



A novel chloroplastic isopentenyl diphosphate isomerase gene from *Jatropha curcas*: Cloning, characterization and subcellular localization



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ABSTRACT

Background: *Jatropha curcas* is a rich reservoir of pharmaceutically active terpenoids. More than 25 terpenoids have been isolated from this plant, and their activities are anti-bacterial, anti-fungal, anti-cancer, insecticidal, rodenticidal, cytotoxic and molluscicidal. But not much is known about the pathway involved in the biosynthesis of terpenoids. The present investigation describes the cloning, characterization and subcellular localization of isopentenyl diphosphate isomerase (IPI) gene from *J. curcas*. IPI is one of the rate limiting enzymes in the biosynthesis of terpenoids, catalyzing the crucial interconversion of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP).

Results: A full-length *JcIPI* cDNA consisting of 1355 bp was cloned. It encoded a protein of 305 amino acids. Analysis of deduced amino acid sequence predicted the presence of conserved active sites, metal binding sites and the NUDIX motif, which were consistent with other IPIs. Phylogenetic analysis indicated a significant evolutionary relatedness with *Ricinus communis*. Southern blot analysis showed the presence of an *IPI* multigene family in *J. curcas*. Comparative expression analysis of tissue specific *JcIPI* demonstrated the highest transcript level in flowers. Abiotic factors could induce the expression of *JcIPI*. Subcellular distribution showed that *JcIPI* was localized in chloroplasts.

Conclusion: This is the first report of cloning and characterization of IPI from *J. curcas*. Our study will be of significant interest to understanding the regulatory role of IPI in the biosynthesis of terpenoids, although its function still needs further confirmation.

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1. Introduction

Terpenoids encompass an extraordinary variety of primary and secondary metabolites in bacteria, fungi, plants and animals. They play vital roles in the structure of cells, electron transport, photosynthesis, cell-to-cell signaling and interactions between organisms [1]. In addition, many terpenoids have important commercial values in medicine, industry and agriculture [2]. All terpenoids originate from the head-to-tail condensation of isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP). IPP and DMAPP are both deemed universal precursors of terpenoid biosynthesis (Fig. 1) [3]. Isopentenyl diphosphate isomerase (IPI, EC: 5.3.3.2) catalyzes the crucial interconversion of IPP and DMAPP, and thus regulates the IPP/DMAPP pool. An appropriate molar ratio of IPP to DMAPP is needed for synthesis of various terpenoid classes. So IPI is considered as a key enzyme in the

regulation of terpenoid biosynthesis [4]. The characterization of this enzyme has been first reported in the baker's yeast by Agranoff et al. [5]. Since then, it has been found in most living systems. Kajiwarra et al. [6] have reported that an *IPI* cDNA from *Haematococcus pluvialis* heterologously expressed in *Escherichia coli* could elevate contents of β -carotene and lycopene by 2.7 and 4.5 fold, respectively. Later, several similar results have been reported that overexpressions of plant *IPI*s in engineered *E. coli* lead to increased accumulation of β -carotene [7,8]. Now several *IPI* genes have been isolated from higher plants such as *Clarkia breweri* [9], *Melaleuca alternifolia* [10], *Daucus carota* [11], *Camptotheca acuminata* [12], and *Ipomoea batatas* [8].

Jatropha curcas belongs to the family Euphorbiaceae and is used in traditional folklore medicine to cure various ailments in Asia, Africa and Latin America [13]. It has been used to cure diseases like cancer, snake bites, paralysis, piles and dropsy [14]. Terpenoids are the main active secondary metabolites in *J. curcas*. Till now, at least 25 terpenoids have been isolated from this plant. They have shown a wide array of pharmacological properties including anti-bacterial, anti-cancer, insecticidal, rodenticidal, cytotoxic and molluscicidal [15]. However, some terpenoids (phorbol esters) in *J. curcas* are toxic to animals. They prevent seed cakes containing high content of proteins to be used as animal feed, which thus wastes most protein resource in the seeds [16]. Therefore, increasing the content of active terpenoids

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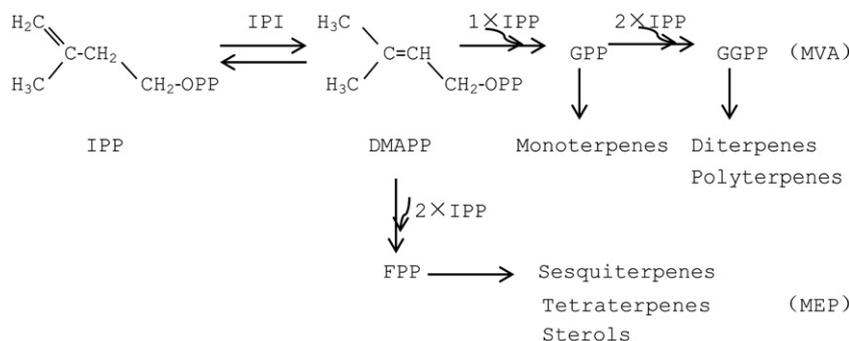


Fig. 1. Overview of terpenoid biosynthesis from IPP and DMAPP. It indicates that the IPP and DMAPP are required for various classes of terpenoids. FPP, farnesyl diphosphate; GPP, geranyl diphosphate; GGPP, geranylgeranyl diphosphate; MEP, methyl erythritol phosphate pathway; MVA, mevalonate pathway; OPP, diphosphate moiety. The block arrows denote multiple steps, and the double arrows reflect the expected equilibrium of the reversible IPP isomerase (IPI) reaction.

or decreasing the toxic ones could enhance the comprehensive utilization of *J. curcas*.

Recent interest generated in the regulation of terpenoid content has prompted many workers to elucidate the biosynthetic pathway. As the biogenesis of terpenoids is yet to be fully understood in *J. curcas*, it is imperative to identify, clone and characterize key pathway genes to understand the regulatory role of various enzymes. Up to now, several key genes involved in terpenoid biosynthesis pathway have been isolated and characterized from *J. curcas*. However, there is little information about the cloning and characterization of *Jatropha IPI*.

