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RESEARCH PAPER

Isolation, characterization, and localization of *AgaSGNH* cDNA: a new SGNH-motif plant hydrolase specific to *Agave americana* L. leaf epidermis

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Abstract

GDSL and SGNH hydrolases are lipases involved in a wide range of functions, behaving in many cases as bifunctional enzymes. In this work, the isolation and characterization of *AgaSGNH*, a cDNA encoding a member of the SGNH-hydrolase superfamily from young leaf epidermis of the monocot *Agave americana* L., is reported. The protein possesses a typical signal peptide at its *N*-terminus that allows its secretion to the epidermis cell wall, as verified by immunolocalization experiments. In addition, the AgaSGNH sequence contains a His-Leu-Gly-Ala-Glu (HLGAE) motif which is similar to that observed in other plant acyltransferases. Expression levels by northern blot and *in situ* localization of the corresponding mRNA, as well as the immunolocalization of the protein in *Agave* young leaves indicate that the protein is specifically present in the epidermal cells. The detailed study performed in different parts of the *Agave* leaf confirms two aspects: first, the expression of *AgaSGNH* is limited to the epidermis, and second, the maximum mRNA levels are found in the epidermis of the youngest zones of the leaf which are especially active in cutin biosynthesis. These levels dramatically decrease in the oldest zone of the leaf, where the presence of *AgaSGNH* mRNA is undetectable, and the biosynthesis of different cuticle components is severely reduced. These data could be compatible with the hypothesis that AgaSGNH could carry out both the hydrolysis and the transfer, from an activated acyl-CoA to a crescent cutin in *Agave americana* leaves and, therefore, be involved in the still unknown mechanism of plant cutin biosynthesis.

Key words: *Agave americana*, epidermis, plant cuticle, plant lipases, SGNH-hydrolases.

Introduction

Hydrolases are enzymes that catalyse bond cleavage by reaction with water, and also condensations (reversal of hydrolysis) and alcoholysis (a cleavage using an alcohol in place of water) even in neat organic solvents (Bornscheuer and Kazluskas, 1999). α/β-hydrolase fold enzymes are a subclass of hydrolases which show similar 3D structures in spite of their low sequence similarity at the amino acid level (Ollis *et al*., 1992). This fold consists of a core of eight mostly-parallel β-sheets, which are surrounded by α-helices (Holmquist, 2000). Both lipases (EC 3.1.1.3) and esterases (EC 3.1.1.1) which belong to this group of enzymes, act by breaking the ester bonds found in some macromolecules in living beings. They are serine esterases because of the presence of a serine (Ser) acting as a nucleophile, together with a histidine (His) acting as a base, and an aspartic or glutamic acid (Asp, Glu) forming the typical catalytic triad of these enzymes (Rubin, 1994). Common esterases possess the catalytic Ser residue inside the highly conserved motif Gly-X-Ser-X-Gly, X being any amino acid and G is glycine (Gly). This motif is located around the middle of the amino acid sequence of the protein, His is normally close to the carboxy terminus and Asp is between both, more or less equidistant.

In 1995, Upton and Buckley described a new subfamily of lipases, the GDS[L]-motif like (pfam PF00657), which showed the residues around the catalytic Ser, 'Gly-Asp-Ser-[Leu]', highly conserved (Upton and Buckley, 1995).

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Moreover, this subfamily possesses other singular and differential characteristics that define it, such as the presence of the catalytic Ser not in the middle of the sequence, but near the *N*-terminus of the protein, and Asp and His arranged close to each other and situated at the *C*-terminus of the sequence (Upton and Buckley, 1995). The identity between GDSL lipases at sequence level is low, but they share five blocks of highly-conserved homology (I–V), which are used to classify them (Upton and Buckley, 1995). This family of enzymes comprises a wide range of bacterial proteins, such as *Aeromonas hydrophila* lipase/acyltransferase (Robertson *et al*., 1994), *Vibrio parahaemolyticus* haemolysin/phospholipase (Shinoda *et al*., 1991) or *Escherichia coli* thioesterase I (EC 3.1.2.2) (Cho and Cronan, 1993), and some plant proteins, such as *Arabidopsis thaliana* and *Brassica napus* proline-rich proteins, with no functional role still assigned (Roberts *et al*., 1993). Some of plant GDSL-motif like lipases have been classified, following a structural criterion, into a new superfamily known as SGNH-hydrolases, due to the presence of four invariant catalytic residues located in critical positions along its amino acid sequence: the catalytic Ser at the *N*-terminus and inside the GDSL motif, a Gly in conserved block II, an Asn in block III, and the catalytic His in block V, at the *C*-terminus of the sequence (Molgaard *et al*., 2000; Akoh *et al*., 2004). In SGNH-hydrolases, last-mentioned His, the Asp situated two residues before, and Ser in the GDSL motif, shape the catalytic triad of common serine hydrolases (Dalrymple *et al*., 1997; Li *et al*., 2000; Lo *et al*., 2003). The four residues that give the name to the superfamily Ser, Gly, Asn, and His (SGNH), are very important for activity, providing a different catalytic mechanism from that known for common α/β-hydrolases (Lo *et al*., 2003). In *Arabidopsis thaliana*, crystallographic studies on the protein product of the At4g34215 gene have shown that conserved Gly and Asn residues are involved in the active site of these enzymes providing hydrogen bonds to the oxyanion hole (Bitto *et al*., 2005).

As some aspects of its structure indicate, many of this type of lipases are extracellular. Secreted proteins possess a short stretch of amino acids at their *N*-terminus that acts as a sorting signal sequence, and targets them to the extracellular matrix (Nielsen *et al*., 1997). The size of this signal peptide is around 25 residues (Shaw *et al*., 1994).

A broad range of substrates has been described for the enzymes of the SGNH-hydrolase superfamily, such as complex polysaccharides (Dalrymple *et al*., 1997; Molgaard *et al*., 2000), lysophospholipids (Lo *et al*., 2003), acyl-CoA esters (Robertson *et al*., 1994), and other compounds (Shaw *et al*., 1994). Some plant GDSL esterases/lipases have been isolated and their hydrolytic activity determined, being involved in fatty acyl-ester hydrolase activity (Beisson *et al*., 1997), degradation of complex polysaccharides of the plant cell wall such as xylose and rhamnogalacturonan (Dalrymple *et al*., 1997), or in root nodulation processes (Arif *et al*., 2004). Plant defence has also been associated with GDSL proteins; in this sense, Oh and co-workers described, using a proteomic approach, a cell wall-secreted lipase with a GDSL-like motif (GLIP1) present in the secretome of *Arabidopsis thaliana* (Oh *et al*., 2005). The authors suggest that this protein, via ethylene signalling, could be a critical component in plant resistance to the necrotrophic fungus *Alternaria brassicicola* by two distinct mechanisms: disrupting spore integrity and activating defence signalling.

The outermost epidermal cell wall of land plants is covered by the cuticle, a hydrophobic membrane which is composed mainly by waxes, hydrolysable compounds (mainly polysaccharides), and a polyester called cutin which, in the majority of plants studied, is principally constituted by ω- and mid-chain-hydroxy C16 and C18 fatty acids interconnected by ester bonds (Walton, 1990). Recent studies have demonstrated that *Arabidopsis thaliana* possesses a particular cutin composition, with an unusual content of α-ω dicarboxylic fatty acids (40%) and is highly similar to suberin, the other most important polyester in plants (Bonaventure *et al*., 2004; Franke *et al*., 2005). From the middle of the 1990s, many studies have been developed focused on structural analysis, biosynthesis mechanisms, and molecular biology of plant cuticle components, specially on waxes and cutin (Negruk *et al*., 1996; Xia *et al*., 1996; Sieber *et al*., 2000; Wellesen *et al*., 2001; Kunst and Samuels, 2003; Broun *et al*., 2004). However, except for the work carried out by Kolattukudy in the 1970s (Kolattukudy, 1970; Croteau and Kolattukudy, 1974), little progress has been made on the discovery of enzymes involved in cutin formation. Despite knowledge on the pathways of cutin monomer biosynthesis, the mechanisms by which these monomers are transported to their site of deposition and polymerize, building the complex matrix of cutin, have still to be elucidated. Kurdyukov and co-workers have recently described an *Arabidopsis thaliana* mutant, BODYGUARD, impaired in the formation of a normal cuticle, which is altered in the protein BDG (Kurdyukov *et al*., 2006*a*). The appearance of the cuticle in *bdg* mutants resembles that of transgenic *Arabidopsis* cutinase-overexpressing plants obtained by Sieber and co-workers that mimic a cutin-deficient plant by degrading cutin from inside itself (Sieber *et al*., 2000). BDG is an epidermis-specific cell wall-located protein and belongs to a new class of extracellular α/β-hydrolase fold proteins involved in cuticle formation. The authors postulate that this protein could have synthase activity and may be responsible for the direct formation of the plant cuticle. In 1974, Croteau and Kolattukudy showed that a particulate extract obtained from epidermis of *Vicia faba* young leaves, possessed an acyltransferase activity capable of incorporating free hydroxy fatty acids into a growing cutin primer (Croteau

and Kolattukudy, 1974). However, to date, no enzyme with such ability has been purified and characterized.

The isolation and characterization of *AgaSGNH* from epidermis of young leaves of the monocot *Agave americana* L. is reported here. This cDNA encodes a member of the plant SGNH-hydrolase superfamily. Expression level studies by northern blot and *in situ* localization of the corresponding mRNA, as well as the immunolocalization of the protein in *Agave* young leaves, were carried out. These results, in correlation with the analysis of cuticle components along the leaf recently described in this species (Guerrero *et al*., 2006), could lead to suggest a hypothetical role of the protein in the metabolism of the epidermis cell wall and/or in the metabolism of the plant cuticle.

Materials and methods

Plant material

Agave americana L. plants were collected from their natural environment in the province of Málaga (Spain) 36°42′ N 4°25′ W. Unfolded leaves were collected from young 30 cm length centre rosettes (Fig. 1A), and leaf epidermis (Fig. 1C) and leaf tissue without epidermis were peeled-off manually, immediately frozen in liquid nitrogen, and stored at –80 °C until used. Roots were taken from the same young plants (Fig. 1B).

