Study on the Regulation Mechanisms of cGMP-dependent Gene Expression of Soybean Chalcone Synthase

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ダイズカルコン合成酵素の cGMP 依存的遺伝子発現調節機構に関する研究

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DEDICATION

I dedicate this thesis to my mother "May". It was through her hard work, sacrifice, suffering and persistence that I was given the opportunity to achieve my academic goals.

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Chapter 1. General Introduction

Living organisms can transduce and decode information. Intracellular signaling molecules often function as intermediates in such transductions. A secondary messenger cyclic nucleotide, 3',5'-cyclic guanosine monophosphate (cGMP), regulates complex signaling cascades in plant as well as animal cells and is critically implicated in many processes including phytochrome signal transduction [1], flowering [2], promotion of seed germination [3], and response to salt and osmotic stress [4]. At the transcriptional level, Pasqualini et al. [5] reported the role of cGMP in ozone- and nitric oxide (NO)-dependent transcriptional regulation of phenylalanine ammonia lyase (*PALa*) and pathogenesis-related protein 1a (*PR1a*), both associated with stress response in plants. In a phytochrome-mediated phototransduction study, cGMP as well as NO were reported to stimulate anthocyanin production and the chalcone synthase (CHS) gene, which encodes a key enzyme in the anthocyanin biosynthetic pathway [1]. Recently, it was reported that cGMP and NO regulate the expression of nine genes besides *CHS*-encoding flavonoid biosynthetic enzymes in soybean (*Glycine max*) [6]. Studies in *Arabidopsis* have also revealed that a number of genes are potentially regulated by cGMP including transcription factors (TFs), transporters and pathogenesis-related proteins [7], and genes responsible for chemical homeostasis, cellular metabolism, and ion transport [8]. In a recent study, cGMP was reported to regulate H_2O_2 accumulation in salt-stressed *Arabidopsis* by stimulating plasma membrane H^+-ATPase gene expression [9]. However, the mechanisms of the transcriptional control of these genes by cGMP remain to be elucidated. Evidence that cGMP is an intermediate of NO signaling has been obtained in several systems [10, 11], and NO can elevate cGMP levels in several plant tissues [6, 12-14]. NO-dependent and cGMP-mediated processes are involved in *Lilium* pollen growth and orientation [15] as well as in gravitropic responses in soybean roots [16]. However, the

molecular mechanisms for the linkage between NO and cGMP in signal transduction are largely unknown, aside from a recent report on a NO-binding molecule with guanylate cyclase activity [17].

Flavonoids/isoflavonoids are a group of plant secondary metabolites synthesized almost exclusively by legumes. They play important roles in the coloration of flowers and other plant organs, UV protection as an antioxidant [18], plant–microbe interactions [19], and defense as antimicrobial phytoalexins against pathogen infection [20]. Isoflavonoids also offer significant health benefits for humans [21, 22]. There are three major isoflavones (genistein, daidzein, and glycitein) in soybean [23]. As depicted in Fig. 1, which shows the flavonoid biosynthetic pathway in soybean, all genes except ANS are both cGMP- and NO-responsive [6] and the key step of the flavonoid biosynthetic pathway is committed by CHS, which uses 4-coumaroyl-CoA as a substrate [24]. Expression of *CHS* is regulated by a wide range of environmental stimuli and developmental control [25]. Despite a high degree of sequence similarity within and across species, CHS genes play diverse roles during plant development or in response to environmental stimuli, for instance, *CHS* is constitutively expressed in floral organs, whereas it is induced by UV light [26], pathogen attack [27], elicitors[28], or wounding [29]. Moreover, *CHS* expression can be regulated by the circadian clock [30]. A single copy of *CHS* has been reported in *Arabidopsis*, parsley, and snapdragon, whereas petunia and soybean contain multiple copies, with soybean containing nine genes (*GmCHS1*–9) [31]. A recent microarray study of soybean has revealed the critical role of *GmCHS7* and *GmCHS8* in isoflavonoid synthesis, confirming that enhanced expression of one or both of these genes during seed development is specifically associated with higher isoflavonoid content in the seeds at maturity [32]. For effective metabolic

engineering of flavonoid biosynthetic pathway and isoflavone content in soybean, it is necessary to understand the mechanisms controlling the expression of these CHS genes.

