Molecular Cloning and Characterization of a Subfamily of UV-B Responsive *MYB* genes from Soybean

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Flavonoid compounds accumulating in the epidermal cell layers of plant tissues are considered to be among the most effective protectants against the ultraviolet-B (UV-B) radiation. To identify a transcription factor involved in the activation of the genes, such as chalcone synthase genes (*CHS*), in the pathway of flavonoid biosynthesis, a pair of degenerate primers was designed to amplify the most conservative region of *MYB* from soybean by polymerase chain reaction (PCR) or reverse transcriptase (RT)-PCR. Both of these amplification products were found to contain molecules of dozens of independent *MYB*-like sequences. The bacterial clones harboring a partial library of the RT-PCR products were differentially hybridized with the amplified cDNA fragments derived from total RNA of UV-B treated seedlings (rMYB/UV-B+) and those from control RNA (rMYB/UV-B-). One clone designated as MYB29 showed a significantly stronger signal with rMYB/UV-B+ hybridization than with rMYB/UV-B-. Starting from the sequence information of MYB29 fragment, an entire sequence containing the complete gene designated as *GmMYB29A1* was obtained by nested PCR of the flanking regions. In the course of this PCR cloning, we identified several independent products closely related to *GmMYB29A1*. In order to amplify the entire protein coding region of the closely related genes, two sets of primers were designed, two upstream primers containing the ATG start codon and the other two downstream primers containing the TGA stop codon. By sequencing those cDNAs amplified with RT-PCR, a total of at least four members were found to comprise the subfamily, designated as *GmMYB29*. UV-B-responsive expression of the members of *GmMYB29* was found to reach its peak within 2 hours after the onset of light exposure while in those of soybean (*Gm*)*CHS* it continued to rise for 6 hours. This finding suggested that these *MYB* transcription factors may activate genes for flavonoid biosynthesis including *GmCHS* in response to UV-B exposure.

Key Words: soybean, chalcone synthase, Ultraviolet-B, *MYB*, flavonoid.

Introduction

Prospective increase in the terrestrial level of Ultraviolet-B (UV-B; 280~320 nm) radiation due to the lasting trend of stratospheric ozone depletion has prompted researchers to investigate protective mechanisms contributing to UV-B tolerance in plants. Flavonoid compounds accumulating in the epidermal cell layers of plant tissues are considered to be one of the most effective protectants against the UV-B radiation (Li *et al.* 1993, Stapleton and Walbot 1994) causing harmful effects on higher plants, such as damage of DNA molecules, leaf chlorosis and reduction of crop yield (Teramura 1983). In fact, mutants of *Arabidopsis thaliana* with deficiency in the flavonoid biosynthesis were extremely sensitive to UV-B radiation and displayed an extensive growth inhibition (Li *et al.* 1993).

Because chalcone synthase (*CHS*; EC 2.3.1.74) catalyzes the first step reaction of the flavonoid biosynthesis, it may be possible to increase the production of UV-B-protective flavonoids by genetically improving the expression of *CHS*. Soybean *CHS* is encoded by a multigene family (*GmCHS*) of at least eight members (*GmCHS1*~*GmCHS8*) (Akada and Dube 1995). We previously reported that the expression of every member of the family was induced by exposure to white light and was enhanced further by supplemental UV-B radiation, except for that of *GmCHS2* (Shimizu *et al.* 1999). Interestingly, the H-box sequence of CCTACC (Loake *et al.* 1992) was very well conserved in the promoter region of every member of the family excluding *GmCHS2*, raising the possibility that the H-box might act as a *cis*-regulatory element for the UV-B responsiveness.

This H-box sequence was first reported to act as a promoter *cis*-element necessary for a feed-forward induction of *CHS* by cinnamate, a substrate for the enzyme reaction. On the other hand, the maize *P trans*-acting factor was found to recognize the identical H-box sequence on the promoter of maize *CHS* (Grotewold *et al.* 1994). Thus we assumed the presence of a soybean homologue of the maize *P*, which would also function through recognition of the H-box. *P* regulates the transcription of genes required for phlobaphene pigmentation in the pericarp including those for CHI (chalcone flavanone isomerase) and A1 (dihydroflavonol 4-reductase, DFR) as well as C2 (chalcone synthase, *CHS*). The molecular structure of *P* is typical of

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the MYB transcription factors which regulate the expression of various genes in animals and plants. In higher plants, maize C1 was the first to have been identified as a MYB-homologous factor (Paz-Ares *et al.* 1987), which was found to regulate the expression of the genes involved in the anthocyanin pigmentation in the kernel aleurone of seeds and to show a significant structural similarity to the animal proto-oncogene c-MYB. The structure of MYB proteins consists of the DNA-binding domain in their N-terminal halves and the transactivation domain in their C-terminal halves. The DNA-binding domain is highly conserved among most MYB proteins, especially the three repetitive sequences of about 50 amino acid residues that contain conserved tryptophan residues in each repeat known as a tryptophan cluster (Kanei-Ishii *et al.* 1990). The MYB proteins containing this domain have been shown to bind to DNA in a sequence-specific manner (Luscher and Eisenman 1990). On the other hand, the transactivation domain is extensively differentiated among MYB proteins and is involved in the activation of transcription in plants (Goff *et al.* 1991).

Using information about the nucleotide sequences of *MYB* genes from a variety of plant species, we designed a pair of degenerate primers to amplify by polymerase chain reaction (PCR) the most conservative region of *MYB*. The PCR products from a total DNA sample and the reverse transcriptase (RT)-PCR products from a total RNA sample contained molecules of dozens of independent *MYB*-like sequences. During the search for a UV-B responsive *MYB*, a library of the bacterial clones containing the RT-PCR products was screened by differential colony hybridization. Here we describe the molecular cloning and structural characterization of a subfamily of *GmMYB29*. Members of *GmMYB29* were found to be induced upon UV-B radiation.

