Plant Physiology and Biochemistry 57 (2012) 181–192

Contents lists available at SciVerse ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Recombinant expression and biochemical characterization of sugarcane legumain

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ARTICLE INFO

Article history:
Received 16 February 2012
Accepted 17 May 2012
Available online 29 May 2012

Keywords:
Vacuolar processing enzyme
Legumain
Sugarcane
Cystatin
Expression analysis
Sugarcane development
Phytohormone response

ABSTRACT

Plant legumains, also termed vacuolar processing enzymes (VPEs), are cysteine peptidases that play key roles in plant development, senescence, programmed cell death and defense against pathogens. Despite the increasing number of reports on plant cysteine peptidases, including VPEs, the characterization of sugarcane VPEs and their inhibition by endogenous cystatins have not yet been described. This is the first report of the biochemical characterization of a sugarcane cysteine peptidase. In this work, a recombinant sugarcane legumain was expressed in *Pichia pastoris* and characterized. Kinetic studies of the recombinant CaneLEG revealed that this enzyme has the main characteristics of VPEs, such as self-activation and activity under acidic pH. CaneLEG activity was strongly inhibited when incubated with sugarcane cystatin 3 (CaneCPI-3). Quantitative analysis of CaneLEG and CaneCPI-3 gene expression indicated a tissue-specific expression pattern for both genes throughout sugarcane growth, with the strong accumulation of CaneLEG transcripts throughout the internode development. Furthermore, the CaneLEG and CaneCPI-3 genes exhibited up-regulation in plantlets treated with abscisic acid (ABA). These results suggest that CaneCPI-3 may be a potential endogenous inhibitor of CaneLEG and these genes may be involved in plant stress response mediated by ABA. Also, the expression analysis provides clues for the putative involvement of CaneLEG and CaneCPI-3 in sugarcane development and phytohormone response.

1. Introduction

Plant legumains (EC 3.4.22.34), also termed vacuolar processing enzymes (VPEs), belong to a family of cysteine peptidases – asparaginyl endopeptidase/legumain family – that is distinct from the papain family and grouped in cysteine peptidase Clan CD [1,2]. These enzymes are synthesized in the endoplasmic reticulum as inactive precursor polypeptides and transported in vesicles to the vacuoles [3]. In their destination site, VPEs mature through an autocatalytic mechanism, because the pro-peptide cleavage sites are flanked by asparagine or aspartic acid residues [2,4]. In general, cysteine peptidases play important roles in plant development, senescence, programmed cell death and defense against pests and pathogens [3].

During seed development, proteins are stored as nitrogen and carbon sources for seedling growth. The precursor forms of these storage proteins are proteolytically processed, resulting in proteins with a specific conformation that prevents their premature proteolysis. In legume seeds, for example, 11S globulins are deposited as hexamers [5]. A study analyzing the proteolytic processing of seed storage proteins found that proglobulin trimers in soybean were assembled in hexamers following cleavage at asparagine residues by VPE, but no properly processed storage proteins accumulated in an *Arabidopsis* triple mutant for VPE [6,7]. Similarly, a VPE was found to play a crucial role in the efficient proteolytic processing of proglutelin in rice [8]. Storage proteins are mobilized during germination and seedling growth. VPEs play roles in the proteolysis of storage proteins, acting in a cooperative way with papain-like cysteine peptidases during the mobilization of storage proteins [9,10]. VPEs are also involved in the proteolytic processing of papain-like pro-peptidases [11].

VPE expression analyses have classified these proteins into seed or vegetative types based on the plant tissues in which they are expressed. Studies report that vegetative VPEs play roles in senescence, stress conditions and programmed cell death during pathogen attacks [12–14]. A study on VPE promoter expression in *Arabidopsis* found that a seed-type VPE (βVPE) was predominantly

Abbreviations: ABA, abscisic acid; AMC, 7-amino-4-methylcoumarin; DAP, days after planting; E-64, trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane; EST, expressed sequence tag; GA3, gibberellic acid; HAT, hours after treatment; JA, jasmonic acid; Z-Ala-Ala-Asn-AMC, benzyloxycarbonyl-Ala-Ala-Asn-7-amide-4-methylcoumaryl; PhyCys, phytocystatin; rCaneLEG, recombinant sugarcane legumain; SA, salicylic acid; VPE, vacuolar processing enzyme.

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0981-9428/$ – see front matter © 2012 Published by Elsevier Masson SAS.
doi:10.1016/j.plaphy.2012.05.020

expressed in dry seeds, embryo axis and cotyledons, whereas vegetative-type VPEs (αVPE and γVPE) were expressed in tissues in the process of programmed cell death as well as roots and senescent leaves [15]. Moreover, some VPEs have been found to exhibit caspase-like activity and their activity appears to be essential to the virus-induced hypersensitive response. The use of caspase inhibitors was found to block the formation of lesions in tobacco leaves infected with mosaic virus and were also able to inhibit VPE activity *in vitro* [12,13].

Plant proteolysis is a complex and well-organized process with the precise regulation of peptidase activities. The different regulation modes of peptidase activities include their synthesis as pro-enzymes, which are activated only when recruited, and the regulation of their activity by endogenous inhibitors, such as cystatins [3,16,17]. Cystatins are reversible inhibitors of cysteine peptidases found in plants and animals. Plant cystatins, also called phytocystatins (PhyCys), are known to be involved in several physiological processes, such as the inhibition of endogenous cysteine peptidases and plant defense mechanisms [18–20]. Cystatins were first described inhibiting cysteine peptidase activity in the papain family [21]. Moreover, animal cystatins are described as being able to inhibit the activity of legumain enzymes through the interaction of the inhibitor with legumain in a novel second reactive site [22]. In plants, some PhyCys with a carboxy (C-) terminal extended region have been described as able to inhibit both human legumain and legumain activity in plant extracts [23].

Sugarcane EST studies have provided insight into gene expression and led to the discovery of several genes that likely play important roles in the plant development and sugar accumulation [24,25]. However, biochemical characterization studies on sugarcane cysteine peptidases, including enzymes from the legumain family, are not yet available. Moreover, there has been no report to date that the sugarcane cysteine peptidase is inhibited by endogenous PhyCys. The aim of the present article is to report the recombinant expression, purification and biochemical characterization of a legumain from sugarcane (CaneLEG) and evaluate its gene expression during plant development and after phytohormone treatments. This paper also describes the inhibition of the recombinant legumain by an endogenous C-terminal extended PhyCys (CaneCPI-3). The CaneCPI-3 expression during plant development and its response to phytohormones were also investigated.

2. Results

2.1. CaneLEG sequence analysis

The clone SCBFRT1071E03 containing the open-reading frame (ORF) from the sugarcane VPE used in the present study (CaneLEG) was identified from an expressed sequence tag (EST) library of the sugarcane variety SP80-3280 [24]. The CaneLEG ORF (GenBank ID: JQ083602) has 1467 bp and encodes a protein with 488 amino acid residues and relative molecular mass of 53.4 kDa. CaneLEG has a putative signal sequence of 22 amino acids, as predicted by the Signal P [26], as well as the N-terminal and C-terminal pro-peptides commonly found in VPEs. The analysis in the PSORT program [27] indicated that the CaneLEG signal peptide probably directs the protein to a vacuole. The amino acid sequence analysis of CaneLEG in the BLASTx program [28] showed that this protein has identity values ranging from 53 to 95% with plant VPEs. The alignment of CaneLEG with other VPEs (Fig. 1) showed that CaneLEG has the conserved catalytic residues cysteine and histidine preceded by a block of four hydrophobic amino acids commonly described for enzymes from Clan CD [29].

2.2. Heterologous expression and purification of CaneLEG

For the initial characterization, CaneLEG cDNA without the signal peptide was cloned in the vector pET29a for recombinant expression in *Escherichia coli* cells. Recombinant CaneLEG protein was expressed in insoluble form in *E. coli* cells, which hindered its purification. Thus, the recombinant CaneLEG was recovered from SDS-PAGE by electroelution (insoluble fraction) and used to produce polyclonal antibodies that were further used in the subsequent experiments. The soluble CaneLEG was obtained by expression in *Pichia pastoris* cells. The protein yield was 4 mg/L of *P. pastoris* culture.

In the initial attempt to obtain recombinant CaneLEG (rCaneLEG) in *P. pastoris*, part of the expressed protein had already been processed. The rCaneLEG processing led to C-terminal pro-peptide cleavage and the consequent loss of His-tag present at the C-terminal, which hindered its purification. To avoid the premature activation of rCaneLEG and consequent loss of C-terminal His-tag, an additional His-tag was inserted at the N-terminal of the protein. Moreover, considering that plant VPEs exhibit optimal activity in acidic pH and that activity is reduced or virtually absent in neutral or basic conditions [4,30], the pH of rCaneLEG expression induction (pH 6.0) was changed to a neutral condition (pH 7.0), which permitted the acquisition of the active purified protein. However, rCaneLEG processing was still observed throughout the induction (Fig. 2a).

After rCaneLEG purification, three major bands with molecular masses of around 60, 50 and 45 kDa were observed (Fig. 2a, lane 6). The 60-kDa band had a greater molecular mass than the expected mass of 51.1 kDa for the unprocessed rCaneLEG. To confirm that the 60-kDa band corresponded to rCaneLEG, immunoblotting was performed using the anti-CaneLEG polyclonal antibodies. This analysis demonstrated the recognition of all bands recovered after purification (Fig. 2b, lane 6), thereby confirming the authenticity of the protein, since the polyclonal antibodies were generated using a CaneLEG produced in bacteria. The 60-kDa band completely disappeared after dialysis in acidic pH (Fig. 2a and b, lane 7), indicating that this band corresponded to the unprocessed rCaneLEG. Furthermore, the observed changes in immuno-reactive bands mobility following purification may be result of the differences on buffer compositions of induced and purified samples (Fig. 2a), since the mass spectrometry of the 60-kDa and 45-kDa purified bands revealed that both corresponded to rCaneLEG (data not shown).

2.3. CaneLEG proteolytic processing

Immunoblotting analysis using monoclonal anti-His-tag antibodies was performed to investigate rCaneLEG processing. The results demonstrated that anti-His-tag antibodies recognized the processed CaneLEG bands, indicating that the protein had only hydrolyzed the C-terminal pro-peptide (Fig. 2c). To confirm that CaneLEG processing was due to self-activation, the cysteine residue of the active site was replaced with a serine residue (CaneLEGΔC^{212}). The proposed scheme of CaneLEG self-activation is displayed in Fig. 3a. In the induction of the mutant CaneLEGΔC^{212} protein, a single band with a molecular mass of 60 kDa was observed (Fig. 3b, lane 1). CaneLEGΔC^{212} was incubated in acidic pH, but even after a long period of incubation, the protein was not activated (Fig. 3b, lanes 4 and 7). However, when CaneLEGΔC^{212} was incubated with the active rCaneLEG, a band of around 45 kDa corresponding to the processed enzyme appeared after 3 h of incubation. This result demonstrates that the processing observed during rCaneLEG expression was due to the self-activation of the enzyme and that the cysteine residue (C212) is essential to CaneLEG activity (Fig. 3).

