RESEARCH ARTICLE Legume Research- An International Journal, Volume 43 Issue 3: 326-331 (June 2020)

Cloning and optimizing the expression of the *DHDPS* gene in the *Medicago truncatula*

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ABSTRACT

Medicago truncatula seeds were cultured and developed in Thua Thien Hue province, Vietnam and they were used as materials for cloning a *DHDPS* gene with the encoding of the isozyme dihydrodipicolinate synthase (*DHDPS*) as well as optimizing the culture conditions for having the highest *DHDPS* gene expression in *Escherichia coli* BL21 StarTM (DE3) cells. The results revealed that the coding region of the *DHDPS* gene from *M. truncatula* was 100% similar with *M. truncatula* 4-hydroxy-tetrahydrodipicolinate synthase 2 (*DHDPS2)* submitted in NCBI (accession number: XM_013589555.2), coding for a long polypeptide of 307 amino acid with the molecular mass of about 33495 Da (Protein ID: XP_013445009.1). The *DHDPS* gene was successfully expressed in the *Escherichia coli* BL21 StarTM (DE3) cells with a pET200 / D-TOPO vector and this produced the *DHDPS2* protein with molecular masses of approximately 33.87 kDa (≈33.5 kDa of *DHDPS2* and 3.7 kDa of fusion fragment of pET 200/D-TOPO vector). The effects of the six different culture mediums of LB, SOB, SOC, YJ, HSG and TB, the induction times of 2h, 4h, 6h, 8h, 10h and 12h and the inducer concentrations of 0.2 mM; 0.5 mM; 0.7 mM; 1.0 mM; 1.2 mM and 1.5 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) were also investigated for the purpose of optimising the expression of *DHDPS2* in *E. coli* cells, and it was found that strong expression of recombinant *DHDPS2* protein *in E. coli*. BL21 (DE3) cells occurred on the TB, HSG and YJ culture mediums after 8 hours with 0.2 mM inducible IPTG (BioRad).

Key words: *DHDPS* gene, Expression, *Medicago truncatula*, 4-hydroxy-tetrahydrodipicolinate synthase 2 (*DHDPS2*).

INTRODUCTION

Medicago truncatula is a member of the Medicago genus and belongs to legumes group. *M. truncatula* is a native plant from the Mediterranean Basin and has also become naturalized in other regions of the world, particularly on the American and Australian continents, following European migrations (Delalande *et al.,* 2007). Up to now, *M. truncatula* was used as model specie for doing research of legume family (Rose, 2008). The famous characteristic of *M. truncatula* is that its populations display good tolerance to drought, salinity (Elmsehli *et al*., 2015) and are grown in a wide range of soils and environmental conditions (Manoj *et al*., 2015). It is cultivated to avoid soil erosion, improve soil fertility and as a source of winter forage (Huguet and Prosperi, 1995). *Medicago truncatula* is annual, diploid (2n=16) and autogamous because of its relatively small genome size (550Mbp) and the short generation time of 8 to 10 weeks is extremely useful for laboratory-scale genetic studies (Frugoli, 2008; Huguet and Prosperi, 1995). The most interesting aspect in *M. truncatula* studies is that its lysine biosynthesis, in which dihydrodipicolinate synthase (*DHDPS*, EC 4.2.1.52) occurs delete as the key regulatory enzyme that participates in this metabolism (Zhu *et al*., 2002; Stepansky *et al*., 2005). *DHDPS* is the first enzyme of the lysine-specific branch of the biosynthetic pathways for aspartate-derived amino acids.

According to the present published documents, *DHDPS* has been isolated from different plant sources such as maize (Frisch *et al*., 1991), wheat (Kumpaisal *et al*., 1987), pigeon pea (Thu *et al*., 2007), pea (Dereppe *et al*., 1992), tobacco (Ghislain *et al*., 1990), *Arabidopsis* (Vauterin *et al*., 1999), rice (Sikdar *et al.,* 2010). The previous study reported that the *DHDPS* gene family in *M. truncatula* consists of at least four isogenes of *DHDPS1, DHDPS2, DHDPS3* and *DHDPS4* which encode four DHDPS isozymes of *DHDPS1* and *4*, *DHDPS2* and *DHDPS3*, respectively. Remarkably, among these DHDPS isozymes, the expression of *DHDPS2* transcripts and specific *DHDPS2* activity was about ten-fold lower specific compared to the *DHDPS1* and the expression of the mature protein in *E. coli* confirmed that a much lower lysine sensitivity of the *DHDPS2* isozyme compared to other plant *DHDPS* (Ellen *et al*., 2003).

