Transcriptional control of anthocyanin biosynthetic genes in the Caryophyllales

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Abstract

Anthocyanins and betacyanins, two types of red pigment, have never been found to occur together in plants. Although anthocyanins are widely distributed in higher plants, betacyanins have replaced anthocyanins in the Caryophyllales. The accumulation of flavonols in the Caryophyllales suggests that the step(s) of anthocyanin biosynthesis from dihydroflavonols to anthocyanins could be blocked in the Caryophyllales. Dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS) cDNAs were isolated from plants of the Caryophyllales. An enzyme activity assay showed that the Caryophyllales possess functional DFR and ANS. The expression profile revealed that DFR and ANS are not expressed in most tissues and organs except the seeds in Spinacia oleracea. Here, the promoter regions of DFR and ANS were isolated from S. oleracea. Analysis of DFR and ANS promoter sequences revealed several putative transcriptional factor-binding motifs. A yeast one-hybrid assay showed that Petunia hybrida AN2 (PhAN2) and JAF13 (PhJAF13), which were the regulators of anthocyanin synthesis in P. hybrida, could bind to the S. oleracea DFR and ANS promoters. However, the transient assay in Phytolacca americana cell cultures and leaves of S. oleracea showed that the promoters were not activated by ectopic expression of PhAN2 and PhJAF13, while the DFR and ANS promoters of Arabidopsis thaliana, an anthocyanin-producing species, were activated. One possible explanation for the lack of anthocyanins in the Caryophyllales is the difference in the promoter regions of DFR and ANS compared with those of anthocyanin-producing species.

Key words: Anthocyanidin synthase, anthocyanin, Caryophyllales, dihydroflavonol 4-reductase, pokeweed, spinach.

Introduction

The red colours of flowers are mainly produced by two types of pigment; anthocyanins and betacyanins. They serve essential functions in plant reproduction as flower and fruit colours by recruiting pollinators and seed dispersers. Although anthocyanins are widely found as flower and fruit pigments in higher plants, betacyanins have largely replaced anthocyanins in the Caryophyllales, except in the families Caryophyllaceae and Molluginaceae. The occurrence of anthocyanins in the betacyanin-producing Caryophyllales has not been reported (Harborne, 1996). Thus, these two red pigments, anthocyanins and betacyanins, have never been demonstrated to co-exist in one plant. Although this curious mutual exclusion has been examined from genetic and evolutionary perspectives (Stafford, 1990, 1994), nothing is known about it at the molecular level. The evolutionary mechanism of the mutual exclusion of these two pathways thus remains a mystery.

Although the biosynthesis of betacyanins is poorly understood, the flavonoid biosynthetic pathway is probably one of the best-studied examples of secondary metabolism in higher plants (Fig. 1). With few exceptions, flavonoid biosynthetic genes have been cloned and analysed, and factors that control transcription of the genes have also been isolated by genetic means. The regulatory mechanism of flavonoid biosynthesis has been revealed in several species (Holton and Cornish, 1995; Forkmann and Martens, 2001; Winkel-Shirley, 2001;
In *Zea mays*, genes for the whole anthocyanin biosynthetic pathway, starting from c2 (encoding chalcone synthase; CHS), are simultaneously activated by MYB and basic helix–loop–helix (bHLH) transcriptional factors, the C1/Pl and the R/B families, respectively (Paz-Ares et al., 1987; Ludwig et al., 1989; Cone et al., 1993). Pigmentation in floral organs of *Z. mays* is controlled by the MYB transcriptional factor P. In contrast to C1 and Pl, P acts independently (Grotewold et al., 1994). MYB and bHLH factors regulating anthocyanin biosynthesis have also been identified by genetic means in a dicot plant, *Petunia hybrida* (Quattrocchio et al., 1993). A MYB transcriptional factor of *P. hybrida*, AN2, activates genes of the late anthocyanin pathway beginning with dihydroflavonol 4-reductase (DFR) in petal limbs (Quattrocchio et al., 1999). In a transient assay, AN2 can interact with either of two distinct bHLH factors, JAF13 or AN1. In *Arabidopsis thaliana*, a MYB factor and a bHLH factor, TT2 and TT8, respectively, were specifically responsible for activation of late flavonoid metabolism and proanthocyanidin production in seeds (Nesi et al., 2000, 2001).

Whereas anthocyanins have not been reported in the Caryophyllales (except in the Caryophyllaceae and Molluginaceae), other flavonoids, especially the major flavonols, are common in the Caryophyllales (Iwashina, 2001). For example, the yellow tepals of *Astrophytum* species contain flavonol glycosides (quercetin 3-O-galactoside and 3-O-rhamnosylglucoside) together with flavonols (quercetin, kaempferol, and isorhamnetin) in the form of spherical crystals (Iwashina et al., 1988). Dihydroflavonols are at the branching point of flavonols and anthocyanins in the flavonoid biosynthetic pathway (Fig. 1). This suggests that anthocyanin biosynthesis from dihydroflavonols to anthocyanins may be blocked in the Caryophyllales. Some insights can be gained from DFR and anthocyanidin synthase (ANS), which are involved in the biosynthetic pathway from dihydroflavonols to anthocyanins.