RNA purification

Total RNA was isolated from frozen leaf epidermis, leaf tissue without epidermis and root tissue from young plants of *Agave americana* L. Two grams of tissue were put in 50 ml polypropylene tubes and suspended in 15 ml of 65 °C preheated 2 M NaCl, 25 mM EDTA pH 8, 100 mM TRIS–HCl pH 8, 2% (w/v) polyvinylpyrrolidone average molecular weight 40 kDa (PVP40), 2% (w/v) hexadecyltrimethylammonium bromide (CTAB), 0.05% spermidine (w/v), and 0.2% (v/v) β-mercaptoethanol buffer. After 2 min incubation, 15 ml of chloroform:isoamylalcohol (CIA) 24:1 v/v were added per tube. After a vigorous vortex, samples were centrifuged 30 min at 6000 *g* at room temperature. The water-soluble upper phase was transferred to a new tube, extracted again with an equal volume of CIA 24:1 v/v, mixed, and centrifuged as before. The process was repeated until no solid was observed between the upper and lower phases. RNA was precipitated with 0.25 vols of 10 M LiCl at –20 °C overnight, and then centrifuged 60 min at 4 °C and 6000 *g*. The pellet was resuspended in 1 ml of the supernatant and transferred to a new 1.5 ml tube. It was then centrifuged for 30 min at 4 °C at 13 000 *g* in a microcentrifuge and the pellet was resuspended in 500 μl of a buffer composed of 1 M NaCl and 5% (w/v) sodium dodecyl sulphate (SDS) preheated at 65 °C. After resuspension the sample was vigorously mixed with an equal volume of CIA 24:1 v/v, and then centrifuged for 5 min at 4 °C at 13 000 *g*. The upper phase was washed several times until clear. The last upper phase (around 400 μl) was mixed with 2 vols of 100% ethanol precooled to –20 °C. Total RNA was precipitated overnight at –20 °C. The mix was centrifuged for 25 min at 13 000 *g*, and the pellet washed with a cold solution of 95% ethanol. RNA was air-dried for 2 min and then dissolved in 100 μl of diethyl pyrocarbonate (DEPC)-treated water.

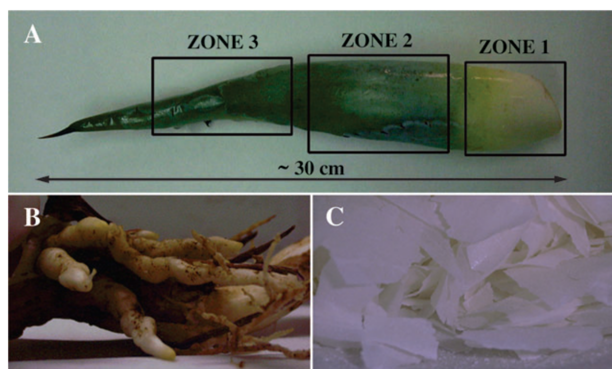

Fig. 1. Parts of *Agave americana* used in this paper. (A) Central cone of a young 30 cm length *Agave americana* plant showing the three different zones taken for analysis. (B) Root of the same plant. (C) Isolated epidermis.

RNA gel blot analysis, northern blot

Total RNA samples (10 μg) were separated in formaldehyde–1.2% agarose gels and blotted to a nylon membrane (Hybond-N, Amersham Biosciences, Little Chalfont, UK). [^{32}P] cDNA probes were synthesized by the random primer method using the High Prime® kit (Roche), and hybridizations were performed at 60 °C overnight in an aqueous solution as described by Sambrook *et al*. (1989). Blots were washed at high stringency in final 0.2× and subsequent 0.1× saline sodium citrate buffer (SSC) solution with 0.1% SDS (w/v), at 60 °C. Hybridization was quantified using a Fuji BAS2000 phosphorimager (Tokyo, Japan). The blot was then exposed to an autoradiographic film between intensifying screens for 2–3 d at –80 °C.

Construction and screening of a cDNA library from leaf epidermis. *pBK-CMV-*AgaSGNH *isolation*

A cDNA library was constructed using leaf epidermis poly(A$^+$) mRNA previously purified from total RNA, using the 'PolyAtract® mRNA Isolation Systems' kit according to the manufacturer's instructions (Promega). This poly(A$^+$) RNA was used for the construction of the directional cDNA library in the *Lambda ZAP Express* phage using the 'ZAP Express® cDNA Synthesis Kit', 'Gigapack® III Gold Cloning Kit', and 'Gigapack® III Gold Packaging Extract' kits according to the manufacturer's instructions (Stratagene, La Jolla, CA). The library was screened using standard procedures (Sambrook *et al*., 1989). The probe used for the screening was a 201 bp PCR product containing a partial open reading frame of the *AgaSGNH* cDNA, obtained by PCR amplification from the cDNA library using the universal T3 (5′-ATT AAC CCT CAC TAA AGG GA-3′), as forward primer and RCUTINO2 (5′-CCI ARI ACI ACI SWI ARI GGI GC-3′) as reverse. After hybridization, the membranes were washed twice in 2× SSC/0.1% SDS solution at 60 °C for 1.5 h , and then exposed to a film between intensifying sheets for 2–3 d. Phagemide split from the putative positive plaques carrying the *AgaSGNH* cDNA cloned (pBK-CMV-*AgaSGNH*) in the Lambda Zap Express vector, was carried out using the ExAssist Interference-Resistant Helper Phage system (Stratage, La Jolla, CA), according to the manufacturer's instructions. The process yielded the phagemid pBK-CMV-*AgaSGNH* in *Escherichia coli* XLORL strain, with the insert cloned between *Eco*RI and *Xho*I sites.

In situ *hybridization*

Agave americana young leaves were divided into three zones: basal (Zone 1), middle (Zone 2), and apical (Zone 3). Tissue samples

(1×0.5 cm^2) were cross-sectioned from each zone, immediately fixed by vacuum infiltration in a 4% formaldehyde, 0.1% Tween-20, and 0.1% Triton X-100 solution overnight at 4 °C, and then embedded in paraffin (Paraplast, Sigma). Tissue sections 10 μm thick were used for *in situ* hybridization studies. Light microscopy treatments and *in situ* hybridizations were performed following the procedure described by Cohen *et al*. (1990) and Quiroga *et al*. (2000). The pBK-CMV plasmid (Stratagene) containing the SGNH cDNA clone from *A. americana* was used to synthesize both sense and antisense DIG-labelled RNA probes, with T3 or T7 RNA polymerase. The plasmid was previously linearized with *Eco*RI and antisense RNA transcribed using T7 RNA polymerase. Sense control probes were synthesized using T3 RNA polymerase after linearization of the construct with *Xho*I. Riboprobes were digested to an average length of 150 nucleotides by mild alkaline hydrolysis in 100 mM carbonate buffer (pH 10.2) at 60 °C for 55 min. About 4% of each labelling reaction in 40 μl hybridization buffer was used as a probe for each slide, and incubated at 50 °C overnight (Ingham *et al*., 1985). After incubation with an alkaline phosphatase-conjugated sheep anti-digoxigenin (anti-DIG) antibody, the signal was detected by alkaline phosphatase assay using 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as substrates, according to the indications of the DIG DNA Labelling and Detection kit (Roche). A positive signal was detected as a blue/purple immunostain.

Immunolocalization of SGNH lipase in A. americana *leaves*

For immunocytochemistry, *Agave americana* young leaves were collected, divided into three zones (Zones 1, 2, and 3) as for gene expression studies, immediately fixed in formaldehyde solution and then embedded in paraffin (Paraplast, Sigma), using the same procedure described in the previous section. After fixation and embedding, tissue sections (10 μm) were cut with a microtome (Microm, HM 340, Heidelberg, Germany), placed onto poly-lysine precoated slides (Polysine™, Menzel-Glaser®) and air-dried overnight at 37 °C. Samples were deparaffinized by washing in Histoclear and rehydrated in an ethanol series, washed in PBS (0.1 M Phosphate Buffer, NaCl 0.15 M, pH 7.4), and blocked with a 5% bovine serum albumin (BSA) solution in PBS. Then, sections were incubated with undiluted primary antibody overnight at 4 °C, using a coverslip and a humid chamber. Rabbit polyclonal antibodies, raised against a 12-residue synthetic peptide designed on the deduced amino acid sequence of *AgaSGNH* cDNA, were used as primary antibody. After several rinses in PBS, samples were washed in a (0.1% BSA, 0.05% Tween-20 in PBS) solution, and then incubated for 2 h at room temperature with Alexa Fluor® 488 goat anti-rabbit IgG antibody (Molecular Probes) diluted 1:1000 in (0.1% BSA in PBS). To eliminate background, slides were rinsed several times in PBS, and washed moderately in 0.1% BSA, 0.05% Tween-20 in PBS three times for 10 min at room temperature. Then, coverslips were mounted on microscope slides using DABCO anti-fading reagent (Sigma), to prevent any attenuation of the fluorescence intensity. Samples were stored at 4 °C until observation. For detection, samples were examined with a Leica TCS-NT confocal laser microscope. In the confocal system, Alexa fluorescence was excited at 488 nm and emission read in the 515–545 nm interval with a green filter (BP530/30). Autofluorescence of the tissue was excited at 568 nm and emission detected with a >590 nm red filter (LP590). Positive images were obtained by overlapping the green and red channels, in order to eliminate autofluorescence of the tissue. The green signal corresponded exclusively to the positive signal; red staining indicated red autofluorescence, and yellow colour showed both red and green autofluorescence. Controls were made by replacing primary antibody with PBS.

Accession numbers

Proteins aligned in Fig. 3 are registered as follows (SwissProt database accession numbers are indicated in parentheses): AthSGNH1 (Q5PNZ0), putative *Arabidopsis thaliana* GDSL-motif lipase/hydrolase protein, similar to family II lipase EXL1 GI:15054382 from *Arabidopsis thaliana*; AthSGNH2 (Q9SVU5), AthSGNH3 (Q8LB81), and AthSGNH4 (Q9M8Y5), putative *Arabidopsis thaliana* GDSL-motif lipase/hydrolase proteins, similar to family II lipase EXL3 (GI:15054386), EXL1 (GI:15054382), EXL2 (GI:15054384) (*Arabidopsis thaliana*); OryAntherSGNH (Q6KAI2), putative *Oryza sativa* cv. *japonica* anther-specific proline-rich protein; OrySGNH1 (Q7F945), putative *Oryza sativa* cv. *japonica* SGNH plant lipase like; OrySGNH2 (Q84Z95), putative *Oryza sativa* cv. *japonica* SGNH plant lipase like; OryEnod1 (Q8H906) and OryEnod2 (Q7XDM8), putative early nodulin proteins; OryEXL4 (Q6K6B3), putative family II lipase EXL4; AgaSGNH (Q5J7N0). GenBank accession numbers for AgaSGNH are AY491975 for nucleotide and AAS75127 for the protein.

Results

Isolation and characterization of AgaSGNH *cDNA from epidermis of young A. americana* leaves

In 1974 Croteau and Kolattukudy reported the presence of an enzyme activity catalysing the formation of a hydroxy-fatty acid biopolymer, cutin (Croteau and Kolattukudy, 1974). This enzyme was present in a particulate extract obtained from epidermis of young *Vicia faba* leaves. According to this work, a preliminary study was carried out to evaluate the possibility of using the epidermis of young leaves of the monocot *Agave americana* L. as a model to study plant cuticle and cutin biosynthesis. The thickness of the *Agave* leaf cuticle, the easy isolation of its epidermis, cuticle, and RNA, and the broad knowledge existing about its cuticular composition represent interesting advantages in this case.

A. americana is a xerophytic monocotyledon whose leaves present a thick cuticle that it is known to contain both cutin and cutan (Wattendorf and Holloway, 1980; Villena *et al*., 1999; Heredia, 2003). Depolymerization studies indicate that *Agave* cuticle corresponds to the C18-type, containing mainly C_{18} fatty acid-derived monomers such as 9,10,18-trihydroxyoctadecanoic acid, 9,10-epoxy-18-hydroxyoctadecanoic acid, and 10,16-dihydroxyhexadecanoic acid (Espelie *et al*., 1982; Deshmukh *et al*., 2005).