M. truncatula seeds were received from Laboratory of Plant Genetics, Institute for Molecular Biology and Biotechnology, Vrije Universiteit Brussel (VUB) to cultivate and used as material in this research. The seeds were successfully germinated and cultivated and the plants do indeed thrive in Thua Thien Hue, Vietnam. This paper deals

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How to cite this article: Hong, H.T.K., Trang, P.T.H.,Long, D.T. and Trang, N.T.Q. (2020). Cloning and optimizing the expression of the *DHDPS* gene in the *Medicago truncatula*. Legume Research. 43(3): 326-331.

Submitted: 26-02-2019 **Accepted:** 29-03-2019 **Published:** 24-05-2019

with the results of the cloning and expressing of sequences encoding for the DHDPS isozyme from three weeks old leaves of *M. truncatula,* as well as optimizing its cultured condition in order to observe its expression in *Escherichia coli* BL21 StarTM (DE3) cells.

MATERIALS AND METHODS

Plant material and chemical preparation

Medicago truncatula seeds were grown and developed according to the method of Ellen *et al*. (2013) with slight modifications. The quality seeds were washed under running tap water and sterilized with 5 ml of concentrated sulphuric acid solution under the sterile laminar flow and washed thoroughly with sterile distilled water. Finally, the seeds were transferred to a petri dish and 5 ml of sterile distilled water were added with a pipette. The petri dish was covered by paraffin and kept it in a culture room (23°C; 16-h/8-h light/dark cycle at 30mmol m^{-2} s^{-1}). Germinating seedlings were transferred to a hydrophonic system. After that, they were transferred to grow under natural environmental conditions. Almost all the plants were developed well in the hydroponic system, using leaf material from three individual plants which were employed to constitute one biological replicate.

DNA isolation and PCR amplification of the *DHDPS* gene

Genomic DNA of *M. truncatula* was extracted from three weeks old leaves of *M. truncatula* via the improved CTAB (cetyltrimethyl ammonium bromide) method (Yan *et al.,* 2018) and used as template in PCR amplification. Primer-BLAST was used to design primers and the specific primers *DHDPSF*: 5′-CACCATGAATGTTAGGAAATCGATTGACG-3′ and *DHDPSR*: 5′-ATACCGACCAACTATGATAAAGTCTTC-3′), which was designed base on the coding DNA sequence (CDS) for the *DHDPS* gene (accession number on Genbank XM_013589555) was used for PCR. The concentrations of DNA were quantified by measuring absorbance using a NanoDrop (ND-1000) at 260-280 nm. To enable directional cloning further, the forward primers contain the CACC sequence (adapter) at the 5′ end. These four nucleotides form a base pair with the GTGG overhang sequence in pET200/D-TOPO vector (Loc *et al*., 2013). For PCR amplification, two µl of DNA extracted genome (20ng/ml) was amplified in a 25 µl reaction mixture containing 1µM of each primer (10 pmol/ml), 12.5 µl of Master mix (Fermentas, Canada) (2.4 mM each deoxyribon- ucleotide) (dNTP) and 0.3 units Taq DNA polymerase (Fermentas, Canada) in the thermocycler (iCycler, BioRad). The thermocycling profile was as follow: initial denaturation at 95°C for 5 minutes; 30 repeated cycles of 95°C for 1 minute, 45°C for 1 minute and 72°C for 1 minute and a final extension of 72°C for 10 minutes and the end at 4°C.

The PCR product was electrophoresed with 1% agarose gel at 80 volts in 1×, TAE buffer and stained with ethidium bromide (0.5 µg/L) for 15 minutes. The stained gel was photographed under UV light using the Gel Documentation system (Bio-Rad).

