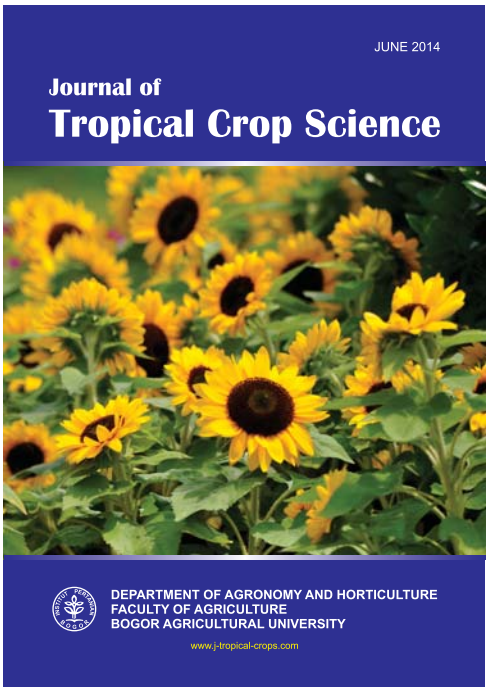

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ON THE COVER

The cover image shows sunflowers by Darda Effendi

SHORT COMMUNICATION

RESEARCH ARTICLES

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Cloning and Characterization of *P5CS1* and *P5CS2* Genes from *Saccharum officinarum* L. under Drought Stress

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Abstract Increasing world sugar demand might be fulfilled with land extensification which include the use of dry area. Development of drought tolerance and high productivity sugarcane variety could be achieved by plant genetic engineering. Under drought condition, proline will be accumulated and functioned as an osmoregulator in plant. Δ^1-*pyrroline*-5-*carboxylate synthase* (P5CS) is one of the important enzymes in proline biosynthesis. This enzyme is encoded by *P5CS* gene family. We cloned two homologous *P5CS* genes from sugarcane, *SoP5CS1* (Accession Number : KF178299) and *SoP5CS2* (Accession Number : KF178300), which encode 729 and 716 amino acid polypeptides. The identity between these two genes was 74% based on nucleotide sequences. The *SoP5CS1* gene had 98% identity with *SbP5CS1* (Accession Number : GQ377719.1) and *SoP5CS2* had 99% identity with *SaP5CS* (Accession Number : EF113257.1). In this experiment, sugarcane plantlets were exposed to medium containing PEG 6000 (40%) for 12, 24, 48, and 72 hours. Proline concentration was measured after treatment and genes expression were analyzed by real time-qPCR. The results showed that the proline concentration was increased 12 folds (9.8 umol.g^{-1}) after 48-hours stress treatment. The highest expression of *SoP5CS1* occured at 24-hours treatment with approximately 16 times from plant without PEG (control plant) and decreased gradually at 48 and 72 hours treatment. The highest expression of *SoP5CS2* occured at 24-hours drought stress with approximately 3.6 folds compared to control. In drought treatment, the expression level *SoP5CS1* was higher than *SoP5CS2* and has increased significantly at 12-hours treatment. It is suggested that the *SoP5CS1* gene contributes more significantly to the production of proline during drought stress than *SoP5CS2*. Hence, *SoP5CS1* could potentialy be used as a marker to screen sugarcane variety for drought tolerance and for the development of transgenic plant tolerant to drought.

Keywords: *cloning, drought, expression, P5CS, sugarcane*

Introduction

Sugarcane is mainly used as raw material for sugar and bioethanol production. This crop productivity and growth has been influenced by enviromental factors such as climate and soil condition. Drought and salinity could decrease sugarcane productivity and growth (Silva et al., 2011). In contrast, the use of marginal and barren land for crop plantation is needed to fulfill high world sugar demand. Research to improve plant stress tolerance is crucial to boost the sugarcane productivity and extend its range. Plant genetic engineering is one of the potential methods for improving plant quality.

