Ethylene- and pathogen-inducible Arabidopsis acyl-CoA-binding protein 4 interacts with an ethylene-responsive element binding protein

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Abstract

Six genes encode proteins with acyl-CoA-binding domains in Arabidopsis thaliana. They are the small 10-kDa cytosolic acyl-CoA-binding protein (ACBP), membrane-associated ACBP1 and ACBP2, extracellularly-targeted ACBP3, and kelch-motif containing ACBP4 and ACBP5. Here, the interaction of ACBP4 with an A. thaliana ethylene-responsive element binding protein (AtEBP), identified in a yeast two-hybrid screen, was confirmed by co-immunoprecipitation. The subcellular localization of ACBP4 and AtEBP, was addressed using an ACBP4:DsRed red fluorescent protein fusion and a green fluorescent protein (GFP):AtEBP fusion. Transient expression of these autofluorescence-tagged proteins in agroinfiltrated tobacco leaves, followed by confocal laser scanning microscopy, indicated their co-localization predominantly at the cytosol which was confirmed by FRET analysis. Immuno-electron microscopy on Arabidopsis sections not only localized ACBP4 to the cytosol but also to the periphery of the nucleus upon closer examination, perhaps as a result of its interaction with AtEBP. Furthermore, the expression of ACBP4 and AtEBP in Northern blot analyses was induced by the ethylene precursor 1-aminocyclopropane-1-carboxylic acid, methyl jasmonate treatments, and Botrytis cinerea infection, suggesting that the interaction of ACBP4 and AtEBP may be related to AtEBP-mediated defence possibly via ethylene and/or jasmonate signalling.

Key words: Acyl-CoA-binding protein, ethylene, pathogen, protein–protein interaction.

Introduction

In Arabidopsis, six genes encode proteins that contain a conserved acyl-CoA-binding domain (Leung et al., 2004). They are the 10-kDa ACBP6 (Engeseth et al., 1996; Xiao et al., 2008), of which homologues are prevalent in eukaryotes, and larger ACBPs (ACBP1 to ACBP5), of which homologues have not been well-investigated in other organisms. Some of the larger ACBPs contain ankyrin repeats (ACBP1 and ACBP2), and kelch motifs (ACBP4 and ACBP5) that can potentially interact with protein partners (Li and Chye, 2003, 2004; Leung et al., 2004). ACBP2 has been previously shown to interact with AtEBP (Li and Chye, 2004). Our observations that various members of the Arabidopsis ACBP gene family consist of additional structural domains other than the conserved acyl-CoA-binding domain, plus their varying affinities for acyl-CoA esters, imply that they do not have redundant roles in plant lipid metabolism (Chye, 1998; Chye et al., 2000; Leung et al., 2004, 2006).

The acyl-CoA-binding domain in each ACBP has been shown to bind acyl-CoA esters, hence these ACBPs may mediate the subcellular transfer of acyl-CoA esters in plant lipid metabolism (Chye et al., 2000; Leung et al., 2004, 2006). Membrane-associated ACBP1 and ACBP2 could possibly maintain an acyl-CoA pool at the plasma membrane and participate in membrane biogenesis (Chye, 1998; Chye et al., 1999, 2000; Li and Chye, 2003; Xiao et al., 2008). We have also shown that transgenic Arabidopsis overexpressing ACBP1 showed enhanced tolerance to Pb(II)-induced stress, implying that ACBP1 could be involved in lipid bilayer membrane repair at the plasma membrane in response to Pb(II) stress (Xiao et al., 2008). ACBP3 has been demonstrated to be
extracellularly-targeted (Leung et al., 2006) while ACBP4 and ACBP5 are predicted to be localized to the cytosol (Leung et al., 2004, 2006). The preference of ACBP4 and ACBP5 in oleyl-CoA binding suggests that they could participate in the transfer of oleyl-CoA esters to the endoplasmic reticulum (ER) from the chloroplasts, in which de novo fatty acid biosynthesis occurs (Leung et al., 2004).

To elucidate the function of ACBP4, the significance of its acyl-CoA-binding domain has been addressed by using site-directed mutagenesis (Leung et al., 2004). The role of its kelch motifs in mediating protein–protein interactions is investigated here because the identification of its interactors will provide a better understanding of ACBP4 function in planta. Kelch motifs, structural repeats first observed in the Drosophila actin cross-linking protein kelch, allow protein folding into a cylindrical ‘β-propeller structure’ (Adams et al., 2000) forming a potential protein–protein interaction domain (Andrade et al., 2001). A bait-containing sequence encoding ACBP4 was constructed for yeast two-hybrid screens using a cDNA library derived from A. thaliana to identify proteins that interact directly with ACBP4. Immunoprecipitation assays were used to confirm the protein–protein interactions. Subsequently, localization of ACBP4 and its interacting protein, AtEBP, was confirmed using transient expression of GFP- and DsRed-tagged fusion proteins in Nicotiana tabacum. When the spatial and temporal expression of ACBP4 and AtEBP was examined by Northern blot analyses, their similar induced expression by the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), methyl jasmonate (MeJA) treatments, and Botrytis cinerea implicate the feasibility of their potential roles in plant defence.

