In Silico analysis of Phospho Enolpyrovate Carboxylase gene

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Abstract

The importance of sugar in many aspects of life, such as food, health and body care products, results in its great demand all around the world. From another hand, the annual production of sugar cannot meet the increasing demand of the growing human population. Therefore, this study focused on one of the most important genes that affect the quantity of sugar production, phosphoenolpyruvate carboxylase (PEPcase). The most important crop used to extract sugar is sugarcane where sugar is stored in the stalk internodes. In this study RNA extraction was performed to produce cDNA for cloning. Sequencing the main genes using the Basic Local Alignment Search Tool (BLAST).

Keywords: *RNA isolation, (PEPcase) analysis, sugar production, sequencing.*

Introduction

The average yield of stalk per year is around 60 tons per hectare, and around 12% of the stalk contains sugar[5].

Sugarcane is an important crap for suger and other products like fibers and ethanol. Besides its important products, sugar characterized with its adaptation to tropical and subtropical areas. Sugarcane is classified under the Andropogoneae tribe; it is a member of the grass family, Poaceae, with maize and sorghum, as the closest relatives[6].

Due to its importance for a lot of food industeris, sugar production does not meet the requirements. The average yield of suger is about 9 tons year[2].

Sugarcane adaptation to climate conditions is due to the prescence of phosphoenolpyruvate carboxylase (PEPcase) enzyme. It helps the plant to uptake more CO2 for photosynthesis, and therefore increasing sugar production ratio[7].

This study aims to understand PEPcase background and therefore find a way to increase sugar production. By using three sugarcane cultivars (GG, RR, RG) to isolate PEPcase gene and analyze it with the help of bioinformatics tools.

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Materials and Methods

Plant Materials

Three different cultivars of sugarcane, *S. officinarum* were selected to isolate the PEPcase gene from the leaves and have been propagated *in vitro* using 20 different culture media. From each cultivar 3 samples have been prepared to be used with each medium from the total 20 media.

The materials were provided by Taman Pertanian, Universiti Putra Malaysia. The three cultivars were: Tebu Gula (has red stem green leaves-RG), which is used in sugar production; Tebu Gagak (has red stem red leaves-RR), which is considered to be a medicinal plant, and Tebu Hijau (has green stem green leaves-GG), which is used to extract the sugar syrup.

Genetic Extraction

RNase Removal

Two different methods were used for removing RNase contamination. First by rinsing the glass ware, mortar and pestle, tips, 1.5ml, 0.2ml and 10 ml tubes in 0.1% DEPC water (Fermentas, Canada). The second

method was to autoclave at 121°C for 20 min, using an autoclaving machine model (Astell, UK).

RNA Extraction

Three methods were applied to extract RNA from the sugarcane leaf:

1. Using easy-BLUE reagent (Intron, Korea)
2. Geneaid Kit
3. Qiagen (RNeasy Mini Kit)

DNase Treatment

RNase-free, DNase1 (Fermentas, USA) was used to remove the contamination of genomic DNA from the RNA sample before RT-PCR.

RNA Quality Estimation

RNA was run on a non-denaturing agarose gel to check the RNA integrity. The gel was prepared as described in[3].

The gel image was captured using on UV transilluminator (Bio-Rad, USA).

Spectrophotometer

The Nanophotometer (Implen, Germany), was used to check the RNA samples quality.

Primer Design

A PEPcase gene sequence was searched from the NCBI database

(http://www.ncbi.nlm.nih.gov/) and blast was performed to find the conserved region with the PEPcase gene sequences of other plant species. These conserved regions were used to design gene specific primer, by using Primer 3 online software (http://bioinfo.ut.ee/primer3-0.4.0/).

In addition, different pairs of primers (P1, P3, P4) from published journal were also used[1].

