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CORRELATION BETWEEN DNA METHYLATION OF THE CHALCONE ISOMERASE GENE IN *LITHOCARPUS POLYSTACHYUS* REHD. (FAGACEAE) AND PHLORIZIN ACCUMULATION

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Abstract

Chalcone isomerase gene (CHI) is an important gene that flavonoid biosynthesis pathway. This study aimed at investigating the effects of DNA methylation in the *CHI* promoter of *Lithocarpus polystachyus* Rehd, gene expression levels and phlorizin accumulation. Primers were designed based on the cDNA sequence of the chalcone isomerase gene (*CHI*) from *Lithocarpus polystachyus* Rehd and the promoter sequence was cloned using TAIL-PCR technology. The CpG Island of the gene promoter was predicted using online software, and the DNA methylation sites of the *CHI* promoter was investigated through bisulfite sequencing. QRT-PCR was used to detect the expression levels of CHI at varying DNA methylation levels and types. Ultra-pressure liquid chromatography (UPLC) was used to determine the phlorizin quantity. The full-length promoter sequence of *CHI* was 2141 bp, with 33 types, 138 cis-acting elements (CREs), 3 CpG islands, 16 methylation sites, and 7 DNA methylation types. DNA methylation of the *CHI* gene promoter region was significantly negatively correlated with gene expression and phlorizin content ($p<0.01$. The successfully cloning of the *CHI* gene promoter and its DNA methylation lays the foundation for further research on gene expression analysis and flavonoid biosynthesis in *L. polystachyus*.

Key words: *Lithocarpus polystachyus* Rehd; *CHI* promoter; DNA methylation; Gene expression level; Phloridzin accumulation.

Introduction

DNA methylation refers to the transfer of a methyl group from the S-adenyl methionine to the 5th carbon of cytosine in a DNA molecule to form 5-methylcytosine and this process is catalyzed by DNA methyltransferase (Zemach *et al*., 2010). DNA methylation of a promoter in functional genes can by preventing transcription factors from binding to the methylated region of the promoter, thereby inhibiting gene expression. This is one of the effective mechanisms of transcriptional regulation in plants (Curradi *et al*., 2002). *Lonicera japonica*, also known as Japanese honeysuckle has been used in traditional Chinese medicine and *L. japonica* var. Chinensis, is a variety of *Lonicera japonica* produced by natural mutagenesis, has identical amino acid sequences of key enzymes that are involved in the synthesis of chlorogenic acid, the main medicinal component of *Lonicera japonica*. However, there are significant differences in the gene expression level and chlorogenic acid content between the two species (Yuan *et al*., 2014). Studies on DNA methylation of specific genes have revealed that the degree of DNA methylation degree in the - 109 bp to - 279 bp region of the phenylalanine ammonia-lyase 2 promoters of *Lonicera japonica* var. Chinensis was far significantly higher compared to that of *Lonicera japonica*, and the DNA methylation degree in the CG site was also significantly different. This indicates that DNA methylation is essential in the regulation of gene expression of key enzyme genes (Liangping *et al*., 2017).

Flavonoids are synthesized through the phenylpropanoid pathway, where phenylalanine is transformed into 4-coumaric acyl – CoA which eventually enters the flavonoid biosynthesis pathway. Chalcone synthases (CHS) is the first enzyme in the flavonoid pathway producing chalcone scaffolds. Chalcone is then converted to dihydrochalcones through an intramolecular cyclization reaction by chalcone isomerase (CHI) (Ferreyra *et al.*, 2012). In plants, *CHI* genes are divided into type I, which is capable of isomerizing 6'-hydroxychalcone to 5-hydroxyflavone, and type II, which is capable of converting both 6'-deoxychalcone and 6'-hydroxychalcone to 5-deoxyflavanone and 5 – hydroxyflavanone (Shimada *et al*., 2003). In our preliminary study, a *Lpr-CHI* gene was cloned and analysis indicated that the expression level determined the content of phlorizin, however, the regulatory mechanism remained unclear (Lin *et al*., 2017). In this study, the *CHI* gene of *L. polystachyus* was further investigated for its promoter DNA methylation. This was achieved by cloning and analyzing the promoter region. Therefore, this study provided valuable insights into the regulation effect of the *CHI* genes in flavonoid biosynthesis in *L. polystachyus*.