In preliminary studies it was detected that an epidermis crude extract of young *A. americana* leaves had esterase and acyltransferase activities. A major protein with an aparent molecular weight of 17 kDa was present and its N-terminus was sequenced. In order to isolate the corresponding cDNA, degenerated oligonucleotides and reverse transcription-PCR were used, yielding a 488 bp partial clone that contained a His-Lys-Val-Met-Glu putative acyltransferase motif, but did not show any significant homology to known proteins (Reina and Heredia, 2001). Several attempts were made to amplify the full-length

cDNA, but failed. For this reason, a cDNA library from the epidermis of *A. americana* young leaves in a pBK-CMV expression vector was constructed, to have an interesting tool to be used for further studies on epidermis and cutin/cuticle metabolism. In order to isolate the full-length cDNA corresponding to the putative acyltransferase whose N-terminal sequence Ala-Pro-Ala/Leu-Ser-Val-Val-Leu-Gly-Ala-Gly/Pro-X-Phe was initially identified from native-PAGE gels (Reina and Heredia, 2001), a series of degenerated inosine-containing oligonucleotide forward primers (CUTINO1: 5′-GCI CCI GCI WSI GTI GTI YTI GG-3′, and CUTINO2: 5′-GCI CCI YTI WSI GTI GTI YTI GG-3′) and their reverses (RCUTINO1: 5′-CCI ARI ACI ACI SWI GCI GGI GC-3′ and RCUTINO2: 5′-CCI ARI ACI ACI SWI ARI GGI GC-3′) were designed and used to amplify the cDNA library as template. Using RCUTINO2 in combination with the T3 cloning primer, a 201 bp PCR fragment was amplified. Based on a first nucleotide-protein BLASTX search (Basic Local Alignment Search Tool; Altschul *et al.*, 1997), the fragment showed homology to a range of enzymes belonging to the GDSL-motif like lipase/acyl hydrolase family (pfam PF00657), a non-conventional family of lipases with some special characteristics, the main being the presence of the catalytic Ser inside the Gly-Asp-Ser-[Leu] (GDS[L]) motif, usually located at the beginning of the amino acid sequence. Rarely, the amino terminal peptide sequence previously chosen to design the cloning primers was not present in this fragment, probably due to the high degree of degeneration of oligonucleotides. However, it was decided to continue with the isolation of the full length of this GDSL-clone because there were some intriguing indications that it is a potentially interesting candidate involved in cuticle metabolism, such as the presence of lipase/esterase motifs in the sequence, the fact that some GDSL lipases may occasionally act as acyltransferases (Robertson *et al.*, 1994), and that the specific pattern of expression of the mRNA is restricted to the epidermis of the leaf. Using the 201 bp fragment as probe, the cDNA library was screened and the corresponding full-length cDNA clone was obtained (Fig. 2). The clone was 1438 bp long and was predicted to have an open reading frame (ORF) of 1104 nucleotides. Conceptual translation of the ORF gave rise to a putative polypeptide of 367 amino acids (ExPASy Translate tool), with an apparent molecular mass of 40.4 kDa and a pI of 8.9 (Gasteiger *et al.*, 2005). A search in the Conserved Domain Database (Marchler-Bauer *et al.*, 2005; http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml), revealed that the amino acid sequence of the clone possessed conserved domains relative to SGNH hydrolases (cd00229) and specifically to SGNH plant lipase-like subfamily (cd01837.2), all of them containing a conserved domain shared with GDSL lipases (pfam00657.12). Moreover, the AgaSGNH sequence possessed conserved domains present in two other subfamilies, the fatty acyltransferase-like (cd01846) and the triacylglycerol lipase-like (cd01847), and shared conserved domains with the phospholipase/lecitinase/haemolysin protein (COG3240.2).

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   1 ctcatccccaacgcacgaggccaatctcatctcctatcatctcattctgcaacaatgagc 60
                                                           M  S  2
  61 agcgtctctaaaattgtcttcatctttctaagtgtttgcctagtagccgtaggtaccctt 120
   3  S  V  S  K  I  V  F  I  F  L  S  V  C  L  V  A  V  G  T  L  22
 121 tcggcttcctcactggcagctcgcgcattcttcgtgtttggtgattctctggttgacaat 180
  23  S  A  S  S  L  A  A  R  A  F  F  V  F  G  D  S  L  V  D  N  42
 181 ggaaacaacaactacctggccacaacggctcgtgctgactctccccttatggcattgat 240
  43  G  N  N  N  Y  L  A  T  T  A  R  A  D  S  P  P  Y  G  I  D  62
 241 tacccgacccaccgcccaactggccgatttcgaatgggctcaacatccctgacattatc 300
  63  Y  P  T  H  R  P  T  G  R  F  S  N  G  L  N  I  P  D  I  I  82
 301 agtgagcacctcggagctgaggccacattgccgtatctgagccctgaccttcgcgggcaa 360
  83  S  E  H  L  G  A  E  A  T  L  P  Y  L  S  P  D  L  R  G  Q  102
 361 aggttgctcgtaggtgccaactttgcatcggctgggatagggatcctcaacgacaccggc 420
 103  R  L  L  V  G  A  N  F  A  S  A  G  I  G  I  L  N  D  T  G  122
 421 atccaattcatcaacatcattaggatttcacggcaaatgcagtactttgagcagtaccag 480
 123  I  Q  F  I  N  I  I  R  I  S  R  Q  M  Q  Y  F  E  Q  Y  Q  142
 481 cagagggtcagcgccttgatcggacaagcgcagatgcggcgcctcgtgaaccgagcactc 540
 143  Q  R  V  S  A  L  I  G  Q  A  Q  M  R  R  L  V  N  R  A  L  162
 541 gtcctcatcaccctcggaggcaatgactttgttaacaactactacttggtgccattctct 600
 163  V  L  I  T  L  G  G  N  D  F  V  N  N  Y  Y  L  V  P  F  S  182
 601 gcacgctctcgccagttctcactgccggactttgttcgctacgtgatctccgagtacaag 660
 183  A  R  S  R  Q  F  S  L  P  D  F  V  R  Y  V  I  S  E  Y  K  202
 661 aagattctcgcaaggctgtacgaattgggagctcgtcaagtcctggtcacggggactgga 720
 203  K  I  L  A  R  L  Y  E  L  G  A  R  Q  V  L  V  T  G  T  G  222
 721 ccacttggctgcgtcccatctgagcttgctcagagaagccgagacgggaattgtgaccca 780
 223  P  L  G  C  V  P  S  E  L  A  Q  R  S  R  D  G  N  C  D  P  242
 781 gaactgcaacgagctggggacctgttcaatcctcaactggtccagattctaaaccaactg 840
 243  E  L  Q  R  A  G  D  L  F  N  P  Q  L  V  Q  I  L  N  Q  L  262
 841 aactcgcagtttggatcaactgtctttcttggtgccaacaccaggagagcgcacatggac 900
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 961 cggccgtacaatgggatcgggctctgcacagtggcatcaaatctatgtcctaacagggac 1020
 303  G  P  Y  N  G  I  G  L  C  T  V  A  S  N  L  C  P  N  R  D  322
1021 ctgtacgcgttctgggatgcattccatcctacgcagaaggcgaaccggataattgtgagc 1080
 323  L  Y  A  F  W  D  A  F  H  P  T  Q  K  A  N  R  I  I  V  S  342
1081 cagttcatgacaggttccaacgagtacatgaccccgatgaacgtgaccagtctcttggcc 1140
 343  Q  F  M  T  G  S  N  E  Y  M  T  P  M  N  V  T  S  L  L  A  362
1141 atgaatgacagcacttgatgaactcaaaccttgatcaccttaaaatcatgtcgtgcgtgt 1200
 363  M  N  D  S  T  *                                              367
1201 ctcaatcttagcttcgcgttgcttgctttctcattgcattgtttttttctttttggcc 1260
1261 aggttgtttattaataatgtctcaaggactgggatgtgtttgaaaattgtttgttttcgg 1320
1321 gtatatagggaaaatgcatcacggaaaatgcatcacgattgatggtgttcaccgatgtgt 1380
1381 agacttggatgaatcaatgaaaatgagatttctagaatgaaaaaaaaaaaaaaaaaaa   1438
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Fig. 2. Nucleotide and deduced amino acid sequences of the *AgaSGNH* cDNA. The deduced amino acid sequence encoded by 367 codons shows a predicted signal sequence at N-terminus from Met1 to Arg30. The five blocks of homology found in SGNH (or GDSL) hydrolases are shown in italics (Block I: Phe33-Asn45; Block II: Leu105-Gly116; Block III: Val163-Asn174; Block IV: Leu211-Pro223; Block V: Phe326-Pro332). Circles mark the oxyanion hole, and the catalytic triad typical of esterases is indicated by triangles. The putative acyltransferase motif is underlined by a single line, and the region where the synthetic peptide for antibody production was designed appears in discontinuous underlined.

The *in silico* analysis of the AgaSGNH protein sequence revealed some interesting aspects. iPSORT analysis (Bannai *et al.*, 2002; http://psort.nibb.ac.jp/) determined a putative signal peptide of 30 amino acids at the *N*-terminus of the protein, containing an Ala-X-Ala motif between Ala29 and Ala31 residues that may serve as a signal peptidase cleaving site (Fig. 2). The location of the protein, using the Wolf PSORT algorithm (Horton *et al.*, 2006; http://wolfpsort.seq.cbrc.jp/), was predicted to be extracellular with a probability of 0.82. Distinct softwares (ScanProsite; de Castro *et al.*, 2006; http://expasy.org/tools/scanprosite/; PROSCAN and Motif-Scan, http://scansite.mit.edu/motifscan_seq.phtml) also predicted three putative *N*-glycosylation sites inside the sequence: from Asn119 to Gly122 (Asn-Asp-Thr-Gly), from Asn356 to Ser359 (Asn-Val-Thr-Ser), and from Asn364 to Thr367 (Asn-Asp-Ser-Thr).

The critical residues for enzyme activity (Fig. 2), assigned by comparison with other SGNH proteins, were the catalytic triad of the enzyme comprising residues Ser38, Asp328, and His331, which appeared in similar positions to those in other lipases of this family; the oxyanion hole comprising residues Ser38, Gly114, and Asn170; and residues that contribute to the stability of the active site (Gly114, Asn170, and Asp328). The analysis of AgaSGNH sequence also revealed the presence of five highly conserved domains shared with SGNH proteins, previously reported by Upton and Buckley in GDSL motif lipases (Fig. 2). Finally, AgaSGNH sequence showed a HLGAE motif at positions His85-Leu86-Gly87-Ala88-Glu89 which appears, as it was referred above, highly conserved in some condensing enzymes such as acyltransferases (Heath and Rock, 1998; Lewin *et al*., 1999) or wax synthases (Beisson *et al*., 2003; Kalscheuer and Steinbuchel, 2003).

