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RESEARCH PAPER

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 autofluoresence-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

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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 oleoyl-CoA binding suggests that they could participate in the transfer of oleoyl-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. Co-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 can^r gal4 gal80 LYS2::GAL1-HIS3, URA3::*(*GAL1$_{UAS}$*17mers)-*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 *Xho*I-*Not*I fragment encoding ACBP4 from pAT181 (Leung *et al*., 2004) into the *Sal*I-*Not*I 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 PJ69-4A, 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 PJ69-4A 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′-CTATTCGATGATG-AAGATACC-3′) and the *ADH1*-terminator reverse primer, JN069 (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 *Hin*dIII-*Sac*I 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 *Eco*RI-*Bam*HI fragment, into the *Eco*RI-*Bgl*II 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 [^{35}S]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 anti-GAL4(TA) antibody (Clontech, USA) was performed following Mongiat *et al*. (2003).

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 and DsRed, respectively (Goodin *et al*., 2002). The 2 kb *Xho*I-*Bam*HI 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 *Xho*I-*Bam*HI *ACBP4* fragment derived from plasmid pAT280 was cloned into the *Xho*I and *Bam*HI 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 Assay 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 RMQTLQLRQELGEAE (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 (2×10^5 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.

Northern blot analysis

Total RNA was isolated from plant tissues following the procedure of Nagy *et al.* (1988). 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′-ACAGAGAAAATGTGTGGCGG-3′ and ML827, 5′-CAAGCATCCACATAT CCACC-3′) and *PDF1.2* (ML741, 5′-TAAGTTTGCTTCCATCATCACCC-3′ and ML742, 5′-TTAACATGGGACGTAACAGATACA-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 2×15 min at room temperature; 0.5× SSC, 0.1% for 2×15 min at 68 °C; 0.1× SSC, 0.1% SDS for 2×15 min at 68 °C).

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 2×10^6 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/cgi-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 PJ69-4A, 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.

Results of X-Gal filter assays are shown in Fig. 1A. Positive protein–protein interaction results in activation of the reporter gene β-galactosidase in yeast cells, which turns yeast colonies blue in filter assays using X-Gal. Without interaction, the yeast colonies remain 'colourless'. As shown in Fig. 1Aa, the GAL4(DB)-ACBP4 fusion interacted with GAL4(TA)-AtEBP, as indicated by the blue colour arising from the production of significant levels of β-galactosidase. No interactions were observed in control yeast cells harbouring GAL4(DB)-ACBP4+GAL4(TA) (Fig. 1Ab) and GAL4(DB)+GAL(TA)-AtEBP (Fig. 1Ac). Therefore, from yeast two-hybrid analysis, AtEBP was identified as a putative protein that interacts with ACBP4.

Corroboration of ACBP4-interacting proteins by co-immunoprecipitation

Proteins, generated from plasmid derivatives of pBluescriptII 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