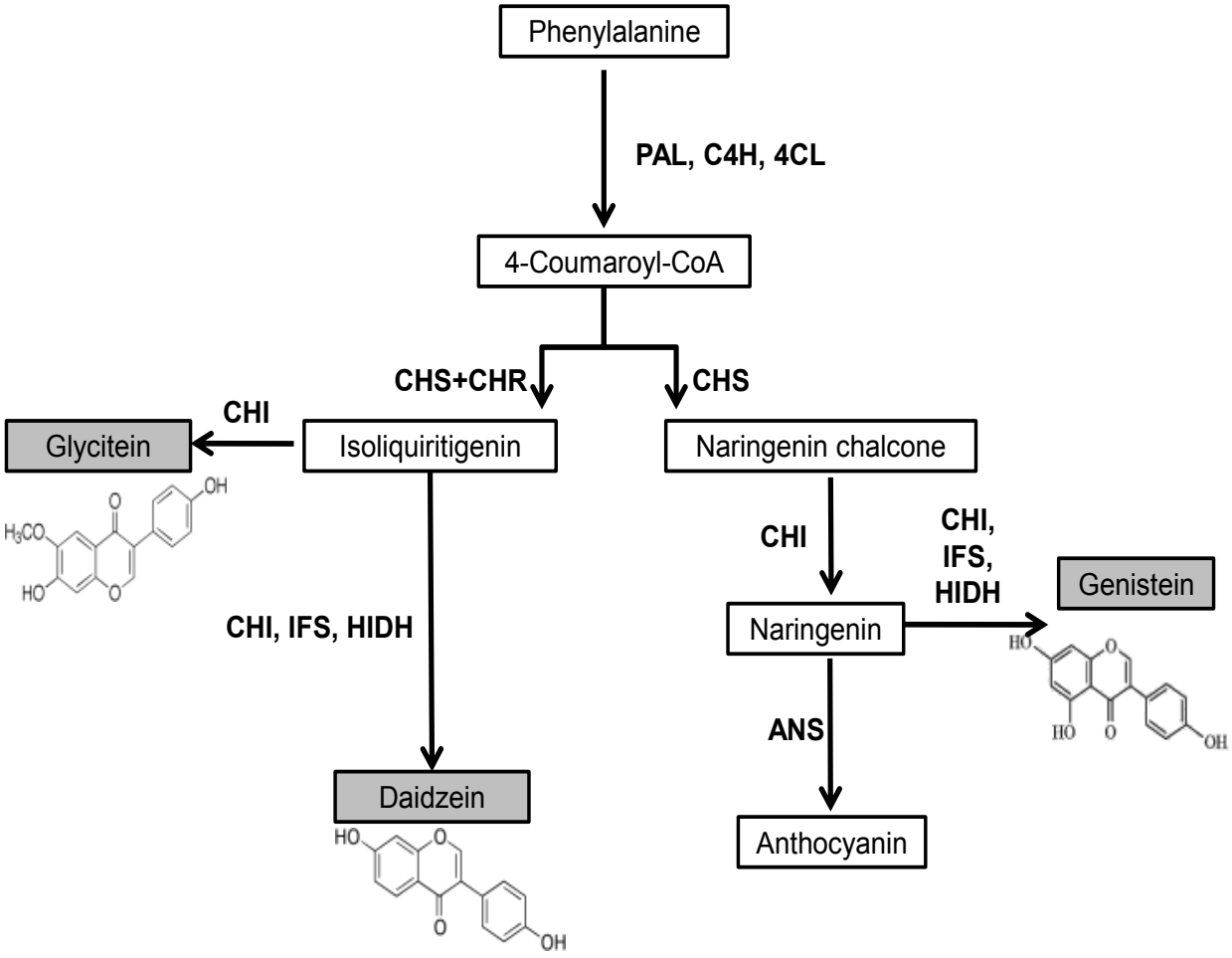

Fig. 1. The flavonoid biosynthetic pathway in soybean. All genes encoding flavonoid biosynthetic enzymes except ANS shown in this figure were both cGMP- and NO-responsive [6]. **PAL** Phenylalanine ammonia-lyase, **C4H**cinnamate 4-hydroxylase, **4CL** 4-coumarate:CoA ligase, **CHS** chalcone synthase, **CHR** chalcone reductase, **CHI** chalcone isomerase, **IFS** 2-hydroxyisoflavanone synthase, **HIDH** 2-hydroxyisoflavanone dehydratase, **IFGT** UDP-glucose:isoflavone 7-O-glucosyltransferase, **IFR** isoflavone reductase, and **ANS** anthocyanidin synthase.

Chapter 2. A cis-element responsible for cGMP in the promoter of the soybean chalcone synthase gene

Part 1. Identification of cGMP responsive *cis*-element in soybean chalcone synthase gene

1.1. Introduction

Many *CHS* promoters contain the CACGTG regulatory motif known as the G-box, which has been found to be important in the response to visible and UV light [33]. In addition to the G-box, other *cis*-elements in the *CHS* promoter involved in light activation of transcription have been identified (Fig. 2). For example, the parsley *CHS* promoter contains Box I (H-box) and G-box-like Box II, together forming one *cis*-acting unit termed Unit-I (Table 1), and both are necessary components of light responsiveness [34, 35]. Moreover, the *Phaseolus vulgaris CHS15* contains G-box and H-box that are required for transcriptional activation in response to fungal elicitors and glutathione [36].The G-box and H-box in the Unit-I sequence are thought to be binding sites for MYC/G-box-binding factors and MYB, respectively [35]. However, there have been no reports on the identification of a cGMP responsible *cis*-element in the promoter of any plant gene using a reliable promoter analysis. The regulation of phenylpropanoid synthesis occurs as a result of coordinated transcriptional regulation of structural genes by several DNA-binding factors including MYB, bHLH, bZIP, WRKY, MADS box, and WD40 TFs [37]. Although a recent study reported the regulation of *GmCHS8* by MYB176 TF [38], the relationship between MYB176 and cGMP signaling is not known.

Here, cGMP-dependent expression of several CHS genes in soybean is described. Promoter analysis of *GmCHS8* showed that Unit-I-like *cis*-element is responsive to cGMP. The roles of

NO and cGMP in the transcriptional control of gene expression for key enzymes in the flavonoid biosynthesis pathway in soybean will also be discussed.

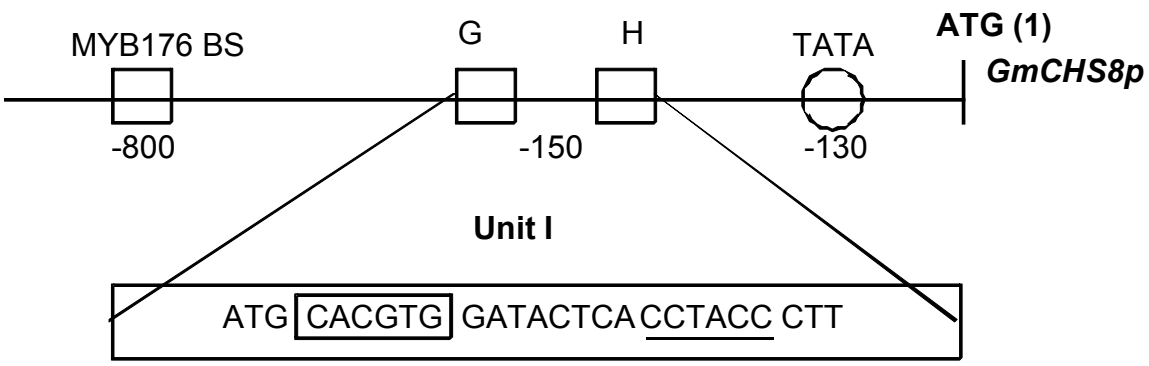

Fig. 2. Soybean *CHS8* promoter. G, G-box, H, H-box, MYB176 binding sequence.

Table 1. Nucleotide sequences of Unit-I and Unit-I-like sequences found in soybean *GmCHS7* and *GmCHS8* and parsley *PcCHS*.

Gene	Nucleotide sequence (5'–3')
GmCHS7	TGCATGCACGTGATACTCACCTACCCTCCCATCCACTCT
GmCHS8	TGCATGCACGTGATGATACTCACCTACCCTTCAACCCTCG
PcCHS	TTATTCCACGTGGCCATCCGGTGGCCGTCCCTCCAACCTAACCTCCCTTGA

Boxed sequence indicates G-box, underlined sequence indicates H-box.
PcCHS indicates parsley CHS [35].

1.2. Materials and methods

1.2.1. Plant material and growth conditions

The soybean (*Glycine max* L. cv. Corsoy) SB-P cell culture [39], a gift of Dr. J.M. Widholm, Department of Crop Science, University of Illinois, IL, USA, was grown photomixotrophically in KN-1 medium containing 10 g/L sucrose at 25°C in continuous light [1, 6]. Seven- to nine-day-old cultures were dark adapted for 2.5–3 days prior to chemical treatment or light illumination. All manipulations were performed at 25°C under green safe light conditions. Exposure of culture to white light and sample collection were performed as described previously [40]. To facilitate the uptake of chemical compounds into the cells, acid loading was performed as described previously [1, 6]. The membrane-permeable cGMP analog 8-Br-cGMP was dissolved in 10% DMSO and added to the cells in 1:1000 dilution. After incubation, cells were collected on filters by suction filtration and immediately frozen in liquid nitrogen.

1.2.2. Isolation of RNA and RT-qPCR

Total RNA was extracted from soybean SB-P cells using Sepasol RNA I Super G (Nacalai Tesque, Inc., Kyoto, Japan) according to the manufacturer's instructions. RNA integrity was verified by running total RNA samples on 1% denaturing agarose gel stained with ethidium bromide. RNA concentration was optically determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc., DE, USA). First strand cDNA was synthesized from 0.3 μg total RNA using the ReverTraAce qPCR RT Kit (TOYOBO Co. Ltd., Osaka, Japan). RT-qPCR assays for quantification of relative gene expression were performed using the primers for *GmCHS2*, *GmCHS7*, and *GmCHS8* (with GenBank accession numbers X65636.1, M98871, and AY237728, respectively). The soybean actin gene *GmACT* (accession number BW652479) was

used as a reference gene. The forward and reverse primer pairs are listed in (Table 2) and were designed using the program Primer3 [41], with melting temperatures ranging from 60 to 70°C, primer length ranging from 20 to 26 bp, and amplicon lengths of 171, 243, 241, and 142 bp for *GmCHS2*, *GmCHS7*, *GmCHS8*, and *GmACT*, respectively. For each primer pair, an estimate of reaction efficiency was derived from a standard curve generated from a serial dilution of pooled cDNA. RT-qPCR was performed in a Rotor-Gene Q (QIAGEN, CA, USA) machine, and results were analyzed ($-\Delta\Delta C_T$ method) using the manufacturer's software (Rotor-Gene Q Pure Detection, v. 2.0.2; QIAGEN, CA, USA). Each reaction mixture was added according to the manufacturer's instructions (Thunderbird^{TM} SYBR^{R} qPCR Mix; TOYOBO) and runs were repeated at least three times. Thermal cycling conditions consisted of an initial denaturation at 94°C for one minute followed by 40 cycles at 94°C for 15 s with a fluorescence reading taken at the end of each cycle, 60°C for 30 s, and 72°C for 25 s. After the final amplification cycle, a melting curve temperature profile was obtained by heating to 95°C, cooling to 65°C, and heating to 95°C at 1°C every five seconds with the continuous measurement of fluorescence at 510 nm. Transcript levels of *GmCHS2*, *GmCHS7*, and *GmCHS8* were finally normalized against the transcript levels of *GmACT*.

