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A 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) gene probably involved in the synthesis of terpenoids in *Chrysanthemum indicum* var. *aromaticum*

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Abstract

Terpenoids are important secondary metabolites in plants. The synthesis of terpenoids involves a key enzyme, 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR). In an aromatic ornamental plant *Chrysanthemum indicum* var. *aromaticum*, terpenoids were found to be an important component in the leaf secretions. In order to study the regulatory mechanism of terpenoids synthesis in *C. indicum* var. *aromaticum*, we cloned the *DXR* gene from *C. indicum* var. *aromaticum*, named *CiDXR*, based on the transcriptome data. *CiDXR* is 1419 bp, encoding a polypeptide chain of 472 amino acids. Comparative and bioinformatic analysis showed that *CiDXR* was highly similar to *DXR*s from other plant species. The protein product of *CiDXR* was analyzed for physical and chemical properties, secondary and tertiary structures. Methyl jasmonate (MeJA) is an effective exogenous elicitor that can impact the synthesis of secondary metabolites by regulating the expression of a series of specific key enzymes. In our study, MeJA treatment was used to study the relationship between *CiDXR* expression and terpenoid metabolism. The expression pattern of *CiDXR* was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Leaves expressed the highest level of *CiDXR* mRNA. In addition, *CiDXR* expression was found to be activated by MeJA, up to 20-fold at 24 h post induction. The MeJA treatment significantly affected relative contents of the secretion products and the induction effect was maximal at 24 h, consistent with the expression of *CiDXR*. This study shows that *CiDXR* may play a key regulatory role in the control of terpenoid synthesis in *C. indicum* var. *aromaticum*.

Keywords: methyl jasmonate; *Chrysanthemum indicum* var. *aromaticum*; terpenoid content; *DXR*;

Introduction

Chrysanthemum indicum var. *aromaticum* is a perennial herb of the Compositae family. Because its flowers, leaves and roots are rich in aroma, *C. indicum* var. *aromaticum* has been used as an aromatic ornamental flower. In preliminary studies, our group has found that the leaves and petals of *C. indicum* var. *aromaticum* are covered with dense glandular trichomes that produce gelatinous secretions containing terpenes and benzenes. The scent of *C. indicum* var. *aromaticum* is largely determined by volatiles in the secretions (He et al. 2015). Terpenoids are a class of compounds, including all the isoprene polymers and their derivatives, constructed using isoprene as the basic building block. At present, the majority of terpenoids are known to be synthesized by two biosynthetic pathways: the mevalonate (MVA) pathway and the plastid-localized 2-C-methyl-d-erythritol 4-phosphate (MEP) pathway (Wölwer-Rieck et al. 2014). The MEP pathway is principally involved in providing isoprenoids for monoterpenes (Lange et al. 2000), sesquiterpenes (Dudareva et al., 2005), diterpenes (Ge and Wu 2005) and carotenoids (Lu et al. 2012). The MEP pathway consists of seven enzymatic reactions, in which DXR is a key enzyme upstream. DXR catalyzes the second-step reaction: the reductive conversion of 1-deoxy-D-xylulose 5-phosphate

(DXP) to MEP and into terpenoids. The level of DXR activity determines the proportion of DXP used for terpenoid synthesis (Zhang et al. 2013). Previous reports also showed that DXR participates in the control of isoprenoid accumulation in plants and plays a key regulatory role in the control of terpenoids in the pathway (Walter et al. 2000; Yao et al. 2008). Overexpression of *DXR* in *Mentha* × *piperita* could promote the synthesis of monoterpenes such as peppermint oil in leaves and increase the amount of essential oil by 50% (Mahmoud and Croteau 2001). The overexpression of *DXR* in the hairy roots of *Tripterygium wilfordii* increased the content of terpenoids, confirming that *DXR* plays an important role in terpene biosynthesis at the transgenic level (Zhu et al. 2014). Overexpression of *DXR* in *Physcomitrella patens* could promote the synthesis of terpenoids, indicating that *DXR* is an effective target for the regulation of terpenoid biosynthesis (Pan et al. 2015). Therefore, as a node of terpenoid synthesis, *DXR* has become a hot spot of research.

Exogenous MeJA can act as a signal transduction molecule and mediate signals in plant cells, induce plants to produce terpenoids, alkaloids, phenols and other secondary metabolites, and improve the content of effective substances (Cho et al. 2008). MeJA is also a kind of important endogenous stress resistance molecule, playing an important role in plant stress protection (Devoto and Turner, 2003). MeJA is able to induce the expression of wound response genes (Xu et al. 1994), which influence the secondary metabolism of plants by opening the coordinated expression of related biosynthetic genes (Endt et al. 2007). MeJA has been reported to selectively stimulate enzymes involved in the biosynthesis of scopadulcic acid B in wild licorice

tissue, such as geranyl geranyl phosphate (GGDP) synthase and GGDP cyclase, which subsequently increase the content of glycyrrhizic acid B (Nkembo et al. 2005). A report has indicated that a treatment with 100 μM of MeJA could significantly increase the content of tanshinones in *Salvia miltiorrhiza*, and could also elevate the expression levels of 3-hydroxy-3-methylglutaryl coenzyme-A reductase (*HMGR*) and *DXR*, the two key enzymes in the biosynthetic pathway of terpenoids (Liang et al. 2012). MeJA has been used to explore the key enzymes, to clarify the pathways, and to determine the regulation of plant secondary metabolite production.

