Degenerated Primers for The KCS Enzyme Gene Encoding Erucic Acid Content in Winged Bean Seeds

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Abstract. Winged bean (Psophocarpus tetragonolobus (L.) DC.) contains moderate to high fat in its seeds including erucic acid (C22:1) which is an important raw-material of various oleochemical industries. β-ketoacyl-CoA synthase (KCS) is one among those enzymes involves in erucic acid biosynthesis pathway by condensation acyl-CoA to β-ketoacyl-CoA. Information about encoding genes of the KCS enzyme involve in erucic acid biosynthesis pathway, therefore, it becomes a prerequisite. Current data show the paired degenerated primers of forward primer 5’-ATNTTCAACCCGACDCCDTC-3’ and reverse 5’-GAGCTTCACCTCCAACATYS-3’. PCR resulted in amplicons of 404 bp and 301 bp which are predicted as putative fragments of the KCS enzyme encoding genes involved in the erucic acid biosynthesis in winged bean.

1. Introduction

Winged beans (Psophocarpus tetragonolobus (L.) DC.) a tropical legume planted from lowland to highland up to 2,000 m altitude; dry weather, temperature of 15-32°C, humidity 50-90%, soil pH 5.50-6.50, rainfalls of 2,500 mm per annum, and full day sunshine [1] People consume its seeds which contain 17.5% fat in various fatty acid compounds [2]. [3] grouping these fatty acids into unsaturated fatty acids (MUFA/monounsaturated fatty acids or PUFA/ polyunsaturated fatty acids).

Erucic acid is one among those PUFA’s characterized with 22 Carbon units and having a double bonds at the cis-13 [4]. The fae1 gene is a specific gene coding for β-ketoacyl-CoA synthase (KCS) enzyme, to condensate biosynthetic of erucic acid within Arabidopsis thaliana, Simmondsia chinensis, Lunaria annua, and some Brassicas [5].

Demands on erucic acid for oleochemical industries has increased annually and forces breeders to provide better quality of winged beans. Information about gene coding of KCS enzyme therefore becomes a prerequisite step as a preliminary work to engineering high erucic acid content winged beans. In doing so, a PCR technique might be applied as an initial phase (polymerase chain reaction).

A suitable primer is an important factor in PCR, however, in the mean time there is no specific primer coding the KCS enzyme in biosynthetic pathway of erucic acid in winged beans available. The availability...
of this primer, therefore, becomes important to engineering high erucic acid content in winged beans. The current study was then purposed to:

1. Designing a specific primer for coding the KCS enzyme gene which involves in the biosynthetic pathway of erucic acid in winged beans.
2. Amplifying DNA fragments of the coding gene of KCS enzyme through PCR technique.

2. Methods

The DNA was extracted from the third leaf from the top of an individual plant. Initially we searched data of the DNA sequences encoding for KCS enzyme in different plant species registered in the gene bank. The data were then adopted to design primers for the KCS encoding gene and so amplifying the DNA fragments.

DNA extraction was performed by applying a modified-CTAB method [6], after which the DNA was checked for quality using electrophoresis on 1% (w/v) agarose gel, and visualized using a UV transilluminator, and checked in a gel-doc computer.

The DNA was also measured for quantity under a nanodrop spectrophotometer at the wavelengths of $\lambda_{230}$, $\lambda_{260}$, and $\lambda_{280}$, then interpreted for purity at $\lambda_{260}/\lambda_{280}$ absorbance. The DNA concentration was then calculated by using the following formula: $\lambda_{260} \times 50 \text{ng/\mu l} \times F$ (dilution factor).

Primers for the KCS encoding gene were designed by using a series of data on nucleotides sequence of KCS gene in various plants accessed from the gene bank (Poaceae, Brassicaceae, and Fabaceae-data are not shown). Primer designing was done by applying a Primer3 program which was accessed through primer3.ut.ee. Designed primer was based on the conservative area of nucleotides data sequence after checking it on the MUSCLE program accessed through http://www.ebi.ac.uk.

