Research Article

**PCR AMPLIFICATION OF TABERSONINE 16-HYDROXYLASE GENE FROM THE LEAVES OF CATHARANTHUS ROSEUS**

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**ABSTRACT**

Hydroxylation of tabersonine by tabersonine 16-hydroxylase is a rate limiting step in the biosynthesis of terpenoid indole alkaloids (TIAs) in *Catharanthus roseus*. Total RNA was isolated from *C. roseus* leaves using three different methods of which isolation with TRIzol® gave the best result. Phusion high-fidelity DNA polymerase amplified t16h gene from *C. roseus* cDNA while Taq polymerase failed due to high A-T content of t16h gene which needs proofreading DNA polymerase to increase the amplification efficacy.

**Keywords:** *C. roseus*, PCR, tabersonine 16-hydroxylase, polymerase, amplification efficacy.

**INTRODUCTION**

*Catharanthus roseus* was found to produce more than 130 monoterpenoid indole alkaloid that have several medical properties1-4. The most important anti-tumor agents, vincristine and vinblastine, are produced in its leaves. Low anticancer alkaloid contents in *C. roseus* has encouraged the intense research for alternative methods for production such as cell cultures, metabolic engineering5-6 semi-synthesis7,8, or even the total chemical synthesis9-13. Total synthesis was found to be difficult due to the structural complexity of the molecules and also the complicated reaction steps that involves the stereochemical reactions. Various semi-synthetic procedures were developed for these alkaloids on the basis of their chemical7 or enzymatic8 coupling of the commercially available catharanthine and vindoline.

Tabersonine is transformed into vindoline by a sequence of seven steps, the first step of which is aromatic hydroxylation, catalysed by tabersonine 16-hydroxylase (T16H) yielding 16-hydroxytabersonine14,15 (figure 1). The activity of T16H was first detected in total protein extracts from young leaves of *C. roseus*, and it was induced by light16.

T16H enzyme was shown to be localized in the endoplasmic reticulum16. Also, it was confirmed that the activity of T16H is located in the epidermal cells of the leaves, identifying these cells as the major site of vindoline biosynthesis17. Study conducted on 50 different cultivars of *C. roseus* showed that only one cultivar accumulated 10 times less vindoline compared to the others and enzymatic studies demonstrated that this low accumulating vindoline cultivar has 10 times lower T16H activity compared to the others18. The aim of this study is to PCR-amplify t16h from the young leaves of *C. roseus*.

**MATERIALS AND METHODS**

Hettich Mikro 220R centrifuge (Tuttlingen, Germany) was used for centrifugation under cooling. SDE-PLAS horizontal electrophoresis unit connected to Consort E865 electrophoresis power supply (Turnhout, Belgium) and a Syngene UV transilluminator (Cambridge, UK) was used for running and visualisation of agarose gels. PCR reactions were done using a FTC4/F02 thermal cycler (Staffordshire, UK). Phusion high-fidelity DNA polymerase kit was purchased from New England Biolabs (Ipswich, Massachusetts, USA). Trizol® reagent and dream Taq master mix were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). RNAsimple total RNA kit and FastQuant RT kit were purchased from Tiangen (Sichuan, China). HyperLadder™ 1kb was purchased from Bioline (London, UK).

**Total RNA isolation**

Young leaves of *C. roseus* (collected from Genetic Engineering and Biotechnology Research Institute, Sadat City University, Sadat city, Egypt, August 2017) were grinded using liquid nitrogen to isolate total RNA using three different methods. The first method (chemical method) followed the previously published RNA isolation protocol which depends on using guanidine thiocyanate in sample homogenization19. The second method is by using RNAsimple total RNA kit following the manufacturer’s manual while in the third method TRIzol® reagent was used according to the manufacturer’s protocol.

**Amplification of t16h-cDNA**

FastQuant RT kit was used to synthesize first strand cDNA from TRIzol® isolated RNA according to the manufacturer’s manual. The 1305 bp fragment of t16h gene was amplified using phusion...
Restriction sites were used in the same amino acid forward primer to decrease primer dimer formation, they encode hairpin formation. Primers and check for primer self used to calculate $R_1$ and 5ʹ-ATGCACTTAAAATTGGTGAA-3ʹ as a forward primer (F1) and 5ʹ-TCAAGCAGGAGAAGGAA- 3ʹ as a reverse primer (R1). Oligo Calc tool (oligonucleotide properties calculator) was used to calculate the annealing temperatures of the designed primers and check for primer self-dimerization and potential hairpin formation. Guanine-12 was replaced by adenine in the forward primer to decrease primer dimer formation, they encode the same amino acid Lys-4. New primers that contain G-C rich restriction sites were used in the amplification to study the effect of increasing G-C content of primers in the efficacy of amplification. The 1328 bp fragment for t10h with G-C rich restriction sites was amplified by plusion high-fidelity DNA polymerase using the primers (5ʹ-CGCGCCATGGAGA TGCACCTAAAATTTGTGAAG-3ʹ, forward F1) and (5ʹ- TATGCGCCCTCAAGCAGGAGAGGA -3ʹ, reverse R2) that contain restriction sites for Ncol and Not1 at the forward and reverse ends of the open reading frame, respectively. Trials for amplification of t10h gene using Taq DNA polymerase were also performed.

![Figure 1: Biosynthesis of vindoline from tabersonine in C. roseus. (OMT) O-methyl transferase; (T3O) tabersonine 3-oxygenase; (T3R) tabersonine 3-reductase; (NMT) N-methyl transferase, (D4H) desacetoxyvindoline-4-hydroxylase; (DAT) deacetylvinodline-4-O-acetyltransferase](image1)

![Figure 2: Agarose gel electrophoresis of the isolated total RNA in triplicate. (A) using chemical method; (B) using RNAsimple total RNA kit; (C) using TRIZol®](image2)
RESULTS AND DISCUSSION

Total RNA isolation

Total RNA isolated using chemical method, RNAsimple total RNA kit and TRIzol® was analyzed by agarose gel electrophoresis to determine which method gave the highest quality and quantity (figure 2). The result indicated that extraction with TRIzol® is the best method for total RNA isolation from plant tissue. It produced high quality and quantity of total RNA (figure 2, panel C) in a single step that maintains the integrity of the isolated total RNA. Extraction with chemical method resulted in low yield (figure 2, panel A), while extraction with RNAsimple total RNA kit produced low quality RNA (figure 2, panel B).

PCR amplification

Phusion high fidelity DNA polymerase succeeded to amplify the A-T rich t16h gene (Caros001600.1, A-T content of t16h = 65.1%) while Taq DNA polymerase failed (figure 3). It is well known that the efficacy of G-C rich or A-T rich fragment amplification increased by using DNA polymerase with 3'-exonuclease activity and using an enhancing agent like DMSO and betaine. Accordingly, the 1305 bp band of t16h gene appeared on agarose gel electrophoresis after using phusion high-fidelity DNA polymerase kit which contains DMSO (figure 3, panel A). The obtained band is consistent with the length of t16h published sequence. Taq DNA polymerase failed in the amplification trails as it does not have 3'-exonuclease activity. Increasing G-C content of the used primers may also increase the amplification efficacy. Slightly higher concentration of t16h was achieved using primers that were designed to include restriction sites for NcoI and NotI at the forward and reverse ends of the open reading frame (1328 bp), respectively (figure 3, panel B).

CONCLUSION

Phusion high-fidelity DNA polymerase amplified the A-T rich t16h gene from C. roseus cDNA. Adding G-C rich restriction sites to the designed primers increased the amplification efficacy.

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