Materials and Methods

Plant materials and light treatment

Seeds of *Glycine max* (L.) Merr. cultivar Williams were germinated at 25°C in darkness on a bed of 1% agar gel in petri dishes. Seedlings grown for three days in darkness were exposed to fluorescent white light (National FL20SS 20W, Japan) supplemented with UV-B light (Toshiba FL20SE 20W, Japan; $\lambda_{\max}$ 312 nm). For the UV-B- treatment, the light was filtered through a 0.1 mm polyester film, which absorbs almost all the radiation below 320 nm. For the UV-B+ treatment, the light was filtered through a 0.1 mm cellulose diacetate film, which allows transmission down to 290 nm (Sisson and Caldwell 1975, Krizek and Koch 1979). After the light treatment, hypocotyl tissues were harvested and snap-frozen in liquid nitrogen for RNA extraction.

DNA/RNA isolation and Degenerate Polymerase Chain Reaction

Total DNA was extracted from the leaves of greenhouse grown Williams using the method of Ausubel *et al.*

(1998) and purified by cesium chloride gradient centrifugation (Sambrook *et al.* 1989). Total RNA was extracted from Williams hypocotyl tissues after UV-B exposure as described previously (Shimizu *et al.* 1999). *MYB*-homologous sequences were amplified by PCR from total DNA or by RT-PCR from total RNA using a pair of degenerate primers (MYBFP; 5'-GTG GRA AAA GTT GYA GRC TNA GWT GG-3' and MYBRP; 5'-CCA RTA ATT YTT RAT TTC RTT RTC SG-3'). These PCR and RT-PCR products were cloned into the bacterial plasmid pBluescript II SK (+) (Stratagene, USA) and were sequenced.

Differential colony hybridization

A nitrocellulose membrane was placed on a 1.5% agar LB medium containing 50 µg/ml ampicillin and was inoculated with the bacterial transformants (rMYB) obtained by shot-gun cloning of the RT-PCR products amplified from total RNA of UV-B+ treated seedlings. The plasmid DNA immobilized on the membrane using the method of Sambrook *et al.* (1989) was hybridized successively with ³²P-labeled RT-PCR products amplified from total RNA of UV-B-treated seedlings (hereafter referred to as "UV-B+ seedlings"), and next with those of seedlings not treated with UV-B (hereafter referred to as "UV-B- seedlings"). Hybridization conditions thereafter were the same as those for the Southern analysis described below. The colonies showing the most conspicuous signal differences (rMYB10 and rMYB29) as well as the least pronounced signal differences (rMYB2, rMYB3, rMYB4, rMYB5, rMYB11, and rMYB13) were selected for sequence analyses.

Nested Polymerase Chain Reaction

Molecular cloning of the entire sequence containing *GmMYB29A1* was performed by using the Cassette and Cassette Primer method (Takara, Japan). A 1 µg aliquot of total DNA was digested either with *EcoRI*, *HindIII*, *Sau3AI*, or *BamHI* and was ligated with the corresponding Cassette using the DNA Ligation Kit Ver. 1 (Takara, Japan). A 100 ng aliquot of the ligated DNA was used as a template for the first PCR amplification with the Cassette Primer C1 and the first *GmMYB29* specific primer (P1; 5'-CTG TTC CCC AGC ATG TCA TG-3', P2; 5'-GTG GTC AGC AAT TGC AGC CA-3', P8; 5'-AAG AGG GAA GAC ATA GAG TCT-3', P9; 5'-ACG CCA ATT GCC ATG ACC AT-3', or P10; 5'-CCA ATT TAA TTC AGT GGA AAC C-3'). A 1 µl aliquot of the first PCR products was used as template for the second PCR amplification with the Cassette Primer C2 and the second *GmMYB29* specific primer (P3; 5'-CAG CTT AAT GAT AGT TTC TTC TT-3', P5; 5'-TTT GTC AAT GTA AGA TGT GAG G-3', P6; 5'-ATG GAG ACA GTA AAG CCT CC-3', or P12; 5'-CTT TCA ACA GCA AAG TGT TGG-3'). The second PCR products were cloned into the plasmid pBluescript II SK (+) and were sequenced. The sequence determination of PCR products was carried out for duplicated or triplicated clones to exclude the possibility of misreading caused by artificial

