Short communication

PCR AMPLIFICATION OF ORNITHINE DECARBOXYLASE (*ODC*) GENE FRAGMENT FROM TOBACCO (*Nicotiana tabacum* L.) cv. TEMANGGUNG

Ira Djajanegara[1], Sabar Pambudi[2], Retno Lestari[2] and Nina Artanti[3]

[1)] Center for the Assessment and Application of Bioindustrial Technology, Agency for the Assessment and Application of Technology (BPPT)
[2)] Department of Biology, Faculty of Mathematics and Natural Sciences,University of Indonesia
[3)] Research Centre for Chemistry, Indonesian Institute of Sciences (LIPI)
Corresponding author: Ira Djajanegara (phone: 62-21-3169539; Fax.: 62-21-3169510; email: iradjajanegara@hotmail.com)

ABSTRACT

In order to create an antisense construct of the gene encoding Ornithine Decarboxylase (ODC) from tobacco (*Nicotiana tabacum* L.) cv. Temanggung, the target gene must be isolated. In this paper, we present the PCR amplification of a fragment from putative gene encoding ODC from tobacco cv. Temanggung. Leaf genomic DNA was isolated and used as the template for PCR. PCR optimization was done by adjusting the annealing temperature and the cycle number. Verification of the fragment obtained was also done using the second primer pairs.

Keywords: Ornithine decarboxylase, tobacco, PCR.

ABSTRAK

Isolasi target gen dilakukan dalam rangka membuat konstruksi antisense dari gen penyandi enzim ODC (Ornithine Decarboxylase). Target gen diisolasi dari tanaman tembakau (*Nicotiana tabacum*) varietas Temanggung dengan metode *PCR cloning*. DNA genomik dari daun diisolasi dan dipakai sebagai cetakan dalam reaksi PCR. Optimisasi proses PCR dilakukan dengan mengubah suhu *annealing* dan jumlah siklus. Untuk lebih memastikan keabsahan fragmen yang didapat uji coba juga dilakukan dengan pasangan primer lain yang menghasilkan amplikon dalam ukuran yang berbeda.

INTRODUCTION

The Polymerase Chain Reaction (PCR) is an *in vitro* method to carry out the amplification of selected nucleotide (DNA or RNA) sequences through a succession of incubation at different temperatures (Hsu *et al.*, 1996). PCR amplification is commonly used as one of the methods to isolate a target gene. In this paper, we describe a PCR amplification to isolate a fragment of the putative gene encoding Ornithine Decarboxylase (ODC) from tobacco (*Nicotiana tabacum* L.). The objective of this experiment was to obtain a full length gene encoding Ornithine Decarboxylase (ODC) from tobacco. Once the target gene is isolated, an antisense construct will be generated and transformed into tobacco. This approach is commonly used to suppress the expression

of a given target gene (Fitchen & Beachy, 1993). Since ODC is a key enzyme in the nicotinic acid biosynthesis pathway (Figure 1.), suppressing the expression of the ODC gene will supress the production of nicotinic acid. In this case, theoretically we can produce a transgenic tobacco that will produce less or zero nicotinic acid and will have a marketable prospect.

In this paper we describe the optimization of the PCR experiment which includes variation of annealing temperature and number of cycles during PCR reaction to obtain a fragment of putative ODC gene

