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Ratio of *Myc* and *Myb* Transcription Factors Regulates Anthocyanin Production in Orchid Flowers

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Abstract. **Many studies have examined anthocyanin gene expression in colorless tissues by introducing anthocyanin regulatory genes of the MYC/R and MYB/C1 families. Expression of the two regulatory genes under the control of a strong promoter generally results in high anthocyanin accumulation. However, such approaches usually have a negative effect on growth and development of the recovered plants. In this study the author used two promoters of different strengths—a weak (*Solanum tuberosum* L. polyubiquitin *Ubi3*) and a strong (double *35S*) promoter—and generated two sets of expression constructs with the *Zea mays* L. anthocyanin regulatory genes Myc_{Lc} and Myb_{C1}. A transient expression system was developed using biolistic bombardment of white *Phalaenopsis amabilis* (L.) Blume flowers, which the authors confirmed to be anthocyanin regulatory gene mutants. Transient expression of different combinations of the four constructs would generate three different Myc_{Lc}-to-Myb_{C1} ratios (>1, 1, <1). The enhanced green florescent protein gene (*EGFP*) was cotransformed as an internal control with the two anthocyanin regulatory gene constructs. These results demonstrate that the ratio of the two transcription factors had a significant influence on the amount of anthocyanin produced. Anthocyanin accumulation occurred only when Myb_{C1} was under the control of the *35S* promoter, regardless of whether Myc_{LC} was driven by the *35S* or *Ubi*3 promoter.**

Novel coloration and patterns of coloration add aesthetic appeal to ornamental plants and therefore have great commercial value to the floral and nursery industries. The red, purple, and blue colors of vegetative and floral organs are the result of anthocyanins, the prominent pigments in higher plants. The biochemistry and genetics of the anthocyanin-generating flavonoid biosynthetic pathway have been extensively studied (Davies, 2000; Griesbach, 2005; Irani et al., 2003; Koes et al., 2005; Winkel-Shirley, 2001). Analysis of *Zea mays* anthocyanin mutants revealed two families of regulatory factors, one encoding an MYC-like transcription factor with a basic helix–loop–helix motif (R family) and the other encoding an R2R3 MYB-like transcription factor (C1 family). The two factors are direct regulators of the anthocyanin structural genes (Spelt et al., 2000). Tissue-specific expression of regulatory genes and the specific response of the cis-element of the downstream structural genes to the regulatory factors (Quattrocchio et al., 1993, 1998) dictate anthocyanin expression pattern and determine, to a large degree, the coloration (Griesbach, 2005). There is increasing evidence that a third transcription factor, WD40, is also involved in anthocyanin regulation (Grotewold et al., 2000; Lesnick and Chandler, 1998).

Both the *Myc* and *Myb* gene families contain members that have arisen by gene duplication (Hanson et al., 1996; Zhang et al., 2000). Structural gene regulation is defined by the diversity among the *Myc* and *Myb* alleles, each of which regulates expression in a different manner. For example Myb_{Ros} from *Antirrhinum majus* L. increases the anthocyanin level in vegetative tissue when expressed in *Petunia ×hybrida* Vilm. and in floral tissue when expressed in *Eustoma grandiflorum* (L.) Cass. (Schwinn et al., 2001). In *P. ×hybrida*, the combination Myc_{An1}/Myb_{An2} induces anthocyanin pigmentation in the flower limb, whereas the Myc_{An1}/Myb_{An4} combination induces anthocyanin pigmentation in the anthers, and the Myc_{An1}/Myb_{Ph4} combination induces vacuolar acidification (Quattrocchio et al., 2006).

The *Myc* and *Myb* regulatory genes have been isolated from many different species (Borevitz et al., 2000; Chandler et al., 1989; Cone et al., 1986; Dellaporta et al., 1988; Elomaa et al., 1998, 2003; Gong et al., 1999; Goodrich et al., 1992; Ludwig et al., 1989; Mathews et al., 2003; Nesi et al., 2000; Paz-Ares et al., 1986, 1987; Perrot and Cone, 1989; Quattrocchio et al., 1998, 1999; Radicella et al., 1991; Spelt et al., 2000; Tonelli et al., 1991). *Zea mays* Myc_{Lc} (R family) and Myb_{C1} (C1 family) are two of the alleles that are the most well studied. They have been expressed in a number of plant species (Bovy et al., 2002; Bradley et al., 1998; Goldsbrough et al., 1996; Lloyd et al., 1992; Quattrocchio et al., 1993) in which

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they either enhanced the amount of anthocyanin produced or activated de novo biosynthesis in unpigmented tissues. However, in the majority of those studies, both regulatory genes were under the control of the cauliflower mosaic virus *35S* promoter, which resulted in deleterious effects on plant growth (Bradley et al., 1998; Goldsbrough et al., 1996). This effect could be the result of either high levels of anthocyanin induced by the increased expression of the two regulatory genes or pleiotropic effects resulting from the accumulation of high levels of the two transcription factors.