2.4. Characterization of CaneLEG

After dialysis, the rCaneLEG was recovered in the processed form (Fig. 2, lane 7), the enzymatic activity was measured only for the processed enzyme. Optimal pH for rCaneLEG was between 6.0 and 6.5, with no activity below pH 4.0 or above pH 8.0 (Fig. 4). Initially, pH 6.0 was chosen for recombinant expression of the protein, but the protein exhibited premature processing and the experimental conditions were changed to pH 7.0. However, the enzyme still exhibited 80% activity at this neutral pH value, which justifies rCaneLEG processing observed during its expression in *P. pastoris* (Fig. 4).

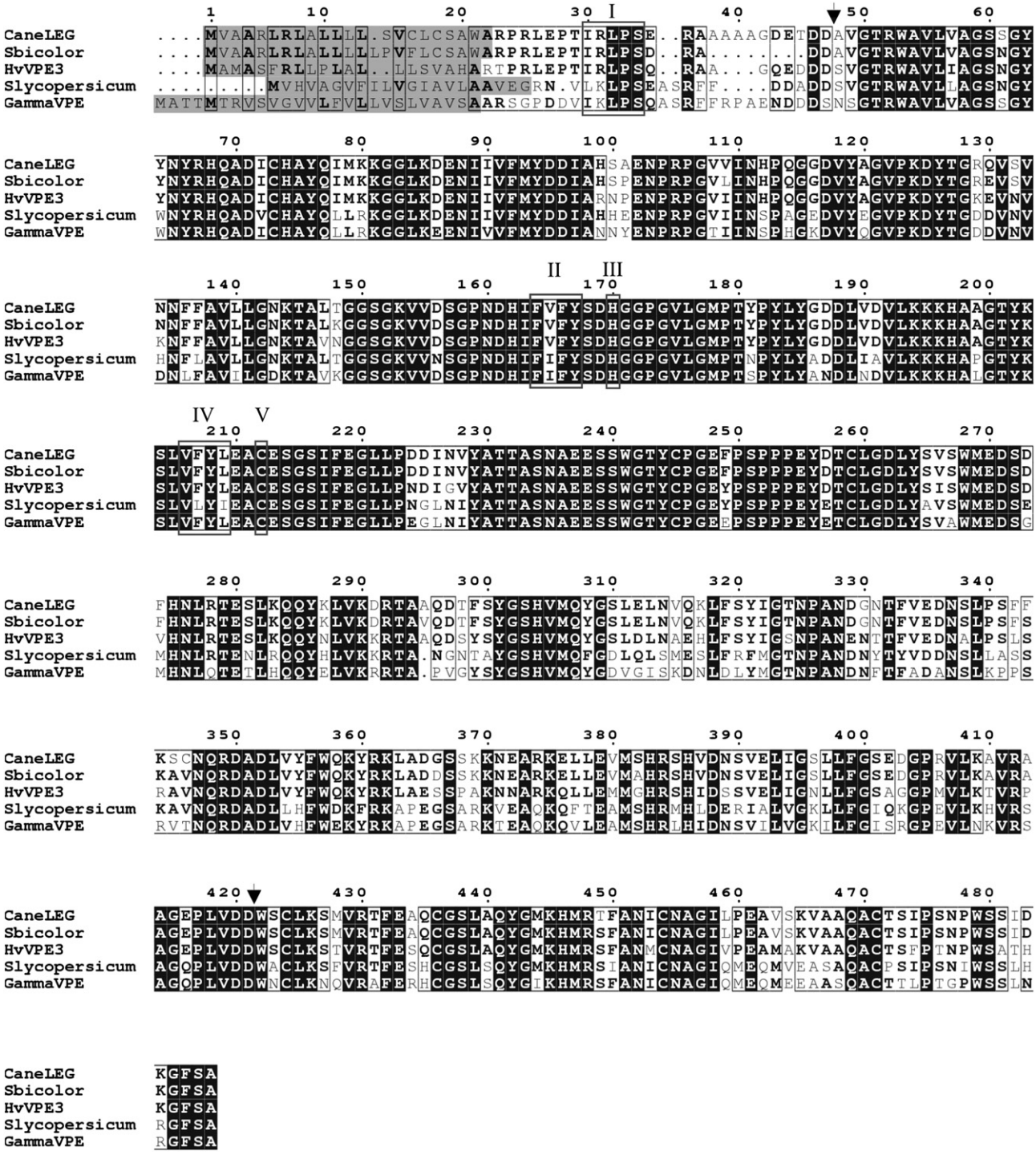

Fig. 1. Alignment between CaneLEG from *Saccharum sp* and other plant legumains. Amino acid sequence alignment was performed with legumain from *Sorghum bicolor* hypothetical protein (EES00922), barley HvVPE3 (CBX26641), *Solanum lycopersicum* VPE (CAH56498) and *Arabidopsis* γVPE (NP_195020). Protein sequence alignment was carried out by MultAlin program. The identical amino acids are shown in black box and similar amino acids are in bold. The signal peptides are shaded. Box I shows the VPE vacuolar target motif described by Jackson et al. [32]. The arrows show the putative sites of cleavage of the N and C-terminal pro-peptides. Boxes II and IV show the blocks of four amino acids preceding the catalytic residues shown in boxes III and V.

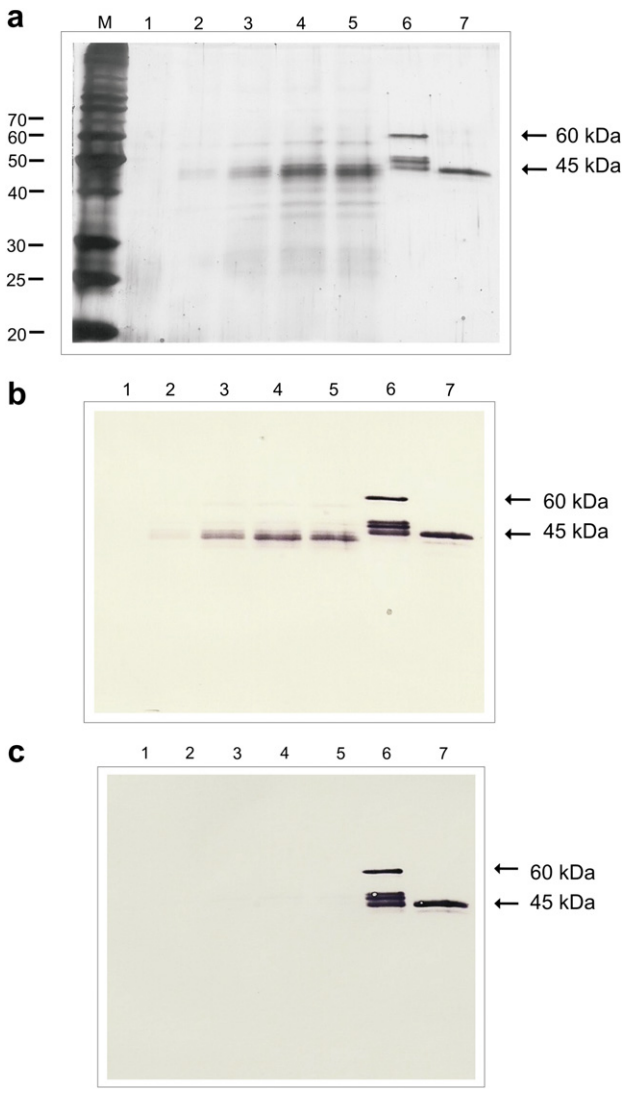

Fig. 2. Analysis of CaneLEG expression in *Pichia pastoris*, purification and immunodetection assay. (a) 12% SDS-PAGE stained with silver, showing induction of CaneLEG by methanol. Lane M molecular weight (MW) marker proteins; Lane 1 supernatant from non-induced culture; lanes 2–5 supernatant from transformed *P. pastoris* culture after induction by methanol at different time points (24, 48, 72 and 96 h, respectively); Lane 6 CaneLEG purified by affinity chromatography in nickel column, protein eluted in 100 mM imidazole before dialysis; Lane 7 CaneLEG purified after dialysis in pH 6.5. (b) Immunoblotting assay with anti-CaneLEG, lanes as in (a). (c) Immunoblotting assay with anti-His-tag, lanes as in (a). Arrows indicate the approximate MW of the corresponding bands.

Optimal rCaneLEG activity occurred at a temperature of 37 °C (Fig. 5a), but high activity (90%) was also observed when the assays were conducted at 30 °C. Moreover, a gradual loss of activity was observed after the prolonged exposure to higher temperatures. The enzyme was less affected when incubated at 4 or 10 °C for 1 h (Fig. 5b), suggesting low thermal stability of the rCaneLEG.

The rCaneLEG catalytic efficiencies (k_{cat}/K_m) were founded to be 33.3 $mM^{-1}\ s^{-1}$ and 65 $mM^{-1}\ s^{-1}$, respectively for the substrates benzyloxycarbonyl-Ala-Ala-Asn-7-amino-4-methylcoumarin (Z-Ala-Ala-Asn-AMC) and Z-Val-Ala-Asn-AMC and the K_m value was 11.69 μM for the standard legumain substrate Z–Ala–Ala–Asn–AMC.

2.5. Inhibition of CaneLEG by endogenous cystatin (CaneCPI-3)

One of the characteristics that differentiate legumains from cysteine peptidases of papain family is that legumains are not inhibited by E-64, which is a potent irreversible inhibitor of papain-like cysteine peptidases. However, legumains are inactivated by iodoacetamide, which is an irreversible inhibitor of all cysteine peptidases that alkylates the thiol groups of the cysteine residues. In the present study, iodoacetamide inhibited rCaneLEG in millimolar order, while E-64 had no effect over enzyme activity (Table 1).

The inhibitory activity of CaneCPI-3, a C-terminal extended sugarcane PhyCys previously described [31], was also investigated against rCaneLEG. CaneCPI-3 displayed strong affinity for rCaneLEG, with a K_i value of 0.28 nM (Table 1). The CaneCPI-3 C-terminal extension (CaneCPI-3Cterm) and N-terminal region (CaneCPI-3Nterm) were individually tested against rCaneLEG. A schematic representation of CaneCPI-3 and its variant forms is shown in Fig. S1. CaneCPI-3Cterm strongly inhibited rCaneLEG and CaneCPI-3Nterm exhibited very weak inhibition against rCaneLEG (Table 1). However, the K_i obtained for CaneCPI-3Cterm was higher than that observed in the inhibition assays with the full CaneCPI-3 (Table 1), suggesting that the C-terminal extension region may be responsible for legumain inhibition and that the full length of the proteinaceous inhibitor is necessary for more effective inhibition.

