AA; Fig. 1B).

As the phytohormones ethylene, JA, and SA are known to regulate plant defense response, ACBP3 expression in response to 1-aminocyclopropane-1-carboxylic acid (ACC; the direct precursor of ethylene), methyl jasmonate (MeJA), and SA was determined. Results from RNA gel-blot analysis revealed that ACBP3 mRNA was induced after treatment with 1 mM ACC, 100 μM MeJA, or 1 mM SA and harvested at 0, 4, 8, 12, and 24 h post treatment for RNA gel-blot analysis. C. ACBP3 expression is induced in response to ACC, MeJA, and SA treatments. Total RNA samples from 3-week-old wild-type Arabidopsis seedlings grown on MS medium were treated with water (−) or AA (+) and harvested at 0, 4, 8, and 12 h post treatment (hpt) for RNA gel-blot analysis. D. The expression of ACBP3 in the wild type (WT), coi1-2, and npr1-1. Total RNA samples from 3-week-old wild-type, coi1-2, and npr1-1 plants treated with water (−) and 100 μM MeJA (+) or 1 mM SA (+) were collected at 8 h post treatment for RNA gel-blot analysis. Ethidium bromide-stained rRNAs are shown below the blots to indicate the relative amounts of total RNA loaded per lane. Blots were repeated with similar results.

**Figure 1.** RNA gel-blot analyses using a DIG-labeled ACBP3 cDNA probe to investigate the expression patterns of ACBP3. A, ACBP3 expression is induced by phytopathogens. Total RNA samples from 3-week-old soil-grown wild-type Arabidopsis seedlings were infected with *B. cinerea* or *P. syringae* DC3000 with 1% Glc and 10 mM MgCl2 as mock controls, respectively, and harvested at 0, 12, 24, and 48 hpi for RNA gel-blot analysis. B, ACBP3 expression is induced by AA. Total RNA samples from 3-week-old wild-type Arabidopsis seedlings grown on MS medium were treated with water (−) or AA (+) and harvested at 0, 4, 8, and 12 h after treatment (hpt) for RNA gel-blot analysis. C, ACBP3 expression is induced in response to ACC, MeJA, and SA treatments. Total RNA samples from 3-week-old wild-type Arabidopsis seedlings grown on MS medium were treated with water, 1 mM ACC, 100 μM MeJA, or 1 mM SA and harvested at 0, 4, 8, 12, and 24 h post treatment for RNA gel-blot analysis. D, The expression of ACBP3 in the wild type (WT), coi1-2, and npr1-1. Total RNA samples from 3-week-old wild-type, coi1-2, and npr1-1 plants treated with water (−) and 100 μM MeJA (+) or 1 mM SA (+) were collected at 8 h post treatment for RNA gel-blot analysis. Ethidium bromide-stained rRNAs are shown below the blots to indicate the relative amounts of total RNA loaded per lane. Blots were repeated with similar results.
SA, suggesting that ACBP3 function may be associated with plant defense. It has been noted that the up-regulation of ACBP3 changes at different time points upon ACC, MeJA, or SA treatment, and this may have been caused by variation in light conditions, because the ACBP3 transcript is regulated by light/dark cycling (Xiao et al., 2009, 2010).

To explore further the relationship between ACBP3 and the JA and/or SA signaling pathways, its expression in the JA signaling-deficient coi1-2 mutant (Xiao et al., 2004) and the SA signaling-deficient npr1-1 mutant (Cao et al., 1994) was examined. Results from RNA gel-blot analyses showed an up-regulation of ACBP3 mRNA in both coi1-2 and npr1-1 (Fig. 1D) mutants in comparison with the wild type, with a marked increase in npr1-1 after SA treatment (Fig. 1D). The slightly JA-inducible expression of ACBP3 in the coi1-2 mutant may be because the coi1-2 mutation is a leaky allele in JA-responsive gene expression as well as in male fertility (Xiao et al., 2004).

Overexpression of ACBP3 Constitutively Activates PR Gene Expression and Promotes Cell Death and Hydrogen Peroxide Production

The expression levels of pathogenesis-related genes (PR1, PR2, PR5, and PDF1.2) in the ACBP3-OE lines (Xiao et al., 2010) were investigated further by RNA gel-blot analysis using PCR-generated digoxigenin (DIG)-labeled PR1, PR2, PR5, and PDF1.2 probes. As shown in Figure 2A, PR1, PR2, and PR5 mRNAs were constitutively activated in all three independent ACBP3-OE lines tested (OE-1, OE-4, and OE-6), while the expression of PDF1.2 was down-regulated. Subsequently, the OE-1 and OE-4 lines were used for further analysis, since they have been phenotypically and biochemically characterized previously (Xiao et al., 2010).

Plants showing SA-dependent constitutive disease resistance are characterized by the occurrence of spontaneous lesions and the generation of high levels of reactive oxygen species (Xia et al., 2004; Glazebrook, 2005). To examine whether ACBP3-OEs show cell death and reactive oxygen species accumulation, the rosettes of OE-1 and OE-4 lines were tested using trypan blue and diaminobenzidine (DAB) staining analyses. Lesions were apparent upon trypan blue staining in the leaves of uninfected ACBP3-OE plants (OE-1 and OE-4) but not in the wild type (Fig. 2B). When DAB staining was used to detect hydrogen peroxide (H2O2) in situ, the uninfected leaves of ACBP3-OE appeared to have generated high levels of H2O2, as detected by a yellow color, in contrast to the uninfected wild type (Fig. 2C).

