

## Promoter activity analysis and transcriptional profile of *Ginkgo biloba* 1-Deoxy-D- Xylulose 5-Phosphate reductoisomerase gene (GbDXR) under abiotic stresses

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### Abstract

Terpene trilactones (TTL) is a pharmacological ingredient in *Ginkgo biloba* and its content has become one of the key indices for medicinal value evaluation of ginkgo. 1-Deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) catalyzes the first step specific for isopentenyl diphosphate production in methylerythritol phosphate pathway, which provide the basic structure required for TTLs biosynthesis. To understand the mechanism controlling the *GbDXR* gene expression, the *GbDXR* promoter sequence was isolated and subjected to transient expression with the green fluorescent protein (GFP) in tobacco plants. Characteristic analysis revealed various *cis*-acting elements that related to light-regulated transcription, hormone signaling (auxin, ethylene), adversity stress and defense signaling (heat/dehydration stress) in the *GbDXR* promoter region. In transient expression assay, deletion of different portions of the upstream *GbDXR* promoter identified that the promoter region -3230bp to -865bp conserve the positive regulation function, which could promote the expression of GFP in the cytoplasm of tobacco leaf epidermal cells. The regulation function of the promoter region -865bp to -262bp remained to be elucidated. EMSA analysis suggested possible interactions of GbERF10 and GbERF17 with the ERF-binding elements in the upstream of *GbDXR* promoter. For abiotic stresses treatment, the expression of *GbDXR* gene could be significantly induced by UV-B and drought stress. In general, the *GbDXR* gene expressed differently in different ginkgo tissues but exhibited the highest transcriptional level in the root, with the maximum TTLs content simultaneously. The positive relationship between gene expression level and TTLs content indicated that the *GbDXR* is responsible for TTLs biosynthesis in *G. biloba*.

**Keywords:** DXR promoter; EMSA analysis; *Ginkgo biloba*; terpene trilactones; transient expression

**Abbreviation:** TTL-terpene trilactones; DXR-1-Deoxy-D-xylulose 5-phosphate reductoisomerase; GFP-green fluorescent proteins; IPP-isopentenyl diphosphate; DMAPP-dimethylallyl diphosphate; MEP-2-C-methyl-D- erythritol 4-phosphate pathway; MVA-mevalonate pathway; DXP-1-Deoxy-D-xylulose 5-

phosphate; Eth- ethylene; EMSA-Electrophoretic mobility shift assay; IPTG-isopropyl- $\beta$ -D-1-thiogalactopyranoside

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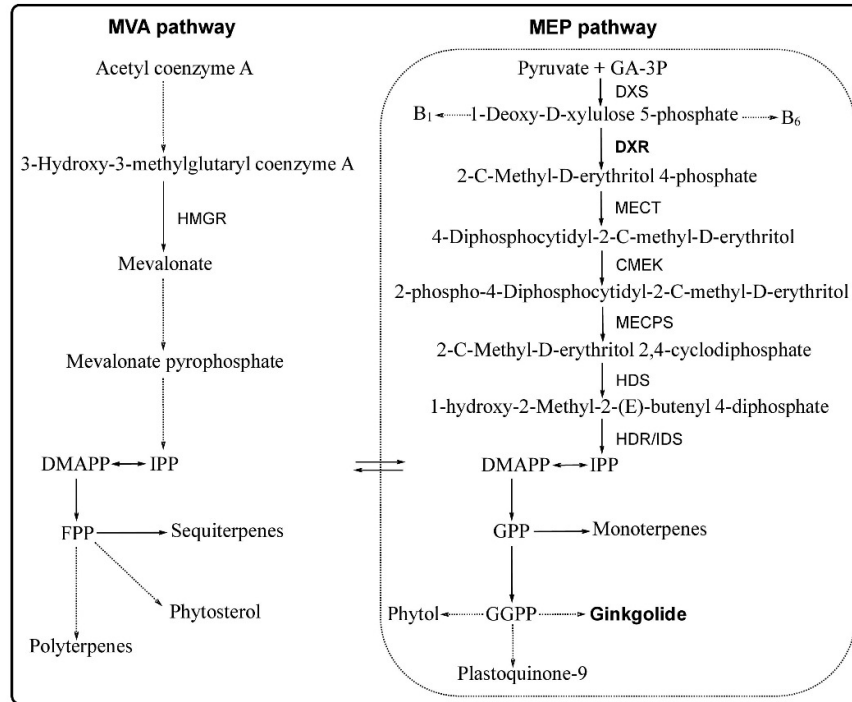
## Introduction

*Ginkgo biloba* L. is a deciduous tree belongs to the division Ginkgophyta in gymnosperm with a very long surviving history, which commonly known as the “living fossil” that flourished during the Mesozoic Jurassic period 170 million years ago (Bowe *et al.*, 2000; Uvackova *et al.*, 2014). Although *G. biloba* is native to China, it has been transplanted worldwide as an ornamental and medicinal tree. Ginkgolides and bilobalide, collectively termed TTLs, are recognized as the medicinal ingredients uniquely exist in *G. biloba* (Strømgaard and Nakanishi, 2004). Their special pharmacological activities have been widely used in diseases treatment, such as reduce the symptoms of memory impairment and old-age dementia, treat the cardiovascular dysfunctions, prevent the vascular disorders and act as the platelet activating factor antagonist (Watanabe *et al.*, 2001; Rodríguez *et al.*, 2007; DeKosky *et al.* 2008). Though the TTLs content in *G. biloba* is low, it is an important index for quality evaluation and standardization of this medicinal materials. Increasing the biosynthesis and accumulation of TTLs through metabolic engineering and artificial planting would have practical significance in the improvement of *G. biloba* quality.

TTLs are highly modified diterpene lactones, and which are derived from the universal five carbon skeleton-isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (Cordoba *et al.*, 2009). Higher plants use two pathways to form IPP and DMAPP, the methylerythritol phosphate (MEP) pathway in the plastid facilitates formation of monoterpenoids and diterpenoids, whereas the mevalonic acid (MVA) pathway in the cytosol facilitates the synthesis of sesquiterpenoids and sterols (Lange *et al.*, 2000). It is inferred that the biosynthetic precursor of diterpenoid ginkgolides is expected to arise from the MEP pathway. In *G. biloba*, the ginkgolides were found present in both leaves and roots, however, genes coding for enzymes of the MEP pathway involved in IPP and DMAPP were highly expressed predominantly in the roots, which suggested that ginkgolides are synthesized in the roots and then transported to the stems and leaves (Kim *et al.*, 2012).