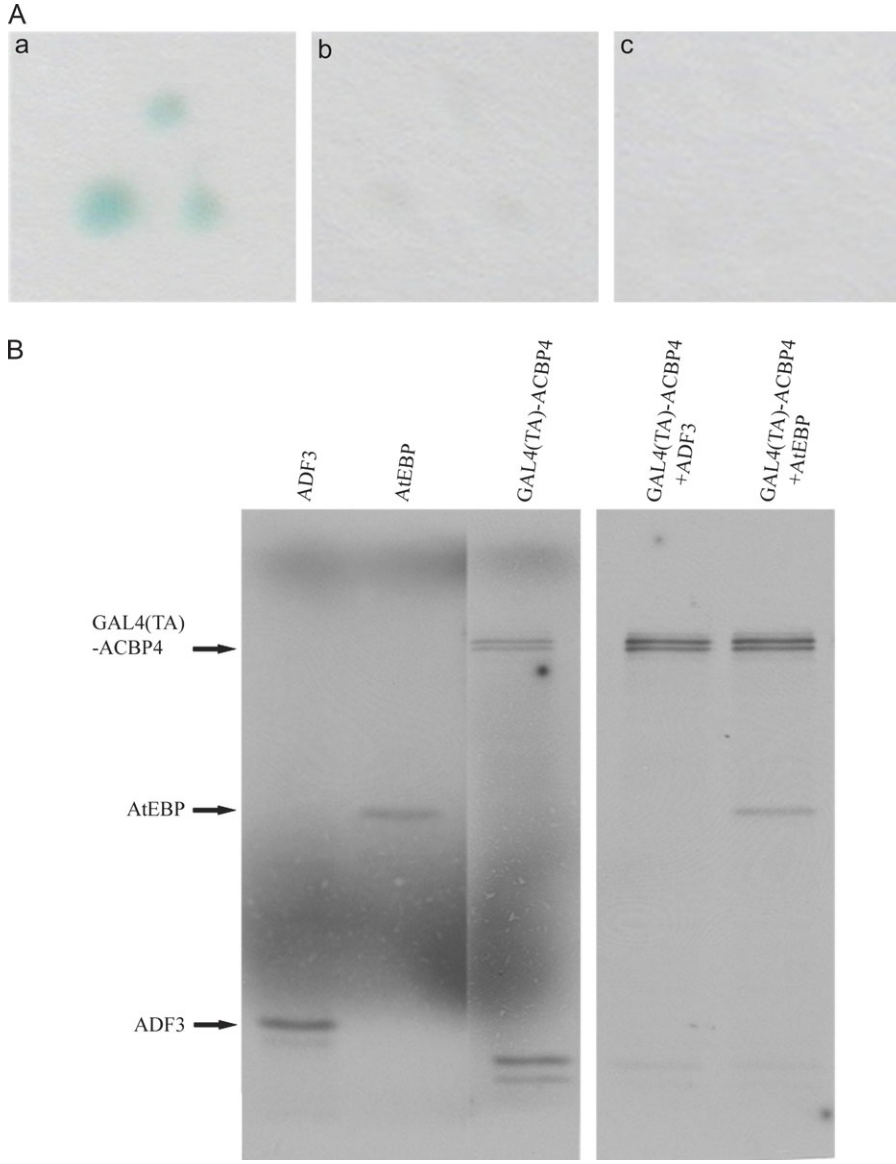

Fig. 1. (A) Colony filter β-galactosidase assays of candidate proteins AtEBP from yeast two-hybrid screens. (a) YPB2/GAL4(DB)-ACBP4+GAL4(TA)-AtEBP; (b) YPB2/GAL4(DB)-ACBP4+GAL4(TA); (c) YPB2/GAL4(DB)+GAL(TA)-AtEBP. (B) Co-immunoprecipitation of ACBP4 and AtEBP using the anti-GAL4(TA) monoclonal antibody. Autoradiograph of a 12% SDS-PAGE (left panel) showing the *in vitro* transcribed and translated ADF3, AtEBP, and GAL4(TA)-ACBP4, respectively, as indicated. The right panel shows the co-immunoprecipitation of equimolar amounts of GAL4(TA)-ACBP4 and ADF3 or AtEBP using the anti-GAL4(TA) antibody. Arrows indicate the positions of these proteins.