Following to this, the designed-primer was also tested for quality through IDT Oligo Analyzer program accessed from the http://sg.idtdna.com/calc/analyzer and tested for its specificity through blasting with the BLASTn program available in the internet site of http://www.ncbi.nlm.nih.gov/blast. Nucleotides sequences of the forward and reverse primers, were substituted with other sequences as obvious in the IUPAC nucleotide code accessed in the http://www.bioinformatics.org.sms/iupac and so called as degenerate-primer. The last form of primer was used in the PCR technique to amplify DNA fragments of KCS encoding gene.

Amplification of KCS gene fragment was done by using PCR machine Primus 25 as suggested by [7]. A total volume of 19 μl PCR mixture consisting of 3 μl DNA template (80 ng/μl), 8.5 μl PCR super mix, 1 μl forward primer (5'-ATNTTCAACCCCGACDCCDTC-3'), 1 μl reverse primer (5'-GAGCTTCACCTCCAACATYS-3'), and 5.5 μl ddH2O was made. PCR cycles were set up as follows: 1 cycle for pre-denaturation of 94°C for 1 minute, and 35 cycles for reaction with 94°C 1 minute pre denaturation, 1 minute and 30 seconds for primer annealing 48.4°C, 2 minutes for extension at 72°C, and 1 more cycle for 10 minutes which set as post extension at 72°C.

Observation was analysed following database of both DNA quality and quantity, quality of the designed-primer, primer’s specificity, and observed DNA fragments. Quality of the obtained DNA extract was done in 1% (w/v) electrophorese gel while its concentration and quality were calculated using a nanno-drop spectrophotometer at A230, A260, and A280 wavelength. Test of designed-primer quality was done using an IDT Oligo Analyzer by counting the contents of guanine and cytocine, melting temperature, hairpins structures, self-dimer and hetero-dimer. The specificity of designed was tested by blasting the new designed -one to the NCBI data of the nucleotide sequences of different plants available in the gene bank.

The DNA fragments were observed using the UV transilluminator at the wavelength of 300 nm connected to a gel-doc computer.
3. Results

The current study succeeded in extracting genomic DNA of winged bean, as well as its positive control, Chinese cabbage (Fig. 1). However, they were contaminated by RNA, phenol or protein [8] shown as smear.

![Visualization of genomic DNA of winged beans short and long pods isolated by CTAB (M= DNA ladder 1 kb, Pk = short pod winged beans, Pj = long pod winged beans, SwP = Positive control)](image)

A smear is a molecule of either destructed DNA or unidentified organic matter [9]. Alternatively, the existence of smear means the isolated-genomic DNA was not in a good condition [9]. A good quality DNA is characterized by its thickness, the thicker the band is the better quality DNA is. [10] stated that the thinner the smear shows the better extraction. In order to minimise the effect of RNA contaminant, current study added 3 μl RNAse to the extracted-genomic DNA before being used as PCR template.

A slight modified-CTAB [6] was used to extract genomic DNA, here we used 3 pieces zeolite an alumina silicate crystalline with microporosity consist of tetrahedral SiO4 and AlO4 which connected one to each other [11], to break the cell wall and allows the cytoplasms come out from the cell, instead of liquid nitrogen which was characterized as inefficient for its high price and volatilization [10]. [12] reported that liquid nitrogen had not significantly different from zeolite in its effectivity to extract DNA of hot chilies.

Genomic DNA extracted from winged bean leaves were then checked for its quantity and quality using a nanno-drop spectrophotometer at Å230, Å260, and Å280 nm wavelengths. The highest concentration of 1224 μg/ml was noted from PK3 (short pod winged bean sample number 3), while the long pod of sample 2 contains 1103 μg/ml (data do not published here). The extracted genomic DNA was highly contaminated due to the extraction steps did not run well. Can be notified if it has scored between 1,7-1,9 at the ratio of Å260/Å280, and score 2 at the ratio of Å260/Å230 [13].

DNA concentration and purity can be affected by several factors like: a process of homogenization with buffer, dissociation of the tissues, and DNA’s precipitation [14]. However, the older the leaf-samples the lower DNA quantity would be [9]. The perfect stage of leave’s development might then important in extracting DNA.

The success of PCR technique depends on several factors namely the availability of primer to amplify DNA fragment, which plays a significant role in PCR especially in maximizing specificity and efficiency of PCR reaction [15].

