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Promoter Analysis and Transcriptional Profiling of *Ginkgo biloba* 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase (*GbHMGR*) gene in Abiotic Stress Responses

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Abstract

The terpene trilactones (TTLs) are believed to be important for the pharmacological properties of *Ginkgo biloba* leaves extract. 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) is a critical enzyme involved in the biosynthetic pathway of TTLs. In this study, an 1.2-kb fragment of 5' flanking region of the *HMGR* gene (*GbHMGR*), was isolated from *G. biloba* by genome walking. Extensive sequence analysis revealed the presence of evolutionarily conserved and over-represented putative *cis*-acting elements in light-regulated transcription, hormone signaling (gibberellic acid, jasmonate and salicylic acid), elicitor and stress responses (cold/dehydration responses), and plant defense signaling (W-box/WRKY) that are common to the promoter region of *GbHMGR*. EMSA analysis suggested possible functionality of W-box in *GbHMGR* promoter region. The behavior of gene transcripts in ginkgo callus upon light, low temperature, MeJA and SA treatments further verified the regulatory function of *GbHMGR* promoter. A significant positive relationship between gene expression level and total TTL contents suggested that *GbHMGR* might be one of key genes involved in TTL biosynthesis in *G. biloba*.

Keywords: EMSA, *Ginkgo biloba*, *GbHMGR*, promoter, terpene trilactones, expression level

Introduction

The maidenhair tree *Ginkgo biloba* L., known as living fossil, has undergone very little evolutionary changes over 200 million years and is considered to be native to China, Korea, and Japan (Singh *et al*., 2008). In recent years, standardized extracts of *G. biloba* leaves have been amongst the top-selling phytomedicines in the world (Gertz and Kiefer, 2004). Active compounds in *G. biloba* extract improve blood circulation, discourage clot formation, reinforce the walls of the capillaries and protect nerve cells from harm when deprived of oxygen (Mohanta *et al*., 2014). Flavonoids and terpene trilactones (TTLs) are believed to be associated with most of the pharmacological properties of *G. biloba* extracts. While flavonoids can be obtained from many other plants, ginkgolides and bilobalide, termed as TTLs, are unique components of *G. biloba* (Liao *et al*., 2011). Though TTLs is considered to play a key role in the active ingredients, the content of TTLs is very low, within 0.06% in dry leaves of *G. biloba* (van Beek and Montoro, 2009). Bioengineering is an ideal way to increase the content of TTLs, but which relies on the overall elucidation of the TTL biosynthetic pathway both at molecular genetics and biochemistry levels in *G. biloba*.

Terpenoids such as ginkgolides are biosynthesized from a universal 5-carbon building block Isopentenyl diphosphate (IPP) (Schwarz and Arigoni, 1999). IPP can be derived from two pathways: One is the classical cytosolic mevalonic acid (MVA) pathway and the other is the plastidial methylerythritol 4-phosphate (MEP) pathway, which is mevalonate independent. The MVA pathway in the cytosol, starting from 3 acetyl-CoA to finally yield IPP, is responsible for synthesizing sesquiterpenoids and sterols. The MEP pathway producing IPP and dimethylallyl diphosphate (DMAPP) from pyruvate and D-glyceraldehyde 3-phosphate (GAP) is mainly responsible for forming monoterpenoids, diterpenoids constituents. The classical cytosolic MVA pathway and the other is the plastidial MEP pathway, which

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is mevalonate independent (Lichtenthaler *et al*., 1997; Zeng *et al*., 2013). But the two pathways are not separated absolutely, in a certain extent, some unknown forms of crosstalk may occurred between them in some particular plants. For example, the biosynthetic precursor of ginkgolides derived from both MVA and MEP pathway in *G. biloba* (Schwarz, 1994; Schwarz and Arigoni, 1999). Therefore, numerous genes in both two pathways could contribute to the low level biosynthesis of ginkgolides. Up to now, many genes encoding enzymes involved in MVA and MEP pathway were isolated from *G. biloba*, including mevalonate diphosphate decarboxylase (MVD; Pang *et al*., 2006), 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGR; Shen *et al*., 2006), 1-deoxy-$_D$-xylulose 5-phosphate synthase (DXS; Gong *et al*., 2006; Kim *et al*., 2006a), 1-deoxy-$_D$-xylulose 5-phosphate reductoisomerase (DXR; Gong *et al*., 2005; Kim *et al*., 2006a), 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate synthase (HDS; Kim and Kim, 2010), 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate reductase (IDS; Kim *et al*., 2008a), 2-*C*-methyl-$_D$-erythritiol 4-phosphate cytidyltransferase (MECT; Kim *et al*., 2006b), 2-*C*-methyl-$_D$-erythritiol 2,4-cyclodiphosphate synthase (MECS; Kim *et al*., 2006c; Gao *et al*., 2006), and 4-(cytidine 5'-diphospho)-2-*C*-methyl-$_D$-erythritol kinase (CMK; Kim *et al*., 2008b).