As one of the structural genes involved in MEP pathway, 1-Deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) catalyzes the critical step by converting 1-Deoxy-D-xylulose 5-phosphate (DXP) into 2-C-methyl-D-erythritol 4-phosphate (MEP), the first step specific for IPP production and which is the basic structure required for isoprenoids biosynthesis (Figure 1). Genes encoding DXR have been cloned and functional identified in *G. biloba* (Kim *et al.*, 2006), however, there is little information about transcriptional regulation of *GbDXR* and no study has been reported regarding the promoter analysis of *GbDXR*.

Promoters are primarily located in the upstream region of genes with various specific *cis*-acting elements that can be recognized by transcription factors to regulate gene expression (Sazegari *et al.*, 2015). In order to explore the physiological role and regulatory mechanism of *GbDXR* involved in TTLs synthesis, we isolated and functional characterized the promoter region of *GbDXR*, the promoter activity was preliminarily identified by promoter deletion analysis with the reporter gene transient expression in tobacco leaf epidermal cells. Furtherly, an interaction assay of AP2/ERF transcription factors with the *cis*-elements on the promoter of *GbDXR* was conducted using a EMSA technology. The expression pattern and functional analyses undertaken may contribute to understand the regulatory role of *GbDXR* promoter in TTLs biosynthesis in *G. biloba*.



**Figure 1.** The cytoplasmic MVA and plastidic MEP pathways in plants (Estévez *et al.*, 2001)

The *DXR* gene catalyzes the step specific for IPP and DMAPP biosynthesis in MEP pathway. *HMGR*, hydroxymethylglutaryl-CoA reductase; *FPP*, farnesyl diphosphate; B<sub>1</sub>, thiamin; B<sub>6</sub>, pyridoxol; *MECT*, 2-C-methyl-D-erythritol-4-phosphate cytidilyltransferase; *CMEK*, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; *MECPS*, 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase; *GPP*, geranyl diphosphate; *GGPP*, geranylgeranyl diphosphate

## Materials and Methods

The different oligonucleotides used in this study were described as follows (Table 1).

**Table 1.** Genome walker and expression analysis primers of *GbDXRP*

Description	Serial number	Sequence (5'-3')
Genomewalker primers	AP1	GTAATACGACTCACTATAGGGC
	AP2	ACTATAGGGCACGCGTGGT
	DXR1	GGACCATCCCACACTTTTCTATCTTCAT
	DXR2	GTTCTATTTCTCATACTAAGCCC
	DXR3	CTAGCTTGTGGATGGATGTAAGTCACA
	DXR4	ACCTTATAAACTCTCTTTTGGCGTG
	DXR5	TCAGGTCAGATTAAGGTCACAAACACAC
DXR6	GCGACTTTGGACAGTTACAAGCAGAGA	
Primers for DXRP1-5 amplification	DPD	gtcag atcta ccatgGACTTTGGACAGTTACAAGCAGAG
	DPU1	cctct agagt cgaccAGGTCAGATTAAGGTCACAAACACA
	DPU2	cctctagagtcgaccTATGACTGTAR TCTCWCAAATCGC
	DPU3	cctctagagtcgaccGACTTACATCCATCCACAAGCTAGA
	DPU4	cctctagagtcgaccTTGGTATCATGTCTAGAGTGGCTTA
DPU5	cctctagagtcgaccACTTATCAATAGTGGAAAGTGGTCC	
Primers for verification	ZJ1	CCCAGGCTTTACACTTTATGCTTCC
	ZJ2	TCAACAAGAATTGGGACAACTCCAG
Primers for qRT-PCR	DXRF	CAGCAATAGAAGCAGGGAAGGAC
	DXRR	ACCTTCTGGTAGACCTTGAATGCAC
	GAPDF	GGTGCCAAAAAGGTGGTCAT
	GAPDR	CAACAACGAACATGGGAGCAT

*Plant materials and treatments*

Ginkgo leaves were obtained from the three-year-old seedlings (*Ginkgo biloba* L. cv 'Jiafoshou') grown in a greenhouse in Wuhan Polytechnic University. Tobacco (*Nicotiana tabacum* L. cv NC89) plants for Agro-infiltration were grown in a growth chamber at 25 °C in 16 h light (2000 lx)/8 h dark (16 L/8 D) for 45 days. Ginkgo seedlings with the same growth and development were subjected to UV-B, drought and ethylene (Eth) treatments. Samples under UV-B treatment were exposed to 1500 J/m<sup>2</sup> UV-B irradiation in a closed chamber, and the control group was placed in a dark chamber. For drought treatment, samples were collected as the relative water content decreased in soil, the whole dehydration stress lasted for 15 days. Eth (100 mg/L) was dissolved in 0.01% Tween 20 and sprayed onto leaves in experimental group, the leaves which were sprayed with the equivalent volume of 0.01% Tween 20 used as control. All samples were collected at different time point according to the arrangement. The harvested leaves were immediately frozen in liquid nitrogen and stored at -80 °C for DNA and RNA extraction.

*Amplification of GbDXR promoter*

Genomic DNA was extracted from leaf samples by an improved cetyltrimethylammonium bromide (CTAB) method described by Cheng *et al.* (2003). To obtain the 5'-upstream promoter sequence, specific primers were designed according to the cDNA sequence of *GbDXR* gene (AY494186, Kim *et al.*, 2006) and the cloning was accomplished using the Universal Genome Walker kit (Clontech Laboratories, TaKaRa). First, the genomic DNA were digested by six restriction enzymes to build different genome walker libraries and which were used as the templates for PCR amplification, then two round PCR were performed continuous with the specific primers GbDXR1, AP1 and GbDXR2, AP2 (Table 1). After three times of extension amplification, an approximate 3230 bp nucleotide sequence was obtained (Figure 2) and confirmed to be the *GbDXR* promoter (designated as GbDXRP) sequence by comparison analysis. The putative cis-elements and the transcription starting site of the *GbDXRP* were analyzed using the PLACE (<http://www.dna.affrc.go.jp/PLACE>) database and the Signal Scan Program PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) database.