Sequence comparison of AgaSGNH with other SGNH proteins

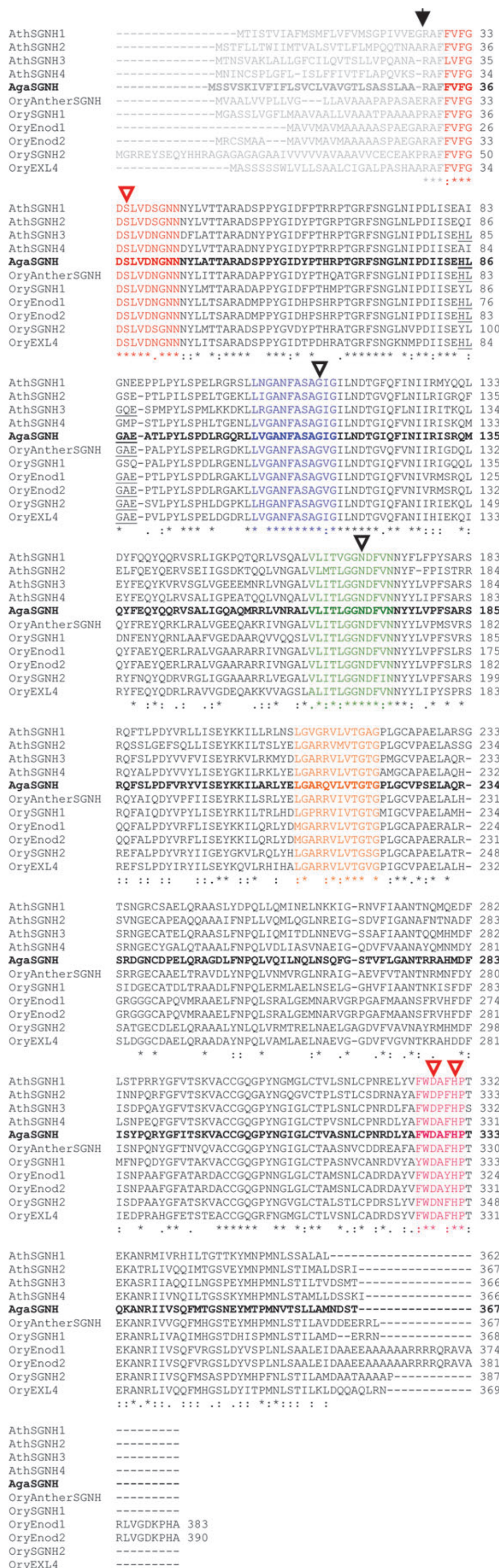
A search looking for proteins homologous to AgaSGNH using the BLASTP algorithm against a non-redundant protein database, showed several sequences (492 hits) exhibiting high levels of *similarity* with the query sequence, most of them from *Arabidopsis thaliana* and *Oryza sativa* cv. *japonica*. Ten of these amino acid sequences, four from *Arabidopsis* and six from rice, were aligned with AgaSGNH (Fig. 3) showing 65–72% identity, and 80–86% similarity. AthSGNH1, AthSGNH2, AthSGNH3, and AthSGNH4 corresponded to SwissProt database accession numbers Q5PNZ0, Q9SVU5, Q8LB81, and Q9M8Y5, respectively, and were described as carboxylic ester hydrolases or putative GDSL-motif lipases/acyl hydrolases. The six *Oryza sativa* cv. *japonica* sequences were named as OryAntherSGNH, OrySGNH1, OrySGNH2, OryEXL4, OryEnod1, and OryEnod2, corresponding respectively to the accession numbers Q6KAI2, a putative anther-specific proline-rich protein; Q7F945 and Q84Z95, described as GDSL lipases; Q6K6B3, a putative family II lipase EXL4 (extracellular lipase 4; a GDSL-like lipase too); and finally Q8H906 and Q7XDM8, two putative early nodulin gene (Enod)-related proteins. These Enod-related proteins have been described as proteins involved in the early stages of nodule formation in legumes (Dickstein *et al*., 2002; Pringle and Dickstein, 2004). The accession number for AgaSGNH in the SwissProt database is Q5J7N0. All aligned sequences possessed the characteristic residues and blocks above described for SGNH (or GDSL) hydrolases such as the active site (Ser, Gly, Asn, Asp, and His), the GDSL motif and the five blocks of homology (Fig. 3). Some sequences presented the putative acyltransferase motif His-Leu-Gly-Ala-Glu previously described (AthSGNH3, AgaSGNH, OryAntherSGNH, OryEnod1, OryEnod2, and OryEXL4). Three of the most similar sequences to AgaSGNH, one from *Arabidopsis thaliana* (AthSGNH3) and two from *Oryza sativa* (OryAntherSGNH and OryEnod1), were used to compare their 2D structure with that corresponding to AgaSGNH using the *in silico* GORIV prediction method (Garnier *et al*.,, 1996; http://npsa-pbil.ibcp.fr); the results are shown in Fig. 4. All the sequences presented high similarity at a structural level, showing similar secondary structures around the important residues. In this sense, catalytic Ser was always predicted to be inside a short strand, followed by a region with high abundance of coil structures. Gly of the oxyanion hole was preceded by two alpha-helixes separated by a coil, and followed by an alpha-helix predominant region until the conserved Asn of the oxyanion hole. The similarity in the remaining regions was also evident (Fig. 4).

The isoelectric points calculated for the secondary structure-predicted sequences showed striking differences between most homologous sequences. For AgaSGNH, the pI was 8.95, 6.22 for AthSGNH3, and 5.74 for rice OryAntherSGNH protein, but for one of the Enod rice protein (OryEnod1) the pI was 9.40.

Finally, the four sequences were predicted to be extracellular (iPSORT software), as the presence of typical signal sequences at their *N*-termini seems to indicate.

Expression level of AgaSGNH *mRNA in* A. americana *tissues: northern blot analysis*

To determine the expression level of *AgaSGNH* mRNA in three different tissues of *Agave americana*, northern blot analysis was carried out using samples of total RNA obtained from young leaf epidermis, young leaf tissue without epidermis, and root meristems (Fig. 5). For the epidermis and leaves without epidermis samples, three regions of differential growth rate were selected on the leaf: basal (Zone 1), the most active in growth and usually etiolated; this zone was the leaf base to the initial green region. Medium zone or Zone 2, that presented a slower growth rate, extended from the beginning of the green zone and was approximately 3 cm long. The apical region, or Zone 3, was the oldest and most inactive growth region on the leaf. It comprised a 2–3 cm length zone from the end of Zone 2 (Fig. 1) Total RNA was purified, electrophoretically fractionated, and transferred onto Nylon membranes (see Materials and methods). The synthesis of a ^{32}P-dCTP radioactive probe was performed by random priming using the 1437 bp clone of *AgaSGNH* cDNA as the template. Ribosomal RNA was used as the loading control after staining with ethidium bromide. Hybridization was carried out at 65 °C, under high stringency conditions. The results of autoradiographic exposure of radiolabelled membranes are shown in Fig. 5. A strong mark around 1.45 kb appeared in the leaf epidermis of the younger zones (Fig. 5, E1 and E2 lines),



but not in roots (R), leaves without epidermis (L1, L2, and L3) nor in the E3 line. *AgaSGNH* was found to be highly expressed in epidermis, but was not present in leaves without epidermis nor in roots. Moreover, the high gene expression level decreased from Zone 1 to Zone 3, where no signal of *AgaSGNH* mRNA was detected.

In situ *hybridization*

To localize further the distribution of *AgaSGNH* mRNA in different tissues, and attempting to find the precise pattern of expression found in leaf epidermis by northern blot, an *in situ* hybribidization experiment was performed as indicated in the Materials and methods. The same three zones of *Agave* leaf analysed by northern blot were used in this analysis: basal (Zone 1), medium (Zone 2), and apical (Zone 3).

As shown in Fig. 6A, in the basal zone of the leaf (Zone 1), *AgaSGNH* expression was visualized as a blue/purple staining that was restricted to the single layer of cells that constituted the epidermis. In detail, the signal was detected in the outline of the epidermal cells and, preferentially, in their apical surfaces. Cells of the mesophyll, localized under the epidermal layer, resulted in a clear negative when hybridized with the antisense probe (Fig. 6D). In the medium zone of the leaf (Zone 2), no signal was present using the antisense probe, except in the neighbourhood of the basal zone where some individual cells with positive staining (indicated by arrows) appeared in the epidermis (Fig. 6B). No other tissues with a positive signal were found in the medium leaf. *AgaSGNH* messenger was not detected in any tissue of Zone 3, the oldest and least active region in growth of the leaf (Fig. 6C). No hybridization with the sense probe was observed in any of the zones studied (Fig. 6D, E, F). The epidermises of the abaxial and adaxial surfaces of the leaf were slightly different in cell morphology, but both showed similar patterns of *AgaSGNH* expression in every zone (data not shown).

Fig. 3. Alignment of the deduced amino acid sequence of AgaSGNH with those of other SGNH proteins. Sequences were aligned using the Clustal software (Thompson *et al.*, 2000). The signal sequences are shown in grey colour at the N termini and the putative cleavage sites are indicated by an arrow. Triangles indicate amino acids of the active site (Ser…Gly…Asn…Asp…His). Red triangles are situated over the typical catalytic triad residues of hydrolases (Ser, Asp, and His). Blocks of homology described for GDSL and SGNH hydrolases by Upton and Buckley are coloured (Block I: red; Block II: blue; Block III: green; Block IV: orange; Block V: pink). The putative acyltransferase motif (HisxxxGlu) is underlined. Asterisks indicate identical residues in all sequences; colons and periods indicate conserved and semi-conserved substitutions, respectively. Ath: *Arabidopsis thaliana*; OryEnod: *Oryza sativa* cv. *japonica*, putative early nodulin gene. AthSGNH1: (Swiss Prot database accession number: Q5PNZ0); AthSGNH2 (Q9SVU5); AthSGNH3 (Q8LB81); AthSGNH4 (Q9M8Y5); OryAntherSGNH (Q6KAI2); OrySGNH1 (Q7F945); OryEnod1 (Q8H906); OryEnod2 (Q7XDM8); OrySGNH2 (Q84Z95); OryEXL4 (Q6K6B3); AgaSGNH (Q5J7N0).