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The Intron cDNA synthesis kit was used to synthesis the template cDNA strand for RT reaction (Maxime RT premix kit). For PCR amplification of the PEPcase gene, three different kits were used and compared, which were Promega Go Taq Flexi DNA Polymerase (Promega, USA), Solis BioDyne HOT FIREPol Blend Master Mix (Solis**,** Estonia), and Intronon Biotechnology (Intron/Korea). There were two types of reactions from biotechnology brand, which the first is a ready to use mixture, which can be used directly to perform both cDNA synthesis and PCR reaction in one reaction (Maxime RTPCR premix kit). The second type was iTaq DNA Polymerase (Cat. No. 25021) which was set in clean sterilized (200 µl) PCR tubes to which the following were added: 3 µl of 0.35 µg/µl RNA, 1 µl of 10 µm forward PEPcase primer, 1 µl of 10 µm Reverse PEPcase primer, 0.25 µl of 5U/µl iTaq DNA Polymerase, 2 µl of 10x PCR buffer, 2 µl of 2.5mM dNTP Mixture, and finally topped up to 20 µl with sterilized distilled water.

The amplification of the gene was performed in the PCR machine (Biometra, Germany) in 40 cycles, Initial denaturation 94° C for 2 min, the temperature of the denaturation step was 94° C for 20 sec, annealing temperature was set according to primers Ta, for 20 sec, and the extension temperature was 72° C for 30 sec. The elongation temperature was 72° C for 3 min, and then holds at 4° C.

The primers used to amplify the PEPcase gene were optimized for a better annealing temperature. First, a touchdown PCR was used to amplify the gene, and then a gradient PCR was used. In addition, proof reading Taq polymerase was used to increase fidelity approximately six times more than that of Taq DNA polymerase. For the primer concentration, different dilutions were tried to get the best result. Three different concentration of the primers were used as follows: 100 pico Mole, 100 nano Mole, 10 µ Mole. The best was 10 µm diluted primer, which was used for the PCR, and the best kit was from Promega with proofreading Taq polymerase.

Gel Electrophoresis of RT- Polymerase Chain Reaction Products The amplified PCR product of PEPcase gene was separated using 1% (w/v) agarose gel at 80 volt for 40 min in 1X TBE buffer (89.0 mM Tris-base, 89.0 mM Boric acid, 2.0 mM EDTA, pH 7.5). The DNA marker used was 1 Kb DNA Ladder GeneRuler (Fermentas, Canada). The gel was stained with ethidium

bromide solution (0.5 □g/ml) for a few seconds and then destained with distilled water. The gel image was captured using UV transilluminator (BioRad, USA).

PCR Product Purification

Mega quick spin purification kit from Intron Biotechnology was used to purify the target PCR product. The purified products were sent to 1st base Sdn Bhd for sequencing using Applied Biosystems 3730xl (DNA Analyzer/USA).

Cloning Preparation

yT & A vector from Yeastern (Yeastern, Taiwan) was used to clone the PEPcase gene (Figure 1). This vector has a LacZ region where there are several restriction enzyme sites found. If the desired gene is inserted into these sites then the LacZ will not be activated, and the host cell cannot hydrolyze the lactase found in the medium. Hence, white colonies were produced instead of blue colonies, these white colonies represented the positive colonies that contain the desired insert.

