Over - expression Effect of Gene Encoding 3-hydroxy-3-Methylglutaryl-CoA Reductase on Production of Taxol in Iranian Hazel (*Corylus avellana* L.)

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Abstract

Background: Sustainable and commercial production of taxol as an anti cancer drug is a critical point to its clinical application. Nowadays, hazel because of rapid growth and wide range distribution is considered as an alternative source of Taxol.

Objective: To increase taxol production the cDNA encoding 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) from Iranian hazel (GeneBank accession number KF306244, showed by CiHMGR) was isolated and over-expressed in pCAMBIA1304 binary vector. The effect of transient over-expression of HMGR in callus and leaf were evaluated on Taxol production.

Methods: The calli was established through the culture of immature cotyledon on Murashige and Skoog basal medium supplemented with 2, 4-D and BA. The first strand cDNA of CiHMGR was synthesized by specific primers. Enzymatic assay of recombinant CiHMGR in *E. coli* were done by western blott and His-tag affinity techniques. Also production of taxol in transformed callus and leaf were evaluated by HPLC analysis.

Results: An Open Reading Frame (ORF) with 1698 bp length and a deduced polypeptide with 566 amino acid residues were amplified. The highest and lowest amount of taxol was 0.016 mg/g.DW and 0.004 mg/gDW in transformed calli and untransformed leaves respectively.

Conclusion: Generally the over-expression of HMGR increase the total isoprenoids yield, therefore to have high production of target secondary metabolites (taxol) we need both of network of transformed genes and elicited cell culture.

Keywords: ***Corylus avellana*, Diterpenoids, Elicitor, Mevalonate biosynthesis pathway**

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Introduction

Paclitaxel is the general name of taxol, a chemotherapeutic agent with a wide range of activity [1, 2, 3]. Like most of the drugs for clinical uses, taxol is produced by semisynthesis, starting from a natural precursor, 10-deacethyl bacatin III that is more readily available from the needles of Yew species as a renewable source [4, 5, 6]. Yew species and entophytic fungi have been considered as the only sources for the commercial supplies of paclitaxel (generally named taxanes) and its precursors [7, 8], instead recent studies have been shown that hazel species possess the metabolic pathway (s) for taxane biosynthesis [9]. Taxol is one of the products of biosynthesis terpenoids pathway, terpenoids are a class of compounds derived from the universal precursor isopentenyl diphosphate (IPP) and its allelic isomer dimethylallyldiphosphate (DMAPP), also called isoprene units. The classic mevalonate (MVA) pathway was discovered in the 1950s and was assumed to be the sole source of the terpenoid precursors IPP and DMAPP (Fig. 1). The MVA pathway is active in bacteria, plants, animals and fungi and functions in the cytosol to generally supply the precursors for production of sesquiterpenes and triterpenes. Other pathway, which is named after the first committed precursor, 2-C-methyl-d-erythritol- 4-phosphate (MEP; the pathway is also sometimes referred to as the DXP pathway), is plastidial in nature and is generally used to supply precursors for the production of monoterpenoids, diterpenoids and tetraterpenoids [9, 10, 11]. Recent studies demonstrating cross-talk between the MVA and MEP pathways suggest that engineering of the MVA pathway for IPP formation could be useful in controlling plastidial terpenoid biosynthesis. Each organism has one of the both MVA and MEP pathways. Generally MVA pathway is affiliated to eukaryotes and MEP is related to prokaryotes. In contrast plants have both pathways but in the separate organelles of cell. Also various Gram-posetive bacteria such as *Staphylococcus aureus, Lactobacillus plantarum* and *L. lactis* have adopted the mevalonate pathway through evolution for isoprenoid biosynthesis [7, 8]. A common strategy for engineering terpenoid synthesis is to focus on enhancing flux to increase the precursor pools of IPP and DMAPP, by cloning the key genes and understanding regulation of both the MVA and MEP supply pathways [11, 12, 13]. The classic MVA pathway is well defined, and numerous studies have demonstrated the importance of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR). HMGR is strictly regulated in plant systems and elicitor induced HMGR genes have been cloned and characterized in numerous plant systems including tobacco, tomato, potato, hazel and euphorbia [14, 15, 16]. Recently two genes of hazel including IPI (Isopentenyl diphosphate isomerase) and GGPP (Geranylgeranyl pyrophosphate) belong to MEP pathway has been cloned, which are key precursors for diterpenes such as taxol [17, 18]. Base on recent studys engineering of the MVA pathway for IPP formation could be useful in controlling plastidial terpenoid biosynthesis [9]. We attempted to manipulate of MVA pathway via overexpression of the 3-hydroxy-3-methylglutaryl coenzyme-A reductase gene from Iranian hazel as a rate limiting enzyme.

