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SHORT COMMUNICATION

Characterization of an Apple Anthocyanidin Synthase Gene in Transgenic Tobacco Plants

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Anthocyanin is the major color pigment in plants. The apple anthocyanidin synthase gene (*ANS*) manifests fruit skin-preferential expression (Kim et al., 2003). To understand the regulatory mechanism for such expression, we isolated and analyzed an apple *ANS* genomic clone. Sequence analysis of ca. 1.4-kb of the *ANS* promoter region predicted several *cis*-elements for MYB, light responsive GT-1, and the ABA Responsive Element (ABRE). Transgenic tobacco plants carrying a chimeric fusion between the *ANS* promoter and the β-glucuronidase gene (*GUS*) showed that GUS was expressed in the receptacles and immature seeds as well as in the floral buds, but not in the vegetative organs.

Keywords: anthocyanidin synthase gene, anthocyanin, apple, GUS, promoter

Color development in flowers and fruits is an important trait in plant fitness. This coloration arises from a blend of chlorophyll, carotenoids, and flavonoids. Anthocyanins are secondary products belonging to a subclass of flavonoids. They function as pigments in flowers and fruits to attract insects for pollination or to aid in seed dispersal (Holton and Cornish, 1995; Springob et al., 2003). Anthocyanins and other flavonoids also can act as protectants against UV light and pathogens or as signal molecules in the interaction between bacteria and plants (Harborne and Grayer, 1994; Holton and Cornish, 1995; Dixon and Steele, 1999; Springob et al., 2003; Kim et al., 2006). Besides anthocyanin, other pigments such as flavonols and carotenoids, as well as metal complex, vacuolar pH, and cell shapes can affect the final pigmentation phenotype (Forkmann, 1994; Holton and Cornish, 1995).

The anthocyanin biosynthesis pathway is well-known for secondary products (Dixon and Steele, 1999). It has been extensively studied in the flowers of petunia (*Petunia hybrida*) and snapdragon (*Antirrhinum majus*), and in the kernels of maize (*Zea mays*) (Holton and Cornish, 1995). Among the genes and enzymes examined in this pathway, anthocyanidin synthase (ANS) is an important component because it converts leucoanthocyanidins to anthocyanidins, the first colored compound in the anthocyanin pathway (Springob et al., 2003). In *Forsythia*, *ANS* transcripts have been detected in the sepals, but not in the anthers or petals (Rosati et al., 1999). In etiolated seedlings of *Arabidopsis*, *ANS* gene is induced by white light to its highest level later on than is the flavonol synthase gene (*FLS*), suggesting the former is a 'late' gene in the biosynthetic pathway (Pelletier et al., 1997). Expression of the *Perilla ANS* gene, which is detected in red leaves of the red forma, is coordinately induced in the forma by high-intensity white light (Gong et al., 1997). Likewise, transcripts of the apple *ANS* gene are detected preferentially in the skin tissues of red fruits, but not green, and are also coordinately induced by light (Kim et al., 2003).

Regulatory genes of the anthocyanin biosynthetic pathway have also been identified (Springob et al., 2003). Among those that are well characterized are the genes that encode MYB and basic helix-loop-helix (bHLH), which influence the intensity and pattern of expression (Holton and Cornish, 1995; Springob et al., 2003). For example, the *Forsythia ANS* gene contains some *cis*-elements involved in the activation and light response of anthocyanin genes (Rosati et al., 1999). In maize, a C1-binding site with a second, adjacent putative site functions in the activation of the *ANS* gene (Lesnick and Chandler, 1998).

In this study, genomic clones of an apple *ANS* gene were isolated and the promoter region was examined *in silico*. To understand the regulatory role of the promoter, a *GUS* gene under the *ANS* promoter was investigated in transgenic tobacco.

MATERIALS AND METHODS

Bacterial Strains

Escherichia coli strains MC1000 and XL-1 Blue MRF' served as hosts for molecular cloning. The f1 helper phage, R408, was used for excision of the pBluescript phagemid from the λUNI-Zap XR vector (Stratagene, USA). The *E. coli* strain XL1-Blue MRA (P2) was used as a host for λphage preparation after the genomic library was screened.