Studies on the functions and regulatory mechanisms of some important enzymes in the synthesis of terpenoids are still in the early stage. To further understand the molecular regulation mechanism of terpenoid synthesis pathways and the key enzymes in *C. indicum* var. *aromaticum*, we isolated and cloned the cDNA of *CiDXR* from *C. indicum* var. *aromaticum*, based on the transcriptome data. Using bioinformatics methods, we analyzed the amino acid sequence of *CiDXR* in *C. indicum* var. *aromaticum*, predicted its physicochemical properties and phylogenetic relationship. We have also measured *CiDXR* mRNA expression in different tissues and in response to MeJA treatments with qRT-PCR. Furthermore, we analyzed the leaf secretions after a MeJA treatment by GC/MS. The aim of this study was to establish the mechanism of *C. indicum* var. *aromaticum* terpenoid synthesis and to provide references for cultivating new aromatic chrysanthemum.

Materials and Methods

Plant Material

The strain of *C. indicum* var. *aromaticum* was originally collected in wild from Shennongjia area of Hubei Province (110 23'57 "E, 31 28'7" N), and was successfully planted in the nursery of Northeast Forestry University. The cuttings from healthy plants were planted in a greenhouse. When roots were grown after planting (two weeks), the seedlings were transplanted in pots. Tests were conducted when the plants have grown to 6-8 leaves.

RNA Extraction and cDNA Synthesis

Leaf total RNA was extracted using TRNzol Universal Reagent (TIANGEN) according to the manufacturer's manual. The RNA quality was assessed by 0.8% agarose gel electrophoresis, and the RNA concentration was measured by a spectrophotometer. RNA samples meeting the quality standard (OD260/OD280≥2.0) were stored at -80℃. An aliquot of RNA (0.5 μg) was reverse transcribed into cDNA with Revert AidTM First Strand according to the manual. The cDNA was saved at -20℃ until use.

Cloning of CiDXR

According to the sequencing data of *C. indicum* var. *aromaticum* transcriptome from a previous study by the group, primers were designed for the ORF upstream *CiDXR*-F (5'-ATGTCTTTGAACACCCTTTCCCCT-3 ') and downstream *CiDXR*-R (5'-TCATACTAGTGCCGGAGTTAAACCA -3') using Primers 5.0. The target

segment was amplified by PCR using the cDNA as the template. The PCR reaction system was 20 μl containing 1 μl of cDNA template, 2 μl of 10× buffer, 2 μl of dNTPs, 0.4 μl of each primer (10 pmol μl^{-1}), 0.2 μl of Blend Taq® (5 U μl^{-1}), and 14 μl of dd-H_2O. The PCR conditions were: first 94℃ for 2 min, followed by thirty-five cycles of amplification (94℃ for 30 s, 54℃ for 30 s and 72℃ for 1 min) and an extension at 72℃ for 5 min, and finally the reaction was held at 4℃. The PCR products were assessed with 1% agarose gel electrophoresis, then purified with the TIANgel Midi Purification Kit and cloned into the pUC-T TA vector for sequencing. The recombinant construct was used to transform *Escherichia coli* DH5α strain for blue-white screening. The *CiDXR* clones were sent to Beijing Bioko Company for sequencing.

Bioinformatic Analysis and Multiple Alignments

CDNA sequence was translated into amino acid sequence using the DNAMAN software, and subjected to similarity comparison by BlastN and BlastX at the NCBI website (http://www.ncbi.nlm.nih.gov/). Phylogenetic trees were constructed using the neighbor-joining method implemented in MEGA 5.1 software. The theoretical isoelectric point (pI), relative molecular mass, and hydrophilicity/ hydrophobicity were calculated using the online software package ProtParam at ExPASy. The secondary structure was predicted with SOPMA2.0 online software (http://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html). SWISS-MODEL (http: //swissmodel.expasy.org/) was used to build 3D models of the

domains using the homology modeling method and the results were viewed with RasMol_2.7.5.2 software.

MeJA Treatment

We selected healthy plants with similar conditions for the experiment. The plants were sprayed with 0.25% MeJA and then covered with plastic bags, while the control plants were sprayed with the same solvent but no MeJA. At 0, 4, 8, 24 and 48 h after treatment, we collected three grams (g) of the third functional leaves from five plants in every group for analysis.

Measuring Gene Expression Levels by qRT-PCR

Plant total RNA was extracted with the Trizol method from leaves, stems and roots. A reverse transcription kit (TOYOBO, Japan) was used for reverse transcription. qRT-PCR was used to detect the relative expression of *CiDXR* mRNA at different times after the MeJA treatment using a primer pair *CiDXR*-qRT-F/R (*CiDXR*-qRT-F: 5'-TTGCTGGAGGTCCCTTTGTTC-3', *CiDXR*-qRT-R: 5'-AGTGTGGCAGAGTCA ACGGTG-3'). The resulting data were shown as the mean ± SD of three biological replicates. TransStart® Top Green qPCR SuperMixq kit was used to establish a 20 μl reaction system containing 10 μl of TransStart® Top Green qPCR SuperMix, 0.4 μl of each primer (10 μM), 8.2 μl of dd-H_2O and 1 μl of cDNA template. The PCR reaction tube was placed in a 96-well plate of Light Cycler® 96 (Switzerland). The thermal program for qRT-PCR was as follows: an initial denaturation (95℃ for 2 min) was

followed by 40 cycles of amplification (94℃ for 30 s, 60℃ for 15 s, and 72℃ for 10 s). For consistency and reliability, we tested different internal controls using the same experimental conditions and selected the most stable and consistent housekeeping gene (*CmEF1α* gene (KF305681)) as the internal control for this study. Relative transcript abundances were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

Whether treatment means differed statistically from one another was assessed using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test (P = 0.05). SPSS v19.0J software was used for all statistical calculations. All analysis was performed based on three biological replicates.

