

Molecular cloning and characterization in eukaryotic expression systems of a sugarcane cysteine protease inhibitor gene involved in drought tolerance

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Abstract: Cystatin responses in sugarcane plants under drought stress have not previously been reported. To test the hypothesis that sugar cane cystatin can function as an osmotic stress tolerance gene, transgenic *Pichia pastoris* (GS115) and *Saccharomyces cerevisiae* (A2279) strains with the ability to express sugarcane cystatin were constructed. The osmotic stress tolerance of the transgenic yeasts was then evaluated, and it was found that the transgenic *Pichia pastoris* (GS115) and *Saccharomyces cerevisiae* (A2279) had increased growth and increased osmotic stress tolerance. To gain a greater understanding of the responses of sugarcane cystatin to drought, 1 drought-susceptible and 3 drought-tolerant sugarcane cultivars were grown in a greenhouse for 3 weeks, exposed to drought stress for 5 days, and rehydrated for 5 days. Semiquantitative RT-PCR was subsequently performed, and the results showed increased sugarcane cystatin gene transcription in stressed plants when compared to the control. There was a greater increase in the drought-tolerant versus the drought-susceptible cultivars. However, the mRNA levels decreased once the plants recovered from the drought conditions. Hence, this study shows that the expression of cystatin in sugarcane is involved in drought stress. This gene may also serve as a target for future breeding programs focused on stress tolerance in sugarcane.

Key words: Cystatin, *Saccharum officinarum*, water deficit, semiquantitative RT-PCR

1. Introduction

Drought is an important factor that limits crop productivity. A yield reduction of up to 60% has been recorded in sugarcane (*Saccharum officinarum* L.) under drought conditions (Robertson et al., 1999). Sugarcane is a major crop in Thailand both for sugar and bioethanol production (Jangpromma et al., 2010a, 2010b). The primary growing areas are located in the northeast, which has a low water-holding capacity and low rainfall (Chetthamrongchai et al., 2001), and drought is a limiting factor for sugar yield in these areas (Jangpromma et al., 2010a, 2010b).

Several strategies have been proposed to improve crop survival and maintain normal growth under drought stress conditions. Drought induces changes in morphological, physiological, and biochemical characteristics (Cellier et al., 1998). Gene expression also changes in response to

drought and modifies the synthesis of several proteins that play important roles in biological functions (Caruso et al., 2009). A large number of drought-induced genes and proteins have been reported in various plant species, such as late embryogenesis abundant (LEA) proteins (Liu et al., 2009), the *Arabidopsis* HARDY gene (Karaba et al., 2007), the *OsDREB2A* transcription factor (Cui et al., 2011), antioxidant enzymes (Turkan et al., 2005; Terzi et al., 2010; Aydın et al., 2014), and certain protease inhibitors (Massonneau et al., 2005; Huang et al., 2007; Zhang et al., 2008).

Proteomics studies have revealed that several proteins expressed in sugarcane leaves exhibit expression changes under drought stress conditions; among these proteins are protease inhibitors (Jangpromma et al., 2010a). Plant protease inhibitors are small proteins that account for

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up to 10% of the total protein content in storage tissues (Habib and Fazili, 2007). The most remarkable of the protease inhibitors involved in abiotic stress responses are the cysteine protease inhibitors, which are also called cystatins.

Cystatins inhibit cysteine proteases by direct (but reversible) binding to the active site (Reis and Margis, 2001; Massonneau et al., 2005). Cystatin expression is involved in several biotic and abiotic stress functions, and cystatins play a defensive role against insects and pathogenic microorganisms (Masoud et al., 1993; Soares-Costa et al., 2002; Habib and Fazili, 2007; Zhang et al., 2008). Recently, cystatins have also been implicated in plant stress responses (Van der Vyver et al., 2003). They have been found to be specifically induced by cold (Van der Vyver et al., 2003; Massonneau et al., 2005; Zhang et al., 2008), heat (Demirevska et al., 2010), salt, and oxidative stress (Zhang et al., 2008).

Drought can induce cysteine protease activity in plants (Zhang et al., 2008; Demirevska et al., 2010). Cysteine proteases respond to different internal and external stimuli and, in some cases, they cause up to 90% of the total proteolytic activity (Grudkowska and Zagdańska, 2004). Increases of cysteine protease activity are involved in the regulation of protein breakdown, and these proteases play a major role in the proteolysis process, which is a necessary component of plant responses to abiotic stress (Grudkowska and Zagdańska, 2004; Demirevska et al., 2010). Protease activity is high during stress conditions, and the activities of these enzymes help regulate programmed cell death, which is a basic biological process that occurs during plant development and during stress responses (Solomon et al., 1999). Cell damage also leads to the synthesis of protease inhibitors, including cystatins, which regulate the activity of cysteine proteases (Zhang et al., 2008). Cystatin responses to drought stress have been reported in rice (Demirevska et al., 2010), maize (Massonneau et al., 2005), *Arabidopsis thaliana* (L.) Heynh (Zhang et al., 2008), and cowpea (Diop et al., 2004). Transgenic plants also demonstrate the defense function of cystatins under abiotic stresses (Van der Vyver et al., 2003; Zhang et al., 2008; Demirevska et al., 2010). In *Arabidopsis thaliana* (L.) Heynh, cystatins were strongly induced by multiple abiotic stresses from high salt, drought, oxidants, and cold. The overexpression of the cystatin genes *AtCYSa* and *AtCYSb* under the control of the strong constitutive promoter in *Arabidopsis thaliana* (L.) Heynh also confirmed that cystatins have defensive functions under abiotic stresses; the transgenic *Arabidopsis thaliana* (L.) Heynh plants showed enhanced tolerance to multiple abiotic stresses (Zhang et al., 2008).

The identification, classification, and expression of cystatins in sugarcane have been reported (Reis and Mar-

gis, 2001; Soares-Costa et al., 2002; Oliva et al., 2004), but studies of their function have been mainly limited to antifungal activity (Soares-Costa et al., 2002). To the best of our knowledge, the cystatin response of sugarcane to drought conditions has not yet been reported. The objectives of this study were to express the sugarcane cystatin gene in the eukaryotic cells of *Pichia pastoris* (GS115) and *Saccharomyces cerevisiae* (A2279) and to investigate the effects of drought stress on cystatin expression.

2. Materials and methods

2.1. Plant materials and drought stress treatments

Four sugarcane cultivars (Khon Kaen 3, 03-4-425, K86-161, and B34-164) were used in this study. Each of the sugarcane stalks with a single bud was germinated on moist paper and transferred to plastic containers 10 cm in diameter and 23 cm in height. The plastic pots were filled with 2 kg of soil; the soil properties were previously described in parallel studies (Jangpromma et al., 2010b, 2012). The soil moisture contents were maintained at 11.5% for the field capacity (FC) and 2.67% for the permanent wilting point.

Two water treatments were assigned as factor A and 4 sugarcane cultivars were assigned as factor B. The treatments were arranged in a 2×4 factorial combination in a randomized complete block design (RCBD) with 4 replications in each greenhouse. Experiments were conducted from May to June of 2010 at the Field Crops Research Station of Khon Kaen University, located in Khon Kaen Province, Thailand.

Water was supplied daily for the FC treatments throughout the duration of the experiment, and stress treatments were imposed on the crops 21 days after planting by withholding irrigation for 5 days. The stressed plants were rewetted after 5 days of drought and maintained at FC (5 days).