The present investigation was aimed at molecular characterization of IPI from *J. curcas*. Using degenerate primers and RACE PCR strategy, we have cloned a full-length cDNA encoding *JcIPI*. The gene was analyzed in detail, expression analysis was performed in specific tissues or under stress conditions, and southern-blot analysis was carried out. *JcIPI* heterologous expression in *E. coli* and subcellular localization in tobacco were also included in this study.

2. Materials and methods

2.1. Plant materials

The *J. curcas* mature seeds, roots, stems, leaves, flowers, barks and yellow fruits were collected from a five-year old plant in Sichuan Province, China, then snap frozen in liquid nitrogen and transferred to a -80°C freezer till further use for comparative tissue specific expression profile. Young seedlings of *J. curcas* germinated from the collected mature seeds were cultivated in a culture room maintained at $26 \pm 2^{\circ}\text{C}$ under a 16/8 h (light/dark) photoperiod. *Nicotiana tabacum* cultivar growing in soil was used for protoplast isolation and transient expression analysis.

2.2. RNA isolation and cDNA synthesis

Total RNA was isolated from the leaves of *J. curcas* using RNAPrep pure Plant Kit (Tiangen, China). The quality and quantity of RNA were checked by agarose gel electrophoresis and by spectrophotometry (NanoVue ND-1000, China) respectively. RNA samples having A260/280 in the range of 1.8 to 2.0 and A260/230 > 2 were selected for cDNA synthesis and were stored at -80°C prior to RT-PCR and RACE analysis. First strand cDNA was synthesized using the PrimescriptTM RT reagent Kit (Takara, China).

2.3. Cloning of full length *JcIPI*

Two degenerate primers (DP1 and DP2, Table 1) designed on the basis of the conserved amino-acid regions of published IPIs from other plants were used for the amplification of the core cDNA fragment of *JcIPI*. Using cDNA as the template, PCR was performed under the

following conditions: 95°C for 4 min, 35 cycles of 94°C for 30 s, 53.5°C for 30 s and 72°C for 50 s followed by a final extension of 72°C for 10 min. A 590 bp DNA fragment was recovered, cloned into the pMD19-T vector (Takara, China) and sequenced (Invitrogen, China). This fragment was subsequently used for designing gene specific primers for the cloning of 3' and 5' ends of *JcIPI* by RACE-PCR.

To obtain the 3'- and 5'-ends of *JcIPI*, the anchored-RACE extension method was used as described by Newton et al. [17]. The initial 3' RACE PCR was carried out using 3P1 and P1 primers. The primary PCR product was used as the template for nested 3' RACE PCR reaction in which 3P2 primer was used along with P2 primer. Both initial and nested PCR reactions were carried out under the following conditions: 4 min at 95°C , 35 cycles (30 s at 94°C , 30 s at 55°C and 1 min at 72°C) and 10 min at 72°C . For 5' RACE PCR, a polyA tail was added to the 3' end of the cDNA using the terminal deoxynucleotidyl transferase according to the manufacturer instructions (Takara, China). Then similar reactions were carried out for 5' RACE-PCR in which 5P1 and P0 primers were used for the initial reaction while 5P2 and P2 primers for the nested PCR reaction. Thermo profile was as follows: 4 min at 95°C , 35 cycles (30 s at 94°C , 30 s at 59°C and 1 min at 72°C) and 10 min at 72°C . The two nested amplified fragments of both 3' and 5' RACE were cloned into pMD19-T vector and sequenced. After assembling the sequences of 3' and 5' RACE products, the full length cDNA sequence of *JcIPI* was obtained.

Table 1

Primers for cDNA cloning, quantitative real-time PCR and recombinant plasmid construction.

Primers	Sequence (5'–3')
DP1	CGHCTYATGTTYGAVGAYGAATGYAT ^a
DP2	CCACCAYYTSA WCAARAARTTRTC ^a
P0	GCTGTCAACGATACGCTACGTAACGGCATGACAGTG(T) ₁₈
P1	GTCAACGATACGCTACGTAACG
P2	TACGTAACGGCATGACAGTG
3P1	CACCTTCTGATGGAAGTGGGG
3P2	AAGGAA AGCAGATGCTGGTGAGGA
5P1	TCCTCACCAGCAGCATCTGC TTTCTTC
5P2	CCCCACTTTC CATC AGAAGGTG
SP1	GCGGGATCCATGCTGTAGCC TCTCG
SP2	GCGGAATCTTAAGTTAACTTGTG
QjclP11	GTAATCCAAACCTAATGA
QjclP12	GTTTGCATGTCAACCACA
18SrRNA1	CAACCATAAACGATGCCGACC
18SrRNA2	CAGCCTTGCACCATACTCCC
SSP1	GCTTAGAATGTCTGTAGCCTCTCG
SSP2	GCGGGATCCAGTTAACTTGTG

^a H: A/T/C; Y: C/T; V: G/A/C; S: G/C; W: A/T; R: A/G. Emphatic sequence of **GGATCC**, **GAATTC** and **TCTAGA** represented the restriction enzyme sites of *Bam*H I, *Eco*R I and *Xba* I respectively.

2.4. Sequence analysis

The nucleotide sequence of *JcIPI* obtained was translated using Translate tool (<http://www.expasy.ch/tools/dna.html>) and the properties of deduced amino acid sequence were estimated using ProtParam (<http://www.expasy.ch/tools/protparam.html>). Structural and functional regions were identified in deduced protein sequence by conserved domain database (CDD) at the National Center for Biotechnology Information (NCBI). Secondary structure was determined by SOPMA (<http://npsa-pbil.ibcp.fr>) program.

2.5. Phylogenetic analysis and prediction of three-dimensional structure of *JcIPI*

Protein sequences were retrieved from the GenBank through the BLAST p algorithm at NCBI using *JcIPI* sequence as a query and several

IPIs with the highest score from different plants were selected. Sequences were aligned using the DNAMAN software package (Version 5.2.9, Canada) using default parameters. Phylogenetic analysis of deduced amino acid alignments was generated using the neighbor-joining method with MEGA 5.1 software [18]. The three-dimensional structure of *JcIPI* was predicted using Phyre 2 web server (<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=inde%20x>).