Materials and methods

Yeast strain

The two-hybrid library screens were performed in the Saccharomyces cerevisiae strain YPB2 [MATa ara3 his3 ade2 lys2 trp1 leu2, 112 can1 gal4 gal80 lys2::GAL1-HIS3, URA3::(GALI-UA37mers)-lacZ] (Kohalmi et al., 1998). Cotransformants were plated on synthetic dextrose agar plates lacking leucine, tryptophan, and histidine [SD-leu-trp-his] supplemented with 10 mM 3-AT (Kohalmi et al., 1998).

Construction of a bait vector of GAL4(DB)-ACBP4 fusion

The bait plasmid pAT188 was prepared by inserting a 2 kb Xhol-NotI fragment encoding ACBP4 from pAT181 (Leung et al., 2004) into the SalI-NorI sites of pBI-880 (a variant of pPC62 as described by Chevray and Nathans, 1992; Kohalmi et al., 1998). All constructs were confirmed by restriction digestion and nucleotide sequence analysis.

Yeast two-hybrid screening

S. cerevisiae strain YPB2 was transformed with bait plasmid pAT188 and transformants were plated on synthetic dextrose agar plates lacking leucine [SD-leu]. An aliquot of transformants was also tested on [SD-leu-his] medium supplemented with 10 mM 3-amino-1,2,4-triazole (3-AT) because an absence of growth on this medium would confirm that the DB-‘bait’ fusion protein is unable to initiate transcription of HIS3. Subsequently, the bait-carrying strain was tested negative for β-galactosidase activity using the X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) colony filter assay. This further showed that the bait was not able to activate transcription of the lacZ reporter gene. The prey vector pBI-771, a variant of pPC86 (Chevray and Nathans, 1992; Kohalmi et al., 1998), was introduced into this strain and its inability to grow on [SD-leu-trp-his] medium supplemented with 10 mM 3-AT and its lack of β-galactosidase activity were confirmed before the bait was further used in cDNA library screening.

To ensure sufficient coverage in the identification of potential proteins interacting with ACBP4, yeast two-hybrid screenings were also performed at the Molecular Interaction Facility, University of Wisconsin–Madison using yeast strains and vectors as previously described by James et al. (1996). For bait preparation, ACBP4 (amino acids 1–669) was cloned in-frame with the GAL4 DNA-binding domain of bait vector pBUTe (a kanamycin-resistant version of GAL4 bait vector pGBDUC1). The resulting vector was subject to DNA sequence analysis to confirm the presence of an in-frame fusion, before use in transformation of S. cerevisiae mating type strain Pf69-4A, followed by testing for autoactivation of the β-galactosidase reporter gene. Library screenings were conducted using the Molecular Interaction Facility Arabidopsis library collection representing cDNAs from flowering Arabidopsis plants. Approximately 50 million clones were screened. Of these, positive yeast clones were tested for interaction by selection on histidine drop-out and β-galactosidase assays. Plasmids were rescued and analysed by restriction endonuclease analysis. Positive prey plasmids were retransformed into the mating type of Pf69-4A and validated in mating and selection assays with the ACBP4 bait, the empty bait vector, and unrelated control baits. Positive clones were subsequently identified by nucleotide sequence analysis using the GAL(TA)-specific forward primer BC304 (5′-CTATTCGATGAGATACC-3′) and the ADH1-terminator reverse primer, JN609 (5′-TTGATTGGAGACTTGACC-3′) (Kohalmi et al., 1998).

Co-immunoprecipitation

To corroborate the interaction from yeast two-hybrid analysis, co-immunoprecipitation studies were performed according to Mongiat et al. (2003). All constructs used in these interaction assays were derivatives of vector pBluescriptII KS(−) (pKS). The HindIII-SacI fragment from pBI-771 carrying GAL4(TA) (amino acids 768–881) was cloned into corresponding restriction sites on pKS. The GAL4(TA)-ACBP4 fusion construct was prepared by inserting ACBP4 cDNA from pAT181, on a 2 kb EcoRI-BamHI fragment, into the EcoRI-BglII sites of pKS-TA with the 5′ of TA-ACBP4 adjacent to the T3 promoter.