Screening of Genomic Library

An apple genomic library was obtained from Dr. Woo-Taek Kim at Yonsei University, Korea. The library was generated from young apple leaves using the *Bam*HI-digested λDASHII vector (Stratagene), according to the manufacturer's instructions. Phages of the library were screened by plaque hybridization with the *MdANS1* probe (Accession number AF117269) labeled with [α-^{32}P]dCTP. After tertiary screening, positive plaques were isolated and the restriction fragment of the recombinant clone was subcloned into pBluescript SK(-) vector (Stratagene).

DNA Sequence Analysis

DNA sequencing was performed according to the dideoxynucleotide chain termination method (Sanger et al., 1977), and with a Thermo Sequenase Cycle Sequencing kit (Amersham, UK) and autosequencer (ABI, USA). The sequences were compared with those in the database, using the BLAST program (Altschul et al., 1990) and DNASIS and PROSIS software (Hitachi, Japan). Putative DNA-protein binding

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sites in the promoter sequence were searched with the MatInspector program and standard settings (core similarity, 0.80; matrix similarity, 0.85).

Analysis of *ANS* Genomic Clone and Construction of *ANS::GUS*

A genomic clone corresponding to *MdANS1* cDNA was isolated and a 1.5-kb *Eco*RI fragment containing the *MdANS1* 5′-flanking region was subcloned into the pBluescript SK(–), generating pANS1.5. A *Sal*I/*Xba*I fragment of pANS1.5 was translationally fused into pBI101.2 (Accession number U12668; Jefferson et al., 1987), generating pSK126. This vector was then transferred into *Agrobacterium tumefaciens* strain LBA4404 by the freeze-thaw method (An et al., 1988).

Tobacco Transformation and GUS Assay

Nicotiana tabacum var. SR1 was transformed with *A. tumefaciens* by the leaf disc transformation method (An et al., 1988). Flowers, floral buds, and leaves of greenhouse-grown transgenic tobacco plants were used for the GUS assay, as described by Jefferson et al. (1987). Tissues were incubated in the dark at 37°C for 10 to 13 h in 100 mM sodium phosphate buffer (pH 7.0) containing 0.1% (w/v) 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Glu), 10 mM EDTA, 0.5% (v/v) Triton X-100, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 20% methanol. After staining, the tissues were cleared with ethanol at 50°C for 5 h, then observed under a microscope with dark-field illumination.

RESULTS AND DISCUSSION

Isolation of an Apple *ANS* Genomic Clone

We previously analyzed the *ANS* gene (*MdANS1*), which is preferentially expressed in the skin tissues of a red apple cultivar, 'Fuji' (Kim et al., 2003). To better understand the regulatory mechanism for this tissue-specific expression, we have now isolated genomic clones. About 3×10^5 plaques of the genomic library were screened by plaque hybridization, using *MdANS1* cDNA as the probe. After tertiary screening, nine recombinant plaques were obtained, and were divided into four groups based on their restriction maps (data not shown). An approximately 12-kbp-long genomic clone, λANS1, was found to correspond to the *MdANS1* cDNA. A 1.5-kbp *Eco*RI subclone of λANS1 (pANS1.5) contained a 5′-flanking sequence with part of the coding region (Fig. 1A). An adjacent 2.0-kbp *Eco*RI subclone, pANS2.0, contained the remaining coding region, with a 194-b intron and a 3′-flanking region. This genomic structure, comprising an intron, is similar to those found in *Arabidopsis*, *Forsythia*, and *Petunia*, but differs from one identified in maize (Furtek et al., 1988; Menssen et al., 1990; Pelletier et al., 1997; Rosati et al., 1999). Our intron comprised consensus GT and AG sequences at the 5′ and 3′ ends, respectively.