In a previous study (Griesbach and Klein, 1993) we developed a transient gene complementation system using biolistics to determine the genetic basis of flower color mutants. We demonstrated that the albescent phenotype of *Phalaenopsis pulcherrima* (Lindl.) J.J. Sm. forma *albescea* (Fowlie) E.A. Christenson was the result of a regulatory gene mutation. In the current study we investigated the effects of different regulatory gene levels on the complementation of the albescent phenotype of *Phalaenopsis amabilis.*

Materials and Methods

Plant material. Several commercial white *P. amabilis* hybrids (Kerry's Bromeliad Nursery, Homestead, FL), *P. stuartiana* Rchb., and *P. schilleriana* Rchb. were used in this study. All plants were grown in commercial orchid greenhouses until flowering. Flowering plants were then held in the laboratory for the duration of the study.

Gene constructs. Promoters, structural genes and terminators were either used directly as received or amplified by polymerase chain reaction (PCR) to create flanking restriction enzyme sites to facilitate subsequent cloning. Polymerase chain reactions were performed with the following primers (restriction sites in bold):

Ubi3 promoter primer sequence: forward 5′CC**AAGCTT**C CAAAGCACATACTTAT3′; reverse 5′-**GGATCC**TTCGCC TGGAGGAGAG-3′

Ocs-Mas super promoter primer sequence: forward 5′**AAGCTT**GGATCCCTGAAAGCGA -CG3′; reverse 5′CC**GGTACC**TAGAGTCGATTTGG3′

Myc_{Lc} primer sequence: forward 5′**GGATCC**ATCGAGTT GTTGTACTCTTCGC3′; reverse 5′**GGTACC**TCTAGAATG CTATGACTTTG3′

rbcS terminator primer sequence: forward 5′-**GGTACC**GC TTTCGTTCGTATCATCGG-3′; reverse 5′**GAATTC**GGAT CGATTGATGCATGTTGTC3′

All PCR reactions were carried out using high-fidelity Vent Polymerase (New England Biolabs, Ipswich, MA) and amplified fragments were subcloned into pCR-BluntII-TOPO vector (Invitrogen, Carlsbad, CA). Amplified fragments were all verified by partial sequencing from both ends.

All the constructs were based on the pUC19 vector. Plasmid S-Myc_{Lc} was a transcriptional fusion of the double *35S* promoter and a 2.2-kb Myc_{Lc} complementary DNA (cDNA) (Lloyd et al., 1992). Plasmid U-Myc_{Lc} was identical to S-Myc_{Lc}, except the *35S* promoter was replaced with the 920-bp *Solanum tuberosum Ubi3* promoter (Garbarino and Belknap, 1994). Plasmid S-Myb_{C1} and U-Myb_{C1} were identical to S-Myc_{Lc} and U-Myc_{Lc}, except that the structural gene of Myc_{Lc} was replaced with the 2.1-kb Myb_{C1} cDNA (Lloyd et al., 1992). The terminator for all four constructs was the ribulose bisphosphate carboxylase (rbcS) terminator from *Pisum sativum* L. The internal transformation control plasmid, SuproEGFP, contained the enhanced green fluorescence protein gene coding region fused to the *Ocs-Mas* super promoter (Ni et al., 1995) and the *35S* terminator.

Particle bombardment. Plasmid DNA was isolated using the HiSpeed Plasmid Midi Kit (Qiagen, Valencia, CA) and quantified using a Shimadzu spectrophotometer ultraviolet-240 (Shimadzu Corporation, Kyoto, Japan). Immediately before bombardment, healthy orchid petals were harvested from the plant and arranged on moist filter paper in the center of a 9-cm-diameter petri dish. Bombardment was carried out using a PDS-1000/He (Bio-Rad, Hercules, CA). For each shot, 50 ng pSuproEGFP and 500 ng each of Myc_{LC} and Myb_{C1} constructs were coprecipitated (Griesbach and Klein, 1993) onto 0.5 mg of 1.0-µm-diameter gold particles (Bio-Rad). Each plate was shot once at a rupture pressure of 3.724 MPa with a vacuum pressure of 94.7 kPa. The distance between the bottom of the rupture disk and the lid of the microcarrier launch assembly was adjusted to 1 cm. The target holder was placed 9 cm below the stopping screen in the bombardment chamber. At least five petals were bombarded for each of the four plasmid construct combinations along with the pSuproEEGFP transformation control. The bombardments were repeated multiple times.

Transient expression assay. Five to 7 d after particle bombardment, the entire area of bombardment was examined for enhanced green fluorescent protein (EGFP) and anthocyanin expression using a stereomicroscope (SMZ1500; Nikon Instruments, Mellville, NY) equipped with an epi-illumination intermediate tube (P-FLA fluorescence attachment; Nikon) and a fluorescence illuminator with a mercury arc lamp. An Endow EGFP 500 Long Pass filter set (Chroma Technology Corp., Rockingham, VT) was placed in the light path. The fluorescent images of the fields with clearly defined EGFP expression were captured at 1 s under 50× magnitude using a digital camera (DXM1200; Nikon) attached to the microscope. Anthocyanin accumulation was also viewed under visible light.

Anthocyanin and EGFP expression was quantitatively measured using WinCAM Pro 1 (Regent Instruments, Quebec, Canada). Color classes were selected to cover the range in the intensity of color resulting from anthocyanin and EGFP expression. The number of pixels of each color class was measured. Enhanced green fluorescent protein expression was used to standardize bombardments. Data were reported as the mean of the number of pixels of anthocyanin color divided by the number of pixels of EGFP color from images taken from five independent transformation events.

