Cloning and functional characterization of an isopentenyl diphosphate isomerase gene from *Tripterygium wilfordii*

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Abstract

Tripterygium wilfordii Hook.F. is one of the most valuable medicinal plants because it contains a large variety of active terpenoid compounds, including triptolide, celastrol, and wilforlide. All of the pharmacologically active secondary metabolites are synthesized from the 2-*C*-methyl-D-erythritol 4-phosphate and mevalonate pathway in the isoprenoid biosynthetic system. The key step in this pathway is the isomerization of dimethylallyl diphosphate and isopentenyl diphosphate, which is catalyzed by isopentenyl diphosphate isomerase (IPI). In the present study, a full-length cDNA encoding IPI (designate as TwIPI, GenBank accession no.KT279355) was cloned from a suspension of cultured cells from *T. wilfordii*. The full-length cDNA of *TwIPI* was 1,564 bp and encoded a polypeptide of 288 amino acids. The bioinformatics analysis showed that the deduced TwIPI sequence contained the TNTCCSHPL and WGEHELDY motif. The transcription level of the *TwIPI* in the suspension cells increased almost fivefold after treatment with methyl jasmonate as an elicitor. A functional color assay in *Escherichia coli* indicated that *TwIPI* could promote the accumulation of lycopene and encoded a functional protein. Volume 00, Number 0, Pages 1–7, 2015

Keywords: *bioinformatics analysis, expression analysis, functional color assay, isopentenyl diphosphate isomerase, Tripterygium wilfordii*

1. Introduction

Terpenoids are among the largest and most widespread natural products, and play crucial roles in cellular membrane composition, photosynthesis, signaling cascades, and defense against predators [1, 2]. All terpenoids are derived from a single building block, isoprene C_5-unit, and the final structures have diverse, but exact numbers of C_5-units. There are two diphosphorylated C_5-units: dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP). The 1′-4 condensations of IPP and DMAPP prolong the chain and successively form the short-chain isoprenoid precursors, such as geranyl diphosphate (GPP, C_{10}), farnesyl diphosphate (FPP, C_{15}), and geranylgeranyl diphosphate (GGPP, C_{20}). Then, these basic carbon chain

Abbreviations: *Amp, ampicillin; Cm, chloramphenicol; crtB, phytoene synthase; crtI, phytoene desaturase; DMAPP, dimethylallyl diphosphate; 2,4-D, 2,4-dichlorophenoxyacetic acid; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; GGPPS&crtE, geranylgeranyl pyrophosphate synthase; GPP, geranyl diphosphate; HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; IBA, indole-3-butytric acid; IPI, isopentenyl diphosphate isomerase; IPP, isopentenyl diphosphate; KT, cytokinin; MeJA, methyl jasmonate; MS, Murashige and Skoog; qPCR, real-time quantitative polymerase chain reaction.*

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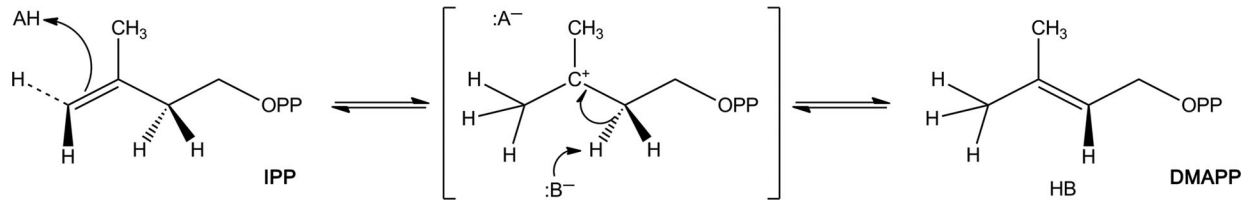

FIG. 1 *The mechanism of the isomerization between IPP and DMAPP.*

precursors are further cyclized and modified, for example, oxidation and rearrangement reaction, to form a wide variety of compounds [3]. Therefore, IPP and DMAPP are the starting materials for the carbon skeleton construction and play a vital role in the biosynthesis of terpenoids. Recent works have demonstrated that the ratio of DMAPP/IPP regulates the isoprene emission in both plants and bacteria [4, 5]. The isopentenyl diphosphate isomerase (IPI) catalyzes the transition between DMAPP and IPP involved a protonation/deprotonation reaction. More specifically, the isomerization of the carbon–carbon double bond is initiated from an antarafacial addition of hydrogen, develops a carbocation intermediate and is completed upon the elimination of hydrogen (Fig. 1) [6].

