

Identification of *P5CS* gene on sugarcane by PCR using heterologous primer

Identifikasi gen *P5CS* tebu dengan PCR menggunakan primer heterologus

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Ringkasan

Usaha peningkatan produktivitas gula dengan pemanfaatan lahan kering, memerlukan tersedianya bibit tanaman tebu (*Saccharum officinarum* L.) unggul yang toleran terhadap cekaman kekeringan. Perakitan tanaman tersebut melalui bioteknologi modern memerlukan ketersediaan gen pembawa sifat ketahanan kekeringan yang sesuai dan informasi molekulernya. Penelitian ini bertujuan untuk mengidentifikasi adanya gen *P5CS* (pyrroline-5-carboxylate synthetase) pada tanaman tebu dan karakteristik molekulernya secara terbatas dalam perspektif usaha perakitan tanaman tebu tahan kekeringan. Identifikasi gen pembawa sifat ketahanan tersebut pada tanaman tebu dilakukan dengan teknik PCR menggunakan primer spesifik. Tingkat homologinya terhadap gen dari spesies lainnya, secara kualitatif diprediksi dengan teknik hibridisasi menggunakan pelacak heterologus yang berasal dari gen *P5CS* tanaman *Vigna aconitifolia*. Kandungan prolin tanaman yang mendapat perlakuan cekaman kekeringan secara *in vitro*, ditetapkan untuk melihat seberapa tinggi akumulasi prolin dapat terjadi oleh induksi ekspresi gen endogenous. Adanya fragmen DNA hasil amplifikasi dengan primer spesifik *P5CS*, membuktikan bahwa pada tanaman tebu terdapat gen *P5CS* yang pada daerah terkonservasi memiliki tingkat homologi yang tinggi. Diperolehnya ukuran yang sama dengan control plasmid pBI-*P5CS* membuktikan bahwa sebagaimana spesies tanaman lainnya, posisi daerah tersebut pada tebu adalah tertentu dan sama. Tidak terjadinya amplifikasi dengan primer dari daerah di luar daerah terkonservasi, menunjukkan bahwa gen *P5CS* tebu tidak sepenuhnya homologous dengan yang berasal dari tanaman *V. aconitifolia*. Terjadinya peningkatan akumulasi prolin pada perlakuan cekaman kekeringan mengindikasikan bahwa gen *P5CS* tersebut juga dapat diinduksi (inducible) oleh stres air.

Summary

An attempt toward increasing the sugar production by using marginal land requires sugarcane (*Saccharum officinarum*) cultivars which tolerant to drought or osmotic stress. Development of such plant through modern biotechnology needs a gene carrying drought tolerance and the pertinent molecular information. When the gene is indigenous property, the planting materials developed will be more valuable. This report describes research results from identification the presence of *P5CS* (pyrroline-5-carboxylate synthetase) gene in sugarcane and limited molecular characteristics related to an attempt to develop sugarcane tolerant to water stress. Identification of the gene in sugarcane conducted by PCR using specific primers. The homology to other species was qualitatively predicted with hybridization using heterologous probe of *Vigna aconitifolia*. Proline contents were examined on the stress-treated and untreated plants as well, to estimate proline accumulated by the induction. The amplified DNA fragment demonstrated that the sugarcane possesses *P5CS* with at least two highly conserved regions as the others reported. The similarity in sizes of amplified DNA fragment suggested that the position of the conserved regions in the tested and reported species is very similar. The lack of amplification using primers of non-conserved regions indicates that the *P5CS* gene of sugarcane was not fully homologous to the gene of the others. Higher content of proline in the stress-treated plants implies that the *P5CS* gene was inducible.

[Key words : pyrroline-5-carboxylate synthetase gene, *Saccharum officinarum*, PCR, heterologous primer]

Introduction

An attempt to improve the sugar production by extension using marginal land requires sugarcane cultivars which tolerant to drought or osmotic stress. Genetic engineering of drought tolerant plants through modern biotechnology needs a suitable gene which carries drought tolerant traits together with its molecular information. If the gene is our own property then the value added to the product will be increased.

