Euphorbia latex biochemistry: Complex interactions in a complex environment

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Abstract

Plant latex is a complex environment. Occurring in hundreds of plant species and contained in a tube system called laticifers, latex is a milky sap with a diverse composition that includes alkaloids, terpenoid compounds, other secondary metabolites and a number of enzymes. These substances are collectively believed to provide an important contribution to plant defence mechanisms by repelling and killing phytopathogens, and sealing wounded areas. This review provides insights of what is currently known about the biochemistry and molecular biology of plant latex, as studied in various model systems, above all the economically important rubber tree, Hevea brasiliensis. Selecting the Mediterranean shrub Euphorbia characias as a complementary experimental model, we have recently begun to disclose the properties of several components of the enzymatic machinery present in its latex. Although the scheme of multi-enzymatic interactions taking place in the E. characias latex depicted to date is certainly incomplete, the emerging scenario suggests that the role played by latex in plants might be significantly less passive than previously believed.

Keywords: Amine oxidase, antiquitin, calmodulin, catalase, Euphorbia, peroxidase

A large number of plant species may exude an often milky, variously coloured sap known as latex. According to Kekwick (2001), latex occurs in some 12,500 species, belonging to 900 genera from about 20 families – most of which are dicotyledons – growing in different ecological settings. Indeed, latex constitutes the cytoplasmic content of laticifers (Hagel et al. 2008), specialised elongated cells or vessel-like series of cells that permeate various aerial tissues of the plant, including sometimes the fruits and also the root system. Laticifers lack chloroplasts, and are generally classified as articulated, that is, composed of a longitudinal series of cells joined or fused together, and as non-articulated, that is, descended from a single cell present in the embryo that has grown in a branched or unbranched manner. These structures and their features are often used as elements to infer taxonomic and phylogenetic relationships between specific plant groups (Rudall 1994; Webster 1994; Vega et al. 2002), although the phyletic distribution of laticifers in the Plantae has not been explained to date (Pickard 2008).

Latex is an emulsion with a diversified composition that includes alkaloids, terpenoid compounds, polymeric substances, such as resins and gums, starch, oils, and a large number of proteins and enzymatic activities (Han et al. 2000; Kekwick 2001; Ko et al. 2003). Currently, no universally shared view exists about the biological role(s) of latex. A function as nutrition or water reserve, or as an excretory product where waste plant metabolites are confined, has been repeatedly proposed. Most authors, however, consider it more likely that latex provides an important contribution to plant defence mechanisms by repelling browsing animals and insects, killing or controlling the growth of microbial phytopathogens and sealing wounded areas (Kekwick 2001; Giordani et al. 2002).

Most of our knowledge on the biochemistry of latex and laticifers stems from studies on Hevea brasiliensis, a member of the Euphorbiaceae and an economically valuable tree as the main source of...
natural rubber. The proteome of *H. brasiliensis* latex has been investigated in some detail also because it contains a range of proteins that can cause allergic reactions in sensitised persons upon regular use of products made from natural rubber, such as healthcare workers wearing examination and surgical gloves (Arif et al. 2004; Wagner et al. 2007). Our knowledge of the protein functions within *Hevea* latex, including lectin-binding proteins, and enzymes involved in the isoprenoid pathway, continues to progress, leading to the recent sketching of a new proposed model for rubber latex coagulation (see Wititsuwannakul et al. 2008). Conversely, relatively little is known on the biochemical features of the latex of plants belonging to the large genus *Euphorbia*, although several authors are working to fill this gap (e.g., Yadav et al. 2006; Mazoir et al. 2008).

We have selected the Mediterranean spurge, *Euphorbia characias* – a shrubby, non-succulent euphorb commonly occurring in various habitats (rocky hillsides, along road verges, in open woods and in olive groves) in vast areas of the Mediterranean basin – as an alternative and complementary experimental model to study the complexity of plant latex biochemistry (Figure 1).

The plant’s latex has been extensively studied with characterisation of a large number of diterpene compounds, responsible for the plant’s irritant effect (Seip & Hecker 1983; Appendino et al. 2000; Corea et al. 2004).