The identification of the isomerization step is of major significance in the overall regulation of terpenoid biosynthesis for both theoretical and applied purposes. The participation of *IPI* in the control of terpenoid accumulation has been supported by previous investigations. The yield of lycopene was enhanced in engineered *Escherichia coli* that contained the exogenous *IPI* gene compared with control strains that possessed the endogenous gene alone [7, 8]. Overexpression of the *IPP* gene in *Eucommia ulmoides* increased the total content of trans-polyisoprenes by threefold to fourfold compared with the wild-type organism [9]. The engineered *E. coli* strain carrying the *IPI* gene from *Kocuria gwangalliensis* and astaxanthin biosynthesis genes showed a twofold increased production of astaxanthin compared with the original strains [10].

One of the immediate focuses in this field is the biosynthesis pathways of active terpenoids from plants, such as taxol in *Taxus chinensis* [11], artemisinin in *Artemisia annua* [12], and tanshinone in *Salvia miltiorrhiza* [13]. These compounds generally show obvious pharmacological activity against intractable and high incidence diseases such as cancer, malaria, and angiocardiopathy. *Tripterygium wilfordii* (Hook.F.) is a vine plant that is also known as the "Thunder of God Vine." *T. wilfordii* belongs to the Celastraceae family and has been widely used as an anticancer, anti-inflammatory, and immunomodulatory medicine and insecticide in China for hundreds of years. The primary bioactive components in the *T. wilfordii* are terpenoids and sesquiterpenoid alkaloids, for example, triptolide, tripdiolide, celastrol, and tripfordine [14]. Various studies have been performed on the pharmacological activities and clinical toxicity of these compounds [15]. Compared with the current standards of therapy (*e.g.*, sorafenib, doxorubin, and daunorubicin), triptolide was more effective against the patocellucar carcinoma [16]. Moreover, the combination of triptolide and cisplatin enhanced the antitumor effects in cisplatin-resistant T24R2 bladder cancer cells [17]. Celastrol can regulate the ratio of pathogenic T helper 17 and protective T regulatory cells in inflamed joints, consequently suppressing autoimmune arthritis [18]. However, limited information is available regarding the biosynthetic pathways of triptolide from *T. wilfordii*.

Triptolide biosynthesis, the enzymes of which are closely related to the genes involved in the biosynthesis of terpenoids, has not been elucidated. Because *IPI* plays an important role in the biosynthesis of terpenoids in *T. wilfordii*, we report here a full-length cDNA of the committed gene (*TwIPI*) from this medicinal plant. Bioinformatics analysis suggests that TwIPI has conserved motif TNTCCSHPL and WGEHELDY. The present study also investigates the comparative expression of *TwIPI* in suspended cells in response to chemical stimuli (*e.g.*, methyl jasmonate [MeJA]). In addition, the function of TwIPI in the terpenoid biosynthesis was characterized and its relationship with terpenoid accumulation was also discussed.

2. Materials and Methods

2.1. Suspension cells

The *T. wilfordii* suspension cells used in the study were cultured axenically in Murashige and Skoog (MS) medium (pH = 5.8) containing 0.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg L^{-1} cytokinin (KT), 0.5 mg L^{-1} indole-3-butytric acid (IBA), and 30 g L^{-1} sucrose at 25 ± 1 °C and shaking at 120 rpm in the dark. Healthy and vibrant cultures were maintained by subculturing 25 mL of fresh medium with 2 g of the previous suspension cells every 20 days.

