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| 著者 Author(s) | Suita, Kenji / Kiryu, Takaaki / Sawada, Maki / Mitsui, Maiko / Nakagawa, Masataka / Kanamaru, Kengo / Yamagata, Hiroshi |
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**Cyclic GMP acts as a common regulator for the transcriptional activation of the flavonoid biosynthetic pathway in soybean**

Kenji Suita, Taka-aki Kiryu, Maki Sawada, Maiko Mitsui, Masataka Nakagawa, Kengo Kanamaru, Hiroshi Yamagata

Laboratory of Biochemistry, Graduate School of Agricultural Science, Kobe University, Nada-ku, Kobe 657-8501, Japan

Address correspondence to: Hiroshi Yamagata, Laboratory of Biochemistry, Graduate School of Agricultural Science, Kobe University, Rokkodai-cho 1-1, Nada-ku, Kobe, 657-8501 JAPAN. Tel & Fax: +81-78-803-5875, E-mail: yamagata@kobe-u.ac.jp

## Abstract

Cyclic GMP (cGMP) is an important signaling molecule that controls a range of cellular functions. So far, however, only a few genes have been found to be regulated by cGMP in higher plants. We investigated the cGMP-responsiveness of several genes encoding flavonoid-biosynthetic enzymes in soybean (*Glycine max* L.) involved in legume-specific isoflavone, phytoalexin and anthocyanin biosynthesis, such as phenylalanine ammonia-lyase, cinnamate 4-hydroxylase, 4-coumarate:CoA ligase, chalcone synthase, chalcone reductase, chalcone isomerase, 2-hydroxyisoflavanone synthase, 2-hydroxyisoflavanone dehydratase, anthocyanidin synthase, UDP-glucose:isoflavone 7-O-glucosyltransferase, and isoflavone reductase, and found that the majority of these genes were induced by cGMP but not by cAMP. All cGMP-induced genes were also stimulated by sodium nitroprusside (SNP), a nitric oxide (NO) donor, and illumination of cultured cells with white light. The NO-dependent induction of these genes was blocked by 6-anilino-5,8-quinolinedione, an

inhibitor of guanylyl cyclase. Moreover, cGMP levels in cultured cells were transiently increased by SNP. Consistent with the increases of these transcripts, the accumulation of anthocyanin in response to cGMP, NO, and white light was observed. The treatment of soybean cotyledons with SNP resulted in a high accumulation of isoflavones such as daidzein and genistein. Loss- and gain-of-function experiments with the promoter of chalcone reductase gene indicated the Unit I-independent activation of gene expression by cGMP. Together, these results suggest that cGMP acts as a second messenger to activate the expression of genes for enzymes involved in the flavonoid biosynthetic pathway in soybean.

**Keywords**

cGMP, Flavonoid biosynthesis, Gene expression, NO, *Glycine*

**Abbreviations**

| | |
|---|---|
| NO | nitric oxide |
| GC | guanylyl cyclase |
| SNP | sodium nitroprusside |
| CHS | chalcone synthase |
| PAL | phenylalanine ammonia-lyase |
| PR | pathogenesis-related |
| LY83583 | 6-anilino-5,8-quinolinedione |
| 4CL | 4-coumarate:CoA ligase |
| IFGT | UDP-glucose:isoflavone 7-O-glucosyltransferase |
| CHR | chalcone reductase |
| IFR | isoflavone reductase |
| HIDH | 2-hydroxyisoflavanone dehydratase |
| C4H | cinnamate 4-hydroxylase |
| CHI | chalcone isomerase |

| | |
|---|---|
| IFS | 2-hydroxyisoflavanone synthase |
| ANS | anthocyanidin synthase |
| GUS | β-glucuronidase |
| LUC | luciferase |
| DEANO | diethylamine NONOate |

The nucleotide sequence for the promoter of the soybean chalcone reductase gene has been deposited in the GenBank database under GenBank accession number EU157917.

## Introduction

Guanosine 3',5'-cyclic monophosphate (cGMP) is an important signaling molecule which controls various cellular functions as a second messenger in many prokaryotes and all eukaryotes (Newton and Smith 2004). In higher plants, the presence of cGMP has been unambiguously demonstrated by mass spectrometry and radio-immunoassay (Newton et al. 1989; Newton and Smith 2004), and the functional involvement of cGMP in several physiological processes such as light signal transduction (Bowler et al. 1994a, b; Neuhaus et al. 1997), response of the cereal aleurone to gibberellic acid (Penson et al. 1996), defense response to pathogen attack (Durner et al. 1998), auxin-induced adventitious rooting (Pagnussat et al. 2003) and stomatal opening (Cousson 2003), pollen tube growth (Prado et al. 2004), regulation of flowering (Szmidt-Jaworska et al. 2004), and gravitropic bending of roots (Hu et al. 2005) has been reported. Several lines of evidence indicate that cGMP acts downstream of NO signaling in some of these physiological processes, although in some cases such as NO-induced cell death and stomatal closure, cGMP is required, but not sufficient for the response (Neill et al. 2003; Wendehenne et al. 2007).

In mammalian cells, many of the cellular effects of NO appear to be mediated by cGMP, although cGMP-independent pathways are also known (Hanafy et al. 2001). One of the

most relevant actions of NO is the activation of soluble guanylyl cyclase (sGC) through interaction with iron in its heme moiety, which induces a conformational change that results in enzyme activation and increasing levels of cGMP (Russwurm and Koesling 2004). In plants, it has also been reported that NO donors such as sodium nitroprusside (SNP) generate an increase in cGMP levels (Durner et al. 1998). However, the molecular mechanisms explaining the precise linkage between NO and cGMP in plant cells remains unclear as the plant candidate guanylyl cyclases, AtGC1 (*Arabidopsis thaliana* guanylyl cyclase 1) and AtBRI1 (*A. thaliana* brassinosteroid insensitive 1), have no NO-binding heme moiety in contrast to the mammalian sGCs (Ludidi and Gehring 2003; Kwezi et al. 2007). The cGMP signaling pathway in plants also remains largely unknown because of the lack of evidence for its biological role or for enzymes involved in cGMP metabolism, although several genes for cyclic nucleotide-gated ion channels have been reported (Newton and Smith 2004). Since little molecular evidence of *bona fide* cyclic nucleotide-dependent kinases (PKA and PKG) in plants is available, specific target molecules of cGMP also remain to be elucidated.

In spite of the occurrence of cGMP and its diverse physiological functions, only a few genes are known to be regulated by cGMP in plants, in contrast to animals, in which the regulation of gene expression by cGMP has been well documented (Pilz and Casteel 2003). In phytochrome-mediated phototransduction, cGMP activates anthocyanin production, and cGMP as well as SNP stimulates the gene for chalcone synthase (*CHS*), a key enzyme in the anthocyanin biosynthetic pathway (Bowler et al. 1994a, b). A *cis*-element in the *CHS* promoter, Unit I, that contains two nuclear factor binding-sites, a Myb-binding site (CCTAAC) and a G-box (CACGTG), was shown to be the target of cGMP signaling (Wu et al. 1996). In the defense response, cGMP as well as NO can induce some defense-gene expression such as phenylalanine ammonia-lyase (*PAL*) and pathogenesis-related protein (*PR-1*) (Durner et al. 1998). Recently, an *in silico* and transcriptomics approach revealed that genes for monovalent cation transporters such as non-selective ion channels and cation:proton antiporters were induced by cGMP in *Arabidopsis* (Maathuis 2006). It is

necessary to extend this kind of study to clarify the genes that are controlled by cGMP for understanding the roles of this molecule in plants other than *Arabidopsis*, because responses to cGMP may be different among plant species, especially in the case that cGMP may affect pathways of secondary metabolism.

Here we report on the role of cGMP in flavonoid biosynthesis in soybean. During the past decade, the increasing knowledge of flavonoid biosynthesis and the important role of flavonoid compounds in plants and in human and animal nutrition have made the biosynthetic pathways to flavonoids and isoflavonoids excellent targets for metabolic engineering (Schijlen 2004). For effective metabolic engineering, it is necessary to understand the mechanisms that control expression of genes for the enzymes involved in flavonoid biosynthetic pathways. We describe that cGMP can regulate the expression of various structural genes of the flavonoid biosynthetic pathway in soybean.