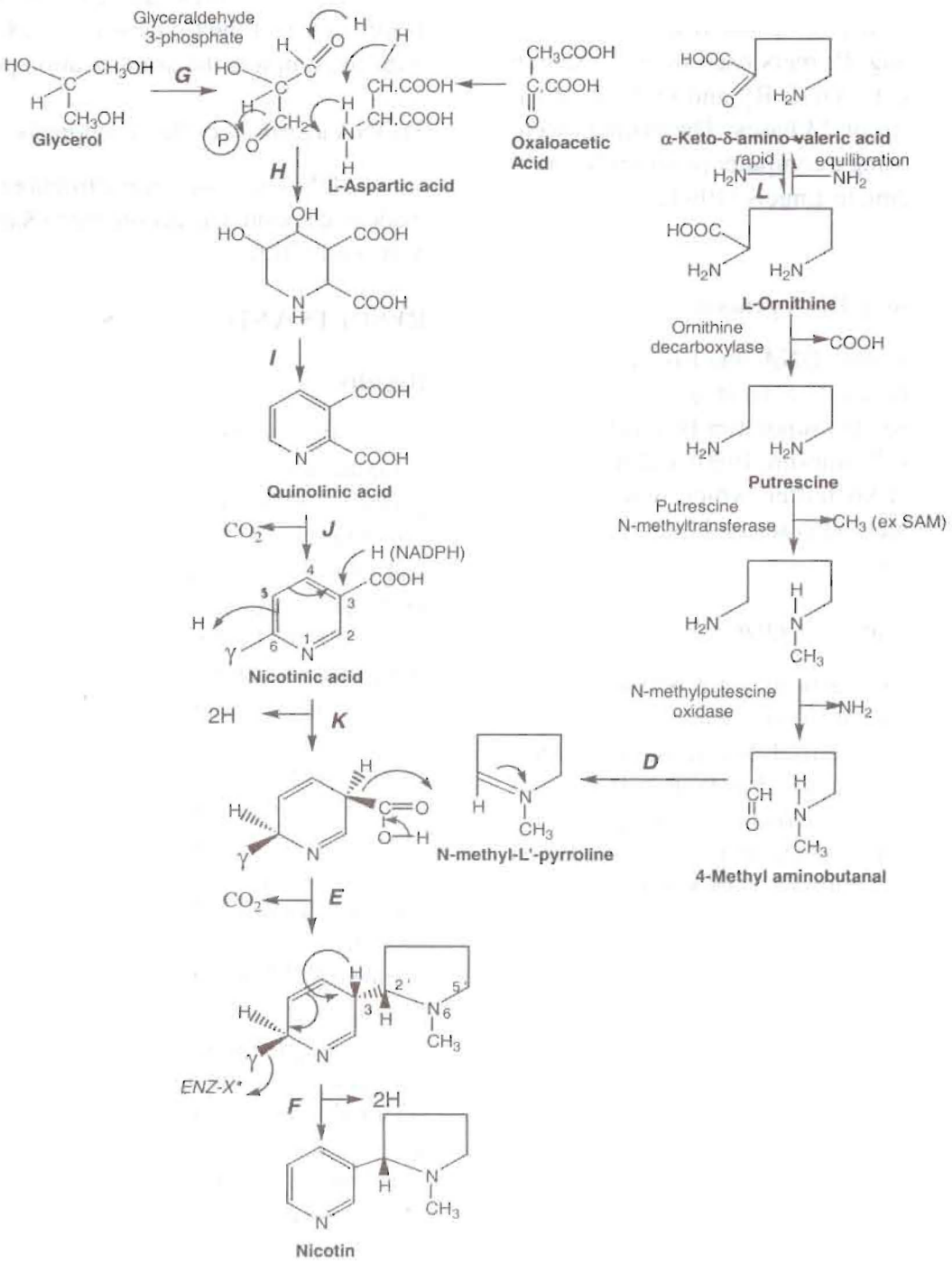


Figure 1. Nicotinic acid biosynthetic pathway (Goodwin & Mercer, 1983)

from tobacco as a preliminary step for the cloning of this gene.

MATERIALS AND METHODS

Materials

Genomic DNA was isolated from leaves of tobacco (*Nicotiana tabacum* L.) cv. Temanggung. Primers used in this research were ODC F_1, ODC R_1, and ODC R_2, each has a length of 23 bases. The primers were designed using a computer program Amplify 2.1 according to Engels (1993).

Methods

1) Isolation of DNA genome

Genomic DNA was isolated by using two methods, the first utilised CTAB (Cetyltrimethylammonium Bromide) buffer (Murray & Thompson, 1980) and the second, without CTAB buffer, which was modified from the method described by Sambrook & Russell (2001).