Table 2. Oligonucleotides used for RT-qPCR and plasmid construction.

Oligonucleotide	Nucleotide sequence (5'–3')
*GmCHS2*F	TCAGCTTGTTTGGACTGCAC
*GmCHS2*R	TCAGAGATTCCCAAGGGTTG
*GmCHS7*F	GCTGTGACTTGTTTTGAGTTC
*GmCHS7*R	GACTTGTCTCACATGCGCTGG
*GmCHS8*F	GCTCCCAGTACTTTAATTGATTTCTG
*GmCHS8*R	GACTTGTCTCACATGCGCTGGAA
*GmACT*F	CGGTGGTTCTATCTTGGCATC
*GmACT*R	GTCTTTCGCTTCAATAACCCTA
*GmCHS8*pF	GGGGTCTAGATGAGCAAGTATACCAACCAT
*GmCHS8*pR	GGGGGGATCCCTTTCCTTCAAATTAAGTGA
*GmCHS8*pΔUnit-IF	GGGGCCGCGGTGAGCAAGTATACCAACCAT
*GmCHS8*pΔUnit-IR	GGGGTCTAGACAATGTACACCAACATATAC
GmCHS8-UnitI+XS	CTAGATGCATGCACGTGATGATACTCACCTACCCTTCAACCCTCGAGCT
GmCHS8-UnitI-SX	CGAGGGTTGAAGGGTAGGTGAGTATCATCACGTGCATGCAT
Unit-I (GH^m)+XS[a]	CTAGATGCATGCACGTGATGATACTCAAAGCAACTTCAACCCTCGAGCT
Unit-I (GH^m)−SX[a]	CGAGGGTTGAAGTTGCTTTGAGTATCATCACGTGCATGCAT
Unit-I (G^mH)+XS[a]	CTAGATGCATGACATGTATGATACTCACCTACCCTTCAACCCTCGAGCT
Unit-I (G^mH)−SX[a]	CGAGGGTTGAAGGGTAGGTGAGTATCATACATGTCATGCAT
*GmMYB176*BS+XS[b]	CTAGAAATAGCGTGAAAATATAGTTAGTATATGCACGTGAGCT
*GmMYB176*BS−SX[b]	CTAATACGTGCATATACTAACTATATTTTCACGCTATTT

[a]Mutated nucleotides are underlined.
[b]Core sequence of MYB176BS is boxed.

1.2.3. Plasmid construction, protoplast isolation, and transient gene expression assay

To measure the relative activity of β-glucuronidase (GUS) in the reporter gene system, the 1.6-kbp promoter region of *GmCHS8* was amplified by PCR using the primers listed in Table 2 containing *Xba*I and *Bam*HI sites, respectively, and introduced into the *Xba*I and *Bam*HI sites of pSKGUS3C [42]. For the preparation of the Unit-I-like sequence found in the promoter of *GmCHS8*, comprising G-box (CACGTG) and H-box (CCTACC) elements, two synthesized oligonucleotides (Table 2) were annealed and introduced into the *Xba*I and *Sac*I sites of a p35S (−46) GUS3C vector [6]. The *GmCHS8* Unit-I-like sequence was further divided into two mutated sequences in either H-Box or G-Box, named Unit-I (GH^m) and Unit-I (G^mH), respectively. Both sequences (Table 2) were prepared and introduced into the p35S (−46) GUS3C vector in the same manner as the *GmCHS8*-Unit-I-like sequence. For the *GmMYB176*-binding sequence (BS), two synthesized oligonucleotides (Table 2) were annealed and introduced into the vector as above. To further investigate the role of the Unit-I-like sequence in cGMP and NO response, a full *GmCHS8* promoter lacking Unit-I-like sequence (*GmCHS8*pΔUnit-I) was amplified by PCR using the primer pair (Table 2) containing *Sac*II and *Xba*I sites, respectively, and introduced into the *Sac*II and *Xba*I sites of the p35S (-46) GUS3C vector [6]. *pBIΩFF* [42] harboring the firefly luciferase (*LUC*) reporter gene under the control of the *CaMV35S* promoter was used as an internal control.

Protoplasts of soybean SB-P cells were prepared as described in the previous paper [43] with some modifications. SB-P cells dark-adapted for 2.5–3 days were collected by centrifugation at 900 *g* for three minutes. An enzyme solution containing 1% cellulase "ONOZUKA" RS, 0.3% Macerozyme R10 (Yakult Pharmaceutical IND. Co., Ltd, Tokyo, Japan), 0.3% pectolyase Y-23 (Kyowa Chemical Products Co., Ltd. Osaka, Japan), 0.8 M mannitol, 20 mM KCl, 20 mM MES–

NaOH, pH 5.7, 10 mM $CaCl_2$, and 0.1% BSA was used for protoplast preparation. Finally, the number of protoplasts was estimated with a hemocytometer and adjusted to 3.0×10^6/ml.

For transient gene expression assays, an equimolar amount of each promoter–GUS construct was added to the isolated protoplasts and gently mixed and 40% PEG4000 (Fluka, Co. Ltd., Tokyo, Japan) was added, followed by 10 min incubation at room temperature. The protoplasts were pelleted by centrifugation at 900 *g* for three minutes at 4°C and resuspended in W5 solution (155 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, and 5 mM glucose) followed by incubation for three to 12 hr at 25°C in the dark. The protoplasts were harvested by centrifugation at 900 *g* for three minutes at 4°C. Fifty microliters of Pica Gene reporter lysis buffer (LCβ/PGC-51; Toyo B-Net, Co., Ltd. Tokyo, Japan) was added to the protoplasts and incubated for 30 min at room temperature. After centrifugation at 12,400 *g* for five minutes, activities of GUS and LUC in the supernatant were measured as described previously [40]. All transient gene expression assays were performed at least in triplicate. All data are presented as the mean ±SE. The statistical significance of differences between two groups was determined by Student's *t* test.