Terpenoid Detection by GC-MS

Samples were pulverized in liquid nitrogen, transferred into flasks of 100 ml, subjected to supersonic extraction (KQ-300DE, Kunshan Ultrasonic Instruments Co., Ltd, China) for 20 min with 100 ml of dichloromethane, filtered and the residue was extracted three more times in the same manner. The extract was poured into a funnel containing 20 g of anhydrous sodium sulfate and filtered into a round bottom flask. The filtrate was concentrated with a rotary evaporator at 40℃. The concentrate was transferred to an amber vial and dissolved in 0.5 ml, placed in a refrigerator at 0℃ until use.

Foliar secretion samples were analyzed with Agilent 7890A-5975C GC/MS equipment. GC conditions were: HP-5MS (30 m × 0.25 mm × 0.25 um) elastic quartz capillary column, injection volume 1 μl, split ratio 5: 1. The sample was maintained at 50℃ for 30 min, heated to 160℃ at a rate of 15℃ min^{-1}, and then to 270℃ at 10℃ min^{-1}, solvent delay 3.5 min. MS conditions were: EI mode, quadrupole temperature 150℃, ion source temperature 230℃, interface temperature 280℃, ionization energy 70 eV, electron multiplier voltage 2100 V, scan range 4-500 u, standard mass spectrometry library NIST08L.

The volatile compounds were analyzed by Turbo Mass 5.4.2 GC/MS software. Each peak of the total ion chromatograms was compared with data from NIST08 database, and then the retention index of a component was compared with that in the Pherobase database. The relative content of each component was calculated using the peak area normalization method.

Results

Cloning and Characterization of CiDXR

The cloned *CiDXR* includes an open reading frame (ORF) of 1419 bp, encoding a polypeptide chain of 472 amino acids. The results of BLASTp alignment showed that the encoded *CiDXR* had three domains (Fig 1). This is consistent with the structure of the DXR in other plants. *CiDXR* belongs to the DXP reductoisomerase superfamily. These enzymes catalyze the formation of MEP from DXP in the presence of NADPH. Compared among DXR sequences, the *CiDXR* sequence is most similar

to those from *Chrysanthemum* × *morifolium* (BAE79548.1), *Artemisia annua* (AAD56391.2), *Tanacetum parthenium* (AER00470.1), *Stevia rebaudiana* (ALJ30090.1) and *Cynara cardunculus* var. scolymus (KVI06333.1), with similarity being 99%, 99%, 98%, 91% and 88% respectively. Therefore, the gene cloned in this study was designated as *CiDXR* and the sequence information was deposited in the GenBank database (accession number: KX932439).

The ProtParam of ExPASy online server predicted that the protein relative molecular mass was 5.07 kDa, theoretical isoelectric point (pI) 6.15, the instability index 32.99 which classifies the protein as stable. The ProtScale online software was used to predict the hydrophilicity of *CiDXR*. The grand average of hydrophobicity (GRAVY) was 0.032. The highest hydrophobicity score was 2.444, assigned to valine (Val) at position 171, and the lowest was -2.244, assigned to serine (Ser) at position 40. The hydrophobic amino acid residues were rather evenly distributed throughout the polypeptide chain and slightly more than hydrophilic amino acid residues in percentage. As a whole, the polypeptide chain appeared hydrophobic, and CiDXR is considered to be a hydrophobic protein.

Phylogenetic Tree Depicting the Relationships among Plants

Since the MEP pathway does not exist in animals and fungi (Lu et al. 2008), we analyzed *DXR* genes in plants. A phylogenetic analysis of *CiDXR* gene was performed using MEGA 5.0 software. Phylogenetic trees were constructed by comparing the amino acid sequences of CiDXR and other 31 DXR proteins of high similarity (Fig. 2).

Clustering analysis showed that CiDXR is closely related to homologs in *Chrysanthemum* × *morifolium*, *Artemisia annua*, *Tanacetum parthenium*, *Cynara cardunculus* var. *scolymus* and *Stevia rebaudiana*, and these DXR sequences were clustered into one group. CiDXR was most similar to the DXR of *Chrysanthemum* × *morifolium* and *Artemisia annua* in the same family, was less similar to those in other species such as *Rosa hybrid* cultivar, *Rosa rugose*, *Rosa* × *centifolia*, *Antirrhinum majus*, *Eriobotrya japonica* and *Pyrus communis*.

Prediction of the Secondary and Tertiary Structures of CiDXR

The secondary structure of CiDXR was predicted with SOPMA2.0. Results showed that the protein is composed of 26.91% α-helices, 25.42% extended strands, 10.17% β-turns and 37.50% random coils, in which random coil and α-helix are the main components (Fig. 3). The amino acid sequence of CiDXR appeared most similar to that of *Artemisia annua* (AAD56391.2), being 99% identical. Three-dimensional homology modeling was conducted using SWISS-MODEL Alignment Mode (Fig. 4). A very tight structural scaffold was predicted with stable domains typically formed by α-helices and β-turns and other secondary structural features. CiDXR consists of three domains, and NADPH-binding motifs were embedded in the domains. Results of structural modeling indicated that CiDXR had many characters commonly found in DXR proteins, and *CiDXR* encodes a functional DXR protein. Thus, molecular characterization of *CiDXR* showed that *CiDXR* was very similar to other *DXRs* of plant origin, indicating that *CiDXR* belonged to DXR superfamily.