DFR, the enzyme that catalyses the first committed step in anthocyanin biosynthesis from dihydroflavonols, catalyses the NADPH-dependent reduction of dihydroflavonols into leucoanthocyanidins. The isolation and functional identification of DFR genes from *Spinacia oleracea* and *Phytolacca americana*, which are non-anthocyanin-producing plants of the Caryophyllales, were previously reported (Shimada et al., 2004). Subsequently, ANS, which catalyses the next step to anthocyanidins, is responsible for the formation of the coloured anthocyanidins from the colourless leucoanthocyanidins. The cDNAs encoding ANS were isolated from *S. oleracea* and *P. americana*. The ability of ANSs of the Caryophyllales to oxidize trans-leucocyanidin to cyanidin is comparable with that of ANSs in anthocyanin-producing plants. Therefore, *S. oleracea* and *P. americana* have functional DFR and ANS. It has been shown that DFR and ANS were not expressed in most tissues and organs except seeds in *S. oleracea*. One possible explanation for the lack of anthocyanin synthesis in the Caryophyllales may therefore be the suppression or limited expression of the DFR and ANS (Shimada et al., 2005).

To gain further insight into the diversification of red pigments in higher plants, the potential for anthocyanin biosynthesis in the Caryophyllales was investigated and the regulatory mechanism behind DFR and ANS expression was examined. Here the isolation and structural analysis of the DFR and ANS promoters of the Caryophyllales are reported. The binding assay was performed in yeast and the transient expression assay in the *P. americana* cell cultures and the leaves of *S. oleracea.*

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**Fig. 1.** Flavonoid biosynthetic pathway of anthocyanins and proanthocyanidins. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone-3-hydrogenase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase; UFGT, UDP-glucose: flavonoid 3-O-glucosyltransferase.
to analyse the interaction with heterologous regulators for anthocyanin synthesis. The comparative analysis of the promoters of DFR and ANS of non-anthocyanin-producing species of the Caryophyllales and those of anthocyanin-producing species is discussed.

### Materials and methods

#### Plant materials

Seeds of *S. oleracea* variety ‘Active’ were purchased from Sakata Seed (Yokohama, Japan) and germinated at 25 °C in the dark for 2 d. After germination, the seedlings were grown in a growth chamber under a 12 h photoperiod at 22 °C. The leaves of *S. oleracea* used in the transient assay were purchased from a market. Suspension cultures were prepared from callus initiated from stem explants of *P. americana*. Cells were subcultured every 7 d in MS medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose and 2,4-dichlorophenoxyacetic acid (2,4-D) as described previously (Sakata et al., 1986). The Arabidopsis thaliana used for RNA isolation were grown in a greenhouse on soil.

#### Isolation of promoter sequences

The *S. oleracea DFR* (SoDFR) and *S. oleracea ANS* (SoANS) promoters from *S. oleracea* were isolated by the inverse polymerase chain reaction (PCR) method. Genomic DNA was extracted with extraction buffer [0.3 M NaCl, 50 mM TRIS–HCl pH 7.5, 20 mM EDTA, 0.5% (w/v) SDS, 14 mM 2-mercaptoethanol] and phenol/chloroform from seedlings of *S. oleracea DFR* variety ‘Active’ were purchased from Sakata Seed (Yokohama, Japan) in a 500 µl reaction volume and used as the template for PCR.

#### RNA isolation and cDNA synthesis

Total RNA was extracted from 2 g of frozen cells with extraction buffer [0.1 M TRIS–HCl pH 9.0, 0.1 M NaCl, 10 mM EDTA, 0.5% (w/v) SDS, 14 mM 2-mercaptoethanol] and phenol/chloroform, and was precipitated with LiCl (Ozeki et al., 1990). Arabidopsis cDNA was synthesized from 2.5 µg of the RNA from Arabidopsis young leaves and seeds with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and used as the template for PCR.