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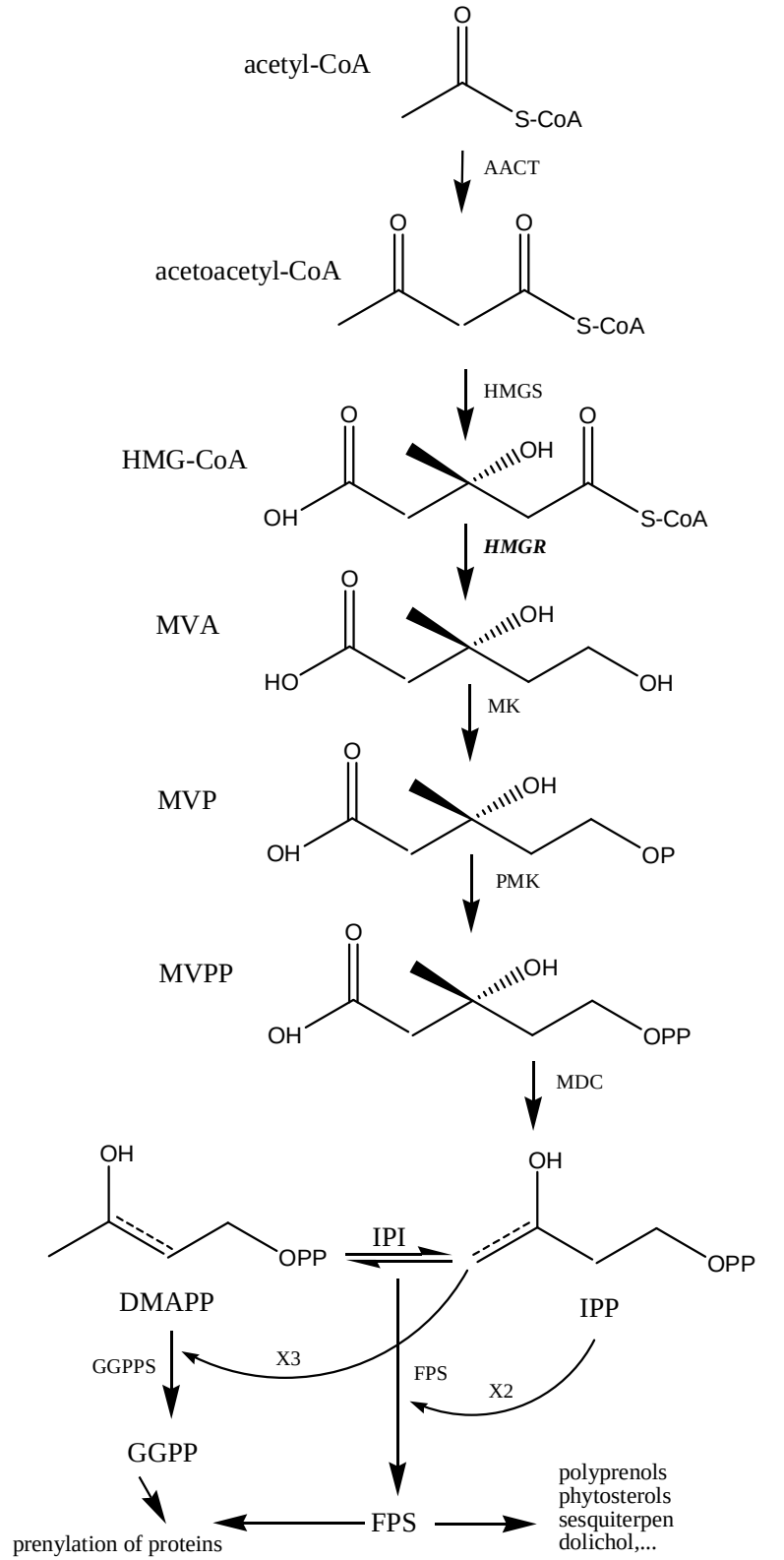


