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Full Length Research Paper

Molecular cloning, characterization and functional analysis of a new isopentenyl diphosphate isomerase gene (IPI) from *Curcuma wenyujin*

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A new isopentenyl diphosphate isomerase gene *cwipi* (GenBank: GU724874) was cloned from the *Curcuma Wenyujin* cDNA library. The full-length cDNA contained a 708 bp open reading frame (ORF) encoding a protein of 235 amino acids with calculated molecular mass of 27.36 kDa and isoelectric point of 5.14. Phylogenetic analysis demonstrated that *cwipi* has a high level similarity to other IPIs belonged to angiosperm group. The protein encoded by *cwipi* was subcellularly localized to the cytoplasm using TargetP prediction software. Semi-quantity RT-PCR results indicated that the transcriptional level of *cwipi* in roots was higher than that of in stems and leaves. *Cwipi* could accelerate the accumulation of β-carotene when it was expressed in *Escherichia coli* harbouring reconstructed carotenoid synthetic pathway. The cloning, characterization and functional analysis of *cwipi* will be useful for understanding the role of IPI in isoprenoid biosynthesis of *C. Wenyujin.*

Key words: Isopentenyl diphosphate isomerase, *Curcuma Wenyujin*, cDNA Library.

INTRODUCTION

Curcuma wenyujin, a perennial plant belonging to the family Zingiberaceae, was an important herbal plant in traditional medicine of China. It can be used to extract several natural products with therapeutical value including curcumenol, curdione, β-elemene. As a kind of sesquiterpene, β-elemene has clear antitumor activity for a variety of tumor types. Pharmaceuticals derived from β-elemene had come into the market several years ago in China. So it was cultivated in large area in China, particularly in Zhejiang Province (Guo, 1983).

Isopentenyl diphosphate (*ipp)* is the molecular "building block" for more than 25,000 distinct isoprenoids, including cholesterol, steroid hormones and terpenoids. The biosynthesis of isopentenyl diphosphate from either the mevalonate (MVA) or the 1-deoxy-D-xylulose 5-phosphate (DXP) pathway would provide the key metabolite for primary and secondary isoprenoid biosynthesis (Dellas and Noel, 2010). The conversion of isopentenyl diphosphate (IPI) to dimethyl-allyl diphosphate (DMAPP), catalyzed by IPI isomerase (IPI), is an important step in the early stages of isoprenoid metabolism (Kalinowska-Tluscik et al., 2010). The investigation of IPI isomerase from *C. Wenyujin* is necessary to study the β-elemene biosynthesis pathway in *C. wenyujin.* In the next step, biosynthesis of β-elemene using microorganism and transgenic plant study will be carried out in our group.

MATERIALS AND METHODS

Plant materials and Total RNA isolation

Plant materials collected from a commercial *C. Wenyujin* plantation of Wenzhou (Zhejiang, China) were used as the starting material for total RNA isolation.

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Table 1. Primers used in this study.

Primers name	Sequence(5' to 3')
SMART IV™ Oligonucleotide	AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGG
CDS III/3'PCR Primer	ATTCTAGAGGCCGAGGCGGCCGACATG-d(T)$_{30}$N$_{-1}$N
5'PCR Primer	AAGCAGTCGTATCAACGCAGAGT
cDNA LD insert Screening F	CTCGGGAAGCGCGCCATTGTG
cDNA LD insert Screening R	TAATACGACTCACTATAGGGCGAATTGGCC
10×Universal Primer A Mix/UPM Long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
10×Universal Primer A Mix/UPM Short	CTAATACGACTCACTATAGGGC
Nested Universal PrimerA /NUP	AAGCAGTGGTATCAACGCAGAGT
o*ipi*-f	AGATCTATGGCGGCAGTGGCGGATGAT
o*ipi*-r	GCGGCCGCTTAAACCAGTTTATGAATCGT
sqCw*ipi*-f	AAAGTTATCAACCACCAGCCTGAA
sqCw*ipi*-r	ATGTTCGAGGACGAATGTATCTTG
sq18S-f	CAACCTGGTTGATCCTGCCAGTAG
sq18S-r	AACTATCCTACCATCGAAAGTTGA
M13-F	GTAAAACGACGGCCAGT
M13-R	AACAGCTATGACCATG

About 1 g young leaves were powdered in liquid nitrogen and used for extraction of total RNA following the protocol of RNAsimple Total RNA Kit (BIOSCI, Hangzhou, China). The quality of total RNA was detected by AstraGene UV/Vis Spectrophotometer (AstraNet Inc., Bath, UK) and 1.5% (w/v) agrose/ethidium bromide gel.

cDNA Library construction and analysis

The double strands cDNA synthesis and library construction were carried out mainly according to the manual of SMART™ cDNA Library Construction Kit (Clontech, Beijing, China).