2.2. RNA isolation and cDNA cloning

Suspended cells were harvested and immediately frozen in liquid nitrogen for RNA extraction. Total RNA was extracted using an optimized cetyltrimethylammonium bromide method [19] and purified. The primers (forward: TTCAATTCAACAAGGGCGTCG and reverse: CAAATAGCCAAACCATAAGCA) were designed according to *TwIPI* sequence in transcriptome data. The *TwIPI* cDNA isolation was obtained through the PrimeScript 1st Strand cDNA Synthesis Kit and PrimeSTAR GXL DNA Polymerase (Takara, Dalian, People's Republic of

China) according to the manufacturer's instructions. The PCR product was purified and cloned into the *pEASY*-T3 vector. The recombinant vector was transferred into *E. coli* Trans5α competent cells. DNA sequencing was used to identify positive clones containing the *TwIPI* sequence.

2.3. Bioinformatics analysis

The nucleotide sequence of *TwIPI* was analyzed for its open reading frame and homology to other *IPI* sequences (http://www.ncbi.nlm.nih.gov/). The deduced amino acid sequence of *TwIPI* was aligned against those of other plant IPIs using the Clustal W method with default parameters (Bioedit; Borland, CA, USA). DNAMAN 8.0 was utilized to perform multiple sequence alignments. A phylogenetic tree was constructed using the neighbor-joining method in MEGA5.1.

2.4. Gene expression analysis

Transcript levels of *TwIPI* were analyzed using real-time quantitative polymerase chain reactions (qPCR). MeJA is a common elicitor used for plant tissue cultures [20–22]. Here, suspension cells of *T. wilfordii* were induced with 50 μM MeJA and subsequently collected from the culture flask at 0, 1, 4, 12, 24, 48, 72, and 120 H. All of the samples were kept at −80 °C until the RNA was isolated. The cDNA obtained from the reverse translation of total RNA was used as the template for the qPCR analysis. The qPCR primers were designed by Primer Premier 5.0 as follows: β-actin (forward: AGGAACCACCGATCCAGACA and reverse: GGTGCCCTGAGGTCCTGTT) and IPI-RT (forward: GTCCCTTCCACCCTAACC and reverse: GCCCAACCACACGATCATTC). The ABI 7500 system and KAPA SYBR FAST qPCR Kits were used to carry out the qPCR analysis. The amplification reaction mixture was composed of 10 μL of 2× qPCR Master Mix, 1 μL of cDNA (approximately 50 ng), 0.4 μL of 50× ROX reference Dye Low, 0.4 μL of each primer (10 μM), and 7.8 μL of ddH_2O. The cycling conditions were as follows: 5 Min at 95 °C followed by 40 cycles of 3 Sec at 95 °C and 33 Sec at 60 °C. The association analysis was then added to detect the fluorescence signal. Each sample was repeated in triplicate to insure the credibility of the data, and the final data were evaluated using the $2^{-\Delta\Delta Ct}$ method with β-actin as the endogenous control [23].

2.5. Construction of expression vectors

The pTrc-*AtIPI* expression vector was kindly provided by Francis X Cunningham Jr. (Gantt) (Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD, USA). pTrc-*AtIPI* was derived from the combination of the *IPI* gene from Arabidopsis thaliana (*AtIPI*), pTrcHisB and pBlueScript SK- [24]. *TwIPI*, spanning from the start codon (ATG) to the stop codon (TAA) was amplified with the primers that included restriction sites (forward: 5′-GGGGTACCATGTCTGTGGTCTCTCGCCT-3′, *Kpn*I site underlined; reverse: 5′-CGGGATCCTTAGGTCAACTTGTGAATTGTT-3′, *Bam*HI site underlined). The PrimeSTAR GXL DNA Polymerase (Takara) and the Gene JET Gel Extraction Kit (Thermo, Beijing, China) were used to amplify and purify the PCR product, respectively. After the restriction enzyme (*Kpn*I and *Bam*HI) digestion of pTrc-*AtIPI*, the Quick DNA Ligase ligated the sticky ends of the cut DNA and plasmid to form the recombinant plasmid pTrc-*TwIPI*. On both sides of the *AtIPI* sequence in pTrc-AtIPI vector, the same *Pst*I site was digested and immediately ligated, yielding the empty vector pTrc. All of the plasmids were enriched, extracted, and stored at −20 °C in preparation for functional analysis.