## Materials and methods

### Plant materials and growth conditions

The soybean (*Glycine max* L. cv. Corsoy) SB-P cell culture (Horn et al. 1983, gift from Dr. J. M. Widholm, Department of Crop Science, University of Illinois, IL, USA) was grown photomixotrophically in KN-1 medium containing 5 g/L of sucrose at 25˚C in continuous light (Bowler et al. 1994a). Ten-day-old cultures were dark-adapted for 2.5 to 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 done as described previously (Yamagata et al. 2001). For UV-B light source, a transilluminator (COSMO BIO Co. Ltd., Tokyo, Japan; λmax=313 nm) with fluence rate of 1.468 μmol $m^{-2}$ $s^{-1}$ was used. To facilitate uptake of the chemical compounds into the cells,

acid loading was performed as described previously (Bowler et al. 1994a, b). 8-Br-cGMP was dissolved in 10% DMSO and added to the cells at 1/1000 dilutions. Since the induction of *CHS* gene in response to 8-Br-cGMP had been observed at all concentrations between 10 μM and 100 μM, and maximal at 50 μM as described previously (Bowler et al. 1994a, b), we used a final concentration of 50 μM of 8-Br-cGMP in this study. These concentrations are highly similar to the concentrations found to be effective in microinjection experiments using tomato seedlings (Bowler et al. 1994a). LY83583 (Wako Pure Chemical Industries, Ltd., Osaka) was added to the cells 30 min before addition of SNP (Nakalai tesque Inc., Kyoto, Japan) for entry of the compound into the cells. Samples were collected on filters by suction filtration and immediately frozen in liquid nitrogen. Seeds of *Glycine max* (cv. Tsurunoko purchased from Takii Seed Co. Ltd., Kyoto, Japan) were allowed to germinate and grown at 25˚C under 12 h light/12 h dark conditions. Cotyledons detached from 10-day old seedlings were used for cotyledon assay.

PCR-based cDNA subtraction

General DNA manipulations were carried out using standard procedures (Sambrook and Russell 2001). Dark-adapted SB-P cells (100 ml culture) were treated with or without 50 μM 8-Br-cGMP for 5 h in the dark. Subsequently, mRNA was isolated from cell cultures using QuickPrep micro mRNA purification kit (GE Healthcare Bio-Sciences Co. Ltd., NJ, USA). PCR-based cDNA subtraction was done using a PCR-select cDNA subtraction kit (Takara Bio Inc., Ohtsu, Japan) according to the manufacturer’s protocol. The mRNA isolated from cell culture treated with and without 8-Br-cGMP was used as a source of tester and driver cDNA, respectively.

Northern-blot analysis

Total RNA was prepared from frozen cells essentially as described previously (Yamagata et al. 1994), and poly(A)$^+$ RNA was purified from total RNA using the Oligotex$^{TM}$-dT(30) mRNA purification kit (Takara Bio). Northern-blot analysis was done as described previously (Bowler et al. 1994b). Equal loading of RNA in each lane was verified by rehybridizing the blots with a $^{32}$P-labeled cDNA fragment of soybean 18S rRNA (GenBank accession number X02623), that was amplified by reverse transcriptase (RT)-PCR using total RNA extracted from SB-P cells as a template and a set of specific primers (Gm18SrRNA-Fw, 5'-CAGCCTGCTAAATAGCTATGTGGAG-3' and Gm18SrRNA-Rv, 5'-GGTTCAGTGGACTTCTCACAACGTC-3'), or staining of rRNAs with ethidium bromide. Soybean cDNA fragments for *PAL* and *CHS* used for preparing random-primed probes have been described (Bowler et al. 1994a), and those for 4-coumarate:CoA ligase (*4CL*) and UDP-glucose:isoflavone 7-O-glucosyltransferase (*IFGT*), were isolated from a soybean cDNA library (Clontech Laboratories Inc., Mountain View, CA, USA) using heterologous probes encoding parsley *4CL* (Kuhn et al. 1984) and *A. majus* UDP-glucose:flavonoid 3-O-glucosyltransferase (Martin et al. 1991). The nucleotide sequences of the isolated cDNA fragments for soybean 4CL (1.2 kb) and IFGT (0.7 kb) have 100% and 90% (score 261 bits) identity with those of Gm4CL14 (GenBank accession number X69954, Uhlmann and Ebel 1993) and GmIF7GT (GenBank accession number AB292164, Noguchi et al. 2007) in the corresponding regions, respectively. For detection of mRNAs for chalcone reductase (*CHR*) and isoflavone reductase (*IFR*), two soybean cDNA fragments, cG2-1 and cG3-10, isolated by PCR-based subtraction as described above, were used as probes. Soybean cDNA for 2-hydroxyisoflavanone dehydratase (*HIDH*, GenBank accession number AB154415) (Akashi et al. 2005) was isolated from the same library by PCR using two primers; *HIDH*-fw, 5’-TGCTTTTGCGTGCAGGTAACGAATC-3’ and *HIDH*-rv, 5’-AACCAGAAAAGAAGCCAAGCGTTTG-3’. In addition, cDNAs for cinnamate 4-hydroxylase (*C4H*), chalcone isomerase (*CHI*), 2-hydroxyisoflavanone synthase (*IFS*), and anthocyanidin synthase (*ANS*), were isolated using the following primers that were designed

based on the corresponding EST sequences; *C4H*-fw,
5'-CCCAGAGATCCAGCAAAAGTTAAG-3'; *C4H*-rv,
5'-CCTCCTTTCTCACTAGTGTCAATC-3'; *CHI*-fw,
5'-ACAGGCATAGGAGTATACTTGGAGG-3'; *CHI*-rv,
5'-TCTCTATCACTGCAGCCTCCTTTTC-3'; *IFS*-fw,
5'-TTCCGAATGCACCCACCACTCCCAG-3'; *IFS*-rv,
5'-GATGCAACGCCGATCCTTGCAAGTG-3'; *ANS*-fw,
5'-GTTGGTGGGATGGAAGAGCTTCTAC-3'; *ANS*-rv,
5'-TCGCAGAACACGGCCCATGATATTC-3'. The sequences of these cDNAs are available upon request.

Measurement of cGMP and anthocyanin content

cGMP levels in SB-P cells were measured by enzyme-linked immunoassay using the cGMP assay system kit (GE Healthcare) as described in the manufacturer's instructions. Samples were acetylated to increase sensitivity.

Anthocyanin content was determined spectrophotometrically as described previously (Bariola et al. 1999). One gram of lyophilized SB-P cells was ground to a fine powder in liquid nitrogen, and then 5 ml of extraction solvent containing 80% (v/v) methanol and 1% (v/v) HCl was added and mixed well. The homogenate was centrifuged at 13,000 *g* for 30 min and 2 ml of chloroform was added to the supernatant. After extraction with chloroform, absorbance of the aqueous/methanol phase was measured at $A_{530}$ and $A_{657}$. Values representing anthocyanin content were calculated using the following equation: $A_{530} - {}^{1}/_{4}A_{657}$. The resulting value was normalized to the fresh weight of each sample.

Cotyledon assay and HPLC analysis

The cotyledon assay was essentially done as described previously (Modolo et al. 2002). A small section (i.d. 0.6 cm) was removed from the surface of each cotyledon of 8 to 10-day grown soybean plants, and the wounded surface was treated with 50 μl of 10 mM SNP or 5 mM 8-Br-cGMP. After the cotyledons were kept in a petri dish containing water-absorbed filter paper in the dark at 26°C for 20 h, they were lyophilized and ground to a fine powder with a mortar and pestle. The products were extracted with 80% (v/v) methanol and analyzed by HPLC in a chromatograph fitted with a diode array detector. The samples were run on a 4.6 mm x 150 mm column (TSK-gel ODS-80TM, Tosoh Corp.,Tokyo, Japan) with a linear gradient from 20 to 60% (w/v) acetonitrile (0.5 ml $min^{-1}$). Daidzein and genistein were identified by calibration with authentic standards (Fujicco Co. Ltd., Kobe, Japan). All compounds were monitored by the absorbance at 280 nm.