CONSENSUS	gKSCRLRW #NyLrp?#+rG?#t??E-?#Ii?Lh??lGN+Ws?iA??lpgr TDNeiKNyW	
ZmMYBP1	 I.....ADVK..NISKE.EDI..K..AT...R..L..SH....	
ZmMYBC1	 L.....NIR..NISYD.EDL..R..RL...R..L..GR....	
ZmMYBP1	 L.....NIK..NISYD.EDL.VR..KL...R..L..GR....	
AtMYBGL1	 M.....S.NVNK.NF.EQ.EDL..R..KL...R..L..KRV... ..QV....	
AtMYB1	 C.Q.N.NLI.NSF.EV.DQA..AA.AIH..K.AV..KL.... ..A...H.	
AtMYB2	 L.....DVR..NI.LE.QFM.LK..SLW..R..K..QY....	
PhMYB1	 T.....DIK..KF.LQ.EQT..Q..AL...R..A..TH..K.	
PhMYB2	 T.....DIK..NF.RE.EDT..Q..EM...R..A..AR....	
PhMYB3	 A.H...NLKK.AF.VE.ERI..E..AK...K.ARM.AQ....	
	MYBFP	MYBRP
GmMYB1 (gMYB1)	I.....DVK..NF.LE.EEN..R..KAL..K..K..SR....	+Intron; 122 bp
GmMYB2 (gMYB2)	M.....DIK..NIAPE.DDL..RM.SLL..R..L..GR....	
GmMYB3 (gMYB7)	L.....DVR..NI.LQ.QIT.LE..SRW..R..K..RH....	+Intron; 93 bp
GmMYB4 (gMYB8)	M.....DIK..NI.PE.DDL..RM.SLL..RR..L..GR....	
GmMYB5 (gMYB10)	V.....GLKK.QL.PL.EEI..E..ATL..K..T..KY.S..	+Intron; 91 bp
GmMYB6 (gMYB13)	M.....NLK..NF.QE.EEC..RM.KKL..R..A..AE....	+Intron; 156 bp
GmMYB7 (gMYB15)	M.....NLKH.HFSVE.EQL.VQ.QQQL..K.AK..RK....	+Intron; 135 bp
GmMYB10 (gMYB16/rMYB10)	I.....DLK..MFSQQ.GDL..S..EVL..R..AQ..AQ....	+Intron; 123 bp
GmMYB8 (gMYB24)	V.....DLK..QI.SQ.ESI.LE..TRW..R..T..RS....	+Intron; 91 bp
GmMYB9 (gMYB25)	I.....DVK..NF.PE.EET..R..KAL..K..K..SR....	+Intron; 121 bp
GmMYB11 (rMYB2)	T.....DIK..KFSLQ.EQT..Q..ALL..R..A..TH..K.	
GmMYB12 (rMYB3)	L.....NVR..NI.LQ.QLL.LD..SRW..R..K..EH....	
GmMYB13 (rMYB4)	L...K.DIK..NISSD.EDL..R..SLL..R..L..GR....	
GmMYB14 (rMYB5)	I.....DVK..NF.PE.EET..R..KAL..K..K..SG....	
GmMYB15 (rMYB11)	A.....DIK..RFSFE.EEA..Q..SVL..K*.T..AN....	
GmMYB16 (rMYB13)	M.....DIK..NI.PE.DDL..RM.SLL..R..L..GR....	
GmMYB29 (rMYB29)	I.....DIK..NF.IE.EET..K..DML..R..A..AK....	

Fig. 1. Alignment of the amino acid sequences deduced from the nucleotide sequences of the PCR products (gMYB or rMYB), and those of the corresponding MYB domain from *Petunia hybrida* (PhMYB 1, 2, and 3; accession numbers Z13996, Z13997, and Z13998 respectively), *Zea mays* (ZmMYBP1; Z11879, ZmMYBC1; X06333, and ZmMYBP1; L19494), and *Arabidopsis thaliana* (AtMYBGL1; M79448, AtMYB1; D01155, and AtMYB2; D14712). The site and the size of the putative intron found in many of gMYB is indicated by a vertical arrow (head on top) and the number of base pairs is indicated on the right side of the sequences. A consensus sequence among the plant MYB proteins as described by Avila *et al.* (1993) is given on top of the alignment. Upper case indicates residues fully conserved in all the proteins used to derive the consensus. Lower case indicates residues identical in at least 80% of the proteins. +, -, #, and ? indicate basic, acidic, hydrophobic, and miscellaneous amino acids, respectively. Horizontal arrows indicate the position of the degenerate primers, MYBFP and MYBRP. The nucleotide sequences of gMYB and rMYB were given to DDBJ under the names of GmMYB with consecutive numbers, and the DDBJ/EMBL/GenBank accession numbers for GmMYB1~GmMYB16, and GmMYB29 are AB029258~AB029273, and AB029274, respectively.

mutation in the process of PCR.

Southern hybridization

For genomic Southern analysis, 10 µg of total DNA digested with either *Hind*III, *Eco*RI or *Xba*I was fractionated by electrophoresis at 2.5 V/cm in a 0.8% agarose gel, transferred onto a nylon membrane Hybond-N (Amersham Pharmacia, USA), and hybridized with a ³²P-labeled probe at 65°C in 6×SSC, 10 mM Tris-HCl (pH 7.5), 2 mM EDTA, 20×Denhardt's reagent, 20 mM sodium phosphate (pH 7.4), 1% sodium N-lauroyl sarcosinate and 200 µg/µl denatured salmon sperm DNA for 12 hours. The membrane was washed in 2×SSC, 0.1% SDS at room temperature for 5 min., in 0.2×SSC, 0.1% SDS at room temperature for 10 min., and finally in 0.2×SSC, 0.1% SDS at 68°C for 1 hour. Signals were visualized by autoradiography using Imaging

Plate (Fuji, Japan). Quantitative analysis of the signals detected was performed by using BAS1000Mac (Fuji, Japan). After autoradiography, the hybridized probe was stripped from the blots by washing in 0.1% SDS at 99°C. The probes specific either for *GmMYB29* group A or B were prepared by PCR amplification from the plasmid clone containing *GmMYB29A1* cDNA or *GmMYB29B1* cDNA with a pair of primers (MYB29-P2; 5'-GTG GTC AGC AAT TGC AGC CA-3' and P11; 5'-TCA TAC CAG AAA TCC ATG CC-3').