Results

Activation of de novo anthocyanin biosynthesis in white *Phalaenopsis amabilis* petals by transient expression of Myc_{Lc} and Myb_{C1}. To test whether the albescent phenotype of *P. amabilis* (Fig. 1A) could be complemented with regulatory gene expression, constructs S-Myc_{Lc} and S-Myb_{C1} were cointroduced into its white petals. Cells expressing Myc_{Lc} and Myb_{C1} were easily observed by the appearance of prominent reddish purple spots (Fig. 1B), characteristic of anthocyanin production (Griesbach and Klein, 1993). This result confirms that white *P. amabilis* can express anthocyanins if Myc_{Lc} and Myb_{C1} are present.

We further tested whether both Myc_{Lc} and Myb_{C1} were required to induce anthocyanin biosynthesis. *Phalaenopsis*

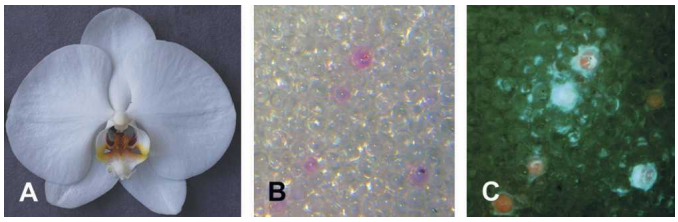

Fig. 1. (**A–C**) Transactivation of anthocyanin synthesis in white *Phalaenopsis amabilis* petals after particle bombardment-mediated cotransformation of the S-Myc_{Lc} and S-Myb_{C1} constructs. (**A**) White *P. amabilis* flowers. (**B**) Anthocyanin accumulation viewed under white light. (**C**) Anthocyanin and enhanced green fluorescent protein accumulation viewed under near-ultraviolet light.

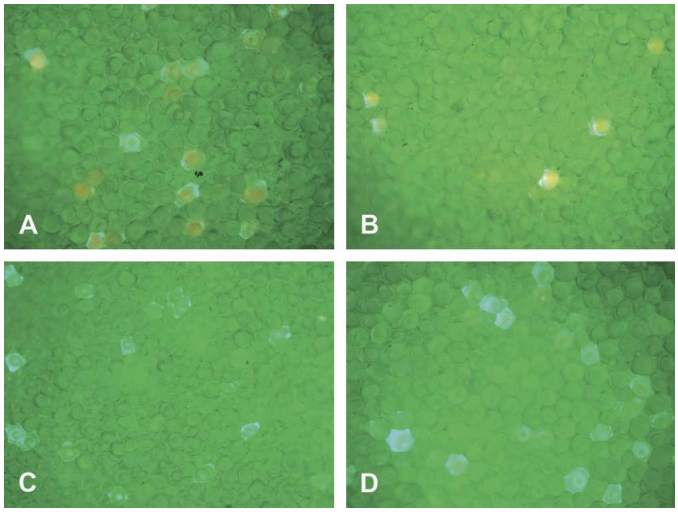

Fig. 3. (**A–D**) Different anthocyanin accumulation levels in white *Phalaenopsis amabilis* petals from transient expression of the two regulatory genes under the control of different promoters. (**A**) S-Myc_{Lc} + S-Myb_{C1}. (**B**) U-Myc_{Lc} + S-Myb_{C1}. (**C**) S-Myc_{Lc} + U-Myb_{C1}. (**D**) U-Myc_{Lc} + U-Myb_{C1}.

amabilis petals were bombarded singularly with either S-Myc_{Lc} or S-Myb_{C1}. No reddish purple spots were observed, which indicated that both regulatory genes are required to activate anthocyanin biosynthesis in white *P. amabilis* petals.

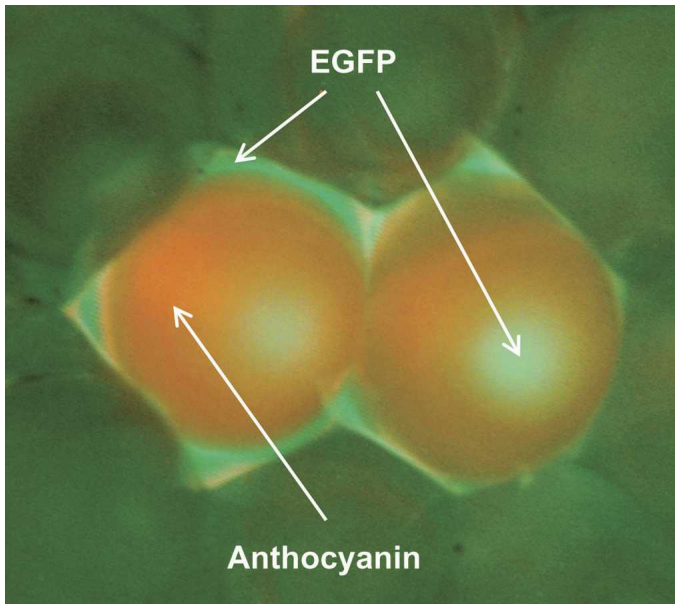

Fig. 2. Simultaneous detection of coexpression of enhanced green fluorescent protein (green, cytoplasm and nucleus) and anthocyanin (orange-red, vacuole) in the cells of white *Phalaenopsis amabilis* petals under near-ultraviolet light.