Resistance to water deficit in plants can occur through proline accumulation. There are two known pathways of proline biosynthesis. In higher plants, the mechanisms of proline accumulation during osmotic stress occur due to *de novo* biosynthesis through glutamate pathway (Delauney & Verma, 1993). The synthesis of proline is initiated from glutamate which is dehydrogenated to glutamyl semialdehyde and spontaneously converted to δ -pyrroline-5-carboxylic acid. Both steps are catalyzed by a bifunctional enzyme pyrroline-5-carboxylate synthetase (P5CS) which also behaves as a limiting step in proline biosynthesis. Overexpression of *P5CS* gene in transgenic tobacco resulted in proline accumulation and osmotic tolerance (Kishor *et al.*, 1995). Alternative pathway of proline biosynthesis on plants is via ornithin hydrolysis which is catalyzed by ornithine aminotransferase enzyme.

There are several aspects to be technically, economically and socially considered in order to choose the best strategy and procedure of plant genetic engineering for better traits. The use of transgene functionally owned of target plants is more beneficial since it has high adaptability. Therefore, the substantial equivalence to be examined for food and biosafety requirement should be more easily fulfilled (Roosens *et al.*, 1998). On the other

hand, the use of such transgene, particularly those with high homology to the endogenous gene of the target plant may not be effectively expressed.

This research aimed to identify the presence of *P5CS* gene on sugarcane together with some of its molecular characterization, especially to study the homology level with the transgene in order to be used as a source gene for genetic engineering of drought tolerant sugarcane.

Materials and Methods

Sugarcane (*Saccharum officinarum*) plantlets cultivars POJ 3016 and PS 851 were used as plant materials. The construct of pBI-P5CS plasmid was kindly provided by Dr. D.P.S. Verma from Ohio State University, USA. Murashige & Skoog (MS) medium which was modified for sugarcane culture provided by Indonesian Sugar Research Institute (P3GI) Pasuruan, East Java. PCR reagent was from Promega (WI, USA). Reagents for Southern blot hybridization and radioisotope ^{32}P -ATP were from Amersham Pharmacia Biotech (NJ, USA). Oligonucleo-tides primer and other chemicals were supplied by Sigma Chem Co. and local companies.

In Vitro Culture of Sugarcane

Plantlets of cultivar PS 851 was used in the experiment of proline assay since these plant materials have good prospect to be developed commercially but have no resistance to drought condition. Embryogenic calli were provided by the process of young leaf roll tissue differentiation. Calli were initiated from leaf meristematic explants which were cultured on basal MS media (Murashige & Skoog, 1962) supplemented with 10%

coconut water, 30 g/L sucrose with 3.0 ppm 2,4-D as a hormone. Subculture was carried out every 2-3 weeks to the same MS media. The calli were then subcultured to MS II solid media with the addition of IAA and dalapon for differentiation. For rooting, plantlets were transferred to MS II liquid media. Drought stress treatment was done by incubating plantlets in 200 mM NaCl at 1 day, 3 days and 7 days.

Genomic DNA Extraction

Genomic DNA was prepared from sugarcane leaves cultivar POJ 3016 using CTAB extraction method. About 3 g leaf tissue was frozen in liquid nitrogen and ground to a fine powder. The powder was then homogenized with 6 mL extraction buffer (150 mM Tris-HCl pH 7.5; NaCl 1M NaCl; 15 mM EDTA pH 8.0; 0.4% Na₂SO₄; and 3% CTAB) followed by incubation at 65°C for 60-90 minutes with agitation. The homogenate was added with chloroform: isoamyl-alcohol (24:1) and gently shaken for 5 – 10 minutes. After centrifugation at 10,000 g for 10 minutes, the upper layer was taken and re-extracted with 4.5 mL chloroform:isoamyl-alcohol. The supernatant obtained was mixed gently with 6 mL isopropanol. Precipitated DNA was hooked up by a Pasteur pipette, rinsed and dissolved in 100 µL TE buffer. DNA extraction of tobacco leaves was also done using the CTAB extraction method but in a small scale total volume.

Homology analysis of sugarcane P5CS gene

The homology of P5CS sequences in sugarcane has been analyzed by PCR using two pairs of primers based on non-conserved and conserved regions of P5CS sequences. Moreover, PCR results were also confirmed by Southern hybridization using DNA probes synthesized from PCR product.