In the case of RT-PCR/Southern analysis, gene specific RT-PCR amplification was carried out using the primer sets of ACTFP; 5'-TGG GAT GAY ATG GAR AAG ATY TGG-3' and ACTRP; 5'-ATC CAC ATC TGC TGG AAG GT-3' for *actin*, FPCHSX2; 5'-AGG CAA GAC ATG GTG GT-3' and RPCHSX2; 5'-GGA ACA TCC TTG AGG

A

GmMYB291
3241 bp

B

	10	20	30	40	50	60	70	80	90	100
A1	ATGGT	AGAG	CTCCT	TGTTG	TGAAA	AGATG	GGATT	GAA	AGG	TGCTT
A2	ATGGT	AGAG	CTCCT	TGTTG	TGAAA	AGATG	GGATT	GAA	AGG	TGCTT
B1	ATGGT	AGAG	CTCCT	TGTTG	TGAAA	AGATG	GGATT	GAA	AGG	TGCTT
B2	ATGGT	AGAG	CTCCT	TGTTG	TGAAA	AGATG	GGATT	GAA	AGG	TGCTT
	110	120	130	140	150	160	170	180	190	200
A1	ATGG	AATTG	CGTG	CCCTC	CCAAAG	CTT	CAGG	CTT	AT	AGG
A2	ATGG	AATTG	CGTG	CCCTC	CCAAAG	CTT	CAGG	CTT	AT	AGG
B1	ATGG	AATTG	CGTG	CCCTC	CCAAAG	CTT	CAGG	CTT	AT	AGG
B2	ATGG	AATTG	CGTG	CCCTC	CCAAAG	CTT	CAGG	CTT	AT	AGG
	210	220	230	240	250	260	270	280	290	300
A1	AGG	CAATTC	TCA	AG	GAAG	AAGA	CGAA	TAT	CATT	AAG
A2	AGG	CAATTC	TCA	AG	GAAG	AAGA	CGAA	TAT	CATT	AAG
B1	AGG	CAATTC	TCA	AG	GAAG	AAGA	CGAA	TAT	CATT	AAG
B2	AGG	CAATTC	TCA	AG	GAAG	AAGA	CGAA	TAT	CATT	AAG
	310	320	330	340	350	360	370	380	390	400
A1	GAC	AATGA	AA	AAAA	AATGT	TGG	CAC	AC	CA	AG
A2	GAC	AATGA	AA	AAAA	AATGT	TGG	CAC	AC	CA	AG
B1	GAC	AATGA	AA	AAAA	AATGT	TGG	CAC	AC	CA	AG
B2	GAC	AATGA	AA	AAAA	AATGT	TGG	CAC	AC	CA	AG
	410	420	430	440	450	460	470	480	490	500
A1	CAT	CAAT	CA	AG	CT	GT	CA	ATTC	CA	CA
A2	CAT	CAAT	CA	AG	CT	GT	CA	ATTC	CA	CA
B1	CAT	CAAT	CA	AG	CT	GT	CA	ATTC	CA	CA
B2	CAT	CAAT	CA	AG	CT	GT	CA	ATTC	CA	CA
	510	520	530	540	550	560	570	580	590	600
A1	ATG	CACT	ACT	TCT	AGT	GA	TTT	CAT	CA	T
A2	ATG	CACT	ACT	TCT	AGT	GA	TTT	CAT	CA	T
B1	ATG	CACT	ACT	TCT	AGT	GA	TTT	CAT	CA	T
B2	ATG	CACT	ACT	TCT	AGT	GA	TTT	CAT	CA	T
	610	620	630	640	650	660	670	680	690	700
A1	CC	TCC	AA	TG	ATGA	AG	CTT	TTGG	CA	AG
A2	CC	TCC	AA	TG	ATGA	AG	CTT	TTGG	CA	AG
B1	CC	TCC	AA	TG	ATGA	AG	CTT	TTGG	CA	AG
B2	CC	TCC	AA	TG	ATGA	AG	CTT	TTGG	CA	AG
	710	720	730	740	750	760	770	780	790	800
A1	CA	CC	CT	CA	ATAC	CA	ATTT	AA	CT	GT
A2	CA	CC	CT	CA	ATAC	CA	ATTT	AA	CT	GT
B1	CA	CC	CT	CA	ATAC	CA	ATTT	AA	CT	GT
B2	CA	CC	CT	CA	ATAC	CA	ATTT	AA	CT	GT
	810	820	830	840	850					
A1	TAT	GAC	ATAT	TCT	CT	CA	AA	TG	AGG	GA
A2	TAT	GAC	ATAT	TCT	CT	CA	AA	TG	AGG	GA
B1	TAT	GAC	ATAT	TCT	CT	CA	AA	TG	AGG	GA
B2	TAT	GAC	ATAT	TCT	CT	CA	AA	TG	AGG	GA

AC-3' for *GmCHS*, and MYB29-P6 and P11 for *GmMYB29*, respectively, and the products themselves were used as probes for hybridization. A 5 µl aliquot of the RT-PCR products with reduced (15~20) cycles of reaction was fractionated by electrophoresis in a 1.5% agarose gel followed by the same procedure of Southern analysis as that described above. The RT-PCR experiments were repeated several times with 25~30 cycles of reaction and similar results were obtained.

Gene-specific amplification of *GmMYB29* subfamily

Based on the sequences of *GmMYB29*, gene specific primers (FPA1; 5'-AGG ACT ATT GAG GTG TGG A-3' and P11 for *GmMYB29A1*, FPA2; 5'-AGG GTT ATT AAG GTG TGG C-3' and P11 for *GmMYB29A2*, FPB1; 5'-GAG CCA TTA AGC CAA AAA TCG-3' and RPB1; 5'-CAT AAG CAT GAT GAC CCT GC-3' for *GmMYB29B1*, and FPB2; 5'-CTC AAT CAG AAC CTG CTC ATC-3' and RPB2; 5'-GTT TGA ATC ATA AGC ATG ACT C-3' for *GmMYB29B2*) were designed. cDNA clones of individual members were used for control experiments to confirm that each set of the gene-specific primers was in fact capable of producing a PCR product specific to the target gene.