Two putative interactors, ADF3 (identified at the Molecular Interaction Facility, University of Wisconsin–Madison) and AtEBP (from a yeast two-hybrid screen in our laboratory) were selected for further studies. Their full-length cDNAs were generated by the Reverse-Transcriptase-Polymerase Chain Reaction (RT-PCR) using the Superscript™ First-strand synthesis system (Invitrogen, Carlsbad, CA, USA). The cDNA fragments were subsequently cloned into pGEM-T Easy (Promega, Madison, WI, USA). Potential ‘ATG’ start codons in the multiple cloning sites of pGEM-T Easy vector upstream of the ADF3 or AtEBP cDNA were eliminated by restriction endonuclease digestion followed by filling-in with Klenow and re-ligation. The cDNAs of both ADF3 and AtEBP were verified by nucleotide sequence analysis.

Subsequently, GAL4(TA)-ACBP4 and each candidate were in vitro transcribed and translated by a TNT quick coupled wheat
germ transcription-translation system (Promega, Madison, WI, USA) in the presence of [15S]methionine (ICN Pharmaceuticals Inc., Costa Mesa, CA, USA), according to the manufacturer’s instructions. The proteins were analysed by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography. Co-immunoprecipitation with monoclonal antibody against GAL4(TA) was performed according to Chye et al. (1999). Total proteins were separated on SDS–PAGE and transferred onto Hybond-C membranes (Amersham). The blots were blocked in TTBS (TBS plus 0.05% Tween 20) containing 5% non-fat milk for 2 h and incubated for an additional 2 h with anti-ACBP4 primary antibodies. The blots were washed three times with TTBS and then incubated with secondary antibody for 1 h. Either the Amplified Alkaline Phosphatase Goat Anti-rabbit Immuno-blot Kit (BioRad) or the ECL Western Blotting Detection Kit (Amersham) was used following the manufacturer’s instructions to detect cross-reacting bands. ACBP4-specific antibodies were generated by rabbit immunization using a synthetic peptide RMQTLQLRQELGAE (corresponding to amino acids 566 to 580 of ACBP4).

**Construction of plasmids used in subcellular localization**

All binary vectors used in this study were derivatives of plasmids pGDG and pGDR which contain genes encoding the autofluorescent proteins GFP andDsRed, respectively (Goodin et al., 2002). The 2 kb Xhol-BamHI fragment encoding the complete ACBP4 peptide was generated by PCR using primers ML350 and ML682 with pAT181 as template, and cloned into pGEM-T Easy vector to generate plasmid pAT280. The 2 kb Xhol-BamHI ACBP4 fragment derived from plasmid pAT280 was cloned into the Xhol and BamHI sites of pGD-DsRed to obtain pAT282 in which ACBP4 is fused to 5’ of DsRed. The plasmid pAT225 in which AtEBP is fused to 3’ of GFP has been previously described (Li and Chye, 2004). The cloning junctions in all constructs were confirmed by nucleotide sequence analysis.

**Transient expression by agroinfiltration**

Tobacco (Nicotiana tabacum var. Xanthi) plants were grown in a greenhouse at 22 °C for 6 weeks. Two days before agroinfiltration, they were maintained in a growth chamber at 22 °C under 16/8 h light/dark as specified by Goodin et al. (2002). Derivatives of Agrobacterium tumefaciens strain LBA4404 containing autofluorescent protein fusion constructs were cultured on LB solid medium supplemented with kanamycin (50 μg ml⁻¹) and streptomycin (25 μg ml⁻¹) at 28 °C for 2 d. For agroinfiltration, Agrobacterium was grown at 28 °C overnight, in LB medium supplemented with kanamycin (50 μg ml⁻¹) and streptomycin (25 μg ml⁻¹). Preparation of Agrobacterium suspension and agroinfiltration of tobacco leaves in planta were carried out following the procedures of Yang et al. (2000).

**Confocal laser-scanning microscopy**

Tobacco leaf epidermal cells from agroinfiltration were examined under a Zeiss LSM 510 inverted confocal laser-scanning microscope (Zeiss, Jena, Germany) following the settings described by Goodin et al. (2002) with minor modifications. Single optical sections were scanned as resulting images for each transient expression. For each plasmid construct, 10–15 cells were imaged with similar results. GFP fluorescence was excited at 488 nm, filtered through a primary dichroic (UV/488/543), a secondary dichroic of 545 nm, and subsequently through BP505–530 nm emission filters to the photomultiplier tube (PMT) detector. DsRed fluorescence was excited at 543 nm, the emission was passed through similar primary and secondary dichroic mirrors and finally through a BP560–615 nm emission filter to the PMT detector. Fluorescence resonance energy transfer (FRET) pairs GFP/DsRed were analysed using a confocal laser-scanning microscope (Zeiss LSM510 META). FRET measurements of DsRed emission with zero contribution from GFP, was accomplished as described by Erickson et al. (2003) using the following settings: excitation at 488 nm and emission filters, BP 505–530 nm for GFP and BP 600–637 nm for DsRed.

