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Full Length Research Paper

Cloning of taxadiene synthase gene into *Arabidopsis thaliana* (ecotype Columbia-0)

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Paclitaxel (Taxol), a complex diterpenoid, produced by yew tree (*Taxus* sp.) is the most important chemotherapeutic agent that is widely used against a variety of malignancies such as ovarian and breast cancers. However, destructive methods for its production from natural resources together with currently used low-yielding industrial production systems via total synthesis or semi-synthesis have led researchers to invent a robust alternative biological production system using biotechnological approaches. The first committed step in taxol biosynthesis pathway is the production of taxadiene from geranylgeranyl diphosphate (GGPP) catalyzed by the plastid-localized enzyme taxadiene synthase (TXS). In this research, an attempt was made to evaluate the effects of the first critical enzyme in the taxol biosynthesis pathway on *Arabidopsis* plant through the expression of taxadiene synthase gene under the control of a dexamethasone-inducible promoter. To achieve this goal, *Arabidopsis* plants (ecotype Columbia-0) were transformed with the construct pTA-TXS-His via floral dip method using *Agrobacterium tumefaciens* AGL1. The transformed plants were confirmed using the PCR reaction amplifying an 800 bp fragment of the cloned gene. Upon these findings, a proposal was made that biotechnological strategies could be utilized for the production of taxol components.

Key words: Paclitaxel, arabidopsis, taxadiene synthase, geranylgeranyl diphosphate.

INTRODUCTION

Paclitaxel (Taxol®), structurally, is one of the more complex members of the taxoid family of diterpenoid that are known as diverse group of secondary metabolites produced by yew (*Taxus*) species (Baloglu and Kingston, 1999). It is the most dominant chemotherapeutic agent commonly used against various cancers such as breast, lung and ovarian cancers (Charles et al., 2001; Francis et al., 1995; Hata et al., 2004) as well as AIDS related Kaposi's sarcoma (Gill et al., 1999). Having stabilized the microtubules cytoskeleton against depolymerization, this antimitotic agent hinders the cell cycle in G_1 or M phase. Based on such unique mechanism of action, it elicits the least side effects compared to similar antineoplastic agents (Arnal and Wade, 1995). Due to these inimitable characteristics, there exists an increasing demand for its applications. However, destructive and low yielding production of taxol from natural resources (bark of the yew tree) is deemed to be unlikely satisfactory for such growing market, thus many challenges have been conducted to establish alternative methods including chemical and biological approaches (Cragg et al., 1993).

Of these, although total synthesis of taxol has been accomplished, commercial scale production of taxol from

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scratch using the available chemical methodologies is laborious and cost prohibitive strategy (Nicolaou et al., 1994). Taxol is manufactured using a semi-synthetic methodology, in which the side-chain section of the molecule is chemically synthesized and added to a plentiful metabolite of the needle of taxus species, 10-deacetyl baccatin en route to the taxol (Ojima et al., 1992). The other promising method is the plant cell culture and induction of the pathway with inducer such as methyl jasmonate and other regulators (Khosroushahi et al., 2006). Furthermore, although taxol producing endophyte fungi has been recently studied, nevertheless the fermentation of the fungi are highly sophisticated with low yielding production that is not commercially feasible (Miller et al., 2008; Strobel et al., 1996). Despite being affordable, all of these alternative methods were faced with cost issue associated with low production yield. Hence, intensive attempt have been conducted toward biological production systems (Jennewein et al., 2001; Walker and Croteau, 2001).

Taxol biosynthesis pathway consists of at least 19 enzymatic reactions from key precursor, geranyl geranyl diphosphate (Hefner et al., 1998). It is cyclized, at the first committed step of the pathway, to taxa- 4(5),11(12)-diene by plastid localized enzyme (that is, taxadiene synthase) to set up the core skeleton (Koepp et al., 1995; Hezari et al., 1995). As seen in Figure 1, the cyclized skeleton is subjected to a series of oxygenation, hydroxylation and acylation reaction prior to production of the mature paclitaxel (Jennewein et al., 2001; Walker and Croteau, 2001). The GGPP substrate used by TXS appeared to be derived mostly from the methylerytritol 4-phosphate (MEP) pathway (Eisenreich et al., 1996), which synthesizes the prenyl diphosphate precursors for the production of isoprenoids in plastids (Rodriguez-Concepcion and Boronat, 2002). Plastidial GGPP is also a precursor for the biosynthesis of phytohormones and photosynthesis related isoprenoids in plants. So the direction of the GGPP pool to the constitutive production of taxadiene synthase seems to be a limiting factor for plant growth and development at primary stage (Figure 1).