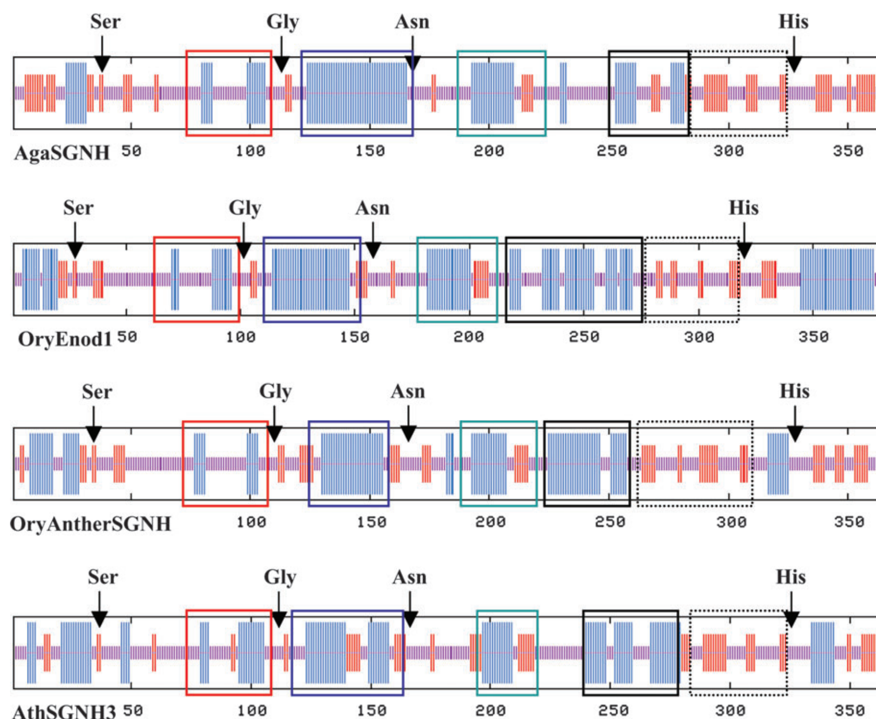


Fig. 4. Secondary structure prediction of the three most similar sequences to AgaSGNH. Program GOR IV (http://npsa-pbil.ibcp.fr; Garnier *et al.*, 1996) was used to predict the secondary structure for AgaSGNH, AthSGNH3, OryAntherSGNH, and OryEnod1 proteins. Blue lines: alpha helix; Red lines: extended strand; Violet lines: random coil. Coloured squares represent regions with similar secondary structure inside the sequences. The amino acids that give name to SGNH-like hydrolases are shown indicating their respective positions along the sequence.

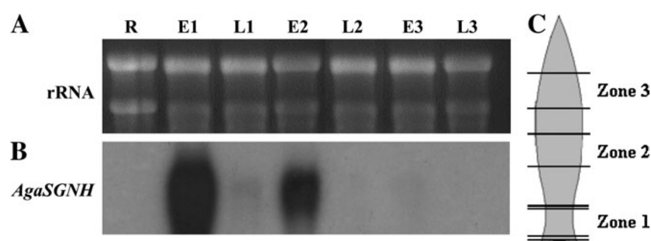


Fig. 5. Expression of *AgaSGNH* in some tissues of *Agave americana* using northern blot. (A) 10 µg of total RNA prepared from roots (R), leaf epidermis (E) and leaf tissue without epidermis (L), were loaded per lane on a denaturing electrophoresis gel. rRNA was used as loading control. (B) Autoradiography of the probed blot for *AgaSGNH* mRNA detection. (C) Scheme showing the distinct zones chosen for RNA and protein expression. Zone 1: E1 and L1; Zone 2: E2 and L2; Zone 3: E3 and L3.

As observed in all the micrographs, the cuticle of *A. americana* leaf usually showed a pink staining, distinct from the blue/purple positive signal. This fact, probably caused by the use of the NBT substrate in the developing reaction, that also stained lipids, allowed the state of the cuticle along the leaf to be observed. A significant and gradual increase in the width and refringence of the cuticle could be observed from the basal to the apical parts, which may be correlated with the age of the leaf (Fig. 6). The *A. americana* leaf is a classic monocot type, that ages from the base to the tip (Wattendorf and Holloway, 1980). It is documented that cuticle structure and composition vary along the ontogeny of the organ, becoming thicker, denser, and more organized as the leaf ages (Kerstiens, 1996), as observed in the images obtained from *A. americana*.

Immunolocalization of AgaSGNH in A. americana *leaves*

In order to analyse the pattern of protein expression, and to correlate with previous results of mRNA distribution obtained by *in situ* hybridization, immunolocalization of AgaSGNH protein was performed on 10 µm semi-thin sections of young *Agave* leaves, monitoring the same zones of the leaf tested for the northern blot and *in situ* hybridization experiments (Zones 1, 2, and 3). For immunolocalization, rabbit polyclonal antibodies raised against a 12 residue synthetic peptide designed on the deduced amino acid sequence of the cDNA were used as the primary antibody (Fig. 2). An Alexa Fluor® 488 goat anti-rabbit IgG (H+L) was used as secondary antibody. For detection, samples were examined with a confocal laser microscope. Fluorescence of the Alexa 488 antibody was excited at 488 nm and emission was read in the 515–545 nm interval using the green filter. Autofluorescence of the tissue was excited at 568 nm and emission was collected at >590 nm in the red channel. Definitive images emerged by overlapping the green and red channels,

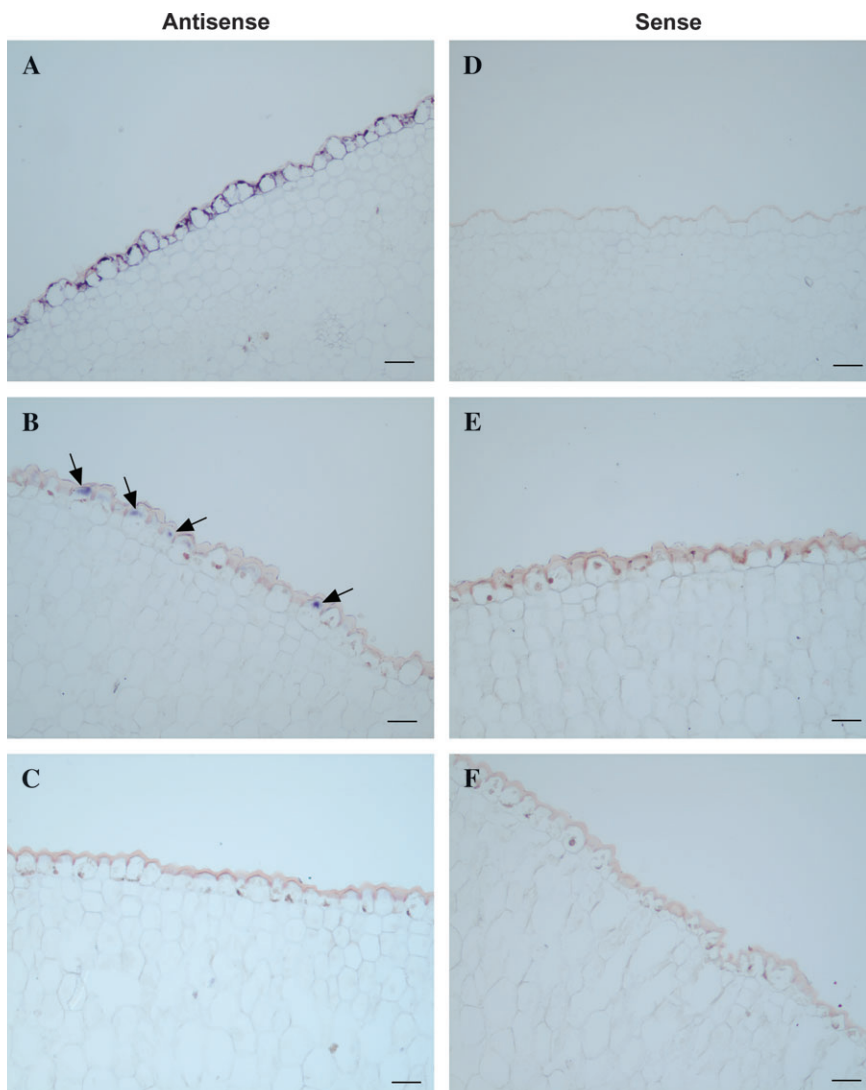


Fig. 6. *In situ* localization of *AgaSGNH* mRNA. Series of images showing *in situ* mRNA expression pattern of *AgaSGNH* in leaf tissue (A–F). Tissue sections corresponding to Zone 1 (A, D), Zone 2 (B, E), and Zone 3 (C, F) previously analysed by northern blot, were hybridized with antisense (A, B, C) and sense (D, E, F) digoxigenin-labelled riboprobes for *AgaSGNH* cDNA. After immunodetection using an alkaline phosphatase-conjugated sheep anti-digoxigenin antibody, sections were observed with a light microscope. Bars=100 μm. Arrows in (B) show positive marks corresponding to low amounts of *AgaSGNH* mRNA. Cuticle appears as a continuous pink-like layer deposited over epidermal cells, in direct contact with environment.

discriminating as the positive signal that which was only present in the green channel and absent in the red one (Fig. 7). Therefore, the green signal observed in the micrographs corresponded with the positive signal; red staining indicated autofluorescence in the red interval, and yellow colour showed structures with both red and green autofluorescence. In the case of the control micrograph (Fig. 7F), the overlay image was observed but not captured, being only available the green channel (showed in this case).

As shown in Fig. 7, protein expression in leaves was found to be limited to the epidermal cells, reaching maximum levels in Zone 2 (Fig. 7B). There, protein accumulated in the outline of epidermal cells and, in particular, inside at the base of these cells in a reticulate structure that could correspond to the endoplasmic reticulum, thus confirming the hypothesis that this protein entered the vesicular pathway as suggested by the presence of a predicted signal peptide. Mesophyll cells were clearly negative to immunostaining in all the zones examined. In Zones 1 and 3 (Fig. 7A, C), SGNH protein amounts decreased appreciably, always being located in the leaf epidermis with the same pattern as observed in Zone 2. Controls produced no positive signal (Fig. 7D, E, F). In all the micrographs (Fig. 7), red staining at the top of the epidermal cells showed the cell wall, and the cuticle proper appeared in yellow. It may be seen how the cuticle penetrates inside the cell wall, clearly delimitating the