In Silico Analysis of the *ANS* Promoter

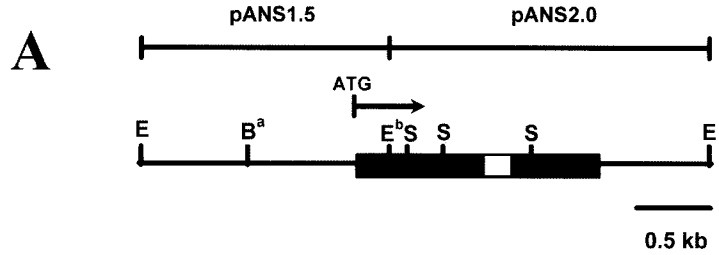
A putative CAAT box sequence, CAAT, and a TATA box sequence, TATAAAA, were found in the 5′-flanking sequence of *MdANS1* at –112/–109 and –91/–85, respectively, from the 5′ end of *MdANS1* cDNA (Fig. 1B). Several *cis*-elements important for light-responsive or flavonoid biosynthesis-specific expression were located as well (Table 1), as were a Myb-class P activator binding site, CCAACC (at –847/–842) and two similar sequences. In the maize pericarp, a P protein activates transcription of the *A1* gene required for 3-deoxyflavonoid biosynthesis (Pelletier and Shirley, 1996). Our gene also contained *cis*-elements for C1 and MYB.Ph3 binding (Martin et al., 1991; Grotewold et al., 1994) in three locations (Table 1). The *cis*-elements for C1, P, and MYB.Ph3 binding have also been reported in the *Forsythia ANS* promoter (Rosati et al., 1999). Furthermore, in the *MdANS1* promoter (Table 1), we found other elements such as a G box (CACGTG) and GT-1 box (GGTTAA) which are present in many light-regulated promoters (Solano et al., 1995; Terzaghi and Cashmore, 1995). GT-1 sites were usually found in tandem, and the spacing between them is critical (Terzaghi and Cashmore, 1995). In the *MdANS1* promoter, a GT-1 site at –1042 is 22-b from a SBF-1 binding site (Gilmartin et al., 1990), a sequence closely related to the GT-1 binding site. The promoter region contains three G box core sequences that are overlapped with ABA-responsive elements (ABRE). Kim et al. (2003) have shown that *MdANS1* is light-inducible; it will be interesting to determine whether these elements are indeed responsible for gene expression. The transcription factors that bind to the putative *cis*-elements might mediate transcriptional activation or inhibition of the *MdANS1* gene. By analyzing the activity of the promoter with mutations in the elements, researchers may be able to identify the elements that affect *MdANS* gene expression.

Expression of the Apple *ANS::GUS* in Transgenic Tobacco Plants

We produced transgenic plants to investigate the role of the *MdANS1* promoter in tissue-specific expression. Because apple is recalcitrant to transformation, a tobacco system was employed. The *ANS* promoter region was translationally fused to the *GUS* gene of the binary vector pBI101.2, generating pSK126 (Fig. 1C). Out of 10 regenerated plants, 6 were confirmed as transgenics based on PCR examination of the *ANS::GUS* construct (data not shown). Expression of this fusion gene was analyzed by histochemical GUS assay.

GUS activity in the transgenic plants was barely detected during the vegetative growth phase, which is consistent with the results from a northern analysis of *MdANS1*. In contrast, at the reproductive phase, GUS activity was detected in several tissues from the reproductive organs: sepals of floral buds, petals of mature flowers, receptacles, and developing seeds (Fig. 2). In the sepals, GUS activity was found mainly in the trichomes and only weakly in the epidermal tissues. Similarly, in the mature petals, activity was greater in the trichomes than in the epidermal tissues. In the petals, vascular tissues also showed strong activity. In the developing fruits, activity was significant in the crenulated epidermal cells of immature seeds, but weak in the placenta. Strong activity was detected in the centers of the receptacles. This high level of activity in the reproductive organs, but low in the vegetative organs, of transgenic tobacco is consistent with the reproductive organ-preferential expression of *MdANS1* described previously (Kim et al., 2003).

GUS activity was remarkably high in the immature seeds of our transgenic tobacco (Fig. 2), which may be related to the anthocyanin production in developing seed coats. In *Phaseolus vulgaris*, the *CHS15* promoter-driven *GUS* gene