First-strand cDNA synthesis was performed using 0.5 μg total RNA, as described in the SMART™ cDNA Library Construction Kit User Manual (PT3000-1version PR043527). The cDNA was amplified employing KOD Plus polymerase (TOYOBO, Osaka, Japan) and primers supplied by the Kit. After the proteinase K digestion, the PCR products were digested with *SfiI* restriction enzyme (Takara, Dalian, China).

DNA fragment larger than 500 bp were extracted from agarose gel by Power Gel Extraction Kit (BIOSCI, Hangzhou, China) and ligated into *SfiI*-digested dephosphorylated bacteriophage λ TriplEx2™ vector (Clontech, Beijing, China). Then the whole ligation systems were packaged using Packagene ® Lambda DNA Packaging System (Promega, Shanghai, China) and amplified. The λ TriplEx-2 phage library was converted to the plasmid form pTriplEX2 library in *E. coli* BM25.8 according to the manufacture's protocols (Clontech, Beijing, China).

Cloning the Isopentenyl Diphosphate Isomerase Gene

By random colony sequencing of cDNA library, we obtained a 3'end completed cDNA fragment (5' RACE ready cDNA) of *ipi*. And then use rapid amplification of cDNA ends (RACE) as described in the SMARTer™ RACE cDNA Amplification Kit User Manual (PT4096-1). We obtain a 5'RACE fragment .The fragment sequencing was completed by Shenggong Inc (Shanghai, China). 5'RACE and the 3'end completed fragment sequence were assembled by Contig Express (Vector NTI Suite 10.0), so full-length cDNA sequence of *ipi* was obtained.

Comparative and bioinformatics analyses

Comparative and bioinformatics analyses were carried out online at the website NCBI (http://www.ncbi.nlm.nih.gov/) and ExPASy (http://cn.expasy.org/). The nucleotide sequence, deduced amino acid sequence and open reading frame (ORF) encoded by *cwipi* were analyzed and alignment using the BLAST program (http://blast.ncbi.nlm.nih.gov/), the subcellular location was predicted by TargetP (http://www.cbs.dtu.dk/services/TargetP/). The phylogenetic analysis of *Cwip*i and IPI genes from other species were aligned using CLUSTAL X (2.1) with default parameters. The phylogenetic tree was constructed using MEGA 5.0 and CLUSTAL X alignments result by the neighbor-joining method with 1000 repeats.

C*wipi's* expression pattern in different tissues

Semi-quantitative RT-PCR analysis was employed to reveal the express profile of *cwipi* in different tissues including leaves, stems and tuberous roots. In RT reaction using ReverTra Ace-α^{R}-kit (TOYOBO, Japan) and the oligo (dT) primer. 1 μg total RNA isolated from different tissues was use as the template. For the next PCR amplification, 2 μl cDNA was used as the template using KOD plus polymerase and specific primer pairs (sqCw*ipi*-r, sqCw*ipi*-f, Table 1). Meanwhile the housing-keep 18S rRNA gene was amplified using specific primer pairs of sq18S-r, sq18S-f (Table 1) as the internal control in order to estimate whether the equal amounts of total RNA of different samples were used in the RT-PCR reaction.

Codon optimization and functional analysis of *ipi* in *Escherichia coli*

For every amino acid which can be encoded by more than one codon, over-expression of recombinant protein in *E. coli* may be severely diminished (to the point of being undetectable) if the ORF