**Western blot analysis**

Protein extracts were prepared by homogenizing Arabidopsis protein from 3-week-old wild-type (Col-0) Arabidopsis rosettes according to Chye et al. (1999). Total proteins were separated on SDS–PAGE and transferred onto Hybond-C membranes (Amersham). The blots were blocked in TTBS (TBS plus 0.05% Tween 20) containing 5% non-fat milk for 2 h and incubated for an additional 2 h with anti-ACBP4 primary antibodies. The blots were washed three times with TTBS and then incubated with secondary antibody for 1 h. Either the Amplified Alkaline Phosphatase Goat Anti-rabbit Immuno-blot Kit (BioRad) or the ECL Western Blotting Detection Kit (Amersham) was used following the manufacturer’s instructions to detect cross-reacting bands. ACBP4-specific antibodies were generated by rabbit immunization using a synthetic peptide RMQTLQLRQELGAE (corresponding to amino acids 566 to 580 of ACBP4).

**Immuno-electron microscopy**

Arabidopsis leaves were fixed in a solution of 4% (v/v) paraformaldehyde and 0.5% (v/v) glutaradehyde in 0.1 M phosphate buffer (pH 7.2) for 20 min under vacuum and then a further 3 h at room temperature. The specimens were then dehydrated in a graded ethanol series, infiltrated in stepwise increments of LR white resin (London Resin, Theale, Berkshire, UK) and polymerized at 45 °C for 24 h. Materials for immuno-gold labelling were prepared according to the procedure of Varagona and Raikhel (1994) with the modification as described. Specimens (90 nm) were sectioned using a Leica Reichert Ultracut S microtome and mounted on formvar-coated slotted grids. Grids were incubated in a blocking solution of TTBS containing 1% (w/v) fish skin gelatin and 1% (w/v) BSA for 30 min. Anti-ACBP4 antibodies diluted 1:50 in blocking solution were added and incubated at room temperature for 2 h. The grids were then rinsed three times, each for 5 min, in TTBS and then incubated with 10 nm gold-conjugated goat anti-rabbit IgG secondary antibody (Sigma), diluted 1:20 with blocking solution. Grids were rinsed three times, each for 5 min in TTBS, following by three 5-min rinses in distilled water. After being stained in 2% (w/v) uranyl acetate for 6 min followed by 2% (w/v) lead citrate for 6 min, the sections were visualized and photographed using Philips EM208s electron microscope operating at 80 kV. Controls were performed excluding the primary antibody.

**Plant materials, growth conditions and treatment**

Tobacco (N. tabacum var. Xanthi) plants were grown in a greenhouse at 22 °C for 6 weeks. Two days before agroinfiltration, they were maintained in a growth chamber at 22 °C under 16/8 h dark/light as specified by Goodin et al. (2002). Arabidopsis thaliana ecotype Columbia (Col-0) was grown under cycles of 8 h dark at 21 °C and 16 h light at 23 °C. For Arabidopsis treatments in northern blot experiments, seedlings were grown on Murashige and Skoog (1962) medium with 2% sucrose in continuous light for 2–3 weeks and then treated with 1 mM 1-aminocyclopropane-1-carboxylic acid (ACC, Sigma-Aldrich, St Louis), 100 μM methyl jasmonate (MeJA, Sigma-Aldrich, St Louis) or water (control). Plant samples were collected at 0, 4, 8, 12, and 24 h post-treatment.