2.2. SPAD chlorophyll meter reading

The SPAD chlorophyll meter reading (SCMR) was measured after plants were stressed under drought stress conditions for 5 days, followed by drought recovery for 1 and 5 days, using a handheld portable chlorophyll meter (Minolta SPAD-502 Meter, Japan). The second fully expanded leaf from the top of each plant was used for these measurements, as previously described (Jangpromma et al., 2010b). In brief, the data points were recorded at 6 positions along the length of the leaf blade and then the data points were averaged into a single value. Sugarcane was taken so as to ensure that the SPAD meter sensor fully covered the leaf lamina and that interference from the veins and midribs was avoided.

2.3. Leaf relative water content

After the SCMR was recorded, the same leaves were used for relative water content (RWC) determination. The leaves were cut into small pieces and placed in sealable plastic

tubes, and the leaf fresh weights (FW) were measured. The samples were then immersed in distilled water for 8 h at room temperature and weighed to gauge the saturated weight (SW). The samples were later oven-dried at 80 °C for 48 h and weighed to determine their dry weight (DW). RWC was calculated according to Barrs and Weatherley (1962) as follows: $RWC (\%) = [(FW - DW)/(SW - DW)] \times 100$.

2.4. Isolation of RNA and first-strand cDNA synthesis

Total RNA was extracted from sugarcane leaves using the SV Total RNA Isolation Kit (Promega, USA); the RNA was used for first-strand cDNA synthesis. This synthesis was carried out according to the RevertAid First-Strand cDNA synthesis kit manual (Fermentas, USA). In brief, 2 µg of total RNA was mixed with oligo (dT)₁₈ primer and incubated at 65 °C for 5 min. The mixture was then combined with the reaction buffer, RiboLock RNase inhibitor, dNTP mix, and RevertAid M-MuLV reverse transcriptase to obtain a total volume of 20 µL. The mixture was stirred gently, centrifuged briefly, and incubated for 60 min at 42 °C. Finally, the reaction was terminated by heating at 70 °C for 5 min.

2.5. Rapid amplification of 3' cDNA ends (3' RACE)

On the basis of 3' RACE, gene-specific primers (3' RACE-GSP1 and 3' RACE-GSP2) and universal primers (NUP and NUP(dT)₃₀) were synthesized. Primers for 3' RACE-GSP were designed based on the corresponding known sequences in GenBank, i.e. *Saccharum officinarum* cystatin mRNA, partial cds (GenBank: AY119689). The sequences of these primers are: 3' RACE-GSP1: 5'-ATGGCCGAGGCACACAACGG-3', 3' RACE-GSP2: 5'-TCGAGAGGCTGGTGAAGGTGAG-3', NUP: 5'-AAGCAGTGGTATCAACGCAGAGT-3' and NUP(dT)₃₀: 5'-AGTGGTATCAACGCAGAGTACTT TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-3'. Amplification was followed by 2 successive PCR runs (Sambrook and Russell, 2001). The first reaction was carried out with a total of 25 µL of PCR reaction mixture consisting of 1X Taq buffer, 1 mM MgCl₂, 0.2 mM dNTP mix, 0.2 µM of each 3' RACE-GSP1 and NUP(dT)₃₀ primer, 1.25 U of Taq DNA polymerase (Fermentas, USA), and 50 ng of Khon Kaen 3 drought-stressed template cDNA. Later, the products of the first PCR run were used as templates for a second nested PCR, which was conducted with a 3' RACE-GSP2 primer that acted as the internal oligonucleotide for the 3' RACE-GSP1 primer, while the second antisense primer was NUP. The total volume and concentration of each component were the same as in the first PCR run.

Both the first and second PCR amplifications were carried out in an MJ Mini Thermal Cycler PCR machine (Bio-Rad, USA), which was programmed for a predenaturation step of 5 min at 94 °C and 35 cycles of 30

s at 94 °C, 30 s at 70 °C, and 1 min at 72 °C, followed by 1 cycle of 10 min at 72 °C. The PCR product of the 3' RACE was analyzed by 2% agarose gel electrophoresis, stained with ethidium bromide, and then photographed under UV light. Subsequently, the 3' RACE band was extracted from the agarose gel using the PCR clean-up gel extraction NucleoSpin Extract II kit (Macherey-Nagel, Germany) and ligated into a pGEM-T Easy vector (Promega, USA). The recombinant vector was then transformed into *Escherichia coli* (DH5α), and positive cDNA clones were chosen and then sequenced.

The nucleotide and deduced amino acid sequence similarities were compared with the GenBank/EMBL database using the BLAST algorithm (Altschul et al., 1997). The pI/MW of the proteins was computed using the Compute pI/MW tool on the ExPASy website.

2.6. Recombinant pPIC9K-sugarcane cystatin and pAUR123-sugarcane cystatin plasmid construction

The primers that were used to amplify the full-length coding region of sugarcane cystatin were Cystatin-F: 5'-ATGGCCGAGGCACACAACGG-3', which had the same sequence as 3' RACE-GSP1, and Cystatin-R: 5'-ATGTTTACTACCTCAGGCGGCC-3'. These specific primers were designed based on the full sugarcane cystatin nucleotide sequences that were obtained from the 3' RACE experiment. A 342-bp blunt-end PCR product was obtained using KOD-plus-DNA polymerase (TOYOBO, Japan). Before the ligation, pPIC9K was cut with *Sna*B I and pAUR123 was cut with *Sma* I to create the expression recombinant plasmids. The blunt-end open circular plasmid DNA was then removed at the 5' phosphates and treated with calf intestinal alkaline phosphatase as part of a dephosphorylation process. Later, a blunt-end ligation was performed between the sugarcane cystatin and each plasmid using the Mighty Cloning Kit (TaKaRa, Japan) according to the manufacturer's instructions. The sequence of the pPIC9K-sugarcane cystatin plasmid was confirmed with 5'AOX1: 5'-GACTGGTTCCAATTGACAAGC-3' and 3'AOX1: 5'-GCAAATGGCATTCTGACATCC-3' primers. The pAUR123-sugarcane cystatin plasmid was confirmed with PAUR123-F: 5'-TCTGCACAATATTTCAAGC-3' and PAUR123-R: 5'-TTCGTTTTAAACCTAAGAGTCAC-3' primers.

2.7. *Pichia pastoris* transformation

The pPIC9K-sugarcane cystatin plasmid and the pPIC9K empty vector were harvested from *Escherichia coli* (JM109). A linearization of recombinant plasmid DNA was performed with *Sal* I to integrate the transgene at the HIS4 locus on the *Pichia pastoris* (GS115) genome. The linear recombinant vector DNA was then transformed into *Pichia pastoris* (GS115) by the lithium chloride transformation method according to the manufacturer's specifications (Invitrogen, Canada). A total of 100 µL

of transformed cells was spread onto MD plates. The inverted plates were incubated at 30 °C for 3–4 days. The recombinant pPIC9K-cystatin-transformed cells were selected with hyperresistant genetics in colony screening and then verified by PCR.