Moreover, inhibitory activity of the mutants CaneCPI-3ΔN^{141}, the CaneCPI-3 full protein with the asparagine residue N141 of the proposed legumain inhibitory site, SNSL, substituted with a lysine residue and CaneCPI-3CtermΔN^{141} (the C-terminal extended region of CaneCPI-3 with the same amino acid substitution) was evaluated (see schematic structure in Fig. S1). The mutants CaneCPI-3ΔN^{141} and CaneCPI-3CtermΔN^{141} had their inhibitory activity completely inactivated by the substitution of the asparagine residue of the SNSL motif with a lysine residue, indicating that N^{141} may be directly involved in the interaction between the CaneCPI-3 inhibitor and CaneLEG (Table 1).

2.6. Tissue-specific expression of CaneLEG and CaneCPI-3 throughout sugarcane development

The tissue-specific expression of CaneLEG and CaneCPI-3 in different sugarcane growth phases was investigated using quantitative real-time PCR (qPCR). RNA samples were prepared from tissues of four developmental stages of sugarcane (Fig. 6a) and the expression data were expressed as CaneLEG or CaneCPI-3 mRNA content normalized to sugarcane polyubiquitin mRNA levels. The tissues analyzed were leaves, leaf roll, root band, germinating bud, apical meristem, internode and lateral bud. The results demonstrated that both CaneLEG and CaneCPI-3 were expressed constitutively in all tissues examined and their expression patterns varied widely throughout sugarcane development (Fig. 6). In growth phase I (germination-establishment phase), CaneLEG exhibited higher expression in leaves than in the leaf roll tissue (Fig. 6b). CaneCPI-3 expression was also greater in leaves, but its expression was lower than CaneLEG in both tissues. In phase II (tillering), the highest CaneLEG expression was detected in the germinating bud and leaves and the lowest expression was found in the apical meristem (Fig. 6c). During the tillering phase, CaneCPI-3 was more detectable in the root band and a low degree of expression was found in the leaf roll (Fig. 6c).

The gene expression patterns of CaneLEG in phase III (grand growth) demonstrated higher transcript abundance in the leaves and internode tissues (Fig. 6d). The highest expression of CaneCPI-3 was found in the apical meristem and low expression was found in the internode (Fig. 6d). At the beginning of phase IV (sugarcane maturation stage), CaneLEG exhibited a strong increase in expression in the internode (4.4-fold) (Fig. 6e). CaneCPI-3 had high transcript levels in the lateral bud, apical meristem, internode and root band, with similar expression level in the two former tissues (Fig. 6e).

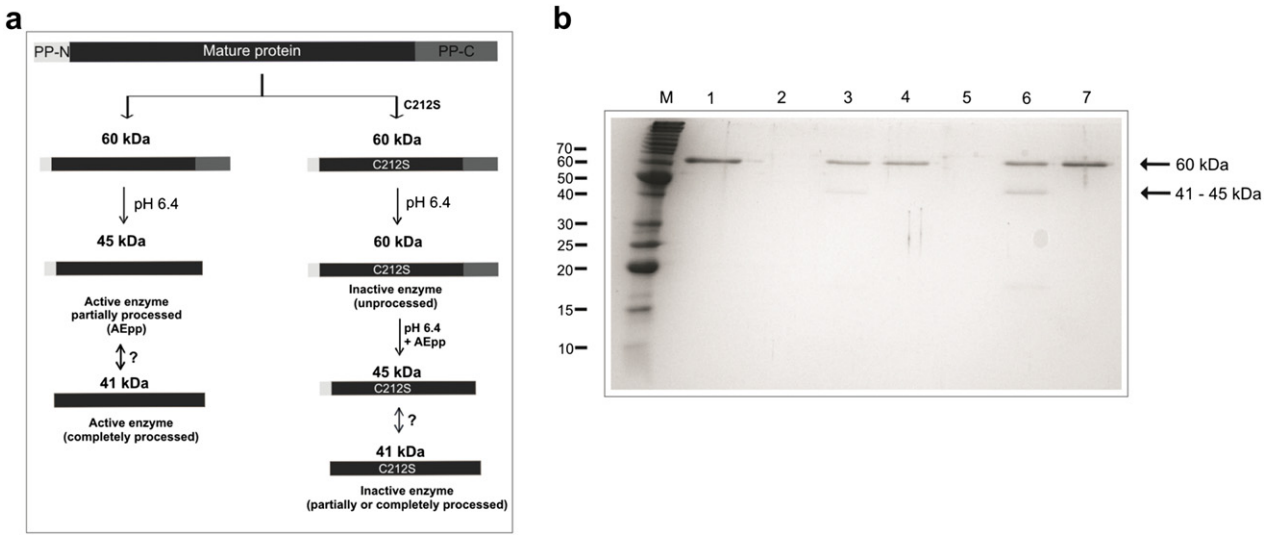

Fig. 3. Processing of recombinant CaneLEG. (a) Schematic representation of the structure of CaneLEG and *in vitro* activation of CaneLEG and CLEGΔC^{212} processing by rCaneLEG. PP-N and PP-C represent N and C-terminal pro-peptides, respectively. (b) SDS-PAGE stained with Coomassie blue showing mutant CaneLEGΔC^{212} incubated with active rCaneLEG. Lane M, molecular weight (MW) marker proteins. Lane 1 purified CaneLEGΔC^{212}. Lane 2 purified rCaneLEG, only visible when silver stained (see Fig. 2). Lanes 3–5: proteins incubated for 90 min. Lane 3 CaneLEGΔC^{212} incubated with rCaneLEG. Lane 4 CaneLEGΔC^{212} without rCaneLEG. Lane 5 rCaneLEG, same amount used in the processing assays not visible in Coomassie blue stained gel. Lanes 6 and 7: proteins incubated for 3 h. Lane 6 CaneLEGΔC^{212} incubated with rCaneLEG. Lane 7 CaneLEGΔC^{212} without rCaneLEG.

2.7. CaneLEG and CaneCPI-3 expression in sugarcane plantlets in response to phytohormone treatments

To study the response of CaneLEG and CaneCPI-3 to phytohormones, qPCR was performed with cDNA synthesized from total RNA isolated from the leaves of sugarcane plantlets treated with gibberellic acid (GA3), abscisic acid (ABA), salicylic acid (SA) and jasmonic acid (JA). The results from the phytohormone treatments were compared to those from plantlets treated with the phytohormone diluent. In plantlets treated with GA3, no significant changes in CaneLEG and CaneCPI-3 expression were found at 3 and 24 h after treatment (HAT) (Fig. 7a). In response to ABA, CaneLEG expression underwent a significant initial decline (0.36-fold) and strong induction (3.6-fold) at 24 HAT (Fig. 7b). ABA treatment led to an increase in expression of CaneCPI-3 through to 24 HAT (1.5-fold at 3 HAT and 2.1-fold at 24 HAT), but at 24 HAT, the CaneLEG expression levels were higher than those of CaneCPI-3 (Fig. 7b). In the SA treatment, CaneLEG underwent decreased expression at 3 HAT, but the control expression levels were recovered at 24 HAT (Fig. 7c). CaneCPI-3 was not significantly responsive to SA treatment even at 24 HAT (Fig. 7c). The JA treatment caused a decrease in CaneLEG and CaneCPI-3 expression at 3 HAT, with CaneLEG exhibiting the stronger decrease (0.165-fold). However, neither CaneLEG nor CaneCPI-3 exhibited significant changes at 24 HAT in the JA treatment (Fig. 7d).

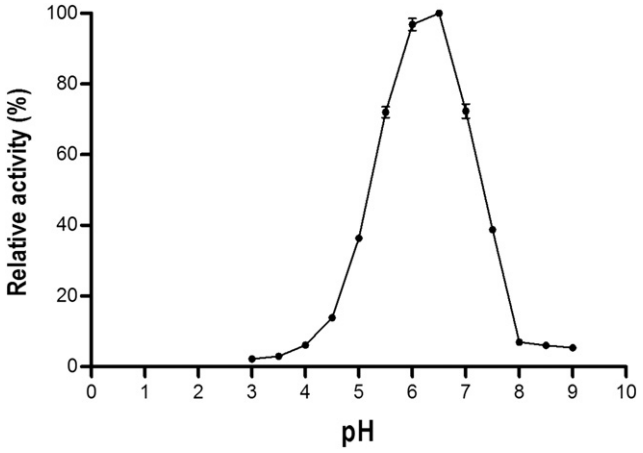

Fig. 4. Determination of optimal pH for CaneLEG activity. pH assays were carried out at 37 °C using the buffers citrate–phosphate buffer (pH range of 3.0–7.0), HEPES 50 mM (pH 7.5) and sodium borate 50 mM (pH range of 8.0–9.0). The Z–Ala–Ala–Asn–AMC substrate was used and each point represents the mean of triplicate determinations ±SE.

3. Discussion

Cysteine peptidases, including enzymes from legumain family, are quite widespread in plants. However, no sugarcane cysteine peptidase has previously been characterized. The present study describes the characterization of a sugarcane legumain and the inhibition of CaneLEG activity by CaneCPI-3 inhibitor was also investigated. Moreover, the expression of CaneLEG and CaneCPI-3 was analyzed throughout the sugarcane development stages as well as in response to the phytohormones.

In this report it was observed that CaneLEG protein sequence presented the characteristic structure from VPEs containing a signal peptide and N-terminal and C-terminal pro-peptides (Fig. 1). Moreover, the CaneLEG protein contains the vacuolar targeting motif IRLPS in the N-terminal pro-peptide and exhibits 99% similarity with the VPE sequence described by Jackson and coworkers [32] suggesting that CaneLEG may be a variant of the sequence described by these authors. Although recombinant VPE expression in a bacterial system has been described for VPE from rice [8], it was not possible to obtain the active CaneLEG in this expression system. The active rCaneLEG was obtained in *P. pastoris* cells and used in the characterization assays (Fig. 2). Some legumains have been produced in yeast, an example is the legumain from *Schistosoma mansoni* that has been expressed in *P. pastoris* [2,33]. It was demonstrated that the mutation of the cysteine residue (C^{212}) of the putative active site was sufficient to inhibit CaneLEG activation (Fig. 3). This result shows that like other VPEs [4,8] CaneLEG is self-processed. The presence of a 60 kDa band following rCaneLEG purification (Fig. 2a) indicates that the recombinant protein may be

