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Short Communication

Molecular cloning and characterization of *GbDXS* and *GbGGPPS* gene promoters from *Ginkgo biloba*

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ABSTRACT. Ginkgolides are key pharmaceutical components in *Ginkgo biloba* leaves. 1-Deoxy-D-xylulose-5-phosphate synthase (*GbDXS*) and geranylgeranyl pyrophosphate (*GbGGPPS*) genes are critical genes involved in ginkgolide biosynthesis. In this study, the promoters of *GbDXS* and *GGPPS*, with 676 and 570 bp in length, respectively, were cloned by chromosome walking. The cis-elements of *GbDXS* and *GbGGPPS* promoters were predicted and analyzed by the plant cis-acting regulatory element (CARE) database. We found some major cis-elements in the sequence of *GbDXS* and *GbGGPPS* promoters. The *GbDXS* promoter has 3 TATA boxes, 10 CAAT boxes, 6 GATA boxes, and 1 I box. The *GbGGPPS* promoter has 1 TATA box, 6 CAAT boxes, 6 GATA boxes, and 4 I boxes. Furthermore, some stress-related cis-elements in the promoters of *GbDXS*

and *GbGGPPS* were found to be light-regulated elements, including sequences over-represented in light-induced promoters (SORLIP1-AT), GATA box, and I box, a gibberellin-responsive element (WRKY), salicylic acid-induced (GT-1), cold- and dehydration-responsive (MYC-Core), and copper-inducible (CURE-Core). Further analyses of these cis-elements will aid in elucidating the molecular mechanisms regulating the expression of the *GbDXS* and *GbGGPPS* genes during ginkgolide accumulation in *G. biloba*.

Key words: Molecular cloning; *GbDXS*; *GbGGPPS*; Promoter; Cis-acting elements; *Ginkgo biloba*

INTRODUCTION

Ginkgo biloba, often called "a living fossil", is one of the oldest surviving plant species and the only living plant in Ginkgoaceae. *G. biloba* leaves contain many natural products, primarily including flavonoids and terpene trilactones. Flavonoids can be obtained from many other plants, but ginkgolides are unique components of *G. biloba* extract. Terpene trilactones mainly contain ginkgolides (ginkgolide A, B, C, and J) and bilobalide. Previous studies have demonstrated that ginkgolides are platelet-activating factor antagonists and strongly inhibit cardiovascular and cerebrovascular diseases, asthma, bronchitis, and arrhythmia (van Beek and Montoro, 2009). Bilobalide protects brain tissue and neurons from ischemic and hypoxic injury, revitalizes the central nervous system, and prevents and treats senile dementia, which may be related to the physiological activity of the nervous system (Strømgaard and Nakanishi, 2004). Although ginkgolides are considered to play a key role in the active ingredients of ginkgo, the concentration of ginkgolides are very low, within 0.25% ginkgolides in *G. biloba* leaves (van Beek and Montoro, 2009).

For the above reasons, terpene trilactone content should be increased in order to improve the medicinal and commercial value of ginkgo leaves. The biosynthetic pathway of ginkgolide has been studied more carefully and is now widely regarded as 2 pathways of mevalonate (MVA) and 2-c-methyl-D-erythritol-4-phosphate (MEP) in plants (Kawoosa et al., 2010). Anabolism of ginkgo terpene trilactones is mainly derived from the MEP pathway. In this pathway, isopentenyl diphosphate and dimethylallyl diphosphate are synthesized, with pyruvate and 3-glyceraldehyde phosphate of the citric acid cycle as their precursors. Afterwards, they are both collectively converted into ginkgolides through a series of enzymatic reactions involving hydroxylation and acylation. Recently, some studies have found that ginkgolide content is closely related to the expression level of critical enzyme genes, such as 1-deoxy-D-xylulose-5-phosphate synthase (*DXS*; Gong et al., 2006), geranylgeranyl pyrophosphate (*GGPP*; Liao et al., 2004), HMBPP reductase (*IDS*; Kim et al., 2008), deoxy-1 wood sugar, 5-phosphate isomerase reduction (*DXR*), and levopimaradiene synthase (*LPS*; Kim et al., 2012). The ginkgolide content is significantly positively correlated with the expression level of these genes (Xu et al., 2011). The primary approach for increasing the ginkgolide content is to enhance the expression of these key genes in the biosynthetic pathway of terpene trilactones.