Results

PCR amplification of MYB-homologous sequences from soybean

Using the sequence information of MYB from *Arabidopsis thaliana*, barley, and petunia, a set of degenerate primers was designed to amplify by PCR the most conservative region in the DNA-binding domain of the plant MYB genes. PCR amplification from a total DNA sample resulted in products with various sizes ranging from about 180 bp to nearly 300 bp. The nucleotide sequences of these genomic PCR products were determined after cloning into the bacterial plasmid pBluescriptII SK (+). We sequenced a total of 19 clones and, excluding some duplications, they were grouped into 10 independent sequences (gMYB). On the other hand, when total RNA samples were used as templates for RT-PCR, a single band of cDNA amplification products with the expected size of about 180 bp was detect-

ed. These cDNA fragments were also cloned into the plasmid vector and analyzed for differential colony hybridization as described in "Materials and methods". The colonies showing the most conspicuous signal differences (rMYB10 and rMYB29) as well as less pronounced signal differences (rMYB2, rMYB3, rMYB4, rMYB5, rMYB11, and rMYB13) were selected for sequence analyses. Alignment of these sequences revealed that the difference in size of the products depended on the absence or presence of introns with various sizes (up to 156 bp) located between the two MYB-coding exons included in the amplified region. Amino acid sequences deduced from the two exons combined were found to share a very high similarity to some other plant MYB genes (Fig. 1).

Molecular cloning and structural analysis of *GmMYB29* subfamily

Using the sequence information of rMYB29, specific primers were designed for molecular cloning of the upstream and downstream sequences by nested PCR amplification as described in "Materials and methods", and we obtained the nucleotide sequence of 3241 bp containing a complete gene, hence designated as *GmMYB29A1*. The open reading frame encoding a polypeptide of 264 amino acids was detected in three exons of 133, 130, and 532 bp, respectively, each of them separated by intron 1 (193 bp) and intron 2 (813 bp) (Fig. 2A).

In the course of nested PCR cloning, we identified several independent products closely related to *GmMYB29A1*. In order to amplify the entire protein coding region of the closely related genes, two sets of primers were designed, that is, two upstream primers, MYB29-NP1; 5'-GTC GGA TCC GAA GAA GAT GGT TAG GGC TCC TTG-3' and MYB29-NP2; 5'-GTC GGA TCC CAC AAA GAT GGT GAG GGC TCC TTG-3' containing the ATG start codon, and two downstream primers, MYB29-CP1; 5'-CAG TGA GCT CAA TTT CAG AAC CCT GGC AAT TC-3' and MYB29-CP2; 5'-CAG TGA GCT CAA CTT CAG AAC TCT AGC AAT TC-3' containing the TGA stop codon. The cDNA fragments amplified by RT-PCR with these primers were cloned and sequenced. As expected, all the sequences contained the entire open reading frame and yet several

Fig. 2. (A) Diagrammatic representation of the genomic sequence containing *GmMYB29A1* and the cDNA structure of the family members. The horizontal solid bar indicates the sequenced region of 3241 bp containing the three protein-coding exons 1, 2, and 3 designated by the hatched (DNA-binding domain) and the open (variable region) boxes. The sizes of the exons and the introns as well as the 5' and the 3' flanking regions are indicated by the number of bp on top of the bar and the exon boxes. Sites of the putative TATA-box (TATA) and the polyadenylation signal (AATAA) are marked with vertical arrows. The short open bars above the exon 3 box indicate the region used as probes for genomic Southern hybridization (g) and that used for RT-PCR/Southern analysis (r). The cDNA of *GmMYB29A1* isolated by RT-PCR using the primers (horizontal arrows) is represented by the exon boxes combined to construct an open reading frame. (B) Alignment of the cDNA sequences of the four members of *GmMYB29* isolated by RT-PCR with the Higgins' multiple alignment program of DNASIS V3.6 (HITACHI SOFTWARE). The nucleotide bases fully conserved among the members are indicated by black boxes and gaps introduced to maximize the alignment are indicated by dashes. The arrows above the alignment show the sequences of primers used for the preparation of probes g and r, and those under the sequences represent the primers used for gene specific PCR. The nucleotide sequence data used in this figure will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB029159, AB029160, AB029161, AB029162, and AB029165.

Table 1. Similarity matrix of the open reading frames of *GmMYB29* members

gene	¹⁾ 5' region/ ²⁾ 3' region (%/ %)			
	A1	A2	B1	B2
A1	100/100			
A2	96/91	100/100		
B1	87/69	87/68	100/100	
B2	88/70	87/70	95/90	100/100

¹⁾ From the 5' to the 345th base.

²⁾ From the 346th base to the 3'.

differences were found among them. These sequences were classified into four independent members, suggesting the presence of four individual genes, hereafter designated as *GmMYB29A1*, *GmMYB29A2*, *GmMYB29B1*, and *GmMYB29B2*, comprising the subfamily of *GmMYB29*. These four genes were further classified into group A (*GmMYB29A1* and *GmMYB29A2*) and group B (*GmMYB29B1* and *GmMYB29B2*) based on their sequence similarity. Comparison of the sequences in the protein coding region of individual members of the *GmMYB29* subfamily shows that while high similarities were observed in the 5' DNA-binding domain, substantial differences were found between groups A and B in the 3' putative transactivation domain (Fig. 2B and Table 1). Homology search of their deduced amino acid sequences using BLASTP program revealed that the amino acid sequences in the N-terminal half, known as the DNA-binding domain, were highly homologous with those of other plant MYB transcription factors, and that the sequences in the C-terminal half containing the putative transactivation domain shows only a limited similarity to a group of proteins classified as subgroup 2 by Kranz *et al.* (1998), including NtMYB1, LeMYB18, PhMYB2, and AtMYB13 (Fig. 3).