1.3.Results

1.3.1. Expression of soybean CHS genes is differently regulated by cGMP and NO

It was previously investigated cGMP-regulated genes in soybean using PCR-based cDNA subtraction and northern blotting and found that 10 genes for flavonoid biosynthetic enzymes including phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate:CoA ligase (4CL), CHS, chalcone reductase (CHR), chalcone isomerase (CHI), 2-hydroxyisoflavanone synthase (IFS), 2-hydroxyisoflavanone dehydratase (HIDH), UDP-

glucose:isoflavone 7-O-glucosyltransferase (IFGT), and isoflavone reductase (IFR) are upregulated by 50 μM 8-Br-cGMP and 100 μM SNP (NO donor) [6]. The soybean genome contains nine CHS genes (*GmCHS1–9*) [38]. Of these, *GmCHS7* and *GmCHS8* have been reported to be involved in the isoflavonoid biosynthesis in embryos [32]. To further investigate the regulatory mechanisms of cGMP- and NO-dependent gene expression, three target GmCHS genes were chosen in this study: *GmCHS7*, *GmCHS8*, and *GmCHS2*. *GmCHS7* and *GmCHS8* have a close relationship, with 67% nucleotide sequence similarity in the 1.6-kbp upstream sequences from their start codons, and contain the same G-box and H-box core sequences, approximately 160 bp upstream from the start codon (Table 1), but *GmCHS2* has 3% and 4% sequence similarity to the upstream sequences of *GmCHS7* and *GmCHS8*, respectively, and has no Unit-I-like sequence in the 1.6-kbp promoter. RT-qPCR analysis confirmed 3.6- and 3.8-fold upregulation of *GmCHS7* and *GmCHS8*, respectively, but not of *GmCHS2*, within three hr after treatment of SB-P cells with 50 μM 8-Br-cGMP, a membrane-permeable cGMP analog (Fig. 3). In contrast, 8-Br-cAMP did not stimulate any GmCHS genes, indicating that the effect of gene expression was cGMP-specific and was a direct effect of cGMP rather than an indirect stress response. Also, the possibility of stimulation by NO via cGMP on the regulation of gene expression of these soybean CHS genes was examined. Treatment of dark-adapted soybean SB-P cells with 100 μM SNP resulted in an increased transcription level of all three GmCHS genes (Fig. 3), although this induction by SNP was lower than that by cGMP. Interestingly, *GmCHS2* was activated by NO, in contrast to the effect of cGMP.

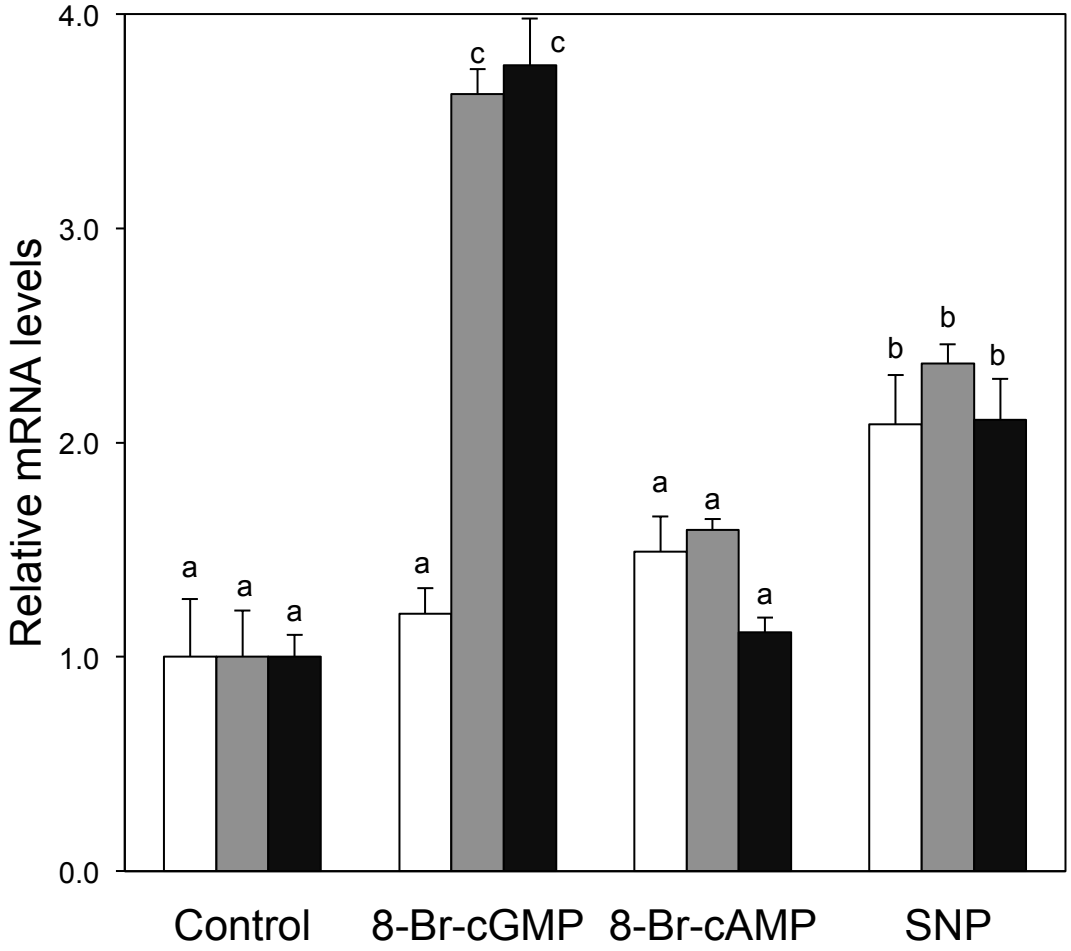

Fig. 3. Effect of 8-Br-cGMP, 8-Br-cAMP, and SNP (NO donor) on relative mRNA levels of *GmCHS2*, *GmCHS7*, and *GmCHS8*. Total RNA isolated from SB-P cells treated with 50 μM 8-Br-cGMP, 50 μM 8-Br-cAMP, and 100 μM SNP was used as a template for qRT-PCR. Relative mRNA levels for *GmCHS2* (white bar), *GmCHS7* (gray bar) and *GmCHS8* (black bar) were measured by qPCR in three biological and three technical replicates for each biological replicate and treatment. Error bars represent SE. Values were normalized against *GmACT* and the average of control levels for each *GmCHS* was taken as 1.0. Bars with different letters are significantly different at $P < 0.05$.

1.3.2. Stimulation of GmCHS genes by cGMP does not require new protein synthesis

To test whether *de novo* synthesized proteins were required for the observed induction of *GmCHS7* and *GmCHS8* by cGMP, 300 μM cycloheximide (CHX) in the presence of 50 μM 8-Br-cGMP were added. At this concentration of CHX, protein synthesis is inhibited without cell toxicity [44]. The results show that CHX had no effect on the expression level of *GmCHS7* and *GmCHS8* in response to 8-Br-cGMP (Fig. 4), indicating that no new cytoplasmic protein synthesis is necessary for 8-Br-cGMP induction of *GmCHS7* and *GmCHS8.*