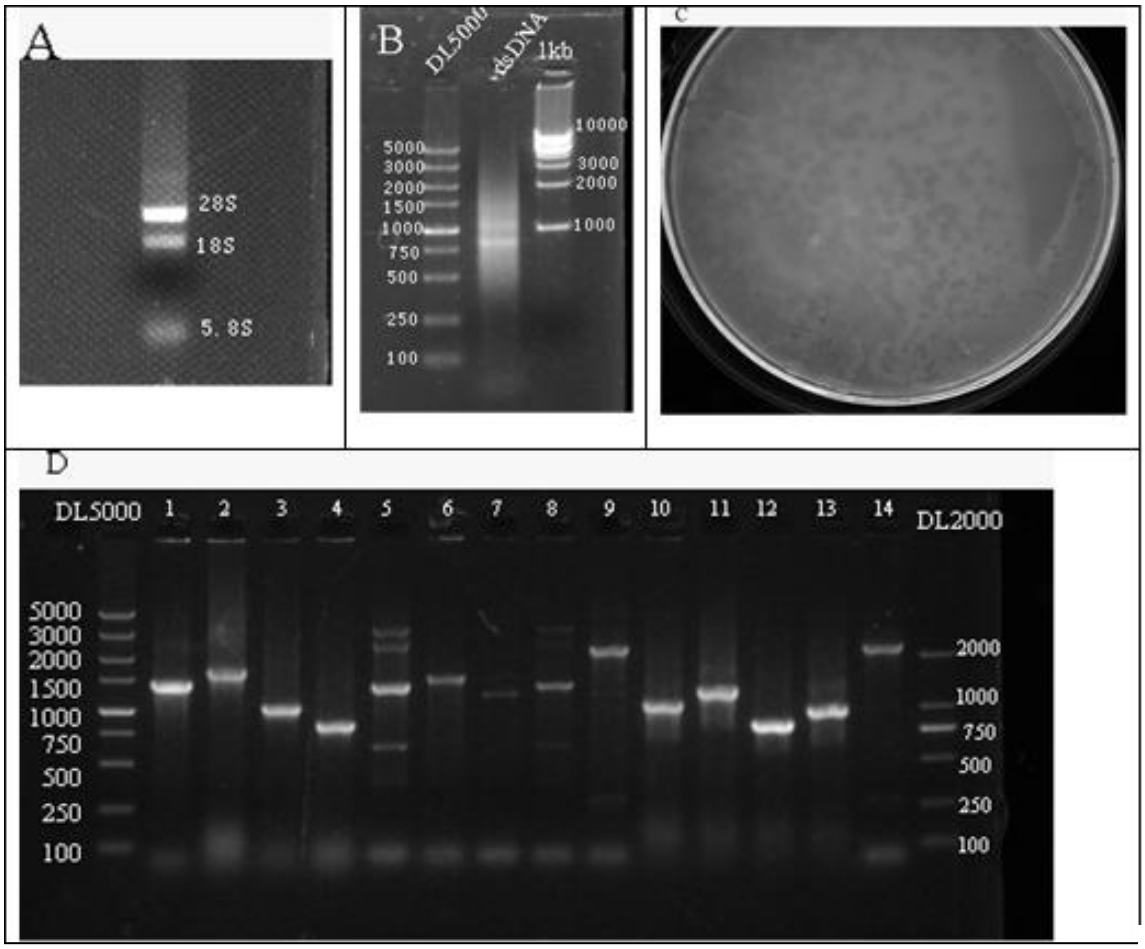

Figure 1. The construction of a cDNA library from *Curcuma Wenyujin*'s young leaves (A, Total RNA isolated from the *Curcuma Wenyujin*'s young leaves. The dsDNA (B) was amplified by LD-PCR, appearing as a smear of 0.5-6kb.The titer of amplified cDNA library was 2×10^{11} pfu/ml by calculation of the plaques (C); thirteen cDNA inserts were detected to confirm the quality of the library by PCR amplification. All the PCR products were larger than 750 bp, and average insert of the recombinants was 1.0 kb long).DNA marker was purchased from TaKaRa (Dalian, China).

that codes for the protein uses "rare" codons that are infrequently used by *E. coli*. In particular, codons for arginine (AGG, AGA, and CGA), leucine (CTA), isoleucine (ATA), and proline (CCC) may be a problem. And the most frequently used codons in *E. coli* are different from those used in *C. Wenyujin*. In order to improve the *cwipi* expressing level in *E. coli*, *cwipi* gene was codon optimized and synthesized by GENEray Inc. (Shanghai, China). And the following function analysis was carried out using the optimized gene (*Ocwipi)*.

For *cwipi*'s function analysis, we introduced color complementation strategy. *E. coli* DH5α was preserved by our laboratory; the plasmid pAC-BETA and pTrc were kindly provided by Dr. RongChen.

Common procedures, including genomic DNA preparation, restriction digestions, transformations, and other standard molecular biological manipulations were carried out as described in the Molecular Cloning (Sambrook, the 3rd edition, 2001).The gene of *Ocwipi* was amplified using KOD plus polymerase and a pair of primers (o*ipi*-f, o*ipi*-r, Table 1), which harbor *Bgl*II, and *Not*I restriction sites, separately. After digestion by *Bgl*II and *Not*I, OCw*ipi* was ligated into pTrc. Then OCw*ipi*-pTrc and the pTrc were transformed into the *E. coli* strain DH5α harboring pAC-BETA, separately. And the positive transformants were selected by ampicillin (50 mg/ml) and chloromycetin (34 mg/ml). In order to observe the β-carotene production of the bacteria, 100 µM IPTG was added in the stroke plate which were cultured at 28°C for 48 h.

