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Molecular and functional characterization of the chalcone synthase gene (*DcCHS1*) promoter in response to hormones

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Abstract

Dragon's blood, which is widely used in traditional medicines, is a red resin that forms in the stem of *Dracaenas* plants after they are wounded. Flavonoids are the main compounds in dragon's blood. Research on dragon's blood has focused mainly on its chemical composition and pharmacological activity, whereas the molecular mechanism of dragon's blood formation is still unknown. Chalcone synthase (CHS) is the first key enzyme in biosynthesis of flavonoids. However, little information is available on regulation of CHS gene expression in *Dracaenas* plants. In this study, a 1404-bp promoter region of *DcCHS1* was cloned from *Dracaena cambodiana*. A typical TATA-box was located at −24 bp to −20 bp upstream from the transcription start site (TSS) of *DcCHS1*. Several typical regulatory elements were found in the *DcCHS1* promoter region, including *cis*-acting elements involved in light responsive, ABRE for responding to abscisic acid, TCA-element for responding to salicylic acid, TGA-element for auxin, and TC-rich repeat elements involved in defense and stress responsiveness. Promoter deletion analysis revealed that the *DcCHS1* promoter induces the specific expression of the GUS gene, and 149 nucleotides upstream of the TSS were sufficient for the expression function. Transient gene expression assay revealed that the expression of *DcCHS1* promoter was positively regulated by methyl jasmonate and 6-benzylaminopurine.

Keywords: Dragon's blood; *Dracaena cambodiana*; flavonoid; hormone; regulatory elements.
Abbreviations: ABA_abscisic acid; 6_BA- 6-Benzylaminopurine; CHS_chalcone synthase; ETH_ethephon; GA_gibberellic acid ; GUS_β-glucuronidase; MeJA_methyl jasmonate; SA_ salicylic acid ; TSS_transcription start site.

Introduction

Dragon's blood is a red resin that forms in the stem of four distinct plant genera: *Croton*, *Dracaena*, *Daemonorops*, and *Pterocarpus* (Fan et al., 2014). In Chinese folk medicine, the resin extracted from the stems of *Dracaena cochinchinensis* has been used as dragon's blood (Luo et al., 2011; Yi et al., 2011). In traditional Chinese medicine, dragon's blood is commonly prescribed to invigorate blood circulation for treatment of traumatic injuries, blood stasis, and pain. Dragon's blood has antibacterial, antispasmodic, anti-inflammatory, analgesic, antidiabetic, and antitumor activities, and it is also known to enhance immune function, promote skin repair, stop bleeding, and enhance blood circulation (Fan et al., 2014).

Flavonoids are the main compounds in dragon's blood (Masaoud et al., 1995; Dai et al., 2012; Zheng et al., 2012; Mei et al., 2013; Fan et al., 2014). Flavonoid biosynthetic pathway has been well-established in *Arabidopsis* (*A. thaliana*), petunia (*Petunia hybrida*), snapdragon (*Anfirrhinum majus*), and maize (*Zea mays*) (Petroni and Tonelli, 2011; Pourcel et al., 2012). This pathway is initiated by condensation of one molecule of 4-coumaroyl-CoA with three molecules of malonyl-CoA by the chalcone synthase (CHS) enzyme. The successive steps catalyzed by chalcone isomerase, flavonoid 3-hydroxylase, flavonoid 3'-hydroxylase, dihydroflavonol-4-reductase(DFR), anthocyanidin synthase, and 3-glycosyltransferase enzymes lead to production of anthocyanin pigments. The actions of flavone synthase and flavonol synthase lead to branching of the flavonoid pathway to flavones and flavonols, respectively (Forkmann and Martens, 2001; Carbone et al., 2009). CHS is considered a key enzyme in regulating flavonoid biosynthesis (Koes et al., 2005; Ferreyra et al., 2012). *CHS* has been well-characterized in many plant species because of its essential role in flavonoid biosynthesis. Its transcription is regulated by heredity, hormones, and abiotic and biotic stress (Cheng et al., 2009). Transcription of *CHS* is under a complex system of regulation, in which a number of potential regulatory elements have been identified (Hartmann et al., 2005). In *Arabidopsis*, the ACGT-containing element (ACE), R-recognition element, and MYB recognition element can be bound by transcription factors HY5, TT8 (transparent Testa8), and PAP1 (production of anthocyanin pigment 1), respectively, to direct tissue-specific and light-responsive gene expression (Hartmann et al., 2005; Lee et al., 2007; Dare et al., 2008). In tobacco (*Nicotiana tabacum*), two basic helix-loop-helix transcription factors (TFs) (i.e., NtAN1a and NtAn1b) interact with R2R3-MYB TF (NtAn2) and activate the promoters of *CHS and DFR* for anthocyanin regulation (Bai et al., 2011). The interaction between the expression of regulatory elements upstream of the *CHS* gene and trans-acting factors clearly merits further investigation as it will help regulate the accumulation of *Dracaena cambodiana* flavonoids. Thus, understanding the regulation of flavonoids in *D. cambodiana* is critically important in determining the mechanism of dragon's