Screening of the latex of *E. characias* has also revealed the presence of numerous enzymes, some of which might well be directly or indirectly involved in plant defence mechanisms. The present review reports on the isolation and characterisation of several of these molecules (and/or of their genes), namely a peroxidase regulated by the Ca\(^{2+}\)/calmodulin system, copper amine oxidase, catalase and antiquitin (Atq), and describes how these could interact between themselves and with other latex substances to assure some form of plant protection against invading pathogens and/or environmental stresses. It is our belief that what is learned on the biochemistry of *E. characias* latex will ultimately contribute to a broader understanding of the metabolism and function of this plant product.

**Peroxidase and calmodulin**

The superfamily of haem-containing peroxidases (EC 1.11.1.7, donor: hydrogen peroxide oxidoreductase) is a widely distributed group of enzymes found in bacteria, fungi, plants and animals that utilise hydrogen peroxide or other peroxides to catalyse a number of oxidative reactions (Welinder 1992). Non-animal peroxidases can be divided into three classes on the basis of their amino acid sequence: Class I contains...
bacterial, fungal and plant intracellular enzymes from mitochondria and chloroplasts, such as ascorbate peroxidase and cytochrome c peroxidase; Class II consists of secreted fungal peroxidases, good examples of these being manganese peroxidase and lignin-degrading peroxidase; Class III is made up of secreted plant peroxidases, with horseradish peroxidase (HRP) as its best-known member.

Plant peroxidases – for which crystal structures and a number of site-directed mutants have become available in several cases along the years – are found in the cytosol, vacuole, apoplast or cell wall, and participate in crucial physiological events, such as development and growth induction, polymerisation of cell wall lignin and suberin precursors, auxin catabolism, wound healing and defence against pathogen infection (Passardi et al. 2005). Typically, Class III peroxidases may exist under an extremely high number of isoforms within the same species, potentially implicated in different functions (Veitch 2004). The HRP isozyme C, for example, one of the more than 30 isoforms of HRP, classified as acidic, neutral and basic forms, has been the archetypal example of Class III higher plant peroxidases. HRP-C is a single glycosylated polypeptide chain containing high-spin Fe\(^{3+}\) in a protoporphyrin IX pentacoordinated to a “proximal” histidine ligand that functions to stabilise the higher oxidation states of the iron atom (Poulos et al. 1993). Another histidine, known as the “distal”, functions as an acid-base catalyst to accept one proton from the peroxide. Two mol of Ca\(^{2+}\)/mol of enzyme are also present in native HRP-C, and the Ca\(^{2+}\)-binding sites are known as the proximal and distal site, respectively, according to their location relative to the porphyrin plane. Calcium ions have been proposed to play a role in maintaining the integrity of haem pocket structure, which is the key to high catalytic activity.

A Class III peroxidase (ELP) was isolated and characterised from the latex of *E. characias* (Medda et al. 2003). ELP is a single glycosylated polypeptide chain of 347 residues with a relative molecular mass of 47 kDa. It contains a ferric iron–protoporphyrin IX in a quantum mechanically mixed-spin state, pentacoordinated to a “proximal” histidine ligand. The ELP sequence (GenBank accession number AY586601) permits to identify two highly conserved histidine residues coordinated to the haem (His\(_{50}\) and His\(_{179}\), distal and proximal, respectively). Like other secreted plant peroxidases, purified ELP has one mol of Ca\(^{2+}\)/mol of enzyme and two calcium binding sites (Figure 2). The proximal Ca\(^{2+}\) ion is strongly bound to Thr\(_{480}\), Asp\(_{560}\), Thr\(_{559}\), Ile\(_{262}\), Asp\(_{256}\), and plays a critical role in conferring structural stability to the haem environment and in retaining the enzyme active site geometry (Mura et al. 2005). The Ca\(^{2+}\) ligands at the distal site are Asp\(_{51}\), Val\(_{54}\), Gly\(_{56}\), Asp\(_{58}\) and Ser\(_{60}\).

