ISSN 0006-2979, Biochemistry (Moscow), 2007, Vol. 72, No. 5, pp. 501-508. © Pleiades Publishing, Ltd., 2007.
Published in Russian in Biokhimiya, 2007, Vol. 72, No. 5, pp. 622-630.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM06-318, February 18, 2007.

Catalase and Antiquitin from *Euphorbia characias*: Two Proteins Involved in Plant Defense?

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Received December 4, 2006
Revision received January 15, 2007

Abstract—Here we report the cDNA nucleotide sequences of a calmodulin-binding catalase and an antiquitin from the latex of the Mediterranean shrub *Euphorbia characias*. Present findings suggest that catalase and antiquitin might represent additional nodes in the *Euphorbia* defense systems, and a multi-enzymatic interaction contributing to plant's protection against biotic and abiotic stresses is proposed to occur in *E. characias* laticifers.

DOI: 10.1134/S0006297907050069

Key words: *Euphorbia characias*, catalase, antiquitin, aldehyde dehydrogenase, calmodulin, latex

Euphorbia characias is a Mediterranean shrub belonging to the Euphorbiaceae family. Characteristic of all Euphorbiaceae is the presence of laticifers [1, 2], specialized cells forming vessel-like structures containing latex, a milky sap with a complex composition, which includes alkaloids, terpenoid compounds, as well as a number of enzymes that collectively are believed to provide an important contribution to plant defense mechanisms by repelling and killing phytopathogens and sealing wounded areas [3, 4].

Previous studies conducted by our group have confirmed the presence of several enzymatic activities in the latex of *E. characias*, and some of the relevant proteins have been purified and characterized. One of these is a cationic peroxidase named ELP (*Euphorbia* latex peroxidase), a member of class III secreted plant peroxidases [5, 6]. These enzymes can oxidize a variety of aromatic molecules in the presence of hydrogen peroxide [7]. The latter then participates directly to plants' defense responses mediated by the oxidative burst, and is also believed to act as a second messenger for the induction of plant defensive genes [8, 9]. Intriguingly, ELP activity was recently shown to be regulated by its interaction with calcium/calmodulin (CaM) complexes, the first example of a CaM-binding peroxidase [6].

In plants, as in all eukaryotes, other enzymes can play a central role in the modulation of a number of H_2O_2 signaling pathways involved in defense and in the control of H_2O_2 homeostasis, contributing to both the positive and negative regulation of its levels. One of these enzymes is the well-known heme-containing redox enzyme catalase, which promotes the dismutation of hydrogen peroxide to water and molecular oxygen. Another example is amine oxidase, which is also present in *Euphorbia* latex [10], a copper-containing protein oxidizing primary amines with the formation of the corresponding aldehyde, ammonia, and hydrogen peroxide:

$$R\text{-}CH_2\text{-}NH_2 + O_2 + H_2O \rightarrow R\text{-}CHO + NH_3 + H_2O_2.$$

Aldehydes, on their side, can be toxic for most organisms, and therefore it is important that metabolic aldehyde levels are strictly controlled. Many biologically important aldehydes and some exogenous ones are metabolized to their corresponding carboxylic acids by the superfamily of $NAD(P)^+$-dependent aldehyde dehydrogenases (ALDH). ALDHs exhibit four different enzymatic functions, i.e. detoxification, intermediary metabolism, NADPH generation, and osmotic protection, and a recent survey of 145 sequences from various organisms

Abbreviations: ALDH) aldehyde dehydrogenases; CaM) calmodulin; EAq) *Euphorbia* antiquitin; ECat) *Euphorbia* catalase; ELAO) *Euphorbia* latex amine oxidase; ELP) *Euphorbia* latex peroxidase; nt) nucleotide; RACE) rapid amplification cDNA ends; RT-PCR) reverse transcription polymerase chain reaction.

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revealed that they cluster into at least 13 subfamilies and two main phylogenetic trunks [11]. A more recent analysis highlighted the presence of some 555 distinct ALDH genes occurring in archaea, eubacteria, and eukaryotes, with the eukaryotic enzymes comprising 20 gene families [12]. Within the ALDH superfamily, antiquitin is an evolutionarily conserved protein that mainly catalyzes the oxidation of acetaldehyde, although other aliphatic and aromatic aldehydes are also substrates. This enzyme was first identified in plants, and later found to be expressed in mammals (including humans) and fishes [13, 14]. Overall, the evidence available points towards its role in the synthesis of organic osmoprotectors, and hence in the maintenance of osmotic balance and restoration of cellular turgor during period of water deficit. It has been claimed that antiquitin might also participate in the detoxification and removal of toxic and reactive aldehydes formed by lipid peroxidation under chemical and drought stresses [14].

In this paper, we demonstrate the presence of catalase and antiquitin in *E. characias* latex starting from the detection and sequencing of the relevant encoding cDNAs. The potential interaction of these two proteins with the other enzymatic systems previously described in *E. characias* latex is discussed.