Transient coexpression of anthocyanin and enhanced green fluorescent protein. We noticed considerable variation in the number of transformed cells from one bombardment to another. It is well-known that not all transformation experiments will have the same efficiency because of variation in factors such as DNA coating (Sanford et al., 1993). Without an internal transformation control, it would not be possible to determine whether the difference in anthocyanin expression was the result of variations in transformation efficiency or actual differences in regulatory gene expression. To ensure a more accurate comparison, we selected EGFP as the internal control for the biolistic process. Under near-ultraviolet light, EGFP appears as green fluorescence in the cytoplasm, and anthocyanin appears as orange fluorescence within the vacuole (Fig. 2). By comparing anthocyanin expression with EGFP expression, differences in gene expressions that were the result of fluctuations in transformation efficiency were minimized.

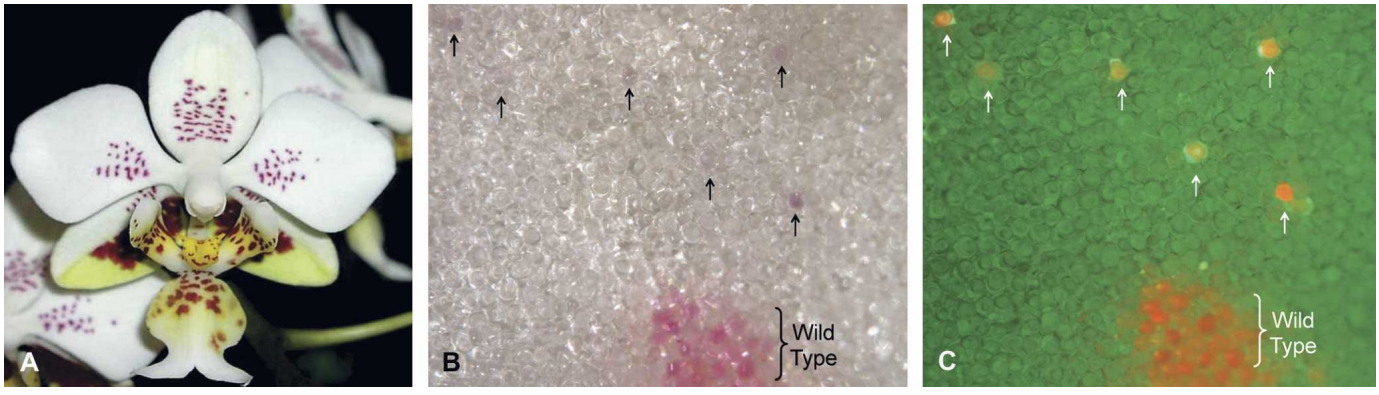

Fig. 4. (**A–C**) Activation of anthocyanin production in the unpigmented areas of *Phalaenopsis stuartiana* petals after introduction of S-Myc_{Lc} and S-Myb_{C1} constructs. (**A**) *Phalaenopsis stuartiana* flower. Induced anthocyanin expression (arrows) was viewed under white light (**B**) and near-ultraviolet light (**C**). A cluster of wild-type anthocyanin-expressing red cells were seen at the bottom of images B and C.

Table 1. Anthocyanin and green fluorescent protein expression in bombarded *Phalaenopsis pulcherima* petals using different regulatory gene constructs.

Genes	Expression [mean ± SE (%)]
S-*EGFP*	2.2 ± 1.0
U-*EGFP*	0.25 ± 0.17
S-Myc_{LC} + S-Myb_{C1}	40.30 ± 19.00 a[z]
S-Myc_{LC} + U-Myb_{C1}	0.03 ± 0.01 b
U-Myc_{LC} + S-Myb_{C1}	32.40 ± 25.00 a
U-Myc_{LC} + U-Myb_{C1}	0.08 ± 0.02 b

[z]Means followed by the same letter are not significantly different according to the Dunn test ($P \leq 0.05$).

Anthocyanin expression was measured by dividing the amount of enhanced green fluorescent protein (EGFP) by the amount of anthocyanin. Enhanced green fluorescent protein was used as an internal standard. The amount of EGFP and anthocyanin was measured using the WinCAM software (Regent Instruments Inc., Quebec, Canada). S- and U- indicate that the gene is under the control of the *35S* or *Ubi3* promoter respectively.

Because cotransformation frequencies are usually about 75% to 90% (Lee et al., 2002; Schocher et al., 1986; Yepes et al., 1995), we can assume that most of the cells expressing the EGFP gene should also express the anthocyanin regulatory genes. In *P. amabilis* petals that were cobombarded with the EGFP and anthocyanin regulatory genes, the number of cells showing both EGFP (green fluorescence in the cytoplasm) and anthocyanin (orange fluorescence in the vacuole) expression was very high (compare Fig. 1B with Fig. 1C). Therefore when comparing gene expression within the different constructs, we were able to compare the ratio of the number of cells expressing EGFP with the number of cells expressing anthocyanin.

