Two R2R3-MYB Genes, Homologs of Petunia AN2, Regulate Anthocyanin Biosyntheses in Flower Tepals, Tepal Spots and Leaves of Asiatic Hybrid Lily

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Anthocyanins are secondary metabolites that contribute to colors of flowers, fruits and leaves. Asiatic hybrid lily (Lilium spp.) accumulates cyanidin anthocyanins in flower tepals, tepal spots and leaves of juvenile shoots. To clarify their mechanisms of regulation of anthocyanin pigmentation, two full-length cDNAs of R2R3-MYB (LhMYB6 and LhMYB12) were isolated from the anthocyanin-accumulating tepals of cultivar ‘Montreux’. Analysis of the deduced amino acid sequences indicated they have homology with petunia AN2, homologous sequences of which had not been isolated in species of monocots. Yeast two-hybrid analysis showed that LhMYB6 and LhMYB12 interacted with the Lilium hybrid basic helix–loop–helix 2 (LhbHLH2) protein. Transient expression analysis indicated that co-expression of LhMYB6 and LhbHLH2 or LhMYB12 and LhbHLH2, introduced by a microprojectile, activated the transcription of anthocyanin biosynthesis genes in lily bulb scales. Spatial and temporal transcription of LhMYB6 and LhMYB12 was analyzed. The expression of LhMYB12 corresponded well with anthocyanin pigmentation in tepals, filaments and styles, and that of LhMYB6 correlated with anthocyanin spots in tepals and light-induced pigmentation in leaves. These results indicate that LhMYB6 and LhMYB12 positively regulate anthocyanin biosynthesis and determine organ- and tissue-specific accumulation of anthocyanin.

Keywords: Anthocyanin pigmentation || basic helix–loop–helix (bHLH) || Cyanidin 3-O-β-rutinoside || Lilium spp. || Tepal spots || Transcription factor.

Abbreviations: 3-AT, 3-amino-1,2,4-triazole; bHLH, basic helix–loop–helix; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; ORF, open reading frame; RACE, rapid amplification of cDNA ends; RT–PCR, reverse transcription–PCR.

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Introduction

The genus Lilium comprises >90 species (Asano 1989) and is classified into sections (Comber 1949, Smyth et al. 1989). Most species of section Sinomartagon, such as L. dauricum, L. maculatum, L. concolor, L. leichtlinii, L. davidii and L. cernuum, are distributed in East Asia. The Asiatic hybrid lily, one of the most popular ornamental plants world-wide, is derived from interspecific crosses of species of section Sinomartagon (Leslie 1982). Asiatic hybrid lily cultivars have a large variation in tepal colors: yellow, orange, red, pink and white. Carotenoids accumulate in yellow and orange tepals; yellow carotenoids such as antheraxanthin, violaxanthin and lutein are in yellow tepals (Yamagishi et al. 2010), and capsanthin is the major pigment in orange tepals (Deli et al. 1998). Pigments accumulated in pink tepals are anthocyanins (Nørbaek and Kondo 1999, Abe et al. 2002). Accumulation of both anthocyanins and capsanthin pigments the tepals red (Yamagishi et al. 2010). Asiatic hybrid lily cultivars often have dark red spots on the interior surfaces of their tepals (Fig. 1). Pigments accumulated in tepal spots are anthocyanins (Abe et al. 2002).

Anthocyanins are found widely in plant species and are responsible for the purple, blue and pink coloration of plant parts. Anthocyanins provide color to flowers and fruits needed to attract pollinators and seed-dispersing animals (Winkelman et al. 2001, Schaefer et al. 2004, Grotewold 2006). Anthocyanins help to protect plants from various stresses, such as strong sunlight and active oxygen species (Nagata et al. 2003, Gould 2004). There are three basic groups of anthocyanin pigments in higher plants: derivatives of pelargonidin, of cyanidin and of delphinidin (Schwinn and Davies 2004). Lilium species contain cyanidin 3-O-β-rutinoside as a major anthocyanin and cyanidin 3-O-β-rutinoside-7-O-β-glucoside as a minor anthocyanin (Nørbaek and Kondo 1999). Asiatic hybrid lily cultivar ‘Montreux’, which was mainly used in this study, contains only cyanidin 3-O-β-rutinoside in tepals and tepal spots (Abe et al. 2002).
Inheritance of tepal anthocyanin and anthocyanin spots was analyzed in Asiatic hybrid lily using F₁ plants derived from a cross between cultivars ‘Montreux’ (pink tepals with spots) and ‘Connecticut King’ (yellow (no anthocyanin) tepals without spots) (Abe et al. 2002). F₁ plants with or without tepal anthocyanin segregated in a ratio of 1:1, indicating that a single major gene controls this trait, and the number of anthocyanin spots showed continuous distribution in the F₁ population, indicating that this trait is a quantitative trait. After trait loci mapping, the loci of tepal anthocyanin and of anthocyanin spots were mapped on the different linkage groups of Asiatic hybrid lily (Abe et al. 2002). These results indicate that these pigmentations are controlled independently even though tepal anthocyanin and anthocyanin spots consist of the same pigment. Because yellow tepal cultivar ‘Connecticut King’ has the ability to biosynthesize anthocyanin in organs other than tepals, such as in anthers and light-exposed bulkscales, and can express anthocyanin biosynthesis genes *chalcone synthase* (*CHS*) and *dihydroflavonol 4-reductase* (*DFR*), transcription factors may mainly regulate tepal anthocyanin and anthocyanin spots in Asiatic hybrid lily (Nakatsuka et al. 2003).