Proline accumulation is an adaptive response when plants are exposed to abiotic stresses. Proline acts as osmotic adjustment and osmoprotectant which function to maintain homeostasis condition, stabilize cell structure, and prevent protein degradation (Verbruggen and Hermans, 2006; Taiz and Zeiger, 2006). Glutamate (Glu) and ornithine (Orn) are the proline precursors (Verbruggen and Hermans, 2006; Roosens et al., 1998). Glutamate pathway is the main route for proline biosynthesis during abiotic stresses. In glutamate pathway, proline is synthesized from glutamate via ɣ-glutamic semialdehyde (GSA) and Δ^1-pyrroline-5-carboxylate. This process is catalyzed by two enzymes, Δ^1-pyrroline-5-carboxylate synthethase (P5CS) and Δ^1-pyrroline-5-carboxylate reductase (P5CR) (Verbruggen and Hermans, 2008; Hare and Cress, 1997; Kishor et al., 2005).

P5CS, a rate-limiting enzyme, plays an important role in proline biosynthesis. P5CS is a bifunctional enzyme that exhibits ɣ-glutamyl kinase and glutamic-ɣ-semialdehyde dehydrogenase activities. This enzyme is encoded by two homologous *P5CS* genes (Verbruggen and Hermans, 2008; Hare and Cress, 1997; Kishor et al., 2005). The *P5CS* genes had been cloned and characterized from several plants such as *Arabidopsis thaliana* (Savoüre et al., 1995), *Solanum licopersicum* (Fujita et al., 1998), *Oryza sativa* (Igarashi et al., 1997; Hur et al., 2004; Zhu et al., 1998), and *Sorghum bicolor* (Su et al., 2011). In some species, these genes showed different expression patterns. Both of *P5CS* genes had different functions in plant development stage and stress response (Verbruggen and Hermans, 2008).

In *A. thaliana*, the expression of *AtP5CS1* was induced by drought and salt stress while *AtP5CS2* was ubiquitously expressed in dividing and differentiating cells (Savoure et al., 1995). *S. licopersicum* had two closely related *P5CS*

genes, *tomPRO1* and *tomPRO2*. The expression of *tomPRO1* was induced by salt stress, whilst the *tomPRO2* transcript was undetectable (Fujita et al., 1998). In *O.sativa*, *OsP5CS1* gene was a housekeeping gene, while OsP5CS2 was induced by salt, drought, and cold stress (Igarashi et al., 1997; Hur et al., 2004; Zhu et al., 1998). In *S. bicolor*, the *SbP5CS1* transcript was significantly up-regulated under abiotic stress while the *SbP5CS2* was a housekeeping gene which function in proline biosynthesis under normal condition (Su et al., 2011). Previous study showed that overexpression of *VaP5CS*from *Vigna aconitifolia* increased stress tolerance and proline content on transgenic tobacco (Kishoret al.,1995).

This study aims to clone and characterize two homologous P5CS genes and find the more potential canditate gene that might be used to improve drought tolerance in sugarcane. In this research, two homologous P5CS genes had been cloned from sugarcane followed by its characterization using bioinformatic analysis. We studied the expression pattern of *SoP5CS* genes during drought stress and demonstrated its relation with proline accumulation to find the more potential candidate among those genes for sugarcane genetic engineering.

Material and Method

Plants materials and drought stress treatment

For cloning and full-length isolation of *SoP5CS1* and *SoP5CS2* genes, five month old after acclimatization of sugarcanes plants of PSJT 941 variety were not watered in seven days in order to simulate drought conditions. The sugarcanes were planted in polybags containing a mixture of soil, sand and manure. The control plants were watered until sampled

Three-month old sugarcane planlets were used for expression study of *SoP5CS1* and *SoP5CS2* genes under drought stress and proline measurement. The planlets were incubated in [MS+PEG] medium in 12, 24, 48, and 72 hours in order to simulate drought stress. We used PEG 6000 with 40% (w/v) concentration. For control, the planlets were incubated in MS medium in 12, 24, 48, and 72 hours. The leaf samples were collected after incubation with three biological replicates for each treatment.

Isolation of total RNA and cDNA synthesis

Total RNA was extracted from leaf samples using Total RNA (plant) Isolation Kit Geneaid following manufacturer's intruction. cDNA was synthesized using Revert-Aid cDNA synthesis Kit (Fermentas) and Super Script III cDNA Synthesis Kit Invitrogen according to the manufacturer's instruction.