ACBP3-OEs Show Enhanced Protection against a Bacterial Pathogen

Given that ACBP3 expression appeared to be associated with the plant defense response (Fig. 1A), an investigation was conducted to examine whether a change in ACBP3 expression would affect plant disease susceptibility. Wild-type and ACBP3-OE (OE-1 and OE-4) seedlings were inoculated with the virulent strain of the bacterial pathogen P. syringae pv tomato DC3000 (Fig. 3A). Four days after inoculation (dai), the leaves of wild-type plants showed severe chlorosis in response to infection (Fig. 3A), while the leaves of OE-1 and OE-4 lines displayed little yellowing (Fig. 3A), suggesting that the ACBP3-OE plants were conferred resistance to this pathogen. When the inoculated plants were analyzed further, it was revealed that bacterial growth, as measured by counting bacterial numbers at 4 dai in the ACBP3-OE lines, were about 3-fold lower than in the wild type (Fig. 3B), confirming...
that an overexpression of Arabidopsis ACBP3 had culminated in an enhanced resistance to P. syringae infection.

The responses of the ACBP3-KOs, including the acbp3 T-DNA insertion mutant and the ACBP3 RNA interference (RNAi) transgenic line RNAi-1 (Xiao et al., 2010), to bacterial pathogen inoculation were tested next and compared with wild-type and OE lines. As shown in Figure 4A, the bacterium-infected acbp3 mutant and RNAi-1 plants displayed higher bacterial counts than the wild type at 4 dai (Fig. 4A). The 2.6- and 2.7-fold increases in bacterial count in the acbp3 mutant and RNAi-1 plants, respectively, were deemed significant \((P < 0.05\) by Student’s t test; Fig. 4A). To investigate whether the expression of plant defense genes PR1, PR2, and PR5 were affected in the acbp3

![Figure 3: ACBP3-OEs show enhanced resistance to P. syringae DC3000 infection. A, Three-week-old wild-type (WT) and ACBP3-OE (OE-1 and OE-4) plants were inoculated with P. syringae DC3000. Plants were sprayed with bacterial suspension (OD\(_{600} = 0.01\)) and photographed at 0 and 4 dai. The yellowing in the wild-type leaves at 4 dai indicates infection. The experiment was repeated with similar results. B, Bacterial growth in wild-type, OE-1, and OE-4 plants at 0 and 4 dai. Data points represent means of three replicates (each replicate was pooled with three leaf discs) taken from three independent plants, and bacterial counts were determined on a per cm\(^2\) basis. (WT) = 0.05 by Student’s t test.)

![Figure 4: The ACBP3-KOs show increased susceptibility to P. syringae DC3000 infection. A, Bacterial growth in wild-type (WT), acbp3 mutant, and ACBP3 RNAi-1 plants at 0 and 4 dai. Data points represent means of three replicates (each replicate was pooled with three leaf discs) taken from three independent plants, and bacterial counts were determined on a per cm\(^2\) basis. B, qRT-PCR analyses showing the expression of PR1, PR2, and PR5 in wild-type, acbp3 mutant, and ACBP3 RNAi-1 plants before and after infection. Rosettes of 3-week-old wild-type, acbp3 mutant, and ACBP3 RNAi-1 plants were syringe infiltrated with bacterial suspension (OD\(_{600} = 0.001\)) and harvested at 0, 24, and 48 hpi. Expression levels for each gene in the wild type, acbp3 mutant, and ACBP3 RNAi-1 plants at 0, 24, and 48 hpi were normalized to uninfected (0-hpi) wild-type, acbp3 mutant, and ACBP3 RNAi-1 values, respectively. cfu, Colony-forming units. Letters above bars are as follows: * significant difference \((P < 0.05\) by Student’s t test) between the wild type, acbp3 mutant, or ACBP3 RNAi-1 at 24 and 48 hpi.)
mutant and RNAi-1, their transcripts were measured using quantitative real-time reverse transcription (qRT)-PCR analysis. The results indicate that the expression of all three PR genes in the inoculated leaves of wild-type plants showed greater induction following pathogen inoculation at 24 and 48 hpi, in comparison with the acbp3 mutant and RNAi-1 (Fig. 4B).

To determine the possible link in SA levels to the activated defense phenotype observed in ACBP3-OEs and ACBP3-KOs, we measured the SA contents using HPLC. As shown in Figure 5, the endogenous SA levels in OE-1 and OE-4 were elevated 1.5-fold in comparison with that of the wild type. In contrast, no significant difference was observed between ACBP3-KOs and the wild type (Fig. 5). Taken together, our observations suggest that ACBP3 expression seems important for induction of the plant defense response.