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 -408  caattaagtttgtaaaccaataattaacttaattttgatagtcaaacacctcatataaagtggaaaaccaagaaccggaagcatttactaaggcggtta
 -309  ggggtatccatgcacgctttcaacttttccagtcaaacctcggtcgcatgatgaaagtctggtgggtgagatgtcacatgtacaacagccacatgcctac
 -210  tcgaaaagaaagaacacattcttcactctccaacacgctcacttactcttgctagtggtagctagcagcagtattttcccttctaaatttgtaaaccc
 -111  aatttggaaccaactactcattataaaatcaacgttacattatcaatgcccgttaaaccattgcttcatcaacccaccacaaaaaaaaaaaaaagagtgc
  -12  agggcctgcattgttatcgcaaaaaaaaaaaaaaaaaaaaacaaacgagtaatatactagctgagatagtatattcttgttgtcgaaagccagctccaa
   88  aaATGGTGAGCTCTGATTCAGTGAATTCAAGGGTTGAGACCTTGGCCGGCAGTGGAATCTCAACCATCCCAAAAGAGTACATCAGACCTAAAGATGAGC
         M  V  S  S  D  S  V  N  S  R  V  E  T  L  A  G  S  G  I  S  T  I  P  K  E  Y  I  R  P  K  D  E  L   33
  187  TCGTAAACATTGGTGACATCTTCGAACAAGAGAAGAACAACGAAGGGCCTCAAGTTCCCACCATCGATTTGAAGGAGATAGAGTCTGATAACGAAAAAG
         V  N  I  G  D  I  F  E  Q  E  K  N  N  E  G  P  Q  V  P  T  I  D  L  K  E  I  E  S  D  N  E  K  V   66
  286  TGAGAGCAAAATGCAGGGAGAAGTTGAAGAAGGCAGCTGTGGACTGGGGTGTCATGCACCTTGTGAACCATGGCATCTCCGACGAGCTCATGGACAAGG
         R  A  K  C  R  E  K  L  K  K  A  A  V  D  W  G  V  M  H  L  V  N  H  G  I  S  D  E  L  M  D  K  V   99
  385  TCAGGAAGGCCGGTAAGGCCTTCTTTGACCTTCCCATTGAGCAGAAGGAGAAGTATGCCAATGACCAGGCCTCTGGTAAGATTCAAGGCTATGGAAGCA
         R  K  A  G  K  A  F  F  D  L  P  I  E  Q  K  E  K  Y  A  N  D  Q  A  S  G  K  I  Q  G  Y  G  S  K  132
  484  AGCTTGCAAACAATGCATCTGGGCAGCTTGAGTGGGAGGACTACTTCTTCCACTGTGTATACCCAGAGGACAAGCGTGACTTGTCAATTTGGCCTCAAA
         L  A  N  N  A  S  G  Q  L  E  W  E  D  Y  F  F  H  C  V  Y  P  E  D  K  R  D  L  S  I  W  P  Q  T  165

  583  CACCTGCTGATTACATgtaagtacttaaaaatcttagcatgcttaattggtgtcacaatcataaaccctagcctaaaatttttaattatatccccgaact
         P  A  D  Y  I                                                                                      170
  682  gattaaaattgtcaatagttttatatatatttgtaaaaggccctcctccccacaagtaattctggttccgccgcttactcttgttaacatcaatcttta
  781  ataccttggcagTGAGGCAACCGCCGAGTATGCTAAGCAATTGAGGGAGCTAGCAACCAAGGTACTGAAAGTTCTGTCACTTGGCTTGGGATTGGATGA
                    E  A  T  A  E  Y  A  K  Q  L  R  E  L  A  T  K  V  L  K  V  L  S  L  G  L  G  L  D  E   199
  880  AGGGAGGCTGGAGAAAGAAGTTGGTGGACTTGAAGAGCTCCTCTTGCAAATGAAAATCAACTACTACCCAAAATGCCCTCAGCCGGAGCTTGCACTTGG
         G  R  L  E  K  E  V  G  G  L  E  E  L  L  L  Q  M  K  I  N  Y  Y  P  K  C  P  Q  P  E  L  A  L  G   232
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 1078  GTGCGTTCCAAATTCCATCGTCATGCACATTGGGGATACACTTGAGATTTTGAGCAATGGGAAGTACAAAAGTATACTCCACAGGGGCATGGTGAACAA
         C  V  P  N  S  I  V  M  H  I  G  D  T  L  E  I  L  S  N  G  K  Y  K  S  I  L  H  R  G  M  V  N  K   298
 1177  GGAAAAGGTGAGGATTTCATGGGCTGTTTTCTGTGAGCCACCAAAGGAGAAGATCATCCTTAAGCCACTGCCGGAGACCGTGTCTGAGGACGAGCCGGC
         E  K  V  R  I  S  W  A  V  F  C  E  P  P  K  E  K  I  I  L  K  P  L  P  E  T  V  S  E  D  E  P  A   331
 1276  AATGTTCCCACCACGAACTTTTGCTGAGCACATTCAGCACAAGTTGTTCAGGAAGAGCCAAGAGGCTTTGCTCCCCAAGtgaagcgggttgtgctaaat
         M  F  P  P  R  T  F  A  E  H  I  Q  H  K  L  F  R  K  S  Q  E  A  L  L  P  K  *                    357
 1375  tataatattgttgtgaactatcgtcctaataaatctgtttatggcatagttggtatgatatatcttagtttccatagtatgtgggtttccttttgcac
 1474  ttggctaagctactcctgtccgcctgctttgttgaataattattgattttatgtccttgtacctgtgatgaatggctatttttcaagtcaagatttcaata
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 1672  ttaaagattatttttatataaaaaatcaaaatcgagatttttgttgtagtcctatttgattcggaatgtaattgtgtaaatcctagtatatcttagaat
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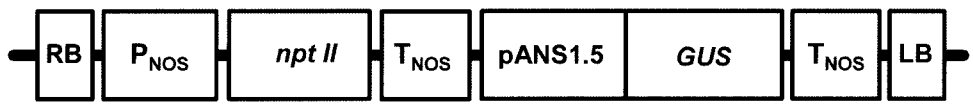