Southern analysis and gene-specific amplification of GmMYB29 subfamily

Southern analysis was carried out to detect four copies of the *GmMYB29* subfamily in the soybean genome (Fig. 4). When southern blots of the total DNA were hybridized with the group A probe, two bands were detected in the lane of *Xba*I digests. On the other hand, at least four bands were detected in the lane of *Hind*III digests and five bands in the lane of *Eco*RI digests. Taking into account the fact that there is an internal site for *Hind*III and *Eco*RI in the probe regions of group A genes, these multiple bands could be best explained by assuming the presence of at least two copies of the genes in the genome. When the same blots were hybridized with the group B specific probe, two bands were detected in the lanes of *Hind*III and *Xba*I digests and a single band in the lane of *Eco*RI digests.

To further demonstrate the presence of each family member, the gene specific primers described in "Materials and methods" were used for PCR amplification of individual genes from total DNA. A single PCR product was identified in each reaction and was also found to be identical

with the target gene by sequence analysis. These results supported the previous findings in which four copies of genes were detected in the *GmMYB29* subfamily.

Enhanced expression of GmMYB29 subfamily in a rapid response to UV-B radiation

Expression of *GmCHS* was induced by white-light exposure and enhanced further by supplemental UV-B radiation (Shimizu *et al.* 1999). Assuming that *GmMYB29* proteins function as transcription factors of *GmCHS* in their UV-B response, expression of the members of the *GmMYB29* subfamily may be up-regulated prior to that of *GmCHS*. RT-PCR analysis was carried out to verify this assumption. Total RNA samples from seedlings grown for three days in darkness that were not irradiated with UV-B or irradiated with UV-B for 1, 2, 4, and 6 hours were used as templates for RT-PCR amplification of the gene transcripts of *actin*, *GmCHS*, and group A (*GmMYB29A1* and *GmMYB29A2*) of the *GmMYB29* subfamily. Southern blots of these products were hybridized with the corresponding RT-PCR products as probes. While the accumulation of *GmMYB29* group A mRNA was very low in the dark, it increased rapidly in response to UV-B irradiation and reached its peak within 2 hours after the onset of UV-B irradiation while the accumulation of *GmCHS* mRNA was still increasing (Fig. 5). These results suggested that the members of *GmMYB29* may regulate the expression of some target genes, such as *GmCHS* in response to UV-B irradiation as a transcription factor.

In order to analyze the expression of individual members of *GmMYB29*, gene-specific primers were used for RT-PCR amplification of cDNA from total RNA isolated from mature plant leaves not irradiated with UV-B or irradiated with UV-B for 2 hours. The results clearly indicated that every member of the *GmMYB29* subfamily was up-regulated in response to UV-B as well, and the expression level of the *GmMYB29* group B genes was found to be even higher than that of the group A genes (Fig. 6).

Discussion

It was reported that plant MYB transcription factors regulate various aspects of plant biological processes including anthocyanin and phlobaphene biosynthesis (Dooner *et al.* 1991), trichome differentiation (Oppenheimer *et al.* 1991), epidermal cell shape determination (Noda *et al.* 1994), responses to environmental stresses such as dehydration and salinity (Urao *et al.* 1993), gibberellin-regulated gene activation (Gubler *et al.* 1995), and so on. However, genes encoding MYB transcription factors involved in UV-B response have not yet been thoroughly characterized. We have isolated and characterized a subfamily of *GmMYB29* genes whose expression was found to be significantly up-regulated upon UV-B irradiation. *GmMYB29* consists of at least four closely related genes, which were classified into two groups based on their sequence similarity; group A,