Activated Expression of PR Genes and Enhanced Pathogen Resistance in ACBP3-OEs Are Dependent on NPR1

To determine whether the constitutively induced expression of PR genes (Fig. 2) and enhanced plant disease resistance (Fig. 3) in ACBP3-OEs are related to an activation in the NPR1- or COI1-mediated signaling pathway, the expression of PR1, PR2, and PR5 in wild-type and ACBP3 OE-1 plants as well as OE-1 in combination with a npr1-5 (Shah et al., 1999) or coi1-2 (Xiao et al., 2004) background was subsequently examined. The npr1-5 (Shah et al., 1999) and coi1-2 (Xiao et al., 2004) mutants were used because they are deficient in SA and JA signaling, respectively (Xiao et al., 2010). Our results from qRT-PCR (Fig. 6A) showed elevated expression in all PR genes (PR1, PR2, and PR5) in OE-1. However, these PR genes were significantly suppressed in the OE-1npr1-5 line when OE-1 was combined with the npr1-5 mutation. The OE-1npr1-5 line now resembled the npr1-5 single mutant (Fig. 6A).

Figure 5. Endogenous SA contents in wild-type (WT), ACBP3-OE, and ACBP3-KO plants. Total SAs were extracted from leaf samples of 4-week-old wild-type, ACBP3-OE (OE-1 and OE-4), and ACBP3-KO (acbp3 and RNAi-1) plants and analyzed by HPLC. Data represent means of three samples from three independent plants. The experiment was repeated with similar results. FW, Fresh weight. ** P < 0.05 by Student’s t test.

Figure 6. Activated PR gene expression and enhanced P. syringae resistance phenotypes in ACBP3-OEs are dependent on NPR1. A, qRT-PCR analyses of the expression levels of ACBP3, PR1, PR2, and PR5 in 4-week-old wild-type (WT), OE-1, OE-1npr1-5, npr1-5, OE-1coi1-2, and coi1-2 rosettes. Expression levels for each gene in all genotypes were normalized to the wild type. ** P < 0.01 by Student’s t test. B, Bacterial counts in the wild type, OE-1, OE-1npr1-5, npr1-5, OE-1coi1-2, and coi1-2 at 0 and 4 dai. Three-week-old plants were sprayed with bacterial suspension (OD600 = 0.01), data points represent means of three replicates (each replicate was pooled with three leaf discs) taken from three independent plants, and bacterial counts were determined on a per cm² basis. The experiment was repeated with similar results. cfu, Colony-forming units. * P < 0.05, ** P < 0.01 by Student’s t test.

Consistent with previous findings (Ren et al., 2008), our data also revealed that the expression of PR genes was significantly elevated in the coi1 mutant in comparison with the wild type (Fig. 6A). Interestingly, the PR1 and PR2 genes showed an additive induced expression in the OE-1coi1 double mutant (Fig. 6A), which may have resulted from a combination of ACBP3 overexpression and the coi1-2 mutation. Furthermore, measurement of bacterial counts in plants following P. syringae inoculation showed that the OE-1npr1 plants no longer exhibited an enhanced resistance to P. syringae infection, similar to the npr1-5 mutant (Fig. 6B). As in previous findings (Kloek et al., 2001), the coi1-2 mutant was more resistant to P. syringae infect-
demonstrate that the enhanced expression of response of the KO on NPR1.

Response of ACBP3-OEs and ACBP3-KOs to B. cinerea Infection

To determine the response of ACBP3 to the necrotrophic fungal pathogen B. cinerea, the detached rosette leaves from the wild type, ACBP3-OEs (OE-1 and OE-4), and ACBP3-KOs (acbp3 and ACBP3-RNAi) were inoculated with Botrytis spores. As shown in Figure 7, the necrotic spots at the sites of inoculation were evident in wild-type leaves at 2 dai, and necroses in ACBP3-OE lines were more severe than in the wild type (Fig. 7A). In contrast, the spots in ACBP3-KOs were restricted (Fig. 7A). Similar results were observed by spraying Botrytis suspension (concentration of 2 × 10^5 spores mL^{-1}) onto wild-type, ACBP3-OE, and ACBP3-KO plants (Fig. 7B). At 7 dai, chlorosis and necrosis were evident in most rosette leaves of OE lines, but those of KO lines were relatively unaffected in comparison with the wild type (Fig. 7B), suggesting that ACBP3-OEs were more susceptible than ACBP3-KOs to Botrytis infection.

Figure 7. Response of ACBP3-OEs and ACBP3-KOs to B. cinerea infection. A, Representative rosettes of 3-week-old wild-type (WT), ACBP3-KO (acbp3 and ACBP3-RNAi), and ACBP3-OE (OE-1 and OE-4) plants showing disease manifestation after inoculation (by 10-μL dotting using 1 × 10^5 spores mL^{-1} suspension) with the fungus B. cinerea. Photographs were taken at 4 dai. B, Phenotypes of 3-week-old ACBP3-OE (OE-1 and OE-4) plants sprayed with the pathogen B. cinerea (2 × 10^5 spores mL^{-1}) or with water as a control (H2O) and photographed at 7 dai. The experiments were repeated with similar results.

The ACBP3-GFP Protein Is Degraded upon Pathogen Infection, and Inoculation of Intracellular Fluids from ACBP3-OE Plants Activates the Expression of PR Genes (PR1 and PR2)

To investigate further the molecular mechanism of ACBP3 in the plant defense response, an ACBP3-GFP construct was generated using the pBI-GFP plasmid (Xiao et al., 2008b) to facilitate tracking ACBP3 following pathogen infection. The resultant plasmid was used to produce transgenic plants expressing ACBP3-GFP (Xiao et al., 2010) that were verified by confocal microscopy before being subjected to P. syringae DC3000 infection. Consistent with our previous reports (Leung et al., 2006; Xiao et al., 2010), ACBP3-GFP was confirmed to be localized to the apoplast and the endomembranes in the primary root of ACBP3-GFP transgenic plants (data not shown). Confocal microscopy revealed further that after P. syringae infection, ACBP3-GFP showed either altered localization (possibly to the endoplasmic reticulum or vesicles) or degradation at 72 h in leaf epidermal cells (Fig. 8B) and at 30 min in primary root cells (Fig. 8D). Such changes were not seen in leaf or root following control treatment using MgCl2 (Fig. 8, A and C, respectively).