Figure 1- Overview of classic Mevalonate biosynthesis pathway in plants

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Material and Methods

Plant material and *invitro* conditions

The immature seeds of Iranian taxol-producing hazel provided from plant collection of institute of medicinal plants (IMP) Karaj, Iran. For surface sterilization, seeds were soaked in 70% (v/v) ethanol for 1 min and rinsed 3 times with sterile distilled water, and then seeds were soaked in 70% (v/v) ethanol for 1 min and rinsed 3 times with sterile distilled water and then seeds were immersed in 2.5 % sodium hypochlorite for 8 min and again rinsed 3 times with sterile water. For callus and somatic embryogenesis the leaves and Immature hazel embryo pelts out and cultured on Murashige and Skoog medium (pH 5.8 and 3% sucrose) [22] supplement with 0.5-4 mg/L 2,4-D, 0.5-4 mg/L BA. The cultures were holed in darkness at 25°C.

DNA and RNA isolation

To increase the expression of *HMGR* gene the leaves were treated with 2 mM methyl jasmonat. After 4 days total RNA was isolated by SDS method according to Wang protocol [20]. The quality and quantity of the RNA were evaluated by agarose gel electrophoresis and nano-drop.

Synthesis of total cDNA and isolation of *HMGR* cDNA frgment

The first strand of total cDNA was synthesized by RevertAid H Minus Reverse Transcriptase kit (fermentase EP0451). The *HMGR* cDNA from total cDNA was amplified by two special primers, forward (5-CC**ATG**GTAGATCTA*CATCATCATCATCATCAT* GAC GTA CGC CGG CGA TCG AC-3) and reverse (5'-ATCAG CTTAAG AAA CTT TAT TGC CAA ATG TTT GAA CGTTA AGA GGA AAC CTT GGA AAC-3) containing His-tag sequence (it's shown in italic), restriction enzyme site, *Bgl*II and *Afl*II and kozak sequence. Primers designed based on the cDNA sequence of *HMGR* gene (EF206343) from hazel (*Corylus avellana* L. Gasaway). The target fragment was amplified by *pfu* polymerase and PCR program was carried out under the following condition: 2: 30 s of denaturation at 94°C, 30 s of annealing at 45.5°C, 3 min of extention at 72°C for 32 cycles. PCR program ended by 7 min for final extension phase. The PCR products were first separated on 1% agarose gel and then purified and sequenced (Eurofins, Germany).

Expression assay of ci*HMGR* in *Escherichia coli.*

The *E. coli* strain DH5α was used as the host strain to test the function of CiHMGR. The PCR products were subcloned into the *Bgl*II-*Afl*II sites of the pCAMBIA1304 vector (Fig. 2) to generate the plasmid pCAMBIA-CiHMGR which was verified by sequencing. The recombinant vector was transformed into *E. coli* DH5α. Also empty vector pCAMBIA1304 was transformed to *E. coli* DH5α as control. For isolation of recombinants *E. coli*, the cultur were grown on solid LB medium containing kanamycin (60 mg/L) and hygromycin (400 mg/L) at 37°C. After 2 days isolated plasmid from *E. coli* clones digested by *AFl*II and electrophoresis on 1% agaros gel. An overnight 3 ml culture from a single colony was grown at 37°C in LB medium containing kanamycin and hygromycin, and was then transferred to 25 ml LB. After incubation at 37°C, gene expression was induced by the addition of 0.3 mM IPTG