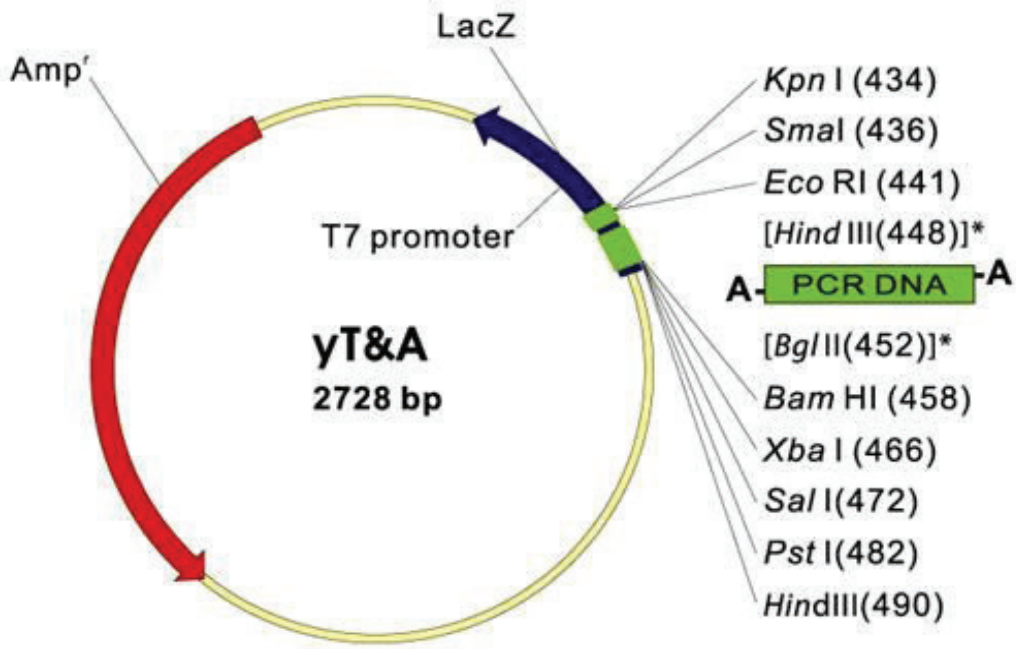


Figure 1: YT&A (2728bp) cloning vector (Yeastern, Taiwan)

LB Medium Preparation

One liter of LB medium was prepared by mixing 10 g of bacto tryptone, 5 g of bacto yeast extract and 5 g of NaCl. The pH is adjusted to 7 with NaOH. Then 0.1 M of Isopropyl b-D-1-thiogalactopyranoside (IPTG) is prepared by dissolving 1.2 g of IPTG into distilled water and topped up to 50 ml, before sterilizing by filtration and storing at 4°C. Then 2 ml of X-Gal is prepared by dissolving 100 mg of 5-bromo-4-chloro-3-indolyl-B-D-galactoside in 2 ml of N,N‟-dimethyl-formamide and covered with aluminum foil and stored at -20° C. Then 15 g agar was added to 1 liter of the LB medium and autoclaved, before allowing the culture medium to cool down to 50°C. Ampicillin was added to the final concentration of 100 □g/ml, and about 30 ml of the medium was poured into 85 mm Petri dishes, and left to solidify before use. The remainder was stored at 4°C for up to one month or kept at room temperature for up to one week. Then 100 □l of 100 mM of IPTG and 20 □l of 50 mg/ml of X-Gal was spread over the LB plate which contained ampicillin and allowed to absorb for 30 minutes at 37° C prior to use.

Ligation

The yT&A cloning vector and PCR products were centrifuged to collect the contents at the bottom of the tubes. The ligation buffer was vortexed vigorously before use.

Glycerol Stock Preparation

The 80% glycerol was prepared with sterile water and autoclaved, and then left until cool. 500 □l of LB broth containing the transformant was added with 500 □l glycerol in a 1.5 ml microcenterfuge tube and kept at -70° C.

Polymerase Chain Reaction

Colony PCR was performed for the screening of the positive colonies to confirm that target gene was inserted into the host cell, and to obtain many copies of the PEPcase gene, which were stored in the refrigerator for a long time for future use by storing in glycerol stock solution.

Results

RNA Extraction

RNA was extracted from the leaf of three sugarcane cultivars (ten individuals for each cultivar) by using three different kits. The quality of these products was checked using Nanodrop spectrophotometer and electrophoresis. The results are shown in (Table 1).

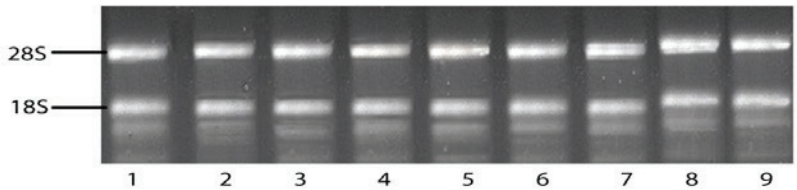
Table 1: Rna extraction quantification

Kit	concentration	A260/A280	A260/A230
Easy blue	1.146µg/µl	1.9	1.8
Genead kit	0.026 µg/µl	1.8	1.7
Qiagen	0.35 µg/µl	2	2

RNA integrity was checked by non- denaturing agarose gel electrophoresis. RNA samples were extracted using the Qiagen kit. The other kits also gave good concentration and purity of RNA, but did not produce intact RNA.