Furthermore, when western-blot analysis was carried out using intracellular fluids (IFs) from ACBP3-GFP rosettes infected with P. syringae, the ACBP3-GFP protein appeared to be subject to degradation in samples taken at 48 and 72 h post infection (Fig. 8E). It is noted that relatively weaker signals were detected with full-length ACBP3-GFP bands (Fig. 8E, arrow), while GFP-degraded forms appeared much stronger (Fig. 8E, asterisk), perhaps arising from differences in anti-GFP antibody specificities to ACBP3-GFP fusion protein and the GFP domain alone. When the IF extracted from uninfected ACBP3 OE-1 plants and P. syringae-infected ACBP3 OE-1 plants were inoculated on wild-type rosettes, the expression of PR1 and PR2 mRNAs was induced in the inoculated (local) rosette leaves but not in uninfected (systemic) rosette leaves (Fig. 8F). PR1 and PR2 mRNAs seemed to be more highly expressed when IF was extracted from the infected ACBP3 OE-1 plants than the uninfected plants. As a control, the use of wild-type IF did not induce PR gene expression in both local and systemic leaves (Fig. 8F).

Microarray Analyses of ACBP3 OE-1

To establish further the potential links between the molecular function of ACBP3 and other pathways, we performed microarray analysis on the wild type and ACBP3 OE-1. The expression of 54 genes was observed to be up-regulated in OE-1 plants (Supplemental Table S1). Following functional classification, most (21) genes were found to be associated with biotic/abiotic stresses and leaf senescence. Consistent with the results of Figure 2, the expression of pathogenesis-related genes, including PR1, PR2, and PR5, in OE-1 showed...
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Figure 8. Effect of P. syringae DC3000 infection on ACBP3-GFP. A to D. Change in localization of the ACBP3-GFP fusion protein in transgenic Arabidopsis after P. syringae treatment. A and B, Confocal images showing leaf epidermal cells of 3-week-old ACBP3-GFP transgenic plants at 72 h following inoculation with 10 mM MgCl₂ (A) or syringe-infected P. syringae DC3000 (10⁸ colony-forming units mL⁻¹; B). Bars = 20 μm. C and D, Confocal images showing primary root cells of 1-week-old ACBP3-GFP transgenic plants treated with 10 mM MgCl₂ (C) or with P. syringae DC3000 (10⁸ colony-forming units mL⁻¹) for 30 min (D). Bars = 20 μm. E, Western-blot analysis using GFP-specific antibodies of IFs from ACBP3-GFP transgenic plants infected with P. syringae DC3000 at 0, 48, and 72 h. The arrow indicates the ACBP3-GFP fusion protein, and the asterisk shows degraded protein derivatives. At bottom is an identically loaded gel stained with Coomassie blue. F, Inoculation of IF from OE-1 plants activates the expression of PR1 and PR2 in wild-type (WT) Arabidopsis. IFs extracted from wild-type, uninfected, and P. syringae-infected OE-1 (OE-1 P.s) plants were inoculated on wild-type rosettes. OE-1 was infected with P. syringae DC3000 for 24 h. The inoculated (local) and systemic rosette leaves were harvested at 48 hpi for total RNA isolation. PR1 and PR2 expression was analyzed by RNA gel blotting using DIG-labeled cDNA probes. At bottom is a ethidium bromide-stained gel before blotting showing rRNA bands. The experiments were repeated with similar results.

The expression of P. syringae avrRpt2-inducible gene AIG1 (Reuber and Ausubel, 1996) and KTI1, encoding a trypsin inhibitor in modulating PCD in plant-pathogen interactions (Li et al., 2008b), showed 3.9- and 4.4-fold increases, respectively (Supplemental Table S1). Twelve genes related to stress responses, including oxidative stress, heat, Glc stress, wounding, and heavy metal stress, were up-regulated in OE-1 (Supplemental Table S1). In particular, the expression of ARAIBIDOPI-SIS THALIANA PLEIOTROPIC DRUG RESISTANCE12 (AtPDR12) increased 2.0-fold. Since AtPDR12 mRNA is induced by lead [Pb(II)] treatment in both roots and shoots (Lee et al., 2005) and overexpression of AtPDR12 conferred resistance to Pb(II), the ACBP3-OE plant may possibly show enhanced tolerance to Pb(II); this would be tested in the future given that ACBP1-overexpressors are Pb(II) tolerant (Xiao et al., 2008a) and that human ACBP has been reported to be a molecular target for Pb(II) (Smith et al., 1998). Another stress-related up-regulated gene in OE-1 is At1g28480, which encodes a member of the glutaredoxin family, GRX480, that is known to interact with TGA transcription factors and suppress the transcription of JA-responsive PDF1.2 (Ndumukong et al., 2007). This finding is consistent with our results in northern-blot analysis showing the down-regulation of PDF1.2 in ACBP3-OEs (Fig. 2A).