3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGR, EC: 1.1.1.34) catalyzes the conversion of HMG-CoA to MVA, which is a rate-limiting enzyme in isoprenoid biosynthesis via MVA pathway (Hunter, 2007; Buhaescu and Izzedine, 2007). In view of its significance in isoprenoid metabolism, genes encoding HMGR have been isolated and extensively characterized from many plants, including the medicinal plants *Catharanthus roseus* (Maldonado-Mendoza *et al*., 1992), *Taxus media* (Liao *et al*., 2004), *Corylus avellana* (Wang *et al*., 2007), *Euphorbia pekinensis* (Cao *et al*., 2010), and *Salvia miltiorrhiza* (Dai *et al*., 2011), *Picrorhiza kurrooa* (Singh *et al*., 2013), *Withania somnifera* (Akhtar *et al*., 2013), and *Cyanotis arachnoidea* (Wang *et al*., 2014). At present, a HMGR gene (*GbHMGR*) has been cloned from *G. biloba* (Shen *et al*., 2006). However, both expression pattern and the promoter region of *GbHMGR* gene have not yet been clearly identified.

In the present study, we report the isolation and characterization of promoter region of *GbHMGR* gene to understand molecular regulation mechanism of *GbHMGR* expression underlying TTL biosynthesis in *G. biloba*. The transcript level of the *GbHMGR* and TTL content was examined after environmental stresses and plant hormone treatments. The correlation between transcript levels and TTL content in *G. biloba* callus further substantiated putative role of *GbHMGR* gene in regulating TTL biosynthesis.

Materials and methods

Plant materials and growth condition

Seeds of ginkgo were harvested from 14-year-old grafts of *G. biloba* cultivar 'Jiafoshou' grown in Botanical Garden of Yangtze University, China in October, 2012. The seeds were used when the embryos were at the cotyledonary stage.

Callus induction of G. biloba and treatments

The embryo-derived callus of *G. biloba* was induced using the method described by Zhang *et al*. (2011). The cultures were incubated in the light (100 μmol m^{-2} s^{-1}) with a 16/8h light/dark photoperiod at 24 ± 1 °C as the control. For dark treatment, four-week-subcultured callus were covered with a cardboard box to keep in complete darkness and the samples were harvested at 24h from start of the treatment. For low temperature treatment, four-week-subcultured callus were grown at 15 ± 1°C and samples were harvested at 48 h after the treatment for analysis of gene transcription level and TTLs content. Methyl jasmonate (MeJA) and salicylic acid (SA) with the concentration 2.0 mM were added to two-week callus cultured MS, and control cultures were untreated during cultivation. The samples was harvested and measured at 48h after MeJA and SA treatments.

Construction of genomic library and isolation of promoter region of GbHMGR gene

Genomic DNA was extracted from ginkgo seed using modified CTAB method (Xu *et al*., 2008a). Ginkgo Genome walker libraries were constructed using the Genome Walker Universal Kit (Clontech, USA). To clone the promoter region of *GbHMGR*, two round PCR were performed using gene-specific primers (GbHMGRP1 and GbHMGRP2) that were designed according to the sequence of *GbHMGR* gDNA (AY741133; Pang *et al*., 2006), and the adapter primers (AP1 and AP2) of the kit. Their sequences were shown in Table 1. After the nested PCR was carried out, amplified fragments were cloned and then sequenced. The sequences that extended upstream of the gDNA of *GbHMGR* were isolated as the 5'upstream region of *GbHMGR* gene and used for further analysis. The isolated 5'-upstream sequence was analyzed for the putative *cis*-acting regulatory elements using the PLACE (http://www.dna.affrc.go.jp/PLACE) and the Signal Scan Program PlantCARE (http://bioinformatics.psb.ugent.be/webtools/*plantcare/html/*) database.

Expression of purification of GbWRKYs protein in E. coli

To obtain the WRKY protein, the open reading frame (ORF) of *GbWRKY1* and *GbWRKY2* of *G. biloba* (*GbWRKY1* and *GbWRKY2* Sequences in Supplemental Figure S1 and S2) was cloned into the expression vector pET-28a(+), yielding His-tagged GbWRKY1 and GbWRKY2. After sequence confirmation, the resulting recombinant plasmid was transferred into the *E. coli* strain BL21 (DE3) cells with heat shock method. A single colony of *E. coli* strain BL21 (DE3) cells harboring the plasmid pET28a-GbWRKY1/GbWRKY2 was inoculated in LB medium at 37 °C containing kanamycin (50 mg L^{-1}), and was grown with 200 rpm shaking at 37 °C until the optical density (OD600) reached about 0.6. Expression of the recombinant protein was induced by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG) and cells were harvested at 3h. The recombinant protein was extract and purified using Nickel-CL agarose affinity chromatography (Bangalore Genei, India) and used for electrophoretic mobility shift assay.

Electrophoretic mobility shift assay

The W-box sequence in the promoter sequence of *GbHMGR* was as TTTGAC using the PLACE and the Signal Scan Program PlantCARE database. Oligonucleotides of W-box sequence were synthesized and labeled with biotin (Shanghai Sangon Biotech Co., Ltd., China) for chemiluminescence using a LightShift® Chemiluminescent EMSA Kit (Pierce Biotechnology, Thermo Fisher Scientific Inc., Rockford, USA). After labeling,

complementary labeled strands were mixed together in an equimolar ratio and annealed at 25 °C after denaturation at 90 °C. Gel mobility shift assays were performed by incubating 0.5 ng of labeled probe with recombinant GbWRKYs protein and competing oligonuleotides in binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 1mM EDTA, 5% glycerol, and 1μg/μL poly(dI•dC)) at 25 °C for 20 min. Mixtures were size-fractionated on a non-denaturing 46% polyacrylamide gel followed by drying and transfer to nitrocellulose membranes and detection by streptavidin-HRP/chemiluminescence for biotin-labeled probes.