*Construction of the GbDXR promoter: GFP plasmid*

To identify regulatory regions involved in transcriptional control of the *GbDXR* gene expression, the cloned full-length *GbDXRP1* together with its 5'-deletions *GbDXRP2*~*GbDXRP6* were fused to GFP by replacing the CaMV 35S promoter in the vector pCAMBIA1304, respectively (Figure 3). The 5'-deleted promoter fragments were amplified by designing specific primers that contain *Pst*I restriction site at the 5'-end of the forward primer and *Nco*I restriction site at the 5'-end of the reverse primer (Table 1), and ensuring that each fragment contains discrete elements of interest as can as possible. Therefore, six promoter fragments *GbDXRP1* (-3230bp~+1bp), *GbDXRP2* (-2562bp~+1bp), *GbDXRP3* (-2024bp~+1bp), *GbDXRP4* (-1457bp~+1bp), *GbDXRP5* (-865bp~+1bp) and *GbDXRP6* (-262bp~+1bp) were obtained using PCR and separately subcloned into pCAMBIA1304, of which the CaMV 35S promoter has already been removed by *Pst*I and *Nco*I digestion. The fragments and linearized vector were linked by In-Fusion HD Enzyme Premix. The nucleotide sequencing results showed that all deletion constructs pCAMBIA1304+*GbDXRP1*~*GbDXRP6* were generated and prepared.