Although several studies regarding ginkgolide biosynthesis and important related genes have been performed, associations between the promoters of these genes and terpene trilactone biosynthesis have not been reported. Plant promoters are important cis-acting ele-

ments at the core of transcriptional regulation, and they play an important role in the regulation of gene expression in plants. The cloning and functional studies related to the promoters of the key genes in ginkgolide biosynthesis will aid us in understanding its signal transduction pathways and gene expression patterns. In this experiment, we cloned the promoters of *GbDXS* and *GbGGPPS* from *G. biloba* leaves and performed sequence analysis and functional prediction of cis-acting elements. Our cloning and function prediction provides a basis for further study to determine the optimal approach for increasing ginkgolide content in *G. biloba* leaves.

MATERIAL AND METHODS

Plant material

Young leaves were selected from 12-year-old grafts of *G. biloba* grown in an orchard at Yangtze University in China. Young leaf samples were harvested and immediately frozen in liquid nitrogen followed by storage at -80°C until use.

Construction of DNA library

Total DNA was extracted from leaf samples by the modified cetyltrimethylammonium bromide (CTAB) method (Xu et al., 2011). Genomic DNA was digested with restriction endonucleases *Dra*I, *Eco*RV, *Pvu*II, and *Ssp*I (New England Biolabs Inc., Ipswich, MA, USA), respectively, and recovered using a polymerase chain reaction (PCR) Extraction Mini Kit (Watson Biotechnologies, Inc., Shanghai, China). Then, they were ligated separately to the adaptor protein (AP; provided in the kit) to construct the genomic walking libraries. Details of this procedure are described in the Genome Walker Universal Kit User Manual (Clontech, Mountain View, CA, USA).

Amplification of *GbDXS* and *GbGGPPS* promoters

Two pairs of specific primers (GbDXS-5/GbDXS-5N and GbGGPPS-5/GbGGPPS-5N) were designed and synthesized (Shanghai Sangon, Shanghai, China) based on the nucleotide sequences of *GbDXS* and *GbGGPPS* genes, and the adapter primers (AP1 and AP2) were designed from the gene sequences that were previously obtained. The primer sequences are given in Table 1. The *GbDXS* and *GbGGPPS* promoters were cloned with the method of chromosome walking described in manufacturer instructions for the Advantage 2 PCR Kit (Clontech). GbDXS-5/GbGGPPS-5, AP1, and the templates of the constructed DNA libraries were used in the first step of PCR amplification with the following conditions: 94°C for 3 min, followed by 7 cycles of amplification at 94°C for 25 s, 72°C for 3 min, followed by 32 cycles of amplification at 94°C for 25 s, 67°C for 3 min, and at 67°C for 7 min. The product was diluted 10-fold and was used in nested PCR, which was performed using AP2 and GbDXS-5N/GbGGPPS-5N as nested primers under the following conditions: 94°C for 3 min followed by 5 cycles of amplification at 94°C for 25 s, 72°C for 3 min; then, 32 cycles at 94°C for 25 s and 67°C for 3 min, followed by an extension for 7 min at 67°C. The second PCR product was purified, cloned into the pMD18-T vector (TaKaRa, Dalian, China) and sequenced. Subsequent basic local alignment search tool (BLAST) results confirmed that the amplified products were partial fragments of the *GbDXS* and *GbGGPPS* genes.

Table 1. Primer sequences used in experiment.