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

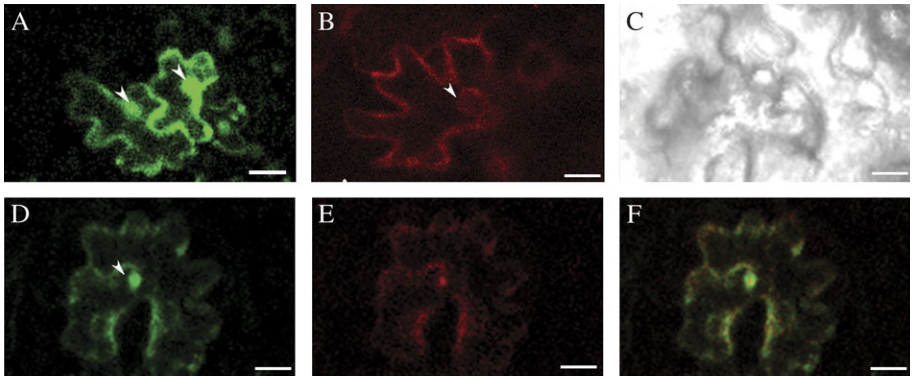

Fig. 2. Confocal images indicating co-localization of ACBP4:DsRed and GFP:AtEBP fusion proteins transiently-expressed in tobacco leaves. Representative tobacco leaf epidermal cells are shown by laser-scanning confocal microscopy following agroinfiltration of plasmid pAT282 or pAT225. (A) GFP:AtEBP expressed from pAT225 in a tobacco leaf. (B) ACBP4:DsRed expressed from pAT282 in a tobacco leaf. (C–F) FRET detection in tobacco leaf epidermal cells co-expressing GFP:AtEBP and ACBP4:DsRed; (C) differential interference contrast image of D-F; (D) green channel shows GFP:AtEBP; (E) red channel shows FRET signal of ACBP4:DsRed; (F) co-localization of two signals is indicated by a yellow colour in merged images of (D) and (E). Arrowheads indicate the position of nuclei. Bar=20 μm.

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* 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.