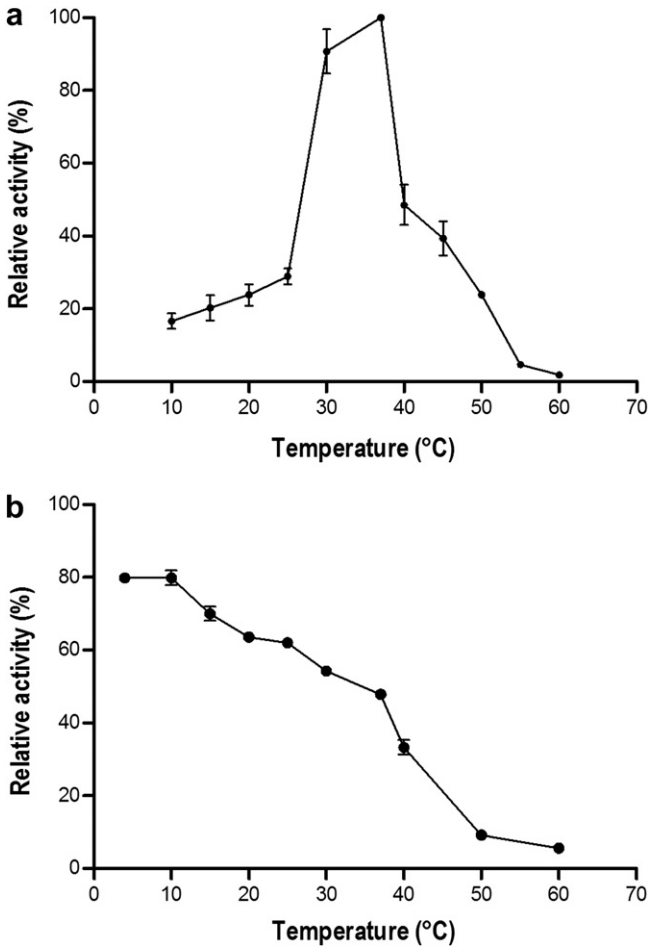

Fig. 5. Determination temperature effect on CaneLEG activity. (a) Optimal temperature of CaneLEG. Assays were performed using citrate–phosphate buffer at pH 6.5 at the specified temperatures in the range of 10–60 °C (b) Thermal stability of CaneLEG. The enzyme was incubated at temperatures in the range of 4–60 °C for 1 h in citrate–phosphate buffer pH 6.5, after which the residual activity was assayed at 37 °C and compared to control (non-incubated enzyme). In all assays the Z–Ala–Ala–Asn–AMC substrate was used and each point represents the mean of triplicate determinations ± SE.

glycosylated by *P. pastoris*, since the CaneLEG protein has a potential glycosylation site at asparagine residue 143. Although VPEs purified from plants are not glycosylated [1], it has been reported that VPEs are glycosylated when expressed in *Saccharomyces cerevisiae* and this glycosylation does not affect the enzyme activity [2,4]. The immunoblotting analysis revealed that, even after incubation periods in acidic pH, rCaneLEG only cleaved the C-terminal pro-peptide (Fig. 2c). It was reported that VPE C-terminal pro-peptide acts as an auto-inhibitory domain and the removal of the C-terminal pro-peptide under acidic conditions is enough to activate the *Arabidopsis* γVPE expressed in insect cells [4]. Moreover, it is possible that pH lower than 5 may be necessary for complete CaneLEG activation, as observed for the human legumain which removes its N-terminal pro-peptide at pH 4.5 [34]. In kinetics experiments, the K_m values showed that rCaneLEG had a higher affinity for Z–Ala–Ala–Asn–AMC substrate than legumains from kidney bean (*Phaseolus vulgaris*) and jack bean (*Canavalia ensiformis*) which showed K_m values of 56 and 33 μM, respectively [30,35]. The catalytic efficiency was calculated to determine the substrate preference of rCaneLEG. The results showed that this enzyme has a preference for valine residue in P3 position of the substrate which corroborates with the substrate preference for valine instead of alanine residue in P3 position reported for the kidney bean VPE [30]. In experiments under acid pH conditions (4.5), CaneLEG was able to hydrolyze a substrate containing Asp at P1 site (acetyl–Tyr–Val–Ala–Asp-7–amino-4-methylcoumarin) (data not shown), as described for human and plant legumains [2,36,37].

The inhibition assays showed that the endogenous sugarcane cystatin (CaneCPI-3) was the most efficient inhibitor of rCaneLEG activity (Table 1). Cystatins are natural inhibitors of cysteine peptidases that were first described as potent inhibitors of the papain-like cysteine peptidase [21]. In plants, Martinez et al. [23] studied a 23-kDa barley cystatin and found the presence of a C-terminal extended region capable of inhibiting the human legumain as well legumain activity in barley extracts. CaneCPI-3 is a sugarcane cystatin very similar to barley C-terminal extended PhyCys and it has been reported that CaneCPI-3 inhibits papain-like enzymes [31]. The present study demonstrates that CaneCPI-3 is an inhibitor of VPE, with the asparagine of the motif SNSL at the C-terminal extension putatively responsible for this inhibitory activity, as demonstrated by the loss of inhibitory activity when the asparagine residue was substituted in the CaneCPI-3 mutants (Table 1). There is evidence of endogenous protease regulation by protease inhibitors and the disturbance of this proteolysis control may affect various aspects of plant development [18,38,39]. Thus, the present results suggest CaneCPI-3 to be a potential endogenous inhibitor of CaneLEG, likely acting in the activity regulation of the enzyme in the plant.

The analysis of CaneLEG and CaneCPI-3 expression throughout sugarcane development (Fig. 6) demonstrated detectable expression levels for both genes in all analyzed tissues (Fig. 6). In the tillering phase, which starts around 40 days after planting (DAP), the plant was provided with additional stalks originating from buds in the primary internode. High CaneLEG transcript levels were found in the germinating bud in this phase. It has been reported that VPEs act in protein mobilization during seed germination and seedling growth through the direct digestion of these proteins or via the processing of other endopeptidases [9,11]. This result is consistent with the fact that sugarcane bud germination is an active stage in which the mobilization of nutrients to the developing shoot occurs [40]. Moreover, the lateral bud germination may share a resemblance with seed germination, since storage proteins previously deposited in protein storage vacuoles are mobilized during seed germination and used for seedling growth [41]. The higher transcript level of CaneCPI-3 in the apical meristem in the tillering phase is in agreement with findings reported by Gianotti et al. [31], who observed higher protein levels of this cystatin in the apical meristem of sugarcane plant than in leaves and roots.

A general tendency was observed for the maintenance or decrease in CaneLEG expression in all tissues from tillering phase to

Table 1
Inhibition constant (K_i) values of synthetic inhibitors, cystatin and its mutated variants for rCaneLEG. rCaneLEG was tested for activity with the synthetic fluorogenic substrate Z–Ala–Ala–Asn–AMC. The enzyme was preincubated in reaction buffer in the presence of each inhibitor for 10 min at 37 °C before substrate addition.

Inhibitor	K_i (nM)
Iodoacetamide	1.5×10^5
E-64	n.i.[a]
CaneCPI-3	0.28
CaneCPI-3Cterm	0.56
CaneCPI-3Nterm	3.2×10^2
CaneCPI-3ΔN^{141}	n.i.[a]
CaneCPI-3CtermΔN^{141}	n.i.[a]

[a] No inhibition.

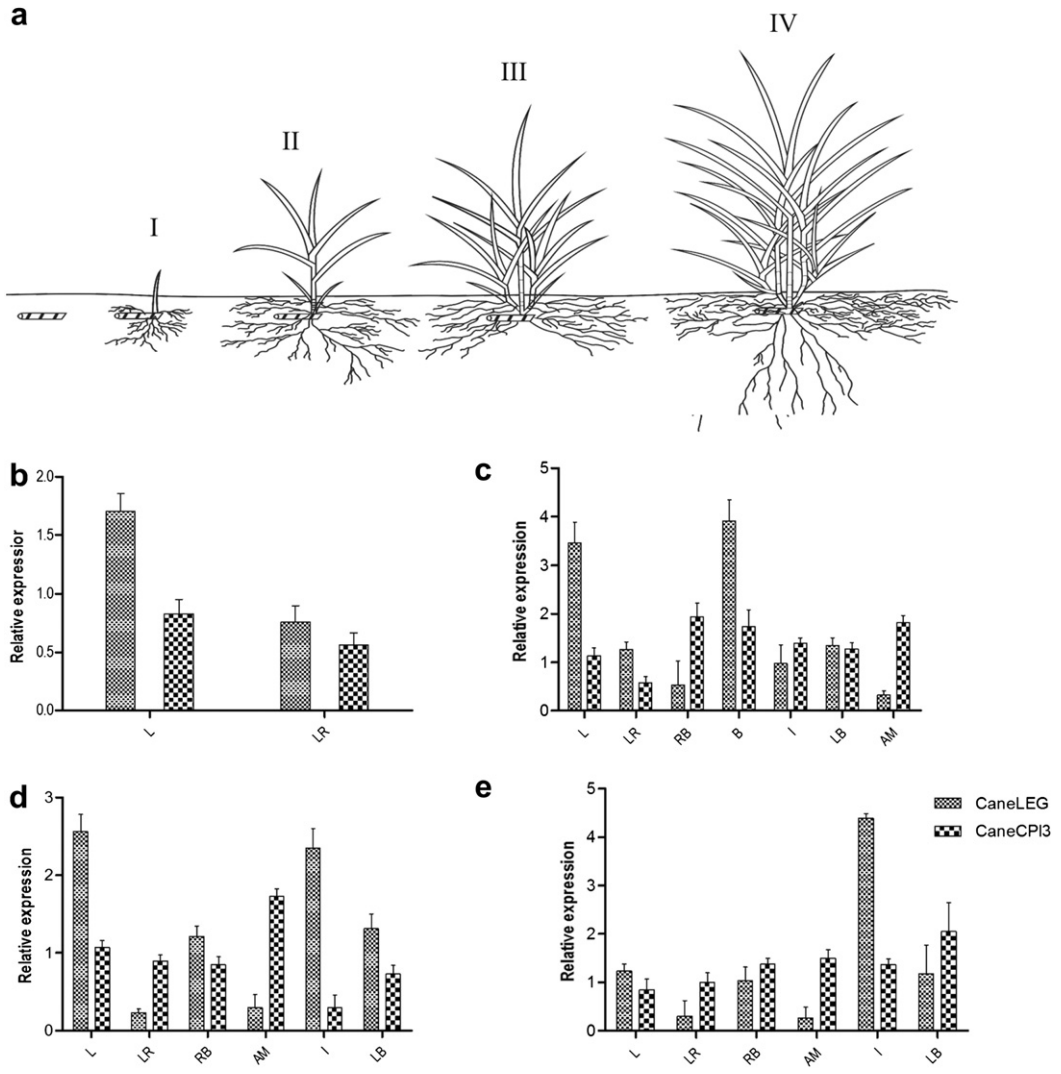

Fig. 6. Tissue-specific expression of CaneLEG and CaneCPI-3 throughout sugarcane development relative to endogen control polyubiquitin. (a) Schematic representation of sugarcane growth phases: I – establishment phase (25 DAP); II – tillering (90 DAP); III – grand growth (180 DAP); and IV – onset of maturation (270 DAP). (b) Expression patterns of CaneLEG and CaneCPI-3 in Phase I. (c) Expression patterns of CaneLEG and CaneCPI-3 in Phase II. (d) Expression patterns of CaneLEG and CaneCPI-3 in Phase III. (e) Expression patterns of CaneLEG and CaneCPI-3 in Phase IV. L: leaves; LR: leaf roll; RB: root band; B: germinating bud; I: internode; LB: lateral bud; AM: apical meristem. Error bars were calculated as described by [67].

the grand growth and initial maturation phases, whereas expression gradually increased in the internode. CaneLEG up-regulation in internode tissue throughout plant development indicates the potential involvement of this gene in internode maturation. This is consistent with previous analyses, which report that a VPE EST was up-regulated in the mature internode of 12-month-old plants of three sugarcane varieties [25]. Moreover, Rae et al. [42] report a rapid increase in VPE expression between the very immature internode and the most mature one. It has also been reported that γVPE in *Arabidopsis* is involved in the degradation of a vacuolar invertase in aging tissues and the mutation of the γVPE gene was found to lead to increased levels of glucosidase, α-mannosidases and an α-galactosidase, suggesting that these proteins may be processed or degraded by VPEs [43]. In sugarcane, a soluble acid invertase is directed to the vacuole and the N-terminal sequence of the mature protein is immediately preceded by an asparagine residue, which may by processed by the VPE [44].