2.6. Southern blot analysis

Genomic DNA was isolated from the leaves of *J. curcas* using Plant Genomic DNA Kit (Tiangen, China). About 10 µg genomic DNA was completely digested with *Kpn I*, *Xba I*, *BamH I* and *EcoR I/BamH I* respectively, separated by electrophoresis on a 0.8% (W/V) agarose gel and then transferred to a Hybond-N + nylon membrane.

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ATTAAATCAATAATCAAAATTGCATAAGAATCACAAGTTAAAGAAAAAAAAAATGCTT
CCATTCATAATTCGCGGATACTTCCACAACCTCCATATATACACTTCCAAGTAATTGGAAAA
ATATATTAACATAAAAACAAAAAGAGGAAAA
1  ATGCTCTGTAGCCTCTCGCATGCTAAATACCCCTACCACCACCAGACTCTCTCTCACTT
1  M S V A S R M L N T P T T T R L S S S L
61  ACCTCCTGTGCTTCATCTCGCTCTCCTCTGTTTCTTTCTTCCAATTTGCCCCTCCATCT
21  T S S A S S R S P L F L S S N F A L P S
121  CTTCTTGACTGCCTCCCATCTTAAATCTTCTCCCTCCCTTCTCGAGGGTTTTTTCG
41  L L R L P P I L K S S S P P F S R V F S
181  TCTTCTGTGCCCTCAACCTCCGCTACTACCTCCATGGGTGACGCTCCAGATGCTGGCATG
61  S S L P S T S A T T S M G D A P D A G M
241  GACGCTGTCCAGAGACGCCCTTATGTTTGAGGATGAATGCATTCTAGTGGATGAGAATGAT
81  D A V Q R R L M F E D E C I L V D E N D
301  AAAGTCGTTGGTCATGCTCCTCAATATAGCTGTGCTTATGGGAAAATATTTTGAAAGGG
101  K V V G H V S K Y S C H L W E N I L K G
361  AACTGTTACACAGAGCCCTTAGTGTATTCTCTTCAACACAAAATATGAGCTCCTACTT
121  N S L H R A F S V F L F N T K Y E L L L
421  CAGCAACGCTCTGCAACAAAGGTCACATTCGCCCTTGTGTGGACAAACACTTGTGCGAGT
141  Q Q R S A T K V T F P L V W T N T C C S
481  CATCCTTTGTACCGTGTATTCTGAGCTTATCGAAGAGAATGCTCTTGGTGTGAGGAATGCT
161  H P L Y R D S E L I E E N A L G V R N A
541  GCACAAAGGAAGCTTTTGGATGAGCTTGGTATCCCTGCTGAAGATGTTCCAGTTGATCAG
181  A Q R K L L D E L G I P A E D V P V D Q
601  TTTACCTCATTTGGGGGTATACTATAACAAGGCACCTTCTGATGAAAGTGGGGAGAGCAT
201  F T S L G R I L Y K A P S D G K W G E H
661  GAACTTGATTATCTGCTCTTCATAGTTCTGATGTTAATGTAAATCCAAACCTTAATGAG
221  E L D Y L L F I V R D V N V N P N P N E
721  GTAGCTGATATCAAGTACGTTAACCAGATCAGTTGAAGGAACCTATTAAGGAAAGCAGAT
241  V A D I K Y V N R D Q L K E L L R K A D
781  GCTGGTGAGGAAGGTTTGAAGCTGTCACCATGGTTTAGACTAGTTGTTGACAACCTCTTG
261  A G E E G L K L S P W F R L V V D N F L
841  TTCAAATGGTGGGAACATGTTGAGCAGGGGACACTCAAGGATGTGGTTGACATGCAAAACA
281  F K W W E H V E Q G T L K D V V D M Q T
901  ATTCACAAGTTAACTTAA
301  I H K L T *
GATTTTAAAGCAAATTTGGGACAAAGTTTGGATTGTGGTATCACCTGTAGCTTTATTTA
AATAAATTTGGTCATTTAGGAGTTCTAAGCAAGTTAGCTTCCGTATCCATTGTAACTT
CAATATTTCCGAAGAGAGTTTATTTAGCCTGGTTATGCTGAATTTGAGTTATTACCT
GAAACCAGCTGGGCATAACGCTTTGTATTATAATCTAGAGTTGGCTTTTATGATTTAAGA
ACTTAATAAAGACTTTTATACTTGCATTAAAAA

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Fig. 2. Nucleotide and the deduced amino acid sequence of *JcIPI* from *J. curcas*. The ATG start codon at position 1 and the TAA stop codon at position 916 are in red. The 5' untranslated region is 154 bp and 3' untranslated region is 283 bp. The blue box represents the NUDIX motif which is the characteristic of IPIs. The vertical lines show the position of introns. The Poly-A is underlined.