Quantitative Real-Time PCR analysis of transcript levels

The transcription levels of *GbHMGR* of *G. biloba* callus were determined at different stress or hormone treatments. RNA was isolated from the ginkgo callus at different treatments using CTAB methods (Cai *et al*., 2007). First-strand cDNA synthesis was performed in triplicate for each sample according to the instructions of the manufacturer (PrimeScript™ RT Reagent Kit, Dalian TaKaRa, China). Quantitative Real-Time PCR (qRT-PCR) was carried out using a Applied Biosystems 7500 Real-Time PCR System with SYBR® Premix Ex Taq™ II Kit (Dalian TaKaRa, China) according to the protocol of the manufacturer. The primers of *GbHMGR* and *G. biloba* house-keeping gene *18S* (GenBank accession no. D16448) for qRT-PCR are listed in Table 1. Reactions were performed in triplicate using 10 μL of SYBR®Premix Ex Taq II, 0.8 μL of each primer, 0.4 μL ROX Reference Dye II, 2 μL of diluted cDNA, and nuclease free water to a final volume of 20 μL. The PCR reaction conditions were pre-incubated at 95 °C for 30 s, followed by 30 cycles of amplification (95°C for 5 s, 60 °C for 34 s), with melt curve stage (95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s). Fluorescence was measured at the end of each annealing step. Raw data were analyzed with Applied Biosystems 7500 software, and expression was normalized to *18S* gene to minimize the variation in the cDNA template levels. Real-time PCR data were technically replicated with error bars, representing mean ± SE (n =3).

Extraction and determination of TTLs

Ginkgolide A (GA), ginkgolide B (GB), ginkgolide C (GC) and bilobalide (BB) were extracted and determined using gas chromatography with a wide bore capillary column (Liao *et al*., 2008). The content of TTLs was the sum of the contents of GA, GB, GC, and BB and expressed as $\mu g \cdot g^{-1}$ DW. All the tests were carried out in triplicate, and data represent the means ± standard errors (SE).

Statistic analysis

Data were analyzed with one-way ANOVA using SPSS 11.0 (SPSS Inc., Chicago, Illinois) for Windows and means were compared with Duncan's multiple range test at $P < 0.05$.

Results and discussions

Cloning and analysis of GbHMGR promoter

The promoter sequence of *GbHMGR* was obtained by constructing a Genomic Walker DNA library from *G. biloba* leaves after 2 rounds of nested PCR using chromosome walking techniques. The promoter sequence of *GbHMGR* gene was 1,264 bp in length (Fig. 1). The *cis*-acting elements of *GbHMGR* promoter was predicted using PlantCARE and PLACE database. Various *cis*-acting elements, along with their functions and location in the promoter of *GbHMGR*, are shown in Table 2 and Fig. 2. We found that the *GbHMGR* promoter sequence contains many important *cis*-regulatory elements such as TATA boxes, CAAT boxes, ACGTT boxes, W boxes, E boxes, etc. Specifically, *in silico* analysis showed that one TATA boxes are present within the promoter region of *GbHMGR*, at position 1233, i.e. 32 bp upstream of the transcription start site. The TATA box is necessary in promote gene transcription by combination to RNA polymerase II, and affects the rate of transcription (Smale and Kadonaga, 2003). Another conserved eukaryotic *cis*-element, CAAT boxes, were also observed at position 1109, i.e. 124 bp upstream from TATA box. The CAAT box controls the transcription initiation frequency and impacts conversion rates of target genes (Edwards *et al*., 1998). The changes in these basic elements within the promoter region will greatly affect the level of transcription of target genes. In addition to these essential *cis*-acting elements, other corresponding *cis*-element with roles in regulation of gene expression in the promoter region of *GbHMGR* were also predicted and listed in Table 2. These include *cis*-elements associated with hormone regulations and found in other plant gene promoters, in detail including one ABRERATCAL motif function as Ca^{2+}-responsive element (Kaplan *et al*., 2006), one CATATGGMSAUR element involved in the induction of gene by Auxin (Xu *et al*., 1997), two Arabidopsis Response Regulator 1 type B (ARR1AT) transcription factor recognition sequences involved in cytokinin signaling and two sequences critical for cytokinin-enhanced binding (Sakai *et al*., 2000), one MYBGAHV element and three GT1 motifs known as to be GA and SA-responsive elements (Gubler *et al*.,1999; Zhou, 1999), respectively. We also found the presence of eight Dof (DNA binding with one finger) transcription factor recognition core sequences, which could be involved in auxin, jasmonate or ethylene responsiveness as previously reporter (Baumann *et al*., 1999; Yanagisawa and Schmidt, 1999; Nakano *et al*., 2006). In addition, the *GbHMGR* promoter contains *cis*-elements previously associated with low-temperature and light responsiveness, including three GT1 motifs (Zhou, 1999), one CRTDREHVCBF2 element (Xue, 2003) and one T box (Chan *et al*., 2001). Interestingly, we also found one MYB-box (MYB1AT), three W-box and four E-boxes in promoter region of *GbHMGR*. The W-box and MYB-box have shown to be WRKY (Eulgem *et al*., 2000) and MYB (Abe *et al*., 2003) transcription factor binding sites, respectively, involved in plant defense signaling. The consensus E-box sequence has been shown to be recognized by the basic helix-loop-helix (bHLH) proteins and involved in light regulation (Hartmann *et al*., 2005). The bioinformatic analysis also revealed the presence of root motif for root specific expression in *GbHMGR* promoter region (Elmayan and Tepfer, 1995). Previous study has shown that the transcript of *GbHMGR* was present specifically in roots (Shen *et al*., 2006). Three ROOT-motif elements (positions 184, 407 and 753) were identified as root-responsive *cis*-elements, which were consistent with the expression pattern of *GbHMGR* gene. Based on the predictive identification of putative *cis*-acting element, it could be hypothesized that the transcriptional activity of the *GbHMGR* promoter is regulated by different signals.