		10	20	30	40	50	
GmMYB29A1	1	MVRAPCEKM	GLKKGPTPE	EDQILMSYIQ	KHGHGNWRAL	PKLAGLLRCG	50
GmMYB29A2	1	MVRAPCEKM	GLKKGPTPE	EDQILMSYIQ	KHGHGNWRAL	PKLAGLLRCG	50
GmMYB29B1	1	MVRAPCEKM	GLKKGPTPE	EDQILTSYID	KHGHGNWRAL	PKQAGLLRCG	50
GmMYB29B2	1	MVRAPCEKM	GLKKGPTPE	EDQILTSYIQ	KHGHGNWRAL	PKQAGLLRCG	50
LeMYB18	1	MGRAPCEKM	GLKKGPTPE	EDNILTSYIQ	NNGHGNWRAL	PKLAGLLRCG	50
NtMYB1	1	MVRAPCEKM	GLKKGPTPE	EDQILISYIQ	TNGHGNWRAL	PKQAGLLRCG	50
PhMYB2	1	MGRAPCEKM	GLKKGPTPE	EDQILVSYIE	KHGHGNWRAL	PKLAGLLRCG	50
AtMYB13	1	MGRAPCEKI	GLKKGPTPE	EDRILINYS	LHGHGNWRAL	PKLAGLLRCG	50
		60	70	80	90	100	
GmMYB29A1	51	KSCRLRWINY	LRPDIKRGNF	SSEEEETIK	MHELLGNRWS	AIAAKLPGR	100
GmMYB29A2	51	KSCRLRWINY	LRPDIKRGNF	SSEEEETIK	MHELLGNRWS	AIAAKLPGR	100
GmMYB29B1	51	KSCRLRWINY	LRPDIKRGNF	TIEEEETIK	LHDMGNRWS	AIAAKLPGR	100
GmMYB29B2	51	KSCRLRWINY	LRPDIKRGNF	TIEEEETIK	LHEMLGNRWS	AIAAKLPGR	100
LeMYB18	51	KSCRLRWINY	LRPDIKRGNF	TKEEETIK	LHEMLGNRWS	AIAAKLPGR	100
NtMYB1	51	KSCRLRWINY	LRPDIKRGNF	TKEEETIK	LHEMLGNRWS	AIAAKLPGR	100
PhMYB2	51	KSCRLRWINY	LRPDIKRGNF	TREEDTIQ	LHEMLGNRWS	AIAAKLPGR	100
AtMYB13	51	KSCRLRWINY	LRPDIKRGNF	TPHEEDTIQ	LHQLGNRWS	AIAAKLPGR	100
		110	120	130	140	150	
GmMYB29A1	101	DNEIKNVWHT	HLKKRLMN	SD	TNKRVSQPR	IKRSDNSST	150
GmMYB29A2	101	DNEIKNVWHT	HLKKRLMN	SD	TNKRVSQPR	IKRSDNSST	150
GmMYB29B1	101	DNEIKNVWHT	NLKKRLK	SDQSK	PSSKRAIKPK	IKRSDNSST	150
GmMYB29B2	101	DNEIKNVWHT	NLKKRLK	SDQSK	PSSKRAIKPK	IKRSDNSST	150
LeMYB18	101	DNEIKNVWHT	NLKKRLK	PCQNSKRHSK	TSHVSNKQPT	TSESSNSDL	150
NtMYB1	101	DNEIKNVWHT	NLKKRLK	PPQNSKRHSK	SKNHDSKQPT	TSESSNSDL	150
PhMYB2	101	DNEIKNVWHT	NLKKRLK	PPQNSKRHSK	NNH-DSKAPS	TSKMLDSE	150
AtMYB13	101	DNEIKNVWHT	HLKKRLHH	SDDQ	NNKEDFVSTT	AAEM	150
		160	170	180	190	200	
GmMYB29A1	151	LTOSEPT	SSS	GCTTS-SDFS	SFSEGTKNMD	NMIKRE	200
GmMYB29A2	151	LTOSEPT	SSA	CTTSLSDFS	SFSEGTKNMD	NMIKRE	200
GmMYB29B1	151	ITQSEPDNFN	FREMDTITSS	ACTTSSSDFS	SVTVGDSK	NMIKRE	200
GmMYB29B2	151	ITQSEPDNFN	FREMDTITSS	ACTTSSSDFS	SVTVGDSK	NMIKRE	200
LeMYB18	151	STSTKQHIK	IAPNSPQ	LSSS-EMS	SVTLVVD	DNQMVIIKEE	200
NtMYB1	151	TIINTQKID	SPVLAPNSPQ	ISSS-TEMS	TVTLVVD	DHQMVIKQEE	200
PhMYB2	151	SFSTIQENIN	EPMTGPNSPQ	RSSSESSTVT	ADSLAATDVT	DDQTFIKHE	200
AtMYB13	151	PTSPQ	QQSSSSADIS	AIT	TLG	NNNDISNSNK	200
		210	220	230	240	250	
GmMYB29A1	201	DIESM-ETVK	PIIDESFWPQ	ETVDYESSTM	MQSSNSWTIS	NELAPPQVQF	250
GmMYB29A2	201	DIESV-ETIM	PIIDESFWSE	ATVDYESSTM	M-TNSNSWTIS	NELAPPQVQF	250
GmMYB29B1	201	DTEST-ETM	PVIDESFWSE	AAIDDETPTM	-SSQSLTIS	NEM-RLQYPF	250
GmMYB29B2	201	DIESM-ETM	PVIDESFWSE	AAIDDETPTM	-SSQSLTIS	NEM-RLQYPF	250
LeMYB18	201	KIESSSEYF	PKIDESFWAD	ELSTENN	MIGH	DQEIQV	250
NtMYB1	201	VMESS-EYF	PEIDESFWTD	ELTTDNWNS	STDHVMVAA	NQELQVLPF	250
PhMYB2	201	EMDSY-ENF	PEIDESFWTE	DLSMGDNL	DLDMEVA	GEKSQVQFPY	250
AtMYB13	201	DSATSSDVL	AIIDESFWSE	VVLTDCLIS	GNEKNEKKI	EN	250
		260	270	280	290	300	
GmMYB29A1	251	NSVE-TFQQQ	SVG-YNDSKF	D-DGMDFWYD	IFIKSGESI	ELPEF*	300
GmMYB29A2	251	NSVE-TFQQQ	SVD-YNGSND	DHDGMDFWYD	IFIKSGESI	ELPEF*	300
GmMYB29B1	251	ANYEETFQOG	HHA-Y-DSNF	D-DGMDFWYD	IFTRTNDIS	ELPEF*	300
GmMYB29B2	251	ANYEETFQOG	H-A-Y-DSNF	D-DGMDFWYD	IFTRTNDIS	ELPEF*	300
LeMYB18	251	REED	NVDIFTTSSK	MEEDMDFWYN	VFIKT	DLPELPEF*	300
NtMYB1	251	S-SKEE	NVDILAT-K	MEEDMDFWYN	VFIKT	DLPELPEF*	300
PhMYB2	251	SH-DMKEQ	SVDVGA-K	LEDDMDFWYN	VFIKA	DLPELPEF*	300
AtMYB13	251	WEG	SLDRNDKG	YNHDMDFWFD	HLTSSCIIG	EMSDISEF*	300

Fig. 3. Comparison of the amino acid sequences deduced from the cDNA sequences of individual members of the *GmMYB29* subfamily with those of a group of proteins including *Nicotiana tabacum* NtMYB1 (accession number U72762), *Lycopersicon esculentum* LeMYB18 (X98308), *Petunia hybrida* PhMYB2, and *Arabidopsis thaliana* AtMYB13 (Z50869). The sequences were aligned using the Higgins' multiple alignment program of DNASIS V3.6 (HITACHI SOFTWARE). The amino acid residues represented by black boxes indicate a high similarity, and dashes indicate the gaps introduced to maximize the alignment.