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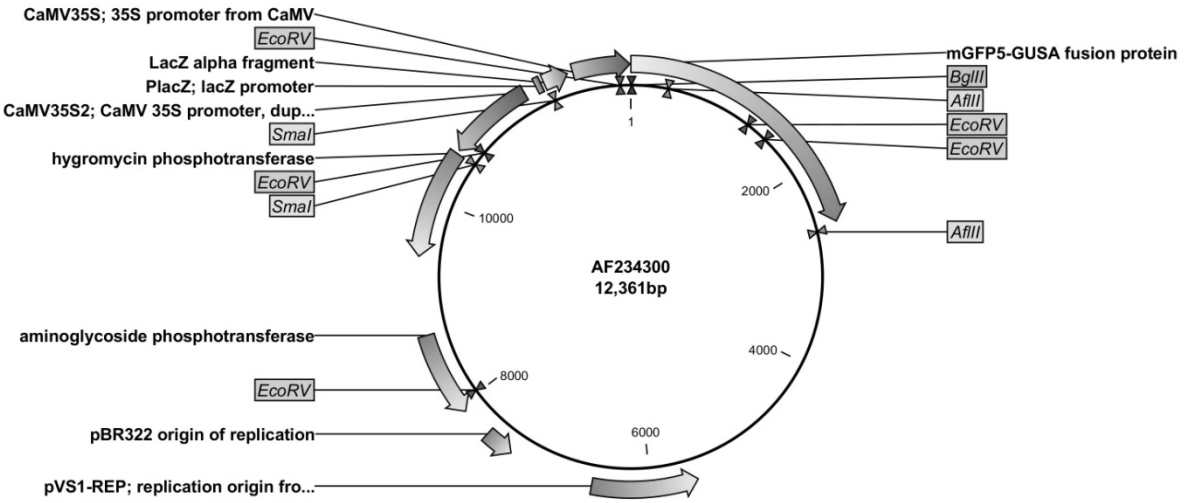


Figure 2 - Restriction map of pCAMBIA1304 by CLC work bench software

to the cultures [21]. To protein isolation Frozen cells obtained from 5 mL of the cultures were thawed and suspended in 0.3 mL of lysis buffer (100 mM Na-phosphate and Tris-HCl (pH 8.0) containing 6M guanidine hydrochloride (GuHCl)). The solutions were supplemented with 1% NP40 (nonyl phenoxypolyethoxylethanol). Cell suspensions were incubated for 1h at room temperature and centrifuged. The obtained supernatant was separated from GuHCl by TCA (trichloro acetic acid) precipitation method [22]. The isolated proteins were separated using SDS-PAGE technique. The separated proteins were blotted onto PVDF membrane (Roche applied science Cat. No. 3 010 031) and blocked with 20 ml of blocking buffer (5 mM Tris/HCl, 15 mM NaCl, 0.01 % (v/v) Tween 20, pH 7.5) for 90 min at room temperature. Blocked membrane was washed three times with TBST buffer (50 mM Tris/HCl, 150 mM NaCl, 0.1 % (v/v) Tween 20, pH 7.5) for 5 min respectively and incubates the block in 10 ml blocking buffer whit 10µl Anti-His_6-Proxidase solution (50 U/ml) for 60 min at room temperature. Membrane was then washed three times with TBST for 5 min respectively. The blot was incubated in 5 ml BM blue POD-substrate solution membrane (Roche applied science Cat. No. 1 442 066) till the protein bands became visible.

Introduction of pCAMBIA-ciHMGR to *Agrobacterium* and agroinfilteration

The pCAMBIA-CiHMGR was isolated from *E. coli* by High Pure Plasmid Isolation Kit from Roche according to the manufacture instruction and introduced to *Agrobacterium tumefaciens* LBA4404 strain with the freeze-thaw method. *A. tumefaciens* harboring the above-mentioned construct and empty pCAMBIA1304 vector were resuspended in the infiltration medium (WPM medium,10 mM MgCl2, 10 mM 2-(4-morpholino) ethanesulfonic acid (MES), 100µM acetosyringone,10% sucrose, 0.005 silwet L-77, pH 5.8). Two types of hazel explants (callus, and mature leaves) were infiltrated by

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vacuum pump (400mmHg, 2min for three times). For evaluation of transient expression of recombinant HMGR gene the leaves and callus sample transferd into MS medium containing methyl-jasmonat (150µM), chitosan (50 mg/L), sucrose (60 g/L), kanamycin (60 mg/L) and hygromycin (400 mg/L). Recombinant clones of *A. tumefaciens* (with one cutting site) were recognized by enzymatic digestion with *Afl*II, non recombinant clones has two cutting site (Fig. 3).