PCR Amplification

The method to amplify DNA fragment with PCR was principally described on Roosens *et al.* (1998). The thermal program was set up as follows denaturation : 94°C, 30 second; annealing: 58°C, 30 second; extension: 72°C, 3 minutes. The reaction mixture consisted of with 50 ng each primer, 100 ng DNA template, 5 mM each dNTP, 1 unit of DNA *Taq* polymerase and H₂O until the total volume 25 µL. The PCR reaction was set up on 35 cycles in the Perkin Elmer Thermal Cycler (PE 2400).

Southern Blot Hybridization

A DNA probe labeled with ³²P was synthesized based on standard procedure for random primed labeling (Sambrook *et al.*, 1989). As a template, PCR product of plasmid DNA was purified with *MicroSpin HR 300 Column* kit (Amersham Pharmacia Biotech). About 20 ng DNA template was used to label with *Rediprime random labelling kit* (Amersham Pharmacia). The DNA probe was then used in Southern blot hybridization as described in Sambrook *et al.* (1989). The DNA samples which were blotted onto the membrane were the PCR products of sugarcane and tobacco genomic DNA amplified using P5CS conserved region primer pair.

Proline Analysis

Proline content was determined according to the method of Bates (1973). About 0.5 g leaf sample was extracted with sulfosalicylic acid 3%. Coloring reaction was proceed using Ninhidrin solution and glacial acetic acid and boiled for 1 hour. After addition of toluene, the chromophore was measured with a spectrophotometer at λ 520 nm.

Results and Discussion

The identification of *P5CS* gene in sugarcane was conducted directly based on the homology of the gene of several species using specific *P5CS* primer and the conserved region of the *P5CS*. Searching for molecular information through database using BLAST (Basic Local Alignment Search Tool) program via internet was done to investigate sequence homology of *P5CS* transgene between *V. aconitifolia* and other plant species. The result of BLAST search is

presented on Figure 1, panel A. Area with black bold lines indicate high sequences homology among several species in the database, whereas the stripe lines indicate the gap within the sequences. The region flanked between 500 bp and 1750 bp was considered as conserved region for *P5CS* coding sequences. Based on this result, two pairs of primers were designed to synthesize hybridization probe and PCR. The primers sequences are presented on Figure 1, panel B. The *P5CS* start- *P5CS* stop primers covered the full-length coding sequence of