The biochemistry and enzymology of the anthocyanin biosynthesis pathway are well understood (Winkel-Shirley 2001, Schwinn and Davies 2004). The activity of anthocyanin biosynthesis enzymes is mainly controlled at the transcriptional level and is regulated by interactions between R2R3-MYB and basic helix–loop–helix (bHLH) transcription factors (Mol et al. 1998, Schwinn and Davies 2004, Koes et al. 2005), such as between *ZmC1* (R2R3-MYB) and *ZmR* (bHLH) in maize kernels (Dooner et al. 1991), *GhMYB10* (R2R3-MYB) and *GhMYC1* (bHLH) in *Gerbera hybrida* flowers (Elomaa et al. 1998, Elomaa et al. 2003), and *PhAN2* (R2R3-MYB) and *PhAN1* (bHLH) in petunia flowers (Spelt et al. 2000). To determine whether R2R3-MYB and bHLH proteins regulate anthocyanin biosynthesis in Asiatic hybrid lily, we isolated and characterized bHLH genes in a previous study (Nakatsuka et al. 2009) and R2R3-MYB genes in this study.

In Asiatic hybrid lily, two bHLH genes, *LhbHLH1* and *LhbHLH2*, are expressed in some organs, including tepals; *LhbHLH1* is a *PhJAF13* homolog and *LhbHLH2* is a *PhAN1* homolog. Petunia JAF13 is a second bHLH protein that interacts with PhAN2, but is unlikely to participate directly in regulating the transcription of anthocyanin biosynthesis genes because *PhJAF13* does not complement petunia *an1* mutants (Quattrocchio et al. 1998, Spelt et al. 2000). In Asiatic hybrid lily, *LhbHLH2* is predominantly correlated to anthocyanin biosynthesis because of the transcriptional profile in organs accumulating anthocyanin (Nakatsuka et al. 2009). However, *LhbHLH2* is not responsible for the tepal color difference among the cultivars because the transcription pattern of *LhbHLH2* in ‘Montreux’ and ‘Connecticut King’ tepals is nearly the same (Nakatsuka et al. 2009).

R2R3-MYB transcription factors that regulate anthocyanin pigmentation are often divided into two subgroups due to their sequence homology: one subgroup includes PhAN2 in petunia and PAP1 in Arabidopsis (hereinafter referred to as the AN2 subgroup), and another subgroup includes ZmC1 and ZmPl in maize (hereinafter referred to as the C1 subgroup) (Stracke et al. 2001, Allan et al. 2008). The AN2 and C1 subgroups are respectively categorized as subgroups 6 and 5 in the Arabidopsis R2R3-MYB gene family (Kranz et al. 1998, Stracke et al. 2001) and as subgroups N09 and N08 in the R2R3-MYB

![Fig. 1 Tepal appearances of Asiatic hybrid lily cultivar ‘Montreux’ at tepal developmental stages 1–5. Both the abaxial (left) and adaxial (right) sides of the inner tepals at each developmental stage are shown. Note that spots became visible at stage 2 in the basal part of the adaxial side, and tepal pigmentation began at stage 3. Numerals indicate tepal developmental stages. Bar = 1 cm](image-url)
gene family of Arabidopsis and rice (Jiang et al. 2004). Most R2R3-MYB genes that regulate anthocyanin biosynthesis in flowers and fruits of eudicot species are in the AN2 subgroup, and R2R3-MYB genes in monocots that regulate anthocyanin pigmentation in leaves and kernels of Poaceae (Gramineae) species and flowers in orchid Oncidium (OgMYB1, Chiou and Yeh 2008) all belong to the C1 subgroup (Allan et al. 2008). To the best of our knowledge, the An2 subgroup R2R3-MYB genes from kernels and flowers of monocot species have not been isolated previously.