2) Amplification of *ODC* gene

PCR reactions were performed in a total volume of 50 µl. Each reaction was composed of 5 µl DNA template (DNA Genome) (10 ng/ µl), 5 µl PCR buffer 10x, 3 µl 50 mM $MgCl_2$ (final concentration 1.5 mM), 1 µl 10 mM dNTP (final concentration 0.2 mM), 0.25 µl *Taq* DNA polymerase (5 U/µl) (final concentration 1.25 Unit), 2.5 µl of the mixture ODC F_1 and ODCR_1 primers or a mixture of ODC F_1R_2 primers (final concentration 0.5 mM), and addition of ddH_2O to a total volume of 50 µl.

The sequences of the primers used in this experiment are as follows:

Amplifications of target DNA were done at first using annnealing temperature of 50°C (30 cycles) and secondly using annealing temperature of 48°C (50 cycles). Each PCR cycle consisted of 3 phases: denaturation at 94°C for 1 minute, annealing at 48°C or 50°C for 30 sec, and polymerization phase at 72°C for 3 mins. Temperature of the final cycle is 72°C for 10 mins to complete the amplification process.

3) DNA agarose gel electrophoresis

DNA agarose gel electrophoresis was made and conducted according to Sambrook & Russel (2001).

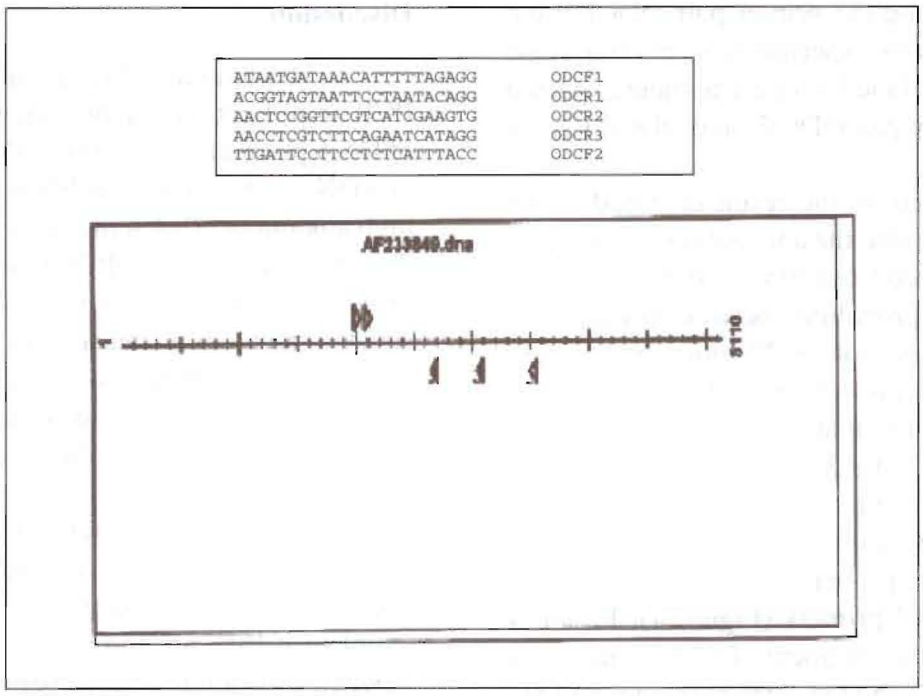
RESULTS AND DISCUSSION

Results

Primer designed using the Amplify 2.1 indicates that there are 2 possible forward primers (ODC F1 and ODC F2) and 3 possible reverse primers (ODC R1, ODC R2 and ODC R3) that can be used to isolate the *ODC* gene fragment (Figure 2).