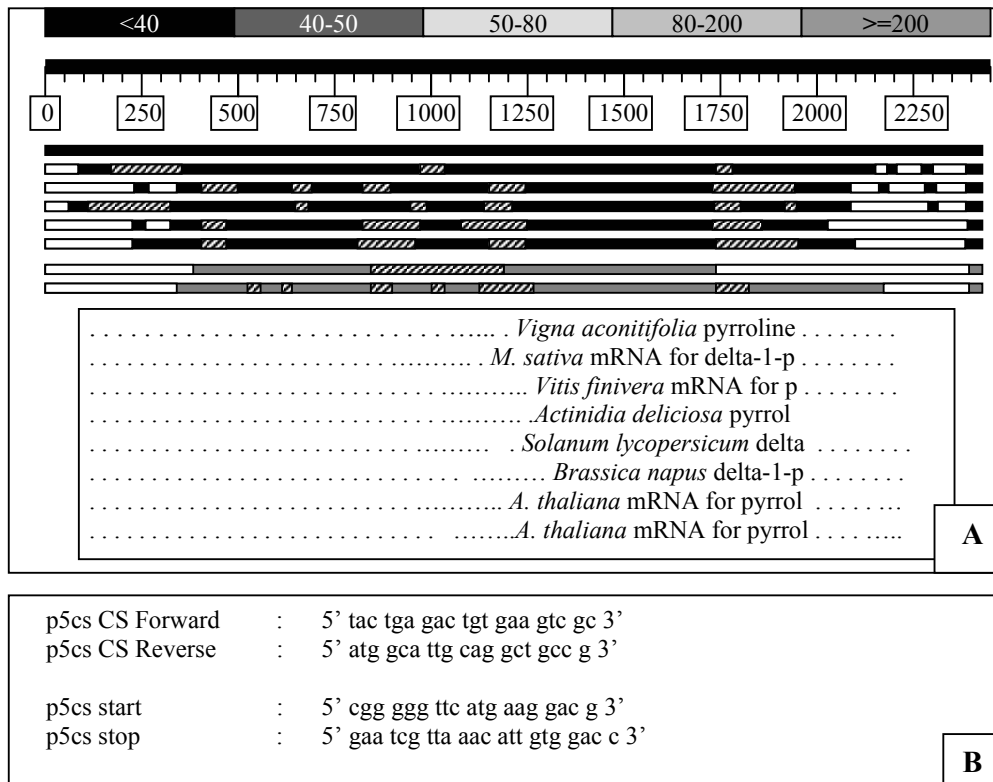


Figure 1. BLAST search of pyrroline-5-carboxylate synthetase. Panel A is a homology diagram of *P5CS V. aconitifolia* within other species. Panel B, DNA primers designed based on conserved region and full length cDNA sequences.

Gambar 1. Pyrroline-5-carboxylate sintetis dengan menggunakan BLAST. Panel A, adalah diagram homologi *P5CS V. aconitifolia* dengan spesies lainnya. Panel B, primer DNA dirancang berdasarkan daerah terkonservasi dan sekuen penuh cDNA.

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P5CS gene (1-2250 bp), and the P5CS CS Forward-P5CS CS Reverse primers will amplify area within the conserved region of *P5CS* gene (500-1750 bp).

Both primers had been utilized to amplify specific DNA fragment by PCR technique. DNA templates used were from several sources such as genomic DNA of sugarcane, tobacco and pBI-P5CS plasmid. Gel electrophoresis showed that the sources of template DNA were in good condition quantitatively and qualitatively. The presence of a single and strong big DNA band in corresponding lanes indicates that the integrity of genomic DNA of sugarcane was good (data not shown). With such good quality of DNA template, PCR reaction was then carried out. To optimize the PCR condition, the first parameter tested was the annealing temperature. The reaction was conducted using Biometra Gradient Thermocycler with temperature varied from 55 – 65°C. Gel electrophoresis of amplified fragments showed that 60°C was the best annealing temperature. At this condition, there is only one single band appeared with strong intensity (4th lane of Figure 2). At lower temperature, more DNA fragments

appeared which implied that the primer had annealed to nonspecific regions. However, at higher temperature, DNA fragment was relatively veint.

Using the optimum condition, PCR was then carried out with the three kind DNA templates. The amplification products were then fractionated with agarose gel electrophoresis. The separation of DNA fragment is depicted on Figure 3. Using primers of the *P5CS* cDNA (full length), PCR reaction only gave amplification on pBI-P5CS plasmid with the size of 2.4 kb. Amplification using primers CRF-CRR showed DNA fragment amplified from the three DNA templates, sugarcane, tobacco and pBI-P5CS plasmids. The size of the fragment from each templates was 1.2 kb appeared on those three samples except on sugarcane DNA preparation two which indicated that the quantity of genomic DNA on the second preparation was very low. The size of this DNA fragments is in accordance with what was predicted from BLAST search (Figure 1, panel A). This data also indicated that the conserved region of *P5CS* gene of sugarcane had a high

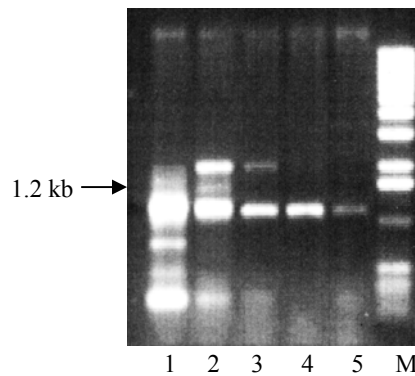


Figure 2. Electrophoresis profile of DNA fragments amplified from sugarcane genomic DNA at temperature gradient PCR. Lanes 1-5 are amplification products with annealing temperatures of 55, 57, 58, 60, 65°C. M is 1-kb ladder molecular weight marker