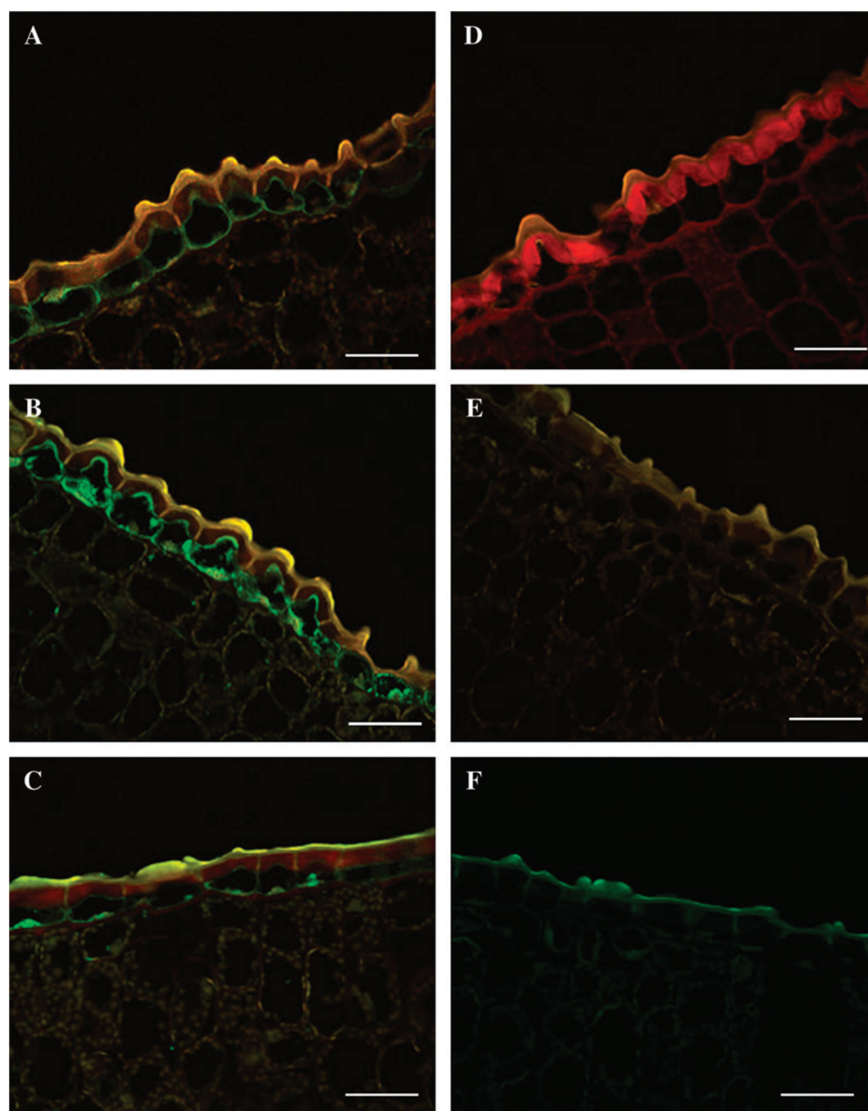


Fig. 7. Immunolocalization of AgaSGNH. Cross-sections of basal (Zone 1, A, D), medium (Zone 2, B, E), and apical (Zone 3, C, F) parts were made from paraffin-embedded external leaf tissue and incubated with rabbit anti-*Agave* GDSL motif polyclonal antibodies (A, B, C). An Alexa Fluor® 488 goat anti-rabbit IgG antibody was used as secondary antibody. Images were taken with a Leica TCS-NT laser scanning confocal microscope. Negative controls (D, E, F) were incubated only with the secondary antibody. Fluorescence of Alexa antibody was excited at 488 nm and emission read with the green filter (515–545 nm interval). Tissue autofluorescence was excited at 568 nm and collected at >590 nm in the red channel. Images were obtained by overlapping the green and red channels. (F) Image was collected only with the green filter. Cuticle appears as a yellow-orange fluorescent continuous layer. Specific green fluorescent signal was only detected in anti-*Agave* GDSL motif incubated preparations (A, B, C) around the perimeter of epidermal cells. No signal was observed in the corresponding negative controls. Red and yellow-orange signals correspond to autofluorescence of the tissue. The sample shown in (F) was also observed with the red filter with no positive signal detected (image not shown). Bars=50 μm.

separation between adjacent epidermal cells. Epidermal cells exhibit polarity, as cited in the literature (Efremova *et al*., 2004), with their outermost cell walls being thicker than adjoining cell walls, and the cuticle and waxy lipids deposit onto the exposed surface.

In situ hybridization and immunolocalization experiments confirmed that *AgaSGNH* expression was leaf epidermis-specific, as previously detected by northern blot. However, some differences were observed in expression level between *in situ* hybridization and immunolocalization in the leaf. Epidermal cells showed the highest *in situ* hybridization signal in Zone 1 (Fig. 6), while the strongest signal for AgaSGNH protein was detected in Zone 2 (Fig. 7). These discrepancies between mRNA and protein expression patterns could be due to a post-transcriptional regulation process of AgaSGNH expression. However, this finding is preliminary and requires further study. This pattern has also been observed for other proteins as BDG, for example, an *Arabidopsis* extracellular alpha/beta hydrolase with putative synthase activity, which is also

epidermis specific, and is involved in cuticle formation (Kurdyukov *et al*., 2006*a*).

The fluorescence signal in immunosections was observed only in the inner surface of the cell wall, with no presence in the outer portion or the cuticle (Fig. 7A, 7, C). This fact should be explained by the high hydrophobicity of cuticle structure, as well as by cutinization of the outermost portion of the cell wall, that could harden and obstruct the access of antibodies to their recognition sites. However, antigen masking or lower protein levels in these areas cannot be excluded. Similar patterns of expression have been shown also for BODYGUARD in *Arabidopsis* (Kurdyukov *et al*., 2006*a*); however, although several genes implicated in these processes have been ascribed specifically to the epidermis at mRNA level (Efremova *et al*., 2004; *FIDDLEHEAD*, Pruitt *et al*., 2000; *HOTHEAD*, Kurdyukov *et al*., 2006*b*; *LACS2*, Schnurr *et al*., 2004; *CUT1*, Millar *et al*., 1999), only some of their final products as BODYGUARD have been localized at a subcellular level so far.

Discussion

The leaf of the monocot *Agave americana* L. presents a prominent epidermis with a thick cuticle, making it an appropiate model to study the mechanisms involved in the biosynthesis of plant cuticle (Reina and Heredia, 2001; Villena *et al*., 1999). In the present work, the isolation and characterization of a novel cDNA encoding a SGNH-motif hydrolase from *A. americana* leaf epidermis were carried out, as well as the description of its patterns of expression at mRNA and protein levels in some tissues. Further potential relationships of the biosynthesis of cuticle components are discussed.

AgaSGNH is a new member of a multifunctional family of plant esterases

AgaSGNH, the *A. americana* polypeptide encoded by *AgaSGNH* cDNA described here, possesses a typical signal peptide at its *N*-terminus that allows its secretion to the epidermis cell wall, as it was verified by immunolocalization experiments (Fig. 7). The protein also presents some conserved domains shared with proteins of the SGNH plant lipase subfamily (cd01837.2), which is included in the SGNH-hydrolases superfamily (cd00229.3). This group of proteins, which includes the carbohydrate esterase family 12 as a distinct subfamily, is different from that of the classic alpha/beta-hydrolase family (Wei *et al*., 1995). Members of the SGNH-hydrolase superfamily, like some alpha/beta-hydrolases, facilitate the hydrolysis of ester, thioester and amide bonds in a wide range of substrates including complex polysaccharides, lysophospholipids, acyl-CoA esters, and other compounds (Roberts *et al*., 1993; Dalrymple *et al*., 1997; Nardini and Dijkstra, 1999). AgaSGNH protein also exhibits conserved domains with a high degree of similarity with a fatty acyltransferase-like family (cd01846.2) which also belongs to the SGNH-hydrolases superfamily mentioned above. The *Arabidopsis* SGNH proteins aligned in Fig. 3, which share 65–72% identity with the AgaSGNH sequence, have no mutants with physiological function assigned to date. Recently, two insertional mutans of *Arabidopsis* (*glip1-1* and *glip1-2*) have been described, altered at the locus At5g40990, that corresponds to the coding region of a GDSL protein identified in the secretome of cell cultures of the same species (Oh *et al*., 2005). The gene product of At5g40990, GLIP1, is a protein that seems to be involved, via ethylene signalling, in plant resistance against the necrotrophic fungus *Alternaria brassicicola*. The protein is located in the epidermal cell-wall space of the leaf, and the recombinant protein possesses lipase and antimicrobial activities, disrupting fungal spores integrity and/or producing lipid-derived defence signalling molecules (Oh *et al*., 2005). Comparison between AgaSGNH and GLIP1 reveals that they share 32% identity and 49% similarity at the amino acid level, and shows that GLIP1 also belongs to the SGNH hydrolase family, because of the presence of the conserved domain of this family. Although identity between AgaSGNH and GLIP1 is much lower than that found with the other four aligned *Arabidopsis*-corresponding sequences with no clear function ascribed (Fig. 3, AthSGNH1, 2, 3, 4), the similarity between both amino acid sequences and their molecular masses (41.71 kDa for GLIP1, and 40.47 kDa for AgaSGNH) could lead to suggest a possibly role of AgaSGNH in a defence mechanism against fungal pathogens, in a similar manner to GLIP1. However, interestingly, their isoelectric points are very different, ranging from 6.6 for GLIP1 to 8.95 for AgaSGNH. This fact could imply a different catalytic mechanism or a different substrate preference, although both proteins are epidermis-specific and extracellular (cell wall-associated). AgaSGNH also shares high similarity with two Enod proteins from rice (OryEnod1 and OryEnod2 in Fig. 2). These kinds of proteins are expressed in the root nodules developed in legumes under infection conditions, and are associated with the structural development of the nodule (Pringle and Dickstein, 2004). In this sense, these proteins could play a structural function related to AgaSGNH, being involved in the initial formation of a cutin-like structure in the nodule similar to suberin, the main structural biopolymer present in roots, and usually associated with pathogenesis-related processes. The tertiary fold of the SGNH plant-specific hydrolases is substantially different from that of the alpha/beta-hydrolase family and unique among all known hydrolases; its active site closely resembles the Ser-His-Asp (Glu) triad found in common lipases, but they are characterized by having four conserved blocks, previously described, each containing one of the catalytic conserved

residues (Fig. 3): serine, glycine, asparagine, and histidine, respectively. Each of the four residues plays an important role in the catalytic function (Molgaard *et al*., 2000). Some 3D structures of members of SGNH family have been elucidated to date (Li *et al*., 2000; Molgaard *et al*., 2000; Bitto *et al*., 2005). In Fig. 4, the resemblance of the 2D structure predicted for the four sequences AgaSGNH, AthSGNH3, OryAntherSGNH, and OryEnod1 can be observed. All of them show a similar pattern of distribution of alpha-helix and beta-strand domains, which are distributed along the sequences and around the catalytic amino acid residues (SGNH) in similar positions.

Some condensing enzymes, such as glycerolipid acyltransferases (Heath and Rock, 1998; Lewin *et al*., 1999) or wax synthases (Beisson *et al*., 2003), show a conserved His-Xn-Glu/Asp (n=3 or 4) motif inside their sequences that is not usually present in the SGNH hydrolases. In AgaSGNH sequence, there is a His-Leu-Gly-Ala-Glu motif at positions His89-Leu90-Gly91-Ala92-Glu93 (Fig. 2), which is similar to that observed in the acyltransferases above described. The alignment with the sequences of *Arabidopsis* and rice (Fig. 3) indicates that some of these proteins also have this putative acyltransferase motif (Fig. 3: AthSGNH1, OryAntherSGNH, OryEnod1, OryEnod2, and OryEXL4). The involvement of AgaSGNH in the transfer of acyl moieties to the cuticle is one of the points to be considered, specially taking into account that AgaSGNH could belong to a new class of transferases, included in the SGNH family, with an enzymatic mechanism possibly different from other glycerolipid or phospholipid acyltransferases. On the other hand, it could be a new member of plant hydrolases playing its role in the epidermal cell wall of the *Agave* leaf. However, all of these hypotheses are very speculative and need further studies.