**Pathogen infection**

Three-week-old wild-type Arabidopsis plants were inoculated with Botrytis cinerea by spraying with a spore suspension (2×10⁷ spores ml⁻¹) in a solution containing 1% glucose or with water containing 1% glucose as a control. After inoculation, the plants were placed in a growth chamber with high humidity (100%) at 22 °C under a 16/8 h light/dark photoperiod as described by Xiao et al. (2004). Plant samples were collected at 0, 24, 48, and 72 h post-inoculation.
Total RNA was isolated from plant tissues following the procedure of Nagy et al. (1998). Northern blot analysis was performed as described previously (Xiao et al., 2004). Briefly, 30 μg of total RNA were separated on a 1.5% agarose gel containing 6% formaldehyde and transferred to Hybond N membranes (Amersham). To generate probes for use in Northern blot analyses, specific primers were designed for PCR-amplification: ACBP4 (ML350, 5'-CCTCGAGAATGGCTATGCCTAGGGC-3' and ML682, 5'-GGATCCACAAGGCGAATCATCATCT-3'), AtEBP (ML826, 5'-ACAGGAAAATGTTGCGGGC-3' and ML827, 5'-CAAGCTCACCACATCCACC-3') and PDF1.2 (ML741, 5'-TAAAGTTTCGC-176TTCCATCATCACCC-3' and ML742, 5'-TTACATGGGACGTACACAGATAAC-3'). Templates used in PCR were plasmid pAT282 (consisting of the ACBP4 cDNA) and the first-strand wild-type pool of cDNAs (for AtEBP and PDF1.2). The fragments were labelled with the PCR Digoxigenin Probe Synthesis Kit according to the manufacturer's instructions (Roche, Germany). Hybridization and detection were performed according to the standard procedures as advised by the manufacturer (Roche). The blots were washed under conditions of high stringency (2× SSC, 0.1% SDS for 20 min at 68°C). Yeast transformation mixture on [SD-leu-trp]. A total of 100 putative positives were selected following transformation and plating of an aliquot of the yeast YPB2 harbouring plasmid pAT188. The number of independent transformants was determined to be 2×10⁶ following transformation and plating of an aliquot of the yeast transformation mixture on [SD-leu-trp-his].

**Results**

**Yeast two-hybrid screening**

The yeast YPB2 transformed with the bait GAL4(DB)-ACBP4 could not grow on [SD-leu-his] and was tested negative on X-Gal colony filter assays (data not shown), suggesting that the pAT188 bait alone could not activate the transcription of reporter genes HIS3 and lacZ and was deemed appropriate for two-hybrid screens. A GAL4(TA) tagged A. thaliana cDNA library was introduced into the yeast YPB2 harbouring plasmid pAT188. The number of independent transformants was determined to be 2×10⁶ following transformation and plating of an aliquot of the yeast transformation mixture on [SD-leu-trp]. A total of 100 putative positives were selected on [SD-leu-trp-his] supplemented with 10 mM 3-AT medium. When these putative positives were further screened for β-galactosidase activity using the X-Gal colony filter assay, nine yeast clones that appeared blue, at varying intensities, were identified as putative clones encoding interactors. Putative library plasmids were retrieved and their nucleotide sequences were searched against the BLAST server http://www.ncbi.nlm.nih.gov/egi-bin/BLAST. Only one clone was in-frame to GAL4(TA), encoding a full-length ethylene-responsive element binding factor (ERF) protein AtEBP (Arabidopsis genome locus: AT3G16770). An AP2/EREBP (ethylene-responsive element binding protein) domain is present in AtEBP at amino acids 76–143 (Okamuro et al., 1997). In another independent yeast two-hybrid screen using the Molecular Interaction Facility (University of Wisconsin–Madison), six putative positives were identified following selection on histidine drop-out and β-galactosidase assays. Subsequently, they were used to retransform yeast mating type strain Pf69-4A, and were validated in mating and selection assays using the ACBP4 bait, the empty bait vector, and unrelated baits. Five clones were tested positive and further identified by nucleotide sequence analysis. Results from analysis using the BLAST revealed that only one clone was in-frame and it encoded a full-length actin-depolymerizing factor 3 (ADF3, At5g59880) protein. However, this was not investigated further in this study because it did not bind ACBP4 in subsequent co-immunoprecipitation, possibly due to the absence of some as yet unidentified essential cofactor(s) for binding in an in vitro co-immunoprecipitation reaction.

**Corroboration of ACBP4-interacting proteins by co-immunoprecipitation**

Proteins, generated from plasmid derivatives of pBlue-scriptII KS using in vitro transcription/translation, were analysed by 12% SDS-PAGE. An autoradiograph of the gel showed that the estimated molecular masses of the in vitro translation products of GAL4(TA)-ACBP4, AtEBP and ADF3, were 84 kDa, 28 kDa, and 16 kDa, respectively, according to their calculated molecular masses (Fig. 1B).

Co-immunoprecipitation of in vitro transcription/translation products to the GAL4(TA)-ACBP4 fusion protein, immobilized to protein A/agarose beads, using monoclonal antibody against GAL4(TA), showed that the GAL4(TA)-ACBP4 fusion protein significantly binds AtEBP (Fig. 1B). However, no binding of GAL4(TA)-ACBP4 to ADF3 was observed (Fig. 1B), perhaps due to the lack of cofactors which must be present for their in vitro interaction.