Materials and Methods

L. polystachyus leaves were sampled from Bama Yao Autonomous County of Guangxi Zhuang Autonomous Region, China. The identification of the plant materials was performed by one of the authors, Prof Zhaobin Xing.

RNA isolation and cDNA cloning: A total of ten *L. polystachyus* plants of the same age and grown under the same growth conditions were selected. The total RNA extraction from leaves was performed and the RNA prep pure Plant Kit (Thermo Scientific) instructions used to

determine the purity of the extracted RNA. Nanodroplet spectrophotometer (Thermo Scientific) was used to estimate the RNA concentration while the integrity was assessed by electrophoresis and visualization under UV light. A total of 1 1μL total RNA were used for RNA reverse transcription using the RevertAid Frist Strand cDNA synthesis kit (Beijing Bayerdi Biotechnology) following the manufacturer's instructions.

TAIL-PCRE cloning: A cDNA sequence of the *CHI* gene was cloned by our research group (Lin *et al*., 2017), using Primer 5.0 software design of the three sets of specific Primer sequences that are presented in table 1. The first set of sequences included CHIBY2, CHIBY3, and CHIBY4 which were paired with the universal primer AD1; the second group included CHIBY5, CHIBY6, and CHIBY7 which were paired with the universal primer AD6; the third group included CHIBY10, CHIBY11, and CHIBY12 which were paired with the universal primer AD3. The 25μL reaction mixture contained 1 μL template, 5 μL dNTP, 12.5 μL KOD Buffer, 0.5 μL KOD Fx, 0.5 μL specific primers, 2.5 μL universal primers, 3 μL ddH_2O. During the first round PCR, the genomic DNA of *L.polystachyus* was the template, in the second round, the template used was 10 times the first round of PCR products, while in the third round it was 100 times the second-round PCR products. The above TAIL-PCR reaction conditions were adjusted as described by Liu & Chen (2007). The PCR products were analyzed by ethidium bromide-stained 1% agarose gel and the corresponding band sequenced at the Nuosai Genome Research Center (Beijing).

Sequence analysis: PlantCARE database was used to analyze the cis-acting regulatory sequence motifs in the *CHI* promoter. The transcription start site (TSS) was predicted using Neural Network Promoter Prediction 2.0 and BDGP. The Li Lab online software was used to predict the CpG island, and the predicted results used to design primers for bisulfite sequencing (Table 1: CHIjqS1 to CHIjhX3).

Bisulfite sequencing: The *L. polystachyus* DNA sample was subjected to bisulfite treatment using the DNA Bisulfite Conversion Kit according to the manufacturer's instructions. This was followed by PCR amplification using the Methylation-specific kit according to the manufacturer's instructions. A 25 μL reaction mixture containing 1 μL of methylation DNA, 1 μL of CHIjhS1 (CHIjhS3), and 1 μL of CHIjhX1 (CHIjhX3), 0.4 μL MSP DNA Polymerase, 2 μL 10×Msp PCR Buffer and 13 μL ddH_2O was prepared. Amplification was performed using the following cycling conditions: 94℃ 5 min, (94℃ 20 s, 55℃ 30 s, 72℃ 20 s (10 s)) ×35 cycles, 72℃ 5 min.

The DNA of *Lpr-CHI* was also PCR-amplified using a methylation-specific PCR kit. The reaction mixture contained 1 μL of CHIjqS1 (CHIjqS3), 1 μL of CHIjqX1 (CHIjqX3) and 1 μL DNA, 9.5 μL ddH_2O, 12.5 μL 2×Pfu Mix. The PCR condition was as follows: 94℃ 3 min, (94℃ 30 s, 53°C (55°C) 30 s, 72℃ 15 s (40 s)) ×35 cycles, 72℃ 1 min. The PCR products were analyzed by ethidium bromide-stained 1% agarose gel and the corresponding band sequenced at the Nuosai Genome Research Center (Beijing). Primer sequences are shown in Table 1.