Cloning of CHR promoter and primer extension analysis

The promoter region of the *CHR* gene was amplified by inverse PCR based on the sequence of *CHR* cDNA (GenBank accession number X55730). Soybean genomic DNA (1 μg) prepared from SB-P cells using cetyltrimethylammonium bromide as described previously (Yamagata et al. 2002) was digested with *Nco* I, and the DNA fragments were self-ligated. This circularized DNA was used as a template for 1st PCR using a forward primer (Gmchr1, 5’-AGCTCTCAAGGAAGCTATCCATCTTGG-3’) and a reverse primer (Gmchr2, 5’-ATTCCAACCACTGGCATCCTCTGTTGG-3’). A second PCR using a forward primer (Gmchr3, 5’-TCTCTTGCCTTTTGACGTGAAGGGTGTG-3’) and a reverse primer (Gmchr4, 5’-GAGGAGTTTGGAAACACTATTGTGGGG-3’) was carried out with 1st -PCR product as a template. The amplified fragment (1.1 kb) was subcloned into pBluescript II $SK^-$ by TA-cloning (pGmCHRproA) and sequenced. Since the DNA sequence of pGmCHRproA that is available from GenBank under the accession number EU157917, has 77 and 98% identity with the corresponding regions of cDNAs with GenBank accession

number X55730 and EST number BI316573, respectively, we considered the pGmCHRproA as the gene corresponding to the cDNA with EST number BI316573, but not for the cDNA clone (cG2-1) isolated by PCR-subtraction, *i.e.*, GenBank accession number X55730. To isolate the upstream sequence of cG2-1, TAIL-PCR (Sambrook and Russell 2001) was carried out using three gene-specific primers, Gmchr1, Gmchr2, and Gmchr5 (5'-TTCTAACTCTCAAAGGGTTGACAAG-3') for 1st PCR, 2nd PCR, and 3rd PCR, respectively, and a random primer (5'-(A/T)GTGNAG(A/T)ANCANAGA-3'). A 0.8-kb product from the 3rd PCR was subcloned (pGmCHRproB) and sequenced. Since the cGMP-responsiveness of pGmCHRproB containing a 0.8-kb region upstream from the ATG start-codon was weaker than that of pGmCHRproA in the transient expression analysis (data not shown), we used pGmCHRproA for further analyses.

Primer extension analysis was carried out as described by Sambrook and Russell (2001). The synthetic oligonucleotide Gmchr2-ex-flu (5'-CCCATTCCGATCACAGGCAC-3') was 5'-end-labelled with fluorescent IRD800 (ALOKA Co. Ltd., Tokyo, Japan) and then used as a primer for a reverse transcriptase reaction by PrimeScript (Takara Bio) with poly(A)$^+$ RNA prepared from SB-P cells that had been treated with 50 μM 8-Br-cGMP for 3 h. The extended product was compared with the sequence ladder obtained from the soybean genomic DNA fragment, that was prepared by PCR using two primer sets (Gmchr5, 5'-CCTGATAGACTGTATAAGCAATAACAG-3' and Gmchr6, 5'-AAAGAGGTCTTGGCGGGTGACT-3'), with the same synthetic oligonucleotide (Gmchr2-ex-flu) by a DNA sequencer (model 4000L; LI-COR, Lincoln, NE, USA).

Particle bombardment

Various lengths of soybean *CHR* promoter, *Arabidopsis CHS* promoter, and tomato *CAB7* promoter (Bowler et al. 1994a) were amplified by PCR using two primers containing *Xba*I and *Bam*HI sites at their upstream- and downstream-ends, respectively, and introduced into

*Xba*I-*Bam*HI sites of pSKGUS3C (Yamagata et al. 2002). Two synthesized oligonucleotides for both strands of CaMV 35S minimal-promoter (-46 to +8), 35S(-46)+XB (5'-CTAGACGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGAC ACGCTG-3') and 35S(-46)-BX (5'-GATCCAGCGTGTCCTCTCCAAATGAAATGAACTTCCTTATATAGAGGAAGGGTC TTGCGT-3') were annealed and introduced into *Xba*I-*Bam*HI sites of pSKGUS3C (denoted as p35S(-46)GUS3C). For the gain-of-function analysis, the upstream region of the *CHR* promoter between nucleotides -600 to -186 relative to the transcription start site was amplified by PCR using the primers, GmchrproA-Fw1; 5'-GGGGTCTAGATACCATATTTGGATTTG-3' and GmchrproA-Rv4; 5'-GGGGTCTAGAACAAATGTTTTCAACATT-3' with pGmCHRproA as a template, and the product was subcloned into the *Xba*I site of p35S (-46) GUS3C, after which the direction of the promoter was verified by sequencing. pBIΩFF (Yamagata et al. 2002) harboring the firefly luciferase (LUC) reporter gene under the control of the CaMV 35S promoter was used as an internal control. Gold particles (1.0 μm in diameter) were coated with 2.5 μg of pBIΩFF and an equimolar amount of each promoter-GUS construct by ethanol precipitation according to the manufacturer's protocol (Bio-Rad Laboratories Inc., Hercules, CA, USA). Dark-adapted SB-P cells were incubated in KN-1 medium containing either 50 μM 8-Br-cGMP or 8-Br-cAMP at 25˚C for 3 h in the dark. The treated cells were harvested on filters by suction filtration, put on a plate of KN-1 agar (0.8% w/v) medium and bombarded with gold particles (500 μg per shot) as described previously (Yamagata et al. 2002). After bombardment, the sample was moistened with KN-1 medium containing 50 μM 8-Br-cGMP or 8-Br-cAMP and incubated in darkness for an additional 10 h at 25˚C.

**Results**

Expression of several genes for flavonoid-biosynthetic enzymes is increased by cGMP, NO,

and white light

We first attempted to identify cGMP-regulated genes in soybean other than previously reported cGMP-induced genes such as *CHS, PAL*, and *PR-1* using a PCR-based subtraction technique. Three and two genes were isolated as up- and down-regulated genes by cGMP, respectively. Of these up-regulated genes by cGMP, two cDNA fragments, cG2-1 (410 bp) and cG3-10 (193 bp) were further analysed. The nucleotide sequence of cG2-1 has 99% identity with that of a cDNA for soybean CHR with GenBank accession number X55730 (Welle et al. 1991). cG3-10 encodes IFR, and its nucleotide sequence has 93% identity with that of a soybean cDNA with GenBank accession number AF202183. CHR is an enzyme that co-acts with CHS to produce 4,2′,4′-trihydroxychalcone (isoliquiritigenin), the first compound for synthesis of the 5-deoxy series of flavonoids and isoflavonoids (Welle et al. 1991) (Fig. 1). IFR catalyzes the reduction of the heterocyclic ring (C-ring) of isoflavone to form isoflavanone, an intermediate of pterocarpan phytoalexins. Northern-blot analysis confirmed that mRNA abundance of *CHR* and *IFR* (as well as *CHS*) increased within 3 h after treatment of SB-P cells with 50 μM of 8-Br-cGMP, a membrane permeable cGMP analog (Fig. 2). In contrast, 8-Br-cAMP did not stimulate these genes indicating that the effect on gene expressions was cGMP specific, and it was a direct effect of cGMP rather than an indirect stress responses. In the *Leguminosae*, anthocyanins and isoflavonoid-derived phytoalexins are the end-products of two major branched-pathways (Fig. 1). The findings that the genes involved in these pathways such as *CHS*, *CHR* and *IFR* were induced by cGMP, prompted us to further examine the general effect of cGMP on the expression of other genes encoding structural-enzymes involved in flavonoid biosynthesis including *PAL, C4H, 4CL, CHS, CHR, CHI, IFS, HIDH, ANS, IFGT,* and *IFR* (Fig. 1). Expression of all these genes was dramatically stimulated by 8-Br-cGMP except for *ANS,* whose transcript level was constitutively high even in dark-adapted cells without chemical treatments (Fig. 3). These results suggested that cGMP is likely to be a common regulator for the transcriptional

activation of flavonoid biosynthetic pathway in soybean.