CaneCPI-3 demonstrated somewhat distinct expression pattern to that observed for CaneLEG in the final sugarcane growth stages. A study of C-terminal extended PhyCys from *Arabidopsis thaliana* indicates that this cystatin plays a critical role in seed germination as well as seedling growth [45]; the authors suggest that this inhibitor may regulate the activity of endogenous papain-like and legumain-like cysteine peptidases. Thus, considering the *in vitro* CaneLEG inhibition by CaneCPI-3 and the occurrence of these gene transcripts in all tissues analyzed, CaneCPI-3 may be involved in the regulation of CaneLEG activity as well as papain-like peptidases throughout sugarcane development.

The present study also analyzed the expression of CaneLEG and CaneCPI-3 genes in response to phytohormones (Fig. 7). In sugarcane plantlets treated with GA3 neither CaneLEG nor CaneCPI-3 expression was significantly affected until 24 HAT, suggesting that the expression of these genes is not controlled by GA3. Protein levels of rice seed VPE REP-2β are reported to have declined rapidly after GA3 treatment, whereas the VPE REP-2α was found to be unaffected [46], demonstrating that different VPEs are regulated differently by this hormone. On the other hand, earlier reports found that the levels of cystatins in *Arabidopsis* and barley were decreased by treatment with gibberellin [47,48]. However, the non-response of CaneCPI-3 to GA3 may be due to the different plant

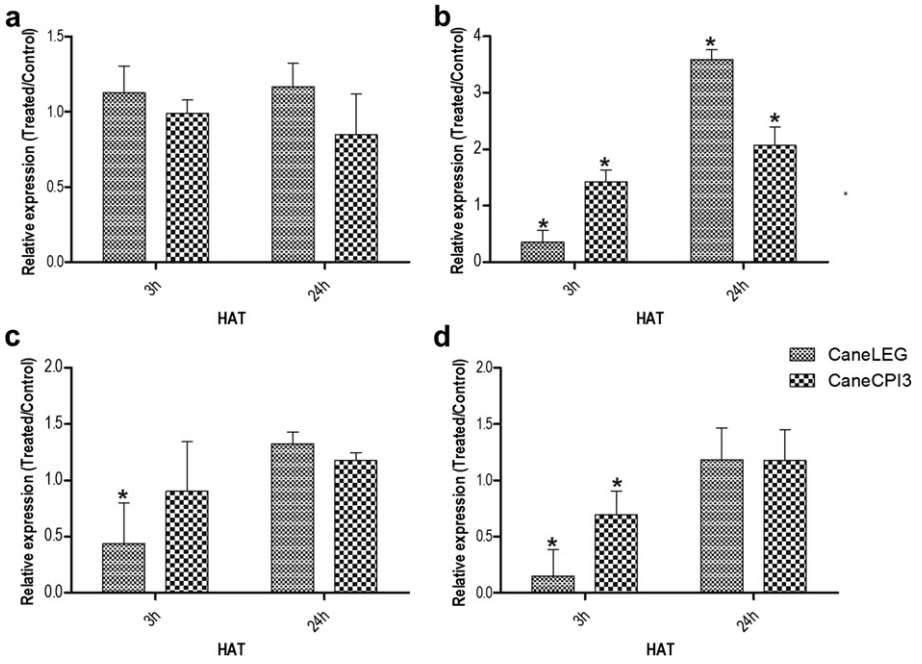

Fig. 7. Expression patterns of CaneLEG and CaneCPI-3 in sugarcane plantlets under various phytohormone treatments. (a) 100 μM of GA3. (b) 100 μM of ABA. (c) 100 μM of SA. (d) 100 μM of JA. Data on each time-point represent the mean of three replications. Plantlets treated with the phytohormone diluent were used as control. Error bars were calculated as described by [67]. Asterisks indicate the significant values ($p < 0.05$) according to analysis in the REST program.

structures analyzed, as leaves were investigated in the present study and expression in seeds was studied in *Arabidopsis* and barley. Moreover, plants have various cystatins, each of which may play a distinct role and be regulated by different signals. In *Arabidopsis*, VPEs have been found to be up-regulated in leaves treated with SA and wound-induced γVPE expression depends on the action of SA [15,49]. However, if CaneLEG and CaneCPI-3 are involved in sugarcane defense responses, their expression may not be induced by the SA pathway. Regarding the effects of JA treatment on sugarcane plantlets, the CaneLEG and CaneCPI-3 transcripts initially down-regulated, but later recovered, demonstrating that the expression of these genes may not be required in response to wound or may be regulated through another pathway. A previous analysis of the exogenous application of JA regarding VPE expression in *Arabidopsis* found no effect on the expression of αVPE or γVPE [15,49]. Some plant cystatins related to plant wound response are reported to be up-regulated by JA [50]. However, there has been no description of the up-regulation of a C-terminal extended cystatin by JA in leaves.

CaneLEG and CaneCPI-3 expression were strongly induced by ABA treatment. Since this hormone is related to many abiotic stress responses [51], the accumulation of CaneLEG and CaneCPI-3 transcripts suggests that these genes may be involved in stress responses related to ABA. Other plant cystatins transcripts also accumulated after ABA treatment, suggesting the involvement of PhyCys in stress responses mediated by this hormone [45,52,53]. Interestingly, the function of this inhibitor class in the ABA-regulated stress response is reinforced by the presence of ABA-responsive elements in the promoter region of some PhyCys, including a C-terminal extended PhyCys gene [45,54]. The gradual increase in CaneLEG expression throughout internode development and the response to ABA also suggest that CaneLEG may be involved in the sugar pathway. In sugarcane, some pathways associated with internode development may overlap with stress signaling pathways, as genes associated with sucrose content are also reported to be responsive to abiotic stress and ABA [25,55]. Therefore, more studies need to be done in order to elucidate the legumain involvement in sugar content, because until now there is insufficient data to explain its role in this pathway.

In summary, although many studies have been carried out on sugarcane transcriptome, there are few researches addressing the characterization of the genes discovered. The present study first describes the characterization of a sugarcane legumain and demonstrates that this enzyme was successfully inhibited by a sugarcane C-terminal extended PhyCys. The expression analyses demonstrate that these genes have temporal tissue-specific expression and, based on the differential expression, these genes may play roles in plant development. The CaneLEG and CaneCPI-3 response to ABA suggests that these genes may be involved in plant stress responses. This initial characterization of CaneLEG may shed some light on the understanding of the roles of this VPE in sugarcane. Further investigation is expected to reveal the involvement of CaneLEG in sugar content in sugarcane internodes.

4. Materials and methods

4.1. Plant materials, growth conditions and treatments

The sugarcane (*Saccharum* spp.) cultivar RB867515 was used in all experiments. To evaluate CaneLEG and CaneCPI-3 gene expression throughout sugarcane development, sugarcane stalk sets were germinated in 32-L plastic pots containing Tropical soil (Oxisol). The plants were grown in a greenhouse with natural light at room temperature. The sugarcane tissues were collected in the following growth phases: germination-establishment phase or phase I (25 DAP); tillering phase or phase II (90 DAP); grand growth phase or phase III (180 DAP); and initial maturation phase or phase IV (270 DAP) [56]. The tissues were harvested, frozen in liquid nitrogen and stored at −80 °C until use.

Phytohormone treatments were performed using sugarcane plantlets grown *in vitro* as described by Lee [57]. The phytohormones ABA, JA, SA and GA3 were added to the medium at a final

concentration of 100 μM, as previously described for phytohormone treatments performed in sugarcane plantlets [58,59]. The control plantlets were treated with the corresponding volume of diluent (sterile 100% ethanol). The plantlets were maintained in a growth room with a 16-h photoperiod at 37.5 $\mu E\ S^{-1}\ M^{-2}$ of light intensity. The growth temperature was kept between 25 and 28 °C. The leaves of the plantlets were collected three and 24 h after treatment, immediately frozen in liquid nitrogen and stored at −80 °C.

4.2. Construction of expression plasmids

The coding region for CaneLEG without the signal peptide was amplified by PCR from the sugarcane clone SCBFRT1071E03.g (GenBank ID: CA131429), using the oligonucleotides ScLEG-F and ScLEG-R for insertion in the pET29a vector (Novagen). The oligonucleotides ScLEGhis-F and ScLEG-R were used for cloning in the pPICZaA vector (Invitrogen). All oligonucleotides used in this study are listed in Table 2. The *Eco* RI and *Not* I sites were included in the primer sequences for subsequent digestion and insertion in the expression vector. The PCR was optimized for a 25-μL reaction mixture containing 10 ng of template DNA, 0.4 μM of each primer, 1 U of *Taq* DNA polymerase (Fermentas), 1× PCR buffer, 0.2 mM of dNTPs and 1.5 mM of $MgCl_2$. The amplification conditions were 94 °C for 3 min, followed by 35 cycles of 94 °C for 90 s, 52 °C for 2 min and 72 °C for 90 s and finally 72 °C for 10 min. The PCR product was purified and digested with the restriction enzymes *Eco* RI and *Not* I and the fragments were cloned in the expression vectors pET29a and pPICZαA, previously digested with the same enzymes. *E. coli* cells were transformed with these constructs and the resulting plasmids were isolated and purified. The recombinant pPICZαACaneLEG and pET29aCaneLEG constructs were confirmed by DNA sequencing.

4.3. Site-directed mutagenesis of CaneLEG and CaneCPI-3

Mutagenesis was performed using the GeneTailor Site-Directed Mutagenesis System (Invitrogen). The CaneLEG mutant (CaneLEGΔC^{212}), which had the cysteine of the active site replaced by a serine, was generated from the plasmid pPICZαACaneLEG using the primers MutCLEG-F and MutCLEG-R (Table 2). The mutagenesis procedure was performed following the manufacturer's instructions. In addition, the asparagine residue 141 of the CaneCPI-3 motif SNSL was replaced with a lysine residue (ΔN^{141}), as described above, using the primers MutNCPI3-F and MutNCPI3-R (Table 2). The plasmid pET28aCaneCPI-3 described previously by Gianotti et al. [31] was used to generate the mutant pET28aCaneCPI-3ΔN^{141}. The pET28aCaneCPI-3CtermΔN^{141} mutant was generated from the pET28aCaneCPI-3Cterm plasmid, previously constructed in our laboratory (see Acknowledgments). All the mutant plasmids were confirmed by DNA sequencing.