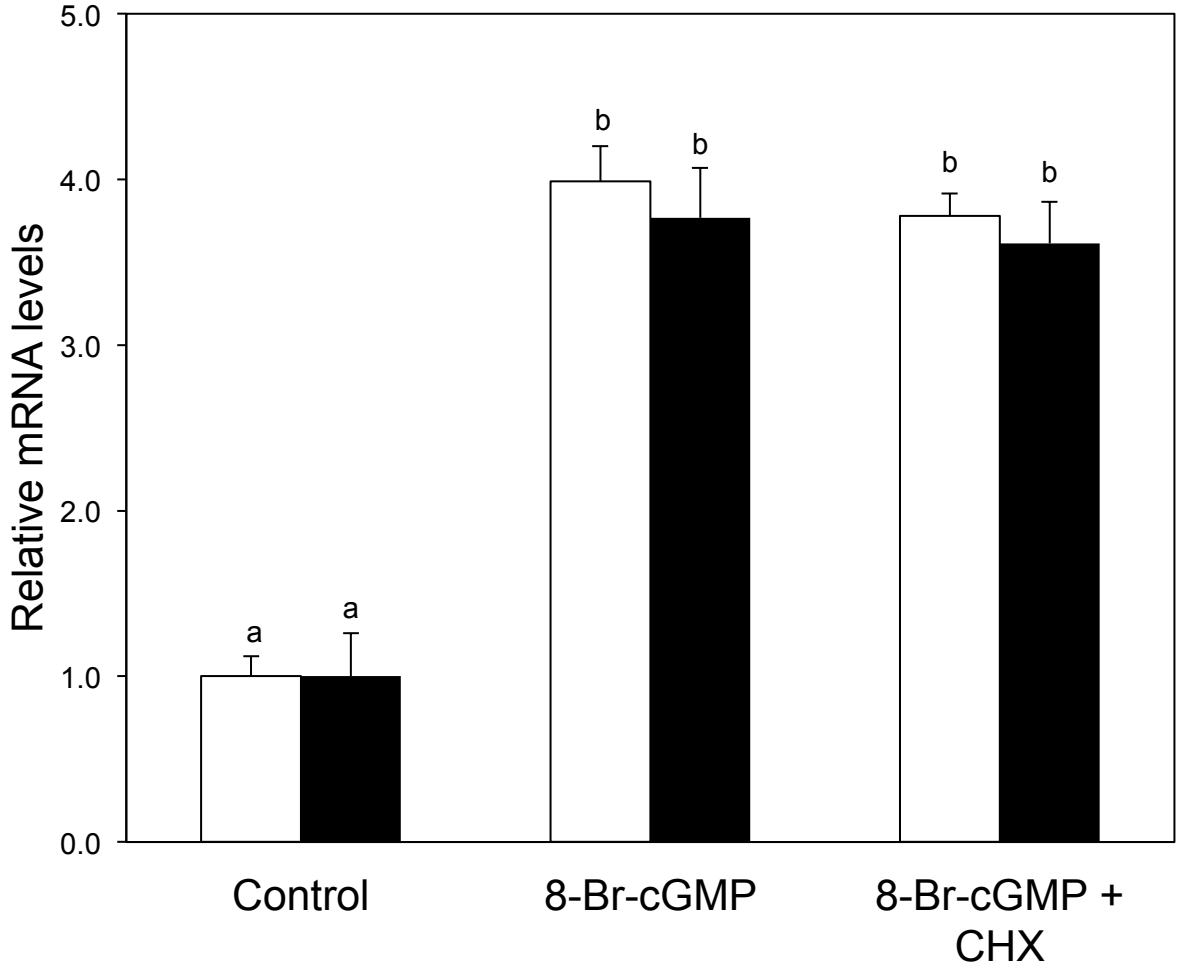

Fig. 4. Effect of 50 μM 8-Br-cGMP in the presence of 300 μM CHX on *GmCHS7* and *GmCHS8* relative mRNA levels. Relative mRNA levels for *GmCHS7* (gray bar) and *GmCHS8* (black bar) were measured by qPCR in three biological and three technical replicates for each biological replicate and treatment. Error bars represent SE. Values were normalized against *GmACT* and the average of control levels for each *GmCHS* was taken as 1.0. Bars with different letters are significantly different at $P< 0.05$.

1.3.3. GmCHS genes display transient activation under white light

The cGMP-dependent pathway of phototransduction regulates *CHS* expression in a specific manner: in response to light, *CHS* mRNA abundance increases rapidly, reaches a maximum after exposure for three hr, and then declines sharply, by 10 hr, to the basal levels found in dark-adapted soybean SB-P cell cultures [1, 6]. Expression levels of the three GmCHS genes in this study were also transiently increased by white light, as shown in Fig. 5. It is also worth noting that the activation due to light responsiveness of *GmCHS7* and *GmCHS8* was similar to that under 8-Br-cGMP, except that gene inductions under 8-Br-cGMP were not transient, probably because 8-Br-cGMP is a nonhydrolyzable analog. In contrast, *GmCHS2* was induced only by white light and not by 8-Br-cGMP, suggesting different response mechanisms among *GmCHS7*, *GmCHS8*, and *GmCHS2* for light-dependent gene induction.

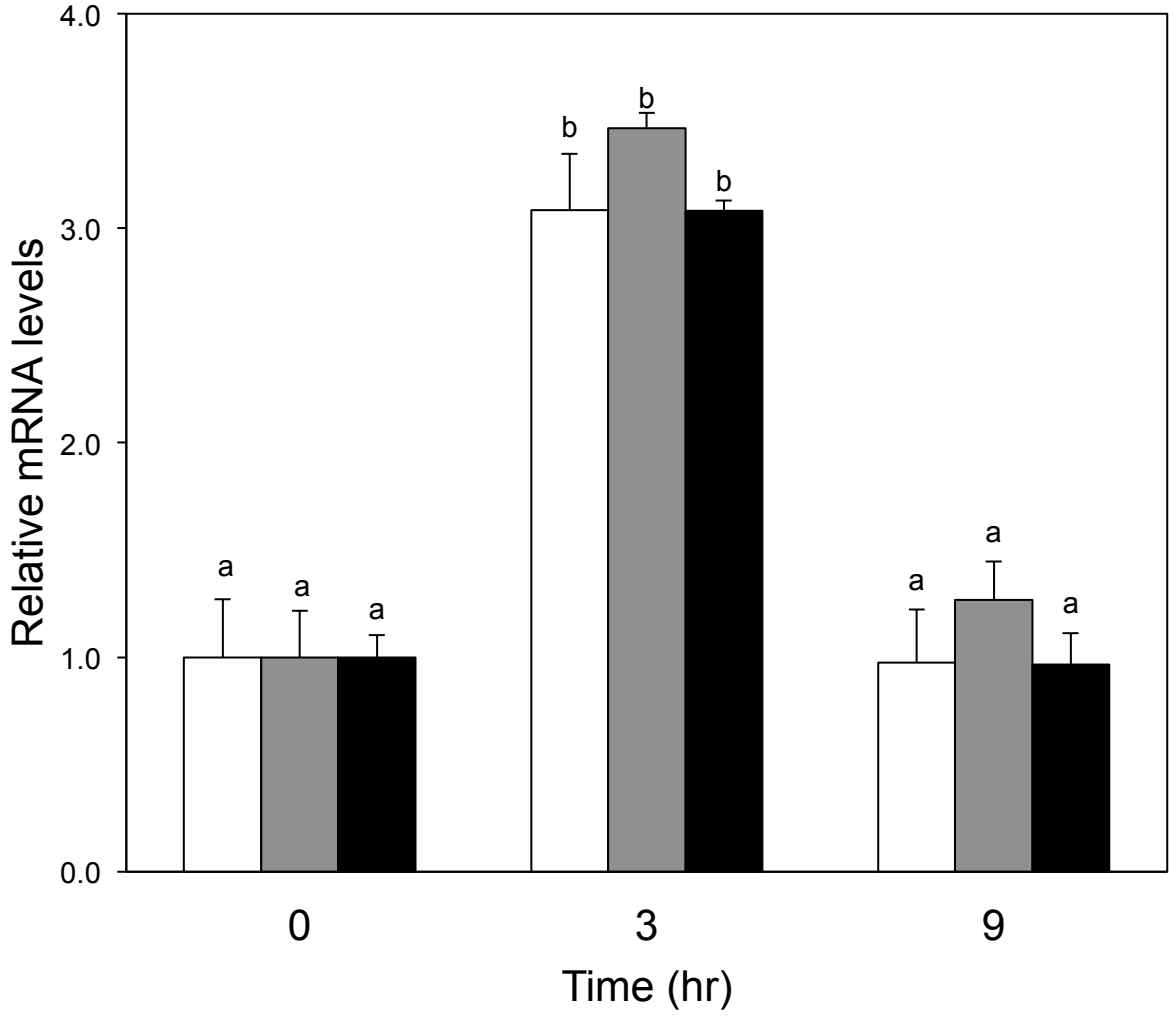

Fig. 5. Effect of white light at different exposure periods on the mRNA levels of *GmCHS2*, *GmCHS7*,and *GmCHS8* in soybean SB-P cells. Dark adapted SB-P cells were illuminated by white light as described previously [40] for three and nine hr. Total RNA isolated from SB-P cells was used as a template for qRT-PCR. Relative *GmCHS2* (white bar), *GmCHS7* (gray bar), and *GmCHS8* (black bar) gene expression was measured by qPCR in three biological and three technical replicates for each biological replicate and treatment with SE shown. Values were normalized against *GmACT*. Bars with different letters are significantly different at $P < 0.05$.