Table 1. Primer sequence.

Primer name	Sequence (5`-3`)	Expected length of amplification
CHIBY2	GACCCAACAGCTTTAAGGATCGGT	
CHIBY3	CCGCCGAGGAACAGTGTGTTGGTT	
CHIBY4	GGAAGACGCTGCTAGGACCATT	
CHIBY5	GAATGAGGTAGGTTGAATGGACGG	
CHIBY6	GTGAGCTTTGGGGTTAGGCACTTT	
CHIBY7	CATGTTGACTCCTACCATTTTGAG	
CHIBY10	TTATGATGGACCACTTTAACCGG	
CHIBY11	GTGGTCCTAATTTAACTCACTC	
AD1	NTCGASTWTSGWGTT	
AD3	WGTGNAGWANCANAGA	
AD6	CAWCGNCNGANASGAA	
CHIBY12	GTACCACACCGATGACCACTTTA	
CHIjqS1	TGCCTTCAGGAGCATCTCTACCAT	215 bp
CHIjqX1	AAAGGCCAGCAGATCACTTACTCAC	215 bp
CHIjqS3	CTTGCAACTCCTTTGAGGGTGGT	214 bp
CHIjqX3	ATAGAATGGTCAGGGCAACAGACTC	214 bp
CHIjhS1	TGTTTTTAGGAGTATTTTTATTAT	215 bp
CHIjhX1	AAAAACCAACAAATCACTTACTCAC	215 bp
CHIjhS3	TTTGTAATTTTTTTGAGGGTGGT	214 bp
CHIjhX3	ATAAAATAATCAAAACAACAAACTC	214 bp
DCHIrtS2	ATGGTCCTAGCAGCGTCTCTTCC	231 bp
DCHIrtX2	AGTCAACTCCTCCGCACTCTTTC	231 bp
DGrtS1	GGTCAAGATCGGAATCAACGGAT	221 bp
DGrtX1	CGACGGACTTCTCACCAAAGAGA	221bp

Rt-qPCR analysis

Quantitative analysis of the *CHI* gene in the leaves of *L. polystachyus* was performed by fluorescence quantitative PCR as described by Lin *et al*., (2017), but with slight modifications. The reaction mixture contained 2×Talent qPCR PreMix 5 µL, forward primer 0.3 µL, reverse primer 0.3 µL, DNA 0.5 µL, 50×ROX Reference Dye 1 µL, RNase-Free H_2O 29 µL. The Rt-qPCR conditions were as follows: 94℃ 3 min, (94℃ 5 s, 55°C 30 s, 72℃ 15 s) ×40 cycles, 72°C 15 s. The *CHI* gene primers DCHIrtS2-DCHIrtX2 and the internal reference gene primer DGrtS1-DGrtX1 sequences used in this experiment are shown in Table 1.

UPLC analysis of phlorizin accumulation in *L. polystachyus*: Phlorizin content was determined using the method described by Sun *et al*., (2015), but with slight modifications. Weighing of 0.2 g of leaves of each sample of *L. polystachyus* was done. The samples were dried at 60°C, ground, and 5 mL of 95% ethanol solution added. The ground samples were subjected to 400 W ultrasonic vibrations for 40 min and centrifuged at 8000 r·min^{-1} for 5 min. The supernatants were collected and used for further tests. The samples were filtered using 0.22 µm microporous membranes and 10 µL of the filtered sample was used for UPLC analysis. UPLC was performed at 30°C using acetonitrile/water cyanide (27:28, v/v) as the mobile phase at a flow rate of 0.2 mL min^{-1} and an ultraviolet/visible detector at 285 nm. All calculations were performed using the following regression equation: y = 6E+07x + 195578, R^2 = 0.999.