In addition, 15 genes related to metabolism, nine genes in transcription, transportation, and signaling, and eight genes of unknown function showed more than 2-fold up-regulation in OE-1 (Supplemental Table S1). In contrast to the 54 up-regulated genes, only 14 genes were down-regulated in OE-1 (Supplemental Table S2). Interestingly, most of these have been predicted to be localized in the extracellular space and many have unknown functions, while some (At1g57750, At1g66850, At1g58430, and At2g42990) are likely associated with lipid metabolism.

qRT-PCR was carried out further on ACBP3-OEs to validate the microarray data. The results of the increased expression of biotic- or abiotic stress-related genes (PR1, PR5, AIG1, KTI1, GRX480, PR2, FMO1, and AtPDR12) or senescence-associated genes (SAG13 and YSL9) in ACBP3-OEs (OE-1 and OE-4) support the microarray results (Fig. 9). Similarly, the down-regulation of genes corresponding to At1g57750, At1g58430, and At2g42990 are likely associated with lipid metabolism.

The microarray data and qRT-PCR results may possibly show enhanced tolerance to Pb(II); this would be tested in the future given that ACBP1-overexpressors are Pb(II) tolerant (Xiao et al., 2008a) and that human ACBP has been reported to be a molecular target for Pb(II) (Smith et al., 1998). Another stress-related up-regulated gene in OE-1 is At1g28480, which encodes a member of the glutaredoxin family, GRX480, that is known to interact with TGA transcription factors and suppress the transcription of JA-responsive PDF1.2 (Ndumukong et al., 2007). This finding is consistent with our results in northern-blot analysis showing the down-regulation of PDF1.2 in ACBP3-OEs (Fig. 2A).

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qRT-PCR was carried out further on ACBP3-OEs to validate the microarray data. The results of the increased expression of biotic- or abiotic stress-related genes (PR1, PR5, AIG1, KTI1, GRX480, PR2, FMO1, and AtPDR12) or senescence-associated genes (SAG13 and YSL9) in ACBP3-OEs (OE-1 and OE-4) support the microarray results (Fig. 9). Similarly, the down-regulation of genes corresponding to At1g57750, At1g58430, and At2g42990 was confirmed by qRT-PCR (Fig. 9). The differences in fold change of some genes, such as AIG1, KTI1, FMO1, and YSL9 in OE-1, between the microarray data and qRT-PCR results may possibly have arisen from differences in the developmental stages of the plant samples used in the two analyses. Taken together, our findings herein suggest that Arabidopsis ACBP3 has diverse functions in both plant development and the protection of plants from biotic or abiotic stress.
DISCUSSION

We have previously demonstrated that Arabidopsis ACBP3 plays an important role in regulating leaf senescence by modulation of ATG8 protein stability and in autophagosome formation in the autophagy pathway (Xiao et al., 2010; Xiao and Chye, 2011). In this study, the function of Arabidopsis ACBP3 in plant defense was established by phenotypic analyses of ACBP3-OE and ACBP3-KO lines in response to biotrophic and necrotrophic pathogen infections as well as by using bioinformatics data showing downstream up-regulated expression of many biotic and abiotic stress-related genes in an ACBP3-OE line in comparison with the wild type.

Many secondary signal molecules, including SA, ethylene, and JA, produced during plant-pathogen interactions initiate various defense responses in plants. Recently, the roles of lipids and lipid-related proteins in plant disease resistance as well as plant-microbe interactions have been extensively studied (Shah, 2005). Among them, JA and its derivatives are well-studied lipid-derived signal molecules of plants in response to wounding during herbivory or pathogen infection (Shah, 2005). However, by analyses of ACBP3-OE in SA signaling mutant npr1 (Shah et al., 1999) and JA perception mutant coi1 (Xiao et al., 2004) backgrounds, our data suggest that the ACBP3-mediated defense response relies on the NPR1-mediated (not the COI1-
mediated) signaling pathway, indicating a possible role for lipid-derived signals in JA-independent early events in the plant response to biotic and/or abiotic stresses. There is mounting evidence that supports the involvement of genes affecting membrane lipid biosynthesis in plant defense. For example, the Arabidopsis FATTY ACID DESATURASE7, SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY1 (SFD1), and SFD2 genes are essential for SA-dependent, but not JA-dependent, systemic acquired resistance (Nandi et al., 2004; Chaturvedi et al., 2008), suggesting that although membrane lipids provide fatty acid for JA synthesis, an intact JA signaling pathway is not essential for the lipid-associated plant defense pathway. Although the expression of ACBP3 was induced by both exogenous JA and SA treatments, ACBP3-OE plants exhibited constitutive PR gene expression and the enhancement in P. syringae resistance was completely dependent on NPR1. The rosette leaves of ACBP3-OEs were characterized by cell death that was accompanied by the demonstration of an enhanced resistance to the biotrophic pathogen P. syringae. In contrast, these OE lines were more susceptible to the necrotrophic pathogen B. cinerea. Given the fact that mutual suppression cross talk occurs between the SA and JA signaling pathways, our observations are not unexpected, because such phenotypes have been frequently seen in many other mutants of SA-dependent defense genes (Glazebrook, 2005). However, in contrast to the NPR1-dependent PR gene expression and defense response in ACBP3-OEs, our results have revealed that the SA-induced expression pattern of ACBP3 was unaffected in the npr1-1 mutant. Instead, its expression level was somewhat up-regulated by SA treatment in the npr1-1 mutant, suggesting that NPR1 is a negative regulator of ACBP3 gene expression. Given the findings that SA levels usually increase in npr1 mutants, a negative feedback loop for NPR1 in regulating SA synthesis through ISOCHORISMATE SYNTHASE1 has been suggested (Delaney et al., 1995; Shah et al., 1997; Shah, 2003). Therefore, up-regulation of ACBP3 in the npr1-1 mutant can be explained by an elevation of endogenous SA, and it seems that ACBP3 involvement in plant defense could be associated with NPR1 protein function.