J.curcas	MSVASRLNT	PTTTRLSSSL	TSSASSRSP-	----LFLSS	NFALPSLLRL
R.communis	MSLTSRLNT	PATTRLSSSL	SSSSSSSSPS	PSPKRYFLHS	NITPPSLLRF
T.cacao	---MSSLINT	ASAFRLSRSL	ASSSSFSFCPQ	PR--LFLSSS	FALPFRPIPH
V.vinifera	MTLSSRLY-T	FASPKLPKHC	ARPQISPSFP	L-----K	YPPILTRSLP
C.avellana	MSVTPHLL-K	TTATRLSCSP	ALTSKSRRLS	S-----S	AFAPRTRLRL
G.barbadense	---MLMLLNT	PYALSLSRSL	ASSS-FPSPP	TRRLFLSST	FTLPPPRFPH
H.brasiliensis	-----	-----	-----	-----	-----
M.truncatula	MAQSS-----	-IVATLSRHF	KR-NITPTLS	-----A	HSSFRSRSYS
P.trichocarpa	-----	-----	-----	-----	-----
J.curcas	PPILKSSSPP	FSRVFSSSLP	STSATTS---	MGDAPDAGMD	AVQRRLMFED
R.communis	PLILKLSSSP	FSRVFSSSRS	TTASISSNST	MGDAPDAGMD	AVQRRLMFED
T.cacao	FSLTSLRASL	SFRTEAASAT	AT-----	MGDAPDAGMD	AVQRRLMFED
V.vinifera	FASHFRRAS-	--LSFSTRST	A-----	MGDAVDAGMD	AVQRRLMFED
C.avellana	WPSSNSVSP	TARVLSTLNV	A-----	MGDAPDAGMD	AVQRRLMFED
G.barbadense	SSLVYSRVSP	SFRTQASAAT	AT-----	MGDAPDVGMD	AVQRRLMFED
H.brasiliensis	-----	-----	-----	MGDAPDTGMD	AVQRRLMFED
M.truncatula	SLSLFRRKAP	LSASLSTMGE	T-----	PVAAVDAGMD	AVQRRLMFED
P.trichocarpa	-----	-----	-----	MGDAPDAGMD	AVQRRLMFED
J.curcas	ECILVDE ^N DK	VVGHV ^S KYSC	HLWEN ^I LKGN	SLHRAFSVFL	FNTKYELLQ
R.communis	ECILVDES ^T	VVGHV ^S KYSC	HLWEN ^I LKGN	SLHRAFSVFL	FNSKYELLQ
T.cacao	ECILVDE ^N DN	VVGHE ^S KYNC	HLWE ^K ILTKN	MLHRAFSVFL	FNSKYELLQ
V.vinifera	ECILVDE ^N DR	IVGHDTKYNC	HLME ^K IESEN	LLHRAFSVFL	FNSKHELLQ
C.avellana	ECILVDE ^N DS	VVGHDTKYNC	HSWE ^K IESGN	MLHRAFSVFL	FNSKYELLQ
G.barbadense	ECILVDES ^{DN}	VVGHE ^S KYNC	HLWE ^K ILTKG	MLHRAFSVFL	FNTKYELLQ
H.brasiliensis	ECILVDE ^N DR	AVGHASKYSC	HLWEN ^I LKGN	SLHRAFSVFL	FNSKYELLQ
M.truncatula	ECILVDE ^N DR	AVGHE ^S KYNC	HLWE ^K IESEN	WLHRAFSVFL	FNSKHELLQ
P.trichocarpa	ECILVDE ^N DR	VVGH ^S KYNC	HLWEN ^I LKGN	ALHRAFSVFL	FNSKHELLQ
J.curcas	QRSATKVTFP	LVWTNTCCSH	PLYRDESEL ^I E	ENALGVRNAA	QRKLLDELGI
R.communis	QRSATKVTFP	LVWTNTCCSH	PLYRESEL ^I E	EDSLGVRNAA	QRKLLDELGI
T.cacao	QRSATKVTFP	LVWTNTCCSH	PLYRESEL ^I IG	ENFLGVRNAA	QRKLLDELGI
V.vinifera	QRSATKVTFP	LVWTNTCCSH	PLYRESEL ^I D	ENALGVRNAA	QRKLLDELGI
C.avellana	QRSATKVTFP	LVWTNTCCSH	PLYRESEL ^I E	ENALGVRNAA	QRKLLDELGI
G.barbadense	QRSATKVTFP	LVWTNTCCSH	PLYRESEL ^I IS	ENHLGVRNAA	QRKLLDELGI
H.brasiliensis	QRSATKVTFP	LVWTNTCCSH	PLYRESER ^I D	EDALGVRNAA	QRKLLDELGI
M.truncatula	QRSATKVTFP	LVWTNTCCSH	PLYRESEL ^I E	ENALGVRNAA	QRKLLDELGI
P.trichocarpa	QRSATKVTFP	LVWTNTCCSH	PLYRESEL ^I TH	EDALGVRNAA	QRKLFDELGI
J.curcas	PAEDVPVDQF	TS ^L GRILYKA	PSDGKWEHE	LDYLLF ^I VRD	VNVNPNP ^N EV
R.communis	PAEDVPVDQF	TPVGRMLYKA	PSDGKWEHE	LDYLLFMVRD	VNVNPNP ^N DEV
T.cacao	PAEDVPVDQF	IPLSRILYKA	PSDGKWEHE	LDYLLF ^I VRD	VNVHNP ^N DEV
V.vinifera	PAEDVPVDQF	TPVGRMLYKA	PSDGKWEHE	LDYLLF ^I VRD	VNVNPNP ^N DEV
C.avellana	PAEDVPVDQF	IPVGRMLYKA	PSDGKWEHE	LDYLLF ^I VRD	VNVNPNP ^N DEA
G.barbadense	PAEDVPVDQF	VPLSRMLYKA	PSDGKWEHE	LDYLLF ^I VRD	VNVHNP ^N DEV
H.brasiliensis	PAEDVPVDQF	TPVGRMLYKA	PSDGKWEHE	LDYLLF ^I VRD	VNVNPNP ^N DEV
M.truncatula	PAQDVPVDQF	TPVGRILYKA	PSDGKWEHE	LDYLLF ^I VRD	VNVNPNP ^N DEV
P.trichocarpa	PAEDVPVDQF	SPLGRILYKA	PSDGKWEHE	LDYLLF ^I VRD	VSVNPNP ^N DEV
J.curcas	ADIKYVNRDQ	LKELLRKADA	GEEGLKLSPW	FRLVVDNFLF	KWWEHVEQGT
R.communis	ADVKYVNRDQ	LKELLRKADA	GEEGLKLSPW	FRLVVDNFLF	KWWEHAEKGT
T.cacao	ADVKYVNRDQ	LKELLRKADA	GEEGLKLSPW	FRLVVDNFLF	KWWDHVEKGT
V.vinifera	ADVKYVSRND	LKELLRKADA	GEEGLKLSPW	FRLVVDNFLF	KWWDHVEKGT
C.avellana	ADVKYVNRDE	LKELLRKADA	GEEGLKLSPW	FRLVVDNFLF	KWWDHVEKGT
G.barbadense	AEAKYVNRDQ	LKELLRKADA	GEDGLKLSPW	FRLVVDNFLF	KWWDHVEKGT
H.brasiliensis	ADIKYVNRDE	LKELLKADA	GEEGLKLSPW	FRLVVDNFLF	KWWEHVEKGT
M.truncatula	ADIKYVNRDE	LKELLRKADA	GEEGLKLSPW	FRLVVDNFLF	KWWDHLEQGT
P.trichocarpa	ADIKYVNDQE	LKELLRKADA	GEEGLKLSPW	FRLVVDNFLF	KWWDHVEKGT
J.curcas	LKDVVDMQTI	HKLT			
R.communis	LKEATDMKTI	HKLT			
T.cacao	LKEATDMETI	HKLT			
V.vinifera	LEEAADMKTI	HKLT			
C.avellana	LKEAADMKTI	HKLT			
G.barbadense	LKEVSDMETI	HKL-			
H.brasiliensis	LKEAADMKTI	HKLT			
M.truncatula	LKGVIDMKTI	HKLT			
P.trichocarpa	LEEAADMKAI	HKLT			