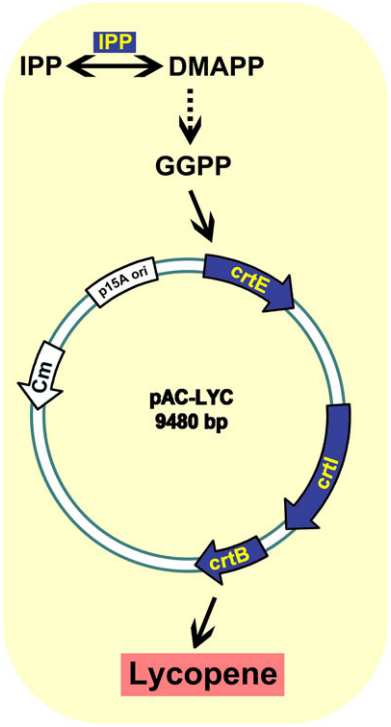

FIG. 2 *The synthesis of lycopene in E. coli with the pAC-LYC vector.*

2.6. Functional analysis of TwIPI in *E. coli*

To determine the function of *TwIPI*, the pTrc and pTrc-*AtIPI* vectors were used as negative and positive controls, respectively, in the functional color assay. The pAC-LYC vectors from professor Francis X Cunningham Jr. (Gantt) contained three key genes involved in the synthesis of lycopene: geranylgeranyl pyrophosphate synthase (GGPPS&*crtE*), phytoene synthase (*crtB*), and phytoene desaturase (*crtI*) [22, 25]. *E. coli* itself can produce the essential precursors IPP and DMAPP, but cannot synthesize lycopenes because of the absence of the lycopene pathway [23, 26]. However, once the pAC-LYC plasmid was transferred to *E. coli*, the bacteria would express the genes in lycopene pathway and yield lycopenes (Fig. 2) [27, 28]. The accumulation of lycopene leads to a visual color change of the colonies. Based on this principle, the pTrc, pTrc-*AtIPI*, and pTrc-*TwIPI* were co-transformed with pAC-LYC into *E. coli Trans*1-Blue cells (TransGen Biotech, Beijing, People's Republic of China), which could serve as an appropriate expression system for