**E-box**

-2270 TGACTGTAGTCTCACAAAATCGCAC**CATATG**GACACCCGATGCAAAAATTTTAAGAGGGC

**MeJA-Re**

-2210 TTTAAGTCGCCTGCCCTCAGAGAATGTCCA**CGTCA**TTTTTTATTA AAAATGATGATGAAT

**Inr-element** **W-box**

-2150 TTGATGAGGTATG**CTCAAT**TTCCAAAATGATTGTCTAAACCTTCTTCTCTCC**TTGAG**AC

**ARF-binding** **I-box**

-2090 **ATGTC**TCCCTTCA**GATAAG**TCCGCGCTTAGCCACATCACTTTCAAATTTAAGCAACCTATC

**MYC-binding** **GT-element**

-2030 AGGCTTCAGATCTCATGAAGCTTTGATAC**CAATGAAAAT**TACAAGAAATGGAATGAAATA

**MYB-binding** **MYC-binding**

-1970 TGGTTTGTA**TAACTG**AAGAATTATA**CATGTG**GATAGAATTCAAATCAAAT**CAC**TTGGTA

**MYB-binding**

-1910 AATGAACTCCCTATTAATAGGGTTGATTAAAAATTACCTTATAAG**AACCTAAC**ACCTA

-1850 ATTACCCAACCTAACCTTAGATTATATTTGAATATTGTCTAAATAAAGTGCAATAATGT

-1790 TCGACACTTATACATTAATAACATTAGAAAATCTAGAAAATTTCTAATGTAACTAATA

**GATA-box** **W-box**

-1730 TTTCCAAACAATAAGGACT**GATA**TAAATAAGAT**TCAG**TAAGGCCGTAATCAACCTAAACC

-1670 ATAACCACACTTATCCCTTTATTTTCATATTGCCATGCCATAAACTGC AAAATAGACATA

**MYB-binding**

-1610 AACACGCCAAAAGAGAGTTTAAGGTCCTGAATCAACCTAAACCA**TAACCA**CACTTATC

-1550 CCCTTTATTTTCATATTGCCATGCCATAAACTGC AAAATAGACATAAACACGCCAAAAGAG

**W-box**

-1490 AGTTTATAAGGTATACTTCCAAGAAATATATATG**TCAGCT**TACATCCATCCACAAGCTAGA

**I-box**

-1430 AATAATTATTATCTAATGAGATATAAGT**GATAA**TCCACAAAATTATAGATATATACGTTA

**SURE** **GT-element**

-1370 **A****GAGAC**CCACTAGTATAAATCAATTAAGCATTTATGCATAAAAGACCAAAGTA**GAAAAT**

-1310 GCCATGTATATATTTAAGACCCCTTTAACCCCTAAATATTTTTTTATTTTAATAATTC

**MYC-binding**

-1250 ATATATCTATCAA**CAATG**CAATCATTCGTAAAAAGGGTAAGTCTTTTGGTATCATGTCT

**SRE** **ARR1-binding** **Inr-element** **I-box**

-1190 AGAGTGGCT**TATCC**AAAAAGAAG**TCGATTTCAATTT**TATTATGGTGGGT**GATAA**AGAGCCT

**MYC-binding** **MYC-binding** **CuRE**

-1130 AATATATTGAAGA**CATGTG**CTTT**CAATG**TGTT**GCTAC**TATCACAATAATGGAACAAG

**MYC-binding** **W-box**

-1070 TGACTTTGTGTAAGGAATGAATGGACTATATTTTA**CAATTG**ATTTGAACA**TCAG**TTATCC

-1010 GTAATAGTCATCACCCATTATCTTCTTTTAGACACAACAATTAGAGCACCATAGAATA

**WRKY** **MYC-binding**

-950 TCACCTCCACTAGAGGATGTATGTAATTTGCA**TCAGCAAAATG**CCTCTATTAGACCACGAGATA

**MYC-binding**

-890 AAAT**CAAAATG**TATCTATAATATACACACCCTATTTCATATTCATACGCTAAATCTTGTT

-830 TTATCTTTTTAATAGAAAACCTTAATTAAGGCCCTTATTTACTAGCTTAATAACAACATTG

**CuRE** **CuRE**

-770 TTTTCACTCATGT**CTAC**TCTCCATCAAAAATAATACTAAGGGTTGCTTT**CTAC**TCAC

-710 CTACATCTTTGTCTAAATTACAATATGTATTTCCATCTAAAATTAATGAATAAAGAAATAG

-650 AGTAAGCTCACAAATTTTATTAAGTGGTGTCAAAGTTTTAAAACTTTTCTCAACCAAAA

-590 ATCAATAAGCAAAAATGATATGAAATGCAATAAATGTCACTTATCAACTATCAATAGTGGA

**AuxRE** **WRKY**

-530 AAGT**GGTCCCA**TTTCTTATTTTTCCAATACAACTTCTAATCTAGTGGTTATA**TCAG**TAA

-470 AGCGCAAAACAATAGGCTTAATAATTGCCACTCAAAGTGGCCGATGTAATTCATTGTGTTT

-410 TGTCCATGAAGACAACCTTGGGCATATACGAGAATAAGATCCTTTTTATTTTTAGAAAA

**DOF-binding** **SEF3-binding**

-350 TG**AAG**ATTAAAAAACATTAACCCATTGGA**CAACCA**TTGGCAAGAGCTGTGATGATGAAC

**WRKY** **WRKY**

-290 TCCATTTCCCTTGAAATTT**TCAG**TTGTTTCTTAACTTTGGTTAAGATGCTAATGCAAA**TCAG**

**MYB-binding**

-230 **CT**TATGTA**TTGGATA**GAGCAAGATACTCTTCTGTTTTGGACTTTAGCGGAATCGGACCTC

**ARR1-binding** **CAAT-box** **SRE**

-170 CTCCGTCTTCCCTACACTCATCTCATCTGTTTCAT**TCAGTTTCAAT**TATCTGTCT**TATCC**TCC

**CAAT-box**

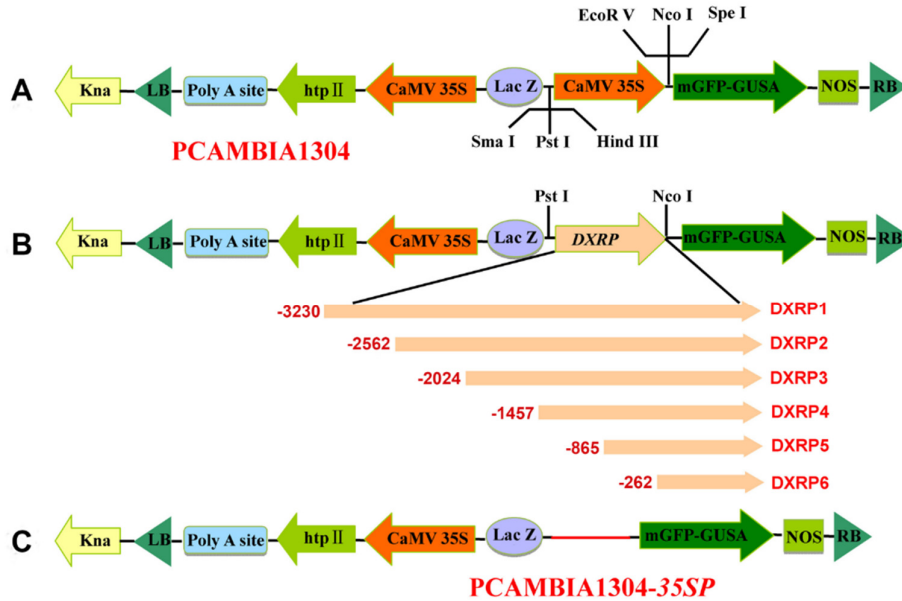
-110 TCCT**CAAT**CCATTATAAACATCTCTTAACCTCTTCGTTGTTCCACATGGTGCCTTCAT

**TATA-box** **+1 Transcription Start**

-50 GCTATTGCACAATCTTCT**TATA**TTTTCTCTGCTTGTAACTGTCCAAAGT**GC**ATCATTTG

+11 AG**ATG**GCTTTGAGAATTGCATCACCGA

**Figure 2.** Nucleotide sequence (part) and some regulatory elements in *GbDXR* promoter are boxed



**Figure 3.** Schematic diagram of the *GbDXR* promoter deletion sequences used for construction of fusion vectors

The pCambia1304 vector was used as the positive control (A). The cloned full-length *GbDXRP1* together with its 5'-deletions *GbDXRP2*~*GbDXRP6* were linked to pCambia1304 by replacing the CaMV 35S promoter respectively, the constructs were fused to the GFP reporter gene in the vector pCambia1304 (B). pCambia1304 without the 35S promoter (pCambia1304-35SP) was taken as the negative control (C)

#### *Agrobacterium-mediated transient expression of deletion constructs in tobacco plants*

In our study, all deletion constructs including pCambia1304-35SP (negative control) and pCambia1304 (positive control) were transferred into the *Agrobacterium tumefaciens* LBA4404 based on the freeze/thaw procedure. Transformants were inoculated into 50 mL YEB plate supplemented with kanamycin (50 µg/mL) and rifampicin (40 µg/mL) and cultured at 28 °C for 48 h. The bacteria liquor was collected and resuspended in infiltration solution (1 mM Glucose, 500 mM MES, 20 mM Na<sub>3</sub>PO<sub>4</sub>·12H<sub>2</sub>O, 1M acetosyringone) and adjusted to an OD<sub>600</sub> between 0.4-0.6 for transient expression assay.

Chose healthy tobacco plants and placed under the white fluorescent lamp for 1-2 h to fully open the stomatas of the leaves. The agroinfiltration at the abaxial surfaces of tobacco leaves with a needleless syringe was performed according to the protocol described by Sparkes *et al.* (2006). Then the tobacco plants were cultivated in a normal growth condition for up to 4 or 5 days for the fluorescence microscopy detecting.

#### *Electrophoretic mobility shift assay (EMSA)*

The putative ERF-binding elements in promoter sequence of *GbDXR* were predicted and identified as TGACY in characterization analysis (Table 2). Oligonucleotides of ERF-binding sequence were synthesized and the distrand DNA were separately labeled with biotin (Biotin 3' End DNA Labeling Kit, Pierce Biotechnology, Thermo Scientific) for non-isotopic detection. To synthetic probe, the complementary labeled strands were mixed together equal amounts and annealed at 25 °C after denaturation at 90 °C. Using a LightShift Chemiluminescent EMSA Kit (Pierce Biotechnology, Thermo Scientific), the binding reaction was performed by incubating 0.5 ng labeled probe, purified AP2/ERF protein and competing oligonucleotides in binding buffer (10 mM Tris-HCl, pH 7.5, 1 µg/µL poly (dI·dC), 50% Glycerol, 1% NP-40, 100 mM MgCl<sub>2</sub>, and 200 mM EDTA) at room temperature for 20 min. Mixtures were electrophoresed on a non-denaturing polyacrylamide gel followed by drying and transferring into nitrocellulose membranes and detecting by Chemiluminescence for Biotin-labeled probes in a CCD camera.

#### *Quantitative analysis of GbDXXR transcription levels*

The transcription level of *GbDXXR* was detected by real-time quantitative PCR (qRT-PCR). The leaves have been through different treatments as well as those ginkgo tissues were collected for RNA extraction. We used PrimeScript™ RT Reagent Kit (Dalian TaKaRa, China) to synthesize the First-strand cDNA and chose SYBR® Fast qPCR Mix (Dalian TaKaRa, China) to perform qRT-PCR reactions, all procedures were in strict accordance with the operation instruction. The primers for qRT-PCR were listed in Table 1 and the PCR reaction conditions were: 95 °C for 30 S, then 40 cycles (95 °C for 5 S, 60 °C for 15 S) of amplification. Each sample was amplified three times to keep the accuracy and all data were analyzed by IBM SPSS Statistics 21.