2.8. *Saccharomyces cerevisiae* transformation

A *Saccharomyces cerevisiae* (A2279) transformation was accomplished by the modified lithium chloride transformation method (Invitrogen, Canada). The pAUR123-sugarcane cystatin plasmid and the pAUR123 empty vector were harvested from *Escherichia coli* (JM109) and then directly transformed into *Saccharomyces cerevisiae* (A2279). For each transformation sample, the reagents were added in the following order: 240 µL of 50% PEG-6000, 36 µL of 1 M LiCl, and pAUR123-sugarcane cystatin recombinant plasmid DNA or pAUR123 plasmid DNA (5–10 µg) with 150 µg of carrier DNA in 50 µL of sterile water. A total of 100 µL of transformed cells was spread onto YPD (dextrose) plus 0.5 µg mL⁻¹ aureobasidin A plates. The inverted plates were incubated at 30 °C for 2–4 days. The size of the pAUR123-sugarcane cystatin recombinant plasmid DNA insert was verified by PCR.

2.9. Sugarcane cystatin protease inhibitory activity assay

Transgenic *Pichia pastoris* (GS115) containing the pPIC9K-sugarcane cystatin plasmid and the pPIC9K empty vector was inoculated into 1 mL of buffered glycerol complex medium (BMGY) and incubated overnight at 30 °C with vigorous shaking (approximately 16–18 h). The 1-mL culture was then used to inoculate 100 mL of BMGY. The overnight cell cultures were then resuspended in 20 mL of buffered methanol complex medium (BMMY) and allowed to grow at 30 °C. Methanol (100%) was added to the culture to a final concentration of 0.5% every 24 h to maintain induction for 3 days. The supernatant was then collected to perform a sugarcane cystatin protease inhibitory activity assay. The inhibitory activity was measured as the capacity to inhibit cysteine protease activity; milk casein was used as a substrate. The reaction mixture consisted of 2 mL of casein solution (1% milk casein in 0.1% phosphate buffer, pH 6.0), 0.2 mL of 10 mM cysteine, 0.2 mL of cysteine protease solution (partially purified ginger protease), and 0, 1.25, 2.50, 3.75, or 5 µg of each sugarcane cystatin sample, which was diluted with double-distilled water in a total volume of 100 µL. The *Pichia pastoris* (GS115) transformed with the empty pPIC9K vector was used as a negative control. The reaction was then allowed to proceed for 30 min at 50 °C. A total of 100 µL of the sample solution was removed after centrifugation at 3000 × g and passed through a spectrophotometer at 280 nm. The protease inhibitory activity was calculated based on $\text{Blank}_{A_{280}} - \text{Sample}_{A_{280}}$ where the blank was the A_{280} value of the following reaction mixture: 2 mL of the casein solution, 3 mL of 5% TCA, 0.2

mL of the 10 mM cysteine solution, and 0.1 mL of double-distilled water. The percentage of inhibition was calculated by comparison with protease inhibitory activity of 0 µg of sugarcane cystatin reaction.

2.10. Two-dimensional electrophoresis

Two-dimensional electrophoresis was performed according to the modified method of O'Farrell (1975), which was previously described by Jangpromma et al. (2010a). The secreted sugarcane cystatin protein in the medium was concentrated by a Vivaspinn apparatus with a 10-kDa cut-off (GE Healthcare, Sweden). The concentrated protein was then cleaned of contaminants by the 2D-Clean-Up Kit (GE Healthcare, Sweden). The first-dimensional isoelectric focusing was performed on a 7-cm immobilized dry strip with a linear gradient of pH 3–10 (GE Healthcare, Sweden) with 100 µg of loading protein. The IEF running conditions were as follows: 250 Vh, followed by 500 Vh and finally 8500 Vh. The second dimension was separated on a 15% SDS polyacrylamide gel. Proteins were stained with silver nitrate (GE Healthcare, Sweden). ImageMaster 2D Platinum software (GE Healthcare, Sweden) was used to calculate the isoelectric point (pI) and molecular weight (MW) of the proteins.

2.11. Yeast osmotic stress tolerance assay

The effects of osmotic stress tolerance on transgenic *Saccharomyces cerevisiae* (A2279) and *Pichia pastoris* (GS115) were investigated according to the method of Zhang et al. (2008). Osmotic stress was initially imposed after the inoculation of a single colony of transgenic *Saccharomyces cerevisiae* (A2279) containing the pAUR123-sugarcane cystatin plasmid and the pAUR123 empty vector into 3 mL of YPD medium plus 0.5 µg mL⁻¹ aureobasidin A at 30 °C with vigorous shaking overnight. Similarly, a single colony of transgenic *Pichia pastoris* (GS115) containing the pPIC9K-sugarcane cystatin plasmid and the pPIC9K empty vector was inoculated overnight in 3 mL of YPD medium that did not contain any antibiotic. A total of 100 µL of each overnight culture was then inoculated into 10 mL of YPD medium plus 0.5 µg mL⁻¹ aureobasidin A for *Saccharomyces cerevisiae* (A2279) and only YPD medium for *Pichia pastoris* (GS115). The cells were incubated at 30 °C with vigorous shaking until the culture grew to an optical density at 600 nm (OD_{600}) of 0.3. Each culture was diluted to 10-, 100-, 1000-, 5000-, and 10,000-fold with the appropriate YPD medium, and 5 µL of each dilution series was then spotted onto YPD agar plates supplemented with 1 M mannitol and 1, 1.5, or 2 M sorbitol. Growth was monitored at 30 °C for 3 days. For the *Pichia pastoris* (GS115) plates, 100 µL of 100% methanol was added to the lid of the inverted plate each day to maintain the induction of the cystatin gene.

The osmotic stress tolerance assay of *Pichia pastoris* (GS115) grown in culture broth was conducted by inoculating a single *Pichia pastoris* (GS115) colony, which

contained pPIC9K-sugarcane cystatin and the pPIC9K empty vector, into 1 mL of BMGY medium. Growth was monitored at 30 °C with vigorous shaking. Next, 3 µL of the overnight culture was inoculated into 20 mL of BMGY medium and grown at 30 °C in a shaking incubator until the culture reached an OD₆₀₀ of 1.0. Subsequently, 4 mL of the culture was dispensed into 4 tubes for each sorbitol concentration. The cell pellet was then resuspended in BMMY media containing different concentrations of sorbitol (0, 1, 1.5, and 2 M sorbitol), and growth continued at 30 °C with vigorous shaking. Methanol (100%) was added to each *Pichia pastoris* (GS115) culture to a final concentration of 0.5% every 24 h. Yeast growth was measured at 600 nm every day for 3 days. All of the assays were repeated at least 3 times.

2.12. Semiquantitative RT-PCR

To evaluate the sugarcane cystatin transcript levels in sugarcane leaves under drought stress and drought recovery conditions, 10 µL samples of a 1:50 dilution of each treatment and each sugarcane cultivar cDNA were used as templates in PCR reactions with the primers Cystatin-RT-F: 5'-TCGAGAGGCTGGTGAAGGTGAG-3' and Cystatin-RT-R: 5'-TTGGCCTCGTACAGCTTCTTGC-3' using Taq DNA polymerase (Fermentas, USA). Glycerol-3-phosphate dehydrogenase (GAPDH) was used as an internal control with the following set of primers: Cane-GAPDH F: 5'-GTGCACGCCACTGGAAGCA-3' and Cane-GAPDH R: 5'-CCACGGGATCTCCTCAGGGT-3'. Each semiquantitative PCR experiment included 30 cycles and was repeated at least twice. The PCR product was separated by electrophoresis on a 2% agarose gel and visualized using ethidium bromide staining with band intensity quantification by Quantity One software (Bio-Rad, USA).