RESULTS

RNA isolation and cDNA library construction

The total RNA from *Curcuma Wenyujin*'s young leaves with the concentration of 130.6 ng/µl, and OD_{260}/OD_{280} value of 2.022 was detected by agarose electrophoresis (Figure 1A). The synthetic double-strand cDNA appeared as a smear of 0.5-6 kb in agarose electrophoresis (Figure 1B). The double-strand cDNAs were ligated into the *Sfi* I-digested λ TriplEx2™ vector as the 3:1 ligation molar ratio. The titer of the amplified library was about

2×10^{11}pfu/ml by calculation of the plaques (Figure 1C). After phage library was converted into bacteria library we randomly picked 14 bacteria colonies from the cDNA library to detect the inserts size by PCR amplification. Figure 1D shows that the inserts of the recombinants have an average length of 1.0 kb.

Cloning of Isopentenyl Diphosphate Isomerase Gene

In order to obtain IPI gene the cDNA library was sequenced by random colony selecting. A 663 bp 3'end completed cDNA fragment was selected by BlastX analysis result which showed high homology to other isopenteny diphosphate isomerase genes (ipi). Based on this 3'end completed cDNA 's sequence the 775bp cDNA fragment was amplified by 5'RACE. By comparing and aligning the sequences of the core fragment, the 5'RACE PCR product and 3' end completed fragment, the full-length cDNA sequence of *cwipi* was deduced .To confirm our inference, RT-PCR was performed using the primers yz*ipi*5-f and yz*ipi*3-r (Table 1), then the PCR products were ligated into p*EASY*-Blunt simple T vector (TransGene Biotech Inc., Beijing, China) and sequenced by Shenggong Inc. (Shanghai, China). The full-length cDNA of 981bp contained a 708bp ORF (GenBank Accession No.GU724874) that encoding a peptide of 235 amino acids with a calculated molecular mass of 27.36 kDa and isoelectric point of 5.14 (Figure 2).

Comparative and bioinformatics analyses of *Cwipi*

Amino acid sequence alignment using Vector NTI suite 10.0 and BLAST (blastp) showed that the predicted *cwipi* had high identities and homologies with other IPIs, such as *Nicotiana tabacum* (BAB40974.1), *Gentiana lutea* (BAE92733.1), *Arabidopsis thaliana* (AAB67741.1) with the identity of 86, 86 and 85%, respectively (Figure 3). The phylogenetic result demonstrated that *cwipi* belonged to angiosperm groups other than gymnosperm groups (Figure 4). The TargetP analysis result showed that the *cwipi* is localization to the cytoplasm.

Tissues specific expression profile of Cw*ipis*

In order to investigate the transcriptional level of *cwipi* in different tissues total RNA from leaves, stems and tuberous root was prepared for semi-quantitative PCR. The results showed that *cwipi* expression could be detected in tuberous roots, stems, leaves, and the expression level was much higher in the tuberous roots and stems than that in the leaves (Figure 5).

Function analysis of *cwipi* in *Escherichia coli*

Expression level of recombinant protein in *E. coli* may be severely diminished (to the point of being undetectable) if the gene contains too much "rare" codons. So codon-optimized *cwipi* was synthesized and used in functional analysis. *E. coli* DH5α harboring plasmid pAC-BETA was used in the color complementation assay. This recombinant strain can produce and accumulate little β-carotene, so the colonies turned yellow in color. When *cwipi* was introduced into this strain the color of colonies turned from yellow to orange-yellow, which indicated that *cwipi* could accelerate the production and accumulation of β-carotene (Figure 6).

