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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 properties[1-4]. 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 engineering[5,6] semi-synthesis[7,8], or even the total chemical synthesis[9-13]. 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 chemical[7] or enzymatic[8] 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-hydroxytabersonine[14,15] (figure 1). The activity of T16H was first detected in total protein extracts from young leaves of *C. roseus*, and it was induced by light[16].

T16H enzyme was shown to be localized in the endoplasmic reticulum[16]. 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 biosynthesis[17]. 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 others[18]. 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 homogenization[19]. 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

high-fidelity DNA polymerase under the following conditions: 98 °C for 30 sec, followed by 35 cycles of: 98 °C for 12 sec, 57 °C for 30 sec, 72 °C for 45 sec, then hold at 72 °C for 7 min using 5′-ATGCACTTAAAAATTGGTGAAG-3′ as a forward primer (F1) and 5′-TCAAGCAGGAGAAGAGGAA- 3′ 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 *t16h* with G-C rich restriction sites was amplified by phusion high-fidelity DNA polymerase using the primers (5′-CGCGCCATGGAGA TGCACTTAAAAATTGGTGAAG-3′, forward F2) and (5′-TATGCGGCCGCTCAAGCAGGAGAAGAGGAA -3′, reverse R2) that contain restriction sites for *Nco*1 and *Not*1 at the forward and reverse ends of the open reading frame, respectively. Trials for amplification of *t16h* gene using *Taq* DNA polymerase were also performed.