GmMYB29A1 and *GmMYB29A2*, and group B, *GmMYB29B1* and *GmMYB29B2*. Expression of the group A members of the *GmMYB29* subfamily was found to reach its peak within two hours after the onset of UV-B irradiation when the accumulation of *GmCHS* mRNA was still increasing. Similar time lag in the induction of an activator and its target genes has been reported in some other cases. For example, the ex-

pression of *Arabidopsis thaliana* *Lhcb3* (Light-harvesting chlorophyll a/b binding protein) is induced by light irradiation for 1 hour and its mRNA accumulation increased even under continuous light irradiation up to 12 hours, whereas in *CCA1*, which encodes a putative transcription factor of *Lhcb3*, the peak of mRNA accumulation was reached after irradiation for 1 hour (Wang *et al.* 1997). Expression of

Fig. 4. Southern analysis of *GmMYB29* groups A and B in the genome of soybean. Southern blots of the *Hind*III, *Eco*RI, or *Xba*I digests of a soybean total DNA sample were hybridized with ³²P labeled probes specific either for *GmMYB29* subfamily A or B. Positions of the DNA size markers are indicated on the left side in kilo bases.

Arabidopsis thaliana rd22 was induced by dehydration, ABA or NaCl treatment, and its mRNA continued to accumulate up to 5 hours after the start of the dehydration treatment (Yamaguchi-Shinozaki and Shinozaki 1993), whereas *rd22BP1*, encoding a transcription factor of *rd22*, showed a rapid enhancement of expression after 10 minutes and reached a peak within 20 minutes after the onset of the dehydration treatment (Abe *et al.* 1997). Therefore, our results suggested that the *GmMYB29A1* and *GmMYB29A2* proteins are likely to function as transcription factors involved in the induction of *GmCHS* by UV-B irradiation.

Starting from the sequence information of rMYB10 which also gave the most conspicuous signal differences in the UV-B(+/-) hybridizations, we isolated by nested PCR a genomic sequence containing the complete protein coding exons. However, the expression of this gene, namely *GmMYB10*, was found to be much less responsive to UV-B and was actually repressed by white light exposure (these

Fig. 5. UV-B induction of the *GmCHS* and *GmMYB29* gene families of group A. Seedlings grown for three days in darkness (D) were exposed to UV-B light (+) for 1, 2, 4, or 6 hours or not exposed to UV-B (-) and the total RNA samples extracted from hypocotyl tissues of the seedlings were used as templates for RT-PCR/Southern analysis of transcripts for *GmCHS*, group A genes of *GmMYB29*, and *actin*.

findings will be reported elsewhere).

By comparing the 17 independent sequences of 42 aa residues with 99 sequences of MYB from many other species, we found that each of these soybean sequences could be classified into different groups of MYB proteins. A close relationship was also detected based on pairwise comparison of the sequences. For example, the sequence of *GmMYB13* (rMYB4) was found to be highly similar to the maize MYB transcription factors C1 and P1 (88.4% and 88.4% identity, respectively) and *GmMYB16* (rMYB13) was found to be similar also to C1 and P1 (79.1% identity each). Among these sequences, the most striking similarity was observed around the latter half of the DNA-binding domain with the stretch of LLGNRWSLIAGRLPGR, which forms a helix-turn-helix structure assumed to bind to the target promoter element. *GmMYB12* (rMYB3) showed a markedly high similarity to *Arabidopsis thaliana* AtMYB2 (81.4% identity) which was reported to be induced by dehydration, a high concentration of salts, or ABA treatment.

Kranz *et al.* (1998) have reported that MYB proteins in plants can be classified into 22 subgroups based on their similarities in the amino acid sequences. LeMYB18, NtMYB1, PhMYB2, and AtMYB13 belong to the subgroup 2, and these proteins contain the amino acid sequences of IDESFWXE/DXLSTD and E/NDDMDFWYNVFI motifs in their C-terminal regions. Because individual members of the *GmMYB29* subfamily contain similar sequence motifs and show an overall similarity to these proteins (Fig. 3), the *GmMYB29* subfamily is also considered to belong to the subgroup 2. Among the members of the subgroup 2, NtMYB1 activates the transcription of *PR-1* which is induced by infection with tobacco mosaic virus (TMV) or

Fig. 6. UV-B induction of individual members of the *GmMYB29* subfamily. Total RNA samples isolated from mature plant leaves exposed to UV-B (+) for 2 hours or not exposed to UV-B (–) were used as templates for RT-PCR using the gene-specific primers for *GmMYB29A1*, *GmMYB29A2*, *GmMYB29B1*, *GmMYB29B2*, *GmCHS*, and *actin*.

treatment with salicylic acid. AtMYB13 is strongly expressed in leaf tissues and is induced by white or UV light exposure, or dehydration stress. Thus, based on the structural relationship with NtMYB1 and AtMYB13, *GmMYB29* may also function in response to a variety of biotic and abiotic environmental stresses.

Although more than a hundred members have been identified in the MYB super family of *Arabidopsis thaliana* (Kranz *et al.* 1998), only a few MYB homologues are included in the subgroup 2, implying that the four-gene subfamily of *GmMYB29* may have been developed specifically in soybean. Although we used gene-specific primers for RT-PCR experiments to demonstrate that every member of the family is strongly responsive to UV-B exposure, there was a significant variation in the levels of expression. We also succeeded in cloning the 5'-untranslated region of *GmMYB29B1*. Alignment of the nucleotide sequences in the promoter regions between *GmMYB29A1* and *GmMYB29B1* revealed that there is a considerable diversity throughout the regions and also that there are some short stretches of identical sequences in the zone upstream of the ATG start codon (data not shown). Although the functions of these promoters are yet to be analyzed, it is suggested that these identical sequences may be involved in transcriptional activation in response to UV-B radiation and sequence diversity in some other parts may contribute to the difference in the expres-

sion levels of the genes. This leads us to speculate that the development of the four-gene family may have been accompanied by differentiation in their physiological role which may have contributed to the evolution of the plants to cope with a variety of environmental stresses. We are currently attempting to determine how each member of the family responds to different kinds of environmental stresses at some distinct developmental stages.

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