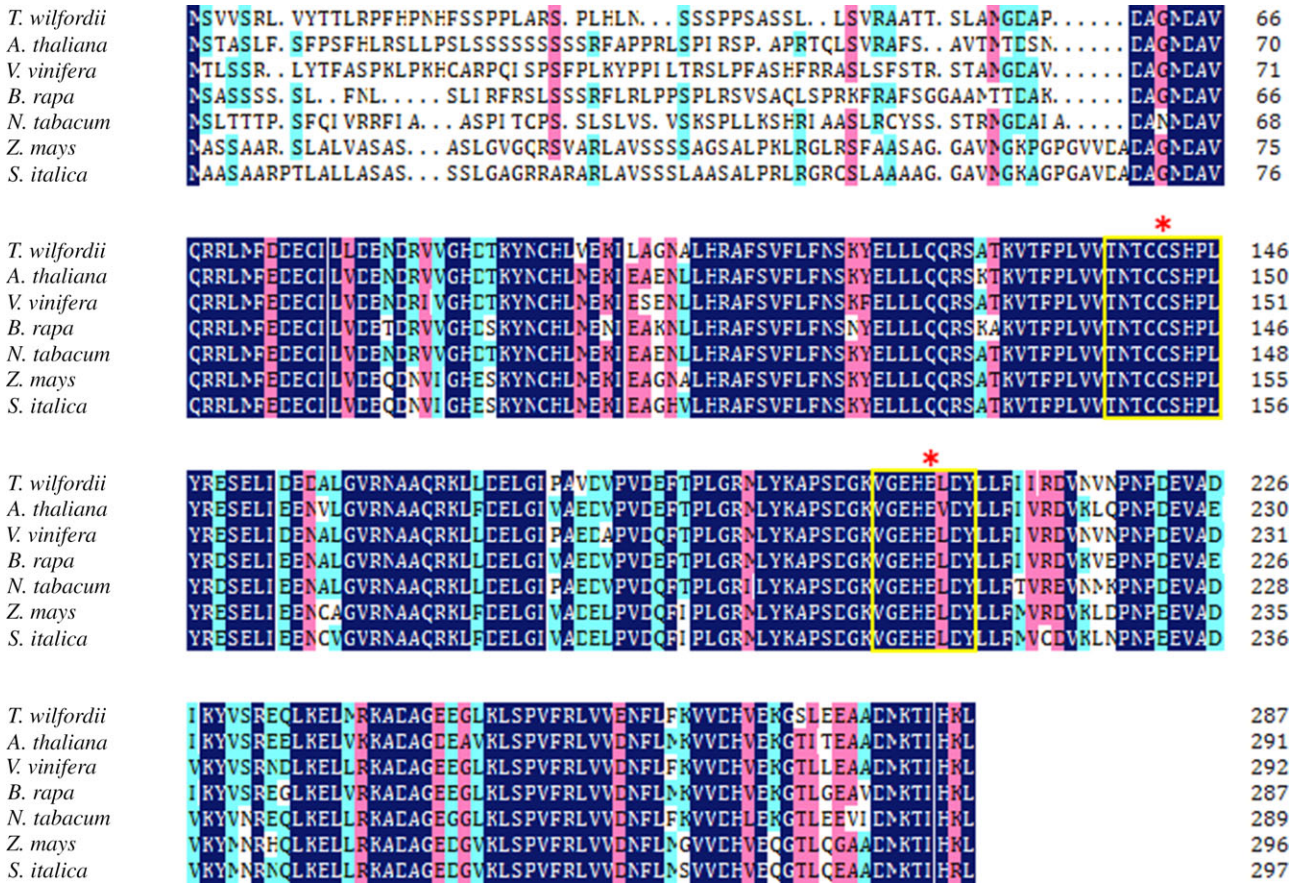

FIG. 3 *Multiple alignments of deduced amino acid sequence of TwIPI with other IPIs Arabidopsis thaliana (NP_197148.3), Vitis vinifera(XP_002277935.1), Brassica rapa (XP_009147323.1), Nicotiana tabacum(BAB40973.1), and Zea mays(XP_008649644.1) are shown here. The conserved residues are colored in dark blue and homologous residues are colored in pink. Yellow boxes show the residues that are critical for the catalytic activities of the enzyme. The conserved C (cysteine) and conserved E (glutamic acid) are indicated with asterisks.*

lycopene [29]. The plasmid pAC-LYC retained a chloramphenicol (Cm) resistance gene, whereas the pTrc-AtIPI plasmid contained an ampicillin (Amp) resistance gene. Therefore, all of the transformation systems were cultured in LB medium containing 100 mg L^{-1} Amp and 20 mg L^{-1} Cm at 28 °C for 3–4 days.

3. Results and Discussion

3.1. Cloning and sequence analysis of *TwIPI* cDNA

The full-length cDNA isolated from the *T. wilfordii* suspension cells comprised 1,564 bp and contained an open reading frame coding a protein of 288 amino acids with a predicted molecular weight of 32.65 kDa and a theoretical isoelectric point of 5.59. Sequence analysis revealed that TwIPI exhibited high homology with reported IPI sequences from other plants, including *Hevea brasiliensi* (BAF98287.1, 91% identity), *Vitis vinifera* (AEP17009.1, 91% identity), *Glycine soja* (KHN40475.1, 90% identity), and *Astragalus membranaceus* (AID51443.1, 90% identity). The deduced protein contained a NUDIX hydrolase domain (AA 107–255), which is an important functional domain of IPI and indicated that the cloned gene was an *IPI* gene from *T. wilfordii*.