In flowers and ornamental plants, flower color is an important consideration in consumer choice. There has been much interest in cultivars bearing flowers with altered color, hues and patterns. Thus, understanding the genetic basis that changes colors, hues and patterns should aid in breeding new cultivars. In this study, two cDNA clones encoding transcription factors are responsible for color differences among the flowers and ornamental plants. In flowers and ornamental plants, flower color is an important consideration in consumer choice. There has been much interest in cultivars bearing flowers with altered color, hues and patterns. Thus, understanding the genetic basis that changes colors, hues and patterns should aid in breeding new cultivars. In this study, two cDNA clones encoding R2R3-MYB transcriptional factors were isolated from pink tepals of Asiatic hybrid lily, and their biochemical properties and spatial and temporal expression were investigated to determine the regulatory system of anthocyanin biosynthesis and which transcription factors are responsible for color differences among the cultivars.

**Results**

Cloning R2R3-MYB transcription factors from lily tepals

A PCR-based method was used to isolate R2R3-MYB transcription factor genes from tepals of Asiatic hybrid lily cultivar ‘Montreux’. We originally designed degenerated primers (dMYBf and dMYBr) based on the conserved amino acid sequences in the C1 and AN2 subgroup R2R3-MYB genes, and amplified eight groups of 128bp sequences showing homology with R2R3-MYB (data not shown). Because two of the eight groups showed homology with the PhAN2 sequence, their full-length cDNA sequences were determined by rapid amplification of cDNA ends (RACE)-PCR. The sequence homologous to the ZmC1 gene was not included in the eight groups.

The two full-length cDNA sequences, LhMYB6 and LhMYB12, encoded 276 and 246 amino acids, respectively. An amino acid alignment of 26 R2R3-MYB sequences in lily and other species (Supplementary Fig. S1) showed that the region of R2R3 repeats was highly conserved, but downstream of this region was divergent in both sequence and length. All AN2 and C1 subgroup sequences and lily sequences contained the motif [D/E]x[K/R]x[Lx]x[Lx]x[R] in the R3 repeat necessary for interactions with R-like bHLH proteins (Zimmermann et al. 2004, Takos et al. 2006). In the variable region, the small motif [K/R]P[Q/R]P[Q/R] was conserved in AN2 subgroup R2R3-MYB and in LhMYB6 and LhMYB12 sequences. This motif was part of the motif previously reported as KPRPR[S/F]F (motif 6, Stracke et al. 2001) and [K/R]P[K/T][F/Y] (Takos et al. 2006). The short conserved sequence KAx[K/R]Cx[S/T] was recognized in the C1 subgroup, but this sequence did not appear in LhMYB6 and LhMYB12 sequences. Incomplete direct repeats were in the variable region of the LhMYB6 sequence, and so LhMYB6 had two [K/R][P[Q/R]P[Q/R]] motifs (Supplementary Fig. S1). A phylogenetic tree, drawn by using full-length amino acid sequences (Fig. 2), showed that LhMYB6 and LhMYB12 formed a cluster with sequences of the AN2 subgroup. Other monocot sequences of R2R3-MYB regulating anthocyanin pigmentation, such as ZmPI and ZmC1 in maize and OgMYB1 in orchid, were all included in the cluster of the C1 subgroup. When only amino acid sequences at R2R3 repeats were used to construct...
a phylogenetic tree, a similar result was obtained, i.e. the AN2 subgroup sequences and the lily sequences formed one cluster, and the CI subgroup sequences formed another cluster (data not shown).

Protein–protein interaction of LhMYB6 and LhMYB12 with LhbHLH2

Transcription of anthocyanin biosynthesis genes is mainly regulated by interactions between R2R3-MYB and bHLH transcription factors (Schwinn and Davies 2004, Koes et al. 2005). Both LhMYB6 and LhMYB12 contained a motif necessary for interactions with R-like bHLH proteins (Supplementary Fig. S1). Thus, whether LhbHLH2 protein (Nakatsuka et al. 2009) interacts with LhMYB6 or LhMYB12 protein was investigated using a GAL4-based yeast two-hybrid system. The GAL4 DNA-binding domain-fused LhbHLH2 and activation domain-fused LhMYB6 or LhMYB12 were introduced into yeast (Fig. 3). Only yeast harboring the combination of LhMYB6 and LhbHLH2 and of LhMYB12 and LhbHLH2 survived on a quadruple drop-out medium. When 3-amino-1,2,4-triazole (3-AT), which is a competitive inhibitor of His3 protein, was added to the medium, the growth of yeast harboring LhMYB12 and LhbHLH2 was lower than that of yeast harboring LhMYB6 and LhbHLH2. Therefore, LhMYB6 and LhbHLH2, and LhMYB12 and LhbHLH2, would form a heterodimer by protein–protein interaction, and the interaction between the former two proteins is stronger than that between the latter two proteins.