#### *Determination of TTLs by HPLC-ELSD*

Different ginkgo tissues were also collected for TTLs extraction, the content of ginkgolide A (GA), ginkgolide B (GB), ginkgolide C (GB), bilobalide (BB) were determined by HPLC-ELSD (Li and Fitzloff 2002). The chromatographic separation was achieved using an Agilent C<sub>18</sub> column (5 μm, 4.6 mm × 250 mm) at 40 °C. The mobile phase was methanol: tetrahydrofuran: H<sub>2</sub>O (20:8:72) and the flow rate was 1 mL/min. The ginkgolide detection was accomplished using the evaporative light scattering detector (ELSD). Each test was carried out in triplicate.

#### *Statistical analysis*

All data were reported as means ± standard error (SE) and analyzed using the Statistical Product and Service Solutions (SPSS 21.0), if  $p < 0.05$ , the results would be viewed as the significant difference.

## **Results**

#### *Identification and characteristics of GbDXXR promoter*

The promoter region of *GbDXXR* was isolated based on the constructed *G. biloba* Genome Walker DNA libraries and gene-specific primers designed from the coding sequence of *GbDXXR*. A 3230 bp fragment containing 5'-upstream sequence of *GbDXXR* was cloned, and bioinformatics analysis of partial of *GbDXXR* promoter sequence was processed using the PlantCARE and the PLACE programs (Table 2, Figure 2). The putative transcriptional start site was located at 12 bp upstream from the ATG codon. The nearest basal TATA-box to the transcriptional starting site was located at -30 bp upstream and the CAAT-box was located at -107 bp, the total 12 TATA-boxes and 22 CAAT-boxes might suggest the strong initiation function of *GbDXXR*. Beyond the basal elements, the *GbDXXR* promoter region also contains various important *cis*-acting elements that related to light-regulated, hormone signaling, adversity stress and defense signaling responsiveness. For example, the GATA-box (GATA), GT-element (GRWAAW) and Inr-element (YTCANTYY) are typical light-responsive elements (Perisic and Lam 1992; Aird *et al.*, 1994; Noguchi *et al.*, 1994), which indicated that the *GbDXXR* may be sensitive to light signaling. The NTBBF1 (ACTTTA) and ERF-binding (TGACY) are plant hormone regulation elements that can response to auxin and ethylene respectively (Altamura, 2004; Phillips *et al.*, 2013). The elements like MYB-box (CNGTTR), MYC-box (CANNTG) and W-box (TGAC) are not only response to plant hormones (Auxin, Abscisic acid, Eth, *et al.*) but also involved in plant defense signaling (Proudhon *et al.*, 1996). Furthermore, the *GbDXXR* also contains high temperature resistance element, like HSE-element (CCAAT), and the disease resistance element As-1 (TTGAC) with three different locations in the promoter region. These varied putative *cis*-acting elements suggested that the transcriptional activity of the *GbDXXR* promoter is regulated by diverse environmental signals.

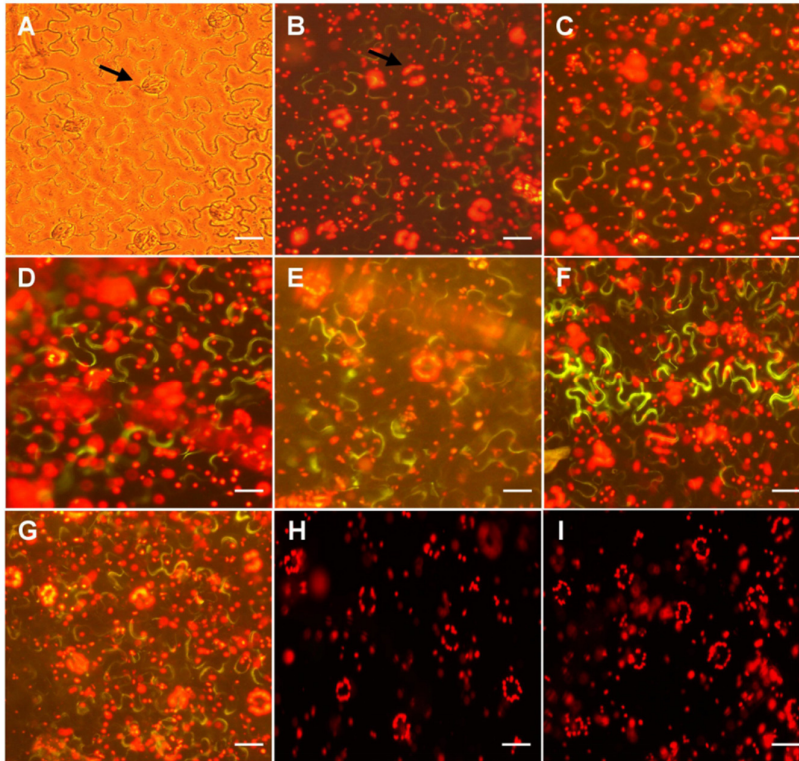
**Table 2.** *Cis*-acting elements analysis of the promoter sequence from *Ginkgo biloba* DXR by PLACE

Name of element	Motif sequence	Copy no. in promoter	Function
TATA-box	TATAA	12	Common <i>cis</i> -acting element in promoter and enhancer regions
GATA box	GATA	14	light regulated, and tissue specific expression
GT-element	GRWAAW	11	Consensus GT-1 binding site in many light-regulated genes
CAAT-box	CAAT	22	Common <i>cis</i> -acting element in promoter and enhancer regions
Inr-element	YTCANTYY	6	initiator; light-responsive transcription
ARR1-binding	NGATT	5	response regulator, ARR1-binding element
E-box	CANNTG	4	Cis-element binding bHLH factor involved in light
T-box	ACTTTG	2	Mutations in the 'T box' resulted in reductions of light-activated
AuxRE	TAACAGA	1	auxin-upregulated proteinase expression
MYBCORE	CNGTTR	6	MYB recognition site involved in dehydration-responsive
MYCATRD22	CANNTG	15	Binding site for MYC, dehydration-responsive gene
DOF-binding	AAAG	18	Transcription factors involved in carbon metabolism
SRE	GAGAC	1	Sugar-responsive element
CURECORECR	GTAC	4	Copper-responsive element
Root-motif	ATATT	13	Root specific expression
W-box	TGAC	15	WRKY binding site, involved in mang physiological processes
NTBBFIARROLB	ACTTTA	3	Required for tissue-specific expression and auxin induction
ERF-binding	TGACY	12	May be involved in activation of ERF3 gene by wounding and
HSE-element	CCAAT	3	Cis-acting element involved in heat stress responsiveness
As-1	TTGAC	3	disease resistance
CATGTG motif	CATGTG	3	drought-responsive cis-element in the early responsive to