blood formation. However, to date, no information is available on the regulation of the flavonoid pathway in *D. cambodiana* (Wang et al., 2014). In this paper, we describe the isolation and functional analysis of the *CHS* in *D. cambodiana (DcCHS1*) promoter regions. Deletion analysis with GUS-reporter gene was performed using an *Agrobacterium*-mediated transient assay to identify the regions of the *DcCHS1* promoter regulating transcription in tobacco leaves in response to hormones.

Results

DcCHS1 genomic sequence analysis and determined on the transcription start site (TSS) of DcCHS1 promoter

A 1277 bp fragment was isolated from the genomic DNA of *D. cambodiana* via PCR strategy. Comparing cDNA to genomic sequences, the *DcCHS1* gene contains two exons and one intron. Two exons (178 and 995 bp in length) were separated by one intron (104 bp in length) (Fig. 1A). The TSS of *DcCHS1* promoter was determined by 5'-RACE using the total RNA extracted from the latex. Six positive clones were sequenced and aligned with the *DcCHS1* gene and promoter (Fig. 1B). The TSS is an adenine (A) at -99 bp upstream of translation initiation codon.

Cis-regulatory element content of the DcCHS1 promoter

A 1404 bp fragment was isolated from the genomic DNA of *D. cambodiana* via PCR strategy. The PLACE (Higo et al., 1999) and PlantCARE (Lescot et al., 2002) databases were used to identify matches in the *DcCHS1* promoter to the *cis*-regulatory elements of other plants species. A typical TATA-box was located at -24 to -20 bp upstream from the TSS. Several typical regulatory elements were found in the *DcCHS1* promoter (Fig. 2). These elements include the following: *cis*-acting elements involved in light responsive, including 3-AF1 binding site, ATCT-motif, Box 4, CATT-motif, GATA-motif, G-box, and GT1CONSENSUS; ABRE for responding to abscisic acid (ABA); TCA-element for responding to salicylic acid; TGA-element for auxin; TC-rich repeat elements involved in defense and stress responsiveness; HSE, which functions in high temperature stress. Additional predicted MBS **(**MYB binding site) was involved in drought inducibility.

Basal expression analysis of the DcCHS1 promoter

Histochemical GUS assays showed that the negative control was not stained, whereas 35S::GUS positive control was strongly stained. The strong activation of the GUS reporter gene by the CHSP1 to CHSP7 deletions was detected in leaves (Fig. 3A). For more precise measurement of GUS expression, we performed quantitative GUS assays. Compared with the CaMV35S-mediated GUS expression (pC1301), plants transformed with constructs CHSP1 to CHSP7 showed higher degree basal expression (Fig. 3B). These data revealed that *DcCHS1* promoter facilitates the specific expression of the GUS gene, and 149 nucleotides upstream of the transcription start site were sufficient for the expression function.