Extraction and quantification of Taxol

After 24 h the samples were collected and taxol was extracted in three replicant from powdered dried calli and leaves by previously described method of Bemani with some modification [23]. The samples were ground and suspended in 10 mL methanol, sonicated and filtered, then the filtrate was air-dried and re-dissolved in methanol: water (1:1, v/v) followed by centrifugation at 5000 rpm. Supernatants were collected, air-dried and re-dissolved in 100 µL methanol (HPLC grade) and filtered by passing through a 0.2 µm syringe filter, before being injected to HPLC. The Taxol content of the extracts was quantified by HPLC system (Knauer, Germany), equipped with a C-18 column (Perfectsil Target, ODS3, 5 µm, 250 × 4.6 mm, Teknokrome, Germany) (Fig. 5). Taxol was eluted with a linear gradient of acetonitrile and water (45:55) at a flow rate of 1 mL/min and was detected at 227 nm using a UV detector (K-250, Knauer, Germany). Identification of taxol was accomplished by comparison of retention times with authentic standard (sigma: T7402) [23].

Results

Calli were induced after two weeks from Cotyledon explants cultured in MS medium supplemented with 2 mg/L BA and 0.5 mg/L 2,4-D. Base on total RNA extracted A 2286 bp sequence was amplified and sequenced that contained 1698 bp ORF encoding 566 amino acids. To evaluate the recombinant HMGR protein in *E. coli*, expression assays were performed using the His-tagged CiHMGR. Whole cell lysate of *E. coli* strain DH5α harboring pCAMBIA-CiHMGR plasmid separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). As expected two bands 61.4 KDa (target protein), 69.5 KDa (control lane) appeared on the membrane (Fig. 4). Based on HPLC analysis in both of transformed tissues (callus and leaf) the amount of taxol was 2.9 and 3.7 fold increased in leaves and calli respectively (Fig. 6). Also ability of taxol production in calli was 1.53 ~ 2 fold higher than leaves. The highest and lowest amount of taxol was 0.016 mg/g.DW and 0.0036 mg/g. DW in transformed calli and untransformed leaves respectively.

Discussion

In other studies have been pointed to the important role of BA and 2,4-D on callus induction and As expected, effective callus induction was accrued in presence of BA [9, 36]. The cDNA of encoding *HMGR* were isolated from several plants including *Hevaea brasiliensis* (gi|AAU08214), *Morus alba* (gi|AAD03789), *Euphorbia pekinensis* (gi|EF562569), *Camptotheca acuminate* (gi|AAB69726), *Ginko biloba* (gi|AY41133) and *Taxus x media* (gi|AY277740). On the amino acid scale there is high homology for HMGRs between plants [24]. Also the complex three dimensional of HMGR has high similarity to human HMGR [25, 26]. The

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HMGR protein was successfully produced by over-expressing in many studies to increase production of secondary metabolites such as sesquiterpenoids, diterpenoids, triterpenoids and carotenoids pigments [27]. In plant the activity of HMGR is regulated by level of its mRNA [29], reversible phosphorylation [30], proteolytic degradation [31], subcellular compartmentation [32], calcium and calmodulin [33]. In some cases the taxol and other taxanes are derivated from cell and tissue culture of hazel but in low yield (1/10 the level of yew) [34] Nevertheless the similar amount of taxol was recovered from elicited hazel cell culture [9].

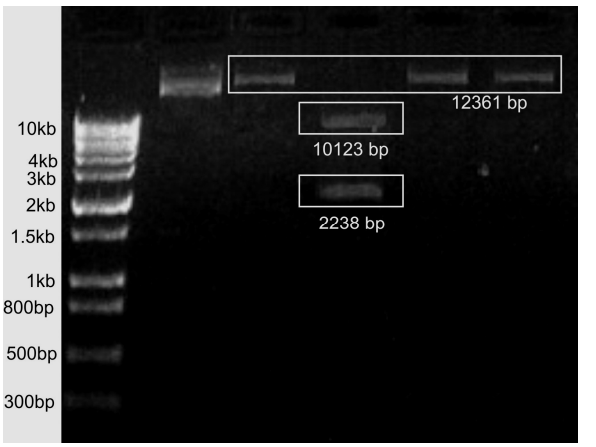


Figure 3 – Digestion *E. coli* clones with *Afl* II restriction enzyme, recombinant clone (pCAMBIA-ciHMGR) has one cutting site and non recombinant clone (pCAMBIA1304) has two cutting site