Table 2
Oligonucleotide primers used in this work.

Name	Sequence 5′–3′[a]
ScLEG-F	CGGAATTCCGCCCACGCCTCGAGCCGAC
ScLEG-R	AAATATGCGGCCGCGGCGCTAAAACCCTTGTCG
ScLEGhis-F	CGGAATTCCATCATCATCATCATCATCGCCCACG CCTCGAGCCGACCATC
MutCLEG-F	GGTCTTTTACCTTGAAGCATcCGAATCTGGGAG
MutCLEG-R	TGCTTCAAGGTAAAAGACCAGGCTTTTGT
MutNCPI3-F	TCAATCCAAGAGAGGTCTAAaTCCCTGTTTCCC
MutNCPI3-R	AGACCTCTCTTGGATTGATTTCACAGCATG
ScCCPI-3Nterm-F	CCCATATGGCCGGCCACGTCCT
ScCCPI-3Nterm-R	CGAATTCTTAGCTGAAATCCTGGAGCTCC
ScCCPI-3Cterm-F	CATATGCACAAAGGGGAGGG
ScCCPI-3Cterm-R	AAGCTTTTATGTAACACAAAC
ScCCPI-3-F	GGTTGTGGGAGGACTTTGC
ScCCPI-3-R	CGGCTGATGCTGGTTTAGC
ScVPE-F	TGGAGTTGCCTCAAGTCCATG
ScVPE-R	GATGCCAGCGTTGCAGATG
Poliub-F	CCCTCTGGTGTACCTCCATTTG
Poliub-R	CCGGTCCTTTAAACCAACTCAGT

[a] Nucleotide substitution in lower case.

4.4. Recombinant expression of CaneLEG, CaneCPI-3 and variants

The plasmids pET29aCaneLEG and pPICZαACaneLEG were used for recombinant expression in *E. coli* and *P. pastoris*, respectively. *E. coli* Rosetta (DE3) cells were transformed with pET29aCaneLEG plasmid and the recombinant protein was produced in 500 mL of LB broth containing 25 μg/mL of kanamycin and 0.4 mM of IPTG at 37 °C for 3 h. For expression in *P. pastoris*, the pPICZαACaneLEG plasmid was linearized with *Pme* I and used to transform *P. pastoris* KM71H competent cells, which were prepared as described by Cregg [60]. The KM71H competent cells were electroporated using a Gene Pulser II (Bio-Rad) under the following conditions: 1500 V, 25 μF and 200 Ω. The transformants were selected by plating in YPDS medium (1% yeast extract, 2% peptone, 2% dextrose, 1 M of sorbitol, and 1.5% bacteriological agar) containing 100 μg/mL of zeocin. Several transformants were confirmed by PCR as described by Akada et al. [61] and screened for expression following the manufacturer's instructions (Invitrogen). The culture supernatants were analyzed by SDS-PAGE stained with Coomassie Blue R-250 or silver nitrate, as described elsewhere [62]. The clone with the highest level of expression was used to scale up the protein production. A single colony of the selected clone was used to inoculate 10 mL of BMGY medium (1% yeast extract, 2% peptone, 100 mM of potassium phosphate buffer, pH 7.0, 1.34% yeast nitrogen base, 4×10^{-5} % biotin, 1% glycerol) that was grown at 30 °C until an OD_{600} of approximately 5. This culture was used to inoculate 500 mL of BMGY medium that was grown until the OD_{600} 4–5. The cells were harvested (5 min, 1500 × g) and transferred to 100 mL of BMMY medium (same as BMGY except that glycerol was replaced with 0.5% methanol) and incubated at 26 °C. To induce the expression, methanol was added every 24 h to maintain a final concentration of 0.75%. After 96 h, the cells were harvested and the supernatant was filtered using a 0.45-μm polyvinylidene difluoride (PVDF) membrane (Millipore). The recombinant CaneLEG was purified by affinity chromatography using Ni-NTA agarose resin (Qiagen). The purified CaneLEG was dialyzed against citrate–phosphate buffer (138.5 mM of Na_2HPO_4, 61.5 mM of citric acid, pH 6.5). For CaneLEGΔC^{212} expression, KM71H cells were transformed with the mutant construct pPICZαACaneLEGΔC^{212}and the recombinant protein was expressed as described above.

The plasmid pET28aCaneCPI-3 containing cDNA that encodes the sugarcane cystatin 3 (CaneCPI-3) was previously constructed by [31]. The complete sequence of CaneCPI-3 cDNA is deposited in the GenBank database under the accession number JQ922266. The pET28aCaneCPI-3 was used to obtain the recombinant protein CaneCPI-3 in *E. coli* cells. For the expression of CaneCPI-3 N-terminal region (from 36 aa to 126 aa) and CaneCPI-3 C-terminal region (from 127 aa to 261 aa), the plasmids pET28aCaneCPI-3Nterm and pET28aCaneCPI-3Cterm were constructed, respectively. Plasmid pET28aCaneCPI-3 was used as a template to amplify the cDNA sequence corresponding to the CaneCPI-3 N-terminal and C-terminal regions using the primers ScCCPI-3Nterm (F and R) and ScCCPI-3Cterm (F and R), respectively (Table 2). The PCR program consisted of 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 55 °C for 30 s and 72 °C for 60 s and finally 72 °C for 10 min. The amplified products were inserted into the pET28a vector (Novagen) using the *Nde* I and *Eco* RI restriction sites for CaneCPI-3 N-terminal, generating the pET28aCaneCPI-3Nterm vector, and *Nde* I and *Hind*

III restriction sites for CaneCPI-3 C-terminal, generating the pET28aCaneCPI-3Cterm vector, which were confirmed by sequencing. The recombinant CaneCPI-3 and its variants were expressed in *E. coli* Rosetta (DE3) cells and purified as described by [31]. All purified proteins were analyzed in 15% SDS-PAGE for CaneCPI-3 and 12% for CaneLEG. Protein concentrations were determined using the BCA Protein Assay kit (Pierce).

4.5. Antibody production and western blotting analysis

Following CaneLEG expression in *E. coli*, the bacterial cells were harvested (5 min, 15,000 g, 4 °C), resuspended in lysis buffer (10 mM of Tris, 50 mM of NaH_2PO_4, 100 mM of NaCl, pH 8.0) and disrupted in the Fisher Scientific Sonic Dismembrator model 500. The insoluble fraction was separated by 12% SDS-PAGE. The band corresponding to the CaneLEG recombinant protein was excised and electroeluted from the gel in a dialysis membrane 14,000 MW (Pierce) with buffer (25 mM of Tris–HCl, 192 mM of glycine and 0.1% SDS), modified from [63]. Electroelution was performed at 100 V for 5 h. The electroeluted protein was quantified with the Bradford reagent (Sigma). The rCaneLEG protein electroeluted from SDS-PAGE was used to immunize mice, as described by Exclude the reference Kuroyanagi et al. [4]. Antibodies were collected from the animals one week following the last immunization.

For the western blotting analysis, the proteins were separated by 12% SDS-PAGE and transferred to the PVDF membrane (Pierce). The PVDF membrane was blocked in a solution of 5% defatted milk in Tris–buffered saline (TBS) (50 mM of Tris–HCl, pH 8.0, 150 mM of NaCl) for 1 h. The membrane was then washed three times with TBS and incubated with the polyclonal anti-CaneLEG or monoclonal anti-His-tag (GE Healthcare) antibodies at a dilution of 1:5,000 or 1:10,000, respectively, for 90 min. The membrane was washed again and incubated with the AP-labeled anti-mouse IgG antibody (Sigma) at the dilution of 1:10,000, as described above. The antigen–antibody interaction was analyzed using the reagent 1-Step NBT/BCIP (Pierce).

4.6. Optimal pH assay

Recombinant CaneLEG activity was evaluated in the pH range 3.0–9.0 in the following buffers: citrate–phosphate buffer (pH range: 3.0–7.0), HEPES 50 mM (pH 7.5) and sodium borate 50 mM (pH range: 8.0–9.0). The optimal pH reaction was performed in a volume of 500 μL containing the recombinant CaneLEG, buffer and 2.5 mM of dithiothreitol (DTT). The reaction was incubated for 10 min at 37 °C and the Z–Ala–Ala–Asn–AMC (Bachem Bioscience, Inc.) was added at a final concentration of 13 μM. Enzyme activity was measured by monitoring the release of AMC at $\lambda_{ex} = 380$ nm and $\lambda_{em} = 460$ nm, using the Hitachi F-2500 spectrofluorimeter.

4.7. Optimal temperature and stability

To determine the optimal temperature, the recombinant CaneLEG was incubated for 10 min in the citrate–phosphate buffer, pH 6.5, containing 2.5 mM of DTT, at temperatures ranging from 10 to 60 °C; after the addition of the Z–Ala–Ala–Asn–AMC substrate, enzyme activity was measured using the Hitachi F-2500 spectrofluorimeter. Optimal temperature was taken as 100% and the enzyme activities assigned to the other temperatures were expressed as percentage of this activity. The thermal stability of CaneLEG was estimated by incubating the recombinant CaneLEG in citrate–phosphate buffer, pH 6.5, containing 2.5 mM of DTT for 1 h in the temperature ranging from 4 to 60 °C, as described by [64]. Following incubation the substrate Z–Ala–Ala–Asn–AMC was added and the enzyme residual activity was evaluated at 37 °C. The residual activity was expressed as the percentage of non-incubated enzyme.

4.8. Determination of Michaelis–Menten constant (K_m) and catalytic efficiency of CaneLEG

The Michaelis–Menten constant (K_m) was determined through the incubation of CaneLEG at 37 °C for 10 min in citrate–phosphate buffer, pH 6.5, containing 2.5 mM of DTT. Enzyme activity was measured using the substrate Z–Ala–Ala–Asn–AMC at concentrations ranging from 0.1 to 32 μM. K_m was determined through non-linear regression using the GraFit program [65]. Additionally, the catalytic efficiencies k_{cat}/K_m of recombinant CaneLEG for the substrates Z–Ala–Ala–Asn–AMC and Z–Val–Ala–Asn–AMC were calculated under pseudo-first-order conditions, in which $[S] \ll K_m$. The k_{cat}/K_m was calculated through non-linear regression analysis using the GraFit program [65].

4.9. Inhibition assays of legumain activity

The inhibition of legumain activity was determined spectrofluorometrically based on the previously described procedure using the fluorogenic substrate Z–Ala–Ala–Asn–AMC. Inhibitory activity was determined by measuring the residual hydrolytic activity of CaneLEG. The enzyme was added to citrate–phosphate buffer, pH 6.5, containing 2.5 mM of DTT. Subsequent incubations at 37 °C were performed with the peptidase inhibitors iodoacetamide (Sigma), E-64, CaneCPI-3, CaneCPI-3Nterm and CaneCPI-3Cterm and respective mutants. The substrate Z–Ala–Ala–Asn–AMC 13 μM was then added and the residual cysteine peptidase activity was determined. The inhibition constant (K_i) was calculated following Morrison's procedure using the GraFit program [65,66].