#### *Deletion analysis of the GbDXR promoter in transgenic plants*

To identify the regulatory activity of the *GbDXR* promoter and its deletions, six GFP fusion constructs were generated as the schematic diagram shown in Figure 3 and implemented to *agrobacterium*-mediated transient expression assay in tobacco leaves. The leaf epidermal cells were observed using the epifluorescence microscope (Nikon, Japan) about four days later after infiltration. The results showed that the epidermal cells and stomatas could be seen clearly in 40 × eyepiece microscope under natural light (Figure 4A). When giving UV-B irradiation, the stomatas and other organizations emitted red fluorescence, but the GFP emitted bright green fluorescence (Burchard *et al.*, 2000). Figure 4B showed the result of the positive control vector pCAMBIA1304, the GFP were expressed normally and located in the cytoplasm of the epidermal cells. The GFP in fusion constructs

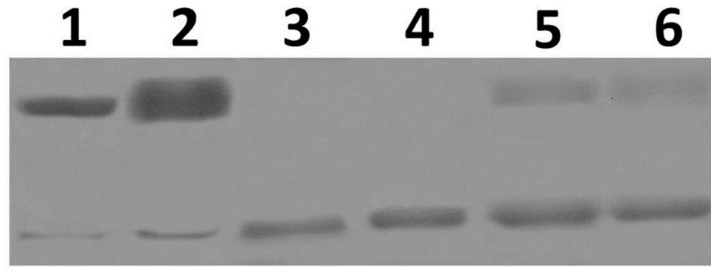
pCAMBIA1304+*GbDXRP1~P3* also expressed in the cytoplasm and emitted bright green fluorescence (Figure 4C-E), which predicted that the promoter sequence *GbDXRP1* (-3230), *GbDXRP2* (-2562) and *GbDXRP3* (-2024) can promote the transient expression of GFP in tobacco epidermal cells. Following, the expression of GFP in fusion constructs pCAMBIA1304+*GbDXRP4~P6* were continue detected, the results showed that *GbDXRP4* (-1457, Figure 4F) and *GbDXRP5* (-865, Figure 4G) still retained the promoter activity and the GFP expressed with relative high levels, but no green fluorescence could be observed in *GbDXRP6* (-262, Figure 4H). pCAMBIA1304 vectors without the 35S promoter (pCAMBIA1304-35SP) were the negative control in this assay, the GFP could not expressed and only the red fluorescence could be observed (Figure 4I).



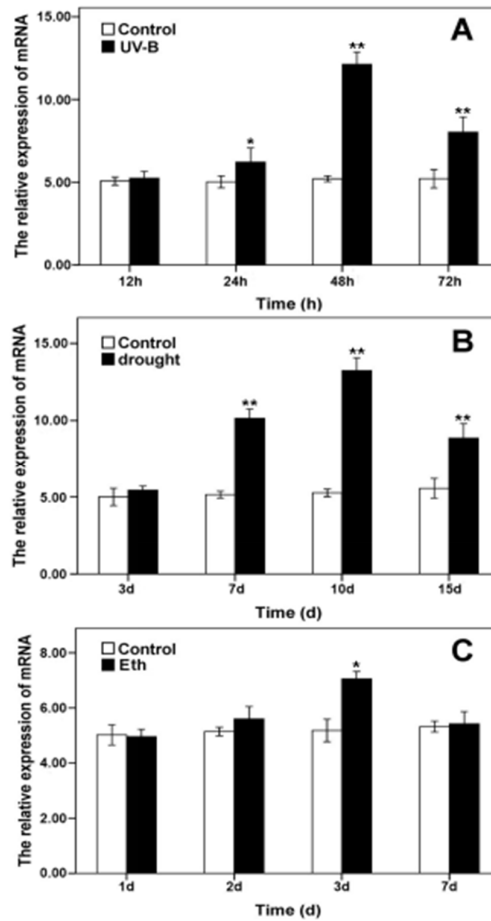
**Figure 4.** Expression of GFP in tobacco leaf epidermal cells detected by epifluorescence microscope. Under nature light, the leaf epidermal cells could be observed clearly at 40×objective (A). Scale bar, 10 μm, the black arrow points to the stomata; The result of the positive control pCAMBIA1304 showed that the GFP expressed and emitted green fluorescence when giving UV-B irradiation (B). Scale bar, 10 μm, the black arrow points to the stomata; The promoter sequences *GbDXXRP1~GbDXXRP5* could promote the expression of GFP respectively, the bright green fluorescence could be observed in the cytoplasm of epidermal cells (C~G). Scale bar, 10 μm; The *GbDXXRP6* could not initiated the expression of GFP and no green fluorescence could be observed (H). Scale bar, 10 μm; In the negative control pCAMBIA1304-*35SP*, the GFP also could not expressed and only the red fluorescence emitted by other organizations (I). Scale bar, 10 μm

#### *EMSA assay of GbDXXRP with AP2/ERF transcription factors*

Characteristic analysis showed that there are at least 12 putative ERF-binding elements in *GbDXXR* promoter sequence, to confirm whether the ERFs proteins could interact with these *cis*-acting elements, the labeled ERF-binding probe together with ERFs proteins were subjected to EMSA assay. Two AP2/ERF transcription factors GbERF10 and GbERF17 from *G. biloba* transcriptome data have already been cloned and which might be related to TTLs biosynthesis. The overexpression of these two ERFs genes were induced by isopropyl-β-D-1-thiogalactopyranoside (IPTG) in *E. coli* respectively. After purified and detection, the molecular weight of ERFs proteins was estimated to be 40-55 kDa with His-tag, and their size agreed with that of the predicted peptide from bioinformatics analysis. The interaction between GbERFs and the *GbDXXR* promoter sequence were assayed with EMSA. GbERF10 and GbERF17 specifically bound with ERF-binding elements (Figures 1, line 1 and 2). No binding bands were detected with crude proteins of *E. coli* without or with the empty vector pET-28a (Figures 5, line 3 and 4), and unlabeled probes competitively inhibited the binding (Figures 5, line 5 and 6). The results preliminary confirmed that GbERF10 and GbERF17 proteins can combine with the ERF-binding elements in *GbDXXR* promoter sequence.