1.3.4. cGMP-dependent *cis*-regulating element in the *GmCHS8* promoter

To identify cGMP-responsive *cis*-elements in the promoter of cGMP-regulated genes, promoter analysis of *GmCHS8* was performed. The *GmCHS8* promoter containing the 1.6-kbp sequences upstream from the ATG start codon as well as a Unit-I-like sequence consisting of the G-box and H-box and individual G-box- or H-box-containing sequences were all subcloned into the p35S (−46) GUS3C vector [6], which has a TATA-box-containing minimal promoter derived from the CaMV-35S promoter, except for the 1.6-kbp *GmCHS8* promoter, which was subcloned into a pSKGUS3C promoterless vector. To test the cGMP responsiveness of the *GmCHS8* promoter, transient reporter gene assay using soybean SB-P protoplasts was performed. As shown in Fig. 6, on treatment of the cells with 50 μM 8-Br-cGMP for 3 hr, the 1.6-kbp *GmCHS8* promoter was activated by 3.4-fold compared with untreated cells. NO and white light also induced *GmCHS8*-promoter-derived GUS expression by 3.1- and 3.4-fold, respectively, in agreement with the RT-qPCR (Figs. 2 and 4). In contrast, 50 μM 8-Br-cGMP had no activation effect on the 1.6-kbp *GmCHS8* promoter lacking Unit-I-like sequence (*GmCHS8*pΔUnit-I) whereas 100 μM SNP activated the same construct by 3.1-fold, suggesting that the *cis*-element responsive to NO is different from the Unit-I-like sequence. Replacing the *GmCHS8* full promoter with Unit-I-like sequence also yielded a significant 3.0-fold increase after cGMP treatment compared with control cells, whereas no significant GUS increase was detected on SNP treatment. In contrast, the Unit-I-like sequences mutated in either H-box or G-box, named Unit-I (GH^m) and (G^mH), were not induced by cGMP. Interestingly, neither GmMYB176 BS nor either mutated version in the core sequences of G-box and H-box of the Unit-I-like sequence responded to any stimulus, indicating that the Unit-I-like sequence in the *GmCHS8* promoter is necessary for the response to white light and cGMP via the combination of G-box and H-box *cis*-elements.

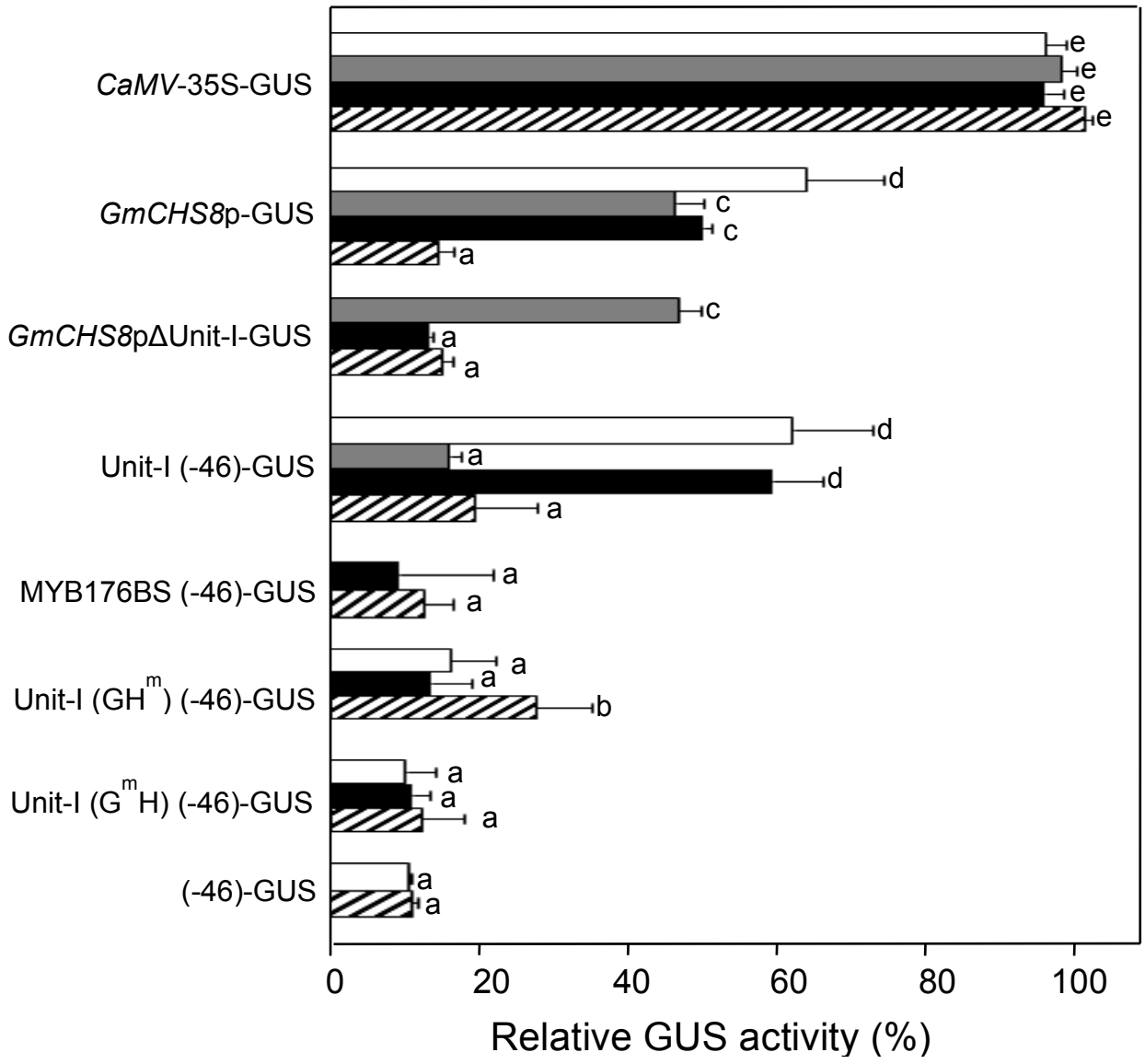

Fig. 6. Analysis of cGMP responsiveness of the *GmCHS8* promoter. Full promoter (*GmCHS8p*), full promoter lacking the Unit-I-like sequence (*GmCHS8*pΔUnit-I),Unit-I-like sequence (Unit-I), mutated versions of G-box and H-box in the Unit-I-like sequence, Unit-I (GH^m) and Unit-I (G^mH), respectively, and *GmMYB176* BS. All sequences were fused to the β-glucuronidase gene (GUS). These constructs were introduced into protoplasts prepared from dark-adapted SB-P cells as described in the Materials and Methods. Cells were incubated with 50 μM 8-Br-cGMP (black bar) or 100 μM SNP (gray bar) in the dark or exposed to white light (white bar) for 3 hr, and GUS activity in the cell extract was measured. Striped bar represents control cells without any treatments. No white bar data for *GmCHS8*pΔUnit-I and *GmMYB176* BS constructs as they were not tested with white light treatment. All GUS values were normalized to LUC values and the average of pBI221 (*CaMV*-35S-GUS-NOS) without any treatment was taken to be 100. Error bars represent SE. Bars with different letters are significantly different at $P< 0.05$.