Generally, the delivery of endogenous proteins to the apoplastic space or the movement of proteins between plant cells occurs either via the secretory pathway or the plasmodesmata (Rojo et al., 2002). Using autofluorescence-tagged ACBP3 fusions, ACBP3 was observed to be extracellularly targeted and the signal peptide in ACBP3 has been proven sufficient for extracellular targeting (Leung et al., 2006; Xiao et al., 2010), suggesting that ACBP3 is distinctive in its localization in comparison with the five other ACBPs. The colocalization of autofluorescence-tagged ACBP3 with fluorescence-labeled Golgi/endoplasmic reticulum complex and vesicles supports an extracellular localization of ACBP3 in association with the secretory pathway (Xiao et al., 2010). Similar to ACBP3, many other apoplast proteins, such as CONSTITUTIVE DIS-
by the autophagy pathway. On the other hand, given our observations of PCD and H$_2$O$_2$ accumulation in the premature leaves of ACBP3-OEs, the constitutive defense phenotype in ACBP3-OE lines could be due to cell death resulting from an elevation in H$_2$O$_2$. Nonetheless, it is also very likely that other associated components (e.g. extracellular lipid signaling) representing autophagy-independent pathways coexist in ACBP3-OEs that can trigger a bacterial resistance phenotype, possibly due to the apoplastic localization of ACBP3.

Given that recombinant ACBP3 binds arachidonyl-CoA esters with high affinity (Leung et al., 2006) and that AA is a well-known lipophilic molecule of fungal origin with elicitor activity (Smith, 1996), ACBP3 may possibly be involved in pathogen recognition by binding lipid molecules secreted by bacterial or fungal pathogens or other pathogen-derived signals released during infection for subsequent transmission into the plant cell that would culminate in strengthening existing defenses and trigger other defense pathways. This idea is supported by the rapid inducible expression of ACBP3 mRNA in response to AA treatment. However, in contrast to rapid mRNA induction, analysis of the ACBP3-GFP fusion protein revealed that it is quickly degraded upon *P. syringae* DC3000 infection. The observation of ACBP3-GFP degradation upon pathogen invasion raises a possible link between ACBP3-mediated lipid signals and CDR1-mediated peptide signals (Xia et al., 2004). One likely explanation in plant-pathogen recognition mediated by ACBP3 may involve its binding to pathogen-secreted lipids (e.g. AA), after which the ACBP3-lipid complex is rapidly degraded by the apoplast protease (possibly CDR1) for initiation of the inducible plant disease resistance. Similar to that of CDR1 overexpressors, the activated expression of *PR* genes in response to the inoculation of IFs extracted from ACBP3-OEs is most likely induced by the SA pathway. On the other hand, the ubiquitination-associated proteins have recently been identified to play important roles in the plant’s response to biotic and abiotic stresses (Zeng et al., 2006). Hence, Arabidopsis ACBP3 can also be targeted by the pathogen-derived E3 ubiquitin ligase for degradation during plant-microbe interaction (Zeng et al., 2006).

The results presented here provide strong evidence of an important role for Arabidopsis ACBP3 in the plant defense response. Taken together with findings reported in our previous studies (Leung et al., 2006; Xiao et al., 2010; Xiao and Chye, 2009, 2011), we propose that ACBP3 is likely involved in both NPR1-dependent bacterial pathogen resistance (by its ability to regulate the autophagy-mediated PCD pathway) and the lipid signaling pathway. The latter may result in a contribution of enhanced recognition of biotrophic and necrotrophic pathogens during the early stages of plant-microbial interaction. In both cases, activated ACBP3 expression will consequently trigger the plant defense system by enhancing the expression of *PR* genes as well as other defense-related genes. Future work on downstream up- or down-regulated genes in ACBP3-OE lines is expected to improve further our understanding of this ACBP in both biotic and abiotic stress pathways.

### MATERIALS AND METHODS

#### Plant Materials, Growth Conditions, and Treatments

All Arabidopsis (*Arabidopsis thaliana*) wild-type (ecotype Col-0), acbp3, and ACBP3-OE (Xiao et al., 2010) lines were grown under light/dark cycles in conditions of 16 h of light, 23°C/8 h of dark, 21°C. For Arabidopsis treatments in RNA gel-blot experiments, seedlings were grown on Murashige and Skog (1962; MS) medium in continuous light for 2 to 3 weeks and then treated with 10 μM AA (Sigma-Aldrich), 1 mM ACC (Sigma-Aldrich), 100 μM MeJA (Sigma-Aldrich), 1 mM SA (Sigma-Aldrich), or water (control). Phenotypic lines were collected at time points indicated in the figures.