3.2. Bioinformatics analysis

The multiple sequence alignment of the deduced amino acid sequence with those of other IPIs revealed that TwIPI has a number of highly conserved regions that are common to IPIs from other green plants. Eukaryotic IPIs contain a conserved cysteine residue within a TNTCCSHPL motif and a conserved glutamate residue within a WGEHExDY motif [30, 31]. These motifs have been shown to be critical for substrate combination and catalysis [32]. Cys139 in the TNTCCSHPL motif from *Saccharomyces cerevisiae* is an active site residue according to the research of Street et al. [30]. TwIPI contained the two conserved active residues: acysteine (142C) in the TNTCCSHPL motif and a glutamate (204E) in the WGEHELDY motif (Fig. 3).

To further explore the evolutionary relationships between TwIPI and other IPIs from diverse organisms (including plants,

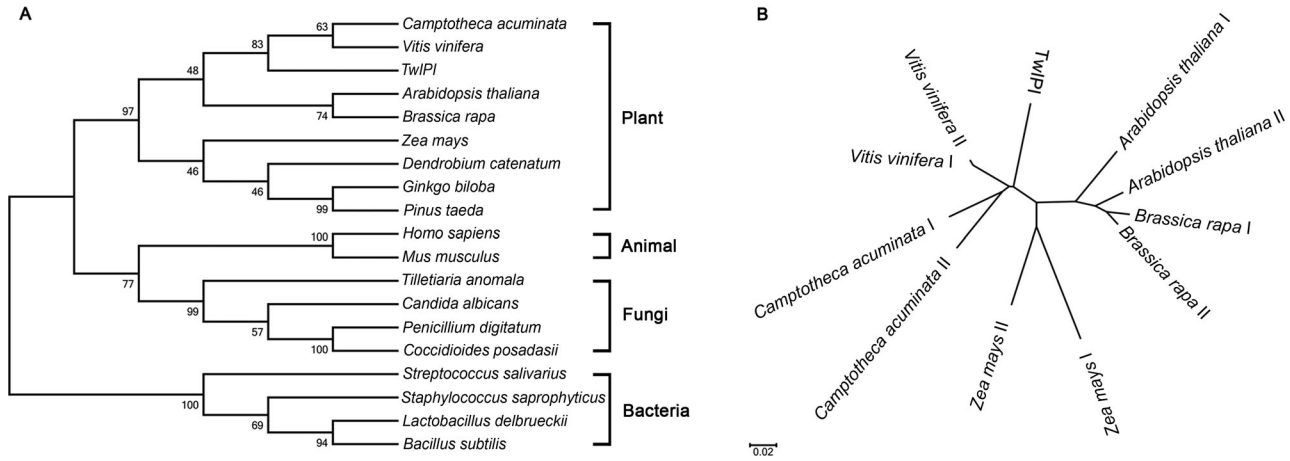

FIG. 4 *Phylogenetic analysis of TwIPI with other IPIs from plants, animals, fungi, and bacteria (A) and with two type IPIs from high plants. The phylogenetic trees were constructed using the Neighbor-Joining method in MEGA 5.1. The protein sequences used in these trees are as follows: (A) Homo sapiens (NP_004499.2), Mus musculus (NP_663335.2), Lactobacillus delbrueckii subsp. bulgaricus ATCC BAA-365(ABJ58480.1), Streptococcus salivarius PS4 (EIC80830.1), Staphylococcus saprophyticus subsp. saprophyticus ATCC 15305 (BAE17701.1), Bacillus subtilis QB928 (YP_006630491.1), Tilletiaria anomala UBC 951 (KDN52937.1), Penicillium digitatum PHI26 (EKV17609.1), Coccidioides posadasii str. Silveira (EFW18288.1), and Candida albicans SC5314(XP_720295.1); (B) Arabidopsis thaliana I(NP_197148.3), Arabidopsis thalianaII (NP_186927.1), Camptotheca acuminata I(AAB94132.1), Camptotheca acuminate II(AAB94133.1), Zea maysI (XP_008649644.1), Zea mays II(ACG24849.1), Vitis vinifera I(XP_002277935.1), Vitis vinifera(AEP17008.1), Brassica rapaI (XP_009147323.1), and Brassica rapaII (XP_009134709.1).*