Figure 1. Nucleotide sequence of *MdANS1* gene and map of *MdANS1*::*GUS* construct. **A**, Restriction map of *MdANS1* genomic clone. A 1.5-kb *Eco*RI fragment designated as pANS1.5 includes the first ATG codon with upstream region, whereas pANS2.0 includes the remaining downstream region. Arrow indicates direction of translation. *MdANS1* coding regions are shown in black boxes, with intron as white box. E, *Eco*RI; B, *Bam*HI; S, *Sac*I. [a]Two *Bam*HI sites are located near each other; another [b]*Sac*I site is located close to and upstream of *Eco*RI. **B**, Nucleotide and deduced amino acid sequences of *MdANS1* gene. Non-coding sequence is in lower case; coding sequence, in upper case. Deduced amino acid sequence of *MdANS1* gene is shown below nucleotide sequence. Numbers on the left and the right indicate respective positions of nucleotide and amino acid. First nucleotide of *MdANS1* cDNA is shown in bold type and referred to as +1. Putative CAAT and TATA boxes in promoter region and polyadenylation signals are underlined. **C**, Chimeric gene construct to express *GUS* under *MdANS1* promoter in transgenic tobacco. This gene cassette was inserted into binary vector pBI101.2 and introduced into tobacco leaf discs by *Agrobacterium*-mediated transformation. RB, right border of T-DNA; P_{NOS}, nopaline synthase promoter; *npt II*, neomycin phosphotransferase gene; T_{NOS}, nopaline synthase terminator; LB, left border of T-DNA.

also shows GUS expression in the epidermal cells of developing seeds from transgenic tobacco plants (Lawton et al., 1991). Here, we could detect only weak activity in the fruit skins of transgenic tobacco, perhaps because of different types of fruit development, i.e., tobacco fruit is a dehiscent capsule that develops from an ovary while apple fruit is indehiscent flesh, being hypanthium developed to fruit parenchyma (Fahn, 1990). Therefore, it would be interesting to examine whether GUS expression in the receptacles of transgenic plants is related to the skin-preferential expression of *MdANS1*in apple. Transformation of apple plants would provide a direct answer for the exact location of *MdANS1* expression, and developing skin-specific promoter will be useful to enhance such biological traits as fruit color in the skin tissues.

Table 1. Putative *cis*-elements observed in the *MdANS1* promoter[a].

P (CC(T/A)ACC)[b]	C1 (AAACGT)	MYB.Ph3 (aaaAaaC(C/G)GTTA or aaaAGTTAGTTA)	I-box (GATAA)	SBF-1(GGTTAA(A/T)(A/T)(A/T)) or GT-1 (GGTTAA)[c]	G-box (CACGTG) or ABRE (ACGT)[d]
CCAACC (-847 to -842)				GGTTAAAAT (-1072 to -1064)	
CCCACC (-250 to -245)[e]	AAACGT (-528 to -523)	ATGAGCTAGTTA (-1312 to -1301)[e]	GATAA (-1278 to -1274)	GGTTAAAGT (-1042 to -1034)	TACGTA (-1316 to -1311)
GCTACC (-154 to -149)[e]		CTAAGGCGGTTA (-319 to -308)		AGTTAAAAG (-865 to -857)	TACGTG (-961 to -956)
				AGTTAACAT (-679 to -671)	AACGTA (-527 to -522)
				GCTTAAAAC (-582 to -574)	
				AGTTAAAAT (-554 to -546)	
				GGTTAAACA (-489 to -481)	
				AGTTAATTA (-388 to -380)[e]	