Table 1. Primer sequences used in the experiment

Name	Sequence (5'–3')	Description
AP1	GTAATACGACTCACTATAGGGC	Reverse primer for promoter amplification, outer
AP2	ACTATAGGGCACGCGTGGT	Reverse primer for promoter amplification, nested
GbHMGRP1	ACGCCCTACTCTTCAACTCCTCCCTTCT	Reverse primer for promoter amplification, outer
GbHMGRP2	ATACGAATGAAGCCAACTGAGCCACGAT	Reverse primer for promoter amplification, nested
HMGRFP	TCTTTGTCATGTTTTTCAGC	Gene specific primer for qRT-PCR, forward
HMGRRP	AGTTTCTCTTTCTCTCTCGC	Gene specific primer for qRT-PCR, reverse
18SFP	ATAACAATACTGGGCTCATCG	Gene specific primer for qRT-PCR, forward
18SRP	TTCGCAGTGGTTCGTCTTTC	Gene specific primer for qRT-PCR, reverse

Table 2. Putative *cis*-acting regulatory elements identified in the promoter of *GbHMGR* using PLACE and PlantCARE database

Factor or Site Name	Position	Signal Sequence	Expected function
ABRERATCAL	7	MACGYGB	Ca^{2+}-responsive up-regulated genes
CRTDREHVCBF2	15	GTCGAC	Low-temperature responsive
CGACGOSAMY3	17 ,1218	CGACG	May function as a coupling element for the G box element
DOFCOREZM	41, 140, 267, 304, 442, 817, 839, 1019	AAAG	Dof1 and Dof2 transcription factors are associated with expression of multiple genes involved in carbon metabolism
GT1 motif	76, 209, 962	GRWAAW	Consensus GT-1 binding site in many light-regulated genes and influence the level of of SA-inducible gene expression
ROOTMOTIFTAPOX1	184,407,753	ATATT	Root specific expression
E BOX	219, 278, 601, 862	CANNTG	*Cis*-element binding BHLH factor involved in light responsiveness and tissue-specific
T Box	241	ACTTTG	Mutations in the 'T box' resulted in reductions of light-activated gene transcription
W Box	282, 483, 855	TGAC	WRKY binding site, involved in many physiological processes
MYB1AT	347	WAACCA	MYB recognition site involved in dehydration-responsive
TATA Box	1233	TATAA	Common *cis*-acting element in promoter and enhancer regions
CURECORECR	334,383,1238	GTAC	Copper-responsive element
CAAT Box	1109	CAAT	Common *cis*-acting element in promoter and enhancer regions
NTBBF1ARROLB	474,993,1127	ACTTTA	Required for tissue-specific expression and auxin induction;
CATATGGMSAUR	601	CATATG	Auxin-responsive
MYBGAHV	737	TAACAAA	Central element of gibberellin (GA) response complex (GARC) in high-pI alpha-amylase gene
ARR1AT	1172,81,448	NGATT	ARR1-binding element
ACGTTBOX	1073,1207	AACGTT	One of ACGT elements

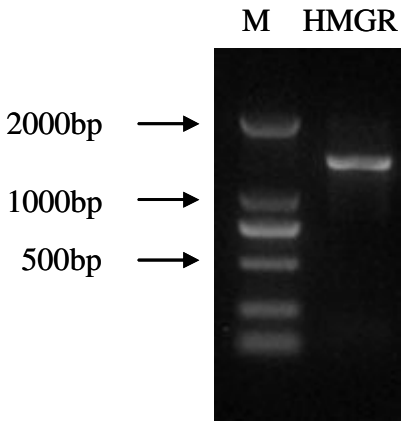

Fig. 1. Promoter sequences of *GbHMGR*. Lane M, DNA marker DL2000; *HMGR*, PCR product of the *GbHMGR* gene promoter

Comparison of the known promoter sequences of the genes such as *GbDXS* and *GbGGPPS* (Xu *et al*., 2013), involved in TTL biosynthetic pathway in *G. biloba*, reveals that *cis*-elements binding Dof proteins. Dof proteins are plant-specific transcription factors with a highly conserved DNA-binding domain, which presumably induces a single C2-C2 zinc finger. Zinc finger proteins like members of the transcription factor IIIA zinc finger protein family (ZCTs) have been previously isolated in medicinal plant *Catharanthus roseus* and were shown to act as transcriptional repressors of TDC and STR promoter activity in the regulation of induced terpenoid metabolism (Pauw *et al*., 2004). Dof transcription factors have been suggested to participate in the regulation of vital processes exclusive to plants such as photosynthetic carbon assimilation, light-regulated gene expression, accumulation of seed-storage proteins, germination, dormancy and response to phytohormones (Yanagisawa 2004). The relative abundance of Dof binding sites in *GbHMGR* promoter suggest that this specific protein could play a significant role of TTL gene expression in *G. biloba*.