The *Euphorbia* enzyme has, at variance with other known plant peroxidases, low specific activity for classical peroxidase substrates, whereas its activity seems to be closely regulated by exogenous Ca\(^{2+}\) ions. After addition of Ca\(^{2+}\), the *k*\(_{cat}\) increased 100 times with a decrease of *K*\(_{M}\) for H\(_2\)O\(_2\). Analysis of the steady state by stopped-flow measurements suggested that the main effect of Ca\(^{2+}\) ions is to favour the oxidation of the ferric enzyme by H\(_2\)O\(_2\) to form Compound I, a reaction intermediate. Calcium binding to the distal low-affinity site probably induces a reorientation of the distal His, thereby changing the almost inactive form of *Euphorbia* peroxidase to a high-activity form (Medda et al. 2003).
Perhaps, the most remarkable property of ELP, is that it contains two distinct calmodulin (CaM)-
binding sites. It is worth recalling that CaM is a small, highly acidic cytosolic protein, with a MW of
16.7–18.8 kDa, involved in the response to fluctuations of the intracellular concentration of Ca$^{2+}$ and
in regulating the activities of multiple proteins. Analysis of the predicted amino acid sequence of
ELP for putative CaM-binding sites was made with the tools provided by the web-based Calmodulin
Target Database (http://calcium.uhnres.utoronto.ca/ctdb). In this method, sequences are analysed for
features such as hydropathy, $\alpha$-helical propensity, residue charge, and hydrophobic residue content,
and a normalised score (0–9) is attributed based on these criteria. Our search revealed the presence of a
putative CaM-binding domain between residues 26 and 39 of ELP, a 14 amino acid sequence
(IQKELKKLFKKDVE) with the characteristics of an IQ-like motif. In addition, a related motif for
CaM-binding, termed 1–8–14, was spotted between residues 79 and 92 (LSLRKQAFKIVNDL) (Mura
et al. 2005). The IQ motif and related sequences are present, often in multiple copies, in diverse families
of CaM-binding proteins, such as myosins, neuro-modulin, neurogranin and brain-specific polypep-
tide PEP-19, and have been shown to bind CaM both in the presence and in the absence of calcium,
depending on the occurrence of particular residues in the sequence. The 1–8–14 motif makes a subclass
of the larger 1–14 motif family, a group of sequences characterised by the presence of two or more bulky
hydrophobic residues spaced by a variable number of amino acids (see Mura et al. 2005 and references
therein). These sequences bind to CaM primarily in the presence of calcium.

Calmodulin plays a pivotal role in physiological processes, as shown by its highly conserved primary
structure in all living organisms. CaMs possess four functional Ca$^{2+}$-binding domains called EF-hand,
numbered I through IV, beginning from the amino- (N) termini of the protein. Between EF-hands I–II
and III–IV is located a solvent exposed $\alpha$-helical region. The binding of Ca$^{2+}$ determines structural
modifications of this protein portion, converting apo-CaM in its active form, a more flexible structure
able to bind to the target protein(s). In this region, some hydrophobic residues (Leu, Ile and Val)
responsible for the interaction between CaM and CaM-binding proteins are particularly conserved.
Also in plants, CaM plays a significant role whose importance is increasingly understood and appreci-
ated (Zielinski 1998; Sathyaranarayan & Poovaiah 2004; Ma & Berkowitz 2007).

Following the finding of ELP as a CaM-binding protein, the next logical step has been to verify
whether this property had a functional significance in the context of latex, that is, whether a CaM-like
protein was constitutively expressed in this tissue. Indeed, the cDNA encoding for an E. characias CaM
was found and sequenced, and its protein product was later detected in the latex (Mura et al. 2005). E. chara-
cias latex CaM (ELCaM) cDNA (785 bp) contains an open reading frame (ORF) of 447 bp which can
be translated into a protein sequence of 149 amino acids (Figure 3). The calculated molecular mass for
the predicted protein is 16.8 kDa, with a pI of 4.1. EF-hand domains are located at positions 21–32, 57–68,
95–105, 130–141 with their clusters of particularly well-conserved, non-contiguous residues. Not
surprisingly, the ELCaM amino acid sequence shows a very high degree of identity (91–100%) and
similarity (99–100%) to CaMs isolated from several other higher plants, including Prunus avium, Elaeis
guinensis, Medicago truncatula, Pisum sativum, Phaseolus vulgaris and Nicotiana tabacum.