In animal cells NO acts upstream of cGMP because it activates GC. We therefore examined whether NO may act via cGMP on the regulation of gene expression of flavonoid-biosynthetic enzymes in soybean. The treatment of dark-adapted soybean SB-P cells with 100 μM SNP resulted in an increased mRNA abundance of all flavonoid-biosynthetic genes except for *ANS* (Fig. 3). However, these inductions by SNP were all transient, and induction levels were lower than those induced by 8-Br-cGMP, *i.e.,* transcripts of these genes increased rapidly and reached a maximum at 3 h, and then declined by 10 h. The NO-dependent induction of these genes was blocked in all cases, except for *C4H,* by 100 μM LY83583, an inhibitor of mammalian guanylyl cyclase, suggesting the presence of mammalian sGC-like enzymes in plants (Fig. 3).

The cGMP-dependent pathway of phototransduction regulates *CHS* expression in a specific manner, *i.e.*, in response to light *CHS* mRNA abundance increases very rapidly, reaches a maximum at around 3 h, and then declines sharply to basal levels found in dark-adapted cultures by 10 h (Bowler et al. 1994a, b). Expressions of all cGMP-induced genes involved in the flavonoid biosynthetic pathway were also induced by white light as already known (Fig. 3). It is interesting that the transient patterns of light responsiveness of these genes are very similar to the reported pattern of light-induced *CHS* expression ( Fig. 3; Bowler et al. 1994a, b). The patterns of light-regulated expression of these genes were also similar to those regulated by NO. These findings suggest that the expression of genes encoding enzymes involved in both anthocyanoid- and isoflavonoid-biosynthesis appear to be controlled by the same NO/cGMP-dependent pathway of phototransduction.

cGMP levels in SB-P cells are elevated by SNP treatment

To further clarify the relationship between cGMP and NO in the transcriptional activation of flavonoid-biosynthetic genes, we investigated the effects of SNP treatment on cGMP levels in

SB-P cells using an enzyme-linked immunoassay (Fig. 4). The basal levels of cGMP in dark-adapted SB-P cells (ca. 0.5 pmol/g FW) was increased 4-fold by 100 μM SNP after 1 h, and declined to basal levels after 2.5 h. cGMP levels in different plants have been shown to vary considerably, with basal levels ranging from 0.05 pmol/g FW in barley aleurone tissue to 2-10 pmol/g FW in tobacco leaves, and cGMP content is generally increased 3- to 10-fold in response to stimuli (Penson et al. 1996; Durner et al. 1998; Neill et al. 2003; Donaldson et al. 2004). The transient increase in cGMP levels by SNP in SB-P cells is consistent with the observed effects on gene expression (Fig. 3), although the increase in cGMP levels was faster than that of gene expression. These findings suggest the possible linkage between NO and cGMP in phototransduction in SB-P cells.

Increase of anthocyanin and isoflavone content in response to cGMP and NO

The findings that cGMP could induce expression of several genes for flavonoid biosynthetic enzymes led us to examine whether cGMP can alter the level of flavonoid-compounds such as anthocyanin. Anthocyanin content in SB-P cells treated with 8-Br-cGMP (50 μM) or SNP (100 μM) for 24 h in the dark was increased 1.4- and 1.3-fold, respectively (Fig. 5a), although the rate of increase of anthocyanin by these chemicals were much less than the increase of mRNA levels of the flavonoid synthetic enzymes (Fig. 3). This discrepancy may be caused by the unknown rate-limiting steps in the anthocyanin synthesis that are not stimulated by cGMP and NO. Instead of SNP, 50 μM of DEANO (Cayman Chemistry Co. Ltd., MI, USA), another NO generator, also could increase anthocyanin content in SB-P cells about 1.6-fold (Fig. 5b). The white- and UV-light-dependent anthocyanin accumulation was also observed (Fig. 5a), as described previously (Kerckhoffs and Kendrick 1997). As shown in Figure 5c, when soybean cotyledons were treated with 10 mM SNP, the isoflavones daidzein and genistein were detected after 20 h of incubation. However, isoflavone content in cotyledons did not change after treatment with 5 mM 8-Br-cGMP (data not shown). Similar results

were also obtained for SB-P cells, *i.e.*, isoflavone content in SB-P cells was increased by SNP (100 μM) but not by 8-Br-cGMP (50 μM).

The *CHR* promoter can direct cGMP-dependent expression

So far, only the Unit I sequence within the light-responsive promoter of parsley *CHS* has been established as a target of cGMP-mediated signal transduction (Wu et al. 1996). To attempt to identify cGMP-responsive *cis*-elements other than Unit I, we investigated different regions of the soybean *CHR* promoter in response to cGMP. The *CHR* promoter containing 1-kb upstream from the ATG start-codon together with a short stretch of the coding region was obtained by inverse PCR using soybean genomic DNA as a template and subcloned (pGmCHRproA). Primer extension analysis of the *CHR* promoter revealed that transcription begins at a cytosine residue located 414 bp upstream of the translational start site (data not shown). The putative TATA box, TATAT, was located 28 bp upstream from this transcriptional start site. The Unit I, however, was not found in the upstream region. Instead, a W-box (TTGACT), known as an elicitor-responsive element and the binding-site for a WRKY transcription factor (Ulker and Somssich 2004), at -215 bp from the transcription start site in the opposite strand, and several I-box core motifs (GATA), reported to be involved in the light-regulated expression of many photosynthetic genes (Terzaghi and Cashmore 1995), were found (data not shown).

To test the cGMP-responsiveness of the *CHR* promoter, we examined reporter gene expression in transient assays. We fused the DNA sequence of *CHR* promoter extending from -600 to +391 bp from the transcriptional start site to the GUS reporter gene (*CHR-GUS*) and introduced it into dark-adapted SB-P cells by particle bombardment with the 35S-LUC construct (pBIΩFF) as internal standard. In contrast to the Northern-blot analyses (Figs. 2 and 3), certain levels of expression of *CHS-GUS* and *CHR-GUS* were observed even in the control cells without chemical treatment (black bars in Fig. 6a), probably because of

mechanical stress of shooting. Nonetheless, when the cells were treated with 8-Br-cGMP, a 2.6-fold increase in relative GUS activity (compared to the untreated control) was found (Fig. 6a), indicating that 600 bp of promoter region is primarily responsible for the cGMP responsiveness of *CHR*. Consistent with previous microinjection experiments using tomato seedlings (Neuhaus et al. 1997), the relative GUS activity of *CHS-GUS* but not *CAB7-GUS* was also increased 2.2-fold by 8-Br-cGMP treatment. On the other hand, 8-Br-cAMP treatment of SB-P cells did not activate reporter gene expression (Fig. 6a).

To broadly determine the region containing the cGMP responsive *cis*-element in the *CHR* promoter, a 5’ deletion analysis of the 600-bp *CHR* promoter was conducted. Relative GUS activities in SB-P cells transfected with *CHR* promoter fragments -600 to +391, and -422 to +391 were increased 2.0- and 1.4-fold, respectively, in response to 8-Br-cGMP (Fig. 6b). By contrast, the -185 to +391 fragment fused to *GUS* showed no significant induction in response to cGMP. Furthermore, the region between -600 and -186 could confer cGMP-responsiveness on the CaMV 35S minimal promoter (Fig. 6b). These results indicate that the *cis*-acting element(s) responsible for cGMP-dependent gene expression is located between nucleotides -600 and -186 relative to the transcription start site in the *CHR* promoter.