**Co-localization of ACBP4:DsRed and GFP:AtEBP**

To verify the subcellular localization of ACBP4 and AtEBP in vivo, ACBP4 was tagged to the N-terminus of DsRed, and the fusion from the CaMV 35S promoter expressed while AtEBP was tagged to the C-terminus of GFP. Following agroinfiltration of tobacco leaves with both ACBP4:DsRed and GFP:AtEBP, observations were carried out by confocal microscopy using a green filter to investigate the fluorescence pattern of GFP.
and a red filter to visualize the fluorescence of DsRed (Fig. 2). GFP:AtEBP was located mainly in the nucleus, with some signals at the cytosol and the plasma membrane (Fig. 2A). ACBP4:DsRed was localized predominantly to the cytosol, inclusive of signals detected in the cytosol surrounding the nucleus (Fig. 2B). Signals of both fusion proteins were common to the cytosol.

In FRET analysis, in cells co-expressing GFP:AtEBP and ACBP4:DsRed, not only GFP:AtEBP green fluorescence (Fig. 2D) but also ACBP4:DsRed red fluorescence (Fig. 2E), which overlapped with the GFP signals (Fig. 2F), were detected, indicating that FRET occurred between GFP:AtEBP and ACBP4:DsRed.

**Detection of ACBP4 protein in Arabidopsis**

Results from Western blot analysis using total protein from 3-week-old Arabidopsis revealed that anti-ACBP4 antibodies cross-reacted with a band of apparent molecular mass of 73.1 kDa (Fig. 3A, lane 3), as previously predicted for ACBP4 (Leung et al., 2004). Immuno-electron microscopy was carried out using transverse sections of leaves of 2-week-old Arabidopsis germinated and grown in MS medium under a 16/8 h
light/dark regime. Although immuno-gold labelling with the anti-ACBP4 antibodies was mostly evident in the cytosol, some signals were detected at the periphery of the nucleus, (Fig. 3B, C). In the control, when the primary antibody was replaced by blocking solution, no significant immuno-gold labelling was observed (Fig. 3D). The immunolocalization of signals at the periphery of the nucleus may have culminated from the interaction of ACBP4 with AtEBP.

ACBP4 and AtEBP show overlapping expression patterns

To address the coexpression of ACBP4 and its interactor, AtEBP, further, their spatial expression patterns were examined. Northern blot analyses were carried out using ACBP4 cDNA and AtEBP full-length cDNA probes, generated in PCR using gene-specific primers. Both ACBP4 and AtEBP accumulated in leaves and stems (Fig. 4, lanes L and S) of Arabidopsis young seedlings, with lower expression in the flowers and siliques (Fig. 4, lanes F and Si). ACBP4, but not AtEBP, showed higher expression in roots (Fig. 4, lane R). Taken together, ACBP4 and AtEBP appear to have some overlapping expression patterns in leaves and stems, which may represent the potential organs for their interaction in vivo.

Expressions of ACBP4 and AtEBP are induced by ACC and MeJA treatments and by Botrytis infection

It has been reported that in young Arabidopsis seedlings, ethephon induces the expression of AtEBP after 12 h, indicating that AtEBP is involved in ethylene signalling (Büttner and Singh, 1997). As ACBP4 was shown to interact with AtEBP in vitro and both displayed some similarity in spatial expression, it was investigated to find whether ACBP4 is regulated by ethylene and/or jasmonates. To this end, 2-week-old Arabidopsis seedlings were treated with 1 mM 1-amino-cyclopropane-1-carboxylic acid (ACC, the direct precursor of ethylene) and 100 µM methyl jasmonate. AtEBP mRNA and ACBP4 mRNA were induced in seedlings at 4, 8, 12, and 24 h (for ACBP4) or 8, 12, and 24 h (for AtEBP) following treatment with ACC and MeJA (Fig. 5A). An ACC-inducible and MeJA-inducible gene encoding plant defensin PDF1.2 (Penninckx et al., 1998) was used as a positive control in these experiments. Induction of AtEBP (At3g16770) and ACBP4 (At3g05420) after ACC and MeJA treatments detected in Northern blot analysis was compared with information available from microarray data analysis on AtEBP expression (www.weigelworld.org/resources/microarray). The expression of both AtEBP and ACBP4 were not inducible in microarrays at 1 h and 3 h after ACC and MeJA treatments and no data were available for a period exceeding 4 h.