Most of the taxol pathway genes have been identified and cloned in various organisms. For example, taxadiene synthase gene was introduced to yeast and *E. coli* for soluble production of enzymes (Engels et al., 2008; Huang et al., 2001). The complexity of genetic modification and paucity of effective protocols for manipulation of secondary metabolites pathways in *Taxus* sp. made the cloning and production of taxol derivatives very challenging. Furthermore, efficient cloning and production of the taxadiene synthase gene which encodes the related enzyme appeared to be an important critical step for production of taxol and its derivatives in the crop plants. Thus, to tackle these problems, in this current investigation, *Arabidopsis thaliana* (ecotype Columbia-0) was used for cloning and efficient expression of taxadiene [taxa-4(5),11(12)-diene], which could be used as an easy and fast growing model plant for further investigation on taxol biosynthesis pathway.

MATERIALS AND METHODS

Material and instruments

Sucrose, agar, sodium hypochlorite, salt and vitamin for preparation of MS media, were all from Merck (Frankfurt, Germany). Pepton, tryptone, beef extract, yeast extract were purchased from Himedia (Mumbai, India). *E-coli* Top 10 F was prepared from Invitrogen (California, USA). Zeatin, tween-20, 2-mercaptoethanol, gelrite, hygromycine-B, acetosyringone and kanamycine sulfate were obtained from Sigma-Aldrich Co. (Deisenhofen, Germany). Primers were purchased from MWG-biotech Co. (Ebersberg, Germany) and all the other reagents used for PCR reaction were from Qiagen (Hilden, Germany). Various size markers were from Fermentas (St. Leon-Rot, Germany). Sillwet L-77 surfactant was from Witco Co. (Endicott, USA). Augmentin tablet 375 mg was from Farabi (Isfahan, Iran) and rifampicin was from Alhavi (Tehran, Iran). Instrument used in this investigation were as follows: OLYMPUS BX51 microscope (Tokyo, Japan) for Agrobacterium microscopic image preparation; gradient thermal cycler pc818 (Astec, Fukuoka, Japan) for polymerase chain reaction; NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) for quantification of nucleic acids and G:Box™ (version 06-2d.1e) gel documentation system (Syngene, Cambridge, UK) for image acquisitions.

Inducible and binary vector construction

Total RNA extraction, cDNA synthesis with specific primers: TXS-forward (5′- ATG GCT CAG CTC TCA TTT AAT G-3′) and TXS-1reverse (5′ TGC CAA TAC AAT AAT AAG TC- 3′), cloning vector assembly and sequence analysis of fragment were carried out according to previously reported methodology (Besumbes et al., 2004). The resulted fragment (2.6 kb) was subjected to a series of excision and sub-cloning processes towards production of pTA-TXS-His construction.

Introduction of construct to the *E. coli, Agrobacterium* and *Arabidopsis*

Construct pTA-TXS-HIS was delivered to the *E. coli* Top 10 F by common freeze and thaw method, after preparation of *E. coli* competent cell by calcium chloride procedure (Tu et al., 2005). The LBA medium supplemented with kanamycin sulfate (50 µg/ml; Fluka) was used for selection of the transformed colonies. Plasmid extraction was carried out according to the Cinnagen's kit instructions.

Cloning of inducible vector in *Agrobacterium tumefacians* AGL1 was carried out by means of triparental mating method, in which *E. coli* HB101 (pAK 2013) was used as a helper bacteria. Then, the grease-like mixture of three types of bacteria was spread on yeast extract broth (YEB) medium containing kanamycin sulfate (50 µg/ml) and rifampicin (25 µg/ml). This approach led to an appropriate primary selection of Agro-pTA-TXS-HIS. Several selected colonies undergone for PCR based amplification of TXS gene (800 bps) using TXS primers: TXS-forward (5′- ATG GCT CAG CTC TCA TTT AAT G-3′) and TXS-2 reveres (5′-TCGTCC CCT GTA TTC ATC AGT A-3′). PCR reaction for amplification of $VirD_2$ gene was performed using the following primers: $VirD_2$-for-ward (5′-ATG CCC GAT CGA GCT CAA GT-3′) and $VirD_2$-reverse (5′-TCG TCT GGC TGA CTT TCG TCA TAA-3′). This confirmed the existence of the Ti plasmid and virulence genes, which are essential for such transformation. The gram staining technique was