**Catalase**

Catalase (EC 1.11.1.6) is a tetrameric haem-containing enzyme promoting the dismutation of
hydrogen peroxide to water and molecular oxygen ($2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$). It is one of the most impor-
tant plant $\text{H}_2\text{O}_2$-scavenging enzymes due to its capacity for degrading $\text{H}_2\text{O}_2$ produced mainly in
the peroxisomes by oxidation of photorespiratory glycylate and in the glyoxysomes by $\beta$-oxidation of fatty acids, without consuming cellular reducing equivalents. Catalase is also an important compo-
nent of the system involved in degradation of $\text{H}_2\text{O}_2$ generated in excess by biotic and abiotic stresses
(Willekens et al. 1995, 1997).

In plants, catalase is present as multiple isoforms, encoded by a small gene family. Catalase gene
expression is well characterised in the monocotyle-
donous Zea mays (Abler & Scandalios 1993; Guan &
Scandalios 1993; Guan et al. 1996) and in the dicot-
yledonous Arabidopsis thaliana and Nicotiana plumb-
baginifolia (Willekens et al. 1994; Frugoli et al.
1996). Each of these contains three active genes
encoding catalase, namely Cat1, Cat2 and Cat3. The
differential expression of these catalase genes in
different tissues and during plant development, as
well as the differential regulation of each gene by
light, suggest the association of each specific gene
product to a particular $\text{H}_2\text{O}_2$-producing process. N.
plumbaginifolia Cat1 is highly expressed in light-
grown leaves and is positively regulated by light,
suggesting that Cat1 might be involved in scavenging
the $\text{H}_2\text{O}_2$ generated during photorespiration. N.
plumbaginifolia Cat2 is the predominant catalase
transcript in stems and vascular tissues and its
expression is not affected by light (Willekens et al.
1994). The rapid induction of Cat2 by ozone, $\text{SO}_2$

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and UV-B suggests an important role in the scavenging of H₂O₂ produced during stress conditions (Scandalios 1990). Cat3 mRNA levels are high in seeds as well as in mature and senescing petals, suggesting a specific role in glyoxysomal fatty acid degradation (Willekens et al. 1994). Based on the expression profiles of genes encoding catalase isozymes in well-known experimental models, plant catalases can be divided into three different classes. Class I is characterised by being highly expressed in photosynthetic tissues; N. plumbaginifolia Cat1 and Z. mays Cat2 can be hosted in this class. Class II catalases, including N. plumbaginifolia Cat2 and Z. mays Cat3, are highly abundant in vascular tissues. Class III, destroying glyoxysomal H₂O₂, is mainly expressed in seeds and young seedlings; this class includes Cat3 from N. plumbaginifolia and Cat1 from maize (Willekens et al. 1995).

We cloned the cDNA encoding for a catalase in E. characias latex (ECat) (Mura et al. 2007) (GeneBank accession number AAX88799). The deduced amino acid sequence consists of 493 residues with a theoretical molecular weight of 56.8 kDa and a pI of 7.12. The amino acid sequence of ECat shows a very high identity (84%) and similarity (91%) with other plant catalases (Manihot esculenta, Prunus persica, Helianthus annuus and A. thaliana) (Mura et al. 2007). Recent studies report that Ca²⁺/CaM may bind to plant catalases and activate them, as found, for example, in Arabidopsis (Yang & Poovaiah 2002). The analysis of the ECat amino acid sequence – obtained by means of a bioinformatics programme, as discussed above for peroxidase and CaM – revealed the presence of a CaM-binding domain stretching 14 residues with the characteristic of an IQ-like motif at position 300–313 (LQEIGRLVLNRNID). In addition, three related motifs for CaM-binding belonging to the subclasses termed 1–8–14 and 1–16 are found between residues 64 and 77 (VHARGASAKGFFQV), 207 and 222 (VNTYTLINKAGKAHYV) and 408 and 423 (IPNAIISGGRRMKTVL).