## Discussion

The data presented here indicate that cGMP and NO play key roles in flavonoid biosynthesis in soybean cells. The evidence in support of this conclusion can be summarized as follows. (i) Soybean *CHR* and *IFR* were identified as cGMP-regulated genes by PCR-based subtraction, (ii) Northern-hybridization analysis confirmed that several genes encoding flavonoid-biosynthetic enzymes were strongly induced by cGMP and NO at transcriptional levels (Fig. 2 and 3), (iii) cGMP levels in dark-adapted SB-P cells were increased 4-fold by SNP treatment (Fig. 4), (iv) Anthocyanins accumulated in response to cGMP/NO (Fig. 5a and b), and (v) The isoflavones daidzein and genistein accumulated in soybean cotyledons in

response to SNP (Fig. 5c). Taken together, these results suggest that cGMP together with NO act as common regulators for the transcriptional activation of flavonoid biosynthetic pathway genes in soybean plants. In contrast to the transcriptional activation, NO and cGMP are not likely to cooperate in some process(es) of isoflavone accumulation, since isoflavone contents in cotyledons and SB-P cells were increased by NO but not by cGMP. Also, the metabolic responses of soybean cells to NO and cGMP were not paralleled to the stimulation of gene activation by them, *i.e.* NO and cGMP strongly induced the expression of genes for the enzymes involved in flavonoid biosynthesis, but only small changes of anthocyanin and isoflavonoid contents could be detected. Since the end products of the isoflavonoid pathway in soybean plants are glyceollins, the daidzein-derived pterocarpans (Modolo et al. 2002), further experiments to detect these products are necessary.

We previously reported that cGMP acts as a second messenger utilized by phytochrome A for the activation of *CHS* expression and whole anthocyanin biosynthetic pathway, based on the facts that cGMP could trigger the *CHS-GUS* expression and anthocyanin production, and inactive cGMP analog, Rp-guanosine 3', 5'-cyclic monophosphorothioate (Rp-cGMPS) could inhibit phytochrome A-mediated *CHS-GUS* activation in tomato hypocotyl cells (Bowler et al. 1994a). Also, several membrane-permeable cGMP derivatives such as 8-Br-cGMP induced *CHS,* but not other genes encoding photosynthetic components in the dark in soybean SB-P cells (Bowler et al. 1994a). However, 8-Br-cAMP has no effect on these responses (Fig. 2; Bowler et al. 1994a). These findings suggest that the observed effects of exogenous 8-Br-cGMP on the induction of several genes for flavonoid biosynthetic enzymes in SB-P cells described here are cGMP-specific and not to be artificial.

The transient patterns of accumulation of transcripts for several flavonoid-biosynthetic enzymes (except for *ANS*) in response to white light were similar to that of *CHS* (Fig. 3), suggesting that these genes are likely to be co-regulated by the same cGMP-pathway of phototransduction that controls *CHS* expression, most likely because of desensitization of photoinduction of these genes as a result of decreasing cGMP levels (Bowler et al. 1994a, b).

When 8-Br-cGMP was added to the cells, expression of these flavonoid-related genes was maintained at high levels for 10 h (Fig. 2 and 3). Because 8-Br-cGMP is nonhydrolyzable, the observation that it stimulates gene expression that does not subsequently attenuate indicates that reductions in cGMP concentrations mediated by light are responsible for desensitization under normal conditions, as discussed previously (Bowler et al. 1994b), although the molecular mechanisms responsible for desensitization remain to be elucidated. In this regard, it is interesting that cGMP levels in SB-P cells were transiently increased by SNP (Fig. 4), similar to the induction of flavonoid-related genes by SNP as well as by light, though the increase of cGMP by SNP was faster (peaking at ~1 h) than gene expression responses to SNP and light (~3 h). These results suggest that the photoinduction and subsequent desensitization of these genes appear to be mediated by cGMP and NO.

There is increasing evidence that NO plays an important role in diverse physiological processes in plants such as disease resistance, abiotic stress, programmed cell death, respiration, senescence, root development, germination, and hormone responses (Lamattina et al. 2003; Neill et al. 2003; Wendehenne et al. 2007). Stimulation by NO of light-induced responses such as seed germination, de-etiolation and inhibition of hypocotyl elongation has also been described (Beligni and Lamattina 2000). Several studies involving large-scale transcriptional analyses have recently provided evidence for the importance of NO in gene regulation (Grün et al. 2006). In animals, the formation of S-nitrosylated proteins seems to be an important mechanism in the regulation of the function/activity of transcription factors. However, no plant transcription factor has been described that is regulated by S-nitrosylation (Lindermayr and Durner 2007). Our findings that expression of all cGMP-induced genes and anthocyanin accumulation in SB-P cells were also stimulated by NO (Fig. 3 and 5a) indicate close relationships between NO and cGMP in the regulation of gene expression of flavonoid biosynthetic enzymes in soybean. It is most plausible that NO and cGMP act in the same pathway for the activation of these genes. Although NO-activated GC has not yet been identified in plants, our findings that LY83583 inhibited all SNP-induced gene

expression except for *C4H* (Fig. 3) and that NO treatment resulted in an increase of cGMP (Fig. 4), suggest the possible existence of mammalian sGC-like enzyme(s) in plants. The presence of NO-inducible GC in plants has also been deduced from the observed effects of various agonists and antagonists of mammalian GC activity on the accumulation of cGMP (Durner et al. 1998).

Since PCR-based subtraction technique is not the global transcript analysis, we performed DNA microarray analysis using *Arabidopsis* cell culture T-87, and found that several hundreds genes were up- and down-regulated by cGMP, respectively (in preparation). Similar results showing that many genes were regulated by cGMP in *Arabidopsis* have also been reported (Maathuis 2006), suggesting that the cGMP is likely to be the general regulator of gene expression in plants. However, in these microarray analyses of *Arabidopsis,* most of the genes for flavonoid biosynthetic enzymes were found to be not regulated by cGMP, suggesting that the role of cGMP as a regulator of gene expression involved in second metabolisms may be different between *G. max* and *A. thaliana.*

Isoflavonoids are a legume-specific subclass of flavonoid secondary-metabolites with roles in plant-microorganism interactions. In soybean roots, the expression of *IFS* is specifically induced by infection of nitrogen-fixing rhizobium, and isoflavonoids in roots (*e.g.,* daidzein and genistein) are essential for nodulation because of their ability to activate rhizobial *nod* genes (Subramanian et al. 2007). Our findings that cGMP and NO can induce several genes of the isoflavonoid biosynthetic pathway (Figs. 2 and 3), together with the observation that NO enhanced the accumulation of daidzein and genistein in soybean cotyledons (Fig. 5c; Modolo et al. 2002), suggest that both cGMP and NO may function in the establishment of symbiosis between leguminous plants and nitrogen-fixing bacteria.

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**References**

Akashi T, Aoki T, Ayabe S (2005) Molecular and biochemical characterization of 2-hydroxyisoflavanone dehydratase. Involvement of carboxylesterase-like proteins in leguminous isoflavone biosynthesis. Plant Physiol 137:882-891

Bariola PA, MacIntosh GC, Green PJ (1999) Regulation of S-like ribonuclease levels in *Arabidopsis.* Antisense inhibition of RNS1 or RNS2 elevates anthocyanin accumulation. Plant Physiol 119:331-342

Beligni MV, Lamattina L (2000) Nitric oxide stimulates seed germination and de-etiolation, and inhibits hypocotyl elongation, three light-inducible responses in plants. Planta 210:215-221

Bowler C, Neuhaus G, Yamagata H, Chua NH (1994a) Cyclic GMP and calcium mediate phytochrome phototransduction. Cell 77:73-81

Bowler C, Yamagata H, Neuhaus G, Chua NH (1994b) Phytochrome signal transduction pathways are regulated by reciprocal control mechanisms. Genes Dev 8: 2188-2202

Cousson A (2003) Pharmacological evidence for a positive influence of the cyclic GMP-independent transduction on the cyclic GMP-mediated $Ca^{2+}$-dependent pathway within the *Arabidopsis* stomatal opening in response to auxin. Plant Sci 164:759‑767

Donaldson L, Ludidi N, Knight MR, Gehring C, Denby K (2004) Salt and osmotic stress cause rapid increases in *Arabidopsis thaliana* cGMP levels. FEBS Lett 569:317-320

Durner J, Wendehenne D, Klessig DF (1998) Defense gene induction in tobacco by nitric

oxide, cyclic GMP and cyclic ADP-ribose. Proc Natl Acad Sci USA 95:10328-10333

Grün S, Lindermayr C, Sell S, Durner J (2006) Nitric oxide and gene regulation in plants. J Exp Bot 57:507-516

Hanafy KA, Krumenacker JS, Murad F (2001) NO, nitrotyrosine, and cyclic GMP in signal transduction. Med Sci Monit 7:801-819