GbWRKYs bind with the W-box sequence of GbHMGR promoter

Several reports have shown that WRKY proteins regulate the expression of gene involved in terpenoid biosynthesis by combining the W-box, and W-box was found in present in gene promoters related with MEP pathway such as *LPS* (Kim *et al*., 2012), *IDSs* (Kang *et al*., 2013), *GbDXS* and *GbGGPPS* (Xu *et al*., 2013). The W-box sequences predicted as TTTGAC were

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1       ATAGGGCACG CGTGGTCGAC GGCCCGGGCT GGTATTATAG AAAGCTCTCC TTATATGTTT
                ABRERATCAL
61      TTGGGTCTTT TGGTTGAAAA TGATTTTATC TTATTCATAG AATATCATCT ATTTTGATGT
                           GT1 motif
121     CTTTTGGGGC ACGCTTCCAA AAGTCAGGGT CCGAACCTTT CTTTTCTAAT AATAGAAATG
                                DOF-Core
181     CAAATATTTA AATTATTGCA TGCAAGATGG AAATTTAACA TCTGCCCTTG GCCATCAAAC
                ROOTMOTIFTAPOX1                 GT1 motif   E-Box
241     ACTTTGTTTA TAATAGCTTA AAATTTAAAG TGCCTATCAT GTGACGTTTA ATTTATTATT
                T-Box                       DOF-Core    E-Box
301     TTAAAAGTTA AACTTTTATT AAACTAAGTG CATGTACTTT TCTTGAAAAC CAAGAGTATT
                DOF-Core                                        MYB1AT
361     TCACTGTATT GGTTTTTCCA TGGTACTATC TTATGCCTTT CAAAATATAT TTTCTATCTC
                                CURE-Core                   ROOTMOTIFTAPOX1
421     TTTCAACAAT AACATCTTGT AAAAGTATGA TTGTTTACTT AATAAATACT CAAACTTTAT
                                DOF-Core                    CACTFTPPCA1
481     TTTGACTATG TATTGGTTGC TGAAATTAAT GAGTATTACA ACTAAATAAA TATGCATATA
                W-box
541     TATACGTGTA TATATATGTG TGTGTGTGTG TATATGTATG TATATATATG TATGCATATA

601     CATATGTATG TATGTATGTA TGCATATACA TATACATGTA TGTATACATA CATGTATATA
                E-Box
661     TATACATGAA TATATATGTG TGTGTGTGTG TATACATACA CACACACACA CACACATATA

721     TATACATGTA TATATATAAC AAATAAATTT TTATATTAAA AATCAAAATA ATAGTTTTAA
                            MYBGAHV             ROOTMOTIFTAPOX1
781     AAAATAAAAA AAATTAAGAA TGGCATTTAA CTTTTAAAAG TCTAAGTATT TGAAATGAAA
                                                    DOF-Core        DOF-Core
841     AGAGAAGCAA TCTTTGACCA ACAACTGCCA TGCACAATAA ATTTTTGAGC TTTTGAGAGG
                            W-box       E-Box
901     ACATTAAACT AATTGCATGG TGCTTTGTGT TGTAAATTGT TAACCGCATA GACCATACCT

961     TGAAAAAAAA TTTATTTAAC CTTCAAATTT AAACTTTAAT TGCATGCAAC TTTCCACAAA
                GT1 motif                                       DOF-Core
1021    AGTATGCGGG ACTGTCCAAG AGAAACTATC TAACTTAGAA TAACACCTTG AAAACGTTTA

1081    GAATGGACTC GCCAGGCGCC GACTTGAACA ATCGTGTAGA GAAACGACTT TATGGCGTAA
                                    CAAT-BOX
1141    ACTACTCGCC AGGCGCCAAC TTGAACAAAT CCGATTGTGT GAAATTTATT GGCATATATC
                CACTFTPPCA1
1201    ATGGCTAACG TTTAAGGCGA CGGGAAACAG CTTATAAGTA CTACTATCCC ATCCAGTTTC
                                            TATA box
1261    ATTGTGTATT TCACCCTTTG CATTGCATTG AAGCTTTTCA CTCCTAGGGT TTTGGAGACG
                +1 Transcription Start
1321    AAAGCAGTCG GTGCATTGCA GTTAGGGAAG ATCACCCGCA CATTTGGGTT AGAGCGAAAC
1381    GAACATGGAA AAGCAGAAGA AGGATTGTAG TTGGAATGAG ATGAGTAGTT ATAAGCAGCT
1441    GGAGATTGCA GGTGGAAAAA GGATGGGTAT TAAGGCTTCG GATACTCTGC CGCTGTCACT
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Fig. 2. The sequence of the *GbHMGR* promoter region. The regulatory elements in the *GbHMGR* promoter are boxed

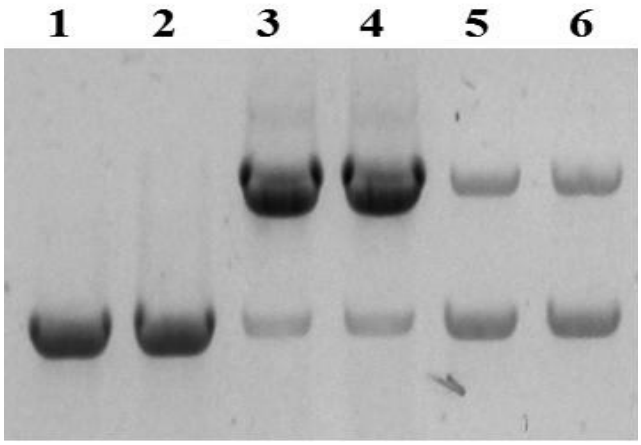

Fig. 3. Gel mobility shift assays for W-box of the *GbHMGR* promoter. 1. *E. coli* protein mixed biotin labeled W-box probe; 2, pET28a protein mixed biotin labeled W-box probe; 3, 4, the recombinant GbWRKY1 and GbWRKY2 mixed biotin labeled W-box probe, respectively; 5,6, GbWRKY1 and GbWRKY2 mixed biotin labeled and unlabeled W-box probes