4.10. In vitro activation of mutant legumain (CaneLEGΔC^{212})

To evaluate the processing of the mutant CaneLEGΔC^{212} protein, it was incubated with the active rCaneLEG at 37 °C for 3 h in a reaction containing 1.75 μg of recombinant CaneLEG protein, 9.3 μg of CaneLEGΔC^{212} protein, citrate–phosphate buffer pH 6.5, and 2.5 mM of DTT in a final volume of 100 μL. Aliquots of the reaction were analyzed in 12% SDS-PAGE. As the negative control, CaneLEGΔC^{212} protein was incubated under the same conditions described above without the CaneLEG recombinant protein.

4.11. RNA isolation, cDNA synthesis and quantitative real-time PCR

The frozen sugarcane tissues were powdered in N_2 liquid and 300 mg were used for RNA extraction. Total RNA was extracted using the Trizol reagent (Invitrogen), following the manufacturer's instructions. After extraction, 3 μg of RNA was treated with 3 U of DNase I (Invitrogen). cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), following the manufacturer's instructions. Two microliters of cDNA were used as the template in a quantitative real-time PCR (qPCR) using either the primers ScVPE-F and ScVPE-R to evaluate CaneLEG expression in different sugarcane tissues or the primers ScCCPI-3-F and ScCCPI-3-R to analyze the expression level of CaneCPI-3. The qPCR was performed in a reaction containing 2 μL of cDNA, 1× Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and 0.4 μM of each primer in a volume of 10 μL. The qPCR program was 50 °C for 2 min, 95 °C for 2 min, 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 40 s. The reactions were performed using the Eco Real-Time System (Illumina). The polyubiquitin gene was amplified using the primers Poliub-F and Poliub-R and used as the

endogenous control. All primers sequences used in the present study are listed in Table 2.

After the qPCR experiment, the melting curve of each reaction was analyzed to confirm the specificity of the amplification. The reactions were optimized to amplification efficiency between 90 and 100%, $r^2 > 0.95$ and slope between −3.0 and −3.6. The amplification reactions were performed in duplicate for three biological replicates. The relative expression of CaneLEG and CaneCPI-3 in different sugarcane tissues was calculated using the ΔCt method, in which ΔCt = Ct (polyubiquitin) − Ct (target) [67]. For the phytohormone treatment assays, relative transcript quantities were calculated by the $2^{-\Delta\Delta Ct}$ method. Using the $2^{-\Delta\Delta Ct}$ method, the fold change in gene expression of untreated plantlets (control) equals one [67]. Data analysis of plants treated with phytohormone was performed using the Relative Expression Software Tool (REST) version 2.0.13 from Qiagen [68].

Acknowledgments

This study was supported by the Brazilian fostering agencies FAPESP (CBME, CEPID Proc. 98/14138-2), CNPq and CAPES. L.K Santos-Silva received a doctoral scholarship from CAPES. F. Henrique-Silva is the recipient of a Research Productivity Scholarship from CNPq (#305655/2009-4). We thank Wendy Martin Rios and Daniel Espinosa for technical assistance in the construction of the plasmids pET28aCaneCPI-3Nterm and pET28aCaneCPI-3Cterm, respectively. We also thank Dr. Isabel Diaz (Universidad Politécnica de Madrid, Madrid, Spain) for suggestions and a critical reading of the manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.plaphy.2012.05.020.

References

[1] I. Hara-Nishimura, K. Inoue, M. Nishimura, A unique vacuolar processing enzyme responsible for conversion of several proprotein precursors into mature forms, FEBS Lett. 294 (1991) 89–94.
[2] N. Hiraiwa, M. Nishimura, I. Hara-Nishimura, Vacuolar processing enzyme is self-catalytically activated by sequential removal of the C-terminal and N-terminal propeptides, FEBS Lett. 447 (1999) 213–216.
[3] K. Müntz, A.D. Shutov, Legumains and their functions in plants, Trends Plant Sci. 7 (2002) 340–344.
[4] M. Kuroyanagi, M. Nishimura, I. Hara-Nishimura, Activation of Arabidopsis vacuolar processing enzyme by self-catalytic removal of an auto-inhibitory domain of the C-terminal propeptide, Plant Cell Physiol. 43 (2002) 143–151.
[5] P.R. Shewry, J.A. Napier, A.S. Tatham, Seed storage proteins: structures and biosynthesis, Plant Cell 7 (1995) 945–956.
[6] R. Jung, M.P. Scott, Y.W. Nam, T.W. Beaman, R. Bassüner, I. Saalbach, K. Müntz, N.C. Nielsen, The role of proteolysis in the processing and assembly of 11S seed globulins, Plant Cell 10 (1998) 343–357.
[7] T. Shimada, K. Yamada, M. Kataoka, S. Nakaune, Y. Koumoto, M. Kuroyanagi, S. Tabata, T. Kato, K. Shinozaki, M. Seki, M. Kobayashi, M. Kondo, M. Nishimura, I. Hara-Nishimura, Vacuolar processing enzymes are essential for proper processing of seed storage proteins in Arabidopsis thaliana, J. Biol. Chem. 278 (2003) 32292–32299.
[8] Y. Wang, S. Zhu, S. Liu, L. Jiang, L. Chen, Y. Ren, X. Han, F. Liu, S. Ji, X. Liu, J. Wan, The vacuolar processing enzyme OsVPE1 is required for efficient glutelin processing in rice, Plant J. 58 (2009) 606–617.
[9] A. Schlereth, C. Becker, C. Horstmann, J. Tiedemann, K. Müntz, Comparison of globulin mobilization and cysteine proteinases in embryonic axes and cotyledons during germination and seedling growth of vetch (Vicia sativa L.), J. Exp. Bot. 51 (2000) 1423–1433.
[10] A. Zakharov, M. Carchilan, T. Stepurina, V. Rotari, K. Wilson, I. Vaintraub, A comparative study of the role of the major proteinases of germinated common bean (Phaseolus vulgaris L.) and soybean (Glycine max (L.) Merrill) seeds in the degradation of their storage proteins, J. Exp. Bot. 55 (2004) 2241–2249.
[11] T. Okamoto, T. Minamikawa, Molecular cloning and characterization of Vigna mungo processing enzyme 1 (VmPE-1), an asparaginyl endopeptidase, Plant Mol. Biol. 39 (1999) 63–73.
[12] N. Hatsugai, M. Kuroyanagi, K. Yamada, T. Meshi, S. Tsuda, M. Kondo, M. Nishimura, I. Hara-Nishimura, A plant vacuolar protease, VPE, mediates virus-induced hypersensitive cell death, Science 305 (2004) 855–858.
[13] E. Rojo, R. Martín, C. Carter, J. Zouhar, S. Pan, J. Plotnikova, H. Jin, M. Paneque, J.J. Sánchez-Serrano, B. Baker, F.M. Ausubel, N.V. Raikhel, VPE gamma exhibits a caspase-like activity that contributes to defence against pathogens, Curr. Biol. 14 (2004) 1897–1906.
[14] I.S. Donnison, A.P. Gay, H. Thomas, K.J. Edwards, D. Edwards, C.L. James, A.M. Thomas, H.J. Ougham, Modification of nitrogen remobilization, grain fill and leaf senescence in maize (Zea mays) by transposon insertional mutagenesis in a protease gene, New Phytol. 173 (2007) 481–494.
[15] T. Kinoshita, K. Yamada, N. Hiraiwa, M. Kondo, M. Nishimura, I. Hara-Nishimura, Vacuolar processing enzyme is up-regulated in the lytic vacuoles of vegetative tissues during senescence and under various stressed conditions, Plant J. 19 (1999) 43–53.
[16] T. Yamada, H. Ohta, A. Shinohara, A. Iwamatsu, H. Shimada, T. Tsuchiya, T. Masuda, K. Takamiya, A cysteine protease from maize isolated in a complex with cystatin, Plant Cell Physiol. 41 (2000) 185–191.
[17] C. Lazure, The peptidase zymogen proregions: nature's way of preventing undesired activation and proteolysis, Curr. Pharm. Des. 8 (2002) 511–531.
[18] D. Rivard, C. Girard, R. Anguenot, L.P. Vézina, S. Trépanier, D. Michaud, MsCYS1 a developmentally-regulated cystatin from alfalfa, Plant Physiol. Biochem. 45 (2007) 508–514.
[19] S.M. Weeda, G.N. Mohan Kumar, N. Richard Knowles, Developmentally linked changes in proteases and protease inhibitors suggest a role for potato multicystatin in regulating protein content of potato tubers, Planta 230 (2009) 73–84.
[20] M. Benchabane, U. Schlüter, J. Vorster, M.C. Goulet, D. Michaud, Plant cystatins, Biochimie 92 (2010) 1657–1666.
[21] V. Turk, W. Bode, The cystatins: protein inhibitors of cysteine proteinases, FEBS Lett. 285 (1991) 213–219.
[22] T. Cheng, K. Hitomi, I.M. Van Vlijmen-Willems, G.J. De Jongh, K. Yamamoto, K. Nishi, C. Watts, T. Reinheckel, J. Schalkwijk, P.L. Zeeuwen, Cystatin M/E is a high affinity inhibitor of cathepsin V and cathepsin L by a reactive site that is distinct from the legumain-binding site: a novel clue for the role of cystatin M/E in epidermal cornification, J. Biol. Chem. 281 (2006) 15893–15899.
[23] M. Martinez, M. Diaz-Mendoza, L. Carrillo, I. Diaz, Carboxy terminal extended phytocystatins are bifunctional inhibitors of papain and legumain cysteine proteinases, FEBS Lett. 581 (2007) 2914–2918.
[24] A.L. Vettore, F.R. da Silva, E.L. Kemper, G.M. Souza, A.M. da Silva, M.I. Ferro, F. Henrique-Silva, E.A. Giglioti, M.V. Lemos, L.L. Coutinho, M.P. Nobrega, H. Carrer, S.C. França, M. Bacci Júnior, M.H. Goldman, S.L. Gomes, L.R. Nunes, L.E. Camargo, W.J. Siqueira, M.A. Van Sluys, O.H. Thiemann, E.E. Kuramae, R.V. Santelli, C.L. Marino, M.L. Targon, J.A. Ferro, H.C. Silveira, D.C. Marini, E.G. Lemos, C.B. Monteiro-Vitorello, J.H. Tambor, D.M. Carraro, P.G. Roberto, V.G. Martins, G.H. Goldman, R.C. de Oliveira, D. Truffi, C.A. Colombo, M. Rossi, P.G. de Araujo, S.A. Sculaccio, A. Angella, M.M. Lima, V.E. de Rosa Júnior, F. Siviero, V.E. Coscrato, M.A. Machado, L. Grivet, S.M. Di Mauro, F.G. Nobrega, C.F. Menck, M.D. Braga, G.P. Telles, F.A. Cara, G. Pedrosa, J. Meidanis, P. Arruda, Analysis and functional annotation of an expressed sequence tag collection for tropical crop sugarcane, Genome Res. 13 (2003) 2725–2735.
[25] R.E. Casu, C.M. Dimmock, S.C. Chapman, C.P. Grof, C.L. Mcintyre, G.D. Bonnett, J.M. Manners, Identification of differentially expressed transcripts from maturing stem of sugarcane by in silico analysis of stem expressed sequence tags and gene expression profiling, Plant Mol. Biol. 54 (2004) 503–517.
[26] J.D. Bendtsen, H. Nielsen, G. Von Heijne, S. Brunak, Improved prediction of signal peptides: signalP 3.0, J. Mol. Biol. 340 (2004) 783–795.
[27] P. Horton, K.J. Park, T. Obayashi, N. Fujita, H. Harada, C.J. Adams-Collier, K. Nakai, WoLF PSORT: protein localization predictor, Nucleic Acids Res. 35 (2007) 585–587.
[28] S.F. Altschul, T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (1997) 3389–3402.
[29] J.M. Chen, N.D. Rawlings, R.A.E. Stevens, A.J. Barret, Identification of the active site of legumain links it to caspases clostripain and gingipains in a new clan of cysteine endopeptidases, FEBS Lett. 441 (1998) 361–365.
[30] V.I. Rotari, P.M. Dando, A.J. Barrett, Legumain forms form plants and animals differ in their specificity, Biol. Chem. 382 (2001) 953–959.
[31] A. Gianotti, W.M. Rios, A. Soares-Costa, V. Nogaroto, A.K. Carmona, M.L. Oliva, S.S. Andrade, F. Henrique-Silva, Recombinant expression purification and functional analysis of two novel cystatins from sugarcane (Saccharum officinarum), Protein Expr. Purif. 47 (2006) 483–489.
[32] M.A. Jackson, A.L. Rae, R.E. Casu, C.P.L. Grof, G.D. Bonnett, D.J. Maclean, A bioinformatic approach to the identification of a conserved domain in a sugarcane legumain that directs GFP to the lytic vacuole, Funct. Plant Biol. 34 (2007) 633–644.
[33] C.R. Caffrey, M.A. Mathieu, A.M. Gaffney, J.P. Salter, M. Sajid, K.D. Lucas, C. Franklin, M. Bogyo, J.H. Mckerow, Identification of a cDNA encoding an active asparaginyl endopeptidase of Schistosoma mansoni and its expression in Pichia pastoris, FEBS Lett. 466 (2000) 244–248.
[34] D.N. Li, S.P. Matthews, A.N. Antoniou, D. Mazzeo, C. Watts, Multistep autoactivation of asparaginyl andopeptidase *in vitro* and *in vivo*, J. Biol. Chem. 278 (2003) 38980–38990.
[35] Y. Abe, K. Shirane, H. Yokosawa, H. Matsushita, M. Mitta, I. Kato, S. Ishii, Asparaginyl endopeptidase of jack bean seeds: purification characterization