Horn ME, Sherrard JH, Widhorm JM (1983) Photoautotrophic growth of soybean cells in suspension culture. Plant Physiol 72:426-429

Hu X, Neill SJ, Tnag Z, Cai W (2005) Nitric oxide mediates gravitropic bending in soybean roots. Plant Physiol 137:663-670

Kerckhoffs LHJ, Kendrick RE (1997) Photocontrol of anthocyanin biosynthesis in tomato. J Plant Res 110:141-149

Kuhn DN, Chappell J, Boudet A, Hahlbrock K (1984) Induction of phenylalanine ammonia-lyase and 4-coumarate:CoA ligase mRNAs in cultured plant cells by UV light or fungal elicitor. Proc Natl Acad Sci USA 81:1102-1106

Kwezi L, Meier S, Mungur L, Ruzvidzo O, Irving H, Gehring C (2007) The *Arabidopsis thaliana* brassinosteroid receptor (AtBRI1) contains a domain that functions as a guanylyl cyclase *in vitro*. PLoS ONE 2:e449

Lamattina L, Garcia-Mata C, Graziano M, Pagnussat G (2003) Nitric oxide: The versatility of an extensive signal molecule. Annu Rev Plant Biol 54:109–136

Lindermayr C, Durner J (2007) S-Nitrosylation in plants- spectrum and selectivity. In: Lamattina L, Polacco JC (eds) Nitric oxide in plant growth, development and stress physiology. Springer, Berlin Heidelberg New York pp 53-71

Ludidi N, Gehring C (2003) Identification of a novel protein with guanylyl cyclase activity in *Arabidopsis thaliana*. J Biol Chem 278:6490-6494

Maathuis FJ (2006) cGMP modulates gene transcription and cation transport in *Arabidopsis* roots. Plant J 5:700-711

Martin C, Prescott A, Mackay S, Bartlett J, Vrijlandt E (1991) Control of anthocyanin

biosynthesis in flowers of *Antirrhinum majus*. Plant J 1:37-49

Modolo LV, Cunha FQ, Braga MR, Salgado I (2002) Nitric oxide synthase-mediated phytoalexin accumulation in soybean cotyledons in response to the *Diaporthe phaseolorum* f. sp. *meridionalis* elicitor. Plant Physiol 130:1288–1297

Neill SJ, Desikan R, Hancock JT (2003) Nitric oxide signalling in plants. New Phytol 159:11–35

Neuhaus G, Bowler C, Hiratsuka K, Yamagata H, Chua NH (1997) Phytochrome-regulated repression of gene expression requires calcium and cGMP. EMBO J 16:2554-2564

Newton RP, Chiatante D, Ghosh D, Brenton AG, Walton TJ, Harris FM, Brown EG (1989) Identification of cyclic nucleotide constituents of meristematic and non-meristematic tissues of *Pisum sativum* roots. Phytochemistry 28:2243–2254

Newton RP, Smith CJ (2004) Cyclic nucleotides. Phytochemistry 65:2423-2437

Noguchi A, Saito A, Homma Y, Nakao, M, Sasaki N, Nishino T, Takahashi S, Nakayama T (2007) UDP-glucose:isoflavone 7-*O*-glucosyltransferase from the roots of soybean (*Glycine max*) seedlings: purification, gene cloning, phylogenetics, and an implication for an alternative strategy of enzyme catalysis. J Biol Chem 282:23581-23590

Pagnussat GC, Lanteri ML, Lamattina L (2003) Nitric oxide and cyclic GMP are messengers in the indole acetic acid-induced adventitious rooting process. Plant Physiol 132:1241-1248

Penson SP, Schuurink RC, Fath A, Gubler F, Jacobsen JV, Jones RL (1996) cGMP is required for gibberellic acid-induced gene expression in barley aleurone. Plant Cell 8: 2325-2333

Pilz RB, Casteel DE (2003) Regulation of gene expression by cyclic GMP. Circ Res 93: 1034-1046

Prado AM, Porterfield DM, Feijó JA (2004) Nitric oxide is involved in growth regulation and re-orientation of pollen tubes. Development 131:2707-2714

Russwurm M, Koesling D (2004) NO activation of guanylyl cyclase. EMBO J 23:4443-4450

Sambrook J, Russell DW (eds) (2001) Molecular Cloning: A laboratory Manual, 3rd ed. Cold Spring Harbor Lab Press, Cold Spring Harbor, NY

Schijlen EGWM, Ric de Vos CH, van Tunen AJ, Bovy AG (2004) Modification of flavonoid biosynthesis in crop plants. Phytochemistry 65:2631-2648

Subramanian S, Stacey G, Yu O (2007) Distinct, crucial roles of flavonoids during legume nodulation. Trends Plant Sci 12:282-285

Szmidt-Jaworska A, Jaworski K, Tretyn A, Kopcewicz J (2004) The involvement of cyclic GMP in the photoperiodic flower induction of *Pharbitis nil*. J Plant Physiol 161:277–284

Terzaghi WB, Cashmore AR (1995) Light-regulated transcription. Annu Rev Plant Physiol Plant Mol Biol 46:445-474

Uhlmann A, Ebel J (1993) Molecular cloning and expression of 4-coumarate:coenzyme A ligase, an enzyme involved in the resistance response of soybean (*Glycine max* L.) against pathogen attack. Plant Physiol 102:1147-1156

Ulker B, Somssich E (2004) WRKY transcription factors: from DNA binding towards biological function. Curr Opin Plant Biol 7:491-498

Welle R, Schröder G, Schiltz E, Grisebach H, Schröder J (1991) Induced plant responses to pathogen attack. Analysis and heterologous expresson of the key enzyme in the biosynthesis of phytoalexins in soybean (*Glycine max* L. Merr. Cv. Harosoy 63). Eur J Biochem 196:423-430

Wendehenne D, Courtois C, Besson A, Gravot A, Buchwalter A, Pugin A, Lamotte O (2007) NO-based signaling in plants. In: Lamattina L, Polacco JC (eds) Nitric oxide in plant growth, development and stress physiology. Springer, Berlin Heidelberg New York, pp 35-51

Wu Y, Hiratsuka K, Neuhaus G, Chua NH (1996) Calcium and cGMP target distinct phytochrome-responsive elements. Plant J 10:1149-1154

Yamagata H, Masuzawa T, Nagaoka Y, Ohnishi T, Iwasaki T (1994) Cucumisin, a serine protease from melon fruits, shares structural homology with subtilisin and is generated from a large precursor. J Biol Chem 269:32725-32731

Yamagata H, Saka K, Tanaka T, Aizono Y (2001) Light activates a 46-kDa MAP kinase-like

protein kinasa in soybean cell culture. FEBS Lett 494:24-29

Yamagata H, Yonesu K, Hirata A, Aizono Y (2002) TGTCACA is a novel *cis*-regulatory enhancer element involved in fruit-specific expression of the *cucumisin* gene. J Biol Chem 277:11582-11590

**Figure legends**

**Fig. 1** The flavonoid-biosynthetic pathway in *Glycine max.*

**Fig. 2** Induction of *CHR*, *IFR* and *CHS* by cyclic nucleotides in SB-P cells. Dark-adapted SB-P cells were incubated with either 50 μM 8-Br-cGMP (**a**) or 8-Br-cAMP (**b**) in the dark. Cells were harvested at the times indicated and transcript levels were analyzed by Northern-blot analysis of total RNA. Equal amounts of RNA (30 μg/lane) were verified by rehybridizing the blots with a $^{32}$P-labeled cDNA fragment of soybean 18S rRNA. Times are indicated in h

**Fig. 3** Induction by cGMP, NO, and white light of several genes involved in flavonoid synthesis, as examined by Northern-blot analysis. Dark-adapted SB-P cells were incubated in the dark with either 8-Br-cGMP (50 μM), SNP (100 μM) with or without LY83583 (LY), or illuminated with white light for the indicated times. Each lane contains 30 μg of total RNA, except for PAL and IFGT, for which 1 μg of poly(A)$^+$ RNA was used. rRNA is a loading control and was detected by ethidium bromide staining of the gel. Times are indicated in h