Effects of phytohormones treatments on DcCHS1 promoter

To verify the role of phytohormone-responsive *cis*-acting elements that were found in the promoter of *DcCHS1*, we tested the CHSP1 constructs of *DcCHS1* with regard to the inducibility of GUS expression in tobacco leaves by treating the plants with methyl jasmonate (MeJA), ABA, gibberellic acid

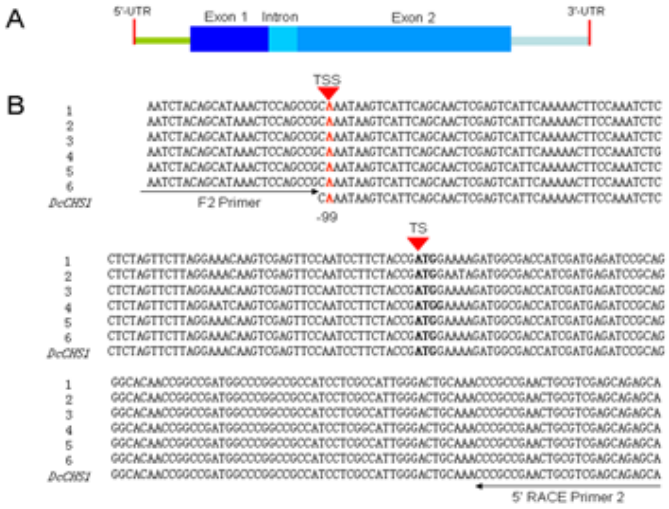

Fig 1. Gene structure of the *DcCHS1* (A) and multiple sequence alignment of the cDNA 5' end of *DcCHS1* transcripts (B). Exons are indicated as blue boxes and introns as grew grid boxes. cDNA ends were amplified using 5'-RACE strategy. The ATG is in black frame. A 99 bp distance upstream of ATG is the putative start of transcription.

Fig 2. Putative *cis*-elements in *DcCHS1* promoter as predicted by PLACE and PlantCARE. The *cis*-elements predicated are underlined at lower position of sequence.

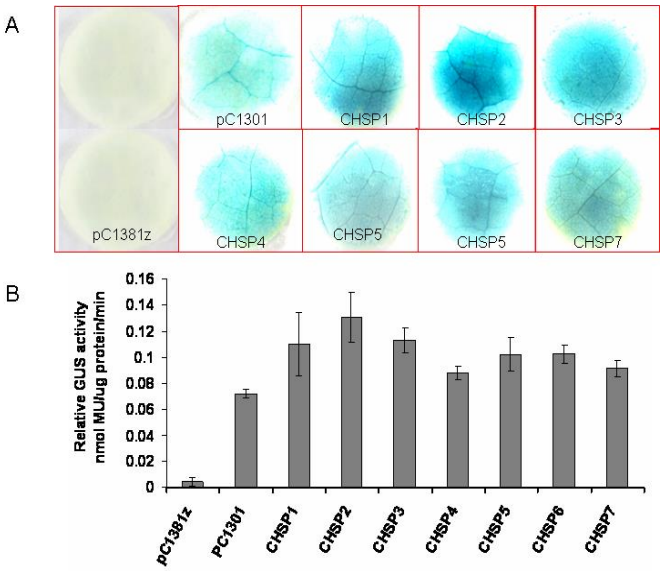

Fig 3. Histochemical and fluorometric analysis of GUS activity in transiently transformed tobacco leaves. (A) Histochemical analysis of GUS activity in transiently transformed tobacco leaves. (B) Fluorometric analysis of GUS activity in transiently transformed tobacco leaves. GUS activity was analyzed fluorometrically and expressed as nmol 4-methylumbelliferone (MU) mg-1 protein min-1. Data are mean values from three independent assays of tobacco leaf extracts, and error bars show the standard deviations of the replicates.