### Construction of plasmids

The reporter plasmids for the yeast one-hybrid assay were constructed by inserting the fragment of the promoter region of *SoDFR* (ProSoDFR), *SoANS* (ProSoANS), *A. thaliana DFR* (ProADFR), and *A. thaliana ANS* (ProANANS) into the multicloning site (MCS) of the pHIS2 vector (BD Bioscience). The fragments containing ~1000 bp upstream of the translational start site and the codons encoding the initial two amino acids of each gene were generated by PCR from *S. oleracea* and *A. thaliana* genomic DNA using the set of primers SoDFRs1/SoDFRas1 and SoANSs1/SoANSas1 promoters (Table 1). The 5' kb DNA fragment was amplified. Sequences of about 1000 bp upstream of the translational start sites of *SoDFR* and *SoANS* were directly sequenced and determined using the PCR fragments. Transcriptional factor-binding sites were searched for using the TFSearch version 1.3 program (http://mbs.cbcrc.jp/research/db/TFSSEARCH.html; Heinemeyer et al., 1998) with a threshold of 80.0 points and the PLACE (http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html; Higo et al., 1999).

### Oligonucleotide primers for PCR

<table>
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<th>Sequence</th>
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<tr>
<td>SoDFR-E3s</td>
<td>5'-GCAGCTCCAAAAGATGGACTGGATG-3'</td>
</tr>
<tr>
<td>SoDFR-E1as</td>
<td>5'-GCTAGCCGCTTACCCCGG-3'</td>
</tr>
<tr>
<td>SoANS-E2s</td>
<td>5'-TTTGAGCGGCGAGGATTATG-3'</td>
</tr>
<tr>
<td>SoANS-E1as</td>
<td>5'-TGGCATGAGGACTTTAAGG-3'</td>
</tr>
<tr>
<td>SoDFR-E1</td>
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<td>SoDFRas1</td>
<td>5'-TAGAGAGGGGTTTATCATGATAGAT-3'</td>
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<tr>
<td>SoANSs1</td>
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<tr>
<td>AtANSs2</td>
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<td>PhDFRs1</td>
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<td>ProSoDFR</td>
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The effector plasmids for yeast one-hybrid assay were constructed by fusing the open reading frames (ORFs) of the *P. hybrida* AN2 (PhAN2), *P. hybrida* A1F31 (PhA1F31), and *A. thaliana* TT2 (AtTT2) and TT8 (AtTT8) to the pGADT7-Rec2 vector (BD Bioscience). The ORFs were amplified without a stop codon by PCR using cauliflower mosaic virus (CaMV) 35S (35S):PhAN2, 35S:PhA1F31 vectors (Quattrocchio et al., 1998), which were a kind gift of Dr Koes (Vrije University, Amsterdam, The Netherlands), and *A. thaliana* cDNA as the template with the primer sets; PhAN2s1/PhAN2as1, PhA1F31s1 and PhA1F31s1/PhA1F31s1, and AtTT2s1/AtTT2as1 and AtTT8s1/AtTT8as1, respectively (Table 1). To construct the vectors to be expressed in yeast as fusions with the GAL4 activation domain (AD), the PCR fragments were digested by EcoRI–SmaI and BamHI, and the digested ORFs were fused in-frame to the GAL4 AD of the pGAD7-Rec2 vector. The
resulting plasmids were named pGAD-PhAN2, pGAD-PhJAF13, pGAD-AIF-T2, and pGAD-AIT-T8, respectively. To construct the vectors to be expressed as fusions with the SV40 nuclear localization signal (NLS), the PCR fragments were digested with KpnI and BamHI/XhoI and the digested ORFs were fused in-frame to the SV40 NLS of pGAD7-Rec2. The resulting plasmids were named pNLS-PhAN2, pNLS-PhJAF13, pNLS-AIF-T2, and pNLS-AIT-T8, respectively.

The reporter plasmids for transient assay were constructed by inserting the ~1000 bp fragment of ProSoDFR, ProSoANS, ProADFR, and ProSoANS into the MCS of the 35S::sGFP (S65T) vector, which was a kind gift from Dr. Niwa (University of Shizuoka, Japan). The fragments were generated by PCR from S. oleracea and A. thaliana genomic DNA using the set of primers: SoDFRs1/SoDFRs2 and SoANSs2/SoANSAs2 and AtDFRs1/AtDFRs1 and AtANSs2/ AtANSAs2, respectively, with restriction enzyme sites added (Table 1). The PCR products of ProSoDFR, ProSoANS, and ProADFR were digested with HindIII and BamHI. The PCR product of ProSoANS was digested with BamHI. The fragments were ligated in-frame to the green fluorescent protein (GFP) gene in the 35S::GFP (S65T) vector, replacing the 35S promoter. The resulting plasmids were named ProSoDFR::GFP, ProSoANS::GFP, ProADFR::GFP, and ProSoANS::GFP, respectively.