In Arabidopsis, both ethylene and jasmonate have been reported to be essential for the induction of a functional defence response towards the necrotrophic fungal pathogen Botrytis cinerea (Thomma et al., 2001; Diaz et al., 2002). Expression of the ethylene downstream regulator ERF1 is up-regulated upon infection by B. cinerea (Berrocal-Lobo et al., 2002). Since ACBP4 and AtEBP mRNAs accumulated in response to ethylene and jasmonate treatments, Arabidopsis plants were subsequently infected with B. cinerea and tested for ACBP4 and AtEBP expression. Both ACBP4 and AtEBP mRNAs accumulated in the infected plants at 48 h and 72 h post-inoculation, while the control plant remained uninduced at these corresponding time points (Fig. 5B). Our findings again suggest that both ACBP4 and AtEBP are probably associated with the ethylene- and jasmonate-mediated plant defence responses.
Discussion

Kelch-motif containing ACBP4 was used as bait in yeast two-hybrid screens from which an interactor (AtEBP) was retrieved. The interaction of AtEBP and ACBP4 was further substantiated by co-immunoprecipitation and by using autofluorescent protein fusions in the transient expression of tobacco leaf epidermal cells. ACBP4 and AtEBP showed overlapping expression patterns in leaves and stems and both were inducible by ACC, MeJA treatment, and infection with the fungal pathogen, Botrytis cinerea.

Co-localization of ACBP4 and AtEBP

AtEBP was predicted to be targeted to the nucleus using the PSORT server for the prediction of the subcellular localization of proteins (http://psort.nibb.ac.jp). However, another server LOCtree (http://cubic.bioc.columbia.edu/services/loctree/; Nair and Rost, 2005) scored the Reliability Index (RI) value of AtEBP nuclear localization to be merely 1, in a range of RI values from 1–10, with 10 denoting the most confident prediction. LOCtree is a novel system of support vector machines that predict subcellular localization by the incorporation of a hierarchical ontology of localization classes modelled onto biological processing pathways (Nair and Rost, 2005). It is significantly more accurate than other traditional networks at predicting subcellular localization (Nair and Rost, 2005). In this study, GFP:AtEBP was not confined to the nucleus but was also detected in the cytosol where it could interact with ACBP4. ACBP4:DsRed, transiently-expressed in tobacco leaves, was predominantly targeted to the cytosol but...
immuno-electron microscopy indicated localization of ACBP4 in the cytosol with signals detected at the periphery of the nucleus, perhaps as a consequence of its interaction with AtEBP. Many protein factors, such as the photoreceptor phytochrome B, COP1, and some bZIP transcription factors demonstrate light-regulated movement between the cytoplasm and the nucleus (Yamamoto and Deng, 1999). Also, some transcription factors such as the Arabidopsis floral identity protein LEAFY (LFY), do move between cells (Wu et al., 2003). Therefore, interactions between ACBP4 and AtEBP at the cytosol, a location common to both may permit their similar translocation across subcellular compartments or between cells.

Interaction of ACBPs and transcription factors

When Arabidopsis cDNA libraries were screened for interacting proteins of Arabidopsis ACBPs by the yeast two-hybrid system in our laboratory, as well as at the Molecular Interaction Facility, University of Wisconsin–Madison, while we could not identify protein interactors for ACBP5, a common interacting protein (AtEBP) was identified for both ACBP2 (Li and Chye, 2004) and ACBP4. AtEBP, containing one AP2/EREBP domain, belongs to the ERF subfamily of AP2/EREBP family of plant transcription factors involved in plant growth and developmental regulation (Riechmann and Meyerowitz, 1998). The conserved AP2/EREBP domain unique to plants, has been reported to be involved in DNA binding (Ohme-Tagaki and Shinshi, 1995) and in mediating protein–protein interactions (Okamura et al., 1997). Proteins of the ERF subfamily have been demonstrated to be mainly expressed in response to biological or physical stress, such as pathogen attack, ethylene or abscisic acid (ABA) treatment, drought, and cold treatment (Zhang et al., 2004; Zhang et al., 2005). Recently, Ogawa et al. (2005) demonstrated that AtEBP conferred resistance to Bax and abiotic stress-induced plant cell death in plant cells overexpressing AtEBP. Furthermore, the function of AtEBP as a transcriptional activator may be related to ethylene signalling based on the analysis of gene expression levels in ethylene-related mutants (Ogawa et al., 2005).