ODC F_1 Primer has 21.73% GC percentage, ODC R_1 has 39.13% GC percentage, and ODC R_2 52.42% GC precentage. Melting temperature (Tm) of ODC F1 is 57.55°C, ODC R1 is 59.19°C, and ODC R2 is 60.42°C. In this experiment 3 primers were chosen to obtain 2 different amplification products. Based on Amplify 2.1 computer analysis, the combination ODC F1 and ODC R1 will yield 1.472 bp fragment, while ODC F1 and ODC R2 will yield a 1.021 bp fragment.

A preliminary experiment was conducted to obtain optimum PCR conditions for amplification using primer pairs described above. PCR parameters being optimised are the annealing temperature and the number of cycles used. The result from first PCR trial using both primer pairs with annealing temperature of 50°C and 30 cycles is presented in Figure 3. The fragment obtained



Notes: triangles pointing to the right are forward primers (ODCF1 & ODCF2); triangles pointing to the left are reverse primers (ODCR1, ODCR2 & ODCR3).

Figure 2. Primer design using Amplify 2.1

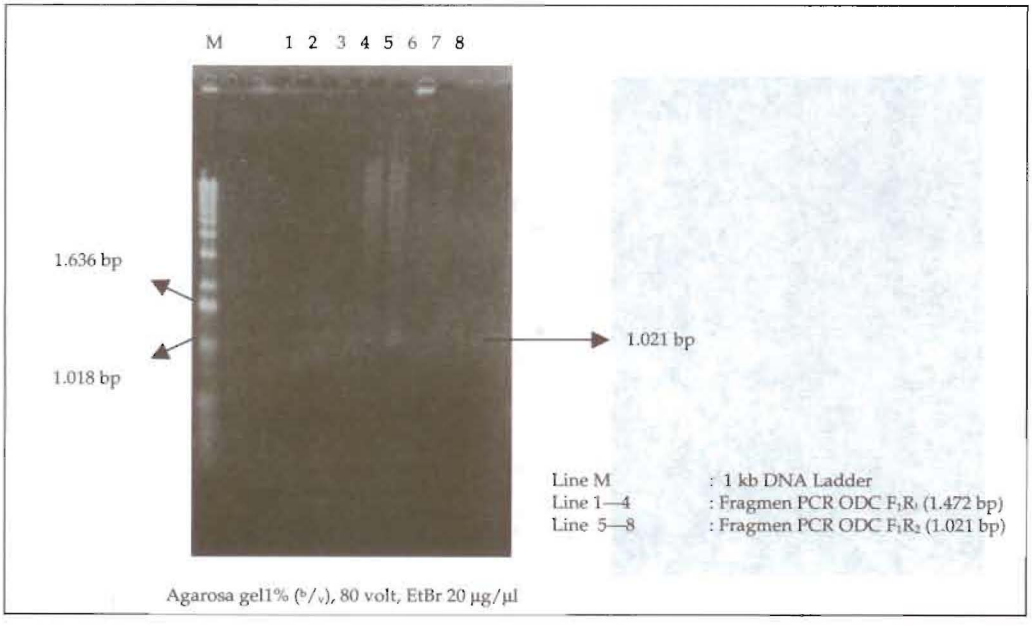


Figure 3. Amplification of fragment *ODC* gene from *Nicotiana tabacum* L. cv. Temanggung at 50°C and 30 cycles

by PCR using the primer pair ODCF1 and ODCR2 in this experiment is very thin (lane 5 – 8) and in lane 1 – 4 the fragment expected using primer pair ODCF1 and ODCR1 is not visible.

Based on the result obtained in the first experiment, the annnealing temperature was decreased from 50°C to 48°C to increase the binding probability between primers and template. Addition of 20 more cycles were done to obtain the desired amplicon in a higher quantity that can be detected clearly in the gel (Figure 3).

Using the improved PCR conditions, amplicons were obtained both using ODC F1 and ODC R1 primer pair as well as ODC F1 and ODC R2 primers (Figure 4). Figure 4 shows that the fragment of *ODC* gene could be amplified and the sizes correspond to the expected sizes based on the Amplify 2.1 analysis.

