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Characterization of pyrophosphate-dependent phosphofructokinase α-subunit gene from sugarcane showing gene without intron

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Abstract. Pyrophosphate-dependent Phosphofructokinase or PFP is an enzyme that regulate sucrose metabolism. It consists of α- and β-subunits, which encoded by *PFPα* and *PFPβ* genes, respectively. Sugarcane PFPα has a strong function in glycolysis and has the potency to be engineered to increase sugarcane yield. Hence, the purpose of this work was to isolate, clone and characterize the sugarcane *PFPα* gene. Total RNA was isolated from leafrolls of TD 91 sugarcane variety. cDNA synthesis followed by DNA amplification of *PFPα* gene were performed using degenerate primers. cDNA and DNA fragments were ligated into pGEM-T Easy vector, which were subsequently introduced to *E. coli* competent cells. *Eco*RI were used to cut the plasmids for sequencing. Finally, homology searching was conducted using BLASTn, and then the nucleotide sequence was translated to a protein sequence using Bioedit. The results showed that *PFPα* cDNA fragment was 900 bp in length. The translated PFPα protein showed binding sites for fructose-6-phosphate and fructose-1,6-biphosphate, which are conserved in all family members of PFP. *In silico* analysis of the DNA fragment showed gene without intron. In conclusion, the *PFPα* gene from sugarcane has been successfully isolated, cloned and characterized.
Keywords: Sugarcane, PFPa gene, sugar metabolic engineering

1. Introduction

Pyrophosphate-dependent phosphofructokinase (PFP) is an enzyme that regulate sucrose metabolism by catalyzing the reversible conversion of fructose-6-phosphate to fructose-1,6-biphosphate using pyrophosphate as the phosphoryl donor [1-3] and fructose-2,6-biphosphate to activate the enzyme. A rise in fructose-2,6-biphosphate stimulates glycolysis in plants by the activation of PFP [4-6]. There are two kinds of plant PFP: α- and β-subunits [7, 8], which encoded by different genes: *PFPα* and *PFPβ*, respectively [9]. The α-subunit has a role as the regulatory component, while the β-subunit performs catalytic activity [7, 10]. The *PFPα* gene of castor (*Riccinus. communis*) encoding PFP α-subunit, is approximately 5.8 kb in length, whereas the *PFPβ* gene is approximately 4.6 kb long [9].


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PFP occur in two forms, β_2 and $\alpha_2\beta_2$ [7, 11], but $\alpha_2\beta_2$ is the most active form [10], and it catalyzes the glycolytic reaction, whereas β_2 catalyzes the gluconeogenic reaction [11]. The association of the α-subunit with the β-subunit in the $\alpha_2\beta_2$ form could increase the activity of the β-subunit, in the absence of fructose-2,6-biphosphate [10]. Because of its function in regulating glycolytic activity of the enzyme, sugarcane PFPα regulates the mass diversion of sugar phosphates in sucrose metabolism that affecting sugar production [3, 12]. Hence, the purpose of this work was to isolate, clone and characterize the *PFPα* gene from sugarcane. The PFPα molecular biology will be useful to increase our understanding of sugar metabolism in sugarcane.

2. Materials and methods

2.1. Materials

Leafroll of TD 91 variety of sugarcane (*Saccharum. officinarum* L.) was obtained from the Indonesian Sugar Plantation Research Center (P3GI) in Pasuruan, East Java. Forward and reverse primers, and pGEM-T Easy vector were ordered from Sigma-Aldrich and Promega, respectively.

2.2. RNA isolation

All glassware and plasticware for RNA extraction were cleaned with 0,1% diethyl-pyrocarbonate (DEPC) water, and subsequently autoclaved before use. Approximately 0.2 gram of sugarcane leafroll was grinded in a mortar and pestle with liquid nitrogen. The RNA extraction was carried out using Trizol® (Invitrogen) extraction kit according to the company's protocol.

2.3. Genomic DNA isolation

Approximately 0.2 gram of sugarcane leafroll was grinded in a mortar and pestle with liquid nitrogen. The fine paste of plant tissue was homogenized in 250 µl of homogenization buffer (200 mM Tris-HCl, 500 mM EDTA, 2.2 M NaCl, 2% CTAB, 0.06% sodium sulfite) pH 8. Five percent N-lauroyl sarcosine, 10% polyvinylpyrrolidone (PVP) and 20% Cetyl Trimethylammonium Bromide (CTAB), each of it 125 µl, was added to the sample and incubated at 65°C for 1 hour. The suspension was cooled down in room temperature and added with 650 µl phenol: chloroform: isoamyl alcohol solution (25:24:1), then inverted and centrifuged at 3000 g for 10 min to mix the solution. The upper phase liquid was taken and put into a new tube and added with the same volume of isopropanol and 125 µl of 6 M NaCl, then incubated at -20°C for 1 hour. The suspension was centrifuged at 3000 g for 15 min. Supernatant was disposed and the pellet was dried at room temperature and resuspended in 125 µl of TE (10 mM Tris-HCl and 1 mM EDTA) pH 8, then stored at -20°C [13].