Gambar 2. Profil elektroforesis fragmen DNA dari genomik tebu dengan gradiasi suhu PCR. Lajur 1-5 adalah hasil amplifikasi dengan suhu annealing 55, 57, 58, 60, 65°C. M adalah bobot molekul 1-kb marker

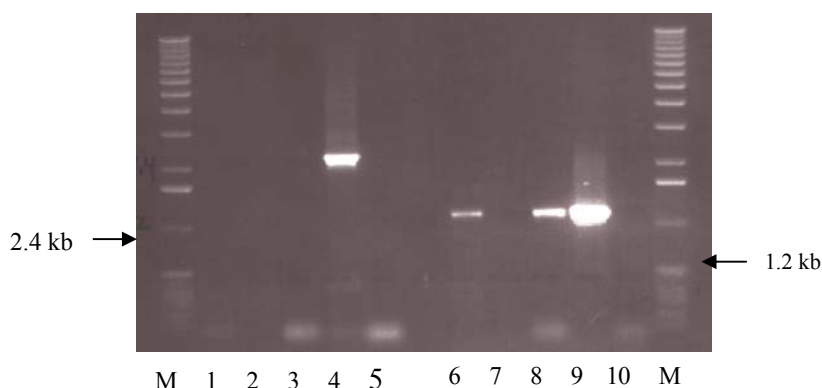


Figure 3. PCR results using primers of start-stop (lanes 1-5) and CRF-CRR (lanes 6-10). Lanes 1 & 6 using sugarcane genomic DNA as template preparation 1; lanes 2 & 7 sugarcane DNA preparation 2; lane 3 & 8 tobacco DNA, lane 4 & 9 pBI-P5CS; and lanes 5 & 10 without DNA template. M is 1-kb ladder.

Gambar 3. Hasil PCR dengan menggunakan start-stop (lajur 1-5) dan CRF-CRR (lajur 6-10). Lajur 1 & 6 menggunakan genomik DNA tebu sebagai 1 preparat tempat; lajur 2 & 7 menggunakan DNA tebu sebagai preparat 2; lajur 3 & 8 DNA tembakau; lajur 4 & 9 pBI-P5CS, dan lajur 5 & 10 tanpa DNA. M adalah marka 1-kb ladder

homology with other species especially with *V. aconitifolia*. However, on the outside region which is on the 5' end and 3' end, the homology is very low.

Positif results of PCR indicate the presence of sequences considerably homologue to the DNA primers. To further analysis for possible homology of the other P5CS regions, Southern blot was conducted using probe derived from the PCR product using CFR – CRR primers of P5CS *V. aconitifolia*. The probe hybridised with the PCR product of sugarcane, tobacco and pBI-P5CS as a positive control. The radiogram on Figure 4 showed that those DNA fragments have quite highly homology as it hybridized with P5CS fragment from *V. aconitifolia*.

Regeneration of drought-tolerant sugarcane through integration of P5CS transgene into its chromosomes leads to the investigation of homology level between the endogenous gene with the transgene. This is to anticipate the occurrence of homology based gene silencing. Among the several mechanisms of gene silencing, homology-dependent silencing (HDS) is a mechanism possibly occurred on transgenic plants.

Based on the homology study of P5CS sequences, there is a chance of HDS to occur especially when transgene was inserted in the region in which antisense transcription occurred. The RNA transcribed causes a gene silencing. This phenomenon was reported by Thierry & Vaucheret on nitrite reductase, which was transcribed at locus 271 on tobacco (Fagard & Vaucheret, 2000).

Gene silencing mechanisms caused by DNA-RNA or RNA-RNA required such high homology up to 80% or more (DeKock, personal communication). Silencing of P5CS transgene on sugarcane will not be a problem since it did not show high homology level based on PCR result using start-stop primers. Moreover, the report of gene transformation of P5CS *V. aconitifolia* on tobacco indicated a positive expression result (Kishor *et al.*, 1995). In this report, showed that homology characteristic between P5CS gene of tobacco was similar to that at sugarcane

The use of transgene with indigenously known function on plant genetic engineering has some additional advantages. The protein is naturally expressed from both the wild plant and transgenic plants. Therefore,