2.13. Statistical analysis

Analysis of variance was performed for RWC, SCMR, cystatin protease inhibitory activity, osmotic stress tolerance assay on media, and semiquantitative RT-PCR analysis according to a factorial in RCBD with 3 replications and the least significant difference (LSD) was used to compare means (Hoshmand, 2006).

3. Results

3.1. Effects of drought on RWC and chlorophyll content

Drought conditions were observed to reduce the RWC for all sugarcane genotypes (Figure 1). The highest reduction in RWC was observed for B34-164 (40%) compared to the control (100%). The reductions in Khon Kaen 3 (60%) and 03-4-425 (60%) were intermediate, whereas the reduction in K86-161 (80%) was the lowest (Figure 1A). However, most of the sugarcane genotypes were not significantly different from the respective control after rehydration for 24 h or 5 days (Figures 1B and 1C).

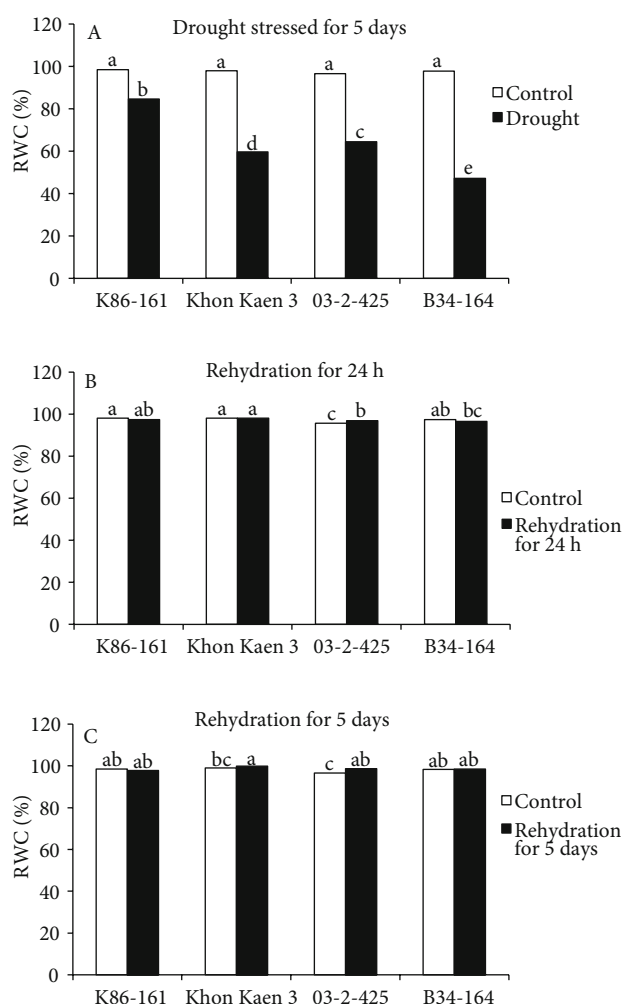


Figure 1. Leaf relative water content (RWC) of 4 sugarcane cultivars after 5 days of drought (A), 24 h of rehydration (B), and 5 days of rehydration (C) under 2 water regimes, control (well-watered) □ and water stress or rehydration ■. The same letters above each bar chart are not significantly different by LSD at P = 0.05.

Drought conditions also reduced the chlorophyll content in all sugarcane genotypes (Figure 2). The reduction was highest in B34-164 (20) when compared to the control (40). The reductions in K86-161 (25) were intermediate, whereas the reductions in Khon Kaen 3 (40) and 03-4-425 (40) were the lowest (Figure 2A). The sugarcane cultivar 03-4-425 fully recovered its chlorophyll content after 24 h of rehydration, whereas B34-164, Khon Kaen 3, and K86-161 did not (Figure 2B). However, these samples could fully recover after 5 days of rehydration (Figure 2C).

3.2. 3' RACE and full-length cystatin gene

Only a partial sequence of the sugarcane cystatin gene is available in GenBank (Accession No. AY119689). Therefore, we used 3' RACE to obtain a full-length

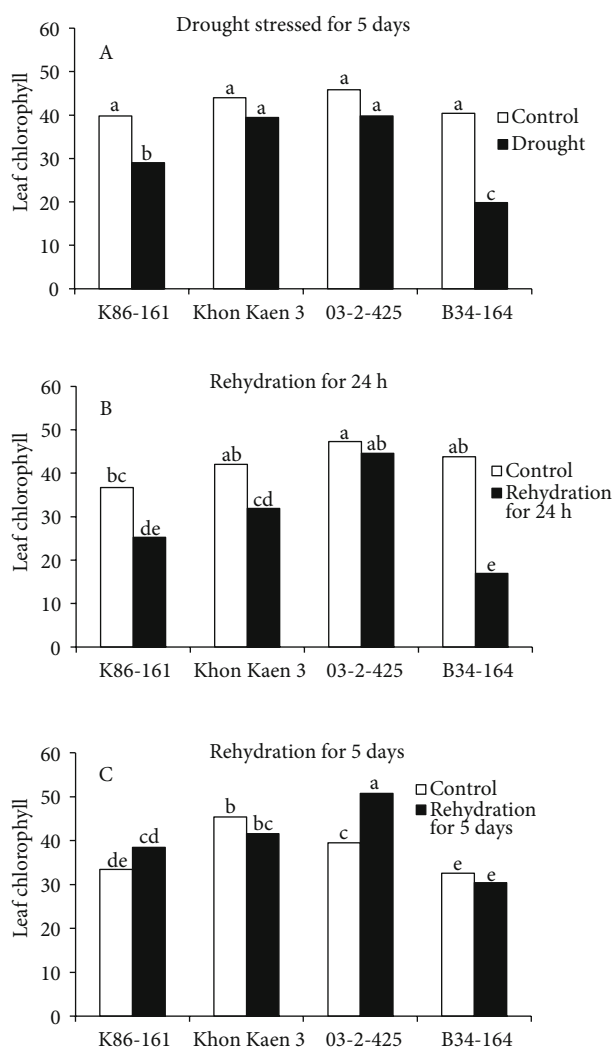


Figure 2. Leaf chlorophyll content (SCMR) of 4 sugarcane cultivars after 5 days of drought (A), 24 h of rehydration (B), and 5 days of rehydration (C) under 2 water regimes, control (well-watered) □ and water stress or rehydration ■. The same letters above each bar chart are not significantly different by LSD at $P = 0.05$.

sequence of the sugarcane cystatin gene from drought-stressed sugarcane leaves. The full-length sugarcane cystatin nucleotide sequence has been submitted to the DDBJ/GenBank databases under Accession No. AB704756. As shown in Figure 3, the start codon of the sugarcane cystatin open reading frame (ORF) is typically ATG and the stop codon is TGA, with a full length of 330 bp containing a 3' UTR of 173 bp. The poly (A) tail is preceded by the putative polyadenylation signal AATAAA. The 330-bp ORF encodes a deduced amino acid sequence of 109 amino acid residues with a calculated MW of 12 kDa and a calculated pI of 6.59. The conserved LARFAVAEH motif in the phytocystatins was found and is highlighted

by the gray shaded region. The α -helix structure is emphasized with a double underline. The conserved putative reactive site interacting with the cysteine protease molecules, namely Q⁵⁹V⁶⁰V⁶¹A⁶²G⁶³, is indicated by a box. This nucleotide and deduced amino acid sequence are very similar to the sequence of *Saccharum officinarum* cystatin (AY119689) deposited in GenBank. The protein deduced sequence shown in this submitted article has only 3 additional alanine residues in the carboxy-terminal end. The cystatins are probably the same, since AY119689 is deposited as a partial coding sequence.