Analysis of CiDXR Relative Expression in Different Tissues and in Response to MeJA treatments

The expression of *CiDXR* was analyzed with qRT-PCR. The data were scaled by setting the expression of *CiDXR* in root at 0 h as 1. The abundance of *CiDXR* mRNA varied among different tissues and organs. The expression of *CiDXR* was the highest in leaves, lower in stems and roots (Fig. 5A). Leaves expressed 5 times higher *CiDXR* mRNA than stem segments did, and nearly 13 times higher than roots did, implying that leaves are the most important tissues for the biosynthesis of terpenoids. After the treatment with 0.25% MeJA, the relative expression of *CiDXR* decreased after increasing firstly, and it reached the highest level at 24 h. The foliar *CiDXR* transcript level almost was same within 8 h , but dramatically increased at 24 h post treatment, up to 20-fold as much as controls (Fig. 5B). Then the foliar *CiDXR* level decreased at 48 h post treatment, but still higher than that at 0 h. This indicates that the expression of *CiDXR* was significantly influenced by MeJA.

Improvement of Terpenoid Production by MeJA Treatment

The GC/MS analysis showed that the surface secretions from leaves of *C. indicum* var. *aromaticum* were mainly terpenes, and benzene. Some alcohols, esters, aldehydes and ketones were also found. Their relative contents especially terpenes were significantly increased by the treatment of MeJA. The content of terpenoids gradually increased and reached its highest level at 48 h after the treatment, but the plant leaves

were obviously damaged at 48 h, which could impede the synthesis of terpenoids and growth of the plants. Therefore, the best time of induction was 24h. The relative contents of benzene and alcohols were the highest at 24 h post treatment, and the contents increased to 33.29% and 15.49% respectively. The relative content of ester compounds did not change significantly during the period examined after MeJA treatment. The optimal effect was obtained at 8 h. Aldehydes and ketones did not change (Fig. 6).

After the treatment with MeJA, 65 compounds were found from the surface secretion of the leaves of *C. indicum* var. *aromaticum*, and 45 compounds have been identified. Seven compounds were common in all treatments. Nineteen compounds disappeared, and 39 compounds were new. The secreted terpenes were mainly 4-methylene-1- (1-methylethyl) bicyclo [3.1.0] 2-hexene, caryophyllene, 1, 3, 8-p-menthatriene, 3-thujene, terpinene, maaliene, guaiene, α-phellandrene, γ-cadinene and epizonarene. A longer treatment time was accompanied with a higher content of 4-methylene-1- (1-methylethyl) bicyclo [3.1.0] 2-hexene and caryophyllene. Several compounds (i. e. 1, 3, 8-p-menthatriene, 3-thujene, terpinene, maaliene and guaiene) were increased first, then decreased or even disappeared, but their relative contents were the highest at 24 h. α -phellandrene, γ-cadinene and epizonarene all reached their highest levels at 24 h post treatment and then decreased. Benzenes were mainly p-/m-cymene with relatively stable content, and the induction effect was good with a treatment duration of 24 h. Alcohols were mainly cis-sabinol and eucalyptol, both of which were not found before the treatment. Cis-sabinol reached its highest level at 24

h post treatment, then gradually disappeared, while the eucalyptol oil increased continuously. Aldehydes and ketones were **(-)**-α-thujone. The relative content of this compound showed a tendency of decrease first then increase after the treatment, but all were lower than that in the control (Table 1). ·

Discussion

At present, MEP and MVA are two important pathways known for the biosynthesis of terpenoids. Enzymes in terpenoid biosynthesis pathway are a hotspot of research. DXR is a key enzyme in the MEP pathway, catalyzing the conversion of DXP to a precursor of terpene synthesis. This reaction is an important rate-limiting step in the MEP pathway and an important regulatory site in the metabolism of terpenoids in the cytoplasm (Takahashi et al. 1998). *DXR* genes have been cloned and characterized from *E. coli* (Kuzuyama et al., 2000; Radykewicz et al., 2000), eubacterium (Grolle et al., 2000) and a few plant species (Rodriguez-Concepcion et al., 2001; Kim et al., 2006). Based on the transcriptome data, we cloned the *DXR* gene, named *CiDXR*, from *C. indicum* var. *aromaticum*. The basic information of *CiDXR*, such as physical and chemical properties, structural domains and function, were predicted through in-silico analysis. These predictions help to learn more about the function of *DXR* gene in *C. indicum* var. *aromaticum*. This study showed that MeJA can induce the biosynthesis of terpenoids and also showed that the transcript level of *DXR* was correlated with the terpenoid content, which could help to elucidate

the expression kinetics and regulation of the key governing enzymes of the terpenoid biosynthetic pathway (Sharma et al., 2015).

Many studies have shown that the expression of key enzymes in a plant secondary metabolic pathway is regulated with tissue- and organ-specificity, in addition to being influenced by elicitors (Chen et al. 2003). Our results showed that *CiDXR* expression could be detected in all tissues, suggesting that *CiDXR* is a constitutively expressed gene but is expressed at different levels in different tissues, with the highest expression in leaves and lower expression in stems and roots (Fig. 5A). The results of this study indicate that leaves are the most important tissues for the formation of terpenoids. This may be related to the density of the glandular trichome distributed on *C. indicum* var. *aromaticum* leaves, and may also be related to the synthesis of terpenoid compounds in different parts. In different species, the expression of *DXR* in response to MeJA treatments was slightly different. In *Amomum villosum*, *DXR* transcripts were also increased up to 9.4-fold at 24 h post MeJA treatment as compared with untreated controls (Wei et al., 2013). A recent work showed that *DXR* transcripts were increased up to 34-fold after MeJA treatment as compared with untreated controls in *Aquilaria sinensis* (Liu et al., 2015). In *Andrographis paniculata*, the expression of *DXR* was highest at 5.0 μM (20.97±1.39) followed by 10 μM concentration(11.00±1.46) of MeJA at 48 h (Sharma et al., 2015). Our results showed that the expression of *CiDXR* increased rapidly post MeJA treatment (Fig. 5B). *CiDXR* transcripts almost was same at 4 and 8 h after the treatment, increased up to 20-fold at 24 h post treatment as compared with the controls. The expression of