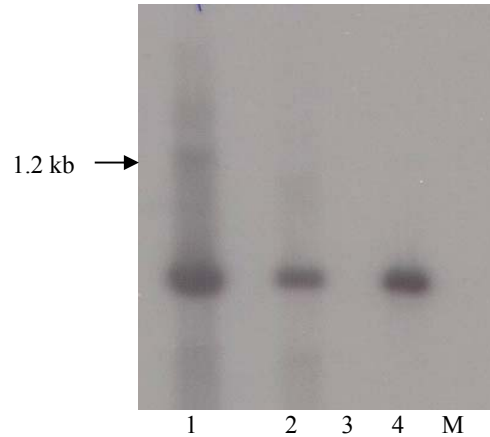


Figure 4. Hybridization using P5CS probe CRF-CRR labeled with ^{32}P . Lanes 1-4 are PCR results of DNA pBI-P5CS, tobacco, sugarcane prep 1, sugarcane prep 2. M is 1-kb ladder.

Gambar 4. Hibridasi menggunakan pelacak P5CS pelabelan CRF-CRR dengan ^{32}P . Lajur 1-4 adalah hasil PCR dari DNA pBI-P5CS, tembakau, prep 1 tebu, prep 2 tebu. M adalah marka 1-kb ladder

evaluation on substantial equivalent for food and biosafety would not be very strict as if the transgene have a completely new function to the plant. Food safety assesment includes allergenic and toxicity properties of the protein expressed from the transgene.

To collect primary information which relevant to the attempt of development for drought tolerant sugarcane using *P5CS* gene, prolin analysis has been done on stress treated and untreated sugarcane plantlets. Data on Table 1 showed that the addition of NaCl could induced prolin accumulation on sugarcane plantlets cv. PS 851, but only up to 10 times. Higher content of proline in the stress-treated plants implies that the *P5CS* gene was inducible. However, the induction was not high enough to evolve the tolerance to water stress. This cultivar has high productivity but sensitive to drought stress. On the other hand, some sugarcane cultivars with high tolerance to drought stress can accumulate higher level of proline on stress condition (Widyasari & Sugiyarta, 1997),

but it has lower productivity. Hence, an attempt to expressed *P5CS* gene on high yield sugarcane to obtain the resistance to drought stress is considerably rational.

The identification of *P5CS* gene on sugarcane by PCR using specific primers which designed based on the conserved sequence of heterologous *P5CS* gene has opened the possibility to clone the gene using more simple screening method. Screening for a gene from DNA libraries is usually carried out by hibridization method using radioisotope labelled probe which is quite laborious. Using PCR method, the screening procedure becomes more simple. By this, an attemp to isolate *P5CS* gene from sugarcane or tobacco should also be easier. Moreover, genomic (Santoso & Budiani, 2001) or cDNA library at particular scale can be constructed simply using plasmid vector. Therefore, plant genetic engineering using input gene from endogenous source is also promising.

Table 1. Proline contents (mM) on sugarcane and tobacco plantlets induced by 200mM NaCl

Tabel 1. Kandungan prolin (mM) dalam planlet tebu dan tembakau yang diinduksi dengan 200mM NaCl

Incubation <i>Inkubasi</i> (day) (hari)	Plantlet <i>Planlet</i>	Untreated <i>Tanpa</i> <i>perlakuan</i>	Treated <i>Dengan</i> <i>perlakuan</i>
1	Sugarcane (<i>Tebu</i>)	0.0251	0.0
	Tobacco (<i>Tembakau</i>)	0.350	1.3651
3	Sugarcane (<i>Tebu</i>)	0.1478	0.330
	Tobacco (<i>Tembakau</i>)	0.2695	1.8502
7	Sugarcane (<i>Tebu</i>)	0.1970	1.162
	Tobacco (<i>Tembakau</i>)	0.1921	1.4133

Conclusions

Identification of *P5CS* gene on sugarcane could be carried out by PCR using specific primers. Sugarcane has an endogenous *P5CS* gene which its conserved region shows high homology to the known *P5CS* gene of several plant species. The identification of sugarcane *P5CS* gene by PCR using heterologous primer has opened the possibility to clone the gene using more simple screening method.

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