Fig. 3. Multiple alignment of the predicted amino acid sequence of *JcIPI* with the corresponding sequences of other plant families: *H. brasiliensis* (BAF98287.1), *P. trichocarpa* (XP_002325469.1), *M. truncatula* (XP_003624178.1), *R. communis* (XP_002514848.1), *T. cacao* (EOY09814.1), *G. barbadense* (ABI94388.1), *V. vinifera* (XP_002277935.1) and *C. avellana* (ABW06959.1). The amino acid residues displayed in blue and red are conserved ones across various plant families.

DNA hybridization was performed according to the standard procedures [19]. The membrane was washed twice at 20°C for 5 min in 2 × SSC, 0.1% (W/V) SDS and then twice in 0.5 × SSC, 0.1% SDS at 65°C for 15 min. DNA probe was the PCR product amplified with QjclPI1 and QjclPI2 primers (ORF sequence from 700 to 899, Table 1), labeled using DIG High Prime DNA Labeling and detected using Detection Starter Kit II (Roche, Germany).

2.7. Tissue specific expression analysis by quantitative real-time PCR (qRT-PCR)

Tissue specific expression of *JcIPI* was analyzed in mature seeds, roots, stems, leaves, flowers, barks and yellow fruits. Total RNA was extracted from the tissues mentioned above, purified by using DNase 1 from DNA contamination and reversely transcribed to the

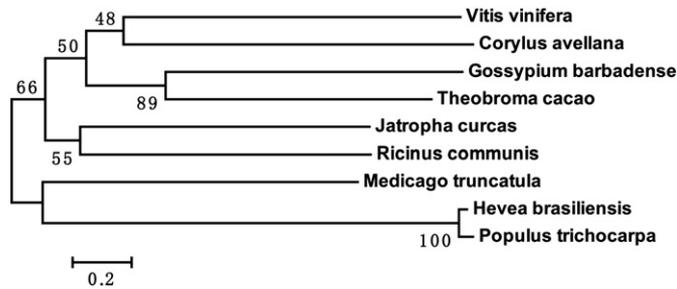


Fig. 4. Phylogenetic tree of IPI protein sequences of various plants: *H. Brasiliensis* (BAF98287.1), *P. trichocarpa* (XP_002325469.1), *M. truncatula* (XP_003624178.1), *R. communis* (XP_002514848.1), *T. cacao* (EOY09814.1), *G. barbadense* (ABI94388.1), *V. vinifera* (XP_002277935.1) and *C. avellana* (ABW06959.1). The construction of the tree was performed with Mega 5.1. Numbers above the branches indicated bootstrap values Phylogenetic tree.

first-strand cDNA by oligo (dT)₁₈ primer. QjCIPI1, QjCIPI2, 18SrRNA1 and 18SrRNA2 primers (Table 1) were designed close to the 3'-end sequence of *JcIPI* and 18S rRNA (an internal control, GenBank accession no. FJ489608.1) respectively. The primers were tested for linearity by constructing standard curves on five serial 10-fold dilutions in order to ensure maximum specificity and efficiency during qRT-PCR. QRT-PCR was performed using a Bio-Rad iCycler MyiQ Real-Time PCR System in a 20 μ L reaction volume containing 1 μ L appropriate diluted cDNA from each sample, 10 μ L SsoFast™ EvaGreen® Supermix (Bio-Rad, USA), 1 μ L forward primer, 1 μ L reverse primer and 7 μ L ddH₂O. PCR conditions were: 95°C for 30 s, 40 cycles of 95°C for 5 s and 50°C for 10 s, followed by a final melt curve profile (65–95°C). Quantification was determined with the comparative C_T method and each datum represented the average of three parallel samples.

2.8. Gene expression of *JcIPI* under stress conditions by qRT-PCR

In order to investigate the gene expression under stress conditions including low temperature (4°C), salinity (NaCl), drought (mannitol) and methyl jasmonate (MeJA), the seedlings of *J. curcas* were cultured on MS solid mediums with the method described by Attaya et al. [20] with a slight modification. The procedures involved the decoating of the seeds, subsequent soaking in distilled water for 4 h at room temperature, surface sterilizing with 70% (v/v) ethanol, immersing in 0.1% (w/v) mercuric chloride (HgCl₂) solution and rinsing by sterile

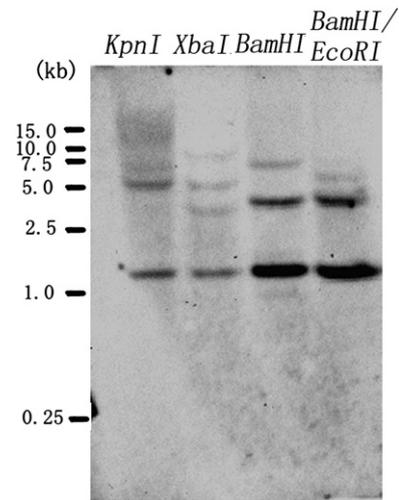


Fig. 6. Southern blot analysis of total DNA using *JcIPI* cDNA as a probe. Total DNA isolated from *J. curcas* was digested with restriction enzymes indicated at the top of panel, electrophoresed, blotted onto a nylon membrane, and subjected to hybridization using *JcIPI* cDNA as a probe. Positions of molecular weight markers were indicated in the left of the panel.

distilled water (four times). These surface sterilized seeds germinated on MS mediums. After 3 weeks, the seedlings were transferred to MS liquid mediums under stress conditions mentioned above for 24 h in the culture room. Leaves were collected, immediately frozen in liquid nitrogen and stored at -80°C before RNA isolation. The method of qRT-PCR was the same with the section of tissue specific expression analysis.

