Isolation and characterization of a cDNA encoding a membrane bound acyl-CoA binding protein from *Agave americana* L. epidermis

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Abstract

A cDNA encoding an acyl-CoA binding protein (ACBP) homologue has been cloned from a cDNA library made from mRNA isolated from epidermis of young leaves of *Agave americana* L. The derived amino acid sequence reveals a protein corresponding to the membrane-associated form of ACBPs only previously described in *Arabidopsis* and rice. *Northern blot* analysis showed that the *A. americana* ACBP gene is mainly expressed in the epidermis of mature zone of the leaves. The epidermis of *A. americana* leaves have a well developed cuticle with the highest amounts of the cuticular components waxes, cutin and cutan suggesting a potential role of the protein in cuticle formation.

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1. Introduction

The plant cuticle is an extracellular lipid membrane that covers the surface of aerial organs of higher plants. It is mainly composed of a matrix of cutin biopolymer, with waxes embedded in (intracuticular) and deposited on its surface (epicuticular) [8].

The biosynthesis of cuticular components takes place inside the epidermal cells, and begins inside the plastid, where their constituent essential fatty acids are synthesized. Then, they are exported to the ER, where they are modified, and later to the plasma membrane [8,12]. Finally, they have to be secreted across the plasma membrane and the cell wall to the outside of the cell, where they will deposit and form the main cuticle components: waxes and cutin. The mechanism of transport of cuticular components from the plastid to the extracellular space is still unknown. Recent studies have suggested the participation of some isoforms of acyl-CoA binding proteins (ACBPs) in the transport of cuticle components from the endoplasmic reticulum (ER) via vesicles to the plasma membrane, and across the plasma membrane to the cell wall [3].

ACBPs are proteins able to bind long chain acyl-CoA esters (C14–C22) with high affinity [11]. There are two classes: cytosolic ACBPs, and those membrane associated. The cytosolic ones (approximately 10 kDa of molecular mass) appear to act as intracellular acyl-CoA transporters, regulating the levels of these compounds inside the cell. Studies in *Brassica* suggest that they could be involved in acyl-CoA transport from the plastid to the ER [9].

Membrane ACBPs are longer proteins, around 30 kDa in plants, that contain a transmembrane region in one of their ends, and an acyl-CoA binding domain [2,15]. In plants, they have been found only in *Arabidopsis thaliana* [2,4,15] and, more recently, in rice [10]. In *Arabidopsis*, several isoforms have been also identified [2,7,14]. Immunolocalization studies in this plant suggest that some of these isoforms could participate in the transport of cutin monomers and waxes via vesicles from the ER to the plasma membrane, as well as across the...
membrane to the cell surface, playing a similar role to that initially ascribed to lipid transfer proteins (LTPs) [3,2].

The leaves of the monocot Agave americana L. present a prominent epidermis, being an appropriate model to study the lipid metabolism involved in the biosynthesis of plant cuticle [17,19]. In the present work, the isolation, characterization and expression of a novel cDNA coding for a membrane ACBP from A. americana leaf epidermis are described. Further relationships to the main cuticle components synthesis are also discussed.

2. Results and discussion

The cDNA library constructed from young leaf epidermis of A. americana was used to isolate ACBP cDNAs by polymerase chain reaction (PCR) using degenerate primers designed on the most conserved regions of other plant ACBPs. The full length clone isolated, named ACBP-1, was 1492 bp long and coded for a polypeptide of 355 residues (~40 kDa) with high identity to other membrane ACBPs deduced from A. thaliana cDNAs (60%). Identity values were lower with other membrane ACBP from bovine brain (47%), and with other plant soluble ACBPs such as those from Brassica napus and Ricinus communis (45%). Percentages of identity were calculated using the BlastP program (http://www.ncbi.nlm.nih.gov/BLAST). At nucleotide level, a BLASTN search confirmed that the Agave sequence had maximum identity with other membrane ACBP cDNAs from plants: 89% to Arabidopsis ACBP-1 (GenBank accession number U75273), 82% to Arabidopsis ACBP-2 (GenBank AF320561), and 80% to rice ACBP (GenBank AK105775).