also found in the promoter sequence of *GbHMGR*. Our group have cloned 31 WRKY genes based *G. biloba* transcriptome data. Our unpublished results indicated two WRKY genes *GbWRKY1* and *GbWRKY2* (ORF sequences in Supplemental Fig. S1. and S2.) might be involved in TTL biosynthetic pathway. *GbWRKY1* and *GbWRKY2* were expressed in *E. coli*, respectively. Upon induction by IPTG, GbWRKY1 (Supplemental Fig. S3, lans 2 and 3) and GbWRKY2 (Supplemental Fig. S3, lan 4 and 5) was expressed as a major protein product in the total celluar soluble protein. The molecular weight of the expressed recombinant GbWRKY1 and GbWRKY2 proteins was estimated to be about 36.7 and 39.7 kDa with the His-tag, respectively, the size of which were in good agreement with that predicted through bioinformatics. The interaction between GbWRKY1 and GbWRKY2 with *GbHMGR* promoter sequence was assayed with EMSA. No binding bands were detected with crude proteins of *E. coli* without or with empty vector pET28a (Fig. 3, lans 1 and 2). GbWRKY1 and GbWRKY2 specifically bind with the W-box sequence, and unlabeled probes inhibit the binding (Fig. 3). These results confirmed that GbWRKY proteins could combine to the W-box sequence of *GbHMGR* gene which is the target gene of WRKY protein. Some studies have also reported the WRKY proteins participated in the control of sesquiterpene and enzylisoquinoline alkaloid biosynthesis and their transcriptional induction by methyljasmonate (Xu *et al*., 2004; Kato *et al*., 2007; Ma *et al*., 2009). Data on EMSA suggested that GbWRKY1 and GbWRKY2 might regulate TTL accumulation through regulating the transcript level of target gene *GbHMGR* in *G. biloba*.

Effect light and low temperature on expression of GbHMGR and TTL content

As shown in Fig. 4A, *GbHMGR* expression level of ginkgo callus was significantly (P<0.05) higher by 281.3% in light as compared to those under dark after 24h treatment. Also, total TTL content of ginkgo callus increased by 18.0% in light as compared to the callus maintained under dark after 24 treatment (Fig. 4B). The above results implied that dark conditions might starve the plants and it is likely that gene expression and TTL content in dark could be a reflection of the effect of carbon limitation. Light would affect carbon pool through photosynthesis and the role of carbon pool in regulating secondary metabolites has been shown in *Pinus sylvestris* (Heyworth *et al*., 1998) and *Hypericum perforatum* (Mosaleeyanon *et al*., 2005). Our previous work also demonstrated that chlorocholine chloride induced the biosynthesis of TTL and expression of key genes related with ginkgolide biosynthesis by promoting the photosynthesis and carbon pool (Xu *et al*., 2011). Also, the possibility exists that light modulated gene expression independent of carbon pool (Fey *et al*., 2005). Moreover, *GbHMGR* expression was in agreement with the promoter data wherein the motifs (e.g. GT1 motif, T box, and E box) for light were present. The role of light in modulating a range of terpenoids and the corresponding transcripts has been documented, but there is no universal behavior and it varies depending upon the metabolite type and the plant species. The same gene, *HMGR* for example, it stimulated by light in *Triticum aestivum* (Aoyagi *et al*., 1993) and potato (Korth *et al*., 2000). Effect of light on the activity of *HMGR* promoter has been reported that explained the light mediated alteration in

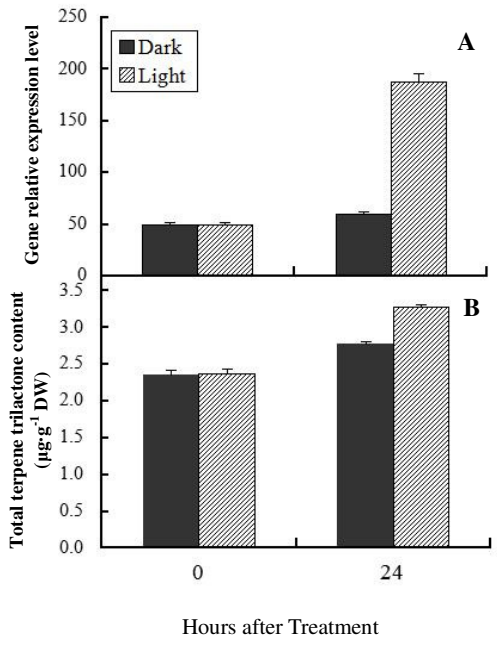

Fig. 4. Effect of light on expression level of *GbHMGR* (A) and the content of total terpene trilactones (B). Total RNA samples were isolated from ginkgo callus and subjected to real-time PCR. Values are the mean of six callus samples and bars represent standard errors.