The sequences reported in this paper have been deposited in the GenBank database (*E. characias* catalase, accession No. AAX88799; *E. characias* antiquitin, accession No. AAX09646).

MATERIALS AND METHODS

Materials. *Euphorbia characias* latex and young leaves were collected in the four seasons at several locations in southern Sardinia (Italy), frozen, and stored at −80°C until use. All chemicals were obtained as pure commercial products and used without further purification.

Isolation of RNA from laticifers and RT-PCR. *Euphorbia characias* branches were sliced and secretory cells content flowed directly into a tube containing 20 ml of 2× RNA extraction buffer (100 mM Tris-HCl, 300 mM LiCl, 10 mM EDTA, 10% SDS, pH 9.5) with 5 ml of RNAlater solution (Sigma, USA) to stabilize and protect RNA. The resultant solution was mixed and centrifuged at 8000*g* for 15 min at 20°C. The supernatant fraction was processed using the TRI Reagent RNA isolation reagent (Sigma) according to the manufacturer's instructions. The quality of purified RNA from latex was verified by gel electrophoresis using 1% denaturing agarose gel stained with ethidium bromide, and by reading the absorbance spectrum from 220 to 300 nm. To obtain cDNAs, *Euphorbia* RNAs were reverse transcribed with an oligo-dT primer using an enhanced avian myeloblastosis virus reverse transcriptase enzyme (Sigma).

PCR amplification with hybrid primers. Oligonucleotide primers for catalase and antiquitin were designed using the consensus degenerate hybrid oligonucleotide primer (CODEHOP) strategy [15], starting from the alignment of multiple catalase and aldehyde dehydrogenases/stress proteins sequences from different plant sources. Four sequences each for both catalase and antiquitin were chosen from the GenBank database and aligned using ClustalW (http://www.ebi.ac.uk/clustalw), and then cut into blocks using the Block Marker software (http://blocks.fhcrc.org/blocks/). Primers were designed using the default parameters at the CODEHOP server (http://blocks.fhcrc.org/codehop.html).

For *Euphorbia* catalase (ECat), the sense primer 5′-CGTGTTCTTCATCCGGgayggnatgaa-3′ was used in association with the antisense primer 5′-GGTCACGTCCAGGGGGtcraartcraa-3′ (Fig. 1). For *Euphorbia* antiquitin (EAq), the sense 5′-GCGGCAACTGCGTGgtntggaargg-3′ and antisense 5′-GGTGCACCGCTGGccngcngtncc-3′ primers were employed (Fig. 2). For both ECat and EAq, each primer presents the consensus clamp given in upper case whereas the degenerate core is in lower case: y = [C, T]; r = [A, G], and n = [A, G, C, T]. PCR was performed in a solution containing 1.5 mM $MgCl_2$, 100 mM Tris-HCl, pH 8.3, 50 mM KCl, 200 μM of dNTP mix, 1 μM sense primer, 1 μM antisense primer, 1 μg of *Euphorbia* cDNA, and 1-3 units of Hot Master Taq (Eppendorf, Germany). Thermal cycles of amplification were carried out in a Personal Eppendorf Mastercycler (Eppendorf) using slightly different programs for ECat and EAq. CODEHOP PCR products were identified by Southern blot using homologous cDNA amplified fragments as probes and standard procedures.

Rapid amplification of cDNA ends (RACE). Rapid amplification of the 5′ end was done using three antisense-specific primers and two anchor primers provided in the RACE kit (Roche Diagnostics, Germany) [16]. To perform 5′ RACE for EAq, the first antisense-specific primer 5′-TTGGTGCACCTTTCCACACAGCGC-3′ was used in a reverse transcription reaction with 2 μg of *E. characias* total RNA. For ECat, the first primer was instead 5′-GGTGTTCACACCCGAGCCGTCCATATG-3′. The first strand of cDNA was purified from unincorporated nucleotides, enzyme, and primers by the High Pure PCR Purification Kit (Roche Diagnostics). A homopolymeric tail was added to the 3′ end of RT-PCR products. The obtained cDNA was amplified by PCR using the oligo dT-anchor primer provided in the RACE kit according to the protocol supplied, a nested antisense-specific primer 5′-CATGTGGAGGCTTTCAGGATGGTGGGAG-3′ for ECat and 5′-CAGCACATGGGAAGTTGAAAGCCG-3′ for EAq. The amplification products for catalase and antiquitin genes were then amplified using a third and last antisense-specific primer and the sense PCR anchor primer provided in the RACE kit. The sequences of these

antisense-specific primers were 5′-GTCTGGAAACTT-CATCCCATCACGG-3′ for ECat and 5′-CTTGAA-CTTCACCGATGCCTTCAGC-3′ for EAq.