1.4.Discussion

Suita et al. [6] have previously demonstrated that cGMP, NO, and white light can induce several genes encoding the flavonoid biosynthetic enzymes in soybean. To identify a cGMP-responsive *cis*-element in the promoter of a cGMP-regulated gene, *GmCHS8* was chosen, which is critically involved in the flavonoid biosynthetic pathway [32], as the primary gene followed by two GmCHS genes to be compared with*GmCHS8*, one (*GmCHS7*) having high (67%) and the other (*GmCHS2*) low (4%) sequence similarity in the promoter region [45]. First, confirmation of 3.6- and 3.8-fold upregulation of *GmCHS7* and *GmCHS8* by cGMP was done using RT-qPCR (Fig. 3), whereas *GmCHS2* transcript levels showed no gene induction by cGMP. There was no gene activation for the three GmCHS genes by cAMP, in agreement with previous studies [1, 6]. SNP induced all three GmCHS genes including *GmCHS2*, suggesting that the mechanisms of transcriptional control by cGMP and NO are different for *GmCHS2*.

The promoter region of *GmCHS8* contains several unique *cis*-elements, including the MYB176 BS [38] and Unit-I-like sequence −800 and −150 bp, respectively, upstream from the start codon. Moreover, the *GmCHS7* promoter contains the Unit-I-like sequence with slight variation (Table 1), unlike GmCHS2 gene which does not contain a Unit-I-like sequence. As shown in Fig. 6, the Unit-I-like sequence is most likely to be the cGMP responsive *cis*-element, since the *GmCHS8* promoter containing the Unit-I-like sequence could be activated by cGMP, whereas the same promoter lacking this sequence could not be activated. Moreover, only Unit-I-like sequence conferred responsiveness to cGMP onthe reporter gene expression. The RT-qPCR experiment showing that *GmCHS7* and *GmCHS8*but not *GmCHS2* could respond to cGMP (Fig. 3) supports this inference. Wu et al. [35] also reportedthat Unit-I sequence in parsley *CHS*

promoter is the target of cGMP signaling. Results showed that Unit-I (GH^m) and (G^mH) could not respond to cGMP on their own, suggesting that the combination of G-box and H-box is necessary for the activation by cGMP (Fig. 6). However, there seems to be no strict restriction on the distance between G-box and H-box, since the spacer length can be varied from 7 bp (for *GmCHS7*) to 24 bp (for *PcCHS*) [35] (Table 1). Suita et al. [6] have previously reported that the *GmCHR* promoter could respond to cGMP despite lacking a Unit-I-like sequence. As already reported, cGMP is an important second messenger involved in a wide diversity of physiological processes in plants, exerting its effects via different *cis*-elements in different genes across different species.

It was found out that all signaling protein components for cGMP-mediated induction of *GmCHS7* and *GmCHS8* were present in nontreated control cells and that no new protein synthesis was necessary for the induction of gene expression by cGMP (Fig. 4). These finding ssuggest that cGMP can activate transcription through the modulation of the existing signaling components or transcriptional machinery. Induction of GmCHS genes by light was attenuated rapidly after three hr (Fig. 5). This attenuation could be explained by a desensitization phenomenon, in which despite continuous stimulation the signal transduction pathway could switch off, returning to basal levels after nine hr of white light exposure as described in the previous paper [1]. cGMP is thought to be a second messenger of this light-dependent control of the expression of *GmCHS7* and *GmCHS8*. In contrast, *GmCHS2* was shown to be activated by NO and white light but not by cGMP (Figs. 2 and 4), suggesting that cGMP is not the second messenger of light signal transduction for *GmCHS2*. The cGMP responsive Unit-I sequence of *GmCHS8* promoter could not respond to NO (Fig. 6), suggesting that the signaling pathways of cGMP and NO for gene activation are different from each other with respect to their target TFs.

Part 2. GmMYB176 transcription factor regulates GmCHS8 gene expression

2.1. Introduction

Flavonoid compounds are secondary metabolites widely accumulated in vascular plants. They accumulate in all organs and tissues, at different stages of development, and depending on the environmental conditions. Beside their multiple roles in plant development and adaptation to the environment, these molecules are of major interest for human nutrition and health [46]. The regulation of phenylpropanoids occurs as a result of coordinated transcriptional regulation of structural genes by several DNA-binding factors, such as MYB, bHLH, bZIP, WRKY, MADS box and WD40 transcription factors [38]. A comprehensive analysis of CHS expression in *Arabidopsis* revealed the involvement of an R2R3 MYB transcription factor for gene activation and demonstrated its effects on flavonoid composition [47].

This part will be describing the relationship between GmMYB176 transcription factor and cGMP on the expression of *GmCHS8* in soybean; also studying the effect of cGMP on *GmMYB176* in soybean.

2.2. Materials and methods

2.2.1. Isolation of RNA and RT-qPCR

Total RNA from soybean SB-P cells was extracted, and cDNA was synthesized as described in part 1. RT-qPCR assays for the quantification of relative expression level of GmMYB176 with GenBank accession number (NM_001249119) were performed using the following forward and reverse primer pairs GmMYB176F (5'-AGTGTTGCTTGAGAATATGGTCC-3') and GmMYB176R (5'-AGAAGGATCAAATTCAGTCTGTT-3') with amplicon size of 119 bp. RT-qPCR was

performed as described in part 1. Transcript levels of *GmMYB176* were finally normalized against those of *GmACT*.

2.2.2. Plasmid construction, protoplast isolation and co-transfection assay

To measure the relative activity of β-glucuronidase (GUS) in the transient gene-expression analysis, the 1.6-kbp promoter region of *GmCHS8* was amplified as described in part 1. For the preparation of GmMYB176 plasmid construct, 836 bp of the promoter region of *GmMYB176* was amplified using the following forward and reverse primer pairs, GmMYB176F (5'- GGGGGGATCCATGTCTCGCGCCTCTTCC-3') and GmMYB176R (5'- GGGGGAATTCCCAAGGACCATATTCTCAA-3') and introduced into *Bam*HI and *Eco*RI sites of pBI221 vector under the control of the CaMV35S promoter. pBIΩFF [44] harboring the firefly luciferase (LUC) reporter gene was used as an internal control.

Protoplast isolation from soybean SB-P cells as well as transient gene-expression assays were performed as described in part 1, with some modifications. In brief, an equimolar amount of triple constructs of GmCHS8 (pSKGUS3C), (pBI221) GmMYB176, and pBIΩFF was added to the isolated protoplasts and gently mixed and 40% (v/v) PEG4000 (Fluka, Co. Ltd., Tokyo, Japan) was added followed by 10 min incubation at room temperature. The rest of the protocol is described in chapter 1. All transient gene expression assays were performed at least in triplicate. All data are presented as the mean ±SE. The statistical significance of differences between two groups was determined by student's *t* test.

2.3. Results

2.3.1. *GmCHS8* is regulated by GmMYB176 transcription factor

A previous report by Yi et al. [38] confirmed the role of GmMYB176 TF in regulating the expression of *GmCHS8* in soybean. In this study the role of *GmMYB176* was confirmed by co-transforming soybean SB-P protoplasts with *CaMV35Sp*::*GmMYB176* as an effector and *GmCHS8p::GUS* full promoter as a reporter gene. As shown in Fig. 7, co-transfecting soybean SB-P protoplasts with 35SCaMVp::*GmMYB176* constitutively expressed yielded a 2-fold increase in *GmCHS8*-prmoter-derived GUS expression in agreement with [38].