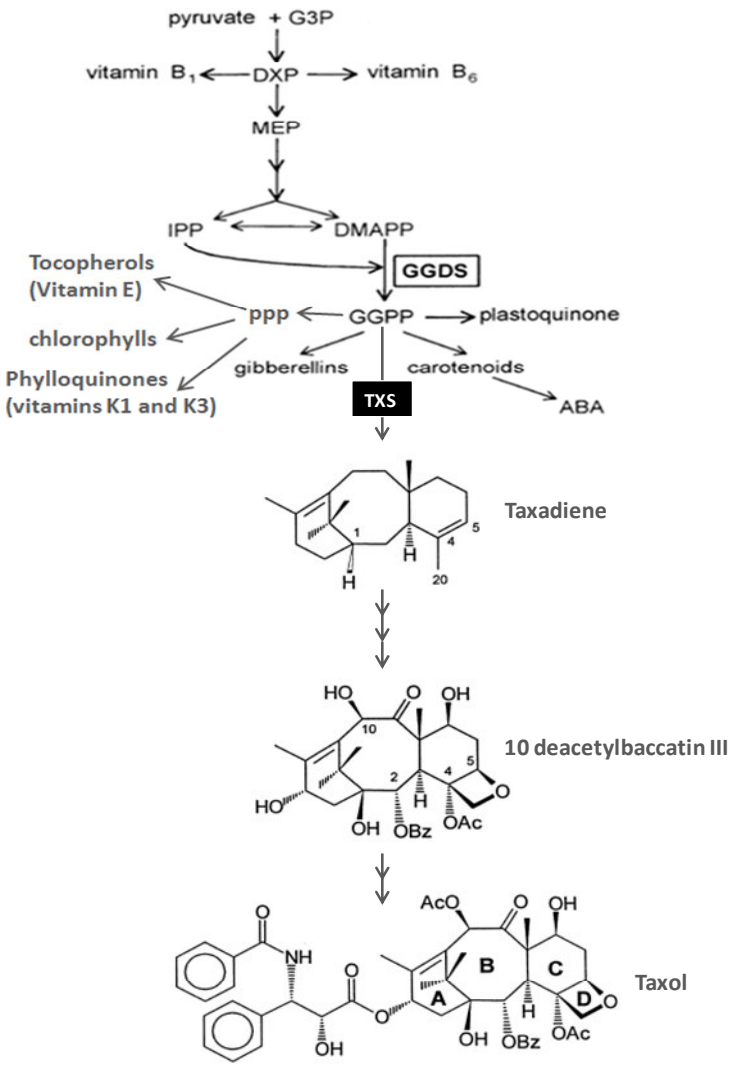

Figure 1. A brief schematic representation of taxol biosynthesis pathway. G3P = Glyceraldehyde 3-phosphate; DXP = deoxyxylulose 5-phosphate; MEP = methylerythritol 4-phosphate; IPP = isopentenyl diphosphate; DMAPP = dimethylallyl diphosphate; GGPP = geranylgeranyl diphosphate; PPP = phytyl diphosphate; ABA= abscisic acid; GGDS = geranylgeranyl diphosphate synthase; TXS = taxadiene synthasea. Adapted from Rodriguez-Concepcion and Boronat, 2002.

recruited for morphological evaluation of transformed/ untransformed bacteria, by which the purity of transformed bacteria could be validated to some extent.