DISCUSSION

The *ipi* genes from several species such as bacterium (Anderson et al., 1989; Hahn et al., 1999; Kaneda et al., 2001; Laupitz et al., 2004; Yamashita et al., 2004), angiosperm (Blanc and Pichersky, 1995; Campbell et al., 1998; Nakamura et al., 2001; Pan et al., 2008), animal (Paton et al., 1997; Xuan et al., 1994) had been cloned and characterized. However there are no reports about the study of IPI gene from *C. Wenyujin* which was an important herb plant. Terpenoids including β-elemene extracted from *C. Wenyujin* have good pharmaceutical applications. IPI was one of key enzymes of terpenoids biosynthesis pathway. So the study of IPI is beneficial for better understanding of β-elemene biosynthesis mechanism. In this article we reported the cloning and characterization the gene encoding the IPI from *C. Wenyujin* for the first time. The deduced amino acid sequence of IPI showed significant similarity to IPI from other species. TargetP analysis showed that the IPI was localized in the cytoplasm. As existing theories sesquiterpenes from plants are usually synthesized in cytoplasm and IPI is one of key enzymes of sesquiterpene biosynthesis pathway. Basing on above results we can conclude that *cwipi* has close relationship with biosynthesis of sesquiterpenes from *C. Wenyujin* (Lichtenthaler et al., 1997). The terpenoid concentration profiles of herb plants varied greatly in different tissues. Compared to leaves and stems roots are main origin of terpenoids in *C. Wenyujin.* But whether the terpenoids such as β-elemene were mainly synthesized in roots or other tissues is unknown now. IPI is key metabolic enzyme of terpenoid biosynthesis. Many reports indicated that terpenoid was biosynthesized in specialized tissue and not transported between tissues. So high transcriptional level of *cwipi* in tuber roots and stems indicated that there were active metabolisms of terpenoid synthesis in these tissues of *C. Wenyujin*. These tissues were indeed sources of terpenoid of biosynthesis and store.

Conclusion

In summary, a new cytoplasmic isopentenyl diphosphate

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1    AAGC AGT GGT ATC AAC GCA GAG TAC ATG GGG GGC CTC ACG CTC CTC    46
47    TCC TCG CCC TCC GCC GTG ACG ATG GCC GCT GTC GCC GAC GAC GCT    91
                                  M   A   A   V   A   D   D   A     8
92    TCC ATG GAC GCC GTC CAG AGG CGC CTC ATG TTC GAG GAC GAA TGT   136
9      S   M   D   A   V   Q   R   R   L   M   F   E   D   E   C    23
137   ATC TTG GTG GAT GAG AAT GAC GTC GTC ATT GGG CAT GAA TCC AAA   181
24     I   L   V   D   E   N   D   V   V   I   G   H   E   S   K    38
182   TAC AAC TGC CAT CTG GTG GAA AAG ATT GAA TCA CTG AAC CTT CTC   226
39     Y   N   C   H   L   V   E   K   I   E   S   L   N   L   L    53
227   CAT AGA GCT TTT AGT GTT TTC CTA TTC AAC TCA AAA TAT GAG TTG   271
54     H   R   A   F   S   V   F   L   F   N   S   K   Y   E   L    68
272   CTA CTT CAG CAA AGA TCT GAA ACA AAA GTA ACA TTT CCT CTG GTT   316
69     L   L   Q   Q   R   S   E   T   K   V   T   F   P   L   V    83
317   TGG ACA AAT ACC TGC TGC AGT CAT CCG CTC TAC CGA GAA TCT GAG   361
84     W   T   N   T   C   C   S   H   P   L   Y   R   E   S   E    98
362   CTT ATT CAG GAG AAC TAC TTG GGG GTT AGA AAT GCT GCA CAA AGA   406
99     L   I   Q   E   N   Y   L   G   V   R   N   A   A   Q   R   113
407   AAG TTG CTG GAT GAG CTA GGC ATT CCT GCT GAA GAT ATA CCT GTT   451
114    K   L   L   D   E   L   G   I   P   A   E   D   I   P   V   128
452   GAT GAA TTC ATT CCT CTC GGC CGC ATG CTT TAT AAA GCT CCA TCC   496
129    D   E   F   I   P   L   G   R   M   L   Y   K   A   P   S   143
497   GAT GGA AAA TGG GGG GAA CAT GAG GTT GAC TAC CTG CTT TTC ATC   541
144    D   G   K   W   G   E   H   E   V   D   Y   L   L   F   I   158
542   GTC CGG GAC GTG AAG CTG CTG CCG AAT CCT GAT GAA GTT GCC GAC   586
159    V   R   D   V   K   L   L   P   N   P   D   E   V   A   D   173
587   GTG AAG TAC GTG AAC CGA GAC CAG CTG AAG GAG CTG CTG AGG AAG   631
174    V   K   Y   V   N   R   D   Q   L   K   E   L   L   R   K   188
632   GCA GAT GCC GGC GAG GAG GGT GTC AAG CTC TCT CCA TGG TTC AGG   676
189    A   D   A   G   E   E   G   V   K   L   S   P   W   F   R   203
677   CTG GTG GTT GAT AAC TTT CTA ATG GAG TGG TGG GCT CGT GTG GAG   721
204    L   V   V   D   N   F   L   M   E   W   W   A   R   V   E   218
722   CAA GGC ACT CTC CTG GAG GCA GCC GAT ATG GAA ACC ATT CAT AAA   766
219    Q   G   T   L   L   E   A   A   D   M   E   T   I   H   K   233
767   CTG GTT TAG GAG TCG TCG TAG GTG CTT CAC GGT ACC TGC CTA ATT   811
234    L   V   *
812   GTG TTG AGA AAG TTC TGC ATT CCG TTG GGC CTG TTG TCC ATC CCT   856
857   CGG ACT GAC TTG TAA TAA GGG TTC TTT AAA CCA TGT CGA AGT GAC   901
902   TGA ATC ATT TGG CAT TAT CGT AAC AAG TGG CTC CGA TAG AAG CTC   946
947   CTG TTC GAC AAA AAA AAA AAA AAA AAA AAA AAA AA  981
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Figure 2. The full-length cDNA sequence and the deduced amino acid of *cwipi.* The start code and the stop code were underlined; the Poly (A) tail was painted with shadow.