Cloning of SoP5CS1 and SoP5CS2 genes

We used two different primer pairs (P5CS1-F: 5'-GAGCCACTTAGCGAGGAAG-3';P5CS1-R: 5'-GGTCCTTCTGCTTGTTACGA-3'; P5CS2-F: 5'-AGAGGGGAGACCGAA GACCAGGAG-3'; P5CS2-R: 5'-TACAGAATGAACCACCAGAATGAT-3') in order to clone the SoP5CS1 and SoP5CS2 genes from sugarcane. Primers were designed using Primer3 (www.frodo.wit.mit.edu). Primer P5CS1-F and P5CS1-R were designed based on EST database number T20007 : 9719 from KEGG (Kyoto Encyclopedia of Genes and Genomes) and nucleotide sequence of *SbP5CS1* gene (Genbank Accession Number : GQ377719.1). Primer P5CS2-F and P5CS2-R were designed based on *SoP5CS* (Genbank Accession Number : EF155655.1). The 50 uL PCR mixture contained 2 uL (250 ng) cDNA, 0.2 mM dNTP, 0.5 uM primers, 5 uL 10X KAPA2G GC Buffer (contained 1.5 mM $MgCl_2$), and 1 unit KAPA2G Robust Hotstart DNA Polymerase. We used touchdown PCR program that was set as followed: initial denaturation at 95°C for 5 minutes, 10 cycles of 95°C for 30 s, 60°C for 30 s, 72 °C for 3 minutes, 10 cycles of touchdown program (95°C in 30 s, annealing temperature between 60°C to 50°C in 30 s, 72 in 3 minutes), 10 cycles for 95°C for 30 s, 50°C for 30 s, 72°C for 3 minutes, folllowed by final extension of 72°C for 7 minutes. PCR were done using Veriti Thermal Cycler Applied Biosystem. The fragment of *SoP5CS1* gene was cloned to pGEM-T easy vector (Promega) while fragment of *SoP5CS2* gene was cloned to pJET 1.2/blunt vector (Fermentas) then transformed to *E.coli* DH5 competent cells. The positive recombinant plasmids were subjected for sequencing.

Bioinformatics

We used several bioinformatic analysis methods in order to analyze the sequences and predict the P5CS proteins. Homology analysis of *SoP5CS* nucleotide sequence was done using BLASTn. Multiple sequence alignment was made by ClustalW. Phylogenetic trees were made using clustalX and MEGA5 using Neighbor-Joining method. Amino acid sequences of *SoP5CS* protein were predicted using Bioedit. In order to predict *SoP5CS* proteins celular localization, we used TargetP and ProtComp from Softberry (www.softberry.com).

Real time quantitative PCR (RT-qPCR) analysis

RT-qPCR was used for expression analysis. The 20 uL RT-qPCR mixture contained 10 uL 2X Power Sybr Green (Applied Biosystem), 0.1 uM primers, and 2 uL cDNA template. We used different pairs of primer for each gene. For GAPDH gene, we used GAPDH-F (5'-TGCACCCATGTT CGTTGT-3') and GAPDH-R : 5'-CCATCAACAGTCTTCTGGGT-3'. *SoP5CS*-spesific primers were used as followed : (P5CSA-F: 5'-GAGCCACTTAGCGAGGAAG-3' ; P5CSA-R: 5'-TTCTGCCCAGTG ACAACAG-3') for *SoP5CS1* and (P5CSB-F: 5'-GGAGACCGAAGACCAGGA-3' and P5CSB-R: 5'-TCACAATGATCACCTCGTACC-3') for *SoP5CS2*. We used relative quantitative Real Time PCR

program that was set as followed : initial denaturation at 95°C in 10 minutes, 40 cycle of RT-qPCR (95°C in 15s and 57°C in 15s), followed by melt curve program with different melting temperature (ΔTm = 0.3°C) at every cycle from 60°C to 90°C. Real time qPCR were done using Applied Biosystem Step One Plus Real Time PCR.

In RT-qPCR, we did three technical replicates for each genes. The relative expression ratio of *SoP5CS1* and *SoP5CS2* gene were calculated with pfaffl method (Pfaffl, 2001). The Pfaffl method was used because of the efficiency of the GAPDH primers as gene reference, was different with the efficiency of P5CS1 and P5CS2 primers. The efficiency of GAPDH, P5CS1, and P5CS2 primers were 1.8, 2.11, and 2.0.