The ACBP-1 peptide from Agave epidermis presented common features observed in other membrane ACBPs such as a potential transmembrane region, uncleavable, at its N-terminus, an acyl-CoA binding domain, in the most conserved zone of the protein, and an ankyrin repeat region at its carboxy terminus (Fig. 1). An aspartic acid-rich region was also identified in the peptide sequence (Fig. 1).

The prediction of non-cleavage for the transmembrane region was obtained using the PSORTWWW server, available on http://psort.nibb.ac.jp. This server also predicted that the protein probably enters the vesicular pathway, and may be located, at subcellular level, at the ER, the plasma membrane and outside the cell.

The hydropathy plot of ACBP-1 from A. americana showed that it contains a hydrophobic region just at its N-terminus (Fig. 2B). This zone was located between residues 8 and 30, and corresponded to the transmembrane region according to SOSUI prediction (http://sosui.proteome.bio.tuat.ac.jp). This domain could serve potentially to anchor ACBP to the membrane of the vesicle, thus permitting the binding of the acyl-CoA ester to the acyl-CoA binding domain of ACBP inside the lumen, and transporting the former to its final destination inside the vesicle.

As occurs with membrane bound ACBP-1 (MotifScan predictions at http://hits.isb-sib.ch/cgi-bin/PFSCAN) and ACBP-2 from Arabidopsis [4,14,15] and rice [10], the peptide from A. americana also presented at its carboxy terminus homology (34–42% identity) to proteins containing ankyrin repeats including Arabidopsis AKR (GenBank Q05753), mouse skeletal muscle ankyrin repeat protein (Q9WY06), human tankyrase (O95271) and several protein phosphatases from mouse and human (Q8BG95, O60237). According to BLASTP predictions, A. americana ACBP contained four consecutive ankyrin repeats at its carboxy terminus (Fig. 1). The ankyrin repeat is one of the most common protein–protein interaction motifs in nature, and has been found in proteins of diverse function as transcriptional initiators, cell-cycle regulators, cytoskeletal, ion transporters and signal transducers [16]. Recently, it has been shown by yeast two-hybrid analysis and transient expression experiments in tobacco that membrane ACBP2 from Arabidopsis interacts via its ankyrin motifs with an Arabidopsis ethylene-responsive element-binding protein AtEBP, probably at the plasma membrane [14].

The comparison with other proteins registered in the PROSITE database (www.expasy.org/prosite) revealed that, as membrane ACBPs described in Arabidopsis and rice, the peptide from A. americana also presented several putative sites of phosphorylation by protein kinase C and casein kinase II, as well as N-myristoylation sites. These results may suggest a possible role of these proteins in the process of signal transduction associated to the process of acyl-CoA transport to the plasma membrane [5].

Fig. 2A shows an alignment of ACBP-1 from A. americana with other ACBPs described in plants. It is noticeable the high degree of conservation at the acyl-CoA binding domain, with identity values around 85% among membrane ACBPs and around 50% with the cytosolic ones.

The analysis of the expression by Northern blot on A. americana leaf revealed that ACBP-1 was preferentially expressed in the epidermis, with levels almost undetectable in leaf without epidermis (Fig. 3). The expression was also monitored in different parts of the leaf. Specifically, A. americana leaf was divided into three parts: zone (1), corresponding to the basal, youngest zone of the leaf, usually eilolated and active in cell division; zone (2), that presented an intermediate growth rate; and zone (3), in the apical, oldest part of the leaf, with no cell division. Fig. 3 clearly shows that the epidermis of the older parts of the leaf (zones E2 and E3), characterized by a thicker and denser cuticle, showed higher levels of the messenger.