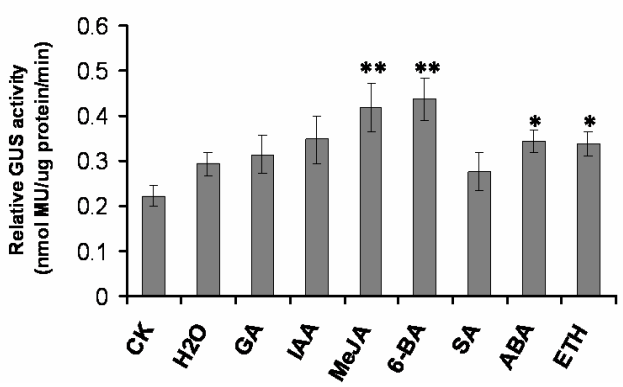

Fig 4. GUS activity driven by *DcCHS1* promoter-GUS chimeric constructs after treatment with phytohormones. Data are mean values from three independent assays of tobacco leaf extracts, and error bars show the standard deviations of the replicates. The difference from mock was assessed by one-sided paired *t*-test (two stars corresponding to P≤0.01; one star corresponding to P≤0.05)

(GA), 6-Benzylaminopurine (6-BA), salicylic acid (SA), and ethephon (ETH). GUS fluorometric assay was performed in agro-infiltrated tobacco leaves after treatment (Fig. 4). After treatment with MeJA or 6-BA, GUS activity from CHSP1 construct showed significant increase (P≤0.01) compared to the mock-treated control. Meanwhile, for ABA or ETH treatment, GUS activity from CHSP1 construct showed significant increase (P≤0.05) compared to the mock-treated control. In GA, IAA, or SA treatment, GUS activity from CHSP1 construct showed no significant induction. We subsequently performed a real-time quantitative PCR analysis to detect the expression patterns for *DcCHS1*.

As showed in Fig. 5A, MeJA treatment significantly increased the expression of *DcCHS1* up to 1 h post treatment, after which the expression declined to less than the control value until 24 h post treatment. However, the expression of *DcCHS1* significantly increased up to 12 h post treatment (Fig. 5B). These data revealed that *DcCHS1* transcription was significantly upregulated by 6-BA and MeJA treatment.

Discussion

CHS expression has been found to be induced by developmental and environmental cues, such as fruit ripening stages, UV light, wounding, and treatment with elicitors (Moriguchi et al., 2001 ; Zhang et al., 2011; Sun et al., 2014). *CHS* promoter region has a number of *cis*-acting elements associated with adversity, hormones, tissue specificity, and other systems with inducible expression apart from key regulatory elements, such as ACE elements, silencers, H-box sequences, and AT-rich units (Weisshaar et al., 1991; Yu et al., 1993). In *P. hybrida* and *Pisum sativum*, the presence of acting elements in the *CHS* promoter is correlated with the expression of adversity, as well as the temporal and spatial specificity (Schmid et al., 1990; van der Meer et al., 1992). The *CHS* gene promoter sequence of *A. thaliana* contains the UV-A/UV-B/blue ray response element, and its expression is strongly induced by blue and ultraviolet rays (Hartmann et al., 1998). In *Ginkgo biloba*, *GbCHS* promoter also contained several environmental factors, the cell cycle inductive response elements, and *cis*-acting elements associated with hormone induction (Li et al., 2014). The activation of petunia *CHSA* promoter has been previously shown to be flower-specific, light-dependent, and UV-inducible, as well as developmentally regulated (van der Meer et al., 1990, 1992). Differential expression patterns of the *CHS* genes are correlated with the types of flavonoid compounds synthesized (Lo et al., 2002). Little information was known about the expressional regulation of *DcCHS1* (Wang et al., 2014). To explore the regulation mechanism of *DcCHS1* expression, we cloned and characterized the 1404 bp promoter of *DcCHS1*, which encodes CHS, the first key enzyme in biosynthesis of flavonoids (Koes et al., 2005; Ferreyra et al., 2012). The TSS is mapped to an adenine at 99 bp upstream of the start cordon and 24 bp downstream of the TATA-box. Some putative regulatory elements, such as *cis*-acting elements involved in light responsive, ABRE for responding to ABA, TCA-element for responding to salicylic acid, and TGA-element for auxin, were also detected. The deletion analysis of the promoter of *DcCHS1* using tobacco plant revealed that the sequence within -149 nucleotides from the TSS was sufficient for the expression function and this region. Plant hormones play a very important role in the process of plant growth, development, and metabolic regulation. MeJA is an important signaling agent and potent elicitor involved in a range of physiological responses (Balbi and Devoto, 2007). Treatment with MeJA stimulates *CHS* expression in *Vitis vinifera* L. (Belhadj et al., 2008) and enhances the antioxidant activity and flavonoid content in blackberries (Wang et al., 2008). In the present study, MeJA was shown to induce pCHSP1-GUS up to 2.1-fold than the control. Moreover, MeJA treatment significantly increased the *DcCHS1* expression. The result suggests that *DcCHS1* is possibly involved in methyl jasmonate-responsive signal transduction. Report showed that 6-BA can stimulate flavonoid production in *D. cambodiana* (Yang et al., 2009). In the present experiment, 6-BA caused the increase of pCHSP1-GUS activity in tobacco leaves to 2.5-fold than the control. 6-BA treatment significantly increased the expression of *DcCHS1*.