Current study applied specific primer which was designed based on sequences of 7 nucleotides from different plant families namely: Brassicaceae, Poaceae, and Fabaceae (data were not published) and cross-checked with the MUSCLE program which were used as the main data to design the primer [16], a designed primer might be applied to the DNA fragment only after being blasted. [17] reported if the more gene sequences and fit to the blasting program they might result in a shorter conserved area which affects primer’s specificity in amplifying the target area. Following to this, a Primer 3 program was being used as also used in many applications [18] for setting the nucleotides sequence which resulted a nucleotides
sequence of 5'-ATTTTCAACCGACACCGTC-3' for forward primer and 5'-GAGCTTCACCTCAACTTCC-3' for reverse one. Both designed-primers have of 20 nitrogen bases each. A good primer, supposed to have 18-30 nitrogen bases which based on random combination of a sequence of particular genome [15]. [19] primer that has lesser than 18/nitrogen bases will be less in its specificity but over 30 nitrogen bases not even increase specificity. Both designed-primers have almost similar melting points of 55.5°C for forward primer and 54.7°C for reverse one. Melting temperature is a temperature where 50% of double bonds DNA would be split at the expected points. [19] stated the correct melting point plays a significant role in annealing of DNA fragments to the templates. If a primer is set at too high melting temperature it might cause a low PCR product, in contrast, if melting point is set at too low temperature will produce nonspecific PCR products due to many mismatch bases [20].

Current primers have different GC content, forward has 50.8% GC, but the reverse one has 52.5% GC. These contents are in between the ideal content as stated by [20] who stated the GC content of a primer varies between 40-60%. A too low GC content may affect the competitiveness of the primer to anneal on the targeted gene area leads to lowering the PCR efficiency [10].

The designed-primers were also subjected to be tested for their quality and specificity, which were checked by an IDT Oligo Analyzer program while the second character was blasted for their nucleotide sequences in a BLASTn program. An IDT Oligo Analyzer analysis was purposed to get some informations related to secondary structures of the primer like hairpins, self-dimer and hetero-dimer which might be appeared on a particular designed-primer. This program gives some predictions on biophysics oligonucleotides and the sequence performance [21].

In order to know the nucleotides available in the gene bank the current primer’s specificity was tested by BLASTn program [21] who reported the BLASTn program in checking a particular site for annealing of the primer’s sequence and a particular sequence within the organism’s genome. The BLASTn program, therefore, is normally used to check the specificity of designed-primer in order to know the fittest of the primer to the targeted DNA [22].

Based on its specificity analysis, both primers forward and reverse, have a quite high similarity with the query coverage of 100% and low E-value (expectation value), but having similarity level of 80-100% (data were not published). Query coverage is a frequency of nucleotide bases similar to the sequence of reference-gene in the gene bank [23]. The query coverage of equal or larger than 25% means the nucleotide sequence is part of the target sequence as in the gene bank [24]. The low E-value shows the specificity of the designed primer to the BLASTn program has highly confidence. E-value of close to 0 (zero) shows a very high confident, contrastingly the E-value of almost 1 shows low in confidence. Meanwhile the sequence similarity with the score between (80-100%) shows the checked sequence of newly designed-primer have high similarity to those of available in gene bank. The higher the similarity score shows the primer might anneal on the target area specifically [17].

Following to test for its quality and so its specificity, the newly designed-primers were then derived to its degenerate form. [25] stated the degenerated-primer is a primer where one or more nucleotides can be filled by several nitrogen bases. In order to get their conserved area, the degenerate primers were blasted with data of early nucleotide sequences. Some nucleotide bases of either primer’s forward or reverse which does not fit to the nucleotide sequences then replaced with other nucleotides’ bases as stated in the IUPAC nucleotide code. The current study noted the nucleotide sequences of newly degenerated forward primer was as follows: 5'-ATNTTCAACCGACDCCDTC-3' and nucleotide sequences of reverse primer was 5'-GAGCTTCACCTCAAACATYS-3'.

4. Discussions
Current degenerate primers were then used to amplify KCS enzyme encoding gene for erucic acid through PCR. Visualization of PCR product using agarose gel of 1 % (w/v), showed amplification of two DNA
bands (multiple bands) on the Pk4 and Pk5 samples after being amplified with the newly designed-degenerated primers. Then upperside DNA band is 404 bp and quite a thick one, however, the lower side one is only 301 bp and thinner than the upper side one (Figure 2). Meanwhile the positive control (Chinese cabbage) showed a DNA size of 487 bp.