CiDXR was dramatically elevated between 8 to 24 h after the MeJA treatment (Fig. 5B). Because MeJA is a provocative inducer, such that MeJA can rapidly induce the expression of its target gene (Wei et al., 2013). This induced effect had also been shown in other species, such as *Camptotheca acuminate* (Yao et al., 2008), *Amomum villosum* (Wei et al., 2013), *Tripterygium wilfordii* (Tong et al., 2015), *Artemisia annua* (Xiang et al., 2015), *Aquilaria sinensis* (Liu et al., 2015), *Andrographis paniculata* (Sharma et al., 2015). These results indicated that the regulation of *CiDXR* was positively influenced by MeJA treatments.

Externally applied jasmonic acid can effectively stimulate the jasmonic acid pathway in plants. Jasmonic acid conjugates can regulate the synthesis of secondary metabolites such as terpenoids, alkaloids and flavonoids by activating or repressing the expression of a series of specific key enzymes in the pathway (Cheong and Choi, 2003; De Geyter et al., 2012; Vom Endt et al., 2002). At present, many scholars have studied the relationship between gene expression and metabolite formation under MeJA stimulation (Kumeta and Ito 2010; Xu et al. 2014). It is reported that MeJA could enhance the expression of *DXR* and the production of 1, 8-cineole in *Alpinia officinarum* rhizomes (Zhang et al. 2012). A recent study indicated that MeJA could increase the expression level of *DXR* involved in the MEP pathway and this is consistent with the increasing artemisinin content after MeJA treatment in *Artemisia annua* (Xiang et al. 2015). Another study demonstrated that *DXR* gene from terpenoid biosynthesis pathways was upregulated by MeJA, which might be connected to triptolide, wilforgine, and wilforine biosynthesis as well (Zhang et al., 2016). In our

study, MeJA treatment was used to study the relationship between *CiDXR* expression and terpenoid metabolism. We found that terpenes, benzenes and alcohols were the main aroma components in the foliar secretion of C. *indicum* var. *aromaticum* after a MeJA treatment. This is consistent with findings by Liu (Liu et al. 1983), and consistent with reports about the volatile oil composition in *C. indicum* var. *aromaticum* (Li et al. 2012; Jian et al. 2014). The main terpenoids induced by MeJA in leaves of *C. indicum* var. *aromaticum* belong to monoterpenes and sesquiterpenes. The levels of α-phellandrene, γ-cadinene and epizonarene from zero reached to the highest at 24 h after MeJA treatment, this is because low concentration compounds produced in a short period of treatment are not easily detected. The contents of maaliene, guaiene and cis-sabinol were the highest at 24 h post treatment and then disappeared at 48 h post treatment of MeJA induction. At 48 h after MeJA spray the plants were obviously damaged, which could impede the synthesis of terpenoids , thus some compounds disappeared. The sudden shift of 4-methylene-1- (1-methylethyl) bicyclo [3.1.0] 2-hexene and maaliene between 8 to 24 h may be due to fact that *DXR* gene is directly involved in the accumulation of these two substances under MeJA treatment. This data was consistent with expression of *CiDXR* between 8 to 24 h. The leaf surface secretion in *C. indicum* var. *aromaticum* changed clearly with time after a MeJA treatment, but the highest induction of terpenoid accumulation was investigated at 24 h MeJA treatment. This result was consistent with the highest expression levels of *CiDXR* at 24 h post treatment. This study indicated that *DXR* played regulation role on the terpenoids pathway in *C. indicum* var. *aromaticum* and the transcription level

of *DXR* was positively correlating with the increase of the terpenoid contents.

This work provides base for further work, including the basic functions of *DXR* gene, regulation of *DXR* gene and biosynthesis of terpenoid natural products. The study reveals merely correlations between the transcript level of *DXR* gene and terpenoid accumulation. This collectively suggests that some degree of variability in secondary metabolism can be linked to changes in the expression of genes in biosynthetic pathways. The study may be of particular value in providing references for cultivating new aromatic chrysanthemum using molecular biology methods. Further experiments are required to decipher the regulation of other genes in the terpenoid biosynthesis pathways and the coordinated actions among the related genes.

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Fig. 1. Alignment of CiDXR with other plant amino acid sequences. The 3 domains of CiDXR are underlined. The sequence details are as follows: CiDXR from *C. indicum* var. *aromaticum*; AaDXR from *Artemisia annua*, AAD56391.2; AtDXR, from *Arabidopsis thaliana*, NP_201085.1; SlDXR from *Solanum lycopersicum,* NP_001234553.2; HbDXR from *Hevea brasiliensis*, ABD92702.1; AmDXR from *Antirrhinum majus*, AAW28998.1).