For the 3′ end, the first strand cDNA was obtained using the oligo dT-anchor primer. cDNAs were then amplified using the sense-specific primer 5′-GCTGAT-ACTCAACGACACCGTTTGGG-3′ for the ECat gene and the sense-specific primer 5′-CAGGCGGTTCAGT-TATAGAGTCTGAGGGG-3′ for the EAq gene. For both genes, the sense-specific primer used was associated to the antisense PCR anchor primer provided in the RACE kit. PCR reactions were performed using 1-3 units of Hot Master Taq under different experimental conditions.

cDNA blotting analysis. cDNA blotting is an efficient technique to study gene expression in plants and is especially useful when applied to recalcitrant material like *E. characias* tissues, where a broad variety of secondary metabolites may interfere with other methods [17]. The cDNAs obtained from total RNA reverse-transcribed samples (5 μg) were run on a 1.2% agarose gel for 10 h at 18 V and then stained with ethidium bromide. After two washes in both denaturing (0.5 M NaOH, 1.5 M NaCl) and neutralizing (0.5 M Tris-HCl, 3 M NaCl, pH 7.5) buffers, the gel was transferred overnight to a positively charged nylon membrane (Roche Diagnostics) by capillary blotting in SSC 20× buffer. The filter membrane was finally hybridized with a full-length ECat or EAq cDNA as a probe.

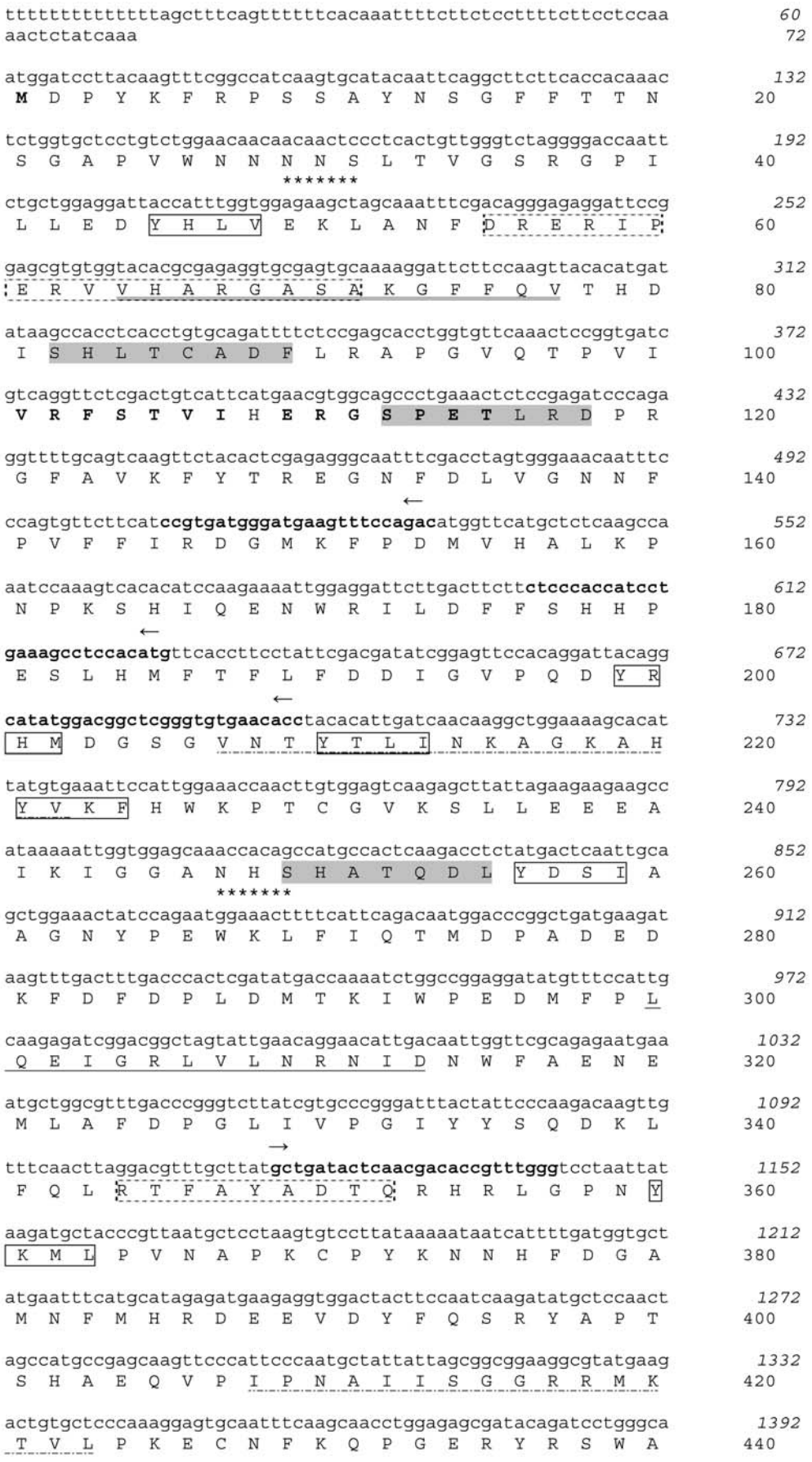
cDNA sequencing and analysis. cDNA sequencing was performed by MWG Biotech (Ebersberg, Germany). Nucleotide and deduced amino acid sequence analyses were performed with the OMIGA v.2.0 software (Oxford Molecular, USA). Translation of nucleotide sequences was done using OMIGA or the ExPASy translate routine software (http://ca.expasy.org/). Similarities were analyzed with the advanced BLAST algorithm, available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/), and with the FASTA algorithm v.3.0 from the European Bioinformatics Institute website (http://www.ebi.ac.uk/fasta33/index.htlm). Sequences were aligned with ClustalW (http://www.exapsy.ch/tools/blast/).