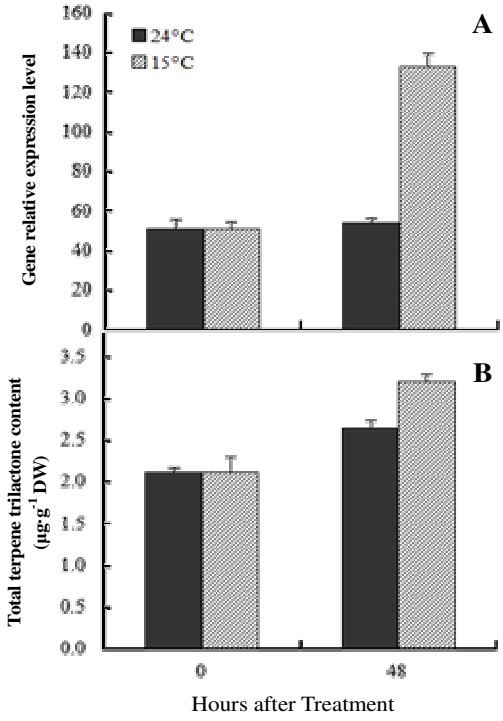

Fig. 5. Effect of low temperature on expression level of *GbHMGR* (A) and the content of total terpene trilactones (B). Total RNA samples were isolated from ginkgo callus and subjected to real-time PCR. Values are the mean of six callus samples and bars represent standard errors

HMGR transcripts (Learned and Connolly, 1997; Kawoosa *et al*., 2010).

In the case of low temperature, both expression of *GbHMGR* and total TTL content were significantly ($P < 0.05$) higher by 143.1% and 21.0%, respectively at 15 °C as compared to those at 24 °C (Fig. 5 A and B). The up-regulation of *GbHMGR* by low temperature is expected because one low temperature responsive motif was identified in *GbHMGR* promoter region. Picrorhiza *HMGR* gene has also been reported to be up-regulated by low temperature due to the motif for low temperature presence in the promoter region of this gene. Likewise, our previous work also found that low temperature could up-regulated the genes such as *GbPAL* (Xu *et al*., 2008a), *GbANS* (Xu *et al*., 2008b), and *GbFLS* (Xu *et al*., 2012) involved in flavonoid biosynthesis in *G. biloba*. Taken together, it can be suggested that secondary metabolite production can be induced by low temperature under the temperature condition of satisfy the growth of ginkgo callus.

Soitamo *et al*. (2008) showed that light at low temperature induced expression genes involved in synthesis of phenylpropanoids, carotenoids, and terpenoids. In Arabidopsis, light-dependent flavonoid (Fuglevand *et al*., 1996) and phenylpropanoid (Hemm *et al*., 2004) biosynthesis has been attributed to upregulation of relevant genes at transcript level, and the involvement of the enhancement of primary metabolite production. Low temperature mediated increase in secondary metabolites in Arabidopsis has mainly been attributed to the transcriptional upregulation of genes of secondary metabolism, which in turn, has been suggested due to the over-expression of relevant transcription factors at low temperature (Hannah *et al*., 2005). It also possible that upregulation of *GbHMGR* by light and low temperature increased carbon partitioning towards terpenoid metabolism resulting in higher TTL content in *G. biloba*.

Effect MeJA and SA on expression of GbHMGR and TTL content

Various signaling molecules that interact with their cognate receptors in the plant plasma memberane activate specific genes, which are responsible for the synthesis of alkaloids among secondary plant metabolites (Menke *et al*., 1999). Among the various signaling molecules, the elicitors MeJA and SA are thought to activate signal transduction pathways that stimulate expression of the enzymes, which form defense compounds such as terpenoids (Martin *et al*., 2003; Pu *et al*., 2009). Thus, this paper study the effects of MeJA and SA on expression of *GbHMGR* and accumulation of TTLs in ginkgo callus. As shown in Fig. 6, MeJA significantly stimulated *GbHMGR* expression and total TTL content by 86.5% and 23.9%, respectively, after 48 h treatment. Similarly, SA treatment at 48h caused increasing expression level of *GbHMGR* expression and total TTL content by 208.6% and 16.5% (Fig. 7), respectively. Response of *GbHMGR* expression to MeJA and SA could provide clue to the function of the enzymes. MeJA- and SA-responsive sites are present in the promoter sequence of *GbHMGR* (Fig. 2 and Table 2). Production of MeJA and SA is involved in plant defense against biotic stresses such as herbivore and pathogen attacks (Robert-Seilaniantz *et al*., 2011). MeJA and SA interact antagonistically against each other to induce transcription of defense-related genes (Koornneef *et al*., 2008).

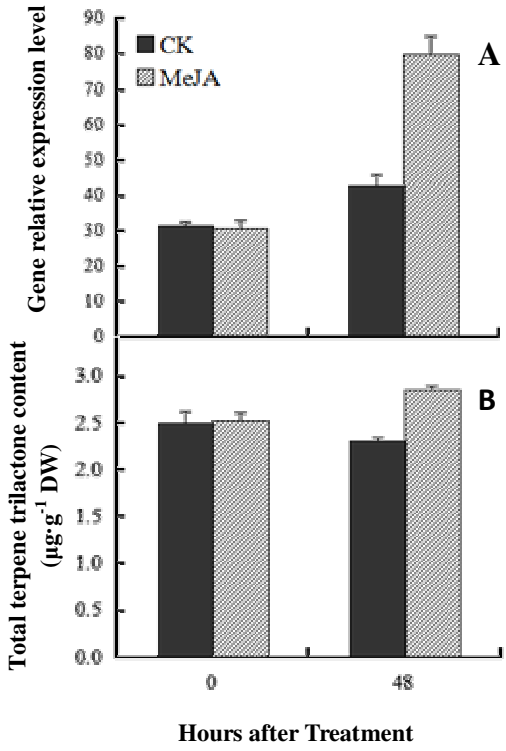

Fig. 6. Effect of methyl jasmonate on expression level of *GbHMGR* (A) and the content of total terpene trilactones (B). Total RNA samples were isolated from ginkgo callus and subjected to real-time PCR. Values are the mean of six callus samples and bars represent standard errors