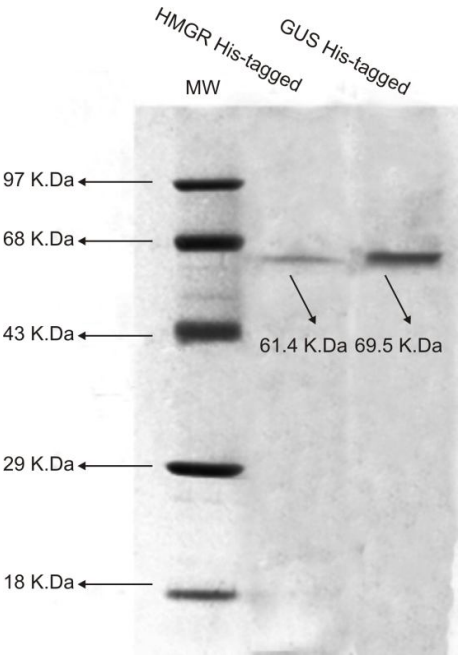


Figure 4- Western blot of expressed HMGR with N-terminal His-tagged (61.4 KDa) and GUS with C-terminal His-tagged (69.5 KDa) using Anti-His-Proxidase and Bm blue POD-substrate

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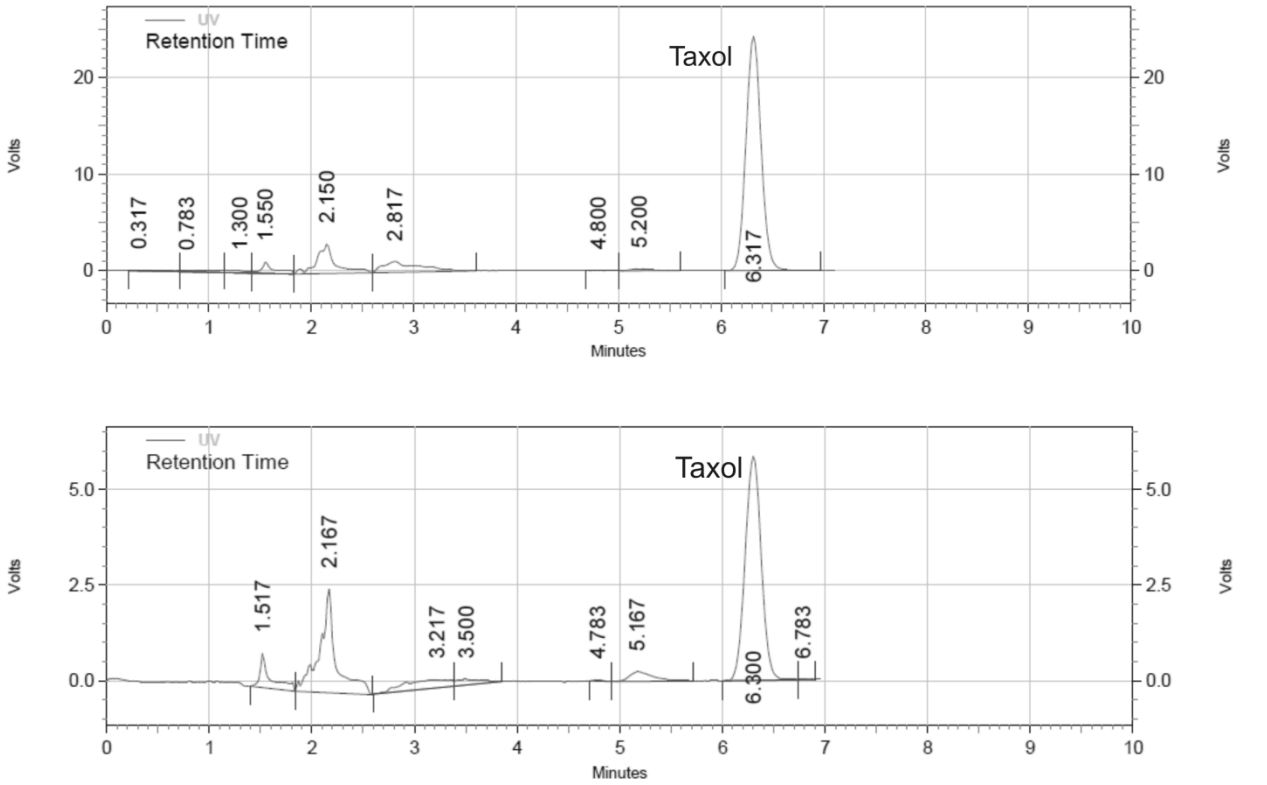