RESULTS AND DISCUSSION

Isolation and characterization of catalase cDNA from *Euphorbia* latex. We cloned the cDNA encoding for *Euphorbia* latex catalase by RT-PCR as described above. Catalase cDNA sequence was determined for both strands using a progressive primer design strategy. The complete cDNA contains an open reading frame of 1479 bp, which extends from the ATG codon at nt 73-75 to the termination codon at nt 1554 (Fig. 1). The 3′-untranslated region includes two potential polyadenylation signals (nt 1695-1700 and 1726-1731), both matching exactly the AATAAA consensus. It seems likely that the second signal represents a near-upstream element associated with polyadenylation of the mRNA 3′-end. The ECat gene encodes a protein of 493 amino acids (Fig. 1). The calculated theoretical molecular mass for the predicted protein is 56.8 kD, with a p*I* of 7.12.

Using a cDNA blotting method [17], the expression of the catalase gene was detectable in latex (data not shown). The transcript length was 1.8 kb, in accordance with the results of sequencing reactions. The deduced amino acid sequence of ECat has high identity (84%) and similarity (91%) with other catalases from different plant sources (*Manihot esculenta*, *Prunus persica*, *Heliantus annuus*, and *Arabidopsis thaliana*). Analysis of the ECat sequence performed through bioinformatics programs (http://elm.eu.org/) reveals the presence of a heme-ligand peptide from residues 344 to 352 and the location of the active site from residues 55 to 71. Protein kinase CK1 phosphorylation motifs are also present at the positions 82-88, 112-118, 249-255. CK1 denotes a family of ubiquitous pleiotropic serine/threonine protein kinases implicated in a number of cellular functions [18]. Two additional phosphorylation sites are found at positions 101-107 and 109-115, both of them showing consensus sequences for protein kinase A (R-X-S/T). Submission of the ECat predicted sequence to the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/) permitted the identification of two generic N-glycosylation motifs at residues 29-31 and 247-249. ECat also contains a tyrosine-based sorting signal Yxx (LMVIF) repeated at six different positions (45-48, 199-202, 211-214, 221-224, 256-259, 360-363), which could theoretically be responsible for the interaction of the protein with the μ-subunits of clathrin-associated adaptor protein complexes [19].

Since activation of catalase by calcium/CaM has been previously reported in *Arabidopsis* [20], we screened the predicted ECat amino acid sequence for putative CaM-binding sites, using the tools provided by the Web-based Calmodulin Target Database (http://calcium.uhnres.utoronto.ca/ctdb). As interaction between CaM and target proteins is known to be mainly hydrophobic in nature, and to require the binding of CaM to short basic peptides in target sequences folded into amphiphilic α-helical structures, this method scans sequences for features such as hydropathy, α-helical propensity, residue charge, and hydrophobic residue content. A normalized score (0-9) is attributed based on these criteria, a string of high values indicating the location of a putative binding site [21]. Through this analysis, the presence of a putative CaM-binding domain between residues 300-313 of ECat, a 14-amino acid sequence (LQEIGRLVLNRNID) with the characteristics of an IQ-like motif, was identified (Fig. 1). In addition, three related motifs for CaM-binding belonging to the subclasses termed 1-8-14 and 1-16 can be spotted between residues 64-77 (VHAR-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S G A P V W N N N N N S L T V G S R G P I 40
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L L E D Y H L V E K L A N F D R E R I P 60
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I S H L T C A D F L R A P G V Q T P V I 100
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N P K S H I Q E N W R I L D F F S H H P 180
←
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←
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H M D G S G V N T Y T L I N K A G K A H 220
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Y V K F H W K P T C G V K S L L E E E A 240
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I K I G G A N H S H A T Q D L Y D S I A 260
****
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K F D F D P L D M T K I W P E D M F P L 300
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→
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F Q L R T F A Y A D T Q R H R L G P N Y 360
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K M L P V N A P K C P Y K N N H F D G A 380
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M N F M H R D E E V D Y F Q S R Y A P T 400
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S H A E Q V P I P N A I I S G G R R M K 420
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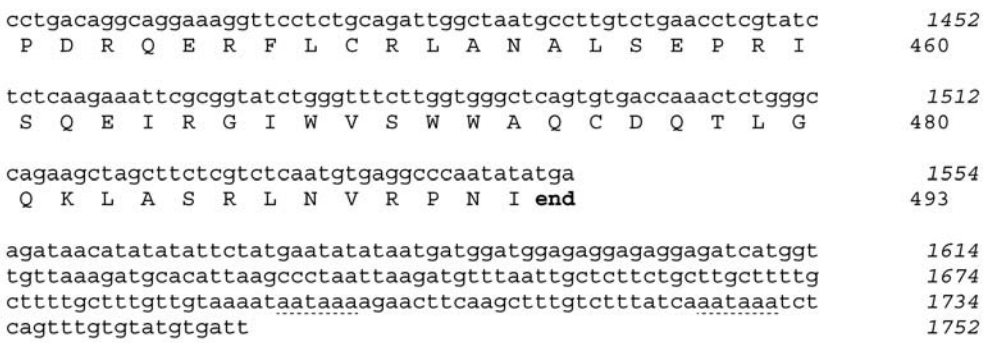