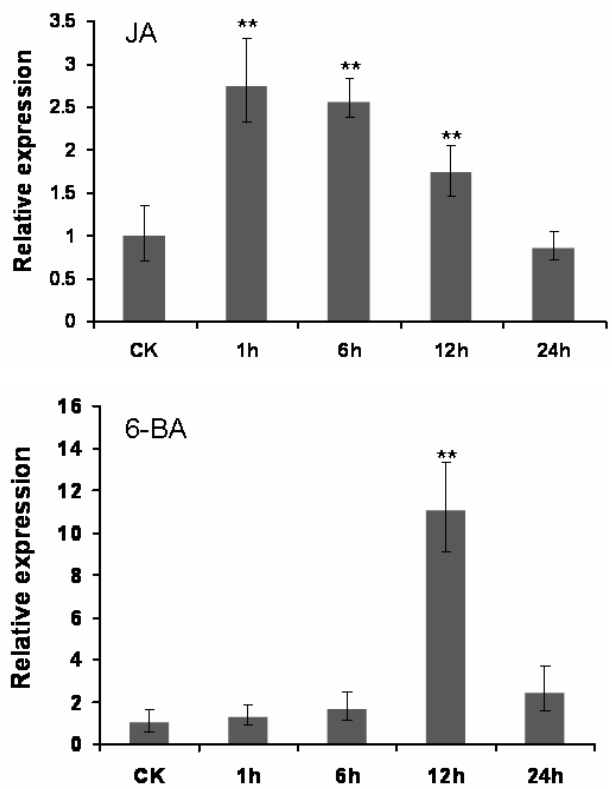

Fig 5. Effect of (A) MeJA and (B) 6-BA on *DcCHS1* expression. The relative expression was normalized to ACTIN gene. Bars indicate the standard errors of the three replicates. The difference from mock was assessed by one-sided paired *t*-test (two stars corresponding to P≤0.01).

Moreover, for ABA or ETH treatment, GUS activity from CHSP1 construct showed significant increased compared to the control. In summary, we can conclude that the *DcCHS1* promoter stimulated GUS activity by MeJA, 6-BA, ABA, or ETH via response to corresponding *cis*-elements. Taken together, the *DcCHS1* promoter sequence possesses ABA responsive elements, but not for MeJA, 6-BA, ABA, or ETH responsive elements. Further deletion analyses will be required to elucidate the *cis*-acting elements of the *DcCHS1* promoter region. Finally, the fragment upstream from the transcription start site of *DcCHS1* may be utilized to drive the transgenic expression in the development of transgenic plants. This study provides useful information for the further study of *CHS* and its regulator effect on the biosynthetic process of flavonoids, thereby allowing researchers to improve the flavonoid contents in *D. cambodiana*.

