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 fluorescent 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 *Ubi3* 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' **CCAAGCTTC** CAAAGCACATACTTAT3'; reverse 5' **GGATCCTTCGCC** TGGAGGAGAG-3'

Ocs-Mas super promoter primer sequence: forward 5' **AAGCTTGGATCCCTGAAAGCGA** -CG3'; reverse 5' **CCGGTACCTAGAGTCGATTGG3'**

Myc_{LC} primer sequence: forward 5' **GGATCCATCGAGTT** GTTGACTCTTCGC3'; reverse 5' **GGTACCTCTAGAATG** CTATGACTTTG3'

rbcS terminator primer sequence: forward 5' **GGTACCGC** TTTTCGTTTCGTATCATCGG-3'; reverse 5' **GAATTCGGAT** 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_{CI}* and U-*Myb_{CI}* 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_{CI}* cDNA (Lloyd et al., 1992). The terminator for all four constructs was the ribulose biphosphate 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_{CI}* constructs were coprecipitated (Griesbach and Klein, 1993) onto 0.5 mg of 1.0- μ m-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 pSuproEGFP 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, Melville, 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 $\times$ magnification 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_{CI}. 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_{CI}* were cotransformed into its white petals. Cells expressing *Myc_{LC}* and *Myb_{CI}* 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_{CI}* are present.

We further tested whether both *Myc_{LC}* and *Myb_{CI}* 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_{CI}* 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_{CI}*. (B) U-*Myc_{LC}* + S-*Myb_{CI}*. (C) S-*Myc_{LC}* + U-*Myb_{CI}*. (D) U-*Myc_{LC}* + U-*Myb_{CI}*.

amabilis petals were bombarded singularly with either S-*Myc_{LC}* or S-*Myb_{CI}*. 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_{CI}* 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 $\pm$ SE (%)]
S-EGFP	2.2 $\pm$ 1.0
U-EGFP	0.25 $\pm$ 0.17
S- <i>Myc_{LC}</i> + S- <i>Myb_{CI}</i>	40.30 $\pm$ 19.00 a ^z
S- <i>Myc_{LC}</i> + U- <i>Myb_{CI}</i>	0.03 $\pm$ 0.01 b
U- <i>Myc_{LC}</i> + S- <i>Myb_{CI}</i>	32.40 $\pm$ 25.00 a
U- <i>Myc_{LC}</i> + U- <i>Myb_{CI}</i>	0.08 $\pm$ 0.02 b

^zMeans 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 *CI*. 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_{CI}*) used the double 35S promoter, whereas the constructs with low gene expression (U-*Myc_{LC}* and U-*Myb_{CI}*) used the *Ubi3* promoter. Different combinations of the constructs resulted in *Myc_{LC}*-to-*Myb_{CI}* ratios > 1 (S-*Myc_{LC}* + U-*Myb_{CI}*), < 1 (U-*Myc_{LC}* + S-*Myb_{CI}*), and equal to 1 (S-*Myc_{LC}* + S-*Myb_{CI}* and U-*Myc_{LC}* + U-*Myb_{CI}*). Together with the internal control SuproEGFP construct, these combinations were delivered into white *P. amabilis* petals.

Both S-*Myc_{LC}* + S-*Myb_{CI}* (Fig. 3A) and U-*Myc_{LC}* + S-*Myb_{CI}* (Fig. 3B) combinations produced high levels of anthocyanin pigmentation, whereas S-*Myc_{LC}* + U-*Myb_{CI}* (Fig. 3C) and U-*Myc_{LC}* + U-*Myb_{CI}* (Fig. 3D) combinations produced little anthocyanin despite efficient expression of cotransformed EGFP control. It appears that low levels of the *Myb_{CI}* protein (U-*Myb_{CI}*), even combined with high levels of *Myc_{LC}* (S-*Myc_{LC}*) resulted in little anthocyanin accumulation, whereas high levels of *Myb_{CI}* (S-*Myb_{CI}*), 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_{CI}* 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_{CI}* expression level. When *Myb_{CI}* is under the control of the *Ubi3* promoter, it is possible that an insufficient amount of *Myb_{CI}* 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_{CI}* 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_{CI}* 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 *CI* 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_{CI}*, suggesting that *Myb_{CI}* 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_{CI}* 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_{CI}* 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_{CI}* 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_{CI}*, 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_{CI}* 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_{CI}* 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_{CI}* 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.

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