Fig. 1. Nucleotide and deduced amino acid sequences of *E. characias* catalase. The nucleotide sequence is numbered in the 5′ to 3′ direction. The terminal tga codon is indicated by "end". "M" corresponds to the N-terminal residue. Arrows indicate specific sense (→) and antisense (←) primers used in RACE experiments. The predicted CaM-binding IQ-like motif (residues 300-313) is underlined, 1-8-14 motif (residues 64-77) is double underlined, and two 1-16 motifs (207-222 and 408-423) are underlined with a dashed-dotted line. The predicted active site (aa 55-71) and proximal heme-ligand peptide (aa 344-352) are in dashed boxes. Protein kinase CK1 phosphorylation sites in positions 82-88, 112-118, 249-255 are shaded in gray. Protein kinase A phosphorylation sites at positions 101-107 and 109-115 are in bold. Asterisks in amino acid sequence indicate N-glycosylation motifs (aa 29-31 and 247-249). Tyrosine-based sorting signals (aa 45-48, 199-202, 211-214, 221-224, 256-259, 360-363) are boxed. The two potential polyadenylation signals aataaa (nt 1695-1700 and 1726-1731) are underlined with a dotted line.

GASAKGFFQV), 207-222 (VNTYTLINKAGKAHYV), and 408-423 (IPNAIISGGRRMKTVL) (Fig. 1).

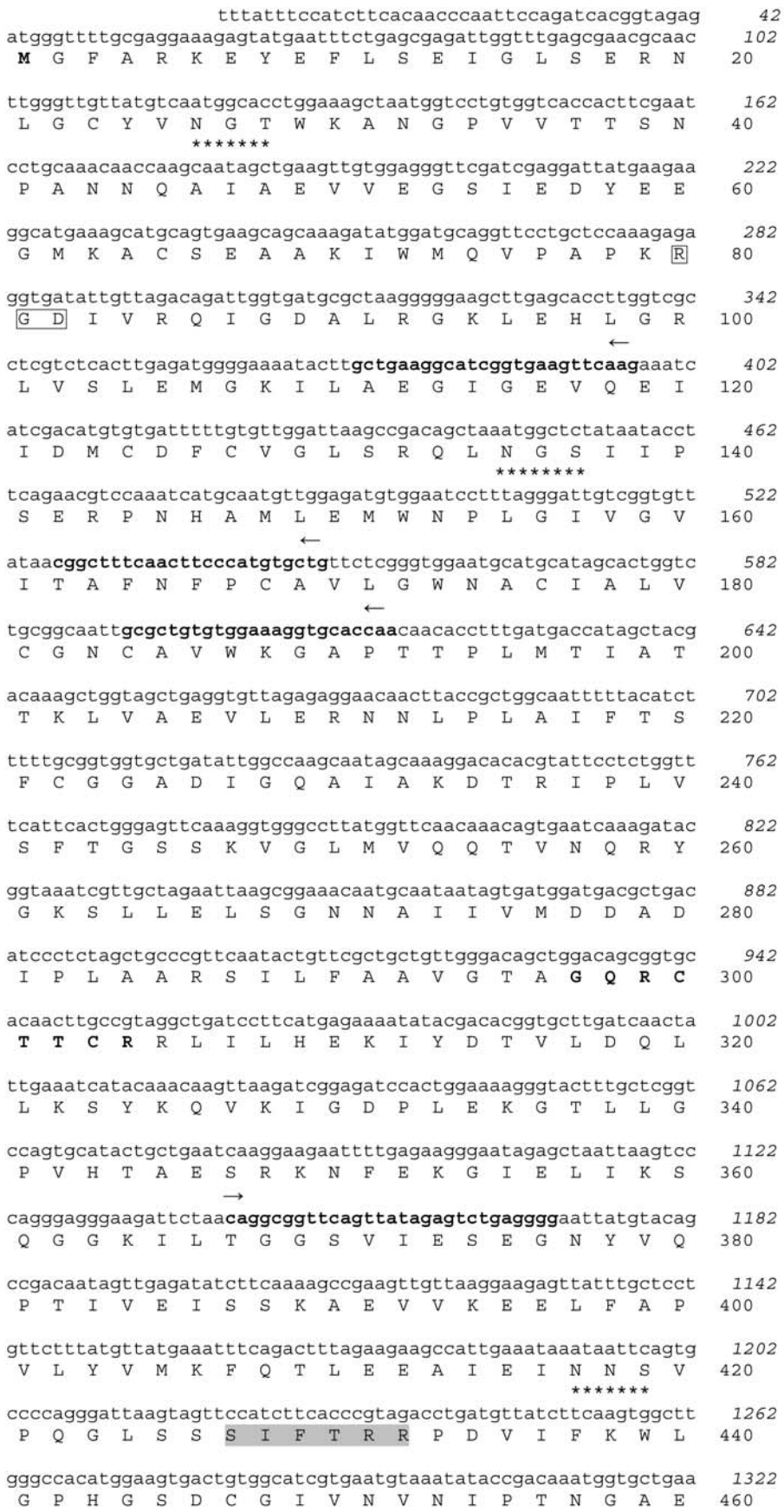
Mean residue hydrophobicity (*H*) and hydrophobic moment (μ) were calculated using the Kyte–Doolittle scale of hydrophobicity [22]. The average propensity for α-helix formation was calculated using the Chou–Fasman values [23].