Name	Sequence (5'-3')
AP1	GTAATACGACTCACTATAGGGC
AP2	ACTATAGGGCACGCGTGGT
GbDXS-5	TGATCGTGTCGAGCAATGGCGTGGCAGGT
GbDXS-5N	CGGCTTTCACCACCATCGGAATTGTTTGC
GbGGPPS-5	CCAGATCCTCCGTTCCTCCTACAAGTTCA
GbGGPPS-5N	AGTGCAGGCTTTGGTAACTTGCTGTCATT

Bioinformatics analysis

The obtained sequences were analyzed using online bioinformatics tools (http://www.ncbi.nlm.nih.gov). The sequence region was analyzed using the plant cis-acting regulatory element (CARE) database (http://www.dna.affrc.go.jp/PLACE and http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

RESULTS AND DISCUSSION

The sequence of *GbDXS* was obtained by constructing a DNA library from *G. biloba* leaves after 2 rounds of nested PCR using chromosome walking techniques. The promoter sequence of the *GbDXS* gene sequence was 676 bp in length (Figure 1A). The transcription start site of *GbDXS* was predicted by the promoter analysis software, as shown in Figure 2. Various cis-acting regulatory elements, along with their functions and location in the promoter of *GbDXS*, are shown in Table 2 and Figure 2. We found that the *GbDXS* promoter sequence contains several important cis-regulatory elements such as TATA boxes, CAAT boxes, GATA boxes, I boxes, etc. Specifically, *in silico* analysis revealed that 3 TATA boxes are present within the promoter region of *GbDXS*, at positions 531, 629, and 657. The TATA box promotes gene transcription in combination with RNA polymerase II, thus affecting the rate of transcription (Smale and Kadonaga, 2003). In the *GbDXS* promoter, the nearest TATA box from the forecasted transcription initiation site is -30 bp upstream, which is in accordance with the characteristics of eukaryotes. Another conserved eukaryotic cis-element, 10 CAAT boxes, were also observed at positions 30, 73, 213, 237, 242, 275, 385, 440, 514, and 643. The CAAT box controls the transcription initiation frequency and impacts conversion rates of target gene (Edwards et al., 1998). The changes in these basic elements within the promoter region will greatly affect the level of transcription of target gene. In addition to these essential cis-acting elements, other corresponding stress-related cis-elements in the promoter region of *GbDXS* were also predicted by the PlantCARE database, such as the I box, MYB-ST, MYC-Core, GT-1, CURE-Core, WRKY, SORLIP1-AT, and others. MYB-ST (position 492 and 457) binding sites (GGATA) and a Myc (position 591) recognition site (CANNTG) were identified. Protein members of both the Myb and the Myc families have been shown to play important roles in plant responses to pathogen infection, low temperature, and drought. Furthermore, several hormones could influence the relevant promoter element. For example, 2 W box motifs were identified at positions 427 and 440, which were involved in the induction of genes by SA, an important component of signal transduction cascades that activates the defense response of plants against pathogen attack (Redman et al., 2002). WRKY (position 520 and 292) is a GA-responsive element and affects ABA signaling (Zhang et al., 2004). Six GATA boxes (position 129, 226, 299, 493, 360, and 457), 6 GT-1 elements (position 252, 262, 79, 327, 606, and 667), 1 I box (position

299), and 2 SORLIP1-AT elements (position 103 and 200) were found and have been shown to be light-responsive cis-elements (Reyes et al., 2004). One CURE-core motif (position 616) and 10 POLLEN-1 motifs (position 251, 285, 310, 377, 425, 466, 476, 566, 625, and 633) were previously identified as copper- and pollen-responsive cis-elements, respectively (Bate and Twell, 1998).