Degree of pigmentation correlated with the expression level of *C1*. Both *35S* and *Ubi3* are constitutive promoters. Constitutive promoters often show different strengths in different tissues. In preliminary work, we examined the promoter strength of *35S* and *Ubi3* in *P. amabilis* petals using *EGFP* as the reporter gene. As expected, the *35S* promoter was stronger than the *Ubi3* promoter (Table 1). Because EGFP expression using the same promoter varied between bombardments, the SE was high.

It is reasonable to assume that different promoter strengths would produce different levels of the regulatory proteins and create different ratios of the two regulatory factors in transient expression. The constructs with high gene expression (S-Myc_{Lc} and S-Myb_{C1}) used the double *35S* promoter, whereas the constructs with low gene expression (U-Myc_{Lc} and U-Myb_{C1}) used the *Ubi3* promoter. Different combinations of the constructs resulted in Myc_{Lc}-to-Myb_{C1} ratios > 1 (S-Myc_{Lc} + U-Myb_{C1}), < 1 (U-Myc_{Lc} + S-Myb_{C1}), and equal to 1 (S-Myc_{Lc} + S-Myb_{C1} and U-Myc_{Lc} + U-Myb_{C1}). Together with the internal control SuproEGFP construct, these combinations were delivered into white *P. amabilis* petals.

Both S-Myc_{Lc} + S-Myb_{C1} (Fig. 3A) and U-Myc_{Lc} + S-Myb_{C1} (Fig. 3B) combinations produced high levels of anthocyanin pigmentation, whereas S-Myc_{Lc} + U-Myb_{C1} (Fig. 3C) and U-Myc_{Lc} + U-Myb_{C1} (Fig. 3D) combinations produced little anthocyanin despite efficient expression of cotransformed EGFP control. It appears that low levels of the Myb_{C1} protein (U-Myb_{C1}), even combined with high levels of Myc_{Lc} (S-Myc_{Lc}) resulted in little anthocyanin accumulation, whereas high levels of Myb_{C1} (S-Myb_{C1}), together with the Myc_{Lc} either at low (U-Myc_{Lc}) or high (S-Myc_{Lc}) levels, resulted in high anthocyanin production. At the high Myb_{C1} level, there was no statistically significant difference in anthocyanin production between the low and high Myc_{Lc} levels (Table 1). These data suggest that the amount of anthocyanin produced was positively affected by the Myb_{C1} expression level. When Myb_{C1} is under the control of the *Ubi3* promoter, it is possible that an insufficient amount of Myb_{C1} is produced to activate the entire anthocyanin pathway.

Pigmentation pattern. The other *Phalaenopsis* Blume species, *P. stuartiana,* displays a striking color pattern of white petals covered with mauve spots (Fig. 4A). To determine whether the unpigmented sections on the petals could express anthocyanin, we delivered constructs S-Myc_{Lc} and S-Myb_{C1} into the petals of *P. stuartiana*. Anthocyanin accumulation in the white tissue areas was easily observed (Fig. 4B, 4C). In addition, *P. stuartiana* responded to the S-Myc_{Lc} and S-Myb_{C1} ratio in the same manner as *P. amabilis* (data not shown).

Discussion

Enhanced green fluorescent protein as an ideal control reporter gene for anthocyanin transient expression. A system for standardizing and quantifying transient genetic complementation was developed using EGFP expression as an internal transformation control. Enhanced green fluorescent protein was selected as the control reporter for the following reasons: First, EGFP and anthocyanin are both nondestructive markers and, under near-ultraviolet light, can be visualized directly (Griesbach, 1990). Second, accumulation of EGFP and anthocyanin is spatially mutually exclusive, with EGFP expression in the cytoplasm and anthocyanin accumulation in the vacuole. Therefore, EGFP and anthocyanin can be detected simultaneously. Third, and last, EGFP expression and regulatory gene-induced accumulation of anthocyanin are cell autonomous (Bowen, 1992; Goodman et al., 2004; Ludwig et al., 1990), which makes it possible to quantify gene expression.

The role of *C1* in conditioning the level of pigmentation. Earlier studies have hypothesized that the role of Myc_{Lc} is to determine the temporal and spatial expression of the pigments (Ludwig et al., 1989). Our results indicate that the degree of anthocyanin pigmentation correlates with the promoter strength, and therefore presumably the level of Myb_{C1}, suggesting that Myb_{C1} plays a role in determining the level of pigmentation. Because the MYC protein has a short half-life (Brody, 1996), its level within the cell is dependent on its rate of transcription, or its promoter strength.

It has been demonstrated previously that Myb_{C1} physically interacts with the MYC-like transcription factor (Grotewold et al., 2000). Domain swap experiments (Hernandez et al., 2004; Sainz et al., 1997) suggested that the Myb_{C1} protein's DNA-binding domain exerts an inhibitory effect on its activation domain. Only when its MYC counterpart is present, does the inhibition become released and the anthocyanin pathway activated. Our results further suggest that Myb_{C1} and Myc_{Lc} are not required in equal amounts to effect transcription.