[a]Putative *cis*-elements observed in a 1398-b region upstream from the 5′ end of *MdANS1* cDNA. [b]The nucleotide sequence is the core consensus sequence reported for the promoters of other plant species. [c]GT-1 boxes and [d]ABRE core sequences are underlined. [e]Sequences found in the reverse orientation compared with the direction of the *MdANS1* open reading frame.

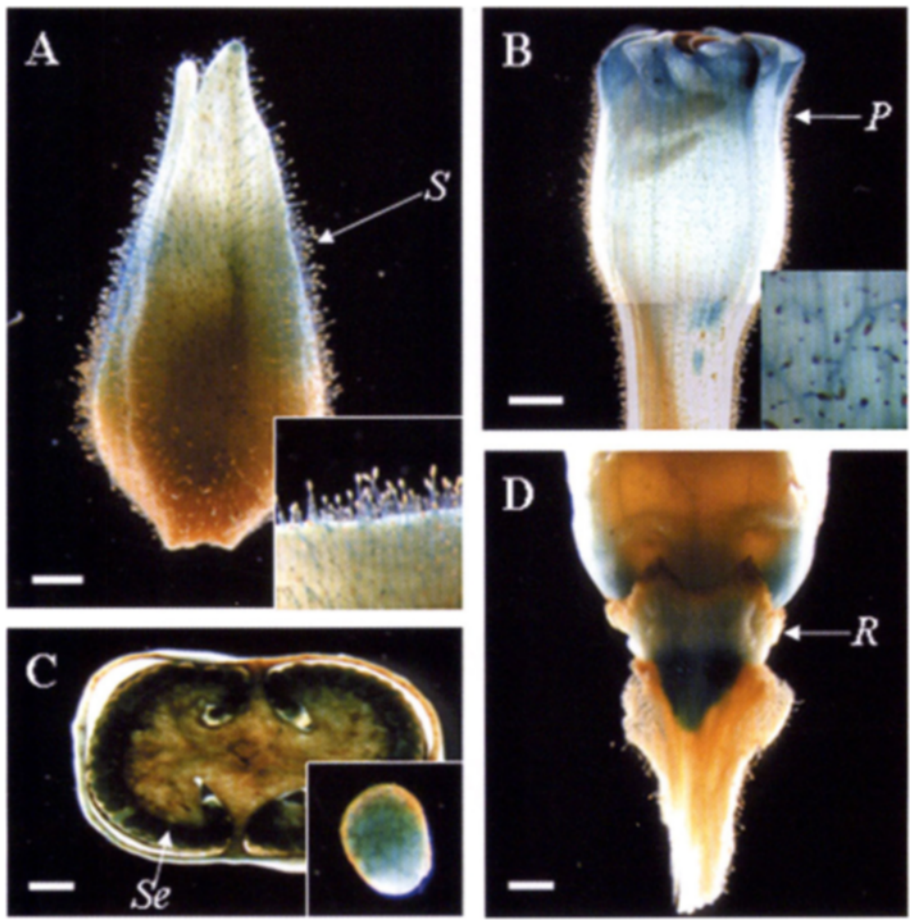

Figure 2. *MdANS1* promoter-driven GUS expression in transgenic tobacco plants. Blue precipitates indicate locations of GUS activity. **A**, Young floral bud. Close-up shows activity in trichomes of sepal. Bar =1 mm. **B**, Flower before anthesis. Close-up shows activity in vascular tissues as well as in trichomes of petal. Bar =2 mm. **C**, Cross section of developing fruit. Close-up shows activity in seed coats. Bar =1 mm. **D**, Lower part of developing fruit. For photo of GUS-expressed region, old sepals were detached from fruit. Bar =1 mm. P, petal; R, receptacle; S, sepal; Se, developing seeds.