Isolation and characterization of antiquitin cDNA from *Euphorbia* latex. The cloned cDNA encoding for *Euphorbia* latex antiquitin contains an open reading frame of 1427 bp, extending from the ATG codon at nt 43-45 to the termination codon at nt 1469 (Fig. 2). The 3′-untranslated region includes a putative polyadenylation signal (AATAAA) which lies 17 residues apart from the poly(A) tail. The predicted 508 amino acid protein has a calculated theoretical molecular mass of 54.6 kD and a p*I* of 5.54. The cDNA blotting method [17] revealed the expression of the EAq gene in latex and confirmed that transcript length was 1.6 kb, detected through sequencing reactions (data not shown).

The EAq amino acid sequence shows a significant degree of identity (72%) and similarity (82%) with antiquitins (aldehyde dehydrogenase family 7, member A1) and stress proteins isolated from several other higher plants (*Arabidopsis thaliana*, *Oryza sativa*, *Brassica napus*, and *Pisum sativum*). Antiquitin also shows ~45% sequence identity with some aldehyde dehydrogenases belonging to different classes. Analysis of the predicted amino acid sequence using ELM reveals the presence of a CK1 phosphorylation site at position 427-432, and two additional protein kinase A phosphorylation sites are found at positions 297-304 and 486-492. Three generic motifs for N-glycosylation are found at residues 26-28, 135-137, and 417-419. The EAq catalytic domain shows a number of conserved residues implied in the catalytic reaction of ALDHs. According to the reaction mechanism suggested by Marchal et al. [24], Cys300 performs the nucleophilic attack on the aldehyde group of the substrate to form the thiohemiacetal. Asn165 stabilizes the intermediate and facilitates the transfer of the hydride to the co-substrate. Glu266 activates the subsequent deacylation step by directed hydrolysis of the thioester bond. The nicotinamide ring of NAD could interact with Gly224, Ser245, and Glu396, as described for other ALDHs [25]. An RGD motif is detectable at position 94-96 (Fig. 2). This motif is found in several extracellular matrix adhesive proteins, where it seems to be involved in the adaptation of plant cells to salt and cold stress [26].

Potential multi-enzymatic interactions in *Euphorbia* latex. In general, very scant information is available for what concerns the physiological role played by plant laticifers and latex biochemistry. In our experimental model system, *E. characias*, two distinct latex enzymatic activities have been characterized so far: i) the H_2O_2-producing amine oxidase (ELAO), a copper/quinone-containing enzyme that catalyze the oxidative deamination of diamines and polyamines to aldehyde and ammonia, concomitantly with a two-electron reduction of dioxygen to hydrogen peroxide [10, 27]; ii) a Ca^{2+}/CaM-regulated class III secreted peroxidase (ELP), probably involved in the activation of plant defense responses and in the homeostasis of H_2O_2 [5, 6].

