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 phyto medicines in the world (Gertz and Kiefer, 2004). Active compounds in *G. biloba* leaf 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...
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), 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 (IDR; Kim et al., 2008a), 2-C-methyl-d-erythritol 4-phosphate cytidylyltransferase (MECT; Kim et al., 2006b), 2-C-methyl-d-erythritol 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). 1,3-dioxy-d-3-dimethylglycerald-Coenzyme A reductase (HMGR, EC: 1.1.1.34) catalyzes the conversion of HMG-CoA to MVA, which is a rated-limiting enzyme in isoprenoid biosynthesis via MVA pathway (Hunter, 2007; Buhaescu and Lizenced, 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), *Tecoma media* (Liao et al., 2004), *Corilus 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 *Cynonotis arachnoidea* (Wang et al., 2014). At present, a HMGR gene (GbHMGR) has been isolated 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 *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.afic.go.jp/PLACE) and the Signal Scan Program PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/databse).

**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 pET28a(+), 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 Geneti, 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 oligonucleotides in binding buffer (10 mM Tris-HCl, pH 7.5; 50 mM NaCl; 1 mM dithiothreitol; 1 mM EDTA, 5% glycerol and 1 μg/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 TRLs

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 TRLs was the sum of the contents of GA, GB, GC, and BB and expressed as μg 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 chromsome 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, ACGT 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 ABRE/ATCATC motif function as Ca2+-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 MYB/BAH/HV element and three GTI 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 CRTDRE/CBF2 element (Xue, 2003) and one T box (Chen et al., 2001). Interestingly, we also found one MYB-box (MYBIA1), 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.
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*.

**Table 1.** Primer sequences used in the experiment

| Name          | Sequence (5'–3')                | Description                                      |
|---------------|---------------------------------|--------------------------------------------------|
| AP1           | GTAATACGACTCTATAGGC             | Reverse primer for promoter amplification, outer |
| AP2           | ACTATAGGGCACGCGTGGT             | Reverse primer for promoter amplification, nested |
| GbHMGRP1      | ACGCCCTACCTCTCCCTCACCCCTTCT    | Reverse primer for promoter amplification, outer |
| GbHMGRP2      | ATACGAATGGCGACCTGACGCAAGAT     | Reverse primer for promoter amplification, nested |
| HMGRFP        | TCTTTGTCTATGTTTTTTTCAGC        | Gene specific primer for qRT-PCR, forward        |
| HMGRRP        | AGTTTCTCTTTCTCCTCTCGC          | Gene specific primer for qRT-PCR, reverse        |
| 18SFP         | ATACAACTGCTGGCTATCG            | Gene specific primer for qRT-PCR, forward        |
| 18SRP         | TCTGCAGTGGTTTGGTCTTTC          | 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        | MACGGYB         | Ca²⁺-responsive up-regulated genes                                                 |
| CRTDREHVVCBF2       | 15       | GTGCAC          | 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 |
| GT motif            | 76, 209,962 | GRWAAW   | Consensus GT-1 binding site in many light-regulated genes and influence the level 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 expression |
| 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                        |
| MYBIAT              | 347      | WAACCA         | MYB recognition site involved in dehydration-responsive                            |
| TATA Box            | 1233     | TATAA          | Common cis-acting element in promoter and enhancer regions                         |
| CURECORCOCR         | 334,383,1238 | GTAC   | Copper-responsive element                                                          |
| CAAT Box            | 1109     | CAAT            | Common cis-acting element in promoter and enhancer regions                         |
| NTBBFIARROLB        | 474,993,1127 | ACTTTA | Required for tissue-specific expression and auxin induction;                        |
| CATATGGMSAUR        | 601      | CATAG           | Auxin-responsive                                                                   |
| MYBGAHV             | 737      | TAACAAA        | Central element of gibberellin (GA) response complex (GARC) in high-pl alpha-amylase gene |
| ARR1AT              | 1172,81,448 | NGATT         | ARR1-binding element                                                              |
| ACGTTTBOX           | 1073,1207 | AACGTG         | One of ACGT elements                                                               |

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

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), *IDS* (Kang et al., 2013), *GbDXS* and *GbGGPPS* (Xu et al., 2013). The W-box sequences predicted as TTTGAC were
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

Fig. 2. The sequence of the GbHMGR promoter region. The regulatory elements in the GbHMGR promoter are boxed.

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, lanes 2 and 3) and GbWRKY2 (Supplemental Fig S3, lane 4 and 5) was expressed as a major protein product in the total cellular 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, lanes 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 enzylosquoline 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 TTL content)
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-regulate 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 membrane 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).
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 upregulation 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.

**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

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.

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

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 coefficient is $r^2 = 0.583$, and a linear curve equation represented by $y = 0.0073 x + 2.098 (P < 0.05)$. 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-
in the 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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