Expression of AgaSGNH *is specific to the epidermis of active growing zones of* A. americana *young leaves*

The northern blot study performed in different parts of the leaf confirms two aspects: first, the expression of *AgaSGNH* is limited to the epidermis; and second, the maximum mRNA levels are found in the epidermis of the youngest zones (Fig. 5; basal and medium, Zones 1 and 2), which are especially active in cutin biosynthesis. These levels dramatically decrease to Zone 3, the oldest zone of the leaf, where the presence of *AgaSGNH* mRNA is undetectable, and the biosynthesis of different cuticle components is severely reduced (Guerrero *et al*., 2006). Expression of AgaSGNH mRNA is observed neither in roots nor in leaf without epidermis. *In situ* hybridization analysis shows that *AgaSGNH* mRNA is located in the outermost layer of cells that constitute the leaf epidermis (Fig. 6), in accordance with northern blot results, and is only present in Zone 1, the most actively growing zone. In *A. americana*, the epidermis of the leaf is composed of a single cell layer. These cells produce and secrete the components of the cuticle, which, once outside the cell, assemble and constitute the cuticular membrane. Epidermal cells of *A. americana* do not contain chloroplasts, and are usually vacuolated. They often present a large vacuole that fills nearly all the cell lumen, and drives the rest of the protoplasm to the opposite pole of the cell. This fact could explain the presence of *AgaSGNH* messenger in the outline of epidermal cells, mainly apical, in the *in situ* experiment; a possible polarization of the secretion could also not be excluded.

The presence of *AgaSGNH* mRNA in Zone 2 detected by northern blot may be due to an incorrect assignation of this part, that could probably correspond to Zone 1 or a transition between Zones 1 and 2. In fact, it is difficult to separate visibly, in a precise manner, the most actively growing zone (basal) from the medium zone.

Immunolocalization of AgaSGNH protein, carried out by confocal laser microscopy, confirmed the specific pattern of gene expression found by *in situ* hybridization and northern blot, and was restricted to the epidermis of the leaf. Although some disparity has been shown between mRNA and protein levels in the different zones of the leaf, these should be attributed to a post-transcriptional regulation of expression. Maximum levels of *AgaSGNH* mRNA in Zone 1 could correspond to gene induction prior to the active process of cuticle component biosynthesis given in this zone, while higher levels of the final product, AgaSGNH protein, in Zone 2 parallels very well the great increase in the distinct lipidic components of cuticle described in this area. The analysis of cuticular components of *A. americana* leaf cuticle has been performed in our laboratory, and the results recently published (Guerrero *et al*., 2006). This study shows that the largest increase in the accumulation of cuticular components, mainly waxes and cutin, occurs between Zone 1 and Zone 2 in the leaf, coinciding with the major accumulation of AgaSGNH protein (Guerrero *et al*., 2006).

Immunolocalization of the protein in an evident and precise manner in the outline of epidermal cells suggests a secretion process at the plasma membrane, which is also compatible with the extracellular final destination of the protein predicted by computing programs and sequence comparison previously described.

Taken together, the presence of the *N*-terminal signal sequence, showing 60% similarity with signal peptides of other extracellular lipases that are cell wall-localized (Oh *et al*., 2005), the predictions made by web-based software (iPSORT), and the results obtained indicating a cell wall location of AgaSGNH protein and an epidermis-specific synthesis of its mRNA observed by northern blot and *in situ* studies, revealed that AgaSGNH is an extracellular protein which probably participates in the cell wall, cuticle or both metabolisms.

Its similarity with other plant proteins belonging to the SGNH-hydrolase family, but different from the classical alpha/beta-hydrolase superfamily, suggests that this kind of protein may catalyse a reaction different from that described for classic lipases and esterases.

One of the putative roles of AgaSGNH in the epidermis of *Agave* may be as a thioesterase enzyme catalysing the hydrolysis of thioester bonds in acyl-CoA molecules acting as activated precursors of cutin monomers. The action of AgaSGHN in the cell wall of epidermal cells could release hydroxy-acyl chains which could be incorporated, by a synthase enzyme similar to BDG (Kurdyukov *et al*., 2006*a*), into a prominent crescent cutin. This function fits well with the obtained results concerning the amounts of cuticular components in the three different zones of the leaf studied in this work (Guerrero *et al*., 2006). The hypothesis given by Kurdyukov and co-workers about the direct implication of BDG in the biosynthesis of cutin in *Arabidopsis* may also be compatible with the idea that AgaSGNH could carry out both the hydrolysis and the transfer, from an activated acyl-CoA to a crescent cutin in *Agave americana* leaves because, as is well known, the composition of *Arabidopsis* cutin is a rarely known case in plants being composed mainly of high amounts of C_{16}- and C_{18}-dicarboxylic acids (Nawrath, 2002, 2006; Bonaventure *et al*., 2004; Suh *et al*., 2005; Kurdyukov *et al*., 2006*a*, *b*); however in *Agave*, the major components of the cuticle structure are the ω- and mid-chain C_{18} hydroxy fatty acids (Villena *et al*., 1999; Heredia, 2003). Although AgaSGNH is similar to other GDSL proteins that are plant defence-related (Enod proteins or GLIP1), in the case of *A. americana*, the messenger, as well as the protein, is synthesized only in the youngest zones of leaf epidermis, where the synthesis of cuticular components is more active, and not in all the zones examined as would be expected if it was involved in the process of leaf protection against plant pathogens.

In order to understand the functional role of this protein, heterologous expression of AgaSGNH in *Escherichia coli* has been assayed under a wide range of experimental conditions with non-conclusive results. In this sense, current work on protein expression in the eukaryotic plant system *Nicotiana benthamiana* is being carried out in order to elucidate protein function (Lu *et al*., 2003). Further studies about the isolation and characterization of the corresponding gene promoter may serve to elucidate some of the potential signals (e.g. wounding, drought, irradiation, pathogenesis, etc) that could control the process in which the protein is involved, as well as also providing an interesting tool to be used for expression assays specifically aimed at the epidermis.

It is important to note that the work described here has been carried out in the non-usual plant model *Agave americana* because of the thickness of its epidermis and cuticle. The knowledge about its cuticular structure also represents an advantage for the isolation of genes and proteins that are epidermis-specific. These genes and proteins, and the information derived from their analyses, could be used in other plant systems such as *Arabidopsis* or tomato fruit (easier to manipulate for transformation experiments) to perform deeper studies about the complex process of plant cuticle biosynthesis.

The results presented in this work, and those from Kurdyukov and co-workers about BDG (Kurdyukov *et al*., 2006*a*), present a scenario in which the involvement of a hydrolase in cutin and plant cuticle biosynthesis, creating the film that covers all aerial parts of higher plants, seems to be more and more certain.

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References

Akoh CC, Lee GC, Liaw YC, Huang TH, Shaw JF. 2004. GDSL family of serine esterases/lipases. *Progress in Lipid Research* **43,** 534–552.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25,** 3389–3402.

Arif SA, Hamilton RG, Yusof F, Chew NP, Loke YH, Nimkar S, Beintema JJ, Yeang HY. 2004. Isolation and characterization of the early nodule-especific protein homologue (Hev b 13), an allergenic lipolytic esterase from *Hevea brasiliensis* latex. *Journal of Biological Chemistry* **279,** 23933–23941.

Bannai H, Tamada Y, Maruyama O, Nakai K, Miyano S. 2002. Extensive feature detection of N-terminal protein sorting signals. *Bioinformatics* **18,** 298–305.

Beisson F, Gardies AM, Teissere M, Ferte N, Noat G. 1997. An esterase neosynthesized in post-germinated sunflower seeds is related to a new family of lipolytic enzymes. *Plant Physiology and Biochemistry* **35,** 761–765.

Beisson F, Koo AJ, Ruuska S, *et al*. 2003. *Arabidopsis* genes involved in acyl lipid metabolism. A 2003 census of the candidates, a study of the distribution of expressed sequence tags in organs, and a web-based database. *Plant Physiology* **132,** 681–697.

Bitto E, Bingman CA, McCoy JG, Allard STM, Wesenberg GE, Phillips Jr GN. 2005. The structure at 1.6 Å resolution of the protein product of the At4g34215 gene from *Arabidopsis thaliana*. *Acta Crystallographica Section D* **61,** 1655–1661.

Bonaventure G, Beisson F, Ohlrogge J, Pollard M. 2004. Analysis of the aliphatic monomer composition of polyesters associated with *Arabidopsis* epidermis: occurrence of octadeca-*cis*-6, *cis*-9-diene-1,18-dioate as the major component. *The Plant Journal* **40,** 920–930.

Bornscheuer UT, Kazlauskas RJ. 2006. *Hydrolases in organic synthesis: regio- and stereoselective biotransformations*, 2nd edn. Weinheim: Wiley-VCH.

Broun P, Poindexter P, Osborne E, Jiang CZ, Riechmann JL. 2004. WIN1, a transcriptional activator of epidermal wax accumulation in *Arabidopsis*. *Proceedings of the National Academy of Sciences*. *USA* **101,** 4706–4711.

Cho H, Cronan Jr JE. 1993. *Escherichia coli* thioesterase I, molecular cloning and sequencing of the structural gene and identification as a periplasmic enzyme. *Journal of Biological Chemistry* **268,** 9238–9245.

Cohen ES, Romero JM, Doyle S, Elliot R, Murphy G, Carpenter R. 1990. Floricaula; a homeotic gene required for flower development in *Antirrhinum majus*. *Cell* **63,** 1311–1322.

Croteau R, Kolattukudy PE. 1974. Biosynthesis of hydroxyfatty acid polymers. Enzymatic synthesis of cutin from monomer acids by cell-free preparations from the epidermis of *Vicia faba* leaves. *Biochemistry* **13,** 3193–3202.

Dalrymple BP, Cybinski DH, Layton I, McSweeney CS, Xue GP, Swading YJ, Lowry JB. 1997. Three *Neocallimastix patriciarum* esterases associated with the degradation of complex polysaccharides are members of a new family of hydrolases. *Microbiology* **143,** 2605–2614.

De Castro E, Sigrist CJA, Gattiker A, Bulliard V, Langendijk-Genevaux PS, Gasteiger E, Bairoch A, Hulo N. 2006. ScanProsite: detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins. *Nucleic Acids Research* **34,** 362–365.

Deshmukh AP, Simpson AJ, Hadad CM, Hatcher PG. 2005. Insights into the structure of cutin and cutan from *Agave americana* leaf cuticle using HRMAS NMR spectroscopy. *Organic Geochemistry* **36,** 1072–1085.

Dickstein R, Hu X, Yang J, Ba L, Coque L, Kim DJ, Cook DR, Yeung AT. 2002. Differential expression of tandemly duplicated *Enod8* genes in *Medicago*. *Plant Science* **163,** 333–343.

Efremova N, Schreiber L, Bar S, Heidmann I, Huijser P, Wellesen K, Schwarz-Sommer Z, Saedler H, Yephremov A. 2004. Functional conservation and maintenance of expression pattern of *FIDDLEHEAD*-like genes in *Arabidopsis* and *Antirrhinum*. *Plant Molecular Biology* **56,** 821–837.