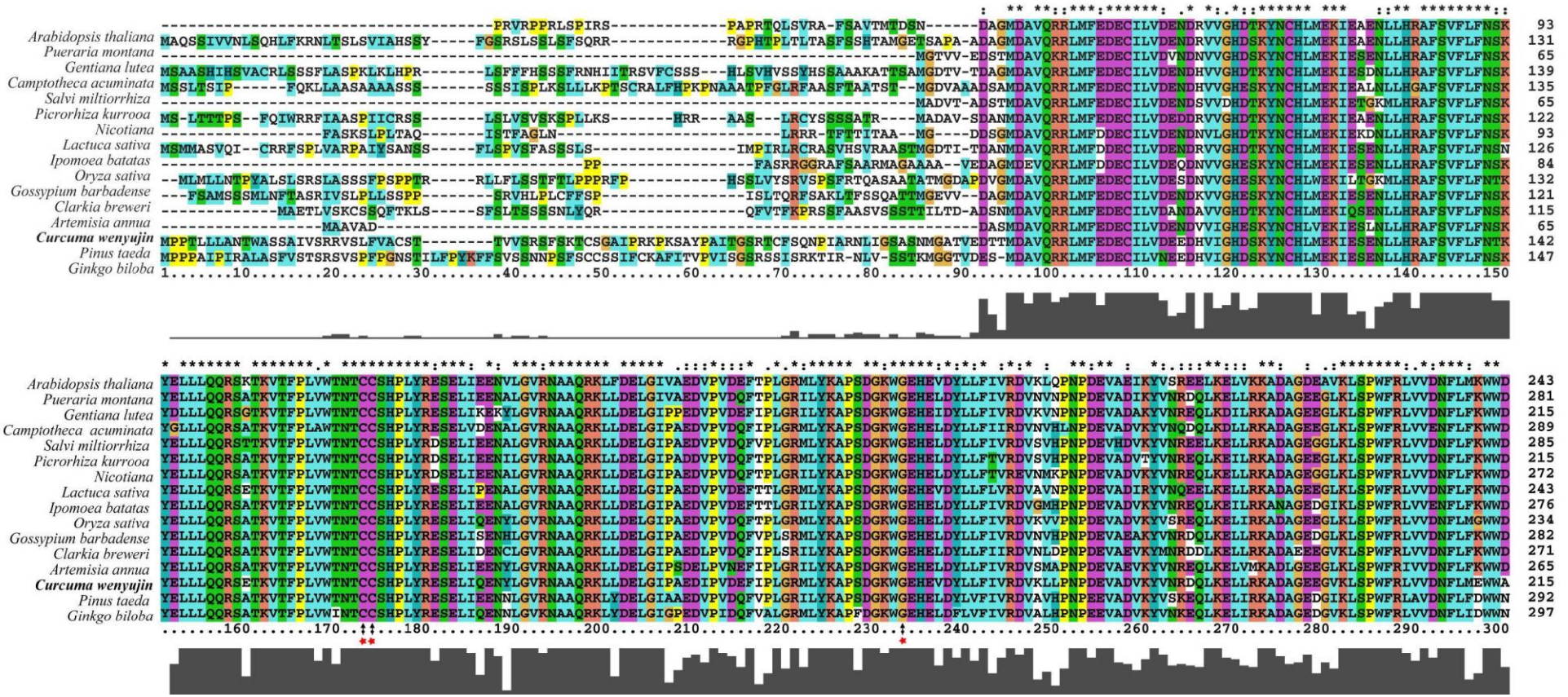

Figure 3. Multi-alignment of amino acid sequences of *cwipi* and other plant IPIs. The alignment quality curve is displayed below the alignment. Asterisks, colons and dots indicate completely, strongly and weakly conserved positions respectively. The aligned IPIs were from *Oryza sativa*(AAF29978.1),*Gentiana lutea*(BAE92733.1),*Ipomoea batatas* (AAZ94730.1) ,*N-icotiana langsdorffii x Nicotiana sanderae*(ABB29847.1), *Camptotheca acuminate* (ABI13583.1),*Arabidopsis thaliana*(AAB67741.1),*Salvia miltiorrhiza*(ABV08818.1),*Clarkia breweri*(CAA57947.1), *Pueraria montana var. lobata*(AAQ84167.1), *Lactuca sativa*(AAF29976.1), *Picr-orhiza kurrooa* (ABO14800.1),*Artemisia annua*(ABG56530.1), *Gossypium barbadense* (ABI94388.1), *Pinus taeda* (ACU56978.1), *Ginkgo biloba* (ACU56979.1).