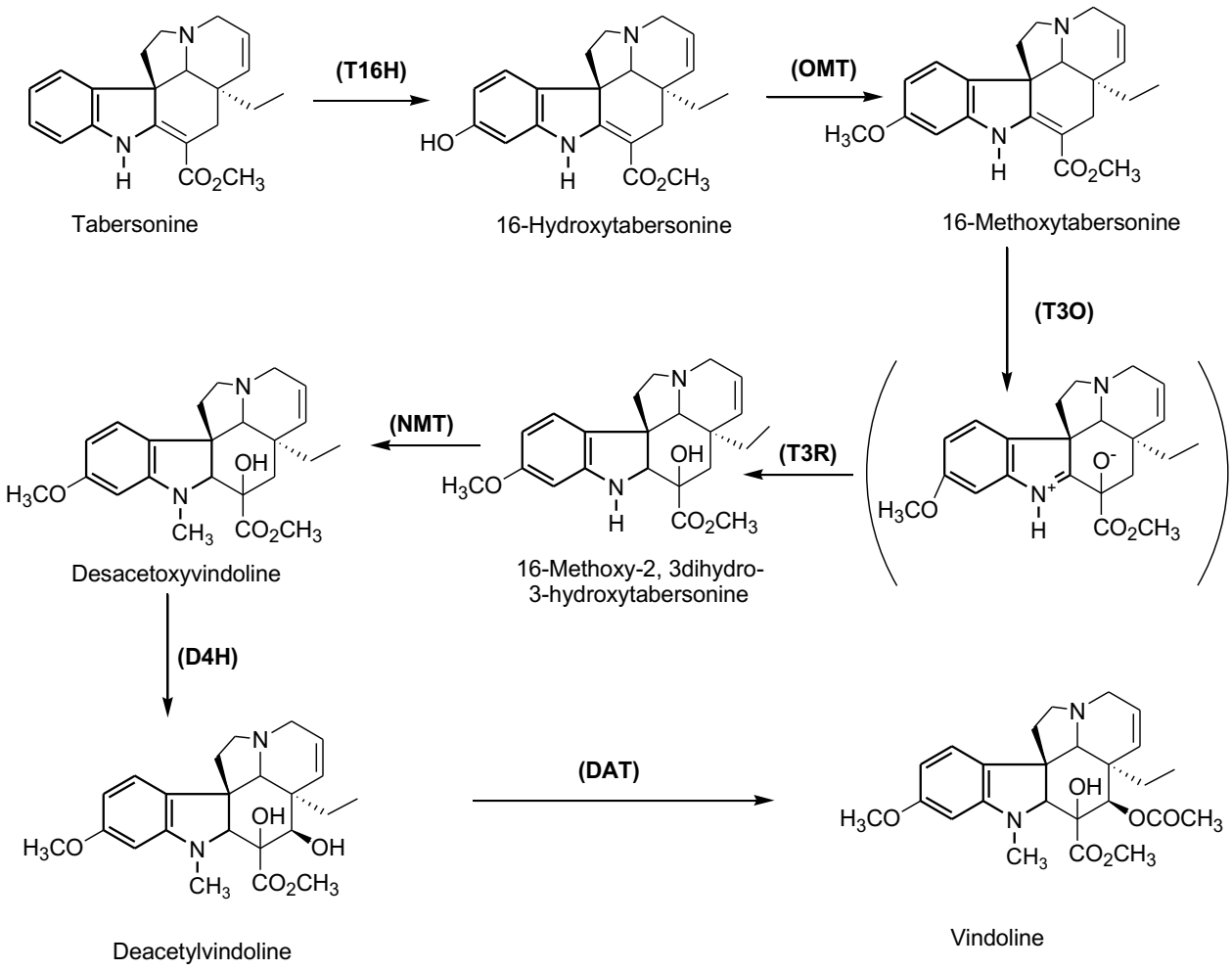

Figure 1: Biosynthesis of vindoline from tabersonine in *C. roseus*. (OMT) *O*-methyl transferase; (T3O) tabersonine 3-oxygenase; (T3R) tabersonine 3-reductase; (NMT) *N*-methyl transferase, (D4H) desacetoxyvindoline-4-hydroxylase; (DAT) deacetylvindoline-4-*O*-acetyltransferase

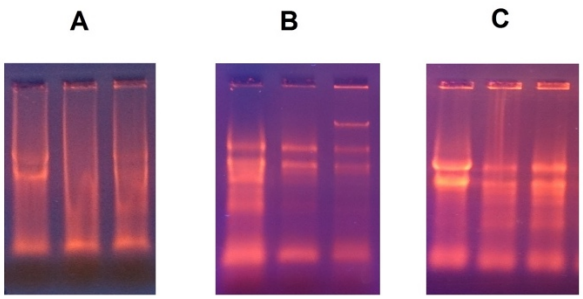

Figure 2: Agarose gel electrophoresis of the isolated total RNA in triplicate. (A) using chemical method; (B) using RNAsimple total RNA kit; (C) using TRIzol®

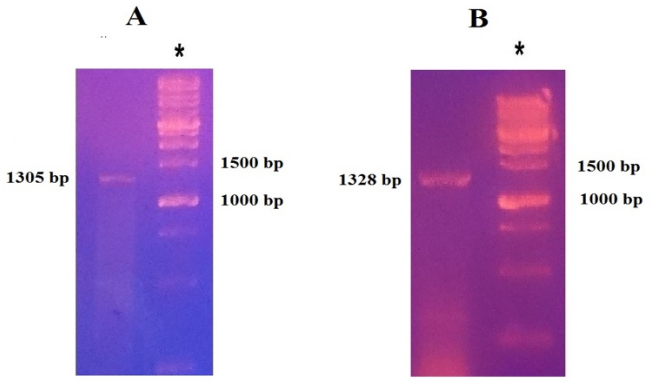

Figure 3: Agarose gel electrophoresis showed amplified *t16h*. (A) using F1 and R1 primers; (B) using F2 and R2 primers. * refers to HyperLadder™ 1kb.

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[20]. 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[20]. 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[21,22] or A-T rich[23] fragment amplification increased by using DNA polymerase with 3'-exonuclease activity and using an enhancing agent like DMSO and betaine[24-26]. 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[27]. Slightly higher concentration of *t16h* was achieved using primers that were designed to include restriction sites for *Nco*1 and *Not*1 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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