Endogenous anthocyanin regulatory gene expression. In contrast to the study of *Z. mays* suspension cells (deMajnik et al., 1998), our results with *Phalaenopsis* indicate that when Myc_{Lc} is at a lower level than that of Myb_{C1}, sufficient amounts of anthocyanin still could be produced, but not vice versa. This difference may suggest that the threshold requirement for the regulatory genes to activate anthocyanin production is

different in different plants. The threshold level is likely the result of either endogenous MYC production or stability.

The explanation for our results might lie in the nature of individual transcription factors. Gene expression profiling on Myc_R/Myb_{C1} transformants (Bruce et al., 2000) has shown upregulation as well as downregulation of cDNA transcripts corresponding to different genes. As expected, gene expression specific to the flavonoid pathway was increased. Those genes with expression that was inhibited included histone, tubulin, and ribosomal proteins. These genes are essential for basic cell function, which might explain the slow growth of transformed cells. The data suggest that Myc_R + Myb_{C1} acted in both activator and repressor capacities.

Gill and Ptashne (1988) described a phenomenon called squelching, which is caused by high-level expression of a transcription factor that subsequently inhibits transcription of certain genes lacking the specific binding site of the transcription factor. Cis-element studies of the anthocyanin structural genes have revealed several critical regions essential for anthocyanin activation (Elomaa et al., 2003; Lesnick and Chandler, 1998; Sainz et al., 1997). The MYB-like transcription factor (C1) binds directly to one of the sites, but there is no evidence so far to show that the MYC-like transcription factor binds to DNA.

We developed an in vivo functional assay system to monitor efficiently the activity of MYC- and MYB-like regulatory factors through anthocyanin production. We showed that low-level expression of the MYC-like regulatory factor can still achieve significant activation of anthocyanin when MYB-like regulatory factor is sufficiently expressed.

The failure to restore anthocyanin production with Myc_{Lc} and Myb_{C1} alone, and the success in inducing anthocyanin production with both genes together suggests that *P. amabilis* either does not have *Myc* and *Myb* anthocyanin regulatory genes or has nonfunctional anthocyanin regulators. Preliminary experiments to test this hypothesis, using reverse transcription PCR (RT-PCR) to compare the expression of two anthocyanin regulatory genes between *P. amabilis* (anthocyanin free) and an anthocyanin-producing species (*P. schilleriana)*, revealed that for *Myc*, not only is the expression level similar between *P. schilleriana* and *P. amabilis*, but the sequences of the *Myc* RT-PCR product derived from each of species was virtually identical (data not shown). On the other hand, RT-PCR produced multiple *Myb* products. The *Myb* that was expressed in *P. schilleriana* was not expressed in *P. amabilis*. A different *Myb* was expressed in *P. amabilis.* Further studies are underway to characterize *Myb* expression in both species to determine whether *Myb* is the key factor in anthocyanin production and its potential target gene(s). Knowledge gained in this study could benefit future studies aimed at creating viable plants with new flower or foliage color.

Literature Cited

Borevitz, J.O., Y. Xia, J. Blount, R.A. Dixon, and C. Lamb. 2000. Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. Plant Cell 12:2383–2394.

Bovy, A., R. de Vos, M. Kemper, E. Schijlen, M. Almenar Pertejo, S. Muir, G. Collins, S. Robinson, M. Verhoeyen, S. Hughes, C. Santos-Buelga, and A. van Tunen. 2002. High-flavonol tomatoes resulting from the heterologous expression of the maize transcription factor genes LC and C1. Plant Cell 14:2509–2526.

Bowen, B. 1992. Anthocyanin genes as visual markers in transformed maize tissues, p. 163–175. In: S. Gallagher (ed.). GUS protocols: Using the GUS gene as reporter of gene expression. Academic Press, London.

Bradley, J.M., K.M. Davies, S.C. Deroles, S.J. Bloor, and D.H. Lewis. 1998. The maize Lc regulatory gene up regulates the flavonoid biosynthetic pathway of petunia. Plant J. 13:381–392.

Brody, T.B. 1996. The interactive fly: A cyberspace guide to *Drosophila* development and metazoan evolution. 27 Apr. 2007. <http://www.sdbonline.org/fly/aimain/1aahome.htm>.

Bruce, W., O. Folkerts, C. Garnaat, O. Crasta, B. Roth, and B. Bowen. 2000. Expression profiling of the maize flavonoid pathway genes controlled by estradiol-inducible transcription factors CRC and P. Plant Cell 12:65–80.

Chandler, V.L., J.P. Radicella, T.P. Robbins, J. Chen, and D. Turks. 1989. Two regulatory genes of the maize anthocyanin pathway are homologous: Isolation of B-utilizing R genomic sequences. Plant Cell 1:1175–1183.

Cone, K.C., F.A. Burr, and B. Burr. 1986. Molecular analysis of the maize anthocyanin regulatory locus C1. Proc. Natl. Acad. Sci. USA 83:9631–9635.