isomerase gene (*cwipi*) was cloned from *C. Wenyujin*. Alignment of *cwipi* with other IPIs of plant origin and has the Cys-149 and Glu-212 (marker with red star) active sites. Semi-quantitative RT-PCR showed the transcriptional level of *cwipi* in roots was higher than in other tissues. Functional recombinant expression showed *cwipi* did encode an enzyme of IPI that could improve the metabolic flux of *β*-carotene biosynthesis in engineering *E. coli.* The cloning, characterization and functional expression of *cwipi* will be helpful to understand the mechanism of the biosynthesis of β-elemene in *C. Wenyujin*.

ACKNOWLEDGEMENT

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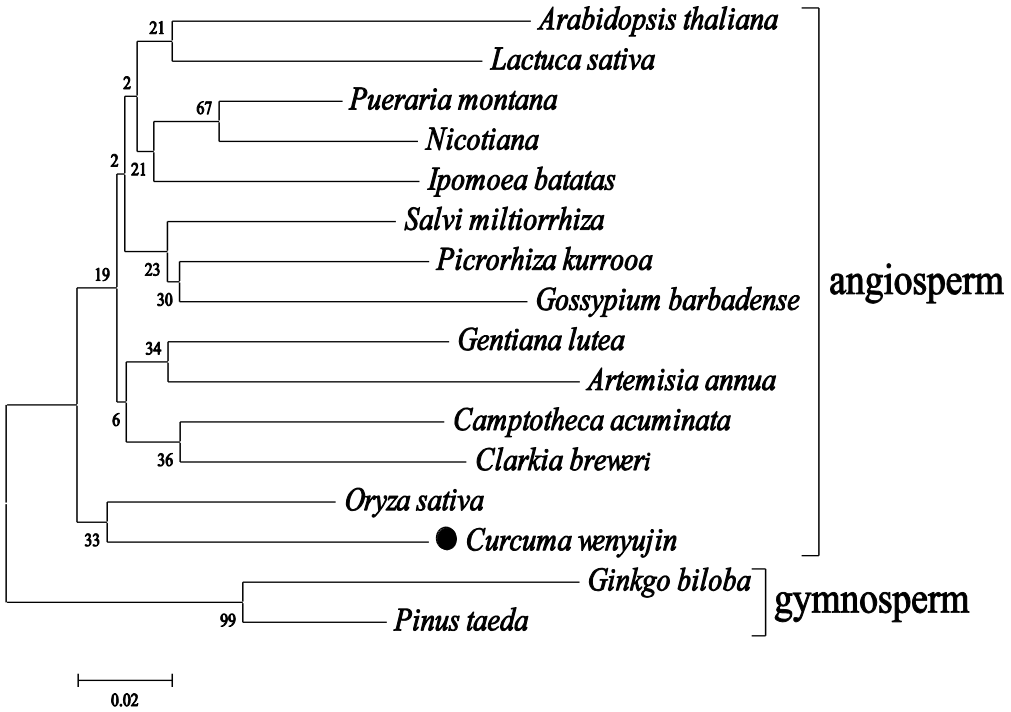