**Fig. 4** cGMP levels in NO-treated SB-P cells. Dark-adapted SB-P cells were incubated with (closed circles) and without (open circles) 100 μM SNP in the dark. At indicated times, samples were collected and analyzed for cGMP content by enzyme-linked immunoassay. Data represent the average of three independent experiments with duplicate measurements for each sample. Standard errors are indicated

**Fig. 5** Increase of anthocyanin and isoflavone content in response to cGMP and NO. **a**. Effect of 8-Br-cGMP, SNP, white light, and UV-B light on anthocyanin content in SB-P cells. Dark-adapted SB-P cells were incubated in the dark for 24 h (bar 1), incubated with

50 μM 8-Br-cGMP (bar 2) or 100 μM SNP (bar 3) for 24 h in the dark, or illuminated with white light for 24 h (bar 4) or UV-B for 2 h followed by incubation for 22 h in the dark (bar 5). Anthocyanin content was determined as described in the text. The average of non-treated cells (dark) was set at 100%. Data represent the mean of three independent experiments. Standard errors are shown. **b**. Effect of DEANO on anthocyanin content in SB-P cells. Dark-adapted SB-P cells were incubated with each concentration of DEANO for 24 h in the dark, and anthocyanin content was determined as **a**. **c**. Accumulation of isoflavonoids in SNP-treated soybean cotyledons. Soybean cotyledons were treated with (**A**) or without (**B**) 10 mM SNP for 20 h. Cell extracts were analyzed by HPLC. Metabolites were identified by comparing their retention times with those of standards. Dz, Daidzein; Gt, genistein

**Fig. 6** Analyses of cGMP responsiveness of several promoters of cGMP-induced genes. a. The promoters of *CHS* (*Arabidopsis thaliana*), *CHR* (*Glycine max*), and *CAB7* (*Licopersicum esculentum*) were fused with the β-glucuronidase gene (*GUS*), and these constructs were introduced into dark-adapted SB-P cells as described in "Experimental Procedures". After bombardment, the cells were incubated with 50 μM 8-Br-cGMP or 8-Br-cAMP in the dark for 10 h, and GUS activities in the cell extract were measured. All GUS values were normalized to LUC values, and the average of pBI221 was taken to be 100. Black bar, control cells without chemical treatment; white bar, 8-Br-cGMP treated cells; gray bar, 8-Br-cAMP treated cells. b. 5'-deletion and gain-of-function analyses of the *CHR* promoter. The 5'-deletion constructs containing various lengths of the *CHR* promoter fused to the promoterless pSKGUS3C plasmid were introduced into dark-adapted SB-P cells. After bombardment, the cells were incubated with 50 μM 8-Br-cGMP, and GUS activities were measured as described in A. The numbers refer to the 5'- and 3'-ends of the *CHR* upstream fragments with respect to the transcriptional start site. For the gain-of-function analysis, the -600 to -186 region of the *CHR* promoter was fused upstream of the CaMV 35S minimal promoter (-46 to +8), and the chimeric promoter was linked to the *GUS* reporter gene.

Data represent the mean (±S.E.) of six independently bombarded samples. NOS, nopaline synthase terminator; 3C, Rubisco 3C (small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase 3C) terminator

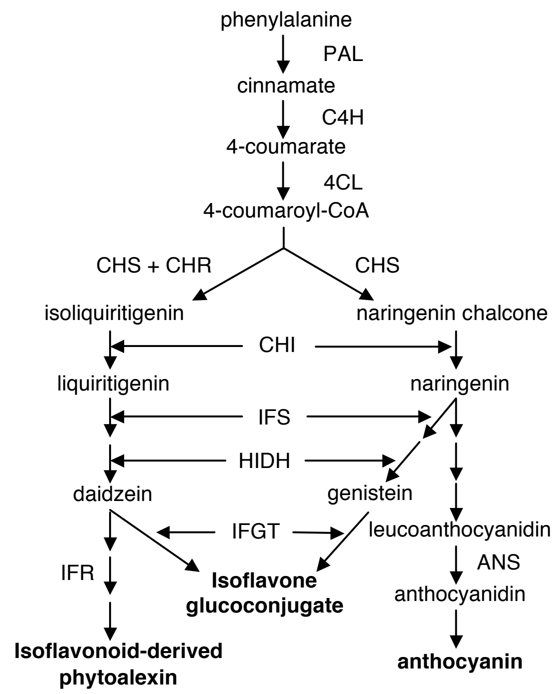



Fig. 1

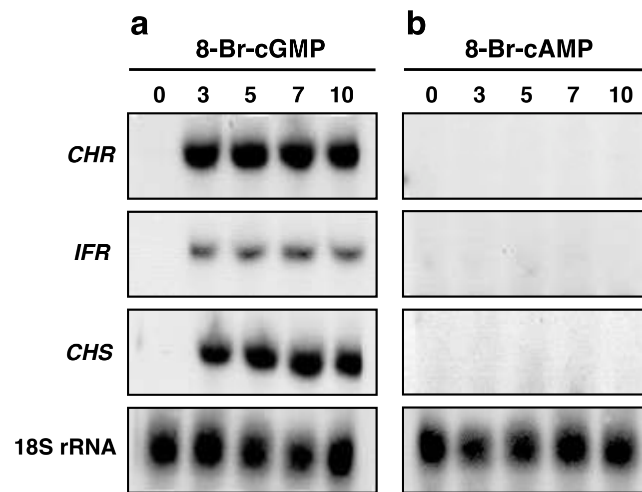
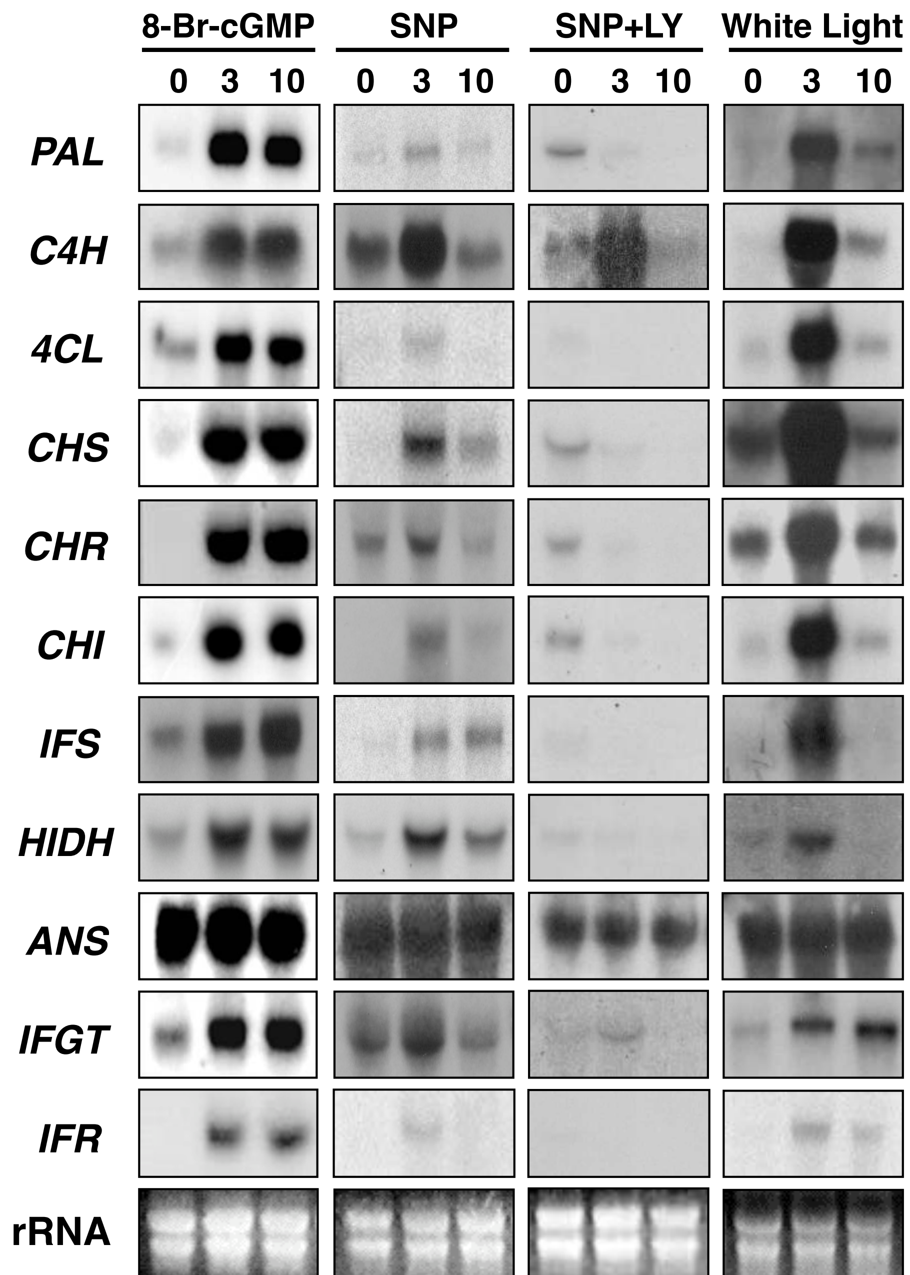