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LITERATURE CITED

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403-411

An G, Ebert PR, Mitra A, Ha SB (1988) Binary vectors, *In* SB Gelvin, RA Schilperoort, eds, Plant Molecular Biology Manual. Kluwer

Academic Publishers, Dordrecht, pp A3/1-A3/19
Dixon RA, Steele CL (1999) Flavonoids and isoflavonoids: A gold mine for metabolic engineering. Trends Plant Sci 4: 394-400
Fahn A (1990) Plant Anatomy. Pergamon Press, Oxford, pp 503-512
Forkmann G (1994) Genetics of flavonoids, *In* JB Harborne, ed, The Flavonoids: Advances in Research since 1986. Chapman and Hall, London, pp 537-564
Furtek D, Schiefelbein JW, Johnston F, Nelson OE (1988) Sequence comparisons of three wild-type *Bronze-1* alleles from *Zea mays*. Plant Mol Biol 11: 473-481
Gilmartin PM, Sarokin L, Memelink J, Chua NH (1990) Molecular light switches for plant genes. Plant Cell 2: 369-378
Gong Z, Yamazaki M, Sugiyama M, Tanaka Y, Saito K (1997) Cloning and molecular analysis of structural genes involved in anthocyanin biosynthesis and expressed in a forma-specific manner in *Perilla frutescens*. Plant Mol Biol 35: 915-927
Grotewold E, Brummond BJ, Bowen B, Peterson T (1994) The myb-homologous P gene controls phlobaphene pigmentation in maize floral organs by directly activating a flavonoid biosynthetic gene subset. Cell 76: 543-553
Harborne JB, Grayer RJ (1994) Flavonoids and insects, *In* JB Harborne, ed, The Flavonoids: Advances in Research Since 1986. Chapman and Hall, London, pp 589-618
Holton TA, Cornish EC (1995) Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell 7: 1071-1083
Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: Beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901-3907
Kim J-S, Lee B-H, Kim S-H, Oh K-H, Cho KY (2006) Responses to environmental and chemical signals for anthocyanin biosynthesis in non-chlorophyllous corn (*Zea mays* L.) leaf. J Plant Biol 49: 16-25
Kim SH, Lee JR, Hong ST, Yoo YG, An G, Kim SR (2003) Molecular cloning and analysis of anthocyanin biosynthesis genes preferentially expressed in apple skin. Plant Sci 165: 403-413
Lawton MA, Dean SM, Dron M, Kooter JM, Kragh KM (1991) Silencer region of a chalcone synthase promoter contains multiple binding sites for a factor, SBF-1, closely related to GT-1. Plant Mol Biol 16: 235-249
Lesnick ML, Chandler VL (1998) Activation of the maize anthocyanin gene *a2* is mediated by an element conserved in many anthocyanin promoters. Plant Physiol 117: 437-445
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
Menssen A, Hohmann S, Martin W, Schnable PS, Peterson PA, Saedler H, Gierl A (1990) The En/Spm transposable element of *Zea mays* contains splice sites at the termini generating a novel intron from a dSpm element in the *A2* gene. EMBO J 9: 3051-3057
Pelletier MK, Murrell JR, Shirley BW (1997) Characterization of flavonol synthase and leucoanthocyanidin dioxygenase genes in *Arabidopsis*. Plant Physiol 113: 1437-1445
Pelletier MK, Shirley BW (1996) Analysis of flavanone 3-hydroxylase in *Arabidopsis* seedlings: Coordinate regulation with chalcone synthase and chalcone isomerase. Plant Physiol 111: 339-345
Rosati C, Cadic A, Duron M, Ingouff M, Simoneau P (1999) Molecular characterization of the anthocyanidin synthase gene in *Forsythia intermedia* reveals organ-specific expression during flower development. Plant Sci 149: 73-79
Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74: 5463-5467
Solano R, Nieto C, Avila J, Canas L, Diaz I, Paz-Ares J (1995) Dual DNA binding specificity of a petal epidermis-specific MYB transcription factor (MYB.Ph3) from *Petunia hybrida*. EMBO J 14: 1773-1784
Springob K, Nakajima J, Yamazaki M, Saito K (2003) Recent advances in the biosynthesis and accumulation of anthocyanins. Nat Prod Rep 20: 288-303
Terzaghi WB, Cashmore AR (1995) Light-regulated transcription. Annu Rev Plant Physiol Plant Mol Biol 46: 445-474