2.4. Primer design

Since *PFPα* from sugarcane is not available in the GenBank, primers were designed based on alignment of *PFPα* mRNA sequences of *Zea mays* (AY103649), *Oryza sativa* (AK121116), *Arabidopsis. thaliana* (NM106305), and *Solanum tuberosum* (M55190). The alignment was carried out using Clustalx. Based on the alignment data, two region of conserved sequences were choosen to design forward and reverse primers as represented in figure 1 and figure 2, respectively.

Forward primer was designed from 851-872 of the alignment consensus sequence and reverse primer was designed from 1729-1750 of the sequence. The gap within the italic sequences of both forward and reverse primers were inserted with any nucleotides. The sequence of forward and reverse primer were $^{5'}$TCTGCACTGATGCTCTTTCTGC$^{3'}$ and $^{5'}$CACCTGGTCCATCAAACTGGAG$^{3'}$, respectively. Both primers were consisted of 22 nucleotides and the GC contain of forward and reverse primer were 50% and 54.5 %, and the 3' end, were cytosine (C) and guanine (G), respectively. Moreover, the nucleotide sequences of both primers did not form dimeric primer.

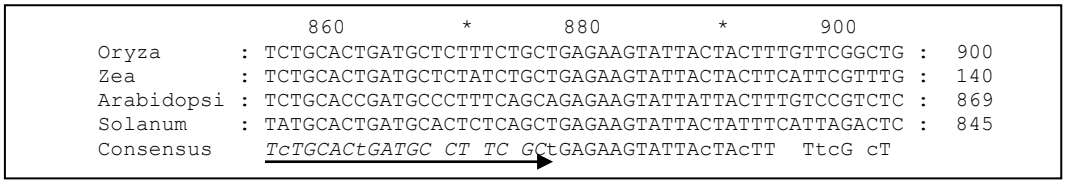

Figure 1. PFPα mRNA sequence alignment of *Z. mays*, *O. sativa*, *A. thaliana*, and *S. tuberosum* for forward primer design.

Figure 2. PFPα mRNA sequence alignment of *Z. mays*, *O. sativa*, *A. thaliana*, and *S. tuberosum* for reverse primer design

2.5. cDNA synthesis with RT-PCR method

Reverse Transcript-Polymerase Chain Reaction (RT-PCR) was carried out by using one step RT-PCR kit (Invitrogen) and a pair of forward and reverse primers. The thermocycling conditions were 1 cycle of reverse transcription at 50°C for 30 min, 1 cycle of denaturation at 94°C for 2 min, 35 cycles of amplification (denaturation at 94°C for 15 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min) and 1 cycle of final extension at 72°C for 7 min.

2.6. DNA amplification with PCR method

Polymerase Chain Reaction was performed using PCR kit (fermentas) and using the same primers as RT-PCR. The thermocycling conditions were 1 cycle of denaturation at 94°C for 5 min, 25 cycles of amplification (denaturation at 94°C for 20 sec, annealing at 55°C for 40 sec, and extension at 72°C for 3.5 min) and 1 cycle of final extension at 72°C for 10 min.

2.7. Ligation of cDNA and DNA to pGEM-T easy vector

The ligation reaction was composed of 40 ng of cDNA (or 50 ng of DNA), 50 ng of pGEM-T Easy vector (Promega), 5 µl of 2x ligation buffer, and 1 µl T4 DNA ligase (5 u/µl). The ligation was conducted at 4°C for 16 hours.

2.8. Introduction of plasmid DNA into E. coli competent cells

The ligation reaction (5 µl) was mixed with *E. coli* DH5α competent cells by using heat shock method. The bacterial suspension was added with 900 ml SOC medium and incubated at 37°C for 3 hours in shaker incubator at 150 rpm. The bacterial suspension (50 ml) was spread on LB medium plate, containing 20 µl of 100 mg/ml ampicillin, 10 µl of 200 mg/ml IPTG and 50 µl of 50 mg/ml Xgal. The plate was incubated at 37°C for 16 hours.