Davies, K. 2000. Plant colour and fragrance, p. 127–163. In: R. Verpoortre and A. Alfermann (eds.). Metabolic engineering of plant secondary metabolism. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Dellaporta, S.L., I. Greenblatt, J. Kermicle, J. Hicks, and S. Wessler. 1988. Molecular cloning of the R-nj gene by transposon tagging with Ac, p. 263–282. In: J. Gustafson and R. Appels (eds.). Chromosome structure and function: Impact of new concepts. Plenum Press, New York.

deMajnik, J., G. Tanner, R. Joseph, P. Larkin, J. Weinman, M. Djordjevic, and B. Rolfe. 1998. Transient expression of maize anthocyanin regulatory genes influences anthocyanin production in white clover and peas. Austral. J. Plant Phys. 25:335–343.

Elomaa, P., M. Mehto, M. Kotilainen, Y. Helariutta, L. Nevalainen, and T.H. Teeri. 1998. A bHLH transcription factor mediates organ, region and flower type specific signals on dihydroflavonol-4-reductase (dfr) gene expression in the inflorescence of *Gerbera hybrida* (Asteraceae). Plant J. 16:93–99.

Elomaa, P., A. Uimari, M. Mehto, V.A. Albert, R.A.E. Laitinen, and T.H. Teeri. 2003. Activation of anthocyanin biosynthesis in *Gerbera hybrida* (Asteraceae) suggests conserved protein–protein and protein–promoter interactions between the anciently diverged monocots and eudicots. Plant Physiol. 133:1831–1842.

Garbarino, J. and W. Belknap. 1994. Isolation of a ubiquitin-ribosomal protein gene (ubi3) from potato and expression of its promoter in transgenic plants. Plant Mol. Biol. 24:119–127.

Gill, G. and M. Ptashne. 1988. Negative effect of the transcriptional activator GAL4. Nature 334:721–724.

Goldsbrough, A.P., Y. Tong, and J.I. Yoder. 1996. Lc as a non-destructive visual reporter and transposition excision marker gone for tomato. Plant J. 9:927–933.

Gong, Z.Z., E. Yamagishi, M. Yamazaki, and K. Saito. 1999. A constitutively expressed Myc-like gene involved in anthocyanin biosynthesis from *Perilla frutescens*: Molecular characterization, heterologous expression in transgenic plants, and transactivation in yeast cells. Plant Mol. Biol. 41:33–44.

Goodman, C.D., P. Casati, and V. Walbot. 2004. A multidrug resistance-associated protein involved in anthocyanin transport in *Zea mays*. Plant Cell 16:1812–1826.

Goodrich, J., R. Carpenter, and E. Coen. 1992. A common gene regulates pigmentation pattern in diverse plant species. Cell 68:995–964.

Griesbach, R. 1990. Flavonoid copigments and anthocyanin of *Phalaenopsis schilleriana*. Lindleyana 5:231–234.

Griesbach, R. 2005. Biochemistry and genetics of flower color. Plant Breed. Rev. 25:89–114.

Griesbach, R. and T. Klein. 1993. In situ genetic complementation of a flower color mutant in *Doritis pulcherrima* (Orchidaceae). Lindleyana 8:223–226.

Grotewold, E., M.B. Sainz, L. Tagliani, J.M. Hernandez, B. Bowen, and V.L. Chandler. 2000. Identification of the residues in the Myb