animals, fungi, and bacteria), a phylogenetic tree was constructed based on the comparison of amino acid sequences of the various IPIs. It was revealed that all of the IPIs appeared to be derived from a common ancestor during evolution and that TwIPI was grouped into a cluster along with the high plants (Fig. 4A). The phylogenetic analysis among the high plants suggested that TwIPI was grouped into a cluster along with those from *V. vinifera*, thus paralleling their evolutionary relationships (Fig. 4B).

3.3. *TwIPI* expression analysis

The results of the qPCR indicated that expression level in the suspension cells was enhanced after the MeJA treatment. The *TwIPI* expression increased with a sharp rise after 48 H, which resulted in an approximately 5.28-fold increase compared with the control group. However, the expression reduced to normal levels at the 120 H (Fig. 5).

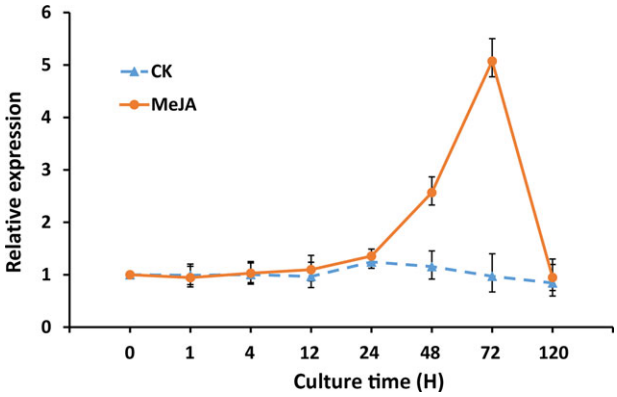

FIG. 5 *Expression of TwIPI in T. wilfordii suspension cells after MeJA induction CK, control group; MeJA, MeJA treatment group. Every data point represents the average of three independent experiments carried out in triplicate.*

3.4. Functional analysis of TwIPI in *E. coli*

The functional color assay was performed to identify the function of *TwIPI*. Engineered *E. coli* harboring only the pAC-LYC vector could produce lycopene only in trace amounts, whereas *E. coli* engineered with two plasmids, that is, pAC-LYC and pTrc-*AtIPI* harboring the Arabidopsis *IPI* gene, could synthesize much higher concentrations of lycopene [33]. It was clear that the *E. coli Trans*1-Blue cells containing pTrc-*AtIPI* and pAC-LYC formed pinker colonies than did the control 2 cells (C2) that were co-transformed with the empty vectors pTrc and pAC-LYC. The bacterial clones of *E. coli* harboring pTrc-*TwIPI* and pAC-LYC were dark pink, which was indicative of abundant lycopene levels. Control 1 (C1) *E. coli* cells that harbored the single vector pAC-LYC failed to grow on LB medium with Amp and Cm because of the lack of the Amp resistance genes (Fig. 6). The light and dark color of the colonies is directly indicative of the yield of lycopene: the more lycopene that is produced by the colonies, the pinker the color is. Hence, it has been shown that the addition of the *AtIPI* gene accelerates the production of lycopene, thus forming dark pink colonies. Similar analysis and results were exhibited in the characterization of *TwIPI*, which demonstrated that *TwIPI* could assume the function of *AtIPI* in the engineered *E. coli* to promote the biosynthesis of lycopene. In summary, the color