The older zones of A. americana leaf usually present an epidermis with a cuticle enriched in waxes, cutin and cutan, a non-hydrolyzable polymer derived from unsaturated fatty acids [19], more than the younger zone mainly constituted by a soft cutin and small amounts of waxes. Concerning the three leaf zones described above, the amount of the different cuticle components was determined and is showed in Fig. 4. It revealed that the amounts of waxes, cutin and cutan from the isolated cuticles of each leaf segment increased from the leaf base up to the tip of the leaf. Cutin and cutan, the major components, reached their maximum between the zones 2 and 3 (Fig. 4).
The expression profile of the ACBP identified in *A. americana* leaf epidermis suggests the hypothesis of an active participation of the protein in the transport of acyl-CoA esters constituent of cuticular components as waxes, cutin and/or cutan monomers, from the epidermal cell to the outside. Using immunogold labeling, Chye et al. [3] showed that an *Arabidopsis* membrane ACBP was localized to vesicles and to the plasma membrane of epidermal cells of the embryo, as well as to the cell wall of outer cells of the seed coat. These authors suggested that ACBP leaves the ER via vesicles and transports lipids to the plasma membrane. Data presented and discussed in this communication give additional information and indicate
A) Alignment among different ACBPs from plants: the soluble ones, shorter in length (*B. napus*, [Q39315](https://www.uniprot.org/uniprot/Q39315); *Gossypium hirsutum*, [Q39779](https://www.uniprot.org/uniprot/Q39779); *R. communis*, [O04066](https://www.uniprot.org/uniprot/O04066)); and those membrane associated (deduced from GenBank ESTs [U75273](https://www.ncbi.nlm.nih.gov/nuccore/U75273) and [AF178947](https://www.ncbi.nlm.nih.gov/nuccore/AF178947), from *A. thaliana*; [AK105775](https://www.ncbi.nlm.nih.gov/nuccore/AK105775), from rice; and [AY650903](https://www.ncbi.nlm.nih.gov/nuccore/AY650903), from *A. americana*). The putative transmembrane regions appear underlined. The acyl-CoA binding domain, highly conserved, is indicated in bold. Residues conserved in all the ACBPs are underlined at the bottom line. Asterisks at the top line denote those residues shown to be involved in acyl-CoA binding in other species. The alignment was performed using the ClustalW program. (B) Hydropathy plot of the peptide deduced from *A. americana* ACBP-1 cDNA clone. Hydropathy coefficient (in Y axis) was calculated according to Kyte and Doolittle [13]. Positive values in Y are considered hydrophobic, and those negative, hydrophilic. The putative transmembrane domain can be observed at the amino-terminus of the protein.
A potential relationship between plant cuticle genesis and the role of the ACBPs. Further research is necessary in order to clarify these relationships.

3. Conclusions

To conclude, we can affirm that the epidermis of leaves of *A. americana* contains an ACBP closely related to other membrane-associated form of ACBPs only previously reported for two species. Moreover, the ACBP gene characterized in this communication is mainly expressed in the mature zone of *A. americana* leaves. A potential relationship between the accumulation of leaf cuticle components and gene expression has been also shown.

4. Methods

4.1. Cuticle isolation and determination of cuticular components

Cuticles from epidermis of leaves of *A. americana* L. plants were isolated following the method previously described by Villena et al. [19]. Cuticular waxes were removed by refluxing in chloroform/methanol (1:1, v/v) for 4 h. The amount of the biopolymers cutin and cutan was determined after the polysaccharide material was removed by immersion of the dewaxed isolates in anhydrous hydrogen fluoride in pyridine for 5 h at 40 °C. Phenolic material present in the cuticles was removed by refluxing the samples in HCl 1 N in 1,4-dioxane. Cutin was depolymerized by saponification of the corresponding samples in 1% (w/v) potassium hydroxide in methanol for 6 hours under reflux conditions. The residual material is cutan [19].

4.2. RNA isolation

Epidermises from *A. americana* L. young leaves (approximately 15 cm long) were peeled, immediately frozen in liquid nitrogen and stored at −80 °C. The material was ground with pestle and mortar in liquid nitrogen just before RNA extraction.