2.9. Plasmid DNA isolation

Based on blue-white screening, white colonies were picked up, and cultured in 5 µl of LB medium containing 100 mg/ml ampicillin. Plasmid DNA was isolated by lysis alkaline method [14]. DNA pellet was resuspended in 500 µl of TE buffer, pH 8 and stored at -20°C.

2.10. Plasmid DNA restriction

Purified plasmids were cut with *Eco*RI endonuclease (Promega). The reaction was composed of 2 µl of 10x H buffer, 1 µl of 2 mg/ml BSA, 6 µl of plasmid DNA/cDNA, 10 units *Eco*RI and 10 µl of de-ion water. The reaction was performed at 37°C for 16 hours.

2.11. Sequencing of PFPα cDNA and DNA

Sequencing were conducted at Biotechnology Laboratory of the Agency for the Assessment and Aplication of Technology (BPPT), Puspiptek, Serpong, Indonesia, using BigDye Terminator v3.1 cycle sequencing kit (Thermo Fisher).

3. Results

Total RNA and genomic DNA have been successfully isolated from sugarcane leafroll. The ratios of $A_{260}/_{280}$ of total RNA and genomic DNA were 1.30 and 1.56, respectively.

The single stranded cDNA has been synthesized using RNA as a template by reverse transcriptase, and then served as a template in the PCR reaction to generate and amplify double stranded cDNA [15]. The size of the synthesized cDNA is about 900 bp (figure 3).

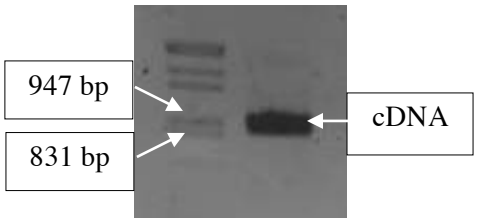

Figure 3. Agarose gel electrophoresis of purified cDNA resulted from RT-PCR.

The cDNA was succesfully inserted into pGEM-T Easy vector, and introduced into *E. coli* indicated by the white colonies after blue-white selection. The recombinant plasmid was succesfully isolated nad cut by *Eco*RI enzyme (figure 4).

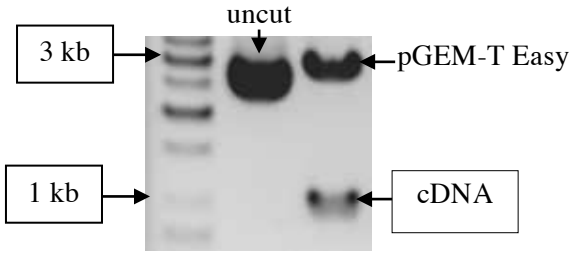

Figure 4. Agarose gel electrophoresis of cut and uncut plasmid cDNA.

The sequencing analysis showed that the insert cDNA had 900 bp in length. The deduction of amino acid sequence of cDNA was performed by using Bioedit program. This amino acid sequence contains binding sites for fructose-6-phosphate and fructose-1,6-biphosphate of PFPα as shown in figure 5.

The DNA of *PFPα* fragment has been succesfully amplified by PCR and ligated into pGEM-T Easy vector. The size of this DNA is 900 bp (figure 6). The alignment of cDNA and DNA of *PFPa* fragment showed that there was no different between cDNA and DNA as shown in figure 7.

```
                240         *       260         *       280         *
S. officinarum : ------------CTDALSAEKYYYFIRLMGRKASHVALECALQSHPNMVILGEEVAASK :  47
S. tuberosum   : ICKVNSQLISNVCTDALSAEKYYYFIRLMGRKASHVALDCTLQSHPNMVILGEEVAASK : 295

                  300         *       320         *       340         *
S. officinarum : LTIFDITKQICDAVQARAEKDKNHGVILIPEGLVESIPELYALLQEIHGLHGQGVSVEN : 106
S. tuberosum   : LTIFDITQQICDAVQARAEHDKNHGVILLPEGLIESIPEVYSLLQEIHGLLRQGVSADK : 354

                  360         *       380         *       400         *
S. officinarum : ISSQLSPWASALFEFLPPFIRKQLLLHPESDDSAQLSQIETEKLLAQLVETEMNRRLKE : 165
S. tuberosum   : ISSQLSPWASALFEFLPHFIRKQLLLHPESDDSAQLSQIETEKLIAHLVETEMNKRLKE : 413

                  420         *       440         *       460         *
S. officinarum : GTYKGKKFNAICHFFGYQARGALPSKFDCDYAYVLGHVCYHIIAAGLNGYMATVTNLKS : 224
S. tuberosum   : GTYKGKKFNAICHFFGYQARGSLPSKFDCDYAYVLGHVCYHILAAGLNGYMATITNLKN : 472

                  480         *       500         *       520         *
S. officinarum : PVNKWRCGAAPISSMMTVKRWSRGPSATQIGKPAVHMASVDLKGKAYDVLRQNSSSFLL : 283
S. tuberosum   : PANKWHCGASPISAMMTVKRYGRGPGKASIGVPALHPATVDLRGKSYELLSQNATKFLL : 531

                  540         *       560         *       580         *
S. officinarum : EDVYRNPGPLQFDGPG------------------------------------------- : 299
S. tuberosum   : DDVYRNPGPLQFDGPGADAKAGSLVVEDADYIGAIKKLQEYLDKVRTIVKPGCSQDVLK : 590
```