The deduced amino acid sequence of sugarcane cystatin was uploaded to BLASTP, which allows users to search for proteins in the NR database, and the results indicated that the deduced cystatin protein is most similar to *Zea mays* L. (maize) cystatin. The sugarcane sequence was 90% identical to that of *Zea mays* L. (GenBank Accession No. CAJ20025.1), 81% identical to that of *Hordeum vulgare* L. subsp. *vulgare* (barley) (GenBank Accession No. BAK03799.1), 74% identical to that of *Oryza sativa* L. (rice) (GenBank Accession No. AAA33911.1), 67% identical to that of *Triticum aestivum* L. (wheat) (GenBank Accession No. BAB18768.1), 62% identical to that of *Brassica rapa* L. subsp. *campestris* (Chinese cabbage) (GenBank Accession No. AAC37479.1), and 60% identical to that of *Arabidopsis thaliana* (L.) Heynh (thale cress) (GenBank Accession No. AAN65082.1).

3.3. Cystatin gene expression and characterization

Sugarcane cystatin gene expression was further studied in eukaryotic systems. Transgenic *Pichia pastoris* (GS115) was induced for cystatin protein expression, and the secreted protein was used to assay biological activity. The protein inhibited partially purified ginger cysteine protease, as shown by a high inhibition (%) that increased with increasing sample concentration. Significant differences between *Pichia pastoris* (GS115) containing the pPIC9K-sugarcane cystatin plasmid and *Pichia pastoris* (GS115) containing an empty pPIC9K vector were observed. The protein induction in *Pichia pastoris* (GS115) containing an empty pPIC9K vector, acting as a negative control, did not show inhibitory activity (Figure 4).

The secreted protein was separated by 2D-PAGE. The 2D-PAGE pattern showed a good separation pattern of secreted proteins. The highly abundant protein spot with a MW of 12 kDa was presumed to be sugarcane cystatin. The MW of this protein is the same as the MW predicted for the deduced amino acid sequence, and the protein had a pI of 4.43 (Figure 5).

3.4. Expression of the cystatin gene in yeast and an osmotic stress tolerance assay

To address the function of sugarcane cystatin, transformations of *Pichia pastoris* (GS115) and *Saccharomyces cerevisiae* (A2279) were performed. The

1	ATG	GCC	GAG	GCA	CAC	AAC	GGG	CGG	CGC	GTG	GGG	33
1	Met	A	E	A	H	N	G	R	R	V	G	11
34	ATG	GTG	GGC	GAC	GTC	CGG	GAC	GCG	CCG	GCG	GGC	66
12	M	V	G	D	V	R	D	A	P	A	G	22
67	CAC	GAG	AAC	GAC	CTC	GAG	GCC	ATC	GAG	CTC	GCG	99
23	H	E	N	D	<u>L</u>	<u>E</u>	<u>A</u>	<u>I</u>	<u>E</u>	<u>L</u>	<u>A</u>	33
100	CGC	TTC	GCC	GTC	GCC	GAG	CAC	AAC	AGC	AAG	ACC	132
34	<u>R</u>	<u>F</u>	<u>A</u>	<u>V</u>	<u>A</u>	<u>E</u>	<u>H</u>	<u>N</u>	<u>S</u>	<u>K</u>	<u>T</u>	44
133	AAC	GCG	ATG	CTG	GAG	TTC	GAG	AGG	CTG	GTG	AAG	165
45	N	A	M	L	E	F	E	R	L	V	K	55
166	GTG	AGG	CAC	CAG	GTG	GTG	GCC	GGG	ACC	ATG	CAC	198
56	V	R	H	<u>Q</u>	<u>V</u>	<u>V</u>	<u>A</u>	<u>G</u>	T	M	H	66
199	CAC	TTC	ACC	GTC	CAG	GTG	AAG	GAG	GCC	GGC	GGC	231
67	H	F	T	V	Q	V	K	E	A	G	G	77
232	GGC	AAG	AAG	CTG	TAC	GAG	GCC	AAG	GTG	TGG	GAG	264
78	G	K	K	L	Y	E	A	K	V	W	E	88
265	AAG	GTG	TGG	GAG	AAC	TTC	AAG	CAG	CTG	CAG	AGC	297
89	K	V	W	E	N	F	K	Q	L	Q	S	99
298	TTC	CAG	CCG	GTC	GGG	GAC	GCC	GCG	GCC	GCC	TGA	330
100	F	Q	P	V	G	D	A	A	A	A	Stop	109
331	GGTAGTAAACATATCACCTTCCAGTGTACGATGTGTTTTTCC 373											
374	CTCAAGGCTCACAAGCTTTTCGCTGGAGGCTGGAGCACAACAA 416											
417	TGAAAGAATTTAGCTGTCCGACATGAAATACATCCCCTGGAA 459											
460	AAGTATATATAATATGATATGATGAATAAACAGCATCTTAACG 503											

Figure 3. The nucleotide and deduced amino acid sequence of sugarcane cystatin. The nucleotide sequence is indicated on the top line, and the deduced amino acid sequence is shown in single letters designated below the nucleotide sequence on the second line. The conserved motif of phycocystatins is shaded in gray, and the α -helix structural region is double-underlined. The amino acid residues that interact with cysteine protease molecules are shown in the box, and the putative polyadenylation signal is underlined. This nucleotide and deduced amino acid sequence is very similar to sequence AY119689 deposited in GenBank. The protein deduced sequence shown in this submitted article has only 3 additional alanines in the carboxy-terminal. The cystatins are probably the same, since AY119689 is deposited as a partial coding sequence.

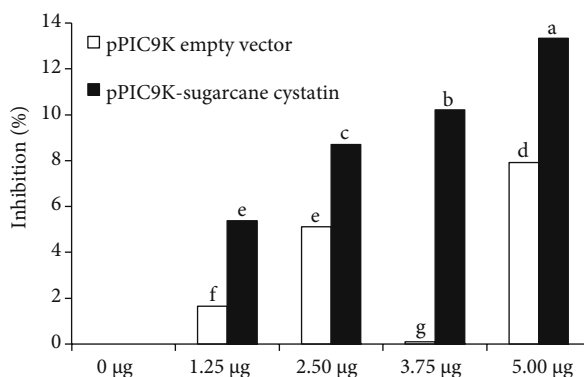


Figure 4. Sugarcane cystatin protease inhibitory activity on partially purified ginger cysteine protease. Partially purified ginger cysteine protease was incubated with 0, 1.25, 2.50, 3.75, and 5.00 µg of each sugarcane cystatin sample. The inhibitory activity was measured by decreased levels of digested peptides of the substrate (milk casein). □ Secreted protein of *Pichia pastoris* (GS115) cells transformed with an empty vector (pPIC9K) and used as a control. ■ Secreted protein of *Pichia pastoris* (GS115) cells transformed with sugarcane cystatin (pPIC9K-sugarcane cystatin). The same letters above each bar chart are not significantly different by LSD at P = 0.05.