Arabidopsis thaliana (ecotype Columbia-0) were cultivated on a mixture of peat moss, vermiculite and perlite (1; 1; 1). Transformation of *A. thaliana* was achieved by means of a modified floral dip method (Bent, 2006). Briefly, the engineered A*grobacterium* (AgropTA-TXS-HIS), glucose 10% and Silwet L-77 0.05% was used as an inoculation medium and then the selection of transformed seed was carried out on Petri dish with solid Murashige Skoog (MS) medium supplemented with hygromycin B (25 µg/ml; Sigma-Aldrich) (Carretero-Paulet et al., 2002). Plants which rooted on hygromycin B were transferred into soil and grown to maturity in the glasshouse. Genomic DNA isolation of the samples was performed using TabDNA Kit (our patented kit). Briefly, about 1 mg of rosette leaf (typically 3 - 5 mg) was ground in liquid nitrogen. These samples were then suspended in extraction buffer, incubated at 55°C for 1 h and extracted with CHCl3-isoamyl alcohol (24:1). They were spun down at 12000 rpm for 5 min; afterward nucleic acid from the aqueous phase was precipitated with equal volume of -20°C isopropanol and incubated at -20°C for 30 min. The extracted and air-dried DNA was eventually eluted in TE (Tris, + EDTA) buffer (Atashpaz et al., 2008; Barzegari et al., 2010; Barzegari et al., 2009).

RESULTS AND DISCUSSION

The production of pharmaceutically important secondary metabolites (e.g., taxol) seems to rely on new biotechnological strategies. For instance, improved production of

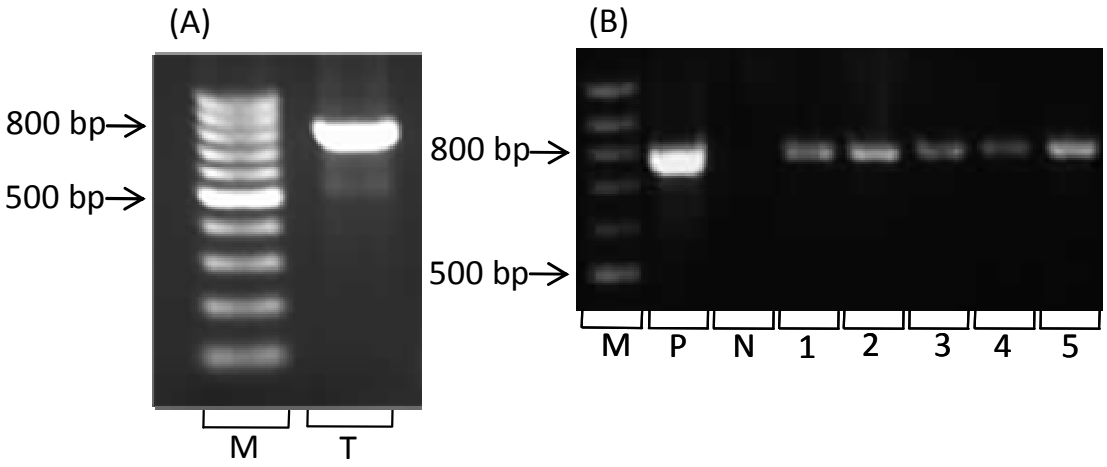

Figure 2. PCR product of the cloned TXS fragment on the 1% agarose gel. A) PCR reaction with the pTA-TXS-HIS construct as a template. B) PCR reaction for confirmation of *E.coli* transformation. M = 100 bp marker, T = amplified TXS, P = positive control, N = negative control, (lane 1-5) different colonies with the band in 800 bp.

the secondary metabolites such as taxol demands better understanding of the related biosynthesis pathway. To achieve such goal, transgenic cells or modified plants could be exploited. *Arabidopsis* as a short life cycle plant with prolific seed production can be easily and efficiently utilized for production of secondary metabolites. These facts together with simple transformation procedure highlighted this plant as an ideal model for introduction of TXS gene through inducible expression system which was used in this investigation.