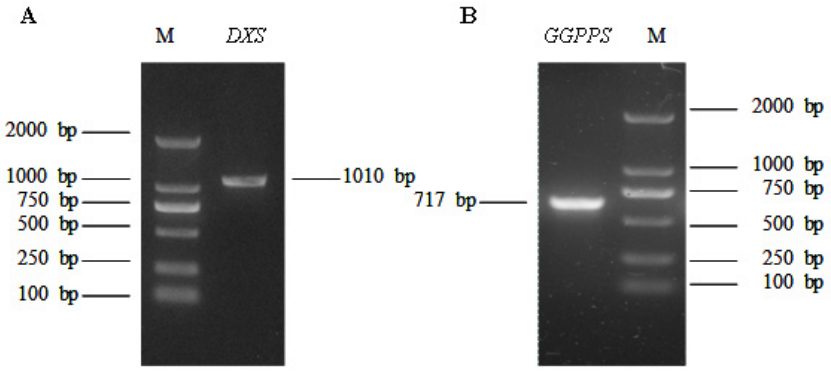

Figure 1. Promoter sequence of *GbDXS* (**A**) and *GbGGPPS* (**B**). *Lane M* = DNA marker DL2000; *DXS* = PCR product of the *GbDXS* gene promoter; *GGPPS* = PCR product of the *GbGGPPS* gene promoter.

Figure 2. Sequence of the *GbDXS* promoter region. The regulatory elements in the *GbDXS* promoter are boxed.

Table 2. Putative cis-acting regulatory elements identified in the promoter of *GbDXS* using the PLACE database (http://www.dna.affrc.go.jp/PLACE).

Element	Position	Signal sequence	Expected function
HEX motif	1	CCGTCG	Regulating the expression of histone H4 promoter
CAAT box	30, 70, 242, 385, 440, 643, 514	CAAT	Common cis-acting element in promoter and enhancer regions
ROOT motif	65, 162, 169, 527, 526, 643	ATATT	Evaluation of the organ specificity and strength of the rol D promoter
SORLIP1-AT	103, 200	GCCAC	Over-represented in light-induced cotyledon and root
GATA box	129, 226, 299, 493, 360	GATA	Part of a light-responsive element
DOF-Core	247, 379, 486, 635, 558	AAAG	Enhances transcription from CyPPDK and a non-photosynthetic PEPC gene
GT-1	252, 262, 79, 327, 606, 667, 252	GRWAAW	Stabilize the FIIA-TBP-DNA complex and the binding site involved in SA inducibility
WRKY	520, 292	TGAC	Encodes a transcriptional repressor of the gibberellin signaling pathway
I box	299	GATAAG	Light-regulated element and binding site of LeMYB1
POLLEN-1	251, 377, 466, 633, 566	AGAAA	Required for pollen-specific expression
MYB-ST	492, 457	GGATA	Transcriptional activator element
W box	519, 292	TTGAC	WRKY-binding site involved in the pathogenesis-activation and negative-regulatory elements
MYC-Core	591, 591	CANNTG	Regulates the transcription in the cold and dehydration-responsive
CURE-Core	616	GTAC	Oxygen deficiency-responsive and recognize the copper-responsive element
TATA box	629, 657	TATA	Core promoter element around -30 of transcription start

The length of the *GbGGPPS* promoter is 669 bp (Figure 1B). The putative transcription start sites and nucleotide sequence of the *GbGGPPS* promoter are shown in Figure 3. Various cis-acting regulatory elements, along with their function and location in the promoter of *GbGGPPS* were forecasted by the PlantCARE database as shown in Table 3 and Figure 3. *In silico* analysis revealed that 10 CAAT boxes (position 17, 183, 212, 224, 266, 355, 462, 515, 429, and 475) were present in the promoter region of *GbGGPPS*. In the position of 167, we predicted a TATA box in the *GbGGPPS* promoter sequence. Furthermore, 11 light-related cis-elements in the promoter region of *GbGGPPS* were also predicted, such as ASF1 motif, GATA box, I box, INRNTP, REALPH, SORLIP-5, T box, 3-AF1-binding site, CATT motif, Sp1, and chs-CMA2a. Moreover, some stress-related cis-elements were also found in the promoter sequences of *GbGGPPS*. One MybCORE (position 38) and 3 Myc-core elements (position 117, 148 and 532) were identified, which have been reported to function as cis-acting elements in cold-, drought-, and ABA-induced gene expression (Planchais et al., 2002). Some hormone-repressive cis-elements were also identified. One ARFAT element (position 308) was enriched in the 5'-flanking region of genes upregulated by both IAA and Brassinolide (Pufky et al., 2003). One TCA element (position 90) and 6 GT-1 motifs (position 10, 21, 163, 411, 517, and 541) were identified, and have been reported to be SA-inducibility cis-acting elements (Park et al., 2004). One CGTCA motif was identified at position 55, which is involved in MeJA responsiveness (Wang et al., 2011). One SREATMSD (position 518) and 1 W-BOXHVISO1 (position 152) were identified as sugar-repressive elements (Tatematsu et al., 2005). One CURE-Core motif (position 345) and 3 WRKY motifs (position 56, 152, 241) were identified as copper- and GA-responsive cis-elements, respectively.