Cloning and sequencing of *DHDPS* gene

The PCR product was eluted from 1% agarose gel and purified by KIT Isolate II PCR and Gel (Bioline, USA) and it was inserted into pGEM®-T Easy vector (Promega, USA) according to the TA cloning method. The ligation component consisted of 50 ng pGEM®-T Easy vector (50 ng/µl), 5 µl buffer, 1µl T4 DNA ligase (3 unit/µl) and 3 µl PCR product (15 ng/µl). Distilled water was added to a make up a final volume of 10 µl. The ligation was incubated at 25°C for 1 hour and at 4°C overnight, and then ligation products were transformed into *E. coli* TOP10 cells (Invitrogen, USA) by the heat-shock method. The presence of the insert was determined by colony direct PCR followed by 1% agarose gel electrophoresis. Positive colonies (white colonies) were cultured on 5 ml of LB medium of 1% peptone, 1% NaCl, 0.5% yeast extract, 1.5% agar, pH: 7.0) supplemented with 50 µg/ml ampicillin, 100 mM Isopropylthio-β-galactoside (IPTG) and 20 mg/ml X-Gal for biomass production. Recombinant pGEM®-T Easy vector was then isolated by EZ-10 Spin Column Plasmid DNA MiniPreps Kit, BS614 (Bio-Base INC). The nucleotide sequence of *DHDPS* gene was analysed by the method of dideoxy terminator and they were compared with nucleotide sequences from the GenBank database using the BLAST program from www.ncbi.nlm.nih.gov/BLAST.

Expression of *DHDPS2* protein

The PCR product from recombinant pGEM®-T Easy vector was eleuted from 1% agarose gel and purified by KIT Isolate II PCR and Gel (Bioline, USA), they were then ligated to a pET200/D-TOPO expression vector harboring T7 promoter (Invitrogen). The ligation component consisted of 20 ng pET200/D -TOPO vector, 1 ml salt solution and 9.6 ng PCR product. Distilled water was added to a final volume of 6 µl. The ligation was incubated at 25°C for 60 min. Recombinant pET200/D - TOPO vector was then transformed into *E. coli* StarTM BL21 (DE3) cells (Invitrogen) according to the manufacturer's instructions. Expression of recombinant *DHDPS2* protein in transformed *E. coli* StarTM BL21 (DE3) was first performed at 37°C on YJ medium culture of 2% glycerol, 1.5% tryptone, 2% yeast extract, 0.25% $K_2HPO_4.12H_2O$, 0.016% KH_2PO_4, 0.05% NaCl and 0.025% $MgSO_4.7H_2O$ (Yang *et al*., 2008), supplemented with 1% glucose and 100 µg/ml kanamycin. The culture was carried out on a rotation shaker with a speed of 200 rpm to an OD600 of 0.8. Isopropyl b-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM for induction. Next assay for optimizing the expression of recombinant *DHDPS2* protein in transformed *E. coli* StarTM BL21 (DE3), the effect of induction time after 2h, 4h, 6h, 8h, 10h and 12h, as well as the concentration of IPTG for induction, were investigated varies from 0.2 to 1.5 mM, respectively.

After optimizing the time and concentration of IPTG induction, the different medium cultures were investigated with (1) LB medium: 0.5% yeast extract, 1% peptone and 1% NaCl and 1.5% agar (Vulfson *et al*., 2001), (2) SOB