For the luciferase assay, the coding sequence of the firefly luciferase (LUC) gene was amplified by PCR with primers LUCs1/LUCas1 (Table 1) from the PGV-B vector (Toyo Ink, Tokyo, Japan). After digestion by BamHI/NcoI and NotI, the PCR fragment was ligated in-frame to each reporter plasmid, replacing the GFP gene. 35S::PHAN2 and 35S::PhJAF13 (Quattrocchio et al., 1998) were used as effector plasmids for transient assay. The plasmid for internal control was constructed by ligating the coding sequence of Renilla reniformis luciferase (RUC) in between the 35S promoter and the nopaline synthase polyadenylation site (nos-ter). The ORF was amplified with the stop codon by PCR using the pRL-null vector (Promega, Madison, WI, USA). The extracts were subjected to the luciferase assay. Firefly luciferase activity was quantified using the PicaGene Luminescence Kit (Toyo Ink), and Renilla luciferase activity was quantified by the Renilla Luciferase Assay System (Promega) in the assay of the DFR promoter in S. oleracea leaves and DFR and ANS promoters in P. americana cell cultures. The PicaGene Dual Luminescence Kit (Toyo Ink) was used in the assay of the ANS promoter in leaves of S. oleracea. Luciferase activity was detected by an AB-2250 luminescence (ATTO, Tokyo, Japan) for 30 s at room temperature. Relative reporter gene activity was calculated as follows: firefly luciferase value (extracts of tissues with transformation of the constructs)–firefly luciferase value (without transformation)/Renilla luciferase value (with normalization construct)–Renilla luciferase value (without transformation)×1000. The given mean was the average of three replications.

**Results**

**Cloning and structural analysis of Spinacia oleracea DFR and ANS promoters**

Southern analysis has revealed that the genomic DNA of S. oleracea contains a single copy of SoDFR and SoANS (Shimada et al., 2005). For analysis of promoters of SoDFR and SoANS, the 5’ upstream regions of the genes were isolated from S. oleracea genomic DNA by an inverse PCR method. By sequencing the amplified fragments, an ~1000 bp segment of the sequence upstream of the translational start site of the genes was determined.

The sequences of isolated SoDFR and SoANS promoters (accessions nos AB246750 and AB246752) are shown in Fig. 2. Analysis of the promoter sequences by the program TSearch ver. 1.3 (http://mbs.cbrj.jp/research/db/TSEARCH.html; Heinemeyer et al., 1998) with a threshold of 80.0 points and the PLACE database (http://www.dna.affrc.go.jp/htdocs-PLACE/signalscan.html; Higo et al., 1999) revealed several elements to which transcriptional factors can bind, in the SoDFR and SoANS promoters. A putative TATA box at position ~27 from the transcriptional start site was located in the SoDFR promoter (Fig. 2A). In the SoDFR promoter, five putative MYB homologous binding sites for P, which regulates the Z. mays phlobaphene pathway (ACWACCNN; Grotewold et al., 1994); two other MYB homologous binding sites for MYB Ph3, which is involved in petal epidermis-specific regulation

**Yeast one-hybrid assay**

The reporter plasmids and effector plasmids were co-transformed into the yeast strain Y187 (BD Bioscience). Transformants were selected on SD medium, as described in the protocol of the BD Matchmaker One-hybrid Library Construction and Screening Kit (BD Bioscience). The transformants were tested for growth on medium lacking histidine and 10 mM 3-aminotriazole (3-AT). Transformants containing the Yeast reporter plasmid pGAD-p53 plasmid, which was supplied in the kit, were used as negative control.

**Transient expression assay in plants**

Transient expression assays were carried out using the PDS-1000/He system (Bio-Rad, Hercules, CA, USA). Tungsten microparticles (1.1 μm; Bio-Rad) were prepared at the concentration of 60 mg ml⁻¹. The reporter plasmid (750 ng) was mixed with each effector plasmid (750 ng) and 35S::RUC (750 ng) in 25 μl of particle suspension and precipitated onto microparticles by the addition of 25 μl of 2.5 M CaCl₂ and 10 μl of 100 mM spermidine-free base, while vortexing. The microparticles were washed with 140 μl of 70% ethanol and resuspended in 24 μl of 100% ethanol. For each bombardment, 10 μl of DNA-coated microparticles were spotted onto each macrocarrier disk (Bio-Rad). The 0.5 ml of packed P. americana suspension cells were incubated for 15 min in MS medium containing 0.5 M mannitol, spread on 3M paper, and placed on the plate. The 1.8 cm leaf discs of S. oleracea were incubated for 30 min in MS medium containing 0.5 M mannitol and placed on 3M paper on the plate. The cells were subjected to bombardment using a pressure of 1100 psi and a 26 mm Hg vacuum. After transformation, the tissues were incubated in the dark for 24 h at 25 °C.