Proline determination

Free proline was extracted and calculated using Bates (1973) method: 0.5 gram fresh leaves was homogenized in 10 mL of 3% sulfosalicylic acid. The homogenate was sentrifuged to separate supernatant (filtrate) and solid molecul. 2 mL of filtate was reacted with 2 mL acid-ninhidrin and 2 mL glacial acetic acid. The mixtures were boiled in 100°C for 1 hour, kept on ice, and then were extracted using 4 mL toluene. Proline absorbance was measured using Life Science Spectrophotometer at λ 520 nm.

Proline concentration was calculated using equation (i) :

$$y = 0{,}0051x - 0{,}0201$$

y = OD_{520}; x = proline concentration (μM). The equation was based on proline standard curve with $R^2 = 0.9987$.

We continued to measure proline concentration using equation (ii) based on Bates (1973) :

$$\frac{[(ug\ proline/ml \times ml\ toluen)/115{,}5\ ug/umole]}{[(gr\ sample)/5]} = \frac{umole\ proline}{g\ of\ fresh\ weight\ material}$$

Results

Sequence Analysis and Phylogenetic Tree

Two sugarcane *P5CS* genes were isolated from 5' UTR to 3' UTR region by RT-PCR and registered in NCBI (Genebank Accession Number : KF178299 and Genebank Accession Number : KF178300). The *SoP5CS1* and *SoP5CS2* encoded 716 and 729 amino acid polypeptides which was predicted would be located in cytoplasm. Sequence analysis showed that *SoP5CS1* shared 75.6% identity in nucleotide sequences and shared 75.7% similarity protein sequences with *SoP5CS2*. BLASTn results showed that *SoP5CS1* shared 96% similarity with *SbP5CS1* (Genebank Accession Number : GQ377719.1; protein id : ACU65226) while *SoP5CS2* had 98% identity with *SoP5CS* (Genebank Accession Number : EF155655), 98% identity with *SaP5CS* (Genebank Accession Number : EU113257), and 93% identity with *SbP5CS2* (Genebank Accession Number : GQ377720).

The two *SoP5CS* protein sequences were aligned with AtP5CS, OsP5CS, and SbP5CS. Multiple sequence alignment result (Figure 1) showed that both of *SoP5CS* protein sequences had conserved regions such as ATP binding site, glutamate-5-kinase (G5K) domain, NAD(P)H binding site, putative leucine zipper domain, and glutamate semialdehyde dehydrogenase (GSA-DH) domain. The conserved Phe (F) residue also found in *SoP5CS1* (position 141) and *SoP5CS2* (position 128) protein sequence. The residue was functioned in proline feedback inhibition regulation (Hong et al., 2000).

Phylogenetic tree (Figure 2) was made based on the P5CS protein sequences in order to study the evolutionary relationship. It was constructed by ClustalX and MEGA5 using NJ method with 1000 bootstraps. It shows that there were two clusters separating monocots and dicots of P5CS protein. Two *SoP5CS* proteins are located within the monocot cluster. However, these proteins were in different sub-clusters which divided monocot P5CS1 and P5CS2. The *SoP5CS1* shared homology with SbP5CS1, ZmP5CS, and OsP5CS2 while SoP5CS2 is similar to SoP5CS, SaP5CS, and SbP5CS2.

Expression Analysis

Real time quantitative PCR (RT-qPCR) was performed in order to analyze the expression of *SoP5CS1* and *SoP5CS2* genes during drought stress. The expression of *SoP5CS* genes on drought treatment plants were compared to control plant. Both genes were induced by drought stress. The expression of *SoP5CS1* were increased significantly up to 11 folds at 12-hours drought treatment then continously increased to achive the highest ratio, 16 folds, at 24-hours treatment. However, *SoP5CS1* transcript gradually decreased to 13 folds and 10 folds at 48-hours and 72 hours drought treatment (Figure 3a.).