Arabidopsis wild-type and OE-1 plants used for microarray gene expression analysis were germinated on MS. The seeds were first placed at 4°C for 2 d and then cultivated in 16-h-light/8-h-dark cycles for 1 week in a tissue culture room. The MS-grown wild-type and OE-1 plants were planted in soil for another 2 weeks in a growth chamber under similar growth conditions (16 h of light, 23°C/8 h of dark, 21°C). After genotyping, rosette leaves from 3-week-old wild-type and OE-1 plants were collected and immediately frozen in liquid nitrogen, and stored at −80°C until use.

#### Culture of Pathogen and Inoculation of Plants

The bacterial strain *Pseudomonas syringae pv. tomato* DC3000 (ATCC No. BBA-871) and fungus strain *Botrytis cinerea* (ATCC No. 11542) were obtained from the American Type Culture Collection. Bacteria were grown on King’s B medium supplemented with rifampicin (50 μg mL$^{-1}$) and incubated at 25°C. One day before the assay, a single bacterial colony inoculated in King’s B liquid medium containing rifampicin (50 μg mL$^{-1}$) was cultured at 25°C overnight with shaking until midlog growth phase (optical density at 600 nm [OD$_{600}$] = 0.134) was obtained. Cells were then collected by centrifugation at 4,000g for 7 min and resuspended in 5 mL of 10 mM MgCl$_2$. Rosette leaves of 3-week-old plants were sprayed with bacterial suspensions (OD$_{600}$ = 0.01 in 10 mM MgCl$_2$). For RNA gel-blot analyses, plant samples were collected at 0, 12, 24, and 48 hpi. For phenotypic observations, plants were photographed at 0 and 4 dai. Determination of in planta bacterial growth was performed as described (Cao et al., 1994; Shah et al., 1997). Three independently infected leaves were used for the wild-type and other plant genotypes, and three leaf discs of the same size (0.6 cm in diameter and area of 0.28 cm$^2$) were taken from each leaf using a hole puncher and pooled as one replicate. The leaf discs were washed three times with distilled water and homogenized in 100 μL MgCl$_2$ using a plastic pestle. Bacterial growth numbers were determined by plating appropriate dilutions from each sample in King’s B medium containing rifampicin (50 μg mL$^{-1}$). For defense gene expression analyses, the rosettes of 3-week-old plants were sprayed infiltrated with bacterial suspensions (OD$_{600}$ = 0.001 in 10 mM MgCl$_2$), and samples were collected at the times indicated in the figures.

The fungus *B. cinerea* was cultured on a potato dextrose agar (BD Difco; catalog no. 213400) agar plate and incubated at room temperature. Collection of conidia and plant inoculation were performed according to previous protocols (Xiao et al., 2004; Li et al., 2008a). The rosettes of 3-week-old Arabidopsis were inoculated with the fungus *B. cinerea* by either inoculating a 10-μL droplet of spore suspension (1 x 10$^6$ spores mL$^{-1}$) or spraying a spore suspension (2 x 10$^5$ spores mL$^{-1}$) in water containing 1% Glc. Water containing 1% Glc was used as a control. After inoculation, the plants were placed in a growth chamber with high humidity (100%) at 22°C under growth conditions of a 16-h-light/8-h-dark photoperiod. Plant samples were photographed at 4 dai (for dotting inoculation) and 7 dai (for spraying inoculation). Determination of in planta fungal growth was performed as described (Leung et al., 2006; Xiao et al., 2008a).

#### RNA Gel-Blot Analysis

Total RNA extraction and RNA gel-blot analysis were carried out as described previously (Xiao et al., 2008a). Briefly, 30 μg of total RNA was separated on a 1.5% agarose gel containing 6% formaldehyde and transferred to Hybond N membranes (Amersham). The gene-specific primers used to generate cDNA probes are summarized in Supplemental Table S3. The cDNA fragments were labeled with the PCR Digoxigenin Probe Synthesis Kit.
according to the manufacturer’s instructions (Roche), and the wild-type first-strand cDNAs were used as templates. Hybridization and detection were performed according to standard procedures (Roche). Blots were washed under conditions of high stringency (2× SSC, 0.1% SDS for 2×15 min at room temperature; 0.5× SSC, 0.1% SDS for 2×15 min at 68°C; 0.1× SSC, 0.1% SDS for 2×15 min at 68°C).

For the experiment concerning ACRP3-OE IF induction of PR gene expression, IFs extracted from wild-type and ACRP3-OE plants as well as from ACRP3 OE-1 plants infected for 24 h with P. syringae DC3000 (10⁹ colony-forming units mL⁻¹) were inoculated on wild-type (Col-0) rosettes. Samples were collected from inoculated local leaves and uninoculated systemic leaves for RNA gel-blot analyses.