Espelie KE, Wattendorf J, Kolattukudy PE. 1982. Composition and ultrastructure of the suberized cell wall of isolated crystal idioblasts from *Agave americana* L. leaves. *Planta* **155,** 166–175.

Franke R, Briesen I, Wojciechowski T, Faust A, Yephremov A, Nawrath C, Schreiber L. 2005. Apoplastic polyesters in *Arabidopsis* surfaces tissues: a typical suberin and a particular cutin. *Phytochemistry* **66,** 2643–2658.

Garnier J, Gibrat JF, Robson B. 1996. GOR secondary structure prediction method version IV. *Methods in Enzymology* **266,** 540–553.

Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A. 2005. Protein identification and analysis tools on the ExPASy server. In: Walker JM, ed. *The proteomics protocols handbook*. Totowa, USA: Humana Press.

Guerrero C, Martín-Rufián M, Reina JJ, Heredia A. 2006. Isolation and characterization of a cDNA encoding a membrane bound acyl-CoA binding protein from *Agave americana* L. epidermis. *Plant Physiology and Biochemistry* **44,** 85–90.

Heath RJ, Rock CO. 1998. A conserved histidine is essential for glycerolipid acyltransferase catalysis. *Journal of Bacteriology* **180,** 1425–1430.

Heredia A. 2003. Biophysical and biochemical characteristics of cutin, a plant barrier biopolymer. *Biochimica et Biophysica Acta* **1620,** 1–7.

Holmquist M. 2000. Alpha/beta-hydrolase fold enzymes: structures, function and mechanisms. *Current Protein and Peptide Science* **1,** 209–235.

Horton P, Park KJ, Obayashi T, Nakai K. 2006. Protein subcellular localization prediction with WoLF PSORT. *Proceedings of the 4th Annual Asia Pacific Bioinformatics Conference*. Taipei, Taiwan, 39–48.

Ingham PW, Howard KR, Ish-Horowicz D. 1985. Transcription pattern of the *Drosophila* segmentation gene hairy. *Nature* **318,** 439–445.

Kalscheuer R, Steinbuchel A. 2003. A novel bifunctional wax ester synthase/acyl-CoA:diacylglycerol acyltransferase mediates wax ester and triacylglycerol biosynthesis in *Acinetobacter calcoaceticus* ADP1. *Journal of Biological Chemistry* **7,** 8075–8082.

Kerstiens G. 1996. Diffusion of water vapour and gases across cuticles and through stomatal pores presumed closed. In: Kerstiens G, ed. *Plant cuticles:an integrated functional approach*. Oxford: Bios Scientific Publishers, 121–134.

Kolattukudy PE. 1970. Cutin biosynthesis in *Vicia faba* leaves. *Plant Physiology* **46,** 759–760.

Kunst L, Samuels AL. 2003. Biosynthesis and secretion of plant cuticular wax. *Progress in Lipid Research* **42,** 51–80.

Kurdyukov S, Faust A, Nawrath C, *et al.* 2006*a*. The epidermis-specific extracellular BODYGUARD controls cuticle development and morphogenesis in *Arabidopsis*. *The Plant Cell* **18,** 321–339.

Kurdyukov S, Faust A, Trenkamp S, Bar S, Franke R, Efremova N, Tietjen K, Schreiber L, Saedler H, Yephremov A. 2006*b*. Genetic and biochemical evidence for involvement of HOTHEAD in the biosynthesis of long-chain alpha-, omega-dicarboxylic fatty acids and formation of extracellular matrix. *Planta* **224,** 315–329.

Lewin TM, Wang P, Coleman RA. 1999. Analysis of amino acid motifs diagnostic for the sn-glycerol-3-phosphate acyltransferase reaction. *Biochemistry* **4,** 5764–5771.

Li J, Derewenda U, Dauter Z, Smith S, Derewenda ZS. 2000. Crystal structure of the *Escherichia coli* thioesterase II, a homolog of the human Nef binding enzyme. *Nature Structural Biology* **7,** 555–559.

Lo YC, Lin SC, Shaw JF, Liaw YC. 2003. Crystal structure of the *Escherichia coli* thioesterase I/protease I/lysophospholipase L1: consensus sequence blocks constitute the catalytic center of SGNH-hydrolases through a conserved hydrogen bond network. *Journal of Molecular Biology* **330,** 539–551.

Lu R, Martín-Hernández AM, Peart JR, Malcuit I, Baulcombe DC. 2003. Virus-induced gene silencing in plants. *Methods* **30,** 296–303.

Marchler-Bauer A, Anderson JB, Cherukuri PF, *et al.* 2005. CDD: a Conserved Domain Database for protein classification. *Nucleic Acids Research* **33,** 192–196.

Millar AA, Clemens S, Zachgo S, Giblin EM, Taylor DC, Kunst L. 1999. CUT1, an *Arabidopsis* gene required for cuticular wax biosynthesis and pollen fertility, encodes a very-long-chain fatty acid condensing enzyme. *The Plant Cell* **11,** 825–838.

Molgaard A, Kauppinen S, Larsen S. 2000. Rhamnogalacturonan acetylesterase elucidates the structure and function of a new family of hydrolases. *Structure with Fold and Design* **8,** 373–383.

Nardini M, Dijkstra BW. 1999. Alpha/beta hydrolase fold enzymes: the family keeps growing. *Current Opinion in Structural Biology* **9,** 732–737.

Nawrath C. 2002. The biopolymers cutin and suberin. In: Somerville C. Meyerowitz E, eds. *The* Arabidopsis *book*. American Society of Plant Biologists.

Nawrath C. 2006. Unraveling the complex network of cuticular structure and function. *Current Opinion in Plant Biology* **9,** 281–287.

Negruk V, Yang P, Subramanian M, McNevin JP, Lemieux B. 1996. Molecular cloning and characterization of the CER2 gene of *Arabidopsis thaliana*. *The Plant Journal* **9,** 137–145.

Nielsen H, Engelbrecht J, Brunak S, von Heijne G. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Engineering* **10,** 1–6.

Oh IS, Park AR, Bae MS, Kwon SJ, Kim YS, Lee JE, Kang NY, Lee S, Cheong H, Park OK. 2005. Secretome analysis reveals an *Arabidopsis* lipase involved in defense against *Alternaria brassicicola*. *The Plant Cell* **17,** 2832–2847.

Ollis DL, Cheah E, Cygler M, *et al*. 1992. The alpha/beta hydrolase fold. *Protein Engineering* **5,** 197–211.

Pringle D, Dickstein R. 2004. Purification of ENOD8 proteins from *Medicago sativa* root nodules and their characterization as esterases. *Plant Physiology and Biochemistry* **42,** 73–79.

Pruitt RE, Vielle-Calzada JP, Ploense SE, Grossniklaus U, Lolle SJ. 2000. FIDDLEHEAD, a gene required to suppress epidermal cell interactions in *Arabidopsis*, encodes a putative lipid biosynthetic enzyme. *Proceedings of the National Academy of Sciences*. *USA* **97,** 1311–1316.

Quiroga M, Guerrero C, Botella MA, Barcelo A, Amaya I, Medina MI, Alonso FJ, de Forchetti SM, Tigier H, Valpuesta V. 2000. A tomato peroxidase involved in the synthesis of lignin and suberin. *Plant Physiology* **122,** 1119–1127.

Reina JJ, Heredia A. 2001. Plant cutin biosynthesis: the involvement of a new acyltransferase. *Trends in Plant Science* **6,** 296.

Roberts MR, Foster GD, Blundell RP, Robinson SW, Kumar A, Draper J, Scott R. 1993. Gametophytic and sporophytic expression of an anther-specific *Arabidopsis thaliana* gene. *The Plant Journal* **3,** 111–120.

Robertson DL, Hilton S, Wong KR, Koepke A, Buckley JT. 1994. Influence of active site and tyrosine modification on the secretion and activity of the *Aeromonas hydrophila* lipase/acyltransferase. *Journal of Biological Chemistry* **269,** 2146–2150.

Rubin B. 1994. Grease pit chemistry exposed. *Nature Structural Biology* **1,** 568–572.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: a laboratory manual*, 2nd edn. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Schnurr J, Shockey J, Browse J. 2004. The acyl-CoA synthetase encoded by LACS2 is essential for normal cuticle development in *Arabidopsis*. *The Plant Cell* **16,** 629–642.

Shaw JF, Chang RC, Chuang KH, Yen YT, Wang FG. 1994. Nucleotide sequence of a novel arylesterase gene from *Vibrio mimicus* and characterization of the enzyme expressed in *Escherichia coli*. *Biochemical Journal* **298,** 675–680.

Shinoda S, Matsuoka H, Tsuchie T, Miyoshi S, Yamamoto S, Taniguchi H, Mizuguchi Y. 1991. Purification and characterization of a lecithin-dependent haemolysin from *Escherichia coli* transformed by a *Vibrio parahaemolyticus* gene. *Journal of General Microbiology* **137,** 2705–2711.

Sieber P, Schorderet M, Ryser U, Buchala A, Kolattukudy P, Métraux JP, Nawrath C. 2000. Transgenic *Arabidopsis* plants expressing a fungal cutinase show alterations in the structure and properties of the cuticle and postgenital organ fusions. *The Plant Cell* **12,** 721–738.

Suh MC, Samuels AL, Jetter R, Kunst L, Pollard M, Ohlrogge J, Reisson F. 2005. Cuticular lipid composition, surface structure, and gene expression in *Arabidopsis* stem epidermis. *Plant Physiology* **139,** 1649–1665.

Thompson JD, Plewnial F, Thierry JC, Poch O. 2000. Rapid and reliable global multiple alignments of protein sequences detected by database searches. *Nucleic Acids Research* **28,** 2919–2926.

Upton C, Buckley JT. 1995. A new family of lipolityc enzymes. *Trends in Biochemical Science* **20,** 178–179.

Villena JF, Domínguez E, Stewart D, Heredia A. 1999. Characterization and biosynthesis of non-degradable polymers in plant cuticles. *Planta* **208,** 181–187.

Walton TJ. 1990. Waxes, cutin and suberin. *Methods in Plant Biochemistry* **4,** 105–158.

Wattendorff J, Holloway JP. 1980. Studies of the ultrastructure and histochemistry of plant cuticles: the cuticular membrane of *Agave americana* L. *in situ*. *Annals of Botany* **46,** 13–28.

Wei Y, Schottel JL, Derewenda U, Swenson L, Patkar S, Derewenda ZS. 1995. A novel variant of the catalytic triad in the *Streptomyces scabies* esterase. *Nature Structural Biology* **2,** 218–223.

Wellesen K, Durst F, Pinot F, Benveniste I, Nettesheim K, Wisman E, Steiner-Lange S, Saedler H, Yephremov A. 2001. Functional analysis of the *LACERATA* gene of *Arabidopsis* provides evidence for different roles of fatty acid omega-hydroxylation in development. *Proceedings of the National Academy of Sciences*. *USA* **98,** 9694–9699.

Xia Y, Nikolau BJ, Schnable PS. 1996. Cloning and characterization of *CER2*, an *Arabidopsis* gene that affects cuticular wax accumulation. *The Plant Cell* **8,** 1291–1304.