The expression of *SoP5CS2* did not significantly increase during drought stress. *SoP5CS2* transcript was induced to achieve the highest ratio, 3.3 folds, at 24-hours drought stress. The expression of *SoP5CS2* transcript was down-regulated to 1.4 folds at 48-hours drought stress. The gene expression was increased again up to 2.8 folds at 72-hours treatment (Figure 3b.). The expression of *SoP5CS1* was compared to *SoP5CS2* of treated plant during drought condition (Figure 4). Hence, the expression of *SoP5CS1* was significantly higher than *SoP5CS2*. The expression pattern of *SoP5CS* genes were similar to *SbP5CS* genes.

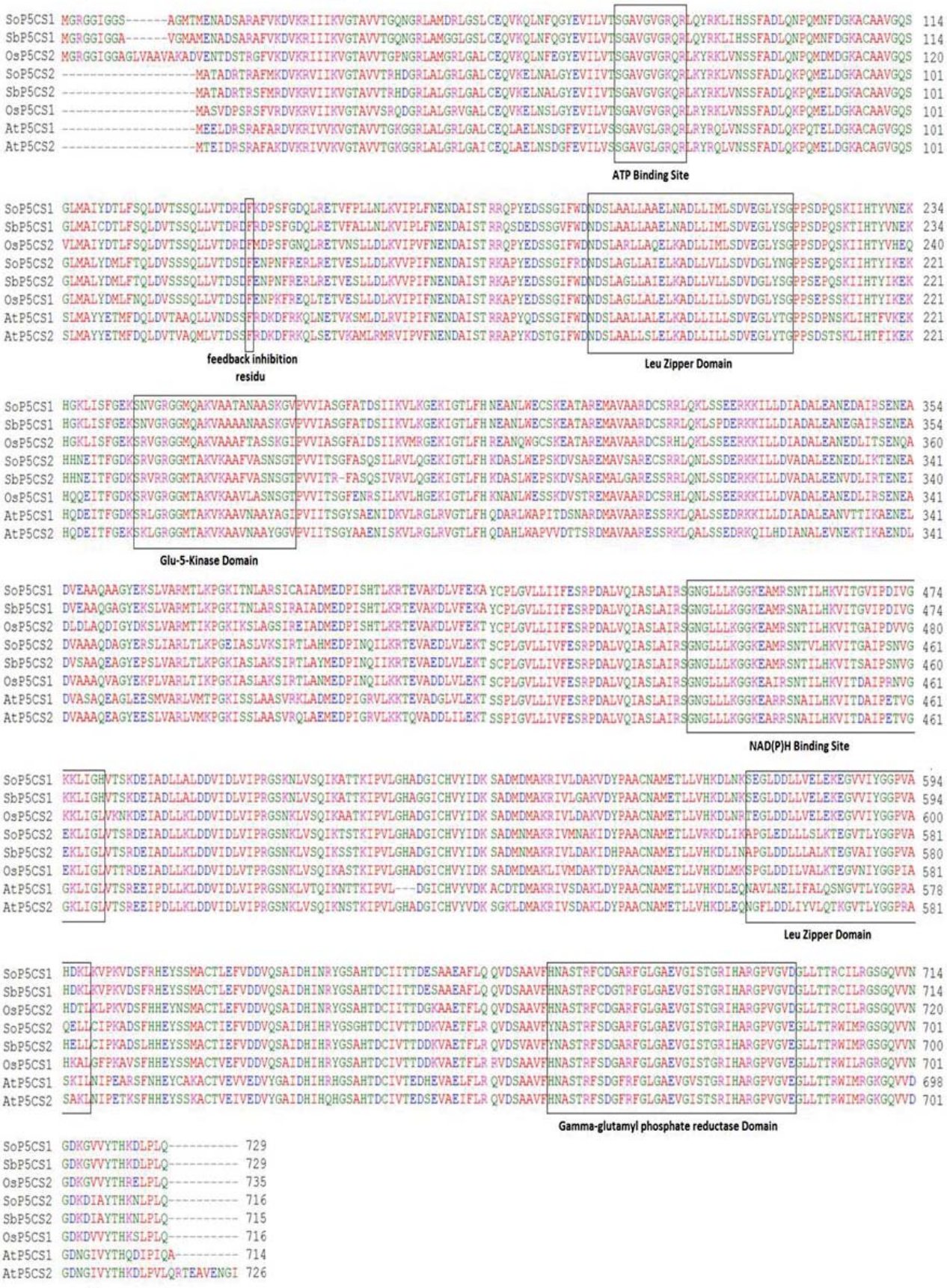


Figure 1. Multiple sequence alignment between two SoP5CS protein sequence with SoP5CS (protein id : ABM30223), SbP5CS1 (protein id : ACU65226), SbP5CS2 (protein id : ACU65227), OsP5CS1 (protein id : BAA19916), OsP5CS2 (protein id : BAG94966), AtP5CS1 (protein id : NP_001198715.1), AtP5CS2 (protein id : NP_191120.2). Boxes ([]) show predicted conserved region.

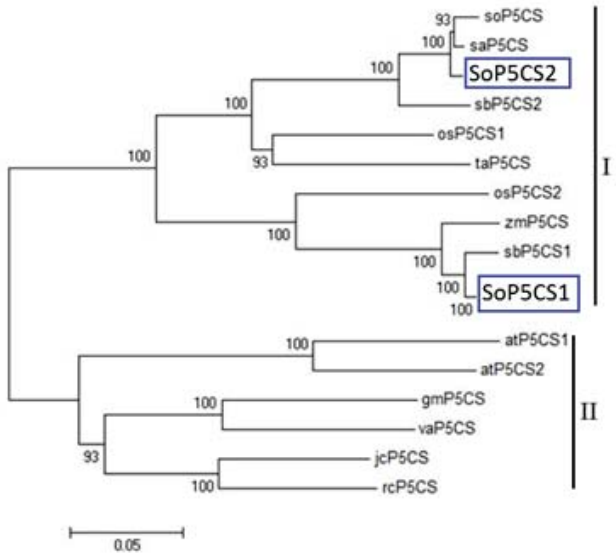
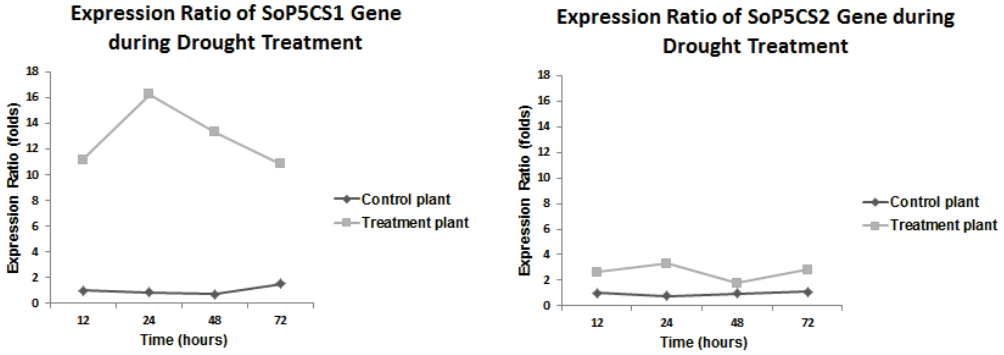


Figure 2. Phylogenetic relationship of SoP5CS1 and SoP5CS2 (boxes) with saP5CS (*S. arundinaceum* P5CS), soP5CS (*S. officinarum* P5CS), sbP5CS2 (*S. bicolor* P5CS1), osP5CS1 (*O. sativa* P5CS1), taP5CS (*T. aestivum* P5CS), OsP5CS2 (*O. sativa* P5CS2), zmP5CS (*Z. mays* P5CS), sbP5CS (*S. bicolor* P5CS1), vaP5CS (*V. aconitifolia* P5CS), gmP5CS (*G. max* P5CS), jcP5CS (*J. curcas* P5CS), rcP5CS (*R. communis* P5CS), atP5CS1 (*A. thaliana* P5CS1), and atP5CS2 (*A. thaliana* P5CS2). The phylogenetic tree had two cluster separating monocots (I) and dicots P5CS (II).