As shown in Figure 2A, PCR reaction with specific primers for amplification of 800 bp of the cloned fragment was used as a method for the confirmation of cloning in pTA-7002 and the result showed the construct harboring the desired fragment (Figure 2A). Cloning of pTA-TXS-His in *E. coli* Top 10F was performed for reproduction of construct for further uses in transformation of *Agro-bacterium* and *Arabidopsis*. Cloning was confirmed by PCR reaction and the result showed that all of the selective colonies have an 800 bp band in gel electrophoresis. For transformation of *Agro-bacterium*, a natural method entitled Triparental mating was used, in which the pillus of helper bacteria were commonly utilized to exchange the construct between *E. coli* and *Agrobacterium* (Bent, 2006). For selection of the transformed *Agrobacterium*, very low amount of the bacterial mixer was used to ensure the colony selection process, which was confirmed by PCR reaction (Figure 3). To tackle the problem encountered with morphological discrepancy of the transformed and untransformed *Agrobacterium*, a microscopic method was used, whose results showed that there exist no significant differences (Figure 3C). For examination of Ti plasmid existence and virulence capacity of *Agrobacterium*, PCR reaction was used with specific primers for amplification of virulence genes. It was witnessed that some of the pTA-TXS-His harboring colonies failed to display the Ti vector, perhaps as a consequence of incubation temperature variation. The $VirD_2$ gene located on Ti vector played a critical role in T-DNA transfer process to the plant cell. The positive bands in PCR reaction of the $VirD_2$ and TXS genes in *Agrobacterium* confirmed its potential for later transfer of TXS gene into *Arabidopsis* which enabled this model plant to express the desired gene, that is, TXS gene (Figure 3). To pursue this concept, the leaves from 8 plants were collected two weeks after germination and used for genomic DNA extraction (Figure 4A), then several first transformed (T1) generation of *Arabidopsis* were examined by PCR reaction upon expression of these genes. As a result, the presence of 800 bp bands in gel electrophoresis confirmed successful transformation and genomic expression of TXS gene (Figure 4B).

It has been reported that, in the biosynthesis path of paclitaxel, the geranylgeranyl diphosphate is shared by many secondary metabolites pathways. Of these, upon constitutive production of taxadiene synthase, it was deemed that some other pathways such as plastidal isoronoid, giberllins, caratonoid biosynthesis and the side chain of chlorophylle may be disrupted, leading to constraint of seedling growth and development at primary stage (Besumbes et al., 2004) as shown in Figure 1. It should be evoked that the high level production of taxadiene synthase is toxic for seedling, perhaps arising from low tolerance of such seedling (Besumbes et al., 2004). The size of modified plants was smaller than the normal ones, nevertheless the resultant seeds were normal. It was speculated that the size differences could be due to the gene leakage and perhaps toxicity of the taxadiene synthase. Production of taxadiene synthase gene in plants tends to have some advantages over the microbial systems. The microbial/fungal systems demand co-transformation of both TXS and GGPP synthase