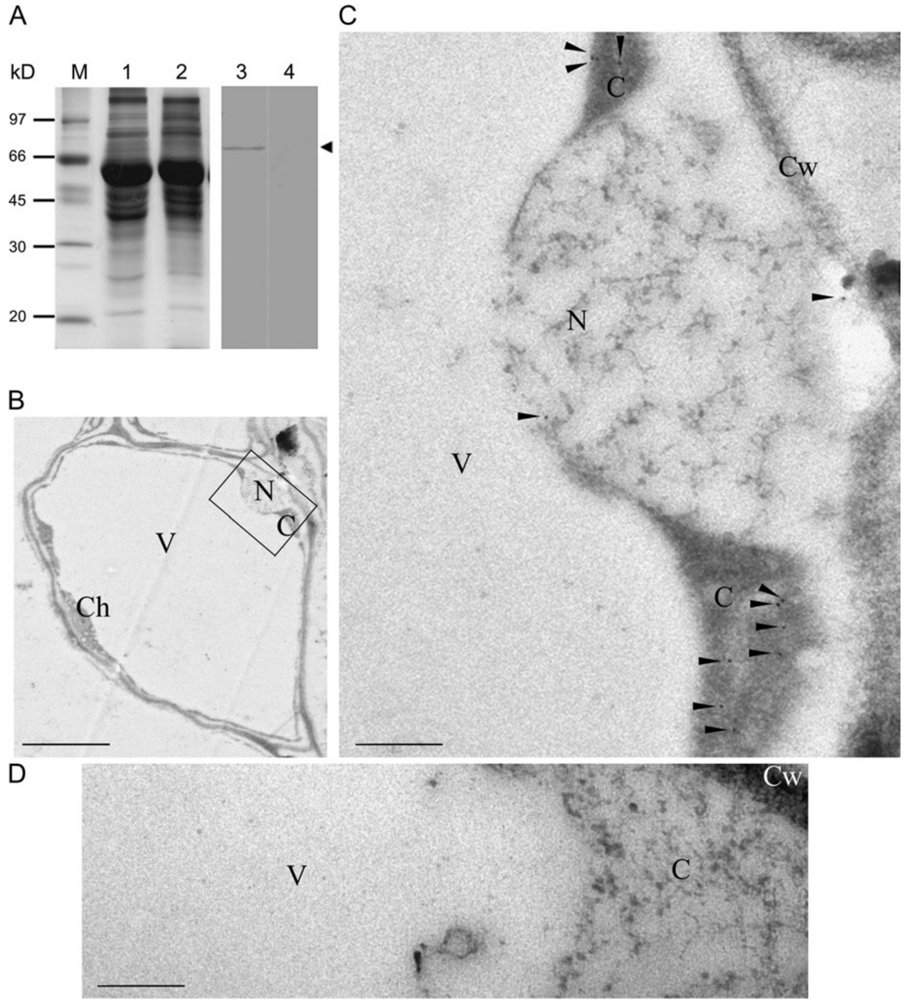

Fig. 3. Localization of ACBP4 in *Arabidopsis* leaves. (A) Western blot analysis using affinity-purified anti-peptide antibodies against ACBP4. Lanes 1 and 2: gel identically loaded as lanes 3 and 4, respectively, stained with Coomassie blue to show the amount of protein blotted in western blot analysis. Lanes 3 and 4: western blot analysis of total protein using anti-ACBP4 antibodies (lane 3) and preimmune serum (lane 4). Lane M, molecular marker in kDa. (B, C, D) Immuno-gold labelling of ACBP4 in an *Arabidopsis* leaf cell using transmission electron microscopy. Transverse sections were stained with affinity-purified ACBP4-specific antibodies. (B) Transverse sections of leaves stained with ACBP4-specific antibodies. (C) Magnification of the boxed area in (B). (D) Control labelling of a leaf cell using secondary antibodies alone. Arrowheads, gold particles. V, vacuole; C, cytosol; Ch, chloroplast; N, nucleus; Cw, cell wall; Bars in (B) represent 2 μm, and in (C, D), 0.2 μm.

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

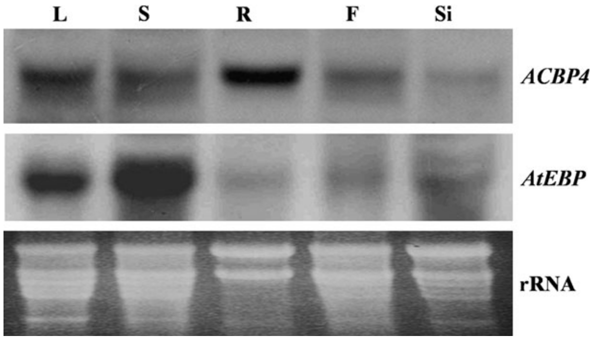

Fig. 4. Expression patterns of *ACBP4* and *AtEBP* in *Arabidopsis* on Northern blot analyses. Total RNAs were extracted from wild-type *Arabidopsis* leaves (L), stems (S), roots (R), flowers (F), and siliques (Si). A gel blot containing about 30 μg total RNA for each lane was hybridized with an *ACBP4*-specific 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.

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 (Okamuro *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*.

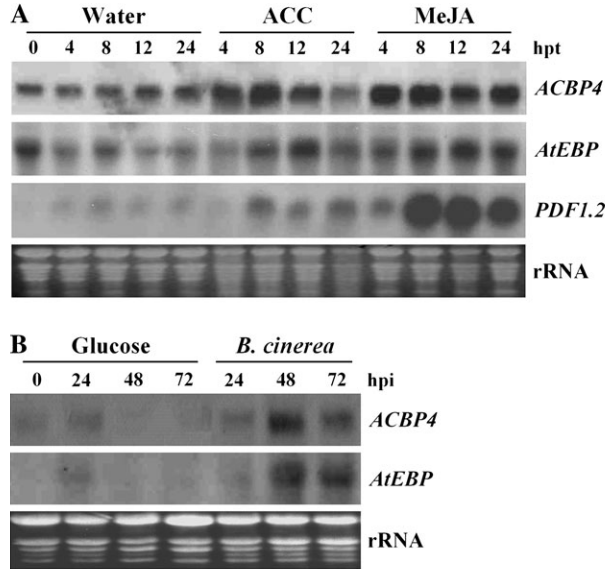

Fig. 5. Northern blot analyses of *ACBP4* and *AtEBP* expression following 1-aminocyclopropane-1-carboxylic acid and methyl jasmonate treatments and *B. cinerea* infection. (A) Accumulation of *ACBP4* and *AtEBP* transcripts in wild-type *Arabidopsis* plants grown in MS media under continuous light following 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 was 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.

(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 (Okamuro *et al*., 1997). The interactions between ACBPs and AtEBP imply that certain ACBPs could be involved in the regulation of 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 $A_2\beta$, 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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