Figure 5. PFPα amino acid sequence alignment of *S. officinarum* and *S. tubersoum* showing binding site for Fru-6-P and Fru-1,6-P_2 in the bold underlined words [8].

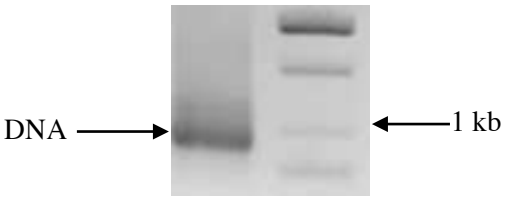

Figure 6. Agarose gel electrophoresis of DNA resulted from PCR.

4. Discussion

4.1. Isolation of total RNA and genomic DNA from sugarcane

Based on A260/A280 ratio, the purity of total RNA and genomic DNA was low. The low purity of this nucleic acid was caused by contaminants such as protein, polysaccharide and polyphenol. This contamination may occur during RNA isolation, since sugarcane contains high concentration of protein, polysaccharide, and polyphenol. Therefore, the RNA is quite difficult to dissolve [13, 16]. Therefore, leafroll or young leaf was used for RNA isolation to avoid these contaminants, since it contains less metabolites than the older one [17].

4.2. Cloning of PFPa cDNA and DNA fragments into pGEM®-T Easy and E. coli competent cells

The successfull of cDNA and DNA cloned into pGEM-T Easy and introduced into *E. coli* were indicated by the white colonies resistant to ampicillin on the blue-white selection medium. The insertion of cDNA or DNA fragments in MCS pGEM®-T Easy located in *lac*Z gene, caused the *lacZ* gene can not be expressed, therefore the bacterial colonies had a white color [18]. This result was confirmed by the digestion of isolated plasmid from white colonies with *Eco*RI. Digestion of recombinant plasmid by *Eco*RI produced 900 bp insertion fragment. The insertion fragment was confirmed by DNA sequencing. Based on DNA sequencing, the size of insertion fragment is 900 bp.