For luciferase assay, the tissues were frozen with liquid nitrogen and ground in Renilla Luciferase Assay Lysis Buffer (Promega, Madison, WI, USA). The extracts were subjected to the luciferase assay. Firefly luciferase activity was quantified using the PicaGene Luminescence Kit (Toyo Ink), and Renilla luciferase activity was quantified by the Renilla Luciferase Assay System (Promega) in the assay of the DFR promoter in S. oleracea leaves and DFR and ANS promoters in P. americana cell cultures. The PicaGene Dual Luminescence Kit (Toyo Ink) was used in the assay of the ANS promoter in leaves of S. oleracea. Luciferase activity was detected by an AB-2250 luminescence (ATTO, Tokyo, Japan) for 30 s at room temperature. Relative reporter gene activity was calculated as follows: firefly luciferase value (extracts of tissues with transformation of the constructs)–firefly luciferase value (without transformation)/Renilla luciferase value (with normalization construct)–Renilla luciferase value (without transformation)×1000. The given mean was the average of three replications.
of the P. hybrida CHSI gene (NRRAGTTAGTTAS; Solano et al., 1995); three core elements for the Dof transcriptional factor, which is involved in tissue specificity and light regulation (AAAG; Yanagisawa and Schmidt, 1999); and the RY motif, which is conserved in seed-specific promoters (CATGCA; Ezcurra et al., 1999), were also found. A site for SBF-1, a silencer/enhancer of a Phaseolus vulgaris CHS gene (KWRTNGTTAWWW; Lawton et al., 1991), was found in the SoDFR promoter.

The light-responsive unit (LRU), which includes a G-box (GBF)-binding site (ACGT) and the region downstream of the MYB recognition element, was not detected in the SoDFR promoter. The light-responsive unit (LRU), which includes a G-box (GBF)-binding site (ACGT) and the region downstream of the MYB recognition element, was not detected in the SoDFR promoter.

The SoANS promoter sequence is shown in Fig. 2B. An intron (at the position from +29 to +68) exists between the transcriptional start site (at position +1) and the translational start site (at position +126) of the SoANS promoter, and a clear TATA box was not found. The light-responsive unit (LRU), which includes a G-box (GBF)-binding site (ACGT) and the region downstream of the MYB recognition element, was not detected in the SoDFR promoter.

The light-responsive unit (LRU), which includes a G-box (GBF)-binding site (ACGT) and the region downstream of the MYB recognition element, was not detected in the SoDFR promoter.
SoANS promoter contained three P-binding sites, two core elements for Dof, two RY motifs, two SBF-1-binding sites, and a GBF-binding site. The LRU, which was not in the SoDFR promoter, was found in the SoANS promoter.

Binding assay of PhAN2 and PhJAF13 in yeast
To analyse the interaction of PhAN2 and PhJAF13, which are heterologous transcriptional factors involved in anthocyanin biosynthesis, with the Caryophyllales DFR or ANS promoters, a one-hybrid assay was performed. The promoters of AtDFR and AtANS were also used as controls in this assay. The reporter plasmids ProSoDFR::HIS3, ProSoANS::HIS3, ProAtDFR::HIS3, and ProAtANS::HIS3 were constructed by fusing ~1000 bp of SoDFR, SoANS, AtDFR, and AtANS promoter fragments to an iso-1-cytochrome c (CYC1) gene TATA box driving the HIS3 reporter gene (Fig. 3). The effector plasmids pGAD-PhAN2, pNLS-PhAN2, pGAD-PhJAF13, and pNLS-PhJAF13 were constructed by inserting PhAN2 and PhJAF13 ORFs downstream of the ADH1 promoter and the GAL4 AD or the SV40 NLS, as shown in Fig. 3. The pGAD-p53 plasmid, which contains the coding region of the p53 protein that does not bind to the promoters in the reporter constructs, was used as a negative control. These constructs were transformed in different combinations into yeast, as shown in Fig. 3. Each transformant was tested for growth on medium lacking histidine, compared with the growth of a negative control. In the yeast strain-transformed SoDFR promoter–reporter construct, a single transformation with AD-PhAN2 and AD-PhJAF13 yielded no positive interaction results. The expression of both PhAN2 and PhJAF13 resulted in growth on medium lacking histidine (Fig. 3A). These results suggested that the interaction of PhAN2 and PhJAF13 should be essential in binding to the SoDFR promoter. In contrast to the SoDFR promoter, the single transformation of AD-PhAN2 and AD-PhJAF13 gave positive interaction results for AtDFR, SoANS, and AtANS promoters (Fig. 3B–D). Co-expression of factors did not affect their binding to these promoters, except for the SoDFR promoter. These results indicate that PhAN2 and PhJAF13 independently bind to these promoters, except the SoDFR promoter.