Easy-Blue kit gave high concentration of RNA and a lysate band on electrophoresis, and Genead kit gave lower concentration of RNA and a lysate band on electrophoresis, but Qiagen kit gave good concentration of RNA and good purity (in gel electrophoresis the Qiagen band was clear and intact). Thus, Qiagen kit was selected for RNA extraction from sugarcane as it was more efficient than the other two kits (Table 2).

Table 2: gel electrophoresis of RNA isolated using Qiagen kit. Lanes (1, 2, and 3) show the intact band of rRNA isolated from GG cultivar. Lanes (4, 5, and 6) show the intact band of rRNA isolated from RG cultivar. Lanes (7, 8, and 9) show the intact band of rRNA isolated from RR cultivar.



(1, 2, and 3) show the intact band of rRNA isolated from GG cultivar. Lanes (4, 5, and 6) show the intact band of rRNA isolated from RG cultivar. Lanes (7, 8, and 9) show the intact band of rRNA isolated from RR cultivar.

Primers

The primers used to amplify the desired gene were taken from [1], as well as own primer designed in this study. Four primers were used to isolate around 3000 bp of PEPcase gene from the three sugarcane cultivars.

Reverse Transcriptase Polymerase Chain Reaction

The RNA from 9 sugarcane samples which were isolated from three replicates of each cultivar GG-PEPcase, RG-PEPcase and RR-PEPcase were converted into cDNA using reverse transcriptase polymerase chain reaction by using Intronon biotechnology kit. Three thousand PEPcase gene base pairs were amplified using 4 pairs of primers. The result of PCR amplification was checked using gel electrophoresis (Figure 2).

PCR products with different sizes were amplified (ranging from 550 bp for Primer 1, 750 bp for Primer 2 and 1000 bp Primer 4 (which is shown in Figure 2.a) and 750 bp for Primer 3 which is shown in (Figure 2.b).