Materials and methods

Plant materials and bacterial strains

D. cambodiana were growing at the experimental farm of the Chinese Academy of Tropical Agriculture. Tobacco (*N. tabacum)* plants were grown in a growth chamber at 25 °C under 16/8 h day/night cycle for six weeks. *Escherichia coli* strain DH5α was used for the cloning and propagation of all recombinant plasmid vectors. *Agrobacterium tumefaciens* strain GV3101 was used for tobacco leaf infiltration.

Cloning of DcCHS1 genomic sequence

Genomic DNA was isolated from *D. cambodiana* leaves by using the cetyltrimethylammonium bromide method, as described by Murray and Thompson (1980). Based on the *DcCHS1* cDNA sequence (Wang et al., 2014), a pair of primers (CHSF: 5′-ATGGTGGCCATCGATGAGATCC-3′ and CHSR: 5′-GTTAGTAGCCACACTGCGCAGC-3′) was designed, which was used to amplify the full-length genomic sequence from genomic DNA templates. Fragment was amplified using LA Taq DNA Polymerase (TaKaRa), following the manufacturer's recommended reaction conditions. The PCR product was cloned into pMD19-T simple vector (TaKaRa), and several clones were sequenced for each reaction.

5′-RACE for identification of the transcription start site of DcCHS

Total RNA from *D. cambodiana* stem was extracted using the RNeasy Plant Mini Kit (QIAGEN) and treated with DNase1 (Thermo), in accordance with the manufacturer's instructions. The *DcCHS1* transcription start site was determined by a 5'-rapid amplification of cDNA ends (RACE) strategy based on a SMART RACE cDNA Amplification Kit (Clontech, USA) protocol. Two specific primers, 5RACE1 (5'-ACATGCGCTT-GAACTTCTCCTTGAG-3') and 5RACE2 (5'-TGCTCTGC-TCGACGCAGTTCGGCGG-3'), were designed from the *DcCHS1* cDNA sequence (Wang et al. 2014). A primary PCR was performed with adaptor primer F1 (5'-AAGCAGTGGT-ATCAACGCAGAGT-3') and specific primer 5RACE1. Fifty times diluted primary PCR products were then amplified using adaptor primer F2 (5'-ACGCAGAGTGGCCATTATGGCC-GGG-3') and nested primer 5RACE2. Second-round PCR products were introduced into the pMD19-T simple vector (TaKaRa) for sequencing. The promoter transcription start site was obtained by aligning the resulting sequences with that of the promoter.

Analysis of Cis-regulatory element content of the DcCHS1 promoter

The promoter region of *DcCHS1* was isolated using PCR-based DNA Walking, in accordance with the manufacturer's instructions for the Universal GenomeWalkerTM Kit (Clontech). The promoter sequence of *DcCHS1* was analyzed using PlantCARE (http://bioin formatics.psb.ugent.be/webtools/ plantcare/html/) and PLACE (http://www.dna.affrc.go.jp).

Construction of DcCHS1 promoter-GUS fusions

For construction of the *DcCHS1* promoter-GUS fusion genes, a series of *DcCHS1* promoter fragments (from -1404 to +84 bp) of various lengths were obtained by PCR-amplification with the use of the forward primers: (P1: 5'- GCGGTCGACT-CTAGAGATTCGAGTACTTTG-3'; P2: 5'-GCGGTCGACGA-AGGGATAGAAAGAATCAAT-3'; P3: 5'-TTAGTCGAC-GTTGGAGGCCTCCCTAAGTAT-3'; P4: 5'-GCGGTCGAC-GTATTTGTTGTTATAATCCAG-3'; P5:5'-GGAGTCGACT-ATGTACCACTGTAGCCTAGT-3'; P6: 5'-GGTGTCGAC-AAGAATTAGCGCTAGGATGG-3'; P7:5'-CATGTCGACA-CTCAAGCCCTTGTAGATAC-3') and the reverse primer: (5'-CTACTGCAGGGGTTTGCAG TCCCAATGGCG-3'). A *Sal*I restriction site was introduced into the forward primers, and a *Pst*I restriction site was introduced into the reverse primer. The amplified sequences were then digested by *Pst*I and *Sal*I and cloned into the *Pst*I /*Sal*I digested binary vector pCAMBIA1381Z (CAMBIA Company, Australia). The seven constructions were described as CHSP1 (-1044 to +84), CHSP2 (-1273 to +84), CHSP3 (-954 to +84), CHSP4 (-736 to +84),