Results

Sequence features of the *CHI* promoter region: In this study, 417 bp, 522 bp, and 1202 bp fragments were obtained from the three rounds of TAIL-PCR (Fig. 1). The full-length promoter of the *CHI* gene was 2141 bp after splicing. Computational analysis using Promoter 2.0 and Neural Network Promoter Prediction (NNPP) databases revealed that the transcription start site of *Lpr-CHI*, with both to be T, which located at 847 nt and 619 nt upstream of the starting codon ATG, respectively. Computational analysis using PLACE databases revealed that *CHI* had typical promoter elements. A total of twenty-five CAAT boxes and sixteen TATA boxes were found. The potential regulatory elements associated with the stress hormone and light-related responses were also found in the promoter region, including the LTR, TGA-element, and the G-box. The important cis-regulatory elements identified in the *CHI* are listed in Table 2.

DNA methylation detection of CHI promoter in *L. polystachyus*: The result of Li Lab prediction showed that the *CHI* gene promoter was rich in GC from 50 to 740 bp, and contained three CpG islands; 259 (-1968~-1709) bp, 124 (-1547~ -1424) bp and 130 (-1411~ -1282) bp (Fig. 2), respectively. BiQ Analyzer 2.0 was used to analyze the sequencing results of DNA sequences after bisulfite treatment and DNA sequencing which revealed that there were 16 DNA methylation sites in the *Lpr-CHI* gene promoter, located at 1815, 1780, 1783, 1761, 1735, 1723, 1694, 1496, 1483, 1480, 1455, 1388, 1380, 1357, 1353, 1341 nt upstream of the starting codon ATG, respectively (Fig. 3). Multiple sample analysis revealed that there were seven DNA methylation types in the *Lpr-CHI* gene promoter region numbered as A to G. JavaSetup8u191 software was used to draw a dot plot of the obtained results. The C loci of 1388 upstream of the ATG in the 7 DNA methylation types was found to be methylated. Furthermore, the DNA methylation in other locis was found to be highly polymorphic.

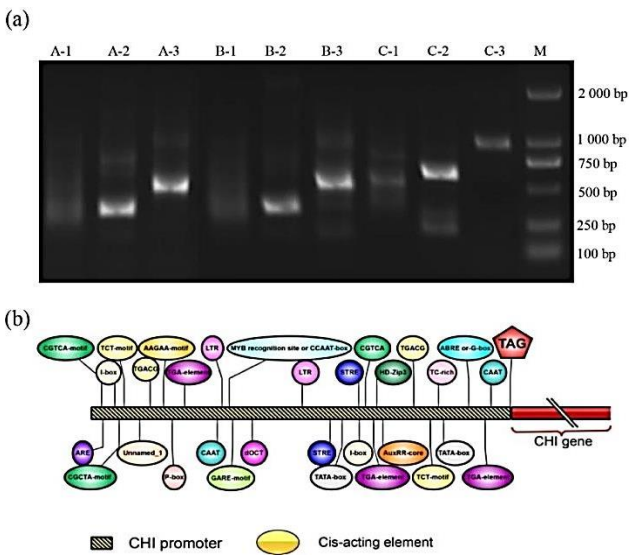

Fig. 1. Cloning and analysis of the *Lpr-CHI* gene promoter. (a): TAIL-PCR amplification of the *Lpr-CHI* gene promoter clone electrophoresis A-1: PCR products of CHIBY2 and AD1; A-2: PCR products of CHIBY3 and AD1; A-3: PCR products of CHIBY4 and AD1; B-1: PCR products of CHIBY5 and AD6; B-2: PCR products of CHIBY6 and AD6; B-3: PCR products of CHIBY7 and AD6; C-1: PCR products of CHIBY10 and AD3; C-2: PCR products of CHIBY2 and AD1; C-3: PCR products of CHIBY2 and AD1. (b): The *Lpr-CHI* gene promoter cis-acting elements distribution map.