In this study, we extended our observations to demonstrate the expression of catalase and antiquitin genes in *E. characias* laticifers. Scheme depicts the potential interactions involving the enzymes currently known to be present in this tissue. One could hypothesize that ELAO controls the level of mono-, di-, and polyamines,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ttgggttgttatgtcaatggcacctggaaagctaatggtcctgtggtcaccacttcgaat 162
L G C Y V N G T W K A N G P V V T T S N 40
****
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P A N N Q A I A E V V E G S I E D Y E E 60
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G M K A C S E A A K I W M Q V P A P K R 80
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←
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←
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←
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T K L V A E V L E R N N L P L A I F T S 220
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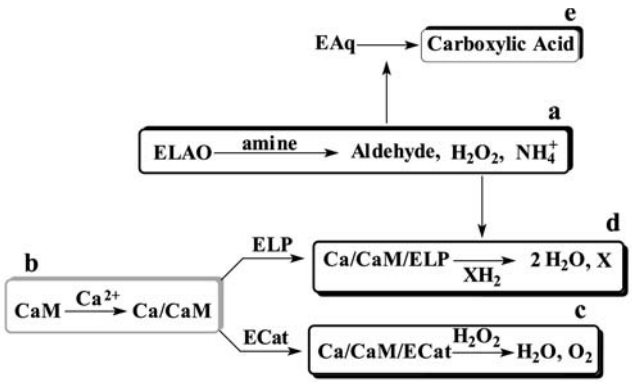
attggtggtgcttttggtggagagaaggcaactggtggcggccgtgaagcaggaagtgac   1382
 I  G  G  A  F  G  G  E  K  A  T  G  G  G  R  E  A  G  S  D   480

tcctggaagcagtatatgcgagcctccacatgcacaatcaactatgggagcgaattacca   1442
 S  W  K  Q  Y  M  R  A  S  T  C  T  I  N  Y  G  S  E  L  P   500

ctagcacaaggtattaacttcggctag                                   1469
 L  A  Q  G  I  N  F  G end                                    508