medium culture: 2% peptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 10 mM $MgSO_4$ (Shen *et al*., 2007), (3) SOC medium culture: 2% peptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose (Shen *et al*., 2007), (4) HSG medium culture: 1.49% glycerol, 0.7% yeast extract, 1.35% tryptone, 0.014% $MgSO_4.H_2O$, 0.15% KH_2PO_4, 0.23% K_2HPO_4 and 0.5% (Miksch *et al*., 2008) and (6) TB medium culture: 1.2% peptone, 2.4% yeast extract, 72mM K_2HPO_4, 17mM KH_2PO_4 and 0.4% glycerol (Shen *et al*., 2007) supplemented with 1% glucose and 100 µg/ml ampicillin. The culture was carried out on a rotation shaker with a speed of 200 rpm to an OD600 of 0.8. The cell biomass from each above medium culture assay was harvested by centrifugation at 15000 rpm/4°C for 1 min. The freezing and thawing at 42°C were performed with three repeats to break cells and total soluble protein was obtained by extraction according to Champion™ pET 200 Directional TOPO® expression kit (Invitrogen, USA). The expression level of 6xHis-DHDPS was assayed by electrophoresis on 15% (w/v) polyacrylamide gel with sodium dodecyl sulfate (SDS-PAGE) at 80V. The gel was then stained with Coomassie Blue R-250 and image was analysed by Quality One software (ver 4.1, BioRad).

RESULTS AND DISCUSSION

Cloning and sequencing of the DHDPS gene

The PCR amplification of the mature peptide coding sequence of *DHDPS* gene from genomic DNA of *M. truncatula* leaf has been shown in Fig 1A. The PCR product had a band with a length of 922 bp detected using bioinformatics software. PCR product was purified and inserted into pGEM®-T Easy vector of *E. coli* cells. The presence of the insert in transformed bacterial cells were determined by PCR amplification with M13 primers of vector and the results is in Fig 1B. DNA bands as shown in Fig 1B.

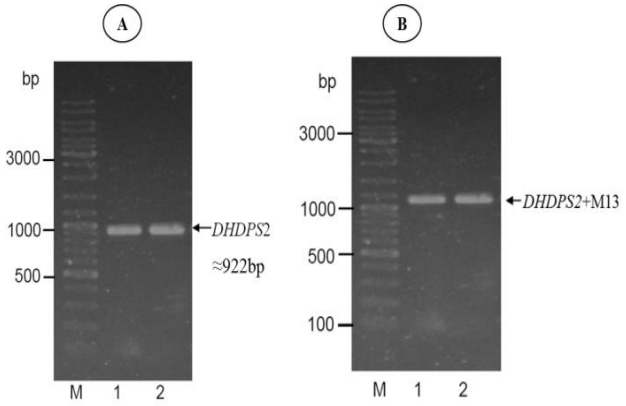

Fig 1: PCR amplification of the mature peptide coding sequence of *DHDPS* gene (accession no: XM_01358955.2) from genomic DNA of *M. truncatula*. M: DNA size marker (100-10000bp, GeneRuler™ DNA Ladder), 1 (A): PCR product of *DHDPS-F/DHDPS-R* specific primer pair, 1 (B): PCR product of M13 primer pair of pGEM®-T Easy vector.

It was ~1123 bp in length (923 bp of insert and 200 bp of primer regions on vector).

Fig 2 display the sequencing profile of the *DHDPS* gene (accession no: XM_01358955.2) with 100% homology with *DHDPS* gene. had about 922 nucleotides. The BLAST search showed that the nucleotide and the amino acid sequences of *DHDPS* gene from *M. truncatula* leaf was 100% homology with *M. truncatula* 4-hydroxy-tetra-hydrodipicolinate synthase 2 (*DHDPS2)* (accession number: XM_013589555 from nucleotide 117 to 1037), coding for a long polypeptide of 307 amino acid with molecular mass of about 33495 Da from NCBI (Protein ID: XP_013445009.1) (Fig 3).

Expression of *DHDPS2* gene

Expression of recombinant *DHDPS2* polypeptides in *E. coli*. BL21 (DE3) cells on YJ culture medium with 0.5 mM IPTG induction, after analyzed through SDS-PAGE has been shown in Fig 4. The protein bands of three repeats transformed *E. coli* cells with 0.5 mM IPTG induction after 5h (lane 1, 2 and 3) from SDS-PAGE gel as shown in Fig 4 revealed that the expression of recombinant *DHDPS* polypeptides in *E. coli*. BL21 (DE3) cells on YJ culture medium was expected to produce proteins of 37.2 kDa (33.5 kDa DHDPS subunit (Fig 3) and 3.7 kDa fusion fragment of the pET200/D-TOTO vector, (Loc *et al*., 2015). For the transformed *E. coli* cells without IPTG induction, a weak protein band of expected sizes were observed (Fig 4, NC), suggesting that induction with IPTG is necessary for optimizing the expression of *DHDPS2* subunit gene.