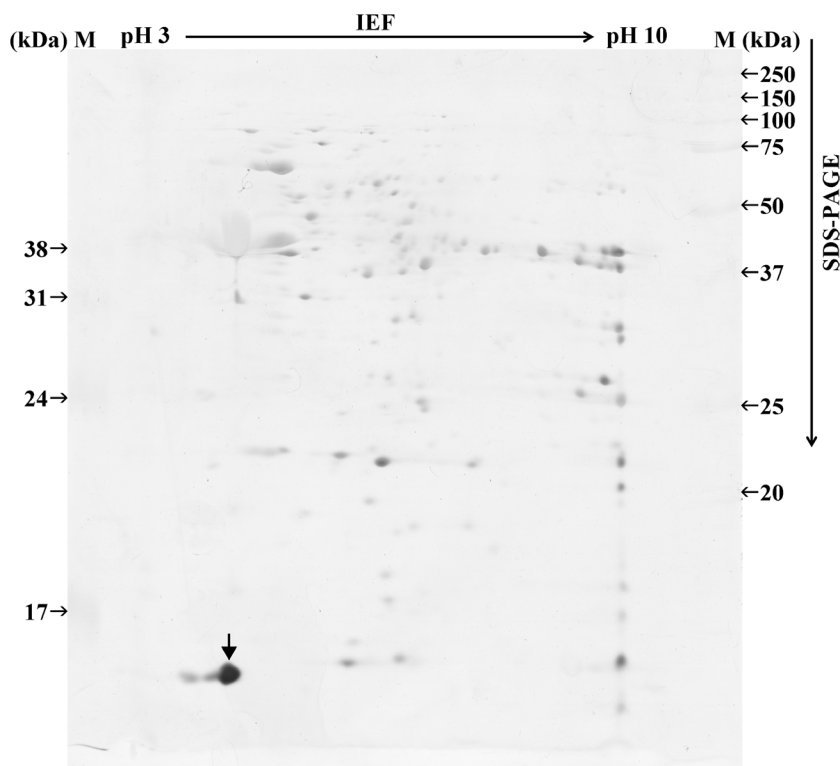


Figure 5. Two-dimensional electrophoresis of secreted proteins from *Pichia pastoris* (GS115). In the first dimension (IEF), 100 μ g of protein was loaded on a 7-cm IPG strip with a linear gradient of pH 3–10. In the second dimension (SDS-PAGE), the protein was separated again by using a 15% polyacrylamide gel and then visualized by silver staining. The arrow indicates the spot proteins predicted as sugarcane cystatin.

expression of sugarcane cystatin via transgenic *Pichia pastoris* (GS115) and *Saccharomyces cerevisiae* (A2279) was compared with that of *Pichia pastoris* (GS115) and *Saccharomyces cerevisiae* (A2279) transformed with an empty vector (control) under mimicked osmotic stress conditions on culture plates. The degree of osmotic conditions was mimicked by increasing the mannitol or sorbitol concentrations.

Under each osmotic condition, the different types of transgenic yeast cells were spotted on the plate at 6 serial dilutions (Figure 6). In the presence of 1 M mannitol and 1, 1.5, or 2 M sorbitol, *Pichia pastoris* (GS115) was more susceptible to osmotic stress than the *Saccharomyces cerevisiae* (A2279) strain, although *Pichia pastoris* (GS115) and *Saccharomyces cerevisiae* (A2279) showed little growth difference on the YPD plate without chemical supplementation (Figure 6).

According to Figure 6A, the transgenic *Pichia pastoris* (GS115) (pPIC9K-cystatin) and the cells transformed with the empty vector (pPIC9K) as a control did not grow well in the presence of mannitol and sorbitol when compared to the control conditions (a YPD plate not supplemented with mannitol or sorbitol). The cell growth decreased when the concentration of sorbitol was increased.

Nevertheless, the pPIC9K-cystatin cells grew at a higher rate than the pPIC9K cells when induced with methanol [(+) MeOH]. This higher growing rate was also evident in the presence of 1.5 M sorbitol, whereas when the cystatin cells were not induced with methanol [(-) MeOH], they were more sensitive to the 1.5 M sorbitol. In the presence of 2 M sorbitol, the pPIC9K-cystatin and pPIC9K cells did not grow under either the (+) MeOH or the (-) MeOH conditions.

The results of *Saccharomyces cerevisiae* (A2279) sugarcane cystatin (pAUR123-cystatin) expression were more similar to those of the transgenic *Pichia pastoris* (GS115) that exhibited enhanced osmotic tolerance than to those of the cells transformed with an empty vector (pAUR123) as a control. However, susceptibility results were evident with 2 M sorbitol (Figure 6B).

These results were again confirmed by studying *Pichia pastoris* (GS115) in medium rather than on plates, which allows for the induction of sugarcane cystatin in methanol (Figure 7). Under normal conditions (0 M sorbitol), the growth of the pPIC9K-cystatin cells was slower than that of the pPIC9K cells. In the presence of different sorbitol concentrations, the pPIC9K-cystatin cells had higher growth rates than the pPIC9K cells for all 3 days of the

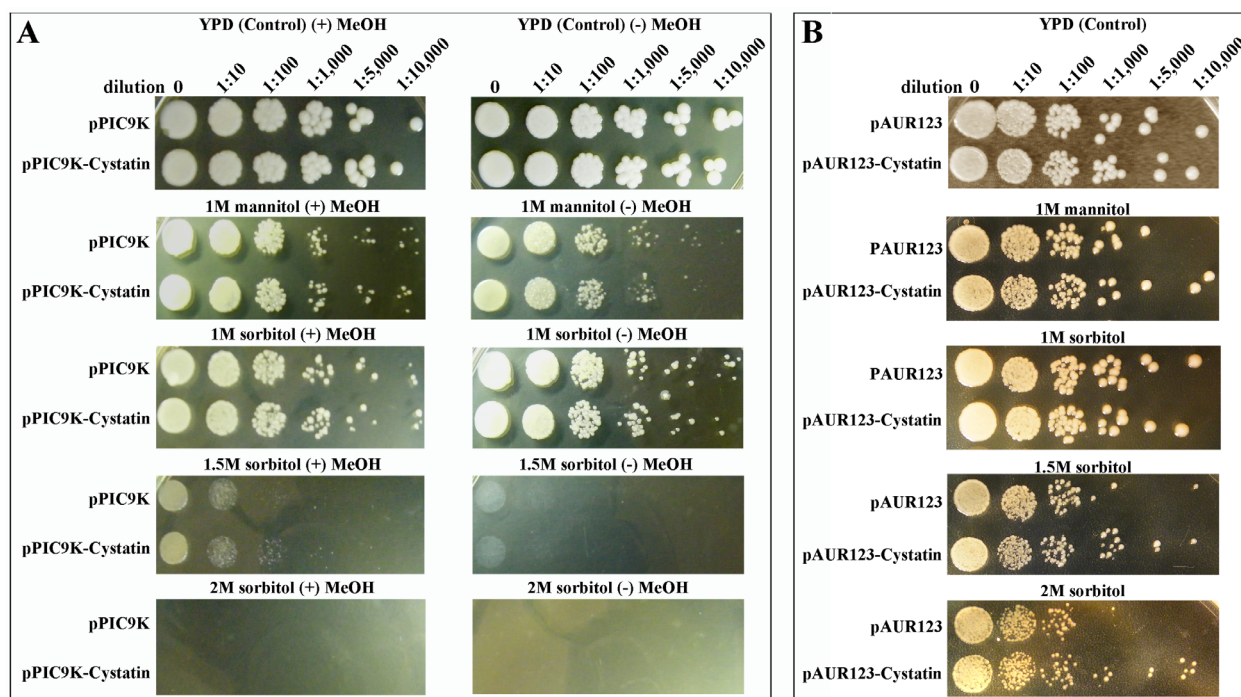


Figure 6. An osmotic stress tolerance assay of transgenic yeasts on plates. Figure 6A shows the osmotic tolerance assessment of *Pichia pastoris* (GS115) expressing sugarcane cystatin (pPIC9K-cystatin), where cells transformed with an empty vector (pPIC9K) were used as a control. The left panel shows an induction of cystatin expression with methanol [(+) MeOH]. In contrast, the right panel is not induced with methanol [(-) MeOH]. Figure 6B shows the osmotic tolerance assessment of *Saccharomyces cerevisiae* (A2279) expressing sugarcane cystatin (pAUR123-Cystatin) along with cells transformed with an empty vector (pAUR123) as a control.

assessment (Figures 7A–7C). The osmotic stress results for *Pichia pastoris* (GS115) and *Saccharomyces cerevisiae* (A2279) on the plates were also similar.