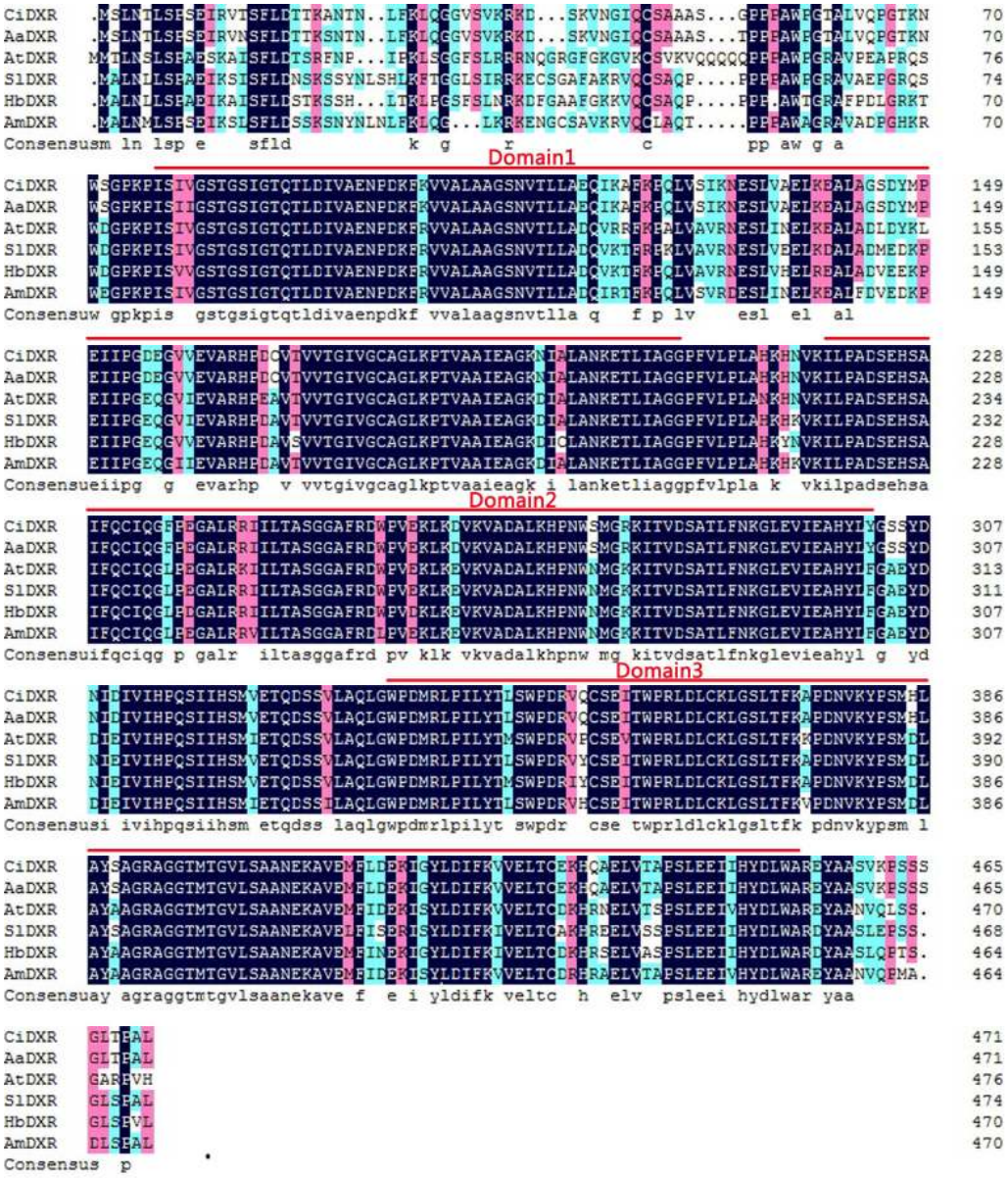

Fig. 2. Phylogenetic relationship of the CiDXR amino acid sequences from *C. indicum* var. *aromaticum* and characterized DXR sequences from other plant species. Amino acid sequences were aligned using the CLUSTAL W program and phylogenetic tree was built using MEGA 5.0 by the maximal parsimony method. Bootstrap values obtained after 1000 replications are shown on the branches. The species and corresponding accession number are as follows. AaDXR (*Artemisia annua*; AAD56391.2); AlDXR (*Arabidopsis lyrata* subsp. *lyrata*; XP_002866510.1); AmDXR (*Antirrhinum majus*; AAW28998.1); AsDXR (*Angelica sinensis*; AJW77397.1); AtDXR (*Arabidopsis thaliana*; NP_201085.1); CaDXR (*Centella asiatica*; AFO64619.1); CcDXR (*Cynara cardunculus* var. scolymus; KVI06333.1); CmDXR (*Chrysanthemum* × *morifolium*; BAE79548.1); CrDXR (*Catharanthus roseus*; AAF65154.1); CsDXR (*Croton stellatopilosus*; ABO38177.1); DhDXR (*Dorcoceras hygrometricum*; KZV52129.1); EjDXR (*Eriobotrya japonica*; AFP43697.1); ElDXR (*Euphorbia lathylris*; AFZ93643.1); GbDXR (*Gossypium barbadense*; ABN13969.1); HbDXR (*Hevea brasiliensis*; ABD92702.1); IrDXR (*Isodon rubescens*; AMW77343.1); LaDXR (*Lavandula angustifolia*; AGQ04155.1); LuDXR (*Linum usitatissimum*; CAF22092.1); MtDXR (*Medicago truncatula*; XP_003608963.2); PcDXR (*Pyrus communis*; AIR72281.1); PkDXR (*Picrorhiza kurrooa*; ABC74566.1); RcDXR (*Rosa* × *centifolia*; AGO04400.1); RgDXR (*Rehmannia glutinosa*; ANW06222.1); RhDXR (*Rosa hybrid* cultivar; AFR79418.1); RrDXR (*Rosa rugose*; AEZ53171.1); RvDXR (*Rauvolfia verticillata*; AAY87151.2); SlDXR (*Solanum lycopersicum*; NP_001234553.2); SmDXR (*Salvia miltiorrhiza*; ACR57217.1); SrDXR (*Stevia rebaudiana*; ALJ30090.1); TkDXR (*Taraxacum kok-saghyz*; AMB19706.1); TpDXR (*Tanacetum parthenium*; AER00470.1); WsDXR (*Withania somnifera*; ADN39416.1).