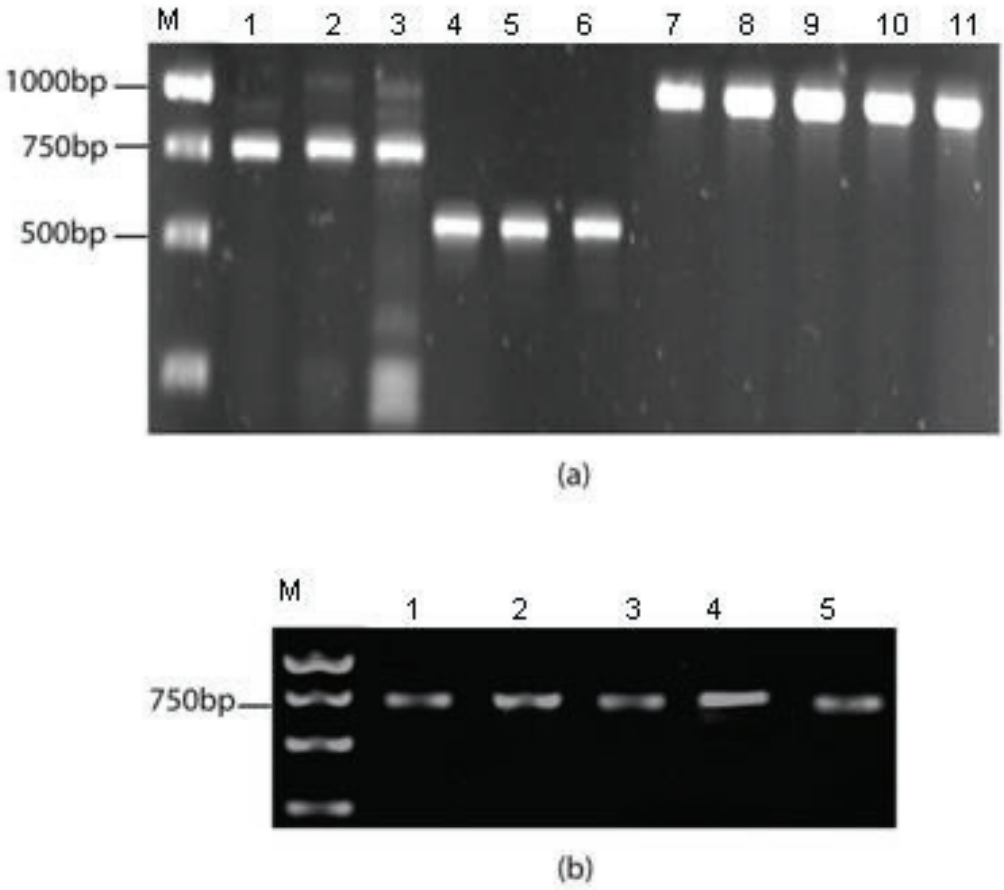


Figure 2: PCR products for PEPcase gene; (a) The first lane is 1 Kb marker, and Lanes: 1, 2 and 3 are GG, RG, and RR respectively for Primer 2, and lanes 4, 5 and 6 are GG, RG, and RR respectively for primer 1, and lanes 7, 8, 9, 10, and 11 are GG, GG, RG, RG and RR respectively for primer 3; (b) The first lane is 1 Kb marker, and Lanes 1, 2, 3, 4, and 5 for GG, GG, RG, RG and RR respectively for primer 4. Lane M: 1kb ladder.

Cloning

After cloning and incubating the plate for 18 hours at 37° C, two types of colonies were formed white and blue (~ 35 white colonies). The white colonies were tested using colony PCR, the gel electrophoresis bands represented the gene inserted into the vector. (Figure 3)

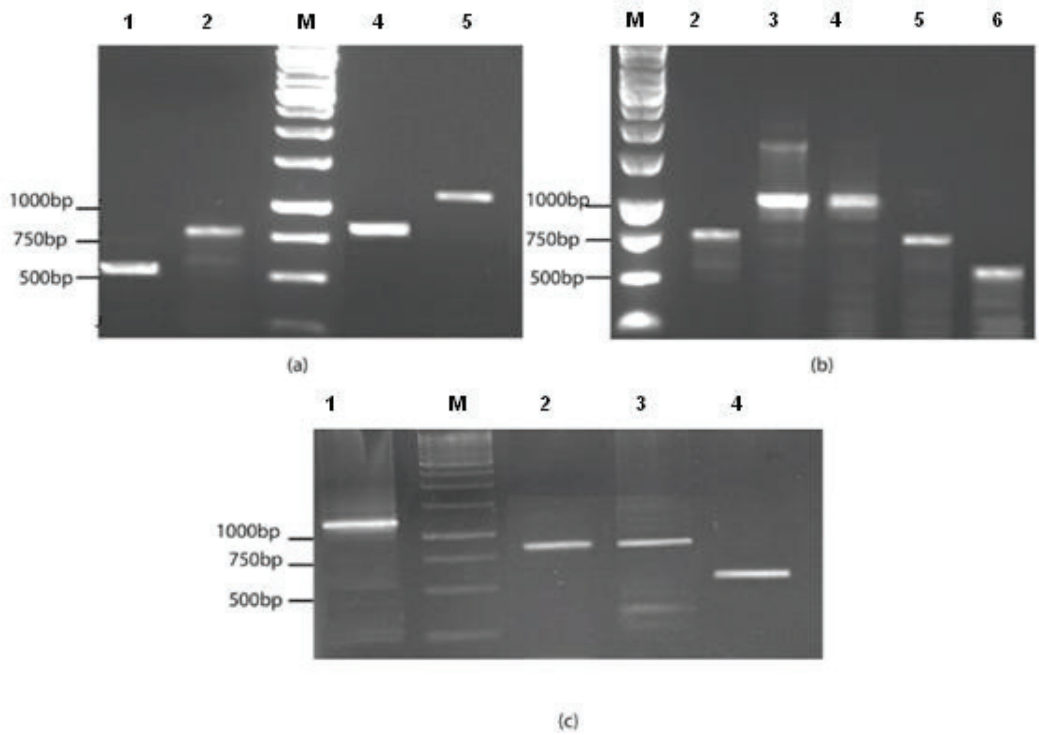


Figure 3: Gel electrophoresis for colony PCR showing the bands of inserted PEPcase gene; (a) Bands for GG: Wells 1, 2, 4 and 5 for PEPcase 1, PEPcase 2, PEPcase 4 and PEPcase 3 respectively, and well 3 indicates for the 1Kb marker; (b) Bands for RG, the numbers are indicating the wells 2, 3, 4, 5, and 6 for PEPcase 2, PEPcase 3, PEPcase 3, PEPcase 4 and PEPcase 1 respectively, and the 1st well is indicating for the 1 Kb marker; (c) Bands for RR: the numbers are indicating the wells 1, 2, 3, and 4 for PEPcase 3, PEPcase 2, PEPcase 4 and PEPcase 1 respectively, and the 2nd well is indicating for the 1 Kb marker.