Binding assay of AtTT2 and AtTT8 in yeast
AtTT2 and AtTT8 co-operatively regulate late biosynthetic genes of proanthocyanidin biosynthesis (Nesi et al., 2001). SoDFR and SoANS are expressed specifically in seeds, in which they may contribute to proanthocyanidin

containing each reporter plasmid were co-transformed with the indicated combinations of the effector plasmids, pGAD-PhAN2, pGAD-PhJAF13, pNLS-PhAN2, and pNLS-PhJAF13. The position of each strain is indicated in the middle. The yeast clones are grown on appropriate media to maintain the expression of the vectors (-Leu, -Trp; left plates) and to test for activation of the HIS3 reporter gene (10 mM 3-AT, -His, -Leu, -Trp; right plates). ProSoDFR::HIS3, promoter of SoDFR; ProAtDFR::HIS3, promoter of AtDFR; ProSoANS::HIS3, promoter of SoANS; ProAtANS::HIS3, promoter of AtANS; CYC1, minimal promoter of the iso-1-cytochrome c gene; AD, activation domain; NLS, nuclear localization signal.
synthesis (Shimada et al., 2005). To compare the binding activity of regulators for anthocyanin synthesis with that for proanthocyanidin synthesis, a one-hybrid assay of AtTT2 and AtTT8 was performed. The binding activity of AtTT2 and AtTT8 to SoDFR and SoANS promoters was analysed using the same method as for PhAN2 and PhJAF13 (Fig. 4). The single transformation of AD-TT2 and AD-TT8 gave positive interaction results for AtDFR, SoANS, and AtANS promoters, but not for the SoDFR promoter (Fig. 4A–D). However, the expression of both TT2 and TT8 gave positive interaction results for the SoDFR promoter. These results were similar to the pattern of binding activity of PhAN2 and PhJAF13.

**Transient expression assay in plants**

To assess the functionality of the SoDFR and SoANS promoter, a transient expression assay was attempted in plant cells. For comparative analysis between the promoters of the Caryophyllales DFR and ANS and those of anthocyanin-producing species, the promoters of AtDFR and AtANS were also used as controls in this assay. Promoters fused in-frame to GFP or LUC genes were used as reporter constructs. In the transient assay in plants, the minimal regions of *Z. mays* DFR and ANS and the *Gerbera hybrida* DFR promoter needed for activation are ≈200 bp (Tuerck and Fromm, 1994; Lesnick and Chandler, 1998; Elomaa et al., 2003). Therefore, in the present experiments, ~1000 bp of the promoter regions was analysed, which may suffice to activate target genes. The effector constructs containing the genes of transcriptional regulators for anthocyanin synthesis, PhAN2 and PhJAF13 (Quattrocchio et al., 1998), and that for internal controls containing the RUC gene driven by the 35S promoter were co-bombarded with reporter constructs into the *P. americana* cell cultures. Reporter constructs with the AtDFR and AtANS promoters were also used as controls (Fig. 5). Any reporter construct alone did not result in significant GFP expression (Fig. 5A) or LUC activity (Fig. 5B). The co-expression of PhAN2 and PhJAF13 with AtDFR and AtANS promoters showed high GFP or LUC expression. Nevertheless, SoDFR and SoANS promoters were not activated by the co-expression of PhAN2 and PhJAF13.

Transient expression in the leaves of *S. oleracea* was also examined (Fig. 6). The co-expression of PhAN2 and PhJAF13 with the AtDFR and AtANS promoters drastically

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**Fig. 4.** Comparative analysis of binding activity of AtTT2 and AtTT8 to the promoter of *S. oleracea* DFR and ANS. The reporter and effector plasmid constructs (as described in the Materials and methods) are shown on the upper part of the figure. (A–D) The yeast strains containing each reporter plasmid were co-transformed with the indicated combinations of the effector plasmids, pGAD-AtTT2, pGAD-AtTT8, pNLS-AtTT2, and pNLS-AtTT8. The position of each strain is indicated in the middle. The yeast clones are grown on appropriate media to maintain the expression of the vectors (-Leu, -Trp; left plates) and to test for activation of the HIS3 reporter gene (10 mM 3-AT, -His, -Leu, -Trp; right plates). ProSoDFR, promoter of SoDFR; ProADFR, promoter of AtDFR; ProSoANS, promoter of SoANS; ProAtANS, promoter of AtANS; CYC1, minimal promoter of iso-1-cytochrome c gene; AD, activation domain; NLS, nuclear localization signal.
increased the LUC activity by 262-fold and 150-fold. Nevertheless, the co-expression of these factors with the SoDFR and SoANS promoters had little or no effect on the LUC activity in *S. oleracea* leaves.