Discussion

The amount of template needed for PCR amplification is approximately 1-10 ng of DNA plasmid or 20 – 300 ng DNA genome or cDNA. According to Elion (1997), too high amount of DNA template will influence the effectiveness of the PCR reaction. In this experiment 50 ng genomic DNA template isolated from tobacco leaves was used. In this case, the ample amount of template was used thus eliminating the amount of template as a factor that will influence the success of PCR amplification.

Generally, the length of primers commonly used is 17-34 base pairs. The primers will have difficulty to bind to the template if they are too short while on the other side the longer the primer will produce non specific amplicons (Elion, 1997). In this case, this factor was eliminated by designing the length of the primers between those

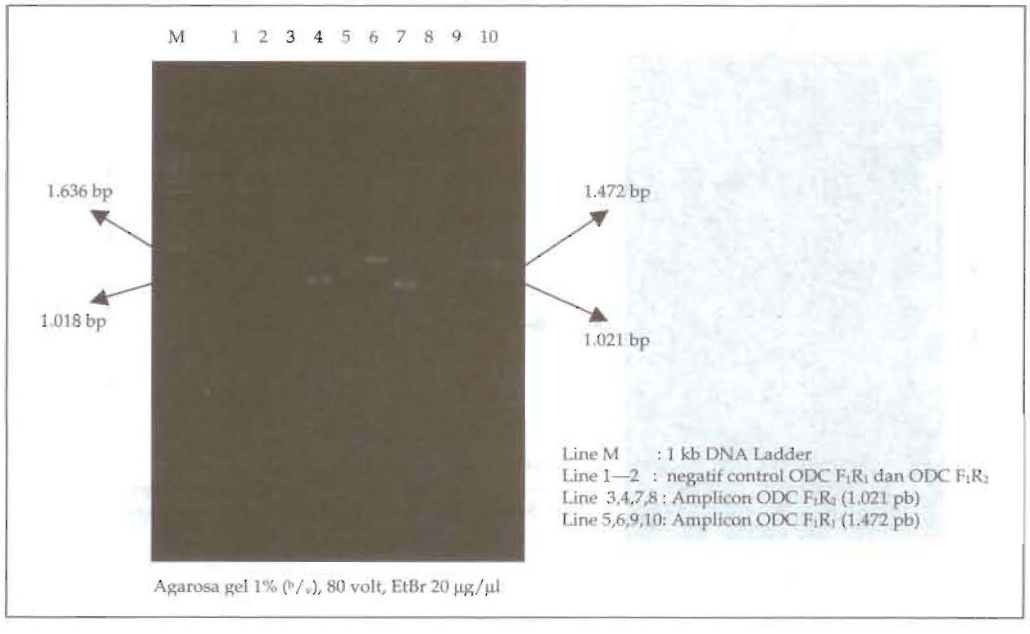


Figure 4. Amplification of *ODC* gene from *Nicotiana tabacum* L. cv. Temanggung at 48°C, with 50 cycles

numbers. ODC F1, ODC R1 and ODC R2 are composed of 23 base pairs. Additionally, those three primers were designed to have GC at the 3' end which enables them to bind better to the template. According to Sambrook & Russell (2001) the ideal melting point difference between primers should not exceed 5°C. Melting point of ODC F1 is 57.55°C, ODC R1 is 59.19°C and ODC R2 is 60.42°C which indicates the melting point difference is less than 5°C.

The amount of a primer in a standard reaction is unlimited. However, the concentration used is usually between 0.5 – 1.0 μM. That concentration is enough for 30 cycles amplification to obtain 1 kb fragment. Too high concentration of a primer will cause mispriming while too low concentration will cause inefficient amplification (Muladno, 2002). In this experiment 0.5 μM primer concentration was used.