The ginkgolides are some of the most important medicinal components of *G. biloba* leaves. *GbDXS* and *GbGGPPS* are key genes in the metabolic pathway of terpene trilactones. The characterization and function analysis of *GbDXS* and *GbGGPPS* will aid in increasing our understanding of the roles of *GbDXS* and *GbGGPPS* in ginkgolide biosynthesis at the molecular level. Several studies have confirmed that cis-acting elements play vital roles in the promoter regulation of plant gene expression (Sawai et al., 2006; de Souza et al., 2009). However, the specific function of some promoters is still not

clear. The process that occurs from reading genetic information to gene expression is complex; whether genes are expressed, expression timing, expression sites, and the functions of the cis-acting elements in the promoters remain to be studied. It is important to determine gene function by isolation and identification of new gene promoters and studying their expression and regulation mechanisms.

Figure 3. Sequence of the *GbGGPPS* promoter region. The regulatory elements in the *GbGGPPS* promoter are boxed.

Table 3. Putative cis-acting regulatory elements identified in the promoter of *GbGGPPS* using the PLACE database (http://www.dna.affrc.go.jp/PLACE).

Element	Position	Signal sequence	Expected function
ARFAT	308	TGTCTC	Up-regulated by both IAA and BL
ASF1 motif	55	TGACG	May be relevant to light regulation
CAAT box	17, 183, 224, 55, 462, 515	CAAT	Common cis-acting element in promoter and enhancer regions
CURE-Core	345	GTAC	Copper-responsive element
GATA box	21, 163, 205, 519, 543, 562	GATA	Part of a light-responsive element
GT-1	10, 21, 163, 411, 517, 541	GRWAAW	Influence the level of SA-inducible gene expression
I box	21, 163, 542	GATAA	Light-regulated transcription element
INRNTP	295	YTCANTYY	Light-responsive transcription element
MYB-Core	38	CNGTTR	Involved in regulation of flavonoid biosynthesis
MYC-Core	117, 148, 532	CANNTG	Function as cis-acting elements in the cold-drought- and ABA-induced gene expression
REALPH	422	AACCAA	Required for phytochrome regulation
SORLIP-5	248	GAGTGAG	Over-represented in both light-induced cotyledon-specific and root-specific genes
SREATMSD	518	TTATCC	Sugar-repressive element
TATA box5	167	TTATTT	Common cis-acting element in promoter and enhancer regions
T box	228	ACTTTG	Reductions of light-activated gene transcription
W box HVISO1	152	TGACT	Binding to the sugar-responsive elements
WRKY	152, 241, 56	TGAC	Transcriptional repressor of the gibberellin-signaling pathway
3-AF1-binding site	87	TAAGAGAGGAA	Light-responsive element
CATT motif	478	GCATTC	Part of a light-responsive element
CGTCA motif	55	CGTCA	Cis-acting-regulatory element involved in the MeJA responsiveness
Sp1	48	CC(G/A)CCC	Light-responsive element
TCA element	90	GAGAAGAATA	Cis-acting element involved in salicylic acid responsiveness
chs-CMA2a	472	GCAATTCC	Part of a light-responsive element

This study aimed to clone and evaluate the functions of *GbDXS* and *GbGGPPS* in the ginkgolide biosynthetic pathway and to confirm the molecular mechanisms that dictate how the cis-acting elements regulate the spatial and temporal expression of these genes and their response to the exogenous factors. The results provide an important basis for our further experiments and also provide the scientific guidance and technical approach to improve ginkgolide content for pharmaceutical production.

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