**Discussion**

Anthocyanins and betacyanins, two types of red pigment, never co-occur in plants. Although anthocyanins are widely distributed in higher plants, betacyanins have replaced anthocyanins in the Caryophyllales. It was previously demonstrated that the Caryophyllales possess functional DFR and ANS by *in vitro* experiments using recombinant proteins (Shimada et al., 2004, 2005). The expression profile revealed that DFR and ANS are not expressed in most tissues and organs, except seeds in *S. oleracea* (Shimada et al., 2005). 5’ Upstream regions of SoDFR and SoANS were isolated using an inverse PCR method. The analysis of the sequences of the promoters revealed that several elements, to which transcriptional factors can bind, were found in the SoDFR and SoANS promoters (Fig. 2). Binding motifs of P, which is a MYB-like transcriptional factor in *Z. mays*, were found in the SoDFR and SoANS promoters. P independently regulates DFR genes for phlobaphene synthesis in *Z. mays* (Grotewold et al., 1994). C1, which activates the anthocyanin biosynthetic genes in *Z. mays*, could bind to a similar sequence of the P motif (Sainz et al., 1997). In fact, C1 heterologously activates the *P. hybrida* DFR promoter with bHLH protein (Quattrocchio et al., 1998).

The LRU containing the G-box and the region downstream of the MYB recognition element was found in diverse genes of the flavonoid pathway. However, the LRU was not found in the SoDFR promoter, but in the SoANS promoter. The RY motifs were highly conserved in promoters for genes with seed-specific expression. Promoter analysis in transformed tobacco plants showed that the RY motif upstream of the *legumin B4* gene causes higher expression in seeds and suppresses expression in leaves (Bäumlein et al., 1992). This motif was found in the SoDFR and SoANS promoters. This observation supports the idea that SoDFR and SoANS are specifically expressed for proanthocyanidin synthesis in seeds (Shimada et al., 2005). However, it is very difficult to explain the regulatory mechanism for anthocyanin synthesis by the specific motifs in their promoter regions.

The conservation of the combinatorial interactions between MYBs and bHLHs from different species for anthocyanin regulation has been demonstrated (Lloyd et al., 1992; Mooney et al., 1995; Bradley et al., 1998).

**Fig. 5.** Activation of the promoter of *S. oleracea* DFR and ANS in *P. americana* cell cultures. The reporter and effector plasmid constructs (as described in the Materials and methods) are shown in the upper part of the figure. (A) Transient expression of the reporter construct, which contains the GFP gene as a reporter gene, with expression of PhAN2 and PhJAF13 in particle-bombarded *P. americana* cell cultures. (B) Transient expression of the reporter construct, which contains the LUC gene as a reporter gene, with or without expression of PhAN2 and PhJAF13 in particle-bombarded *P. americana* cell cultures. The columns and error bars denote the mean and standard error of the activity of each reporter plasmid after bombardment with the combinations 35S-PhAN2 and 35S-PhJAF13. The reporter gene activity, measured as firefly luciferase (LUC) enzyme, is expressed in arbitrary units and has been normalized to *Renilla* luciferase (RUC) activity expressed from a co-bombarded inner control plasmid, 35S-RUC.
The interaction of the MYB factor, PhAN2, and the bHLH factor, PhJAF13, is sufficient for activation of the *P. hybrida DFR*, the *Z. mays DFR*, and the *G. hybrida DFR* promoter (Quattrocchio *et al.*, 1998; Elomaa *et al.*, 2003). The one-hybrid system in yeast showed that *Perilla frutescens* MYB-like protein, MYBP-1, and bHLH proteins, MYC-RP/GP, can bind to the *DFR* promoter (Gong *et al.*, 1999a, b). The one-hybrid assay revealed that PhAN2 and PhJAF13 can co-operatively bind to the *SoDFR* and *SoANS* promoter. However, PhAN2 and PhJAF13 independently did not bind to the *SoDFR* promoter, but to the *SoANS* promoter (Fig. 3).

*SoDFR* and *SoANS* are expressed in seeds, which probably contributes only to proanthocyanidin synthesis (Shimada *et al.*, 2005). AtT2 and AtTT8 are specifically responsible for proanthocyanidin production in seeds of *A. thaliana*. These proteins regulate the late biosynthetic genes of the flavonoid pathway (Nesi *et al.*, 2000, 2001). The results of the one-hybrid assay of AtT2 and AtTT8 showed similar results to those of PhAN2 and PhJAF13 (Fig. 4). However, in both cases, the apparent difference between the pattern of binding of these factors to *SoDFR* and *SoANS* promoters and that to *AtDFR* and *AtANS* promoters was not observed. The yeast one-hybrid assays suggest that these transcriptional factors for flavonoid biosynthesis can co-operatively bind to the *DFR* and *ANS* promoters of the Caryophyllales.