2.9. Heterologous expression of *JcIPI* in *E. coli*

Using first strand cDNA as the template, the entire ORF containing the start codon and the stop codon was amplified by two gene-specific primers (SP1 and SP2, Table 1) containing *BamH I* and *EcoR I* restriction sites respectively. The amplified product was double digested with *BamH I* and *EcoR I* enzymes and ligated into pET-32a vector digested with the same two restriction enzymes. The pET-*JcIPI* construct was transformed into *E. coli* BL-21 (DE3) cells (Merck, Germany).

An overnight culture of *E. coli* BL-21 (DE3) cells containing pET-*JcIPI* construct was grown at 37°C in Luria broth medium (LB). 1% of the

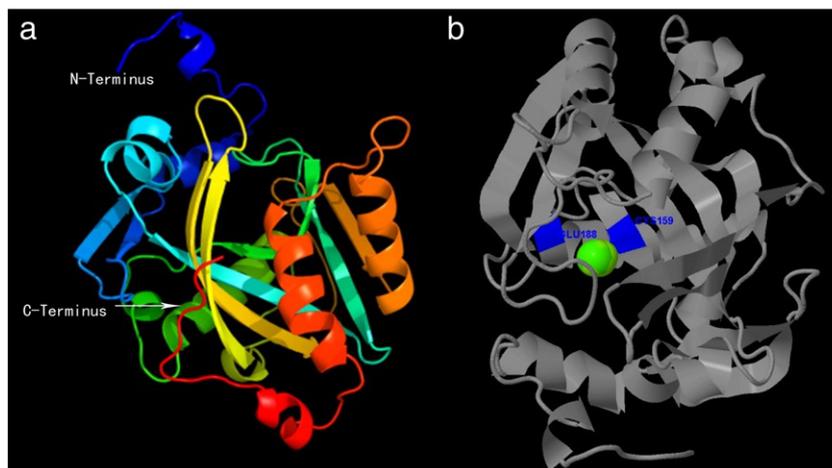


Fig. 5. Three-dimensional models for *JcIPI*. (a) Cartoon display of the 3D structure of *JcIPI*, as predicted by Phyre 2 Web Server using crystal structure of human isopentenyl diphosphate delt-isomerase 2 (PDB ID code: c2pnyA_). (b) Predicted ligand (shown in gray) binding site as predicted by 3DLigandSite Web Server. Active site is predicted to involve amino acids Cys 159 and Glu 188.

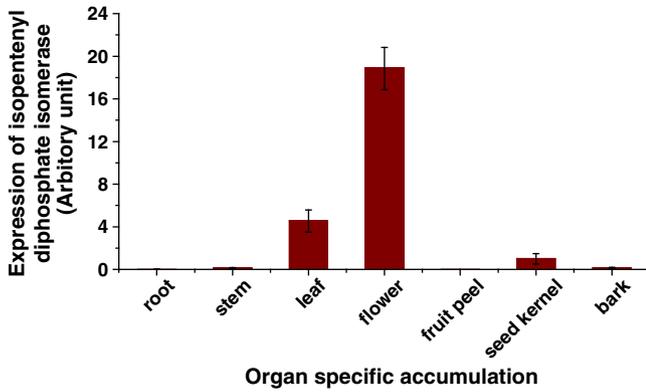


Fig. 7. Comparative expression level of *JcIPI* transcripts in various plant tissues using quantitative real-time PCR. Roots, stems, leaves, flowers, fruits, seeds and barks were collected from Sichuan province, China. 18S rRNA gene was used as an internal control for all the samples. Vertical bars represented the means \pm SE ($n = 3$), not significantly different ($P > 0.05$).

overnight culture was used to inoculate fresh LB medium to obtain exponentially growing culture (OD of 0.5–0.6). 0.5 mM IPTG (Isopropyl- β -D-1-thiogalactopyranoside) was added to exponentially growing cells. Induced culture at 16°C was harvested after 12 h. The harvest 1 mL culture aliquots were centrifuged at 12,000 rpm for 1 min at 4°C and the pellet was resuspended in 1 mL extraction buffer (50 mM Tris-HCl, pH 8.0, 10 M MgCl₂, 20% glycerol and 5 mM 2-mercaptoethanol). Cells were disrupted by sonication on ice for 15 to 20 s burst time period. The lysate was centrifuged at 12,000 rpm for 1 min at 4°C. The supernatant, precipitate and overall lysate were boiled with 2 \times SDS loading dye for 10 min and loaded on (15%) SDS-PAGE gel.

2.10. Protoplast isolation and transient expression

The ORF of *JcIPI* without the stop codon was amplified by SSP1 and SSP2 primers containing *Xba* I and *Bam*H I restriction sites respectively. The amplified product was double digested with *Xba* I and *Bam*H I enzymes and ligated into PBI121-GFP vector digested with the same two restriction enzymes. This *JcIPI*-GFP construct was used for subcellular localization. The isolation and transfection of *N. tabacum* leaf mesophyll protoplasts were conducted as described by Yoo et al. [21]. Briefly, protoplasts were isolated from well-expanded leaves of *N. tabacum* plants. Volumes of *JcIPI*-GFP plastids (10 to 20 μ g) were transfected into 150 μ L protoplasts using a PEG-calcium transfection solution. Protoplasts were incubated overnight at 22 \pm

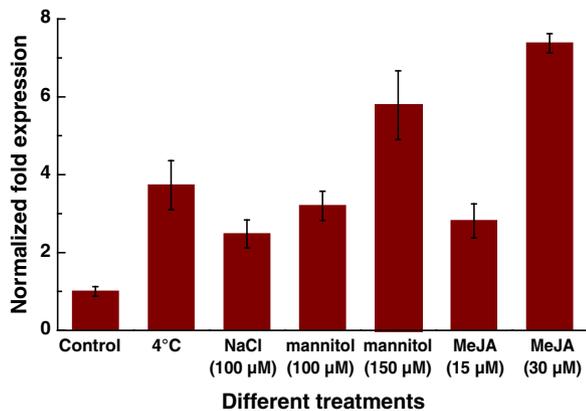


Fig. 8. Transcription pattern of *JcIPI* under stress treatments by quantitative real-time PCR. The leaves obtained from treated seedlings of *J. curcas* were used as samples. 18S rRNA gene was used as an internal control for all the samples. Vertical bars represented the means \pm SE ($n = 3$), not significantly different ($P > 0.05$).