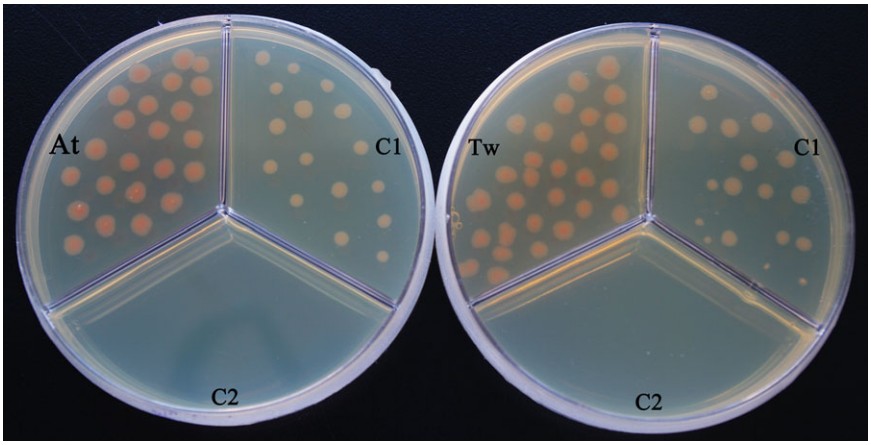

FIG. 6 *Functional identification of TwIPI in E. coli Either pTrc-AtIPI (At), pTrc-TwIPI (Tw) or pTrc (C1) were co-transformed into E. coli XL1-Blue with pAC-LYC. The single transformation of pAC-LYC was used as the C2 control.*

complementation assay confirmed that *TwIPI* encoded the functional IPI protein and played a major role in promoting lycopene production.

The functional color assay has been used to identify the function of several genes in the biosynthesis pathway of terpenoids. In *Amomum villosum*, the1-deoxy-D-xylulose-5-phosphate reductoisomerase gene and the 1-deoxy-D-xylulose-5-phosphate synthase (DXS) gene were characterized via color assay [34]. Additionally, the 3-hydroxy-3-methylglutaryl coenzyme A reductase gene from *Withania somnifera* encodes a functional protein and may play an important role in isoprenoid biosynthesis, as determined by the functional color assay [35]. Moreover, this method helped confirm a gene encoding 2-C-methyl-D-erythritol 4-phosphate cytidyltransferase from hairy roots of *Rauvolfia verticillata* [36]. To date, IPIs from *Camptotheca acuminate*, *Ipomoca batata, Gossypium barbadense*, and *Ganoderma lucidum* have been tested with the functional color assay, which indicated that the function of these IPIs were similar to the *Arabidopsis* IPI [37–40].

As demand for *T. wilfordii* rises gradually, wild resources could not satisfy anymore. The main pharmacological components, like triptolide, tripdiolide, celastrol, and so on, have a low content in *T. wilfordii* (approximately 40–50 μg/g) [41]. Metabolic engineering offers sustainable and alternative methods to improve active terpenoids. A co-overexpression of geraniol-10-hydroxylase and strictosidine synthase improved 1.56-fold yield of camptothecin in *Ophiorrhiza pumila* hairy roots [42]. Overexpression of 3-hydroxy-3-methylglutaryl CoA reductase (HMGR), DXS, and GGPPS can significantly enhance the production of tanshinone. The co-expression of HMGR and GGPPS could realize a highest production of tanshinone (about 2.727 mg/g dw) [43]. Hence, through metabolic engineering will be an efficient and functional way to enhance content of triptolide and celastrol. In the present study, a new functional *TwIPI* gene involved in the biosynthesis of terpenoids in *T. wilfordii* was cloned and characterized. What more, the farnesyl pyrophosphate synthase genes from *T. wilfordii* were also found [44]. We may use these genes to improve the yield of terpenoids in *T. wilfordii* through metabolic engineering methods in the future.

4. Conclusion

In this paper, the full-length *TwIPI* gene was isolated from *T. wilfordii*, and a detailed expression analysis of *TwIPI* was performed. The functional color assay confirmed that *TwIPI* plays a significant role in promoting lycopene pathway flux and encodes a functional protein.

5. Acknowledgements

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