Correlation analysis

The methylation ratios of the 7 DNA methylation types in *L. polystachyus* samples were significantly different ($p<0.01$). Besides, both the phlorizin content and *CHI* gene expression were also significantly different among the different types. The methylation ratios ranged from 50% to 81.25%. The phlorizin content ranged from 5.44 g/100g to 16.84 g/100g, with the highest content being 3 times the minimum content. The *CHI* gene expression level ranged from 0.91 to 5.11, with the maximum expression level being 5.6 times the lowest. A negative correlation was reported between phlorizin content and methylation ratio (R^2 = 0.9788) and *CHI* expression and methylation ratio (R^2 = 0.9606), However, the phlorizin content was positively correlated with the expression of CHI gene: y = 2.0665x + 3.4187 (R^2 = 0.98) (Figs. 3 and 4).

Table 2. Cis-acting elements in the *Lpr-CHI* gene promoter.

Type of cis-elements	Copies	Sequence	Function
CAAT-box	25	CAAT	common cis-acting element in promoter and enhancer regions
TATA-box	16	TATA	core promoter element around -30 of transcription start
TGACG-motif	8	TGACG	cis-acting regulatory element involved in the MeJA-responsiveness
CGTCA-motif	8	CGTCA	cis-acting regulatory element involved in the MeJA-responsiveness
TGA-element	5	AACGAC	auxin-responsive element
ABRE	5	CACGTG	cis-acting element involved in the abscisic acid responsiveness
MBS	3	CAACTG	MYB binding site involved in drought-inducibility
I-box	2	GTATAAGGCC	part of a light responsive element
TCT-motif	2	TCTTAC	part of a light responsive element
LTR	2	CCGAAA	cis-acting element involved in low-temperature responsiveness
G-Box	2	CACGTT	cis-acting regulatory element involved in light responsiveness
GARE-motif	1	TCTGTTG	gibberellin-responsive element
HD-Zip 3	1	GTAAT(G/C)ATTAC	protein binding site
CCAAT-box	1	CAACGG	MYBHv1 binding site
P-box	1	CCTTTTG	gibberellin-responsive element
AuxRR-core	1	GGTCCAT	cis-acting regulatory element involved in auxin responsiveness
TC-rich repeats	1	GTTTTCTTAC	cis-acting element involved in defense and stress responsiveness
ARE	1	TGGTTT	cis-acting regulatory element essential for the anaerobic induction
MRE	1	AACCTAA	MYB binding site involved in light responsiveness
Unnamed__4	21	CTCC	
as-1	8	TGACG	
Myb	4	CAACTG	
WRE 3	3	CCACCT	
MYB	3	CAACCA	
STRE	3	AGGGG	
MYC	2	CATTTG	
Myc	1	TCTCTTA	
AT~TATA-box	1	TATATA	
MYB recognition sit	1	CCGTTG	
dOCT	1	CTCGGATC	
AAGAA-motif	1	GAAAGAA	
Unnamed__1	1	CGTGG	