Copper amine oxidase

Copper/quinone-containing amine oxidases [amine: oxygen oxidoreductase (deaminating) (copper containing); EC 1.4.3.6] (Cu/TPQ AOs) are widely...
distributed throughout nature and are found in bacteria, yeasts, fungi, plants and mammals (Floris & Finazzi Agró 2004). In plants, copper AOs are involved in processes of yellowing, senescence, wound healing and cell wall biosynthesis, and play an important role in cell growth by regulating the intracellular di- and polyamine levels, while the aldehyde products might have a key role in the biosynthesis of some alkaloids (Frébort & Adachi 1995). Well-studied examples of plant AOs include the enzymes isolated from the seedlings of dry pea (Pisum sativum) (Kumar et al. 1996), lentil (Lens esculenta) (Agostinelli et al. 2005) and from E. characias latex (ELAO) (Padiglia et al. 1998).

ELAO shows very similar spectroscopic and chemical features to those of other plant copper AOs. It is a soluble homodimeric protein, and each subunit (MW $\geq$ 74 kDa) contains an active site with a tightly bound Cu$^{II}$ ion and an organic cofactor known as Topaquinone (TPQ), derived from the post-translational modification of a Tyr residue inserted in the polypeptide chain (Janes et al. 1990; Tanizawa 1995). Due to the presence of TPQ, the oxidised form of ELAO has a distinctive pink colour and shows, in addition to the protein absorbance maximum at 278 nm ($\varepsilon_{278} = 378$ mM$^{-1}$ cm$^{-1}$), a broad absorption band in the visible region at 490 nm ($\varepsilon_{490} = 6$ mM$^{-1}$ cm$^{-1}$) (Padiglia et al. 1998).

Similar to the other AOs, ELAO catalyses the oxidative deamination of primary amines to the corresponding aldehydes, with the concomitant reduction of molecular oxygen to hydrogen peroxide. The ping-pong catalytic mechanism can be divided into two half-reactions (Figure 4). The substrate specificity of ELAO is much narrower than that found in lentil and pea seedling AOs, being limited to diamines of critical molecular dimensions, and its activity for the best-known substrate putrescine ($k_c = 34$) is about one-fourth than that of the same reference AOs ($k_c \approx 155$).

The cDNA encoding for an AO has been isolated from young leaves of E. characias and sequenced (GeneBank accession number AF171698) (Padiglia et al. 2002). A single long ORF of 2068 bp encodes a protein composed of 653 amino acids with a molecular mass of about 74 kDa. Alignments of the Euphorbia AO cDNA nucleotide sequence with that of AO from lentil and dry pea seedlings revealed several conserved regions, especially in the C-terminus, with a 90–97% homology. The near 5’-region shows several insertions, deletions and a different nucleotide sequence with ca. 60% homology. The defined active site of Euphorbia AO, as deduced from its cDNA sequence, is apparently very similar to that of other well-known plant enzymes. In particular, the tyrosine residue post-translationally modified in TPQ can be identified as Tyr$_{192}$ inserted in the consensus sequence Asn–Tyr–Asp of the polypeptide chain; the copper atom, buried and not directly accessible from the solvent, can be coordinated by the imidazole groups of three conserved His residues (Padiglia et al. 2002). As recently shown, ELAO and ELP are able to oxidise the important plant intermediate tyramine, and could play a role in its metabolism (Mura et al. 2008).

**Antiquitin**

Aldehyde dehydrogenases (ALDHs; EC 1.2.1.19) make up a superfamily of proteins that share a role in the metabolism of endogenous and exogenous aldehydes, but that at the same time are greatly diversified from the functional point of view. Indeed, belonging to this group are proteins with an assigned detoxification role related to the conversion of aldehydes into the corresponding organic acids using NAD(P)$^+$ Other ALDHs seem to participate in the intermediary metabolism of amino acids and retinoic acid, and in the protection from osmotic stress through the generation of osmoprotectants, such as glycinebetaine (Ishitani et al. 1995). Over 1000 different proteins are up to now ascribed to the ALDH superfamily on the basis of nucleotide and protein sequence homologies. Those showing a >40% identity are considered to belong to the same family and, inside the same family, those sharing a 60% identity form a subfamily. Using these criteria, about 20 families of eukaryotic ALDHs are currently identified (Sophos et al. 2006; further information can be retrieved from the ALDH Gene Superfamily Database at www.uchsc.edu/sop/pharmscience/-alcdbase/aldhcov.html).