![Figure 2](image)

**Figure 2.** Visualization of PCR product of Winged beans DNA amplified by degenerate primers (M1 = DNA ladder 1 kb, Pk4 = short pods winged beans sample No. 4, Pk5 = short pods winged beans sample No 5, SwP positive control/Chinese cabbage)

Data of the current study unfortunately show discrepancies to that of reported by [5] and [27], who reported encoding gene for the KCS enzyme of *Brassica napus* 'maplus' and *Crambe bursa-pastoris* 'galactica' were at the size of 540 bp, but 403 bp noted from *A. thaliana* and *Camelina sativa*. The discrepancy noted in this study was might due to different samples were used, where the current study used quite different from the subject of previous studies. Furthermore, existing data were also different from data of [27], who reported the size of DNA band encoding gene for KCS enzyme of the Nasturtium (*Tropaeolum majus*) at 650 bp. The explanation for this discrepancy might due to the different template was used in these two studies, current study used genomic DNA as its template in contrast with cDNA which was used before. Genomic DNA of a particular organisms has still an intergenic area (intron), whereas, the cDNA does not have any intron. The cDNA therefore, has only coding area to produce functional protein.

The two DNA bands with different sizes which obviously noted from winged beans DNA’s samples could be interpreted as DNA fragments of encoding gene for KCS enzyme, alternatively, they both were not encoding gene for the KCS enzyme. Conclusion if those two DNA bands were a fragment of encoding gene for the KCS enzyme was due to one or more loci of the genomic DNA were amplified clearly, while the locus (ci) contains one single gene for biosynthetic of erucic acid. Genetic study of biosynthetic pathway of erucic acid on *B. rapa* showed if the synthesis of erucic acid was affected by two different main genes (*Bn-fae1.1* and *Bn-fae1.2*) located in locus E1 and E2 [28]. Both loci were known to affect production of erucic acid in various plants [28, 29, 4].

Alternatively the presence of those two DNA bands was fragment of encoding gene for the KCS enzyme was due to the possibility of the newly designed degenerated primers, sticked on the intron (a non-coding nucleotide sequence) of the genomic DNA of winged beans which still have many introns but numbers and size are still unclear. Along the PCR processes, these introns will be amplified together with the DNA fragments and produce non-specific products. These introns are located in either one locus or more loci. [30] stated if the difference in DNA band sizes obtained in this study was merely due to many annealing areas inside the organism genome.
The DNA band of the positive control was 487 bp, the current study predicted the discrepancy on band’s size was due to the newly designed-degenerate primers were having closer nucleotide sequence to Brassicaceae instead of others. Data of nucleotide sequence which close to the winged beans was noted from two species, *Glycine max* and *Medicago truncatula*, the newly designed-primers were then knowing and complementing to the DNA sequences of the positive control rather than the research’s object (winged beans).

Numbers of DNA fragments amplified from both samples and positive control were probably due to there are many spreaded-sites for a primer to anneal in the DNA template. Alternatively, it might due to the amplification process was initiated at more than one site of annealing, therefore, the fragment sizes are quite different in size [31]. Meanwhile, the differences in winged-bean DNA’s intensity were mainly due to primers inability to fully and strongly anneal on the target’s genes fragment. [31] stated annealing process of a primer is affected by its ability in detecting the sequence of its DNA complementary. [32], the differences in intensity might probably due to primers competition to anneal on the DNA template, therefore some nucleotides might anneal in a large number of fragments others anneal on few numbers only. Moreover, [31] explained if the DNA’s intensity might resulted from the purity of the DNA itself. The contaminated DNA’s either from polysaccharides or phenolic compounds and too little concentration might affect the DNA intensity.

5. Conclusions

Current study might conclude:

1. The designed degenerate primers have the following nucleotide sequences of *forward* primer 5’-ATNTTCAACCCGACDCCDTC-3’ and nucleotide sequences of *reverse* primer 5’-GAGCTTCACCTCCAACATYS-3’.
2. PCR amplification resulted in two DNA bands at different sizes of 404 bp and 301 bp and concluded as encoding gene for β-ketoacyl-CoA synthase enzyme which plays a role in biosynthetic of erucic acid in winged beans.

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