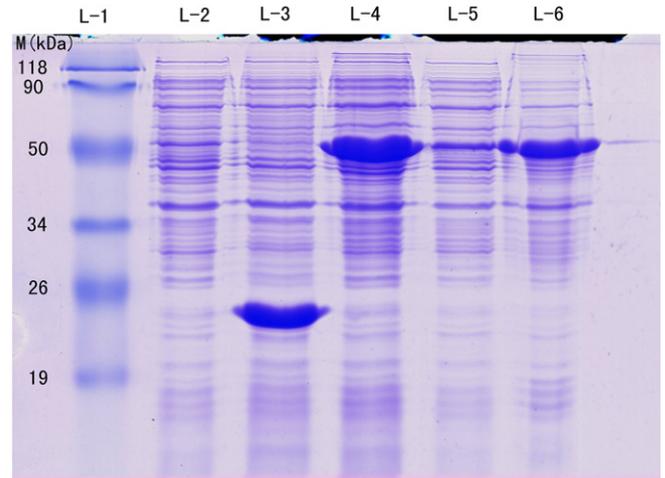


Fig. 9. Heterologous expression in *E. coli* with IPTG induction (0.5 mM) at 16°C for 12 h. Protein molecular weight marker (MW) (L-1), *E. coli* (L-2), *E. coli* harboring empty vector (L-3), overall lysate harboring pET-IPI construct (L-4), the supernatant of lysate (L-5) and the precipitate of lysate (L-6).

2°C under white light to allow expression of the introduced gene. The GFP fluorescence was examined and photographed using a Leica SP5 confocal microscope (Leica, Germany).

3. Results and discussion

3.1. Cloning and sequence analysis of JcIPI

A full length gene designated as *JcIPI* (GenBank accession no. KJ908689) was isolated from *J. curcas* leaves. Based on the conserved amino acid domains of IPIs from other plants, degenerate primers were designed to amplify an initial core fragment of 590 bp. RACE technique was used to obtain the 3' and 5' ends of *JcIPI*. RACE amplicons of 3' and 5' ends were amplified, sequenced and assembled, thus a full length gene sequence of 1355 bp was generated containing an ORF of 918 bp encoding a protein of 305 amino acids. The 5' and 3' untranslated region (UTR) sequences were of 154 and 283 bp respectively. The comparison of the full-length cDNA and the genomic DNA of *JcIPI* revealed that five introns were present in the genomic DNA (Fig. 2).

3.2. Analysis of the deduced protein sequence

Multiple sequence alignments showed a high degree of conservation in the functional region (aa 90–270). It also revealed that *JcIPI* had a significant similarity with many IPIs from higher plants at the amino acid level such as *Hevea brasiliensis* (92%), *Populus trichocarpa* (90%), *Medicago truncatula* (85%), *Ricinus communis* (84%), *Theobroma cacao* (82%), *Gossypium barbadense* (78%), *Vitis vinifera* (77%) and *Corylus avellana* (76%), indicating that *JcIPI* belonged to the IPI superfamily (Fig. 3). In order to investigate the evolutionary position of *JcIPI* among the phylogenetic tree of various IPIs, a phylogenetic tree of IPIs was constructed using MEGA 5.1 software as shown in Fig. 4. Among IPIs, *JcIPI* and *RcIPI* showed the closest relationship because they both belonged to the family Euphorbiaceae.

IPIs have a typical region called NUDIX motif associating with their catalytic function. These NUDIX enzymes classically catalyze the hydrolysis of Nucleoside Diphosphate linked to another moiety (X) by nucleophilic substitution in presence of a divalent cation [22]. CDD search at NCBI indicated the presence of conserved active sites, metal binding sites and NUDIX motif in *JcIPI*, suggesting that the protein may encode for a functional enzyme. The secondary structure analysis of *JcIPI* showed that predicted *JcIPI* protein

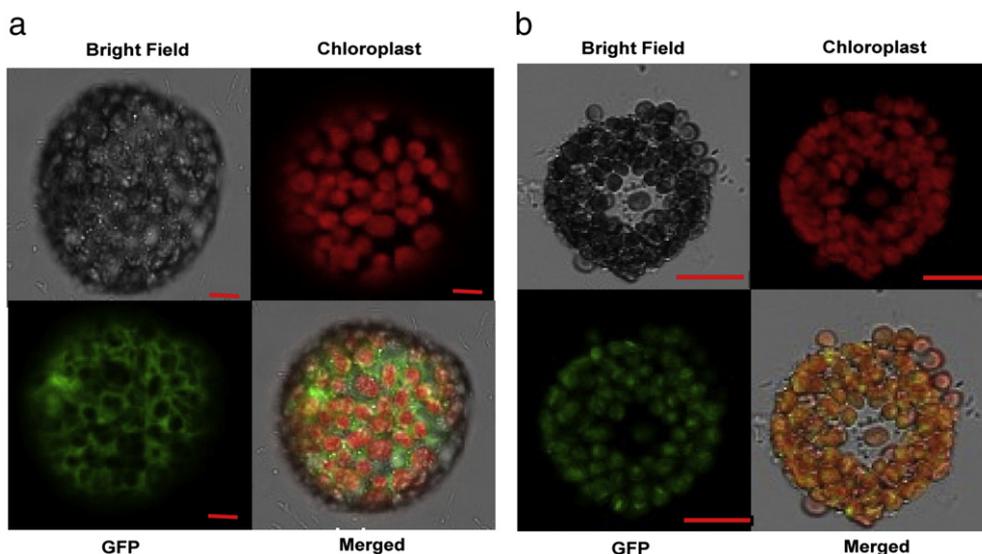


Fig. 10. Protoplast transient expression of JcIPI in tobacco. Subcellular localization of JcIPI was determined via protoplast PEG transfection using JcIPI-GFP fusion construct. JcIPI was localized in chloroplasts. Red color indicated autofluorescence emitted by chloroplasts, green color indicated autofluorescence emitted by GFP. (a) PBI-121 empty vector. Scale bars = 7.7 μm . (b) JcIPI-GFP fusion construct. Scale bars = 15 μm .

consisted of 42.95% alpha helix, 11.80% extended strand, 2.30% beta turn and 42.95% random coil.