Several ERF proteins have been reported to interact with other proteins including a transcriptional factor, a nitrilase-like protein, and an ubiquitin-conjugated enzyme (Büttner and Singh, 1997; Xu et al., 1998; Koyama et al., 2003). AtEBP was reported to interact in particular with an ocs element binding protein (Büttner and Singh, 1997) and ACBP2 (Li and Chye, 2004). A highly conserved motif RAYD element within the AP2/EREBP domain contains a conserved core region that is predicted to form an amphipathic α-helix. This α-helical structure has been implicated in a role in DNA binding or in mediating protein–protein interactions important for RAP2.3 (AtEBP) function (Okamura et al., 1997). The interactions between ACBPs and AtEBP imply that certain ACBPs could be involved in the regulation of

Fig. 4. Expression patterns of ACBP4 and AtEBP in Arabidopsis on Northern blot analyses. Total RNAs were extracted from wild-type Arabidopsis leaves (L), stems (S), roots (R), flowers (F), and siliques (Si). A gel blot containing about 30 µg total RNA for each lane was hybridized with an ACBP4-specific cDNA probe. The membrane was stripped and reprobed with an AtEBP-specific full-length cDNA probe. Ethidium bromide staining of rRNAs shown at the bottom indicates the relative amounts of total RNA loaded in each lane. The blots were washed under conditions of high stringency.

Fig. 5. Northern blot analyses of ACBP4 and AtEBP expression following 1-aminocyclopropane-1-carboxylic acid and methyl jasmonate treatments and B. cinerea infection. (A) Accumulation of ACBP4 and AtEBP transcripts in wild-type Arabidopsis plants grown in MS media under continuous light following treatment with 1 mM ACC and 100 µM MeJA. Thirty micrograms total RNA per lane were hybridized to the respective probes as indicated on the right of the figure. The ACC- and MeJA-inducible PDF1.2 transcript was used as a positive control. Ethidium bromide-stained rRNA is shown below the blots to indicate the relative amounts of total RNA loaded per lane; hpt, hours post-treatment. (B) Inducible expression of ACBP4 and AtEBP transcripts in wild-type Arabidopsis plants infected with B. cinerea. Thirty micrograms total RNA per lane were hybridized to the respective probes as indicated on the right of the figure. Ethidium bromide-stained rRNA is shown to indicate the relative amounts of total RNA loaded per lane; hpi, hours post-inoculation. The blots were washed under conditions of high stringency.
plant development or defence through interactions with the transcription factor AtEBP. Long-chain acyl-CoAs have been demonstrated to regulate gene expression in bacteria, yeast, and mammals (Black et al., 2000). Petrescu et al. (2003) has reported that recombinant mouse ACBP in rat hepatoma cells and in transfected COS-7 cells interacts with the hepatocyte nuclear factor-4α (HNF-4α), a nuclear binding protein that regulates the transcription of genes involved in lipid and glucose metabolism. HNF-4α also catalyses the hydrolysis of bound long-chain fatty acyl-CoAs, and subsequently binds the fatty acid product, thus allowing cross-talk between acyl-CoA-binding sites and free fatty acid binding sites in HNF-4α (Hertz et al., 2005).

**ACBP4 may play a role in plant defence and related signalling pathways**

ACBP4 has been reported to bind oleoyl-CoA esters in vitro (Leung et al., 2004). Enzymes that use acyl-CoA esters but do not contain any acyl-CoA-binding domain could possibly dock to acyl-CoA-binding proteins, via protein–protein interactions at the ankyrin repeats of ACBP1 and ACBP2 or via such interactions at the kelch motifs of ACBP4 and ACBP5, to retrieve acyl-CoA substrates. If ACBP4 were a transporter and pool former of acyl-CoA esters, it would donate acyl-CoA esters to regulatory factors reminiscent of yeast ACBP in the gene regulation of OLE1, in which saturated fatty acids induce OLE1 transcription while unsaturated fatty acids repress its expression (Choi et al., 1996). In plants, fatty acid-derived signals have been implicated in the regulation of plant defence and development (Farmer et al., 1998). Calcium-independent phospholipase A2β, a multifunctional signalling enzyme that catalyses the hydrolysis of saturated fatty acyl-CoAs at physiologically relevant concentrations, is selectively autoacylated by oleoyl-CoA, is protected from autoacylation by Ca^{2+}-activated calmodulin, and is rescued from calmodulin-mediated inhibition of phospholipase A2 activity by oleoyl-CoA (Jenkins et al., 2006).

The present study demonstrates that ACBP4, like its identified protein partner AtEBP, is induced by the defence signals ethylene, and jasmonate, and the fungal pathogen *B. cinerea*. The clear roles of ethylene and jasmonate in plant defence signalling, development, and in environmental stress mitigation is relatively well-established. Our results now suggest a possible role for ACBP4, working in conjunction with AtEBP, in mediating plant defence- and ethylene-related signalling pathways. While the precise roles for ACBP4 and AtEBP need to be addressed further, it appears that the roles of the new family of six Arabidopsis ACBPs (Leung et al., 2004) are not restricted to binding acyl-CoAs in various subcellular compartments in plant lipid metabolism, but may possibly be extended to the transfer of acyl-CoAs in relation to plant defence- and ethylene-related signalling.

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