3.5. Semiquantitative RT-PCR

Significant increases in mRNA intensity were found in K86-161 and B34-164 after 5 days of drought stress (Figure 8). Khon Kaen 3 and 03-4-425 also exhibited a slightly increased mRNA intensity. K86-164 had the highest mRNA intensity (120) under drought conditions, whereas B34-164 had the lowest (90) (Figure 8A).

After recovery for 24 h, the mRNA intensity of the nonstressed controls was significantly higher than that of the stressed plants for all sugarcane genotypes (Figure 8B). After recovery for 5 days, the mRNA intensities of all sugarcane genotypes under drought conditions appeared to be higher than the intensities after 24 h of recovery, but they were still lower than those of the nonstressed controls that were not significantly different (Figure 8C).

4. Discussion

A concentrated effort to improve drought tolerance in crops may be the most economical approach to increasing crop productivity. With this strategy in mind, plant breeders have had the major goal of understanding the mechanisms

of crop adaptations to drought (Rampino et al., 2006; Xiong et al., 2006; Jangpromma et al., 2012). Adaptation mechanisms to water shortage are involved in numerous biochemical and physiological responses in plants (Jangpromma et al., 2010a). Therefore, to understand the metabolic pathways of sugarcane in response to drought, a proteomics strategy was used to identify and characterize proteins that respond to drought stress conditions, as previously described (Jangpromma et al., 2010a). For instance, sugarcane leaf proteins are involved in numerous biological processes under drought conditions, such as antioxidant activity, signal transduction, photosynthesis, protein degradation, plant defense, and lipid metabolism. A number of sugarcane leaf proteins are associated with drought (up-/downregulated or present in stressed plants but absent in controls) when compared to nonstressed plants (control), namely protease inhibitors (PIs). Plant PIs have been shown to participate in various physiological and developmental processes and may also be involved in responses to various biotic and abiotic stresses (Huang et al., 2007), the most notable being cystatin. Consequently, we analyzed sugarcane cystatin to further evaluate its function in drought stress. To accomplish this aim, sugarcane genotypes exhibiting contrasting responses to drought stress were selected.

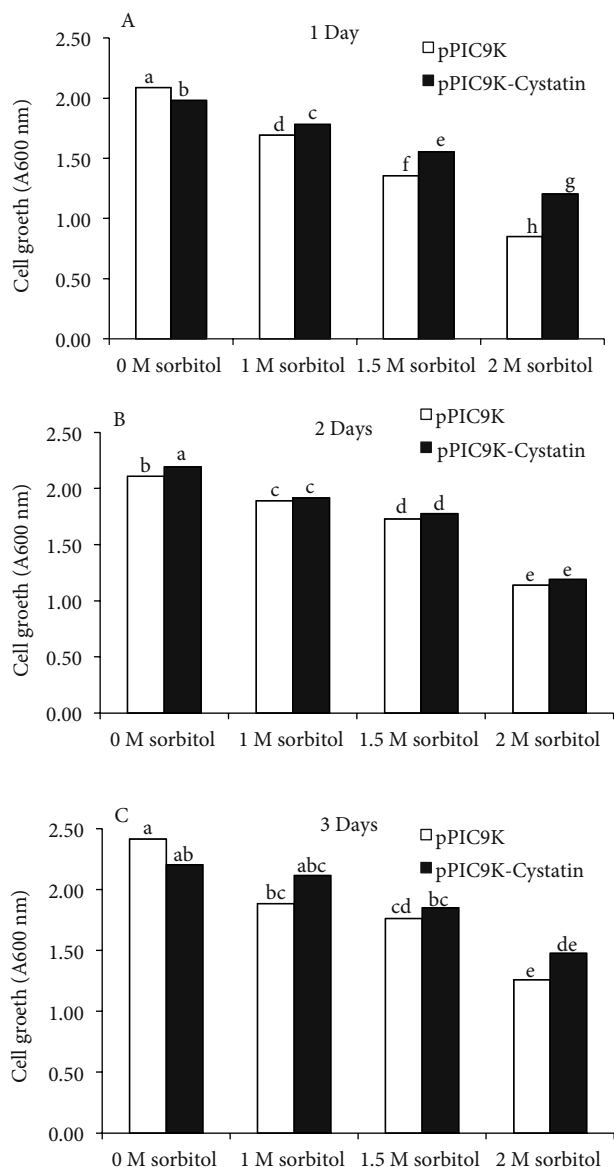


Figure 7. Osmotic stress tolerance assay of transgenic *Pichia pastoris* (GS115) on media. Yeast growth was measured at 600 nm every day for 3 days. Figure 7A shows the osmotic tolerance assessment for 1 day, Figure 7B for 2 days, and Figure 7C for 3 days. □ *Pichia pastoris* (GS115) cells transformed with an empty vector (pPIC9K) as a control. ■ Secretion media of *Pichia pastoris* (GS115) cells transformed with sugarcane cystatin (pPIC9K cystatin). The same letters above each bar chart are not significantly different by LSD at P = 0.05.

Physiological traits are commonly examined to assess drought tolerance in many crops (Gesimba et al., 2004; Songsri et al., 2009; Jangpromma et al., 2010b, 2012; Makbul et al., 2011; Bera et al., 2014). In a previous report, 10 sugarcane cultivars (Uthong 6, Khon Kaen 80, K86-161, Khon Kaen 3, 03-4-425, KU60-1, Phill66-07, B34-

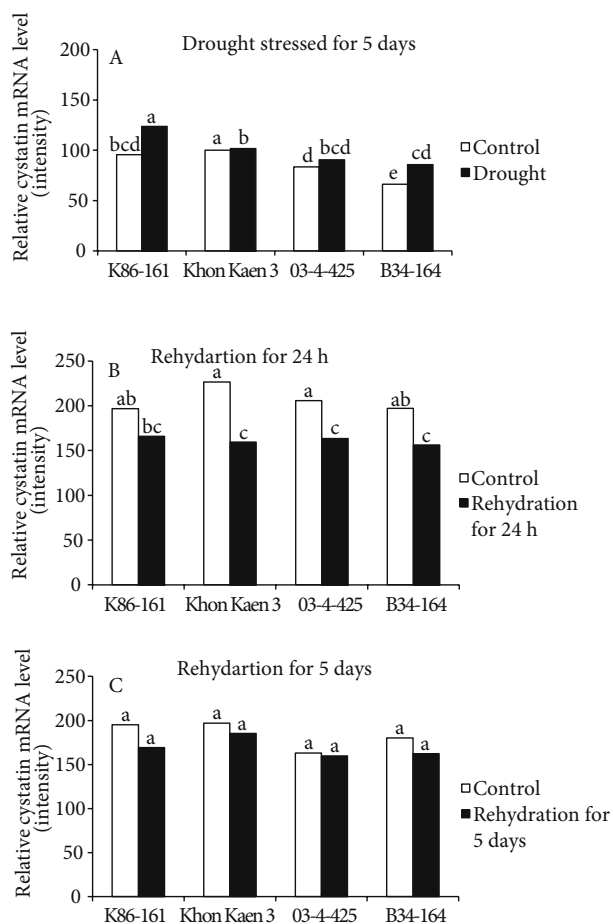


Figure 8. Semiquantitative RT-PCR analyses of cystatin transcript levels in sugarcane leaves under drought stress and drought recovery conditions in comparison to well-watered plants (control □). Figure 8A shows the results for 5 days of drought ■, Figure 8B shows the results for 24 h of rehydration ■, and Figure 8C shows the results for 5 days of rehydration ■. The same letters above each bar chart are not significantly different by LSD at P = 0.05.