The previous study reported that the expression vector with a T7 lac promoter, final IPTG induce time and concentration should be optimized because of its great contribution to recombinant protein expression and serious harm to cell growth (Loc *et al*., 2013). In this research, after adding 0.5 mM IPTG into the medium, the target protein started to be synthesized and induction time was necessary for recombinant protein production. The optimal induction time for expression was examined by analyzing samples after 2h, 4h, 6h, 8h, 10 and 12h induction. The results showed that highest expression of *DHDPS* subunit occurred after 8h of induction (Fig 5). The effect of different concentrations IPTG on the *DHDPS* production was investigated. IPTG concentrations of 0.2 mM, 0.5 mM, 0.7 mM, 1.0 mM, 1.2 mM and 1.5 mM was used to induce expression of *DHDPS* subunits after 8h, and the result was shown in Fig 5. Comparison of the intensity of the bands in SDS-PAGE showed that 0.2mM IPTG is suitable enough for higher expression of *DHDPS* subunit (Fig 6).

The effect of six different culture mediums of LB, TB, HSG, YJ, SOB, SOC with 0.5 mM IPTG induction on the expression of recombinant *DHDPS2* protein *in E. coli*. BL21 (DE3) cells have been shown in Fig 7. In fact, LB is one of the normal culture media to use for determining the expression of the recombinant general protein *in E. coli*. BL21 (DE3) cells (Loc *et al*., 2015, Ellen *et al*., 2013, Miksch *et al*., 2008). LB culture medium was also applied to detect

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Query      1  ATGAATGTTAGGAAATCGATTGACGACATTAGGAGTCTGAGATTGATGACTGCGGTGAAA  60
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Query     61  ACTCCGTACCTACCAAATGGTCAAATTGATCTTGAATCATACGATAACCTAGTAAACATG  120
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Query    181  ATGAGCTTGAATGATAAAGTAATGCTTATTGCTCACACCGTCACCTCTTTCGGCGATAAA  240
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Query    241  GTCAAAGTTATTGGTAATACAGGAAGCAACTCAACATCACTAGCAATTAGTCTAACTGAG  300
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Query    301  CAAGGTTTTGCTGTTGGAATGGATGCATCACTTCAAATAAACCCTTACTATGGAAAAACC  360
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Query    361  TCAATGGAAGGTTTGGTTGCTCATTACAAAAGTGTACTTTCAGTAGGACCTATCATTTTA  420
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Sbjct   1017  TTTATCATAGTTGGGTCGGTAT  1037
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Fig 2: Coding sequence of the *DHDPS* gene (accession no: XM_01358955.2) with 100% homology.

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From 1 to 921.
Translation 307 a.a. MW=33495.58999999996

 M  N  V  R  K  S  I  D  D  I  R  S  L  R  L  M  T  A  V  K  T  P  Y  L  P  N  G  Q  I  D
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 L  E  S  Y  D  N  L  V  N  M  Q  I  A  N  G  V  E  G  I  L  V  A  G  T  T  G  E  G  H  L
CTTGAATCATACGATAACCTAGTAAACATGCAAATTGCAAATGGTGTTGAAGGAATTCTTGTTGCTGGAACAACTGGTGAAGGCCATTTA

 M  S  L  N  D  K  V  M  L  I  A  H  T  V  T  S  F  G  D  K  V  K  V  I  G  N  T  G  S  N
ATGAGCTTGAATGATAAAGTAATGCTTATTGCTCACACCGTCACCTCTTTCGGCGATAAAGTCAAAGTTATTGGTAATACAGGAAGCAAC

 S  T  S  L  A  I  S  L  T  E  Q  G  F  A  V  G  M  D  A  S  L  Q  I  N  P  Y  Y  G  K  T
TCAACATCACTAGCAATTAGTCTAACTGAGCAAGGTTTTGCTGTTGGAATGGATGCATCACTTCAAATAAACCCTTACTATGGAAAAACC