A specific family of ALDH, namely ALDH7, is particularly remarkable as its member proteins from animals and plants show an exceptional nucleotide sequence homology. For example, the identity between human ALDH7B1 and its counterpart in dry pea attains 60% (Lee et al. 1994), which is surprising considering the long evolutionary distance between the two organisms. This finding suggests that the proteins of this family must play an important role in cellular processes, so that their structure has remained substantially unchanged for a long stretch of the evolutionary history of life. Owing to this feature, the name antiquitin (Atq) was assigned to this protein family to underscore its ancient origin. This family is further divided into three subfamilies: ALDH7A, ALDH7B and ALDH7C. The first includes proteins found in animals, the second in plants, while the third is restricted to Drosophila melanogaster (Fong et al. 2006).

In plants, genes encoding for ALDH7 have been now characterised in a number of species, including Pisum sativum (Guerrero et al. 1990), Brassica napus
In all cases, an increase of gene expression was observed in response to dehydration- and salinity-induced stress. Accordingly, plants of *A. thaliana* and *N. tabacum* expressing a soybean homologue Atq gene were shown to display enhanced tolerance to drought and salinity, and also to oxidative stress, suggesting that besides acting in osmoregulation, Atq may be also involved in adaptive responses mediated by a physiologically relevant detoxication pathway in plants (Rodrigues et al. 2006). Although information on the role of Atq in animals and humans is comparatively less abundant, it is generally assumed that the function of the protein in these organisms is similar to that in plants, that is, centred around osmoregulation and/or detoxification. However, evidence – such as the implication of Atq in several human diseases – is emerging that its physiological functions might be more diversified and complex than was thought so far (Fong et al. 2006).

In *E. characias*, Atq cDNA was isolated and characterised from latex (Mura et al. 2007). The cloned cDNA encoding for this Atq (EAAtq) contains an ORF of 1527 bp which can be translated into a protein sequence of 508 amino acids (GenBank accession). The calculated theoretical molecular mass for the predicted protein is 54.6 kDa, with a pI of 5.54. The EAAtq amino acid sequence shows a high degree of identity (72%) and similarity (82%) to Atqs isolated from several other higher plants (Mura et al. 2007).
An enzymatic interactome in *Euphorbia* latex

Two tubing systems have evolved in plants that play a role in storing, moving and physically releasing secondary metabolites: the laticifers and the secretory ducts. In the first case, the secondary metabolites are stored inside the living cell(s) which produce(s) them, while in secretory ducts they are stored in an extracellular space. It is generally agreed that the ecological role of laticifers is to deter insect herbivory and possibly discourage foraging by higher animals. “To achieve these ends, laticifers normally contain sequestered chemicals that discourage their herbivores, and maintain internal pressures high enough to spew these chemicals onto or into herbivores that puncture them,” wrote William Pickard in a recent review on the general physiology and ecophysiology of laticifers and secretory ducts (Pickard 2008). Besides insects, other organisms, including fungi and other microbial pathogens, may represent a potential threat to plant health and integrity, and the laticifers/latex system seems to be equipped to counterfeit these attacks as well. In any cases, selected enzymes, such as chitinases against fungi and cysteine proteases against insects, are either directly involved in defence reactions and/or synthesise biologically active compounds (Graham & Sticklen 1994; Konno et al. 2004).