164, Uthong 2, and LF82-2122) were selected to evaluate their adaptation ability on the basis of the chlorophyll content, root traits, water use efficiency, and growth characteristics of above-ground parts under drought stress and rehydration conditions (Jangpromma et al., 2010b, 2012). We chose 4 sugarcane cultivars, K86-161, Khon Kaen 3, 03-4-425, and B34-164, to conduct a further study of sugarcane cystatin mRNA expression levels. The K86-161, Khon Kaen 3, and 03-4-425 cultivars were selected as drought-tolerant cultivars based on their root traits (i.e. root length, root surface area, and root volume) and high chlorophyll content. Meanwhile, B34-164 was selected as a drought-susceptible cultivar because it has been shown to have poor drought resistance traits. In this study, the RWC

and chlorophyll content of these 4 sugarcane cultivars were determined to confirm their responses to drought. The RWC was selected as a useful indicator of plant water balance and a relevant physiological measure of plant water deficit because it indicates the absolute amount of water required by the plant relative to the water consumed through transpiration (González and González-Vilar, 2001; Hassanzadeh et al., 2009). Additionally, Silva et al. (2007) indicated that chlorophyll content can be used to identify drought tolerance in sugarcane cultivars. Jangpromma et al. (2010b) showed that using a SPAD chlorophyll meter to assess chlorophyll content is more effective and rapid and provides a high positive association with the direct method (extraction with a chemical), which is expensive, laborious, and time-consuming. Therefore, the RWC and the chlorophyll content in sugarcane leaves, as determined by a SPAD chlorophyll meter, were chosen to confirm differences in drought tolerance for the sugarcane cultivars. Our results confirmed that all 3 drought-tolerant sugarcane cultivars had a higher RWC and chlorophyll content in comparison to the drought-susceptible cultivar, thereby providing the basis to classify each genotype as tolerant or susceptible to water shortage.

To obtain the full-length sequence of the sugarcane cystatin gene, a 3' RACE experiment was conducted and a BLASTP search was carried out to confirm that the sequence belonged to cystatin, according to its similarity to cystatins from other plants.

We hypothesized that sugarcane cystatin might function as a drought tolerance gene and might help sugarcane to better survive during water shortages. To test this hypothesis, eukaryotic expression systems were chosen to study the expression of sugarcane cystatin and to further evaluate the osmotic tolerance of transgenic yeast. Following the work of Zhang et al. (2008), we used both *Pichia pastoris* (GS115) and *Saccharomyces cerevisiae* (A2279) because yeast and sugarcane are similar eukaryotic cells; thus, yeast and sugarcane might have identical/similar metabolism pathways. *Pichia pastoris* (GS115) was selected for its various advantages of higher eukaryotic expression systems, such as protein processing, protein folding, and posttranslational modification; this organism is widely used for expressing protein that is secreted into the growth medium (Cregg et al., 2000). Therefore, *Pichia pastoris* (GS115) is very suitable for both small- and large-scale production of sugarcane cystatin, which gives much better yields for characterizing biological activity, and conducting 2D-PAGE. The results from the inhibitory assay suggested that the secreted protein partially inhibited purified ginger cysteine protease. These results provide evidence that the secreted protein is a cysteine protease inhibitor.

After characterization of the secreted proteins, both transgenic *Pichia pastoris* (GS115) and *Saccharomyces cerevisiae* (A2279) were subjected to an osmotic stress tolerance assay. The results indicated that these transgenic yeasts had the same increased tolerance to osmotic conditions because they showed an ability to increase the growth of yeast cells when compared to the control. The higher tolerance in transgenic yeasts might explain why the sugarcane cystatin expression caused the inhibition damage of cells by cysteine protease; this protease was more highly expressed and caused damage to the yeast cells under osmotic conditions. The results of this study support the previous results of Zhang et al. (2008), who demonstrated that the cystatin genes from *Arabidopsis thaliana*, namely *AtCYSa* and *AtCYSb*, can help transgenic *Saccharomyces cerevisiae* (A2279) become resistant to high salt, oxidation, cold, and drought stresses.

In our study, we used both *Saccharomyces cerevisiae* (A2279) and *Pichia pastoris* (GS115) host yeast cells to assay osmotic tolerance on the basis of cystatin expression. Although both transgenic yeasts showed more tolerance to osmotic stress than the controls, we found that only transgenic *Saccharomyces cerevisiae* (A2279) could grow under high osmotic conditions (2 M sorbitol), indicating that *Pichia pastoris* (GS115) is more sensitive to osmotic stress than the transgenic *Saccharomyces cerevisiae* (A2279). This result may be related to an incomplete induction of the expression of secreted protein by transgenic *Pichia pastoris* (GS115) caused by methanol. Therefore, experiments under medium conditions were also carried out. These results confirmed that *Pichia pastoris* (GS115) with sugarcane cystatin expression had high osmotic tolerance and that the stress on the media might be more suitable for transgenic *Pichia pastoris* (GS115) than on the plate.

To clarify the role of sugarcane cystatin under drought conditions, the response of sugarcane cystatin in plants was further studied by semiquantitative RT-PCR, and the results demonstrated that sugarcane cystatin expression levels were higher in drought-tolerant cultivars than in drought-susceptible cultivars. This may occur because the tolerant cultivars have a better mechanism for inducing cystatin to act as a defense protein. In addition, drought stress induced a higher expression of sugarcane cystatin in comparison to the control.

Regarding RWC, it seems that the sugarcane cultivars with a high RWC also had high chlorophyll content and

high expression levels of sugarcane cystatin. Likewise, Rampino et al. (2006) reported that RWC values parallel the expression of *DHN* genes, a gene family belonging to the *LEA* gene group. However, differences were observed between the drought-resistant and drought-susceptible genotypes. The results indicate that the drought-tolerant cultivars have a better mechanism for inducing the cystatin gene, which might result in a greater survival rate when the plants are subjected to drought stress conditions. Moreover, both the physiological traits and the molecular data indicate that sugarcane plants have a complex genetic control for drought tolerance. Hence, the classification of drought-tolerant genotypes cannot be performed with confidence on the basis of a single characteristic or trait. Our results support the currently held view that sugarcane cystatin acts as an important element in plant defense mechanisms and potentially assists in the tolerance of plants to drought. However, the question of how cystatin protects the plant and supports drought tolerance remains to be determined.

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