A B

Figure 3. The Expression of *SoP5CS1* (A) and *SoP5CS2* (B) in treatment and control plant

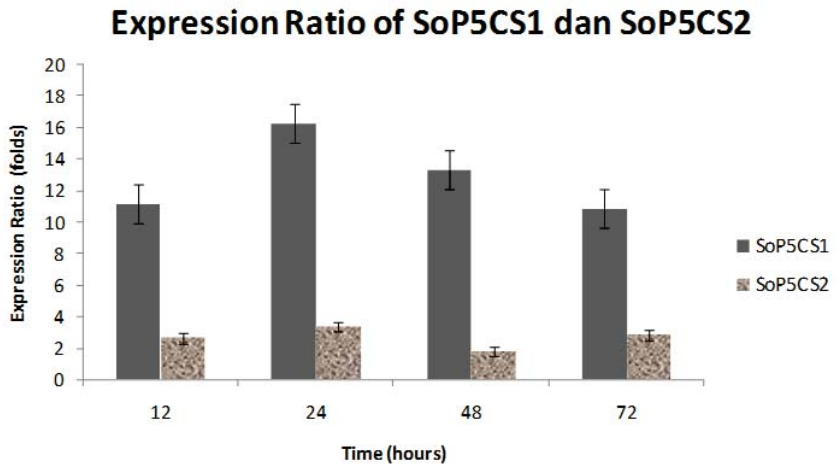


Figure 4. Comparison of two *SoP5CS* genes expression during drought treatment

Proline Concentration

The proline concentration was measured using the same samples for expression analysis in order to determine the corelation between proline accumulation with *SoP5CS* genes transcript level. The proline content of treatment plant was higher than control plant since 12-hours drought treatment (Figure 5). The proline concentration was significantly increased at 48-hours treatment then achieved the highest peak (9.8 umol.gram^{-1}) at 72-hours drought stress.

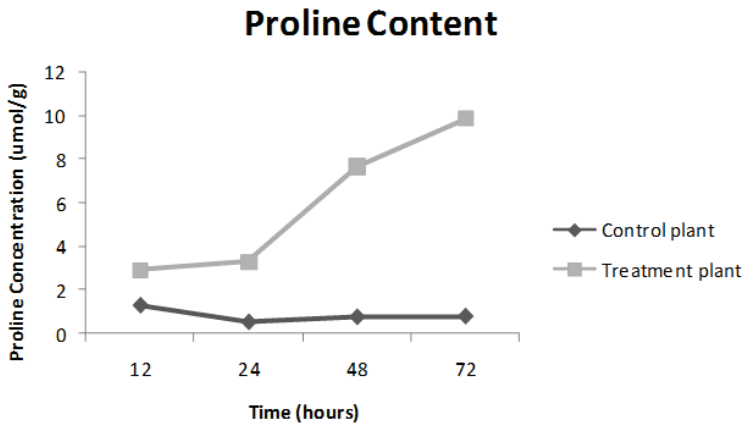


Figure 5. Comparison of proline content between treatment and control plant

Discussion

The P5CS enzyme from sugarcane was encoded by two homologous *P5CS* genes as observed in other plant species. Initial, we cloned the *SoP5CS2* genes that were related with *SoP5CS* and *SbP5CS2* genes on database in NCBI Genebank. We found another *P5CS* cDNA from sugarcane using KGENES Search by KEGG (Kyoto Encyclopedia of Genes and Genome). This data shared high homology with *SbP5CS1* gene from sweet sorghum. Both of the isolated *SoP5CS* genes had complete coding regions which encode two closely related SoP5CS proteins.

The SoP5CS proteins shared high similarity with SbP5CS2 as shown in multiple alignment result. The *SoP5CS1* similar to *SbP5CS1*, whilst *SoP5CS2* had high homology with *SbP5CS2*.These proteins had conserved motif ATP and NAD(P)H binding site, Glu-5-kinase and gamma-glutamyl phosphatase reductase domain, and leucine zipper region that were located at the same position (Su et al., 2011). The result indicated that the *SoP5CS* proteins would have the same expression profile with *SbP5CS* proteins.