and high utility in protein sequence analysis, J. Biol. Chem. 268 (1993) 3525–3529.
[36] S. Halfon, S. Patel, F. Vega, S. Zurawski, G. Zurawski, Autocatalytic activation of human legumain at aspartic acid residues, FEBS Lett. 438 (1998) 114–118.
[37] M. Bosch, N.S. Poulter, R.M. Perry, K.A. Wilkins, V.E. Franklin-Tong, Characterization of a legumain/vacuolar processing enzyme and YVADase activity in Papaver pollen, Plant Mol. Biol. 74 (2010) 381–393.
[38] M. Solomon, B. Belenghi, M. Delledonne, E. Menachem, A. Levine, The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants, Plant Cell 11 (1999) 431–444.
[39] S.F. Sin, E.C. Yeung, M.L. Chye, Downregulation of Solanum americanum genes encoding proteinase inhibitor II causes defective seed development, Plant J. 45 (2006) 58–70.
[40] C. Van Dillewijn, Botany of Sugarcane, Chronica Botanica, Waltham, MA, 1952.
[41] J.D. Bewley, M. Black, Seeds: Physiology of Development and Germination, second ed. Plenum Press, New York, 1994.
[42] A.L. Rae, M.A. Jackson, C.H. Nguyen, G.D. Bonnett, Functional specialization of vacuoles in sugarcane leaf and stem, Trop. Plant Biol. 2 (2009) 13–22.
[43] E. Rojo, J. Zouhar, C. Carter, V. Kovaleva, N.V. Raikhel, A unique mechanism for protein processing and degradation in Arabidopsis thaliana, Proc. Natl. Acad. Sci. USA 100 (2003) 7389–7394.
[44] A.L. Rae, R.E. Casu, J.M. Perroux, M.A. Jackson, C.P. Grof, A soluble acid invertase is directed to the vacuole by a signal anchor mechanism, J. Plant Physiol. 168 (2011) 983–989.
[45] J.E. Hwang, J.K. Hong, J.H. Je, K.O. Lee, D.Y. Kim, S.Y. Lee, C.O. Lim, Regulation of seed germination and seedling growth by an Arabidopsis phytocystatin isoform AtCYS6, Plant Cell. Rep. 28 (2009) 1623–1632.
[46] H. Kato, K. Sutoh, T. Minamikawa, Identification cDNA cloning and possible roles of seed-specific rice asparaginyl endopeptidase REP-2, Planta 217 (2003) 676–685.
[47] M. Martínez, I. Rubio-Somoza, P. Carbonero, I. Díaz, A cathepsin B-like cysteine protease gene from Hordeum vulgare (gene CatB) induced by GA in aleurone cells is under circadian control in leaves, J. Exp. Bot. 54 (2003) 951–959.
[48] J.K. Hong, J.E. Hwang, C.J. Lim, K.A. Yang, Z.L. Jin, C.Y. Kim, J.C. Koo, W.S. Chung, K.O. Lee, S.Y. Lee, M.J. Cho, C.O. Lim, Over-expression of Chinese cabbage phytocystatin 1 retards seed germination in Arabidopsis, Plant Sci. 172 (2007) 556–563.
[49] K. Yamada, M. Nishimura, I. Hara-Nishimura, The slow wound-response of gammaVPE is regulated by endogenous salicylic acid in Arabidopsis, Planta 218 (2004) 599–605.
[50] M. Pernas, R. Sánchez-Monge, G. Salcedo, Biotic and abiotic stress can induce cystatin expression in chestnut, FEBS Lett. 467 (2000) 206–210.
[51] A. Wasilewska, F. Vlad, C. Sirichandra, Y. Redko, F. Jammes, C. Valon, N. Frei dit Frey, J. Leung, An update on abscisic acid signaling in plants and more, Mol. Plant 1 (2008) 198–217.
[52] N.N. Diop, M. Kidric, A. Repellin, M. Gareil, A. d'Arcy-Lameta, A.T. Pham Thi, Y. Zuily-Fodil, A multicystatin is induced by drought-stress in cowpea (Vigna unguiculata (L.) Walp.) leaves, FEBS Lett. 577 (2004) 545–550.
[53] X. Zhang, S. Liu, T. Takano, Two cysteine proteinase inhibitors from Arabidopsis thaliana AtCYSa and AtCYSb increasing the salt drought oxidation and cold tolerance, Plant Mol. Biol. 68 (2008) 131–143.
[54] J.E. Hwang, J.K. Hong, C.J. Lim, H. Chen, J. Je, K.A. Yang, D.Y. Kim, Y.J. Choi, S.Y. Lee, C.O. Lim, Distinct expression patterns of two Arabidopsis phytocystatin genes AtCYS1 and AtCYS2 during development and abiotic stresses, Plant Cell Rep. 29 (2010) 905–915.
[55] F.S. Papini-Terzi, F.R. Rocha, R.Z. Vêncio, J.M. Felix, D.S. Branco, A.J. Waclawovsky, L.E. Del Bem, C.G. Lembke, M.D. Costa, M.Y. Nishiyama Jr., R. Vicentini, M.G. Vincentz, E.C. Ulian, M. Menossi, G.M. Souza, Sugarcane genes associated with sucrose content, BMC Genom. 10 (2009) 120.
[56] V. Diola, F. Santos, Fisiologia, in: F. Santos, A. Borém, C. Caldas (Eds.), Cana-de-açúcar Bioenergia açúcar e álcool Tecnologia e perspectivas, UFV, Viçosa MG, 2010, pp. 25–49.
[57] T.S.G. Lee, Micropropagation of sugarcane (Saccharum spp.), Plant Cell Tissue Organ Cult. 10 (1987) 47–55.
[58] F.R. Rocha, F.S. Papini-Terzi, M.Y. Nishiyama Jr., R.Z. Vêncio, R. Vicentini, R.D. Duarte, V.E. de Rosa Jr., F. Vinagre, C. Barsalobres, A.H. Medeiros, F.A. Rodrigues, E.C. Ulian, S.M. Zingaretti, J.A. Galbiatti, R.S. Almeida, A.V. Figueira, A.S. Hemerly, M.C. Silva-Filho, M. Menossi, G.M. Souza, Signal transduction-related responses to phytohormones and environmental challenges in sugarcane, BMC Genom. 8 (2007) 71.
[59] P.S. Schlögl, F.T. Nogueira, R. Drummond, J.M. Felix, V.E. de Rosa Jr., R. Vicentini, A. Leite, E.C. Ulian, M. Menossi, Identification of new ABA- and MEJA-activated sugarcane bZIP genes by data mining in the SUCEST database, Plant Cell Rep. 27 (2008) 335–345.
[60] J.M. Cregg , Methods in Molecular Biology Pichia Protocols, second ed., vol. 389, Humana Press, Totowa, NJ, 2007.
[61] R. Akada, T. Murakane, Y. Nishizawa, DNA extraction method for screening yeast clones by PCR, Biotechniques 28 (2000) 670–673.
[62] B.R. Oakley, D.R. Kirsch, N.R. Morris, A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels, Anal. Biochem. 105 (1980) 361–363.
[63] P. Jenö, M. Horst, Electroelution of proteins from polyacrylamide gels, in: J.M. Walker (Ed.), The Protein Protocols Handbook, Humana Press, Totowa, NJ, 1996, pp. 207–214.
[64] Y. Nagamori, N. Fujishima, S. Okada, Purification and some properties of dipeptidyl carboxypeptidase from Bacillus pumilus, Agric. Biol. Chem. 54 (1990) 999–1005.
[65] R.J. Leatherbarrow, GraFit Version 5, Erithacus Software Ltd., Horley, UK, 2001.
[66] J.F. Morrison, The slow-binding and slow, tight-binding inhibition of enzyme-catalysed reactions, TIBS 7 (1982) 102–105.
[67] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$, Methods 25 (2001) 402–408.
[68] M.W. Pfaffl, G.W. Horgan, L. Dempfle, Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR, Nucleic Acids Res. 30 (2002) e36.