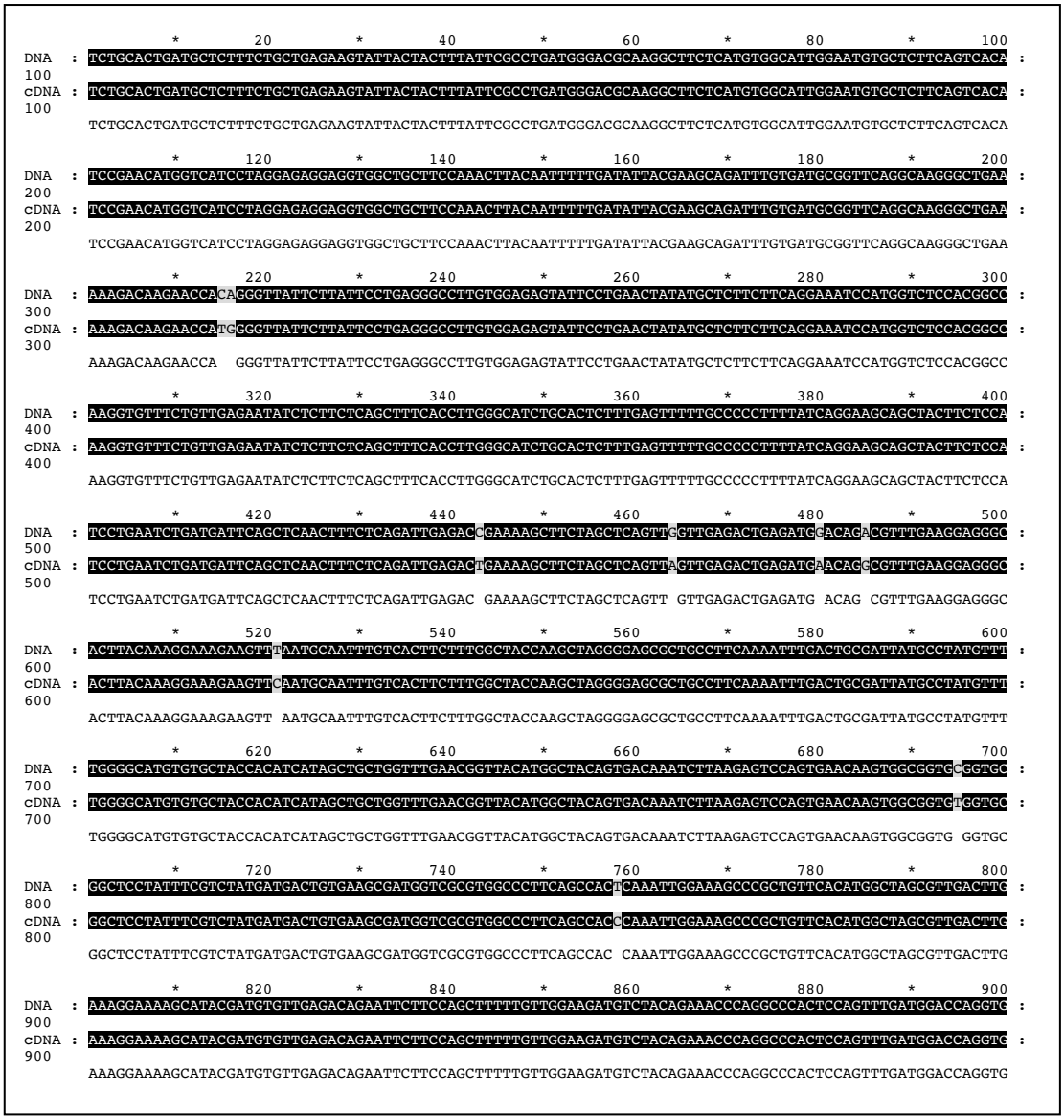

Figure 7. Alignment of *PFPα* cDNA and DNA from sugarcane (*S. officinarum* L.).

4.3. Characterization of PFPa cDNA and DNA from sugarcane

Based on the BLASTx analysis, the isolated cDNA of sugarcane had 96% similarity with *Z. mays PFPα*, 86% similarity with *O. sativa PFPα*, 80% similarity with *S. tuberosum PFPα* and 78% similarity with *A. thaliana PFPα*. Therefore, we conclude that the isolated cDNA was a cDNA of *PFPα* gene fragment. The cDNA sequence of *PFPa* gene fragment from sugarcane has been submitted to DNA Data Bank of Japan (DDBJ) with accession number: AB270695.

Based on the alignment of PFPα amino acid sequence from *S. officinarum* and *S. tubersoum*, it is revealed that binding site for Fru-6-P and Fru-1,6-P_2 are present in PFPα from sugarcane. The binding sites for Fru-6-P and Fru-1,6-P_2 in amino acid sequence of sugarcane PFPa are K (lysine, position 257), MGR (methionine-glycine-arginine, position 17-19), E (glutamate acid, position 78), Q (glutamine, position 100), and S (serine, position 109) as shown in figure 5. The protein motifs are highly conserved in sugarcane PFPa, as well as PFPa from other plants.

The alignment of 900 bp of *PFPa* cDNA and DNA from sugarcane revealed that this *PFPa* gene fragment has no intron as shown in figure 7, since there was no sequence different between cDNA and DNA. This gene without intron occurred possibly caused by inverse transcription of mRNA into cDNA by reverse transcriptase enzyme, then it may insert into the chromosome, and called as retropseudogene. Retropseudogene possesses high sequence similarity (75%) with the original gene.

Those sequences are highly conserved in all family members of PFP [8, 12]. However, it encountered a kind of mutation, thus cannot be transcribed and translated into protein. The retropseudogene is predicted to be one of *PFPa* family member in sugarcane. There was a possibility that the member of *PFPa* retropseudogene in sugarcane was more than the members of *PFPa* gene, therefore, the *PFPa* gene from sugarcane was more difficult to amplify as occurred in *EF-1a* gene [19, 20].

5. Conclusion

The fragment of *PFPα* gene from sugarcane has been successfully isolated, cloned and characterized. The absence of intron in *PFPa* gene should be investigated further, since the cDNA and DNA were partial sequences. Full length cDNA and DNA of *PFPa* from sugarcane can be obtained using primer design from EST database of sugarcane that will be conducted in the future.

Acknowledgements

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