The evidence presented above clearly indicates the coexistence of multiple enzymatic activities within the latex-driving system of *E. characias*. In particular, two main players have been characterised so far in this experimental model, namely the H$_2$O$_2$-producing amine oxidase (ELAO) – a copper/quinone-containing enzyme that catalyses the oxidative deamination of diamines and polyamines to aldehyde and ammonia, concomitantly with a two-electron reduction of dioxygen to hydrogen peroxide; a Ca$^{2+}$/CaM-regulated Class III secreted peroxidase (ELP) – the first example to date of a peroxidase regulated by this classic signal transduction mechanism – probably involved in the activation of plant defence responses and in the homeostasis of H$_2$O$_2$. The later finding that catalase and Atq genes are expressed in *E. characias* laticifers further extends our view of latex biochemistry, and allows us to draw a more detailed map of some of the multi-enzymatic interactions that could potentially take place in this unusual environment.

As shown in Figure 5, one could indeed hypothesise that ELAO controls the level of mono-, di- and polyamines, and presumably participates in cell wall lignification and, through the production of hydrogen peroxide, in the defensive oxidative burst. The oxidation of biogenic amines, on the other hand, may generate biologically active substances, specifically aldehydes. EAtq, therefore, could either catalyse the oxidation of these metabolic and potentially toxic products, or could use them as substrates for the synthesis of osmoprotectants within the laticifer vacuole, thus contributing to turgor control in laticifer cells. As mentioned above, the conservation of sufficient internal turgor pressure as a result of osmotic water uptake, and its rapid recovery after latex discharge upon puncture, is a key element of laticifer function. Studies in *Hevea* have indicated that potassium ions, sucrose, malate and amino acids are the principal osmotically active solute species in latex (Pickard 2008 and references therein), but secondary metabolites may also play a role in this sense, and also in assuring protection from osmotic stress. Glycinebetaine, for example, is a metabolite with osmoprotectant properties synthesised by two enzymes: a ferredoxin-dependent choline monooxygenase which catalyses the oxidation of choline into betaine aldehyde, and glycinebetaine aldehyde dehydrogenase (BADH; EC 1.2.1.8) which converts betaine aldehyde into glycinebetaine. It represents a common compatible solute in higher plants where it accumulates in response to drought and salinity, and to low and high temperature stress. Various other ALDHs (EC 1.2.1.19) are also present in plants, with different substrate specificity (Brauner et al. 2003). Only the Atq gene has been detected in *E. characias* latex so far, and no information is currently available even on the presence/absence of glycinebetaine itself, so it is currently not possible to tell whether BADH is present or not in this peculiar metabolic environment. Future studies should be aimed at exploring further the mechanism through which *E. characias* regulates laticifer osmotic pressure, and how it protects itself from osmotic and other environmental stresses, such as drought and salinity.

Finally, since both ELP and ECat are involved in the regulation of a number of H$_2$O$_2$-signaling pathways related to defence against invading pathogens/environmental stresses, and in the control of H$_2$O$_2$ homeostasis in many better characterised plants, one would expect them to have the same functions in *Euphorbia* laticifers. A particular feature of ELP and, inferentially, of ECat is that they are CaM-binding proteins, which might call for their stance as important nodes in the finely tuned cross-talk between calcium and H$_2$O$_2$ that is increasingly emerging as an important characteristic of plant defence systems (Lamb & Dixon 1997; Demidchik et al. 2002; Yang & Poovaiah 2002; Foreman et al. 2003; Mittler et al. 2004). It is well known that CaM is a cytoplasmatic protein, and, in the ELCaM gene, a sequence that can be translated to a leader sequence with the characteristics of a secretion signal peptide is absent. Thus, its detection in the vacuolar system (latex) could be a particular event related to stress conditions. One could assume that, following plant injury
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and tissue rupture, the latex and cytoplasm contents mix, so that CaM interacts with its target proteins in latex. Another possibility is suggested by the tyrosine-based sorting signal 139YEEF142 found in the ELCaM sequence. The YxxΦ (Φ, bulky hydrophobic residue) peptide has been described in various proteins destined to be internalised by clathrin pathways (Ohno et al. 1995). Normally, YxxΦ is recognised by the µ1 subunit of the heterotetrameric adaptor protein AP1, and the derived complex is then internalised into the vacuole through clathrin-coated vesicles (Brett et al. 2002).

In conclusion, although we have just begun to look into latex biochemistry in a more analytical way than attempted before, the picture we obtain is already a stimulating one, and the perspectives far reaching.

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