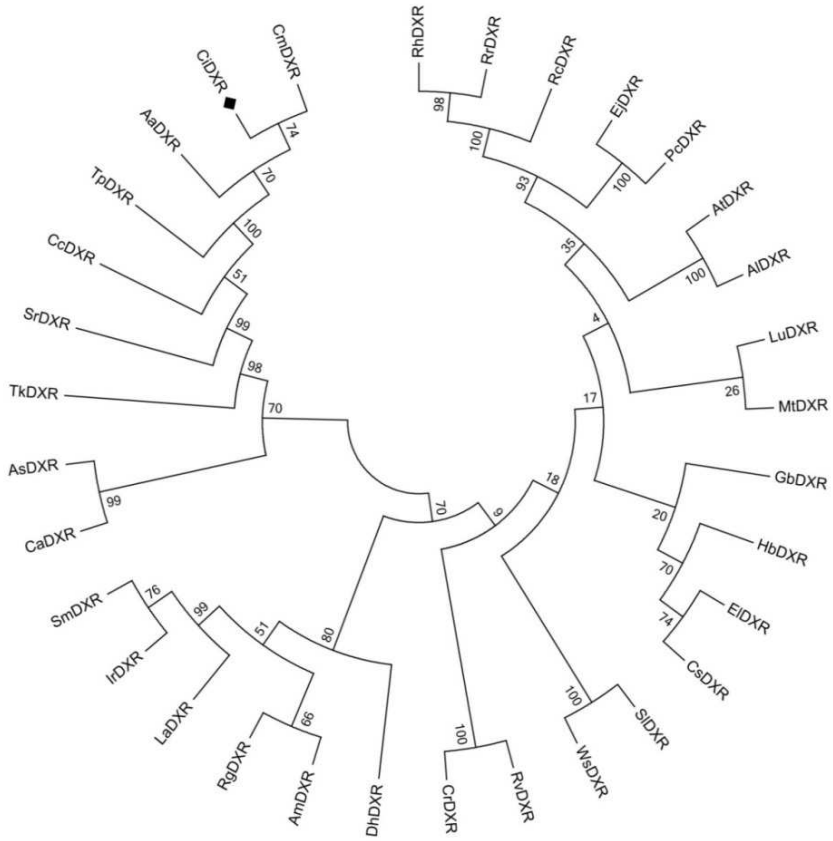

Fig. 3. The deduced secondary structure of CiDXR by SOMPA, α-helices, extended strands, β-turns and random coils are indicated, respectively, with blue, red, green and pink lines.

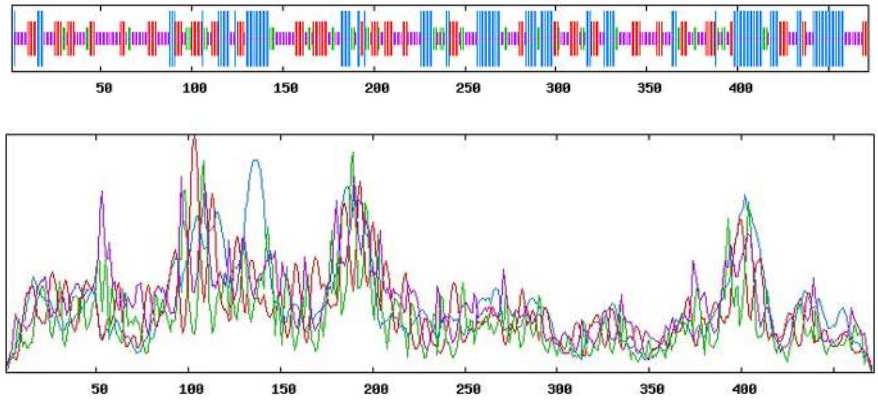

Fig. 4. The deduced 3D structure of CiDXR protein (a, ribbons; b, backbone; c, wireframe; d, ball and stick). Different structures were represented by different colors. Pink represents α-helices. Yellow represents β-turn. White represents extended strand. Blue represents random coils.