Figure 4. Phylogenetic analysis of IPIs from *Curcuma Wenyujin* and other plant species by MEGA5.0 from ClustalX (2.1) alignments. The sequence of *Escherichia coli* were used as outgroup. Bootstrap values greater than 50% (based on 1000 bootstrap replicates) were indicated in parentheses below the branches. The IPIs used in phylogenetic tree analysis were those from *Oryza sativa*(AAF29978.1), *Gentiana lutea*(BAE92733.1), *Ipomoea batata* (AAZ94730.1),*Nicotiana langsdorffii* (ABB29847.1),*Camptotheca acuminatea* (ABI13583.1),*Arabidopsis thaliana*(AAB67741.1),*Salvia miltiorrhiza* (ABV08818.1),*Clarkia breweri* (CAA57947.1), *Pueraria montana var. lobata* (AAQ84167.1),*Lactuca sativa* (AAF29976.1),*Picrorhiza kurrooa* (ABO14800.1),*Artemisia annua* (ABG56530.1), *Gossypium barbadense* (ABI94388.1), *Pinus taeda* (ACU56978.1),*Ginkgo biloba* (ACU56979.1).

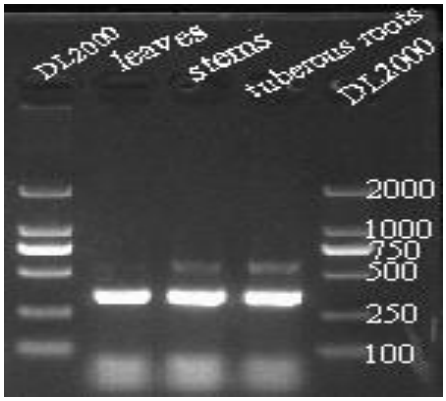

Figure 5. Expression pattern of *cwipi* in different tissues of *Curcuma Wenyujin* by semi-quantitative RT-PCR analysis. Total RNA samples were isolated from tuberous roots, leaves and stems respectively, and subjected to semi-quantitative RT-PCR analysis (upper panel). The 18S rRNA gene was used as the control to show the normalization of the amount of templates in PCR reactions (lower panel).

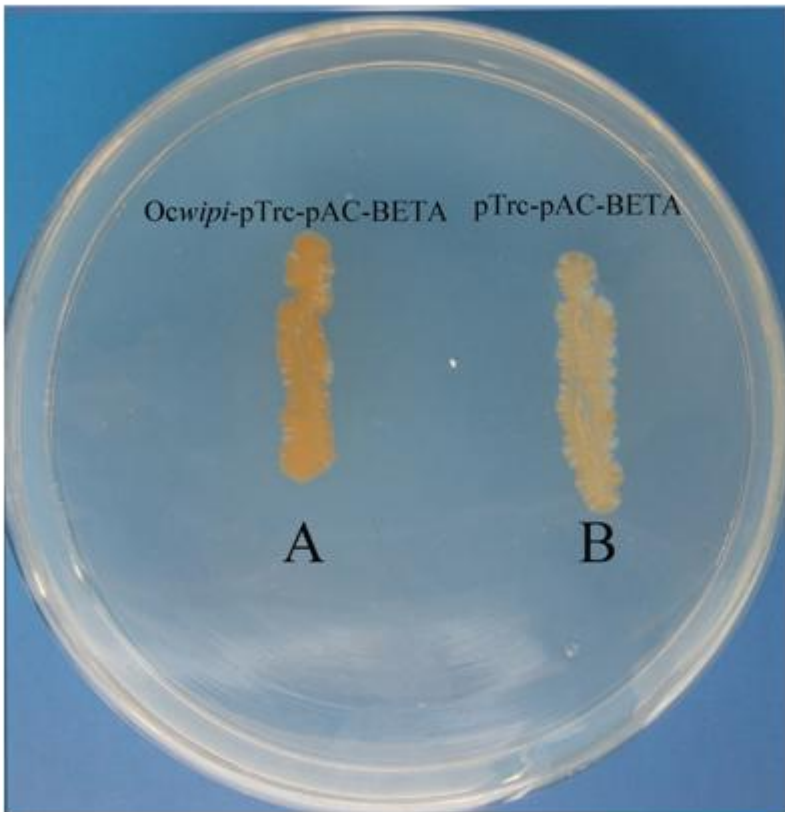

Figure 6. The functional complementation test of *Ocwipi* activity, using strain *E. coli* DH5α harboring pAC-BETA and O*cwipi*-pTrc (A) or pTrc (B).

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