 S  M  E  G  L  V  A  H  Y  K  S  V  L  S  V  G  P  I  I  L  Y  N  N  P  S  R  T  A  Q  D
TCAATGGAAGGTTTGGTTGCTCATTACAAAAGTGTACTTTCAGTAGGACCTATCATTTTATATAACAACCCATCAAGAACTGCTCAAGAT

 I  P  P  S  V  V  E  I  L  A  Q  N  P  N  F  V  G  I  K  E  C  I  A  N  E  R  V  K  K  Y
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 A  S  Q  G  I  F  V  W  T  A  N  Q  K  E  S  H  E  G  A  I  G  D  V  S  L  A  S  N  L  I
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 P  S  L  M  V  K  L  M  N  E  G  M  N  P  S  L  N  S  K  L  I  P  L  F  D  W  L  S  L  E
CCTAGTTTAATGGTGAAACTCATGAATGAAGGAATGAATCCTTCATTGAATTCAAAGCTTATTCCTTTGTTTGATTGGCTTTCTTTAGAG

 V  I  P  I  G  L  N  T  A  L  A  Q  L  G  V  I  K  P  V  F  R  L  P  Y  V  P  L  S  R  E
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 R  R  V  E  F  V  K  L  V  K  Q  I  G  R  D  H  F  V  G  E  K  D  V  E  V  L  D  D  E  D
AGAAGGGTTGAGTTTGTCAAATTGGTGAAGCAAATTGGAAGAGATCATTTTGTTGGAGAAAAGGATGTTGAAGTTCTTGATGATGAAGAC

 F  I  I  V  G  S  V
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Fig 3: Amino acid sequence of 4-hydroxy-tetrahydrodipicolinate synthase 2 (*DHDPS2*) from NCBI (Protein ID: XP_013445009.1).

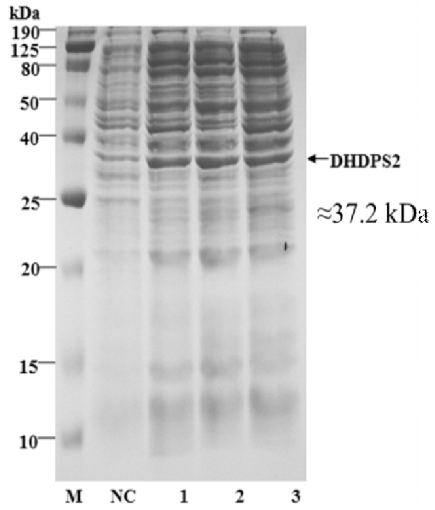

Fig 4: SDS-PAGE analysis of the expression of recombinant *DHDPS2* polypeptides in *E. coli*. BL21 (DE3) cells on YJ culture medium. M: protein weight marker (10-190 kDa, Bioline), NC: transformed *E. coli* cells without IPTG induction. Lands 1, 2, 3: transform which designed to base on the coding DNA sequence (CDS) for the mature peptide of *E. coli* cells with 0.5 mM IPTG induction (three times replicative).

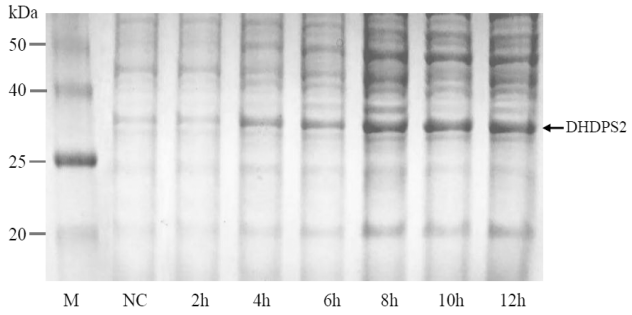

Fig 5: SDS-PAGE analysis of the expression of recombinant *DHDPS* protein *in E. coli*. BL21 (DE3) cells on YJ culture medium. M: protein weight marker, NC: without IPTG. Other ands: with 0.5 mM IPTG at 2, 4, 6, 8, 10, 12 h, respectively.