tcgataaactacacaaaggtgaagcatcttaacttccatattcacagtggagtttcggat   1529
ggggaacatatgtgctaagctttaagttaataacaataataaacaatcatcatgattgta   1589
aaaaaaaaaaa                                                   1600
```

Fig. 2. Nucleotide and deduced amino acid sequences of *E. characias* antiquitin. The nucleotide sequence is numbered in the 5′ to 3′ direction. The terminal tga codon is indicated by "end". The polyadenylation signal aataaa (nt 1567-1572) is underlined (dotted line). "M" corresponds to the N-terminal residue. Arrows indicate specific sense (→) and antisense (←) primers used in RACE experiments. The RGD motif (aa 80-82) is boxed. CK1 phosphorylation site at aa 427-432 is shaded in gray. Protein kinase A phosphorylation sites at positions 297-304 and 486-492 are in bold. Asterisks in amino acid sequence indicate N-glycosylation motifs (aa 26-28, 135-137, 417-419).



Potential interactions between enzymatic systems in *E. characias* laticifers. The oxidation of biogenic amines by *Euphorbia* latex amine oxidase (ELAO) generates hydrogen peroxide, aldehyde, and ammonia (a). Hydrogen peroxide is a substrate for two enzymatic systems: ECat, which promotes the dismutation of H_2O_2 to water and molecular oxygen (c); *Euphorbia* latex peroxidase (ELP), which utilizes hydrogen peroxide to oxidize a second reducing substrate (indicated as XH_2 in (d)). The activity of ELP is enhanced by the Ca^{2+}/CaM activated complex, and since ECat also contains CaM-binding sites, it is likely that also this enzyme is calcium regulated (b). Finally, aldehydes produced by the action of ELAO could be oxidized and detoxified by EAq (e)

and presumably participates to the lignification of cell wall and, through the production of hydrogen peroxide, to the defensive oxidative burst. The oxidation of biogenic amines, on the other side, may generate biologically active substances, specifically aldehydes. EAq, therefore, could either catalyze the oxidation of these metabolic and potentially toxic products, or could use them as substrates for the synthesis of osmoprotectors, thus assisting the turgor control in laticifer cells. Finally, both ELP and ECat are involved in the regulation of a number of H_2O_2-signaling pathways related to defense against invading pathogens/environmental stresses and in the control of H_2O_2 homeostasis in many better-characterized plant organisms, and it is reasonable to expect them to have the same functions in *Euphorbia* laticifers. A particularity of ELP and, inferentially, of ECat is that they are calmodulin-binding proteins, which might call for their stance as important nodes in the finely tuned cross-talk between calcium and H_2O_2 that is increasingly emerging as a substantial characteristic of plant defense systems [20, 28-30]. Whereas ELP represents the first example of a peroxidase regulated by the classic Ca^{2+}/CaM signal transduction mechanism, recent findings have indicated the activation of a catalase from *Arabidopsis* by the same system [20].

This study was partially supported by 60% funds from Cagliari University.

REFERENCES

1. Rudall, P. (1994) *Ann. Missouri Bot. Gard.*, **81**, 270-282.
2. Webster, G. L. (1994) *Ann. Missouri Bot. Gard.*, **81**, 3-32.
3. Kekwick, R. G. O. (2001) *Latex and Laticifers, Encyclopedia of Life Science*, Available at http://www.els.net. Accessed 2006 February 28.
4. Ko, J. H., Chow, K. S., and Han, K. H. (2003) *Plant Mol. Biol.*, **53**, 479-492.
5. Medda, R., Padiglia, A., Longu, S., Bellelli, A., Arcovito, A., Cavallo, S., Pedersen, J. Z., and Floris, G. (2003) *Biochemistry*, **42**, 8909-8918.
6. Mura, A., Medda, R., Longu, S., Floris, G., Rinaldi, A. C., and Padiglia, A. (2005) *Biochemistry*, **44**, 14120-14130.
7. Hiraga, S., Sasaki, K., Ito, H., Ohashi, Y., and Matsui, H. (2001) *Plant Cell Physiol.*, **42**, 462-468.
8. Orozco-Cardenas, M. L., Narvaez-Vasquez, J., and Ryan, C. A. (2001) *Plant Cell*, **13**, 179-191.
9. Kawano, T. (2003) *Plant Cell. Rep.*, **21**, 829-837.
10. Padiglia, A., Medda, R., Lorrai, A., Murgia, B., Pederse, J. Z., Finazzi Agro, A., and Floris, G. (1998) *Plant Physiol.*, **117**, 1363-1371.
11. Perozich, J., Nicholas, H., Lindahl, R., and Hempel, J. (1999) *Adv. Exp. Med. Biol.*, **463**, 1-7.

12. Sophos, N. A., and Vasiliou, V. (2003) *Chem. Biol. Interact.*, **143/144**, 5-22.
13. Tang, W. K., Cheng, C. H., and Fong, W. P. (2002) *FEBS Lett.*, **516**, 183-186.
14. Chan, W-M., Tang, W-K., Chen, C. H. K., and Fong, W-P. (2003) *Comp. Biochem. Physiol. B*, **136**, 443-450.
15. Rose, T. M., Henikoff, J. G., and Henikoff, S. (2003) *Nucleic Acids Res.*, **31**, 3763-3766.
16. Frohman, M. A. (1993) *Meth. Enzymol.*, **218**, 340-356.
17. Jaakola, L., Pirttila, A. M., and Hohtola, A. (2001) *Plant. Mol. Biol. Rep.*, **19**, 125-128.
18. Knippschild, U., Gocht, A., Wolff, S., Huber, N., Loehler, J., and Stoeter, M. (2005) *Cell. Signal.*, **17**, 675-689.
19. Ohno, H., Stewart, J., Fournier, M. C., Bosshart, H., Rhee, I., Miyatake, S., Saito, T., Gallusser, A., Kirchhausen, T., and Bonifacino, J. S. (1995) *Science*, **269**, 1872-1875.
20. Yang, T., and Poovaiah, B. W. (2002) *Proc. Natl. Acad. Sci. USA*, **99**, 4097-4102.
21. Yap, K. L., Kim, J., Truong, K., Sherman, M., Yuan, T., and Ikura, M. (2000) *J. Struct. Funct. Genomics*, **1**, 8-14.
22. Kyte, J., and Doolittle, R. F. (1982) *J. Mol. Biol.*, **157**, 105-132.
23. Chou, P. Y., and Fasman, G. D. (1978) *Annu. Rev. Biochem.*, **47**, 251-276.
24. Marchal, S., Rahuel-Clermont, S., and Branlant, G. (2000) *Biochemistry*, **39**, 3327-3335.
25. Pohl, E., Brunner, N., Wilmanns, M., and Hensel, R. (2002) *J. Biol. Chem.*, **277**, 19938-19945.
26. Canut, H., Carrasco, A., Galaud, J. P., Cassan, C., Bouyssou, H., Vita, N., Ferrara, P., and Pont-Lezica, R. (1998) *Plant J.*, **16**, 63-71.
27. Medda, R., Padiglia, A., and Floris, G. (1995) *Phytochemistry*, **39**, 1-9.
28. Demidchik, V., Shabala, S. N., Coutts, K. B., Tester, M. A., and Davies, J. M. (2002) *J. Cell Sci.*, **116**, 81-88.
29. Foreman, J., Demidchik, V., Bothwell, J. H., Mylona, P., Miedema, H., Torres, M. A., Linstead, P., Costa, S., Brownlee, C., Jones, J. D., Davies, J. M., and Dolan, L. (2003) *Nature*, **422**, 442-446.
30. Mittler, R., Vanderauweram, S., Gollery, M., and van Breusegem, F. (2004) *Trends Plant Sci.*, **9**, 490-498.