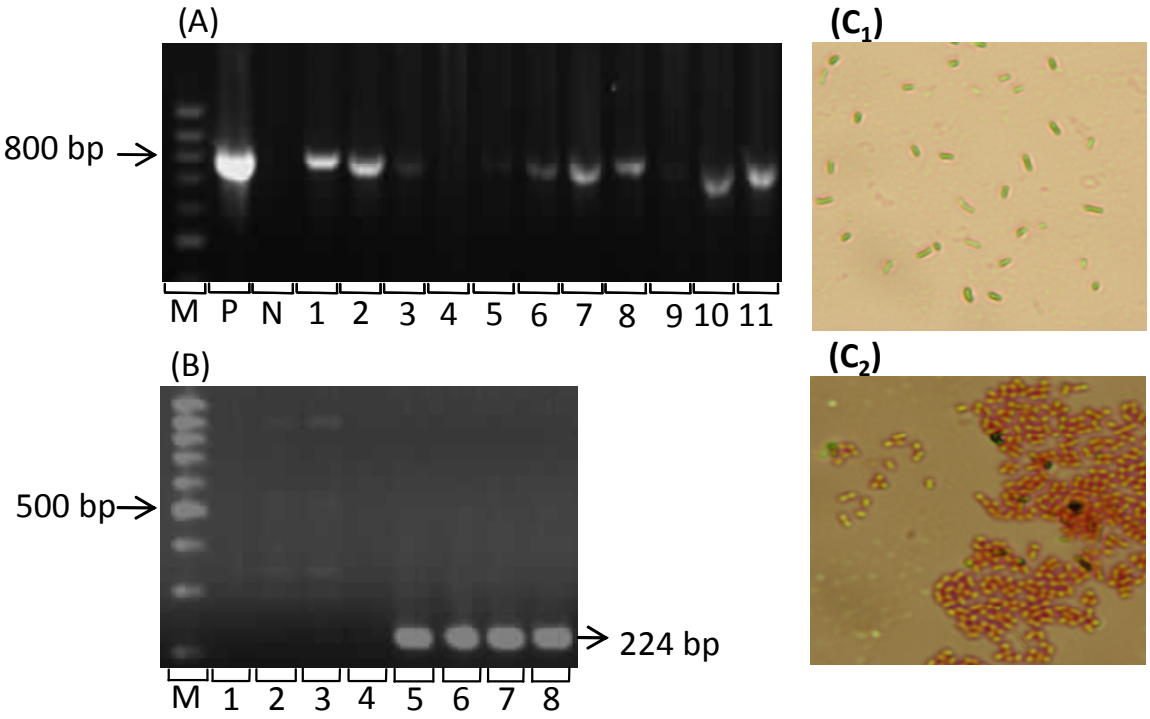

Figure 3. Transformation of *Agrobacterium*. A) Electrophoresis gel image confirming PCR amplification of the cloned fragment in lanes 1, 2, 3, 6, 7, 8, 10, and 11. B) PCR amplification of VirD2 gene; lane (5-8) are PCR positive colonies. P: positive control, M: 100 bp marker, N: negative control. C) Morphological evaluation of *Agrobacterium*. C_1 and C_2 represent the untransformed and transformed *Agrobacterium*, respectively.

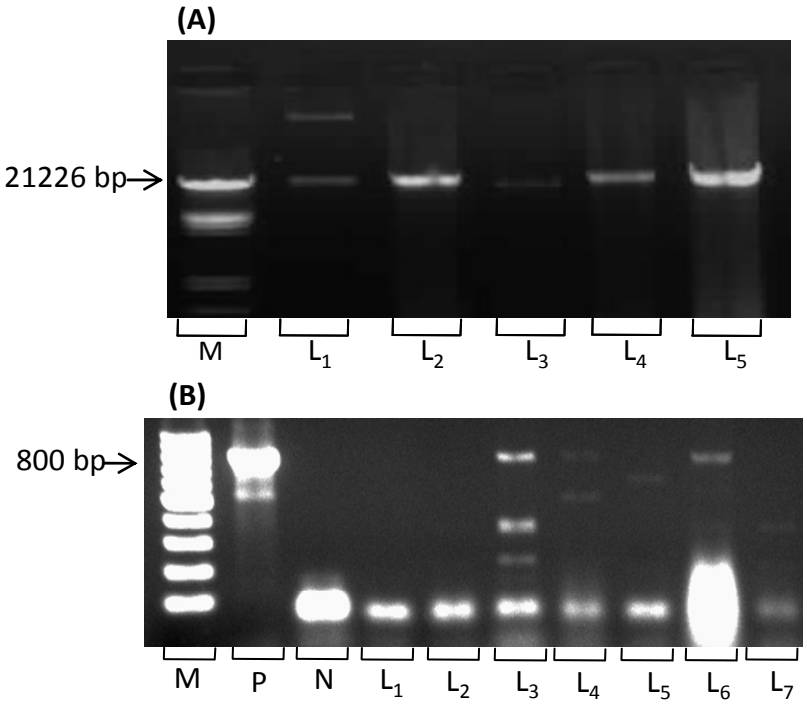

Figure 4. Transformation of *Arabidopsis*. A) Genomic DNA extracted by TabDNA Kit. B) PCR reaction from various line of *Arabidopsis* seedling after transformation. M = 100 bp marker, P = positive control, N = negative control and lanes 3, 4, and 6 represent PCR positive line.

genes for expression and soluble production of TXS (Huang et al., 2001). The results gotten demonstrated that the production of taxol precursors and perhaps some other similar secondary metabolites could be achieved through the inducible expression of related genes in *Arabidopsis* and other crop plants. This could be considered as a platform technology for commercial production of valuable compound through biological systems. As an alternative method to strengthen the level of GGPP, manipulation of MEP pathway could compensate the shortage of this multi-functional substrate. For instance, over expression of the first enzyme in the MEP pathway can afford the abundant quantity of GGPP (vez et al., 2001). Additionally, cloning of taxadiene synthase or other isoprenoid related genes could be implemented in crop plant such as tomato and potato, which have more active secondary metabolism and high production of GGPP-dependant diterpenoid.

Conclusion

To pursue the potential of the model plant '*Arabidopsis*' for transformation with desired customized constructs to produce the target secondary metabolite, in this current investigation, transformation of *Arabidopsis* through inducible expression of pTA-TXS-HIS construct was carried out. Transformation of *Arabido-psis* towards production of TXS was found and it was thus proposed that this model plant and perhaps other crop plants could be exploited for desired genetic modification. This approach may favor the production of scarce diterpenoid intermediates such as taxadiene used for semi-synthesis of taxol in pharmaceutical industries.

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