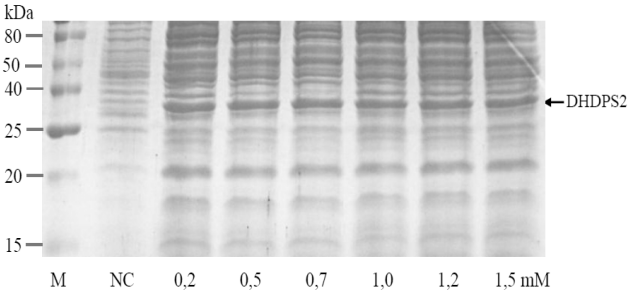

Fig 6: SDS-PAGE analysis of the expression of recombinant *DHDPS2* protein *in E. coli*. BL21 (DE3) cells on HSG culture medium with different concentrations of IPTG induction at 8h. M: protein weight marker, NC: without IPTG Other lands: with IPTG at 0.2, 0.5, 0.7, 1.0, 1.2 and 1.5 mM,respectively.

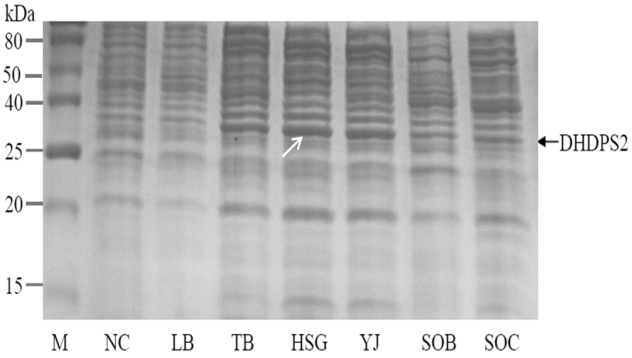

Fig 7: SDS-PAGE analysis of the expression of recombinant DHDPS protein *in E. coli*. BL21 (DE3) cells in different culture mediums. M: protein weigSht marker, NC:transformed *E. coli* cells on YJ culture medium without IPTG induction. Other lands: culture mediums of LB, TB, HSG, YJ, SOB, SOC with 0.5 mM IPTG induction, respectively.

the expression of recombinant general protein *in E. coli*. BL21 (DE3) cells in this assay and the result on SDS-PAGE gel showed a very weak band of molecular weight of about 37.2 kDa (Fig 5, LB) in comparing with other culture media under the same assay condition with 0.5 mM IPTG induction after 5h (see material and method). Among six different investigated culture mediums, the expression of recombinant DHDPS protein *in E. coli*. BL21 (DE3) cells on the TB, HSG and YJ culture mediums shown rather stronger band than SOB and SOC culture mediums (Fig 5).

Taking into account the results obtained from this study, it is possible to suggest that the highest expression of recombinant which designed to base on the coding DNA sequence (CDS) for the mature peptide of *DHDPS* protein in *E. coli*. BL21 (DE3) cells occurred on the HSG culture mediums after 8h of induction with 0.2 mM IPTG (BioRad).

CONCLUSION

In this study, isolation and cloning of the *DHDPS2* gene from the *M. truncatuala* leaf genome using PCR method were done. Nucleotide sequence of the *DHDPS2* subunit coding region of *DHDPS2* gene is 922 bp in length and is 100% similarity with the sequence published on Genbank (accession number: XM_013589555.2). The *DHDPS2* gene was successfully expressed in *E. coli* BL21 (DE3) cells with pET200 / D-TOPO vector for the fusion protein of about 37.2 kDa size (including the size of the fusion 3.7 kDa). The strong expression level of *DHDPS2* protein in *E. coli* BL21 (DE3) cells occurred on the TB, HSG and YJ culture mediums after 8 hours with 0.2 mM inducible IPTG (BioRad).

ACKNOWLEDGEMENT

This work was supported by the National Foundation for Science and Technology Development (NAFOSTED), Vietnam (Code No: 106-NN.02-2014.13). The authors are thankful to Prof. *Geert Angenon' laboratory,* Vrije Universiteit Brussel (VUB), Belgium for providing *M. truncatula* seeds.

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