**Figure 5.** Gel mobility shift assays of the binding reactions in EMSA detection 1,2 is the recombinant GbERF10 and GbERF17 mixed with Biotin-labeled ERF-binding probe, respectively; 3 is *E. coli* protein mixed with Biotin-labeled ERF-binding probe; 4 pET-28a protein mixed with Biotin-labeled ERF-binding probe; 5,6 GbERF10 and GbERF17 mixed with Biotin-labeled and unlabeled ERF-binding probes, competitively inhibiting



**Figure 6.** Quantitative analysis of *GbDXR* transcription levels in response to A: UV-B, B: Drought, C: Eth Relative quantities of *GbDXR* mRNA at different time point in each sample was assayed in triplicate (n=3). Values are the mean of three treated plants and bars represent the standard errors. The *asterisk* along the *x*-axis represents statistically significant differences of mean (\* $p < 0.05$ ; \*\* $p < 0.01$ )

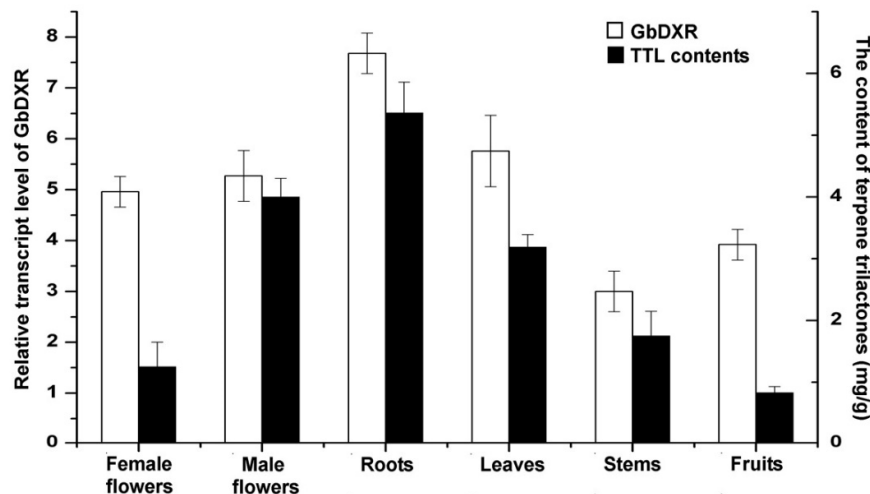
*Changes of GbDXRP transcript level to UV-B, drought stress and Eth stimulants in Ginkgo*

In this study, we used UV-B, drought and Eth at different concentrations to treat ginkgo leaves, and explore the *GbDXR* transcription level change regulated by external environment via *GbDXRP* (Figure 6). The transcription level of *GbDXR* was tested at 12, 24, 48, and 72 h under the UV-B treatment, the results of qRT-PCR showed that the highest transcription level of *GbDXR* appeared at 48 h after treatment, and then declined at 72 h (Figure 6A). The drought stress lasted for 15 days, the continuous dehydration led to an abnormal expression of *GbDXR*, the transcriptional level of *GbDXR* was increased obviously and reached maximum after drought treatment for 10 d. However, as the degree of drought increased, the transcriptional level of *GbDXR* slowly declined (Figure 6B). The effect of Eth treatment on the expression of *GbDXR* was not significant, the transcriptional level increased slightly after 3 d of treatment (Figure 6C). The changes of the transcriptional level in different abiotic stresses indicated that the UV-B and drought stress could significantly induce the expression of *GbDXR*.

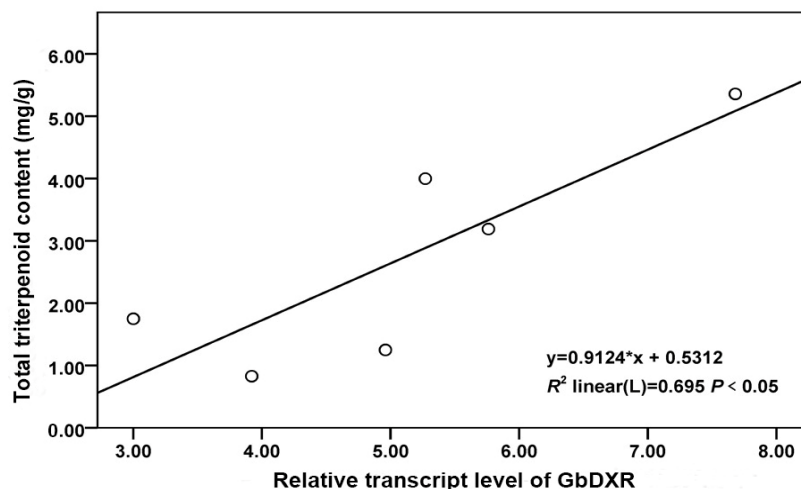
*TTLs content in ginkgo tissues and the relationship with the expression of GbDXR gene*

Previous research showed that *GbDXR* retained a higher transcription level in roots than in leaves and the TTLs content in these two tissues were different (Kim *et al.*, 2006). Here, we extended the expression pattern analysis of *GbDXR* to additional tissues of ginkgo via qRT-PCR. The TTLs content in these tissues were measured simultaneously using the HPLC-ELSD detection (Figure 7). Figure 7 showed the changes of the transcript level of *GbDXR* and TTLs content in all tissues, including the roots, stems, leaves, fruits, male and female flowers. Similarly, the highest transcript level of *GbDXR* was in the roots and with the maximum total triterpenoids content  $5.3633 \pm 0.0862$  mg/g. In male flowers, though the total triterpenoids content was 40% higher than leaves, the transcript level of *GbDXR* was slightly lower. The total triterpenoids content in female flowers was similar to the stems, but the transcript level of *GbDXR* in these two tissues was different.

The linear regression analysis was carried out to study the relationship between the *GbDXR* expression levels and the TTLs content in these tissues. The *GbDXR* transcript level was taken as the independent variable (x) while the total triterpenoids content was the dependent variable (y), the linear curve equation represented as  $y = (0.9124 \pm 0.0036) * x + (0.5312 \pm 0.0887)$ ,  $R^2 = 0.695$  (Figure 8). The relatively high linear correlation coefficient ( $R^2$ ) suggested the positive correlation between the *GbDXR* expression and the TTLs content and which indicated that the biosynthesis of total terpenoids correlate with the changes of *GbDXR* transcript levels.