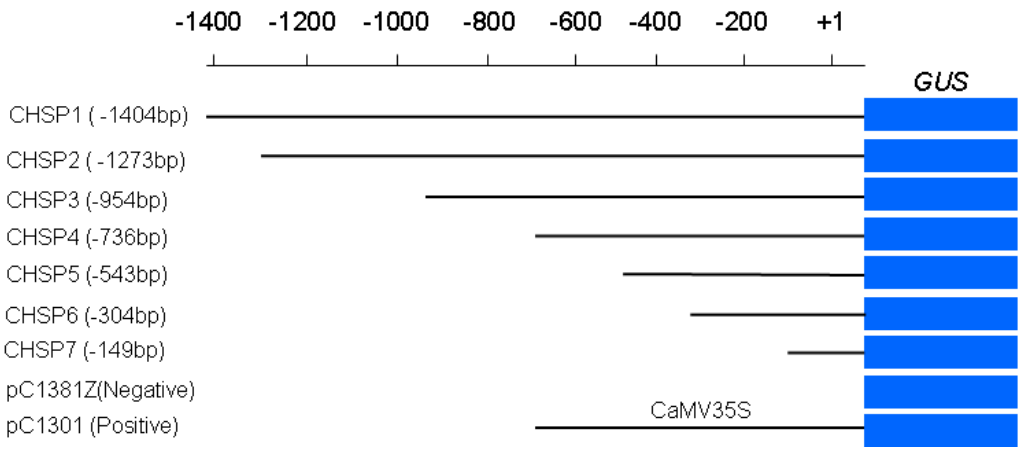

Fig 6. Schematic of *DcCHS1* promoter deletion-GUS constructs for assaying reporter gene transient expression in tobacco leaves. The transcription start site is indicated as +1. Promoter fragments of different sizes were inserted into the pCAMBIA1381Z vector containing the GUS reporter gene. The numbers of each construct indicate the distance from the *DcCHS1* transcription start site. Plasmid pCAMBIA1381Z and pCAMBIA1301 were used as negative and positive controls, respectively.

CHSP5 (-543 to +84), CHSP6 (-304 to +84), and CHSP7 (-149 to +84) (Fig. 6). All of the constructions were sequenced and verified by PCR and digest reactions. Furthermore, pCAMBIA1301 was used as positive control, whereas pCAMBIA1381Z was used as a negative control.

Agrobacterium-mediated transient expression assay

Agrobacterium-mediated transient expression assays were performed as previously described (Xu et al., 2010). A single colony of *A. tumefaciens* strain GV3101 harboring a promoter construct was inoculated into 3 mL of LB liquid medium supplemented with rifampicin (60 μg/mL) and kanamycin (50 μg/mL) at 28 °C for 24 h. A 1-mL culture was transferred into 25 mL of LB liquid medium with the same antibiotic and incubated at 28 °C for 5 h with shaking. *Agrobacterium* cells were harvested after centrifugation at 4,000*g* for 15 min, resuspended in infiltration solution (10 mM MES, pH 5.7, 10 mM $MgCl_2$, and 100 μM acetosyringone), incubated at 28 °C for 3 h, and adjusted to an OD_{600} of 0.8 for infiltration to tobacco leaves. After agroinfiltration at the abaxial surfaces of tobacco leaves with the use of a needleless syringe (Yang et al., 2000), the tobacco plants were maintained in a moist chamber at 16/8 h (day/night) cycle at 25 °C for 48 h. All stress treatments were applied 48 h after agroinfiltration.