domain of maize C1 that specify the interaction with the bHLH cofactor R. Proc. Natl. Acad. Sci. USA 97:13579–13584.
Hanson, M.A., B.S. Gaut, A.O. Stec, S.I. Fuerstenberg, M.M. Goodman, E.H. Coe, and J.F. Doebley. 1996. Evolution of anthocyanin biosynthesis in maize kernels: The role of regulatory and enzymatic loci. Genetics 143:1395–1407.
Hernandez, J.M., G.F. Heine, N.G. Irani, A. Feller, M.-G. Kim, T. Matulnik, V.L. Chandler, and E. Grotewold. 2004. Different mechanisms participate in the R-dependent activity of the R2R3 MYB transcription factor C1. J. Biol. Chem. 279:48205–48213.
Irani, N., J.M. Hernandez, and E. Grotewold. 2003. Regulation of anthocyanin pigmentation. Recent Adv. Phytochem. 38:59–78.
Koes, R., W. Verweij, and F. Quattrocchio. 2005. Flavonoids: A colorful model for the regulation and evolution of biochemical pathways. Trends Plant Sci. 10:236–242.
Lee, M.H., M.K. Min, Y.J. Lee, J.B. Jin, D.H. Shin, D.H. Kim, K.-H. Lee, and I. Hwang. 2002. ADP-ribosylation factor 1 of *Arabidopsis* plays a critical role in intracellular trafficking and maintenance of endoplasmic reticulum morphology in *Arabidopsis*. Plant Physiol. 129:1507–1520.
Lesnick, M.L. and V.L. Chandler. 1998. Activation of the maize anthocyanin gene a2 is mediated by an element conserved in many anthocyanin promoters. Plant Physiol. 117:437–445.
Lloyd, A.M., V. Walbot, and R.W. Davis. 1992. *Arabidopsis* and *Nicotiana* anthocyanin production activated by maize regulators R and C1. Science 258:1773–1775.
Ludwig, S.R., B. Bowen, L. Beach, and S.R. Wessler. 1990. A regulatory gene as a novel visible marker for maize transformation. Science 247:449–450.
Ludwig, S.R., L.F. Habera, S.L. Dellaporta, and S.R. Wessler. 1989. Lc, a member of the maize R gene family responsible for tissue-specific anthocyanin production, encodes a protein similar to transcriptional activators and contains the myc-homology region. Proc. Natl. Acad. Sci. USA 86:7092–7096.
Mathews, H., S.K. Clendennen, C.G. Caldwell, X.L. Liu, K. Connors, N. Matheis, D.K. Schuster, D.J. Menasco, W. Wagoner, J. Lightner, and D.R. Wagner. 2003. Activation tagging in tomato identifies a transcriptional regulator of anthocyanin biosynthesis, modification, and transport. Plant Cell 15:1689–1703.
Nesi, N., I. Debeaujon, C. Jond, G. Pelletier, M. Caboche, and L. Lepiniec. 2000. The TT8 gene encodes a basic helix–loop–helix domain protein required for expression of DFR and BAN genes in arabidopsis siliques. Plant Cell 12:1863–1878.
Ni, M., D. Cui, J. Einstein, S. Narasimhulu, C.E. Vergara, and S.B. Gelvin. 1995. Strength and tissue specificity of chimeric promoters derived from the octopine and mannopine synthase genes. Plant J. 7:661–676.
Paz-Ares, J., U. Ghosal, U. Wienand, P. Peterson, and H. Saedler. 1987. The regulatory c1 locus of *Zea mays* encodes a protein with homology to myb prto-oncogene products and with structural similarities to transcriptional activators. EMBO J. 6:3553–3558.
Paz-Ares, J., U. Wienand, P. Peterson, and H. Saedler. 1986. Molecular cloning of the c locus of *Zea mays*: A locus regulating the anthocyanin pathway. EMBO J. 5:829–833.
Perrot, G. and K.C. Cone. 1989. Nucleotide sequence of the maize R-S gene. Nucl. Acids Res. 17:7092–7096.
Quattrocchio, F., W. Verweij, A. Kroon, C. Spelt, J. Mol, and R. Koes. 2006. PH4 of petunia is an R2R3 MYB protein that activates vacuolar acidification through interactions with basic-helix–loop–helix transcription factors of the anthocyanin pathway. Plant Cell 18:1274–1291.
Quattrocchio, F., J.F. Wing, H.T.C. Leppen, J.N.M. Mol, and R.E. Koes. 1993. Regulatory genes controlling anthocyanin pigmentation are functionally conserved among plant species and have distinct sets of target genes. Plant Cell 5:1497–1512.
Quattrocchio, F., J. Wing, K. van der Woude, E. Souer, N. de Vetten, J. Mol, and R. Koes. 1999. Molecular analysis of the anthocyanin2 gene of petunia and its role in the evolution of flower color. Plant Cell 11:1433–1444.
Quattrocchio, F., J.F. Wing, K. van der Woude, J.N.M. Mol, and R. Koes. 1998. Analysis of bHLH and MYB domain proteins: Species specific regulatory differences are caused by divergent evolution of target anthocyanin genes. Plant J. 13:475–488.
Radicella, J.P., D. Turks, and V.L. Chandler. 1991. Cloning and nucleotide sequence of a cDNA encoding B-Peru, a regulatory protein of the anthocyanin pathway from maize. Plant Mol. Biol. 17:127–130.
Sainz, M.B., E. Grotewold, and V.L. Chandler. 1997. Evidence for direct activation of an anthocyanin promoter by the maize C1 protein and comparison of DNA binding by related Myb domain proteins. Plant Cell 9:611–625.
Sanford, J., F. Smith, and J. Russell. 1993. Optimizing the biolistic process for different biological applications. Methods Enzymol. 217:483–509.
Schocher, R.J., R.D. Shillito, M.W. Saul, J. Paszkowski, and I. Potrykus. 1986. Co-transformation of unlinked foreign genes into plants by direct gene transfer. Biotechnology (N.Y.) 4:1093–1096.
Schwinn, K., V. Alm, S. Mackay, K. Davies, and C. Martin. 2001. Regulation of anthocyanin biosynthesis in *Antirrhinum*. Acta Hort. 560:201–206.
Spelt, C., F. Quattrocchio, J.N.M. Mol, and R. Koes. 2000. Anthocyanin1 of petunia encodes a basic helix–loop–helix protein that directly activates transcription of structural anthocyanin genes. Plant Cell 12:1619–1632.
Tonelli, C., G. Consonni, S. Dolfini, S.L. Dellaporta, A. Viotti, and G. Gavazzi. 1991. Genetic and molecular analysis of Sn, a light-inducible, tissue specific regulatory gene in maize. Mol. Gen. Genet. 225:401–410.
Winkel-Shirley, B. 2001. Flavonoid biosynthesis: A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol. 126:485–493.
Yepes, L.M., V. Mittak, S.Z. Pang, C. Gonzalves, J. Slightom, and D. Gonsalves. 1995. Biolistic transformation of chrysanthemum with the nucleocapsid gene of tomato spotted wilt virus. Plant Cell Rpt. 14:694–698.
Zhang, P., S. Chopra, and T. Peterson. 2000. A segmental gene duplication generated differentially expressed myb-homologous genes in maize. Plant Cell 12:2311–2322.