Fig. 2

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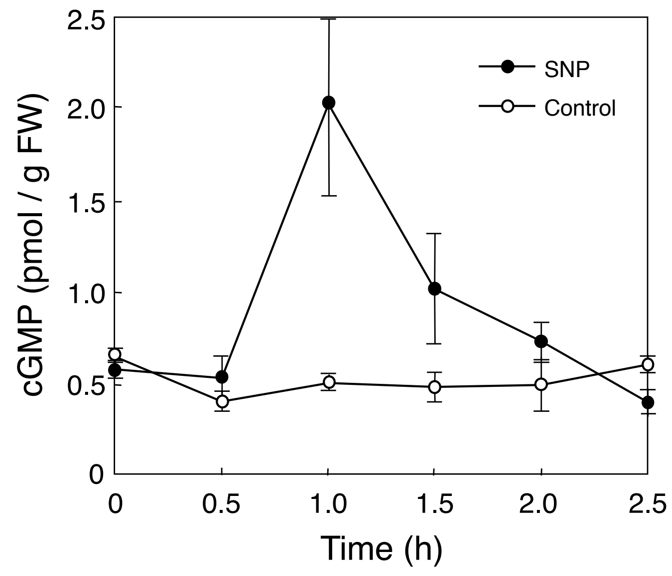


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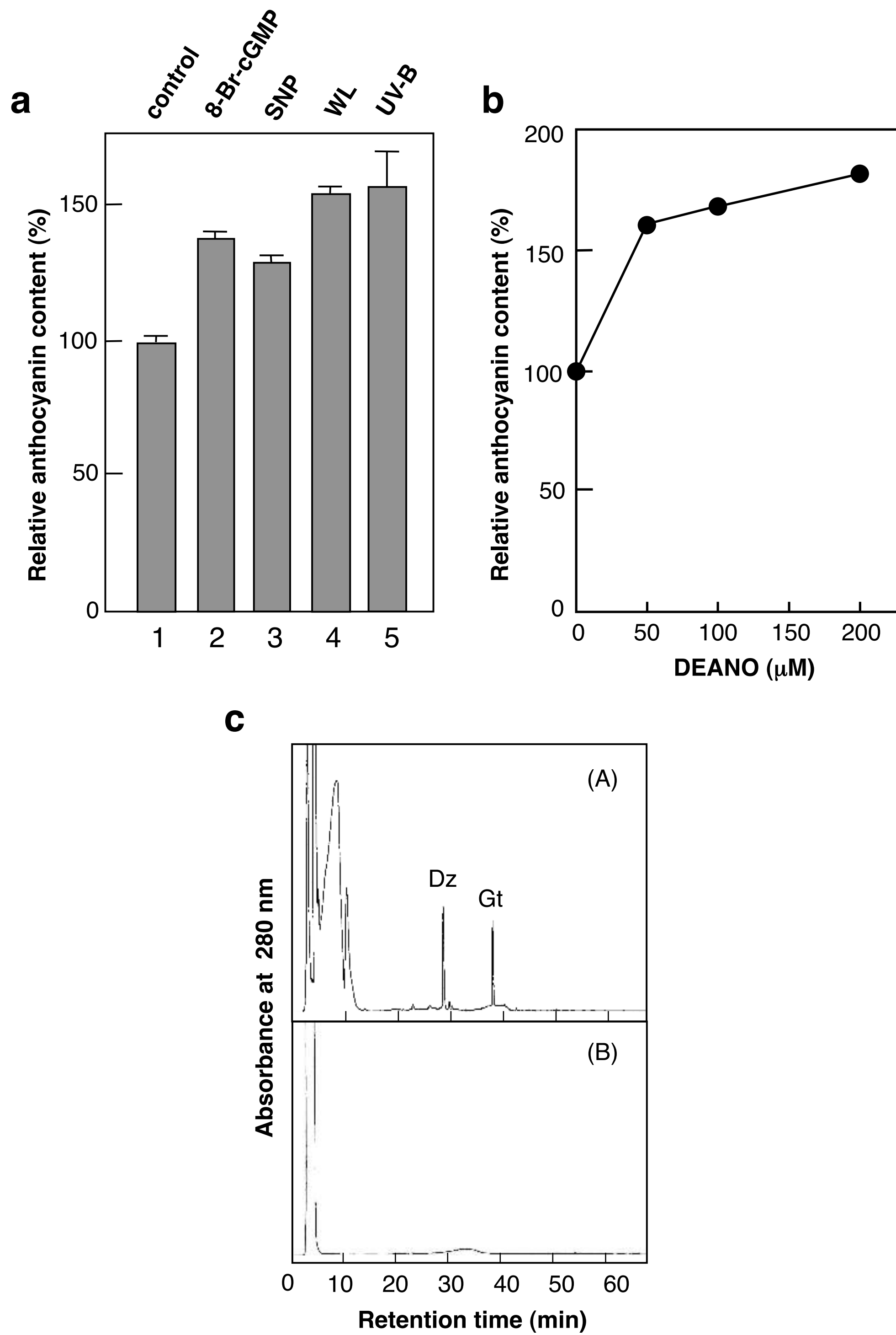


Fig. 5

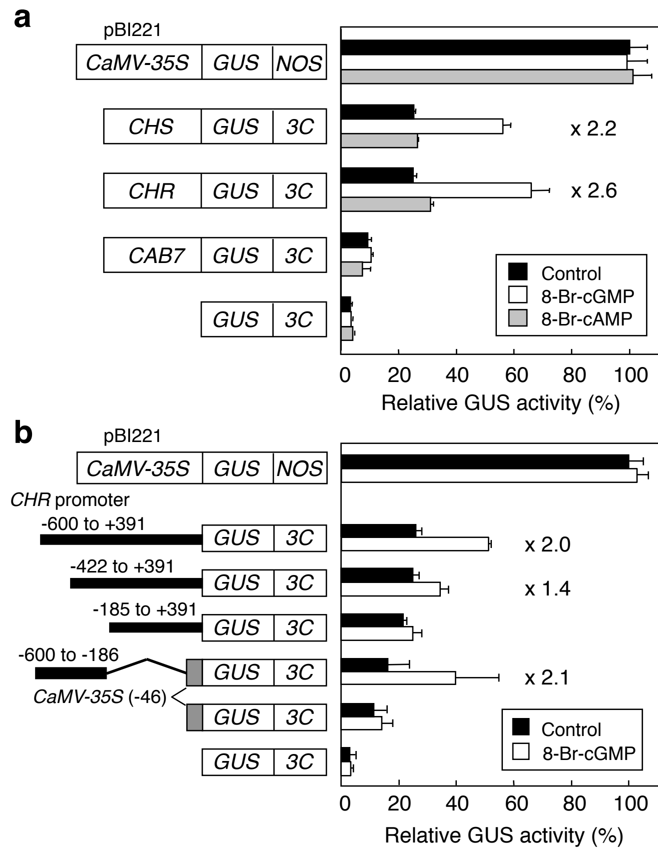


Fig. 6