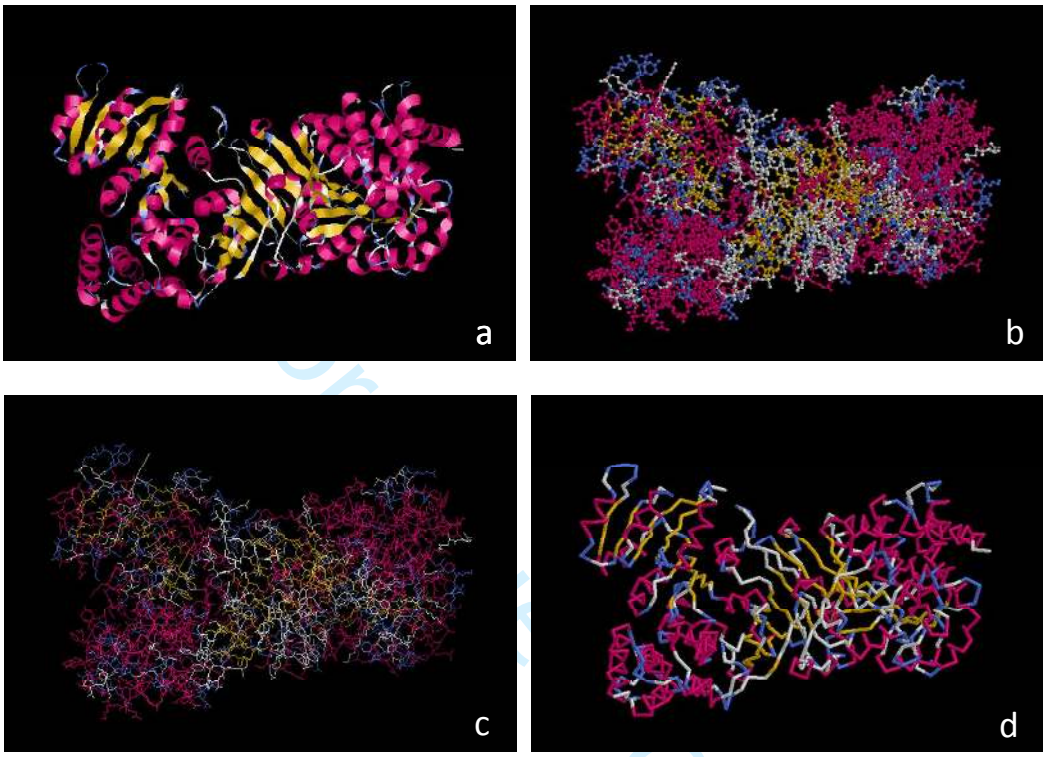

Fig. 5. QRT-PCR analysis of expression patterns of *CiDXR*. A: expression patterns of *CiDXR* in root, stem and leaf. B: expression patterns of *CiDXR* and in response to MeJA treatments. Bars marked with different letters were significantly different (p<0.05) by Duncan's multiple range test.

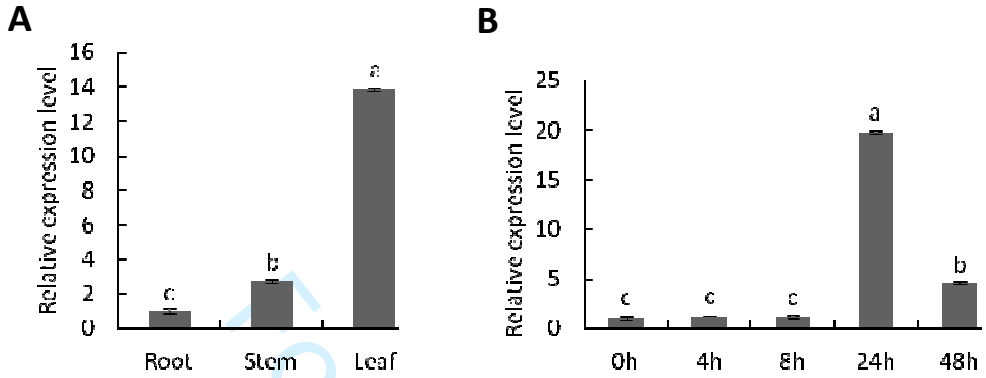

Fig. 6. Leaf surface secretion induced by time-gradients of methyl jasmonate.

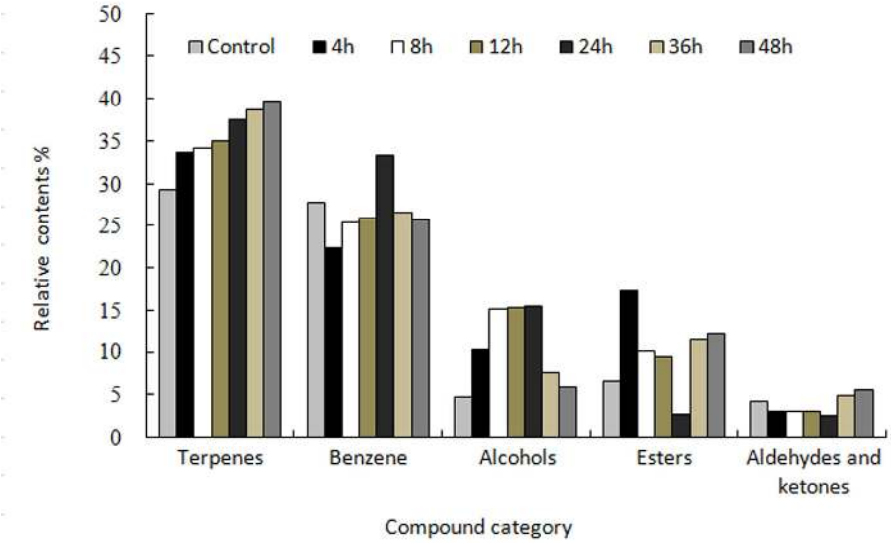

Table 1. Main terpenoids induced by MeJA in leaves of *C. indicum* var. *aromaticum*

Compound	Relative content (%) in samples				
	0 h	4h	8h	24h	48h
4-methylene-1-(1-methylethyl) bicyclo [3.1.0]	0.74	4.13	4.68	4.68 9.72	9.72 12.20 12.20
2-hexene					
Caryophyllene	2.83	2.97	3.26	3.79	4.60
1, 3, 8-p-methatriene	0.19	0.96	1.57	2.35	1.68
3-thujene	0.70	1.03	1.42	1.75	0.77
Terpinene	0.27	0.44	0.57	0.71	0.32
Maaliene	3.00	3.75	3.58	6.28	0.00
Guaiene	0.17	0.73	0.74	0.90	0.00
α-phellandrene	0.00	0.00	0.17	0.30	0.19
γ-cadinene	0.00	0.78	0.87	0.92	0.81
Epizonarene	0.00	0.00	0.00	1.23	0.98
p-/m-Cymene	19.46	20.04	21.24	23.80	23.21
Cis-sabinol	0.00	9.06	10.14	10.19	0.00
Eucalyptol	0.00	0.00	2.94	3.31	3.85
(-)-α-thujone	3.80	3.05	3.10	3.22	3.31