A transient expression assay was attempted in plant cells. The conservation of the combinational interactions between MYBs and bHLHs from different species for anthocyanin regulation has been shown by the ectopic expression of the regulators for anthocyanin synthesis in heterologous plant systems (Quattrocchio *et al.*, 1998; Elomaa *et al.*, 2003). The promoters of anthocyanin-producing species, the *AtDFR* and *AtANS* promoters, were activated by heterologously expressed PhAN2 and PhJAF13 in *P. americana* cell cultures and leaves of *S. oleracea* (Figs 5, 6). In contrast, *SoDFR* and *SoANS* promoters were hardly or not activated by PhAN2 and PhJAF13 even in the cells of Caryophyllales species. Moreover, not only *SoANS* and *SoDFR* promoters but also *P. americana DFR* and *ANS* promoters (accession nos AB246751 and AB246753) were not activated by PhAN2 and PhJAF13 in the *P. americana* cells (data not shown). In the present systems, a transient assay in plant cells showed that the interaction of *SoDFR* and *SoANS* promoters with regulators for anthocyanin

![Fig. 6. Activation of the promoter of *S. oleracea DFR* and *ANS* in *S. oleracea* leaves. The reporter and effector plasmid constructs (as described in the Materials and methods) are shown in the upper part of the figure. (A) Transient expression of the *DFR* gene promoter–reporter construct, which contains the LUC gene as a reporter gene, with or without expression of PhAN2 and PhJAF13 in particle-bombarded *S. oleracea* leaves. (B) Transient expression of the *ANS* gene promoter reporter construct, which contains the LUC gene as a reporter gene, with or without the expression of PhAN2 and PhJAF13 in particle-bombarded *S. oleracea* leaves. The columns and error bars denote the mean and standard error of the activity of each reporter plasmid after bombardment with the combinations 35S-PhAN2 and 35S-PhJAF13. The reporter gene activity, measured as firefly luciferase (LUC) enzyme, is expressed in arbitrary units and has been normalized to *Renilla* luciferase (RUC) activity expressed from a co-bombarded inner control plasmid, 35S-RUC. Positive cont. shows the luciferase activity in the *S. oleracea* leaves bombarded with the 35S-LUC construct as positive control plasmid.
synthesis differed from that of the AtDFR and AtANS promoters. This result suggests that a structural difference between the promoter regions of the genes of Caryophyllales species and those of anthocyanin-producing species may be a key factor in the limited expression of SoDFR and SoANS.

The results suggest that the putative MYB and bHLH transcriptional factors for anthocyanin biosynthesis, like AN2 and JAF13, bind to the DFR and ANS promoters of the Caryophyllales but do not activate these promoters. Co-factors involved in the activation of genes for flavonoid biosynthesis in conjunction with MYB- and bHLH-type transcriptional factors have been reported (Baümlein et al., 2005). It has been reported that TTG1, a WD-repeat protein, might stabilize bHLH–MYB complexes (Baudry et al., 2004). It is possible that the plants lack such a cofactor, which is needed to activate the DFR and ANS promoters in conjunction with transcriptional factors similar to PhAN2 and PhJAF13 in the Caryophyllales cells. However, the AtDFR and AtANS promoters were activated by PhAN2 and PhJAF13 in the Caryophyllales cells in the transient expression assay. The results indicate that co-factors, which are needed for the activation of the promoters by these factors, exist even in the Caryophyllales cells. The other possibility is the presence of a repressor-binding site only in Caryophyllales promoters. Negative regulators for anthocyanin biosynthesis, FaMYB1 and FUS3, have been isolated from Fragaria ananassa and A. thaliana, respectively (Baümlein et al., 1994; Aharoni et al., 2001). The activation of the DFR and ANS promoters of the Caryophyllales by the MYB and bHLH transcriptional factors might be blocked by the binding of a repressor that can bind only to the DFR and ANS promoters of the Caryophyllales and not to those of anthocyanin-producing plants, resulting in limited expression of these genes and the absence of anthocyanin synthesis.

Transient expression assays in Z. mays and P. hybrida have shown that Z. mays C1 and R or P. hybrida AN2 and JAF13 can activate the promoter of Z. mays CHS (c2) but not that of P. hybrida CHS (chsA). These results indicated that regulatory anthocyanin genes are conserved between species and that divergent evolution of the target gene promoters is responsible for the species-specific differences in regulatory networks (Quattrocchio et al., 1998). The modification of SoDFR and SoANS cis-regulatory elements may lead to the limited expression of SoDFR and SoANS, resulting in defective anthocyanin synthesis in S. oleracea. The evolution of cis-regulatory elements has been proposed to be a major source of morphological diversification, as mutations in cis-regulatory elements often lead to dramatic tissue-specific pattern changes while preserving the essential roles of these genes in other processes (Shapiro et al., 2004; Gompel et al., 2005).

A more detailed analysis of the promoters and the characterization of regulators for flavonoid biosynthesis will provide further understanding of the regulatory mechanism of flavonoid biosynthesis in the Caryophyllales and may elucidate why the Caryophyllales do not produce anthocyanins.

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