Bioinformatics Analysis

Several bioinformatics tools had been applied to analyze the PEPcase gene and protein sequences.

Sequence Blast: (Basic Local Alignment Search Tool)

The cDNA sequences of GG-PEPcase, RG-PEPcase and RR-PEPcase were subjected to blastN analysis (http://blast.ncbi.nlm.nih.gov/) which searched nucleotide query against nucleotide database. And this has revealed that there is high similarity to nucleotide sequence of *Saccharum officinarum, Saccharum hybrid,* and *Saccharum spontaneum,* with query coverage percent 98%, 100% and 98% for GG-PEPcase, RGPEPcase and RR-PEPcase, respectively, where the other matchings were PEPcase of *Zea mays*, *Oryza sativia, Arabidopsis thaliana* with lower query coverage percent with E-value 0.0.

Disscusion

RNA Isolation

The success of genetic study is highly dependent on the quality and quantity of the extracted genetic material. Thus, three kits were tested to find the best for this purpose, and has been found that the best was the Qiagen RNA extraction kit, which gave good quality

and quantity of RNA material with minimum amount of protein and carbohydrate contaminations. Furthermore, non-denaturing agarose gel electrophoresis also showed good intact bands on the gel. Whereas the other two kits, Easy-blue and Genead kits either gave low concentration of RNA, less purity or slightly degraded RNA.

Genetic Analysis

Four pairs of primers were used to isolate 3kb of PEPcase genes from three cultivars of *Saccharum officinarum* (GG-PEPcase, RG-PEPcase and RRPEPcase). Primer-1, primer-2, primer-3 and primer-4 were extracted at approximately 550 bp, 750 bp, 1000 bp and 750 bp respectively using Intronon biotechnology kit. The isolated PEPcase genes were compared with the PEPcase gene sequence of *Saccharum officinarum* obtained from NCBI data base (http://www.ncbi.nlm.nih.gov/), and has been found that the gene length and sequence of PEPcase ORF region is almost similar, but not identical, ranging from (2635 bp - 2831 bp). While the molecular weight ranging from 810714.52 Da to 876603.37 Da, whereas the gene length and molecular weight of SC-PEPcase are 2886 bp and 892108.55 Da respectively. There are differences in genetic level because the PEPcase gene was extracted from different cultivars.

The difference in gene length among *Saccharum officinarum* and its cultivars led to differences in protein sequences length and molecular weights. The protein sizes of the three cultivars ranged from (878 a.a - 961a.a) and the protein molecular weight ranged from (97.71 kDa - 105.64 kDa), whereas the protein size of SC-PEPcase was 961a.a. and protein molecular weight was 108.58 kDa.

The PEPcase protein sequences of the three sugarcane cultivars were almost the same as that of *Saccharum officinarum* obtained from NCBI online data base. The differences in gene length is caused by mutations in the genetic level during replication. The result has shown clearly that these changes did not lead to any critical differences in PEPcase enzyme functions, evolutionary tree analysis and 3D model structures. Thus, these changes are considered as only minor and unimportant differences at the functional point of view[4]. Further investigation is required to confirm whether these changes have any effect on sugar production from sugarcane.

Conclusion

The present research attempted to isolate and analyze the phosphoenolpyruvate carboxylase (PEPcase) at the genetic and protein level using in-silico analysis. The study showed that the active sites were conserved among all the cultivars studied here and this result is consistent with previous studies on other C4 plants.

Future work

Studies in the future can focus on the reasons and mechanisms of the effects of PEPcase gene, this gene can be isolated to be inserted into the C_3 plant and investigate its effect on sugar productivity.

Ethical Clearance- all data has been prepared in the department of Department of Biology, Faculty of Science, Universiti Putra Malaysia.

Source of Funding- self-funding.

Conflict of Interest - Nil.

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