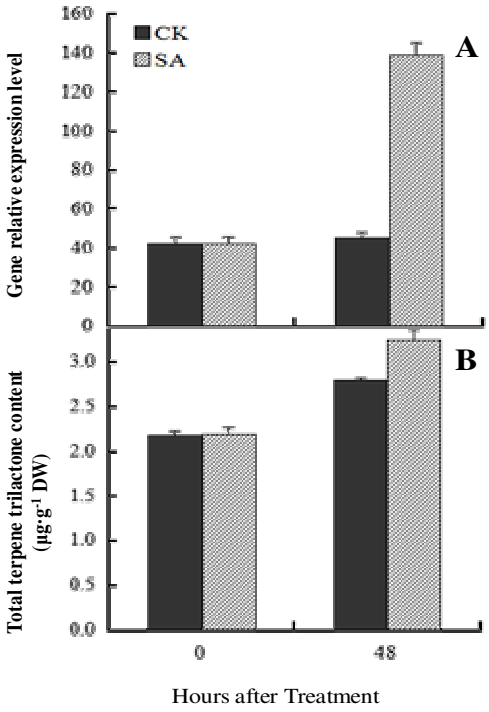

Fig. 7. Effect of salicylic acid on expression level of *GbHMGR* (A) and the content of total terpene trilactones (B). Total RNA samples were isolated from ginkgo callus and subjected to real-time PCR. Values are the mean of six callus samples and bars represent standard errors

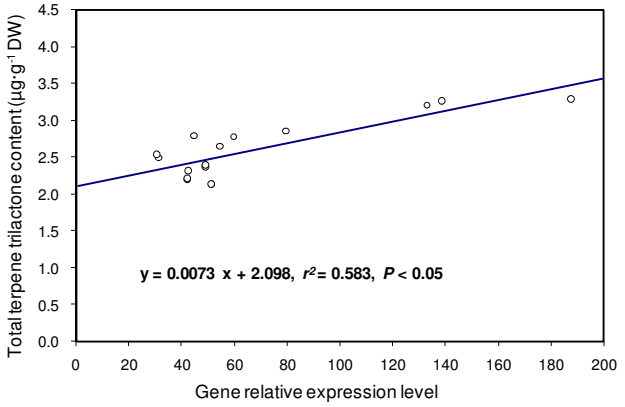

Fig. 8. Relationship between the content of total terpene trilactones and expression level of *GbHMGR* in *Ginkgo biloba* (n = 16)

The *HMGR* genes were also induced by MeJA and SA in *Solanum tuberosum* (Choi *et al*., 1994), *Brassica juncea* (Alex *et al*., 2000), *Artemisia annua* (Pu *et al*., 2009), and *S. miltiorrhiza* (Liao *et al*., 2009; Dai *et al*., 2011). *C. arachnoidea* (Wang *et al*., 2014). The up-regulation of *HMGR* expression by MeJA and SA is of particular interest because of the possible limiting role of this enzyme in terpenoid synthesis. However, MeJA and SA are not specific inducer of the *GbHMGR* gene. They also induces other genes contributing to terpenoid biosynthesis. In *G. biloba*, genes *IDS*, *DXS*, *CMK* have also been reported to be positively responsive toward MeJA and SA treatment (Gong *et al*., 2006; Kim *et al*., 2008a, b). They suggested that the positive response implied the involvement of the gene in ginkgolide biosynthesis. Therefore, it is considered that the increased content of TTLs by MeJA or SA in present study is due to an integrated effect on a cluster of genes related to TTL biosynthesis. Further work will be required to study the effect of MeJA and SA on expression of more related genes involved in TTL biosynthesis.

Relationship between GbHMGR expression and TTL accumulation

To further study relationship between of *GbHMGR* expression and TTL accumulation. We performed linear regression analysis of gene expression level and total TTL content data in ginkgo callus subjected to the light, low temperature, MeJA and SA treatments. The results showed that the relationship between total TTL content (y) and *GbHMGR* expression level (x) was significantly positively linearly correlated (Fig. 8), with a correlation coefficent is r^2 = 0.583, and a linear curve equation represented by y = (0.0073 ± 0.0014) x + (2.098 ± 0.1112). These results indicated that the *GbHMGR* gene is responsible for the TTL accumulation and might played a crucial role in TTL biosynthesis.

Conclusions

In summary, the present study isolated and characterized the MVA pathway *GbHMGR* gene promoter from *G. biloba*. Functional and bioinformatic analyses revealed that the *GbHMGR* promoter contains a number of *cis*-motifs that could bind transcription factors involved in regulation of TTLs. EMSA analysis suggested possible functionality of W-

box in *GbHMGR* promoter region. The behavior of gene transcripts in ginkgo callus upon light, low temperature, MeJA and SA treatments further verified the regulatory function of *GbHMGR* promoter. A positive relationship between gene expression level and total TTL contents implied that *GbHMGR* might be one of key genes involved in TTL biosynthesis in *G. biloba*. Studies on gene promoters in terpenoid biosynthetic pathway constitute a valuable approach to identify new regulatory factors and/or families that could controlled the TTL biosynthesis in *G. biloba* cell culture.

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