Trypan Blue and DAB Staining

Trypan blue staining (Alvarez et al., 1998) and DAB staining (Thordal-Christensen et al., 1997) were carried out as described previously with minor modifications. For trypan blue staining, rosettes from 4-week-old wild-type and ACRP3-OE plants were boiled for 1 min in trypan blue staining buffer (12.5% phenol, 12.5% glycerol, 12.5% lactic acid, 48% ethanol, and 0.025% trypan blue) and incubated for 10 min at room temperature, followed by destaining (five times) in 70% chloral hydrate. For DAB staining, rosettes from 4-week-old wild-type and ACRP3-OE plants were cut and placed in 1 mg mL⁻¹ DAB solution (pH 3.8) overnight (about 8 h) at room temperature and subsequently cleared in 96% boiling ethanol for 10 min.

qRT-PCR

Total RNA (5 µg) extracted using TRIzol reagent was reverse transcribed into cDNA by using the SuperScript First-Strand Synthesis System (Invitrogen; catalog no. 12371-019) according to the manufacturer’s instructions. PCR was conducted on a StepOne Plus real-time PCR system using SYBR Green Mix (Applied Biosystems). The conditions for qRT-PCR were as follows: initial denaturation at 95°C (10 min) followed by 40 cycles of 95°C (15 s) and 56°C (1 min). For each reaction, three experimental replicates were obtained using primer pairs specific to the gene of interest and an Arabidopsis ACTIN internal control as listed in Supplemental Table S4. The relative expression level of each investigated gene was normalized to that of the ACTIN control by subtracting the threshold cycle value of ACTIN from that of the investigated gene. Fold change in expression was calculated according to the comparative threshold cycle method (Schmittgen and Livak, 2008). The data shown in this study represent means ± SD of two independent experiments.

SA Measurement

SA was extracted from 0.3 to 0.5 g of 4-week-old leaves of wild-type, ACRP3-OE, and ACRP3-KO plants according to Morita-Yamamura et al. (2005). After extraction, the dried residues were resuspended in 50% methanol and analyzed by HPLC with a fluorescent detector set at excitation = 295 nm and emission = 370 nm.

Laser-Scanning Confocal Microscopy

For the analysis of ACRP3-GFP localization before and after pathogen infection, an inverted confocal laser-scanning microscope (Zeiss LSM 510) equipped with helium/neon lasers and multitracking was used as described previously (Xiao et al., 2010). GFP fluorescence, excited at 488 nm, was emitted through a primary dichroic filter (UV/486/543), a secondary dichroic filter (545 nm), and BP505-530 nm emission filters to the photomultiplier tube detector. LSM 510 software (Zeiss) was used to process the images obtained.

IF Extraction and Western-Blot Analysis

The IFs from Arabidopsis were extracted according to Xia et al. (2004) with minor modifications. Plant leaves were harvested and completely immersed in extraction buffer (100 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, and 2 mM EDTA). Leaves were infiltrated under vacuum (720 mm Hg) for 2 min followed by rapid release. Infiltration was repeated twice. Subsequently, the leaves were removed from the extraction buffer and placed between two absorbent papers to blot off the surface buffer. Leaves were then placed in a 50-mL Falcon tube containing a supported mesh for the separation and recovery of the IFs from the leaves. IFs were obtained from the infiltrated leaves by centrifugation at 3,000 rpm for 15 min. Total protein was extracted by homogenizing 3-week-old wild-type Arabidopsis rosettes according to Xiao et al. (2008b). Proteins were separated by 12% SDS-PAGE and subsequently transferred onto Hybond-C membranes (Amersham) using a Trans-Blot cell (Bio-Rad). The membranes were blocked for 2 h in 1× Tris-buffered saline plus 0.05% Tween 20 with 5% nonfat milk and incubated for another 2 h with anti-GFP (Invitrogen; 1:3,000) primary antibodies. The blots were washed three times with Tris-buffered saline plus 0.05% Tween 20 and then incubated for 1 h with secondary antibody. The ECL Western Blotting Detection Kit (Amersham) was used following the manufacturer’s instructions for detection of cross-reacting bands.

Microarray Analysis

Total RNA was extracted and purified using the RNeasy Mini Kit (Qiagen) with on-column DNase digestion according to the manufacturer’s instructions. The RNA quality and quantity were examined with the Bioanalyzer 2100 (Agilent Technologies) and MOPS-formaldehyde agarose gels. Each sample contains three biological replicates, and each replicate consists of a pool of RNA from three plants. Affymetrix ATH1 arrays were used for hybridization. GeneSpring GX software (Agilent) and the MAS5 algorithm were used in data collection and normalization. Welch’s approximate f test statistical analysis was followed by fold change and significance analysis between OE-1 and wild-type samples. Significantly differentially regulated genes were subsequently identified using Benjamini-Hochberg multiple testing correction, with P < 0.05 and change > 2.0-fold in OE-1 samples when compared with wild-type samples considered as cutoff values. Gene functional classification was analyzed using online software DAVID tools (http://david.abcc.ncifcrf.gov/) and MapMan (http://pdpb.tc.cornell.edu/dbsearch/searchacc.aspx) according to the Gene Ontology consortium (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NM_001084972 (ACBP3), NM_127025 (PR1), NM_115586 (PR2), NM_106161 (PR5), and NM_123809 (PDF1.2).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Genes up-regulated more than 2-fold in ACRP3 OE-1.

Supplemental Table S2. Genes down-regulated more than 2-fold in ACRP3 OE-1.

Supplemental Table S3. Sequences of gene-specific primers to generate cDNA probes for RNA gel-blot analyses.

Supplemental Table S4. Sequences of gene-specific primers for qRT-PCR.

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LITERATURE CITED


Rojo E, Sharma VK, Kovaleva V, Raikhel NV, Fletcher JC (2002) CLV3 is localized to the extracellular space, where it activates the Arabidopsis CLAVATA stem cell signaling pathway. Plant Cell 14: 969–977