The three dimensional structure of was predicted by Phyre 2 web server (Fig. 5) using *Homo sapiens* isopentenyl diphosphate delt-isomerase 2 as a template. The model covered 76% JcIPI sequence with 100% confidence. It showed that JcIPI was a compact globular protein and belonged to the class of α/β protein. It had two typical structure domains with Cys-159 and Glu-188 active sites respectively, which was consistent to other IPIs. Metal irons are crucial for catalysis and engaged in the interactions with the diphosphate moiety through bindings with the Cys-159 and Glu-188.

3.3. Southern blot analysis

IPI genes have been shown to be duplicated in mammals, many plants and algae [23]. However, as shown in Fig. 6, four bands were detected when the genomic DNA was digested with *BamH I*. The result suggested that JcIPI was encoded by multiple genes. It revealed the presence of an IPI gene family in *J. curcas* genomic DNA. These different members in gene family may be regulated in different manners and the specific role of individual needed to be investigated further.

3.4. Comparative tissue specific expression of JcIPI

Relative transcript analysis of JcIPI from different tissues (mature seeds, roots, stems, leaves, flowers, barks and yellow fruits) was carried out using quantitative real-time PCR. The expression level in all tissues was normalized against the normal transcript level of 18S rRNA, which was taken as a control in this experiment. Results showed that JcIPI expression could be detected only in flowers, leaves and seed kernels (Fig. 7), suggesting that it is not a constitutively expressed gene in various tissues. JcIPI expression in flowers was very high and the expression profile was similar to *Arabidopsis thaliana* [4] and *Catharanthus roseus* [24]. Intriguingly, Wang and Tang [25] found that expression levels of IPI from *C. avellana* were significantly higher in roots than in either stems or leaves. The reason we considered was that flowers had vigorous life activities and high levels of secondary metabolic activities. Also, the difference of subcellular localization between JcIPI and others may lead to the different expression levels.

3.5. JcIPI mRNA expression under stress conditions

Terpenoids in plants have an important role in defense against abiotic stresses [26]. The expressions of many enzyme genes in the biosynthetic pathway can be induced by some environmental factors. To examine the influence of stress conditions like low temperature, salinity, drought and MeJA on IPI expression in *J. curcas*, the total RNA of leaves from treated plants was subjected to qRT-PCR analysis. The results showed that JcIPI gene transcripts of those plants exposed to stress conditions were obviously higher than those of the control plants (Fig. 8). Abiotic factors like salinity, low temperature and drought correlated with JA response. Microassay in *Arabidopsis* revealed the detailed changes in gene expressions [27,28]. Under salt and cold stresses, the expression of JcIPI was increased by 147% and 273%, respectively. With the increasing degree of drought, the accumulation of JcIPI mRNA was enhanced too. It appeared that JcIPI was involved in the response to those stresses.

3.6. Bacterial expression of JcIPI

In order to observe the expression of JcIPI in *E. coli*, the entire ORF of JcIPI was cloned into the expression vector pET-32a, containing a functionally T7 promoter and a Trx tag (about 18 kDa). It was a fusion tag to facilitate subsequent purification of the recombinant protein. The pET-JcIPI construct was sequenced to check for in-frame fusion and then transformed into *E. coli* BL-21 cells. The resultant recombinant JcIPI fusion protein with an approximate size of 50 kDa (including the Trx tag) was observed on the 15% SDS-PAGE gel (Fig. 9, L-4). This was consistent with the predicted protein mass of 34.36 kDa. The induced *E. coli* cells transformed with pET-32a did not show recombinant protein bands at the corresponding point (Fig. 9, L-3). The results showed that JcIPI could highly and correctly expressed in *E. coli* and provided an experimental basis for future tests of enzyme activity.

3.7. Subcellular localization of JcIPI

Transient expression analysis of JcIPI-GFP construct in tobacco protoplasts was used to determine the subcellular localization of JcIPI. Plant cells have two pathways producing IPP/DMAPP: the mevalonic acid (MVA) pathway in cytosols and the methyl-erythritol-phosphate

(MEP) pathway in plastids [21]. Correspondingly, there are two IPI isoforms localized in cytosols and plastids respectively. Furthermore, IPI is also present in other organelles such as peroxisomes [29,30] and mitochondria [4,31]. Terpenoids synthesized in different organelles were different, thus the study on subcellular localization was of great importance. Based on Fig. 10, JcIPI-GFP was localized in chloroplasts. Therefore, this JcIPI we obtained is involved in the MEP pathway in *J. curcas*. It would regulate the subsequent metabolites including mono-, diter- and poly-terpenoids.

4. Conclusions

Pathway laxity and modulation using metabolic engineering are interesting prospects for regulating the terpenoid production in *J. curcas*. Rate-limiting enzymes have been found to play important roles in regulating the metabolic flux in several plants [32]. Based on this backdrop, a new chloroplastic isopentenyl diphosphate isomerase (*JcIPI*) gene was cloned from *J. curcas*. *JcIPI* showed more than 75% homology to IPIs from other plants, with active sites of Cys159 and Glu188. Expression levels of *JcIPI* were highest in flowers of *Jatropha* plants. An *IPI* gene family was present in *Jatropha* genomic DNA. Transient expression revealed that *JcIPI* was localized in chloroplasts and participated in the MEP pathway. These findings will further our fundamental knowledge of key genes involved in the terpenoid biosynthetic pathway of *J. curcas*, and provide a foundation for terpenoid metabolic engineering using the *JcIPI* gene.

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Author contributions

Proposed the theoretical frame: YX, FC; Conceived and designed the experiments: LW, Wrote the paper: LW; Performed the experiments: LW; Analyzed the data: LW, LY, XH.

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