Hormone treatments

To characterize the induction of promoter activity by the stress-responsive hormones, tobacco plants were treated with MeJA, ABA, GA, 6-BA, SA, and ETH. For MeJA, GA, ABA, 6-BA, SA, and ETH treatments, the tobacco plants were sprayed with 100 μM MeJA, 50 μM GA, 100 μM ABA, 50 μM 6-BA, 100 μM SA, and 100 μM ETH in distilled water, respectively. Tobacco plants treated with MeJA GA, ABA, 6-BA, SA, and ETH were then incubated in a vinyl bag. The control plants were sprayed with water. All treated plants were maintained in a moist chamber at 16/8 h day/night cycle at 25 °C. Leaves were used for GUS analysis after 15 h. All experiments were repeated at least three times.

Histochemical and fluorometric assays for GUS activity

Histochemical staining was performed according to Jefferson (1987) by using X-Gluc as a substrate. Tobacco leaves were incubated in GUS staining solution [80 mM sodium phosphate buffer (pH 7.0), 0.4 mM potassium ferricyanide, 0.4 mM potassium ferrocyanide, 8 mM EDTA, 0.5 mg/ml 5-bromo-4-chloro-indolyl-β-D-glucuronide (X-Gluc), and 0.05% Triton X-100] for 24 h at 37 °C, followed by chlorophyll removal from the leaves by successive washes with 70% ethanol at 37 °C. Stained tissues were examined by light microscopy. Transient expression of GUS activity in the treated tobacco leaves was performed as described previously (Jefferson, 1988). Tobacco leaf tissue was homogenized in 600 μL extraction buffer (50 mM sodium phosphate buffer [pH 7.0], 10 mM EDTA [pH 8.0], 0.1% Triton X-100, 0.1% (w/v) sodium dodecyl sulfate, 10 mM β-mercaptoethanol) and centrifuged at 12,000g for 15 min at 4 °C. Approximately 20 μL of the supernatant was mixed with 180 μL of pre-warmed (37 °C) GUS assay solution (1 mM methyl-4-umbelliferyl-D-glucuronide in extraction buffer) and incubated at 37 °C for 40 min. About 50 μL mixture was transferred into 950 μL stop buffer (0.2 M Na_2CO_3). GUS activity was measured with a Glomax Multi+ detection system (Promega) at 365 (excitation) and 455 (emission) nm. Stop buffer and 50 nM to 1 μM 4-methylumbelliferone (4-MU) were used for calibration and standardization. The total concentration of protein extracts from the tested samples was normalized by dilution with extraction buffer as described previously (Bradford 1976). GUS activity was expressed as nM of 4-MU generated per min per mg of soluble protein. The GUS measurements were repeated at least three times with similar results.

Statistical analysis

The experimental results are reported as means±standard error of three replicates. The data were analyzed with one-way ANOVA by using Statistical Product and Service Solutions (SPSS, Version 16.0) at $P \leq 0.01$ or $P \leq 0.05$ (Fisher's protected least significant difference).

Conclusion

In conclusion, we isolated and characterized a *CHS* promoter from *D. cambodiana*. Functional and bioinformatic analyses reveal that the *CHS* promoter contains a series of *cis*-regulatory elements that could respond to hormones. Transient gene expression assay indicated that *DcCHS1* promoter from *D. cambodiana* directs the GUS reporter gene expression. GUS expression was upregulated by MeJA and 6-BA. This study provides information necessary to understand the regulated

expression characteristic of *DcCHS1* in biosynthesis of flavonoids and contribute to elucidation of the mechanism of formation of dragon's blood.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Authors' contributions

HFD WLM SQP conceived and designed the experiments, JYW HLL DG performed the experiments, JYW SQP anlyzed the data, WLM SQP drafted the manuscript. All authors have read and approved the final manuscript.

Acknowledgments

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