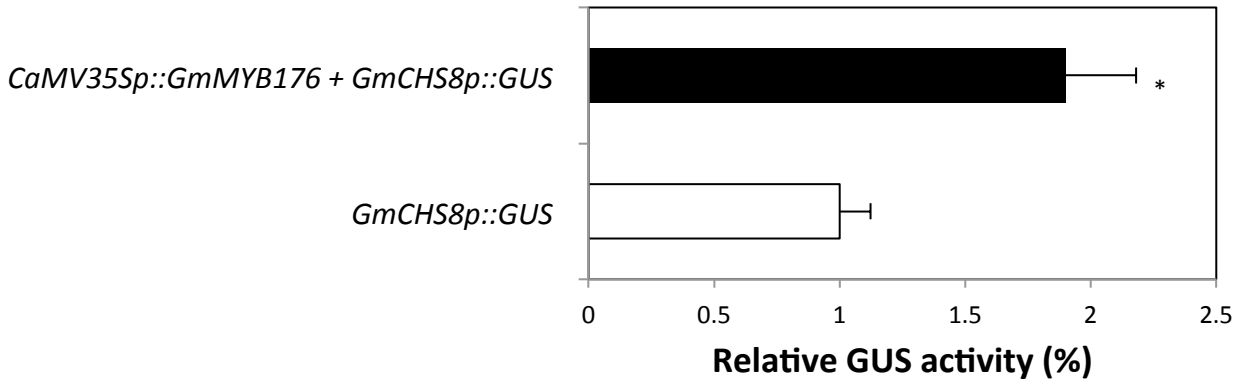

Fig. 7. Analysis of *GmCHS8* full promoter responsiveness to GmMYB176 TF. Transient expression assays were performed by co-transforming reporter (*GmCHS8p*::*GUS*) and effector (35SCaMVp::*GmMYB176*) at equimolar ratios into soybean SB-P protoplasts. Constructs were introduced into protoplasts prepared from dark-adapted SB-P cells as described in the Materials and Methods. GUS activity in the cell extract was measured. White bar represents control cells (*GmCHS8p*::*GUS*) without any treatment, black bar represents co-transfection of *GmCHS8p::GUS* and *35SCaMVp::GmMYB176* constructs. All GUS values were normalized to LUC values and the average of the control was taken to be 1. Error bars represent SE. Bar with asterisk is significantly different at $P < 0.05$.

2.3.2. GmMYB176 gene expression is not affected by cGMP

To investigate the regulatory mechanisms of cGMP dependent GmMYB176 gene expression, 50 μM of 8-Br-cGMP was added to SB-P cells. RT-qPCR analysis (Fig. 8) confirmed that cGMP did not induce *GmMYB176* but reduce to 0.32% within 3 hr after treatment.

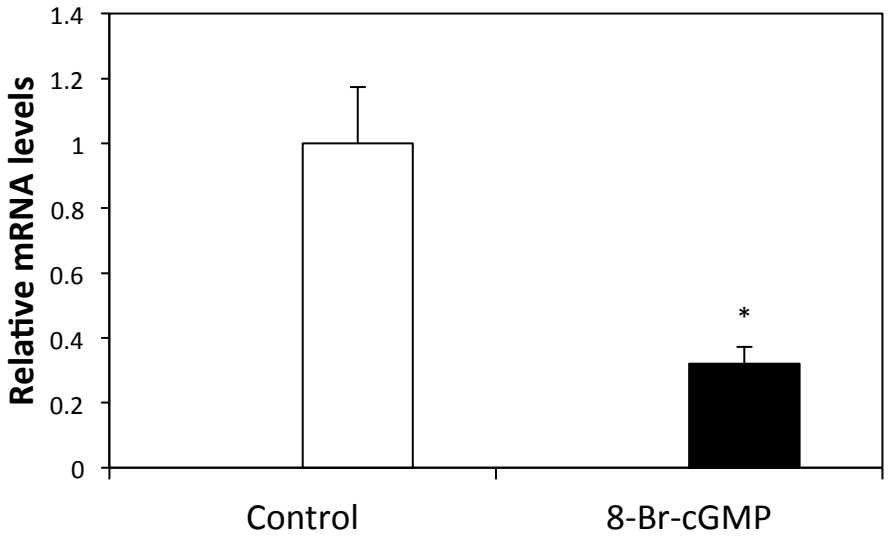

Fig. 8. Effect of 8-Br-cGMP on relative mRNA levels of *GmMYB176*. Total RNA isolated from SB-P cells treated with 50 μM 8-Br-cGMP for 3h, was used as a template for qRT-PCR. Relative mRNA levels for *GmMYB176* without any treatment (control, white bar) and treated with 50 μM 8-Br-cGMP (black bar) were measured by qPCR in three biological and three technical replicates for each biological replicate and treatment. Error bars represent SE. Values were normalized against *GmACT* and the average of control levels for each *GmCHS* was taken as 1.0. Bar with asterisk is significantly different at $P < 0.05$.

2.4. Discussion

Many isoflavonoid genes encoding key enzymes including *CHS* are regulated by MYB transcription factors in Arabidopsis, maize, and several other plants [48]. Here, an attempt to identify the transcription factor responsible for cGMP responsiveness in GmCHS8 gene was made.

The transient gene expression assays using soybean SB-P cells (Fig. 7) demonstrated that GmMYB176 TF is involved in the regulation of GmCHS8 gene in soybean, in agreement with previous report [38]. However, RT-qPCR results showed that the expression of GmMYB176 is not controlled by cGMP, rather downregulated (Fig. 8).

SUMMARY

The cyclic nucleotides cGMP and cAMP have been reported to play key roles in the regulation of plant processes and responses. It has been previously reported that several genes encoding flavonoid biosynthetic enzymes, including chalcone synthase (CHS) in soybean (*Glycine max* L.), were induced by cGMP but not cAMP. The soybean genome contains nine CHS gene copies (*GmCHS1*–9). The responsiveness of several GmCHS genes to cGMP, cAMP, NO, and white light was investigated. Quantitative RT-PCR analysis showed that the transcript levels of *GmCHS7* and *GmCHS8* were increased by 3.6- and 3.8-fold, respectively, with cGMP whereas the transcript levels of *GmCHS2* remained constant. Although cAMP had no effect on the transcript levels of the three genes, NO had an activation effect on all three. White light activated the three genes in a transient manner, with *GmCHS2*, *GmCHS7*, and *GmCHS8*transcript levels increasing 3-fold after three hr and decreasing to basal levels after nine hr. The *GmCHS8* promoter contains several important *cis*-elements, including the G-box and H-box forming the Unit-I-like sequence and the MYB binding sequence, a target of the GmMYB176 transcription factor regulating the expression of *GmCHS8*. A transient gene expression assay revealed the activation of the Unit-I-like sequence, but not of the MYB binding sequence, by cGMP. The combination of G-box and H-box was necessary for cGMP responsiveness. Taken together, these results suggest that the Unit-I-like sequence in the promoters of *GmCHS7* and *GmCHS8* is a cGMP responsive *cis*-element in these genes and that NO exerts its effect via *cis*-elements other than the Unit-I-like sequence.

An attempt to confirm whether GmMYB176 TF is involved in cGMP regulation in soybean is also described. Transient gene expression assay revealed the upregulation of GmCHS8 gene

by GmMYB176 TF. However, results of RT-qPCR showed that GmMYB176 TF is not regulated in cGMP.

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