In phylogenetic tree, the P5CS protein from many plant species were clustered into two groups dividing P5CS on monocots and dicots. The *SoP5CS1* and *SoP5CS2* protein were clustered in different sub-groups within monocots P5CS. This result indicated that sugarcane P5CS protein was encoded by P5CS gene family. Turchetto-Zolet (2009) studied the evolutionary relationship of P5CS protein. In their phylogenetic tree, monocots and dicots P5CS were clustered separately. Further study showed that there were two sub-clusters in monocots P5CS separating P5CS1 and P5CS2 protein. This event could result in one or more copies of a gene in genome which had new function or paralogous gene (Grassi et al., 2008).

There were some previous studies about *P5CS* gene from *A. thaliana*, *O. sativa*, and *S. bicolor* that reported about expression profiles of two homologous *P5CS* genes from the plant species under abiotic stresses such as drought, salt, and cold. Here, we focus on *P5CS* gene expression under drought stress. In *A. thaliana*, *AtP5CS1* had a role in abiotic stress response and prolin accumulation.

This gene expression increased during drought stress. *AtP5CS2* was especially expressed in dividing cell. The gene was ubiquitously expressed both in normal and stress condition. However the expression of *AtP5CS2* was significantly lower than *AtP5CS1* during abiotic stress such as drought. In *O. sativa*, the *OsP5CS1* was highly expressed in reproductive and vegetative organs especially during normal condition. In contrast, the *OsP5CS2* transcript was highly induced by drought stress.

The *SbP5CS* genes had similar expression pattern with *AtP5CS* and *OsP5CS* genes. The *SbP5CS1* gene was highly expressed in mature plant during abiotic stress including drought condition. The *SbP5CS2* transcript was highly detected in dividing cell. The *SbP5CS* was also ubiquitously expressed both in normal and drought condition however the expression of *SbP5CS1* was extremely higher than *SbP5CS2*. Su *et al*., (2011) suggested that *SbP5CS1* had an important role in proline

accumulation during abiotic stress while *SbP5CS2* played a role in regular proline biosynthesis during normal or unstressed condition. Transcriptional regulation is a major key in gene expression. Study about *SbP5CS* promoters showed that both of *SbP5CS* genes had cis-elements such as MYCCONSENSUSAT, WRKY, and MYBCORE. These elements functioned as binding site for transcription factors which involved in cold, wound, and drought stress (Su et al., 2011). Two closely related *S. bicolor* P5CS genes had different function in plant development, growth, and stress response that might be caused by the different regulation of *cis*-element in SbP5CS promoter (Alberts et al., 2008).

Current study reported one type *P5CS* gene from sugarcane (Iskandar et al.,2011; Patade et al., 2012). Iskandar et al. (2011) reported the expression of *P5CS* in young and mature culm internodes of sugarcane after 15-days water deficit treatment. The expression of *P5CS* was higher than control, and generally not significantly different between the young and mature culm internodes.

Expression analysis showed that both of *SoP5CS1* and *SoP5CS2* were induced by drought stress. The *SoP5CS1* transcript was significantly increased, when compared to control, during PEG incubation. However, the expression of *SoP5CS2* was not significantly increased during drought condition. Our study showed that *SoP5CS1* transcript and proline accumulation had different pattern. The expression of *SoP5CS1* had been significantly increased at 12-hours drought treatment then achieved the highest peak at 24-hours drought treatment. *SoP5CS1* transcript level was gradually decreased after 24-hours treatment. In contrast, proline was significantly accumulated at 48-hours drought treatment.The highest proline concentration was achieved at 72-hours drought treatment. The expression and activity of P5CS protein might be inhibited by feed-back inhibition regulation of high proline concentration. The proline was continously produced after 48-hours, when *SoP5CS1* transcript repressed, might be caused by proline biosynthesis by ornithine pathway. Study in *A. thaliana* showed that ornithine pathways played a role in proline biosynthesis during drought and salt stress (Roosens et al., 1998).

Based on the similarity of SoP5CS with SbP5CS genes and expression analysis data, we suggest that the *SoP5CS1* gene contributes more significantly to the production of proline during drought stress than *SoP5CS2*. The *SoP5CS1* could potentially be used as a marker to screen sugarcane variety for drought tolerance and may also potentially be useful for the development of transgenic plants tolerant to drought. Further study about SoP5CS promoters is needed in order to determine the *SoP5CS1* and *SoP5CS2* gene regulation.

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