**Figure 7.** The relative transcript level of DXR and the diverse distribution of TTLs content in different ginkgo tissues



**Figure 8.** Relationship between the content of terpene trilactones and the expression level of GbDXR in *G. biloba* (n=12)

## Discussion

Previous research showed that the gene expression is regulated by the *cis*-elements in its promoter region (Guan *et al.*, 2000; Yamaguchi *et al.*, 2005). The *GbDXRP* also contains various *cis*-elements that can respond to light signaling, temperature, plant hormones, dehydration stress, and disease resistance. Some *cis*-elements were predicted to make the corresponding reaction to multiple environmental stimulants. In particular, by responding of these specific elements to the changes of environment, the transcripts of those structural genes may be promoted or inhibited since to affect the synthesis of some secondary metabolites, the products that can mitigate the stress-damaged start to accumulate and provide the instant protection for plants. The secondary metabolites like anthocyanins, flavonoids are scavenger compounds of plants under adverse conditions (Yang *et al.*, 2012).

We chose the transient expression in tobacco plants to detect the promoter activity of *GbDXRP*. The *GbDXR* 5' region was examined through a series of 5' deletions translationally fused to the GFP reporter gene. The result showed that the series of deletions from -3230 to -865 can promote the expression of the GFP when the stable genetic transformation was generated, but the further deletion -262 nearly terminated the activity, which suggested that the region from -865 to the initiation codon is sufficient for promoter activity. However, the regulation function of the promoter region from -865 to -262 remained to be elucidated. Tebbutt *et al.* (1995) reported that in the deletion analysis of a tobacco pollen-specific polygalacturonase promoter, the two LAT52/56 motifs and the PG box undertake the promoter function in the sequence. Therefore, further study is needed to find these critical regulatory elements in *GbDXR* promoter sequence.

UV-B radiation is an important factor enhancing the production of secondary metabolites in plants. Takshak *et al.* (2014) found that the concentrations of alkaloids, anthocyanins, carotenoids, flavonoids, lignin, phytosterols and saponins were generally increased in *Withania somnifera* under supplemental UV-B radiation. The similar results depicted in *Chrysanthemum morifolium* (Si *et al.*, 2015), *Dendrobium officinale* (Guo *et al.*, 2016), *Eucommia ulmoides* (Fu *et al.*, 2017), and many other medicinal plants. In our study, the UV-B treatment could rapidly improve the expression of *GbDXR*, indicating that the TTLs content increased and might act as protective compounds accumulate in *G. biloba*, the result is consistent with the studies of Vranová *et al.* (2012). Under the induction of UV-B radiation, some transcription factors might activate the promoter of *GbDXR*, and positive regulated the expression of *GbDXR* gene. Accumulation of metabolites often occurs when plants suffer from environmental stresses (Akula and Ravishankar, 2011), the drought stress,

as one of the common environmental stresses, its effect on secondary metabolites has already been reported in several medicinal plant species. Hao *et al.* (2007) have detected the TTLs content in *G. biloba* under different drought phases, the TTLs content has no obvious change under mild drought stress, as the water content declined to moderate stress, the TTLs content increased and accumulated significantly, but when the water content was less than 20%, the content of TTLs declined obviously. Here, the transcriptional level of *GbDXR* exhibited the similar changes to drought stress, we presumed that the *GbDXR* regulate the synthesis of TTLs by responding to drought stress via *GbDXRP*. Additionally, many specific dehydrations stress responsive elements are imbedded in *GbDXR* promoter sequence, including 15 MYCATRD22 (CANNTG) and 3 CATGTG motif (CATGTG) (Kasuga *et al.*, 1999), which suggest that the *GbDXR* gene can response to drought stress sensitively. Together with the result of drought treatment to ginkgo leaves in Zhang's (2005) report, we concluded that moderate dehydration stress may help us to obtain ginkgo leaves with relative higher TTLs content. Studies found that in ethylene-responsive genes promoter region, the GCC box *cis*-acting elements specifically response to Eth (Pirrello *et al.*, 2012). In addition, the vascular wounding responsive elements were also demonstrated to be the Eth responsive elements (Sasaki *et al.*, 2007). It has been reported that the Eth could improve the yield of rubber latices in *Hevea brasiliensis* (Duan *et al.*, 2010). Though the Eth could also induce the expression of *GbDXR* to a certain degree, its effect on the TTLs content in *G. biloba* was not significant. EMSA analysis preliminary confirmed that GbERF10 and GbERF17 specifically bound with ERF-binding elements in *GbDXR* promoter sequence. The AP2/ERF transcription factors belong to one of the largest transcription factor families in *G. biloba*, and their participation in the synthesis and metabolism of terpenoids have been reported in *Artemisia annua* (Yu *et al.*, 2012) and *Salvia multiorthiza* (Sun *et al.*, 2019).

The expression of *GbDXR* and the TTLs content have tissue variability in *G. biloba*. The *GbDXR* could expressed in all tested tissues and the highest transcript level was detected in roots, the similar expression patterns have already been reported in researches of *GbLPS*, *GbIDS* and *GbMVD*, the genes that are committed to the synthesis of TTLs in MEP pathway (Liao *et al.*, 2016). Actually, the roots contain more TTLs than other tissues in our research. These results further proved that the roots are the preferential sites for the TTLs biosynthesis. The total terpenoids content in the leaves and flowers are relative higher than stems and fruits, and the ginkgo leaves are raw materials for extracting total terpenoids and flavonoids in pharmaceutical industry. Statistics analysis results showed that there is a positive relationship between the expression of *GbDXR* and the TTLs content, therefore, we proposed that the *GbDXR* plays a key role in total terpenoids metabolism.

## Conclusions

TTLs are the active ingredients in *G. biloba*, their content in ginkgo leaves is low and will be affected by environmental conditions. The *DXR* gene catalyzes the first committed step specific for IPP production of the MEP pathway since to produce the precursors of TTLs biosynthesis in *G. biloba*. Therefore, the regulation of the expression of *GbDXR* gene is very important for improving the content of TTLs.

Studies on gene promoters' regulation function lay the foundation for in-depth studies of the molecular mechanism underlying TTLs biosynthesis and for the metabolic engineering of bioactive ingredients in *G. biloba*.

## Authors' Contributions

Conceptualization: HY, HC and SC; Funding acquisition: HC and SC; Methodology: HC, LL, LL and HY; Supervision: SC; Writing-original draft: HY, LL and LL; and Writing-review and editing: SC, HC, LL and LL. All authors read and approved the final manuscript.

### **Ethical approval** (for researches involving animals or humans)

Not applicable.

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### **Conflict of Interests**

The authors declare that there are no conflicts of interest related to this article.

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