Specificity of an amplification process is influenced by several factors including the annealing temperature. Annealing temperature depends on the length and GC content of the primers used in the PCR reaction. ODC F1, ODC R1 and ODC R2 have 21.73%, 39.13% and 52.17% GC content, respectively. All of those primer are 23 bases long. According to Saiki (1989), 55°C is a good starting point for primer with 50% GC and 20 bp length. However, in this experiment 50°C was used because the GC content of the primers are relatively low. Identifying the optimum annealing temperature is very important for the success of a PCR reaction because too low temperature will cause unspecified PCR products while too high temperature will cause difficulty for the primer to bind to the template (Hsu *et al.*, 1996).

Generally, the temperature setting for polymerization is between 72°C – 80°C. In this experiment 72°C was used in order to eliminate the polymerization temperature as the limiting factor in the PCR reaction. During polymerization, Taq DNA polymerase initiates its activity on extending the DNA from 3'end. The velocity of the extension by the enzyme is approximately 35 to 100 nucleotides per second depending on the buffer used, pH, salt concentration and the DNA target molecule. In order to obtain the 1.472 bp and 1.021 bp amplicons, 4 mins amplification was used which is equal to 30 cycles. However, based on the first trial in which a very faint band was obtained (Figure 1), the cycle was increased from 30 to 50 cycles (10 mins) and finally visible amplicons were obtained from both ODC F1 and ODC R1 as well as ODC F1 and ODC R2 primer pairs (Figure 2). According to Yu & Pauls (1994), to amplify a 1.0–1.5 kb target fragment, 10–15 mins polymerization period is required to obtain a perfect double stranded fragment.

The first trial of PCR amplification using 50°C and 30 cycles as the annealing temperature showed a very faint band at 1.472 bp using primer pair ODC F1R1 which is the expected size for that primer pair. On the other hand, when the annealing temperature was decreased to 48°C and using 50 cycles amplicons were obtained both from ODC F1 and ODC R1 as well as ODC F1 and ODC R2 pairs (Figure 2). This result indicates that the ODC F1 and ODC R1 as well as ODC F1 and ODC R2 primer pairs require a relatively low annealing temperature to bind to the template. This is probably caused by the nature of those primers.

CONCLUSIONS

The annealing temperature and the number of cycles used in a given PCR are two important factors during PCR process for obtaining a correct and perfect amplicon.

The results from this experiment can be used for further effort to clone the gene encoding ODC from tobacco.

REFERENCES

Elion, E.A. 1997. Constructing recombinant DNA molecules by polymerase chain reaction. http://www.does.org/masterli/cpmbo317.htm, accessed on 3 November 2002.

Engels, W.R. 1993. Contributing software to the Internet: The amplify program. *Trends Biochem. Sci.* 18: 448–450.

Fitchen, J.H. & R.N. Beachy. 1993. Genetically engineered protection against viruses in transgenic plants. *Ann. Rev. Microbiol.* 47: 739 – 763.

Goodwin, T.W. & E.I. Mercer. 1983. Introduction to Plant Biochemistry. 2nd ed. Pergamon Press, Oxford.

Hsu, J.T., S. Das & S. Mohapatra. 1996. Polymerase chain reaction engineering. *Biotechnol. Bioeng.* 55 (2): 359-366.

Muladno. 2002. Seputar Teknologi Rekayasa Genetika. Pustaka Wirausaha Muda, Bogor.

Murray, M.G. & W.F. Thompson. 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acid Res.* 8: 4321–4325.

Saiki, R.K. 1989. The design and optimization of the PCR. In: Erlich, H.A. (ed.). 1989. *PCR Technology: Principles and Applications for DNA Amplification*. Stockton Press, New York: 7-16.

Sambrook, J. & D.W. Russell. 2001. *Molecular Cloning A Laboratory Manual*. 2nd ed. Vol 1. CSHL Press, New York.

Yu, K. & K. P. Pauls. 1994. Optimization of DNA extraction and PCR procedures for random amplified polymorphic DNA (RAPD) analysis in plants. In: Griffin, H.G. & A. M. Griiffin (eds.), PCR Technology: Current Innovations. CRC Press, Boca Raton: 193 – 200.