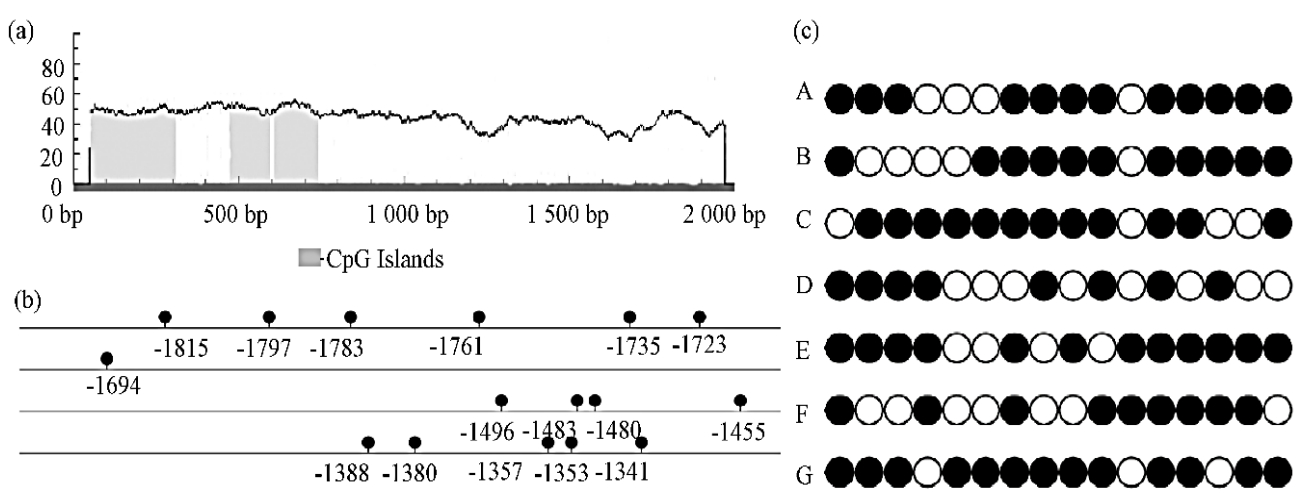

Fig. 1. The *Lpr-CHI* gene promoter prediction and analysis. (a): Prediction of CpG Island of *Lpr-CHI* gene promoter. (b): Schematic diagram of the methylation site distribution of the *Lpr-CHI* gene promoter. ●: Methylated CpGs. (c): DNA methylation type of the *Lpr-CHI* gene promoter. ●: corresponding to methylated Cs, ○: unmethylated Cs. From left to right represents the corresponding sites in (b).

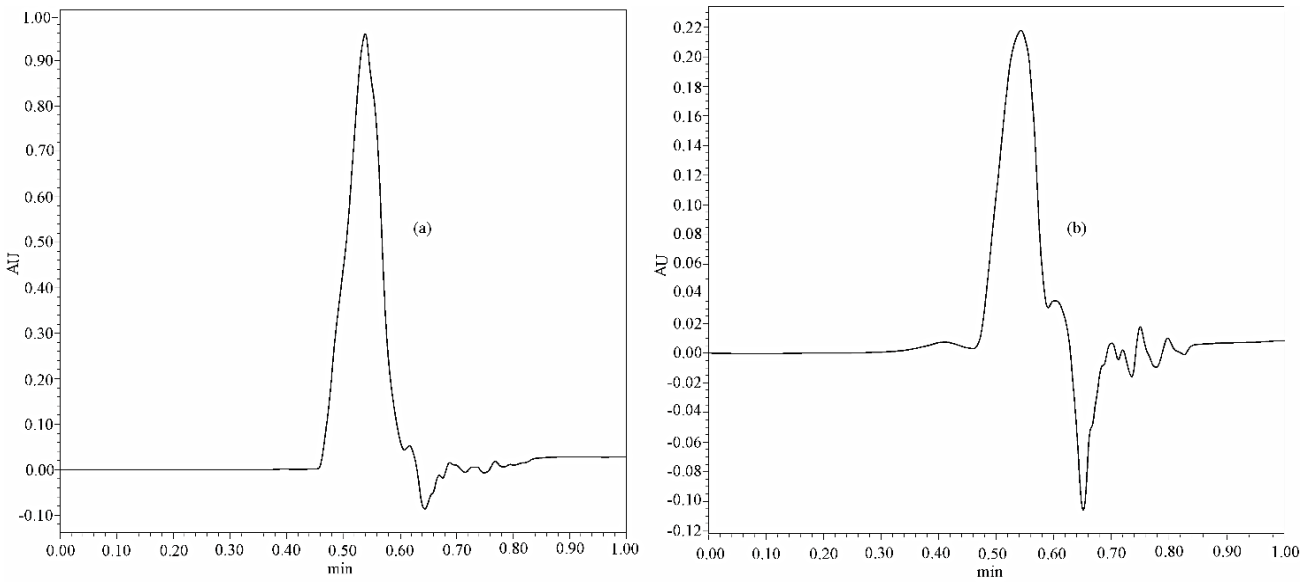

Fig. 3. UPLC chromatogram of phlorizin content of *L. polystachyus* Rehd sample. (a) the highest content of phlorizin; (b) the lowest content of phlorizin.