Figure 5 - HPLC analysis of taxol biosynthesis in callus (a) and leaf explants (b)

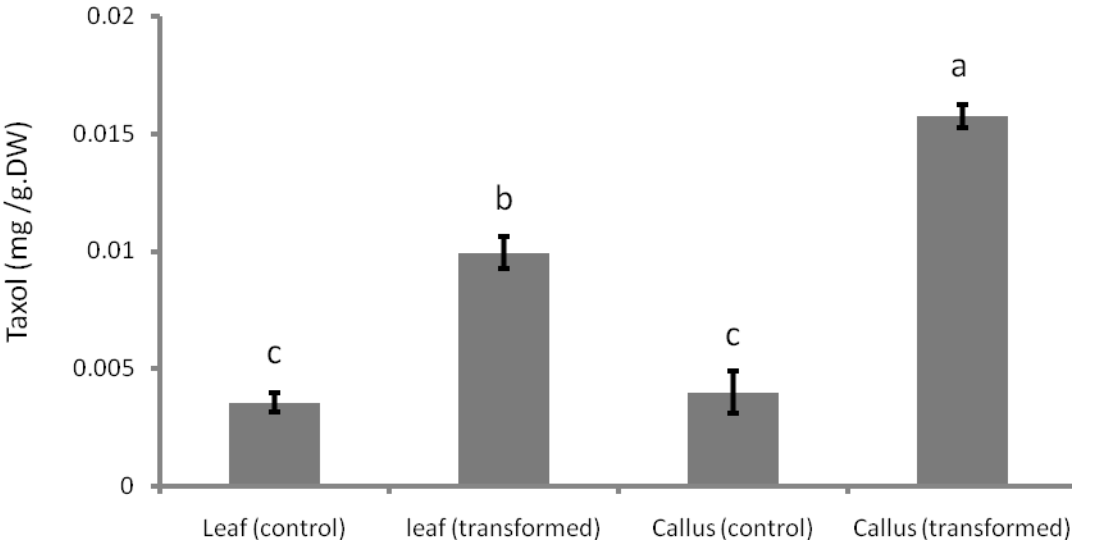


Figure 6 – Total taxol production in transformed calli and leaves

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Conclusion

Generally results shown that the over-expressing of gene encoding HMGR enzyme can enhance the production of downstream metabolites such as taxol in leaves and *invitro* culture of hazel. Over-expressing of HMGR as a rate limiting enzyme upgrade the flux rate of MVA pathway and level of IPI (isopentenyl diphosphate isomerase). Cytosolic IPI increase the pool of the precursor DMAPP (dimethylallyl diphosphate) in plastid. Accumulation of DMAPP in plastid (the first precursor in production of diterpenoids) increases the amount of diterpenoids [10]. For commercial production of taxol several strategies including semi-synthetic from natural precursor (10-deacetylbacatin III), total synthesis by fungi or bacteria, and cell culture have been applied. The cell culture of *Taxus* sp. and *Corylu*s sp. are considered as stable and long-term alternatives for production of taxoids.[9,35]. To increase the efficiency of over-expressed HMGR, manipulation of some downstream genes of MEP pathways such as IPI and GGDS (geranylgeranyl diphosphat synthase) are require. Also elicitation of hazel cell culture systems plays complementary role in biosynthesis of diterpenoids.

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