Total RNA from leaf epidermis was isolated according to the method described by Chang et al. [1]. The same procedure for RNA extraction was used for peeled young leaves.

4.3. Northern blot analysis

Total RNA (10 μg) was denatured in a 20 M MOPS (pH 7), 4 mM sodium acetate, 1 mM EDTA, 6.4% formaldehyde, 49% formamide buffer for 15 min at 55 °C, and electrophoretically resolved in a 1.5% (w/v) agarose gel prepared in 40 mM MOPS (pH 7), 8 mM sodium acetate, 2 mM EDTA, 6.6% formaldehyde buffer. The running buffer was 20 M MOPS (pH 7), 4 mM sodium acetate, 1 mM EDTA. RNA was visualized on the gel by ethidium bromide staining, and then transferred onto Hybond-N (Amersham) membrane and UV fixed. An *A. americana* ACBP-1 specific probe, 619 bp long, containing the 3′-UTR of the cDNA clone was prepared by subcloning, labeled with [α-32P]dCTP by hexanucleotide random priming [15], and subsequently used for Northern blot hybridization. Prehybridization was performed in sodium phosphate 0.3 M (pH 7.2), 7% (w/v) SDS, 1% (v/v) BSA, 1 mM EDTA buffer for 1 h at 65 °C, and followed by 12 h of hybridization at 65 °C in fresh prehybridization solution containing the radiolabeled probe. Subsequent washes were carried out in 2 × SSC, 0.1% SDS for 5 min at room temperature; 2 × SSC, 0.1% SDS for 15 min at 65 °C; 2 × SSC, 0.1% SDS for 30 min at 65 °C, and 2 × SSC, 0.1% SDS for 30 min at 65 °C. The filters were finally exposed at −80 °C to a KODAK film using an intensifying screen.

4.4. Isolation of a cDNA encoding ACBP by PCR

A cDNA library from young leaf epidermis of *A. americana*, cloned in Lambda ZAP Express vector (Stratagene), was previously constructed and available in our laboratory. This library was used as template to amplify ACBP cDNA clones using degenerate primers based on the sequence of conserved regions described in other plant ACBPs [6]. The
sequence of the full length cDNA was obtained in two steps. First, the 5′-end of the clone was obtained amplifying the library using T3 standard primer and ACBPR degenerate primer; and, in a second step, we obtained the sequence of the 3′-end of the cDNA by amplification of the library with T7 standard primer and ACBP-BL specific primer. The sequence of ACBP-BL primer was designed on the sequence of the partial cDNA previously isolated in the first step. Both fragments presented a 100% of identity in a 100-bp overlap.

The sequences of ACBP primers used in the amplifications were as follows: ACBPR degenerate primer, 5′-CT(G/T)CCA (A/T)GC(A/T)T(G/C)CCACTTG 3′; degenerate positions are indicated in brackets. The sequence of Agave ACBP-BL specific primer was: 5′-ACGGAGGGCCCTGTACTAA 3′.

Amplification reactions were performed in a 20 μl final volume containing an appropriate dilution of the cDNA library as template, 2 μM of each primer, 0.1 mM of each deoxynucleotide triphosphate, 1 U of Biotherm™ DNA Polymerase (Gencraft), in a 16 mM (NH₄)₂SO₄, 67 mM Tris–HCl pH 8.8, 1.5 mM MgCl₂, 0.01% Tween-20 buffer. Before PCR, the mixture was heated at 94 °C for 2 min, and after amplification the reaction was extended at 72 °C for 5 min and finally cooled at 4 °C before analysis. ACBP sequences were amplified in a PTC-200 Peltier Thermal Cycler (MJ Research) during 35 cycles using the following program: 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 1 min. After amplification, the samples were resolved by electrophoresis onto 1.5% agarose gels in TAE 1 × buffer. Fragments of interest were isolated from the gel, subsequently cloned in pGEM-T Easy vector (Promega) and sequenced.

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References