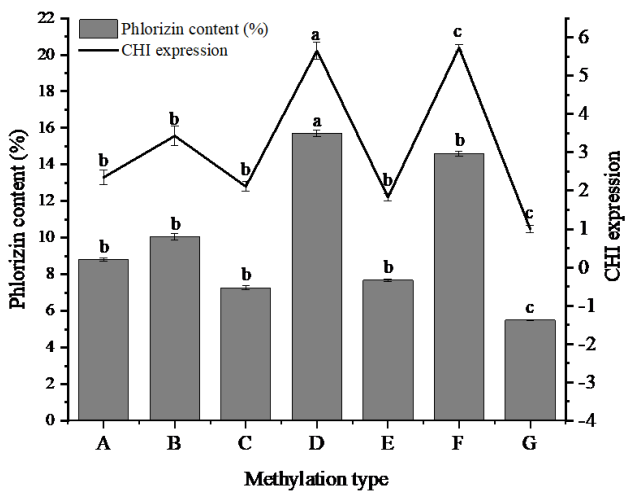

Fig. 2. Analysis of DNA methylation type, phlorizin content and *CHI* gene expression.

Discussion

In this study, the promoter of the *Lpr-CHI* gene (2141 bp) was successfully obtained using TAIL-PCR. The sequence contained several core promoter elements: TATA-box, CAAT-box, etc., which were consistent with the conservative plant gene promoters and regulatory elements. Besides, *L. polystachyus* contained 4 light-responsive elements: G-box, TCT-motif, I-box, and MRE, with a total of 7 regulatory sites. Moreover, in potatoes, tomatoes, Arabidopsis and other species, the promoter region of the *CHI* gene is reported to contain a variety of photo-responsive elements (Chen *et al*., 2015), an indication that light has an important effect on the *CHI* gene expression. A variety of hormone response elements in the promoter sequence of the *CHI* gene were also reported. Lin *et al*., (2020) found that the type I CHIs of five Fagaceae contain MeJA response elements. A study reported that Methyl Jasmonate (MeJA) treatment induced accumulation of Sophora flavescens Aiton CHI transcripts and enhanced the accumulation of pterocarpans (Kim *et al*., 2020). A total of 16 MeJA response elements were detected and more than half of these were hormone response elements. It was speculated that MeJA greatly influences the expression level of the *Lpr-CHI* gene. Saure (1990) showed that high gibberellin levels were not conducive to the production of flavonoids in plants. In this study, the discovery of the gibberellin response site in the *L. polystachyus CHI* gene indicated that its expression level may be affected by gibberellin. Therefore, there is a need for future studies to investigate the mechanism of action of gibberellin on the *CHI* gene and its role in flavonoid biosynthesis.

There are different genomic methylation levels in different species, different organs or tissues within the same species, and in different developmental stages. Genomic methylation plays a major regulatory role in plant gene expression, cell differentiation, metabolism, and other physiological processes (Xiao, 2006). DNA methylation within the promoter region of plant functional genes prevents transcription factors from binding to the methylated promoter, thus inhibiting gene expression (Curradi *et al*., 2002). In this study, there were 16 methylation sites and 7 types of *CHI* gene promoters in *L. polystachyus*, however, only 1388 sites upstream of ATG were methylated in all the 7 methylation types. This study showed a negative correlation between *CHI* gene promoter methylation and CHI gene expression in *L. polystachyus.* This indicated that *CHI* gene promoter methylation is one of the effective regulatory mechanisms of gene expression in this species. Phlorizin is one of the main medicinal components in *L. polystachyus*. A positive correlation was shown between the level of accumulation of phlorizin and *CHI* gene expression levels. This indicates that the content of phlorizin in *L. polystachyus* is also regulated by methylation of the *CHI* gene promoter. Studies have reported that when maize is treated at low

temperature, the DNA methylation level of the *ZmMI 1* gene decreases and is specifically expressed under low temperature (Steward *et al*., 2002). In another study, when tobacco leaves were treated at low temperatures, the *NtGPDL* gene was demethylated and gene expression was up-regulated (Choi & Sano, 2007). This indicates that low temperature reduces the level of DNA methylation and increases gene expression. Therefore, upon the discovery that the CHI gene promoter has a low-temperature response element, it was speculated that the gene could maintain the minimum expression level of the gene through the coordination of low temperature and DNA methylation.

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