Ability of LhMYB6 and LhMYB12 to activate transcription of anthocyanin biosynthesis genes determined by transient expression assay

Because LhMYB6 and LhMYB12 showed homology with the AN2 subgroup R2R3-MYB proteins, whether expression activation of LhMYB6 and LhMYB12 could stimulate transcription of anthocyanin biosynthesis genes was investigated by a transient expression assay. Constructs of 35S-driven LhMYB6 or LhMYB12 were bombarded into white bulb scales of ‘Montreux’ together with or without a 35S-driven LhbHLH2 construct. At 48 h after the bombardment, transcription of LhDFR, LhCHSa, and LhCHSb was detected by reverse transcription–PCR (RT–PCR) in the bombarded organ where the expression of LhbHLH2 and LhMYB6, and LhbHLH2 and LhMYB12, appeared together (Fig. 4). The bombardment of a 35S-driven LhbHLH2 construct only did not activate transcription of LhDFR, LhCHSa, and LhCHSb (data not shown). These results indicate that LhMYB6 and LhMYB12 activate the transcription of anthocyanin biosynthesis genes and that LhbHLH2 protein is necessary for this activation.
Temporal and spatial transcription of LhMYB6 and LhMYB12

To clarify which transcripts of LhMYB6 or LhMYB12 accumulated in anthocyanin-pigmented organs, their temporal and spatial transcription was evaluated by quantitative RT–PCR. The temporal transcription of LhMYB6 and LhMYB12 during tepal development of ‘Montreux’ (basal and upper parts of tepals) and ‘Connecticut King’ (basal part of tepals) was examined. Flowers were divided into five developmental stages based mainly on tepal pigmentation (Fig. 1): stage 1 (bud length of about 3 cm), no pigmentation; stage 2 (about 4 cm), spots visible on the basal part; stage 3 (about 6 cm), tepal pigmentation began; stage 4 (1 d before anthesis, about 7 cm), the content of tepal anthocyanin was highest; and stage 5 (about 8 cm), flowers opened. Nakatsuka et al. (2003) showed temporal changes of anthocyanin contents in tepals. Midribs of tepals showed anthocyanin pigmentation on the abaxial side at all stages, and so midribs were not included in this study when gene expression was analyzed. No anthocyanin pigment was in ‘Connecticut King’ tepals (data not shown), the developmental stages of which were judged based on tepal length.

In LhMYB6, the transcriptional profile of ‘Montreux’ tepals differed between the basal and the upper parts. The transcription was higher at stages 1, 2 and 5 than at stages 3 and 4 in the basal part, and the transcriptional change was low during all tepal stages in the upper part (Fig. 5A). In ‘Connecticut King’ tepals, transcription of LhMYB6 was low and did not change during tepal development (Fig. 5A).

The transcript of LhMYB12 accumulated at tepal stages 3, 4 and 5 and peaked at stage 4 in ‘Montreux’ (Fig. 5B). This transcription pattern correlated well with anthocyanin accumulation in tepals, which began at stage 3 and peaked at stage 4 (Fig. 1) (Nakatsuka et al. 2003). The transcriptional level was higher in the basal part than in the upper part. Nakatsuka et al. (2003) showed that the anthocyanin concentration is also higher in the basal part than in the upper part. No transcription of LhMYB12 was detected in all tepal stages of ‘Connecticut King’. These results indicate that LhMYB12 regulates biosynthesis of tepal anthocyanin.

Fig. 5 Temporal and spatial expression of LhMYB6 (A and D), LhMYB12 (B and E) and LhDFR (C and F). (A–C) Relative expression in flower tepals (developmental stages 1–5) of Asiatic hybrid lily cultivars ‘Montreux’ (basal and upper parts) and ‘Connecticut King’ (basal part). Transcription of LhMYB12 and LhDFR was not detected in tepals of ‘Connecticut King’. (D–F) Relative expression in five flower organs collected from stage 4 flowers and in leaves of ‘Montreux’. LhActin was used to normalize the expression of these genes. Vertical bars indicate the standard error (n = 3).
The transcription of *LhDFR* was investigated (Fig. 5C). In ‘Montreux’, a high level of transcription was detected in tepal stages 3, 4 and 5 in the basal and the upper parts. Tepal stage 1 showed very low amounts and stage 2 showed low amounts of transcripts in the basal part, but no transcription was detected in stages 1 and 2 in the upper part. Transcripts of *LhDFR* were not detected in ‘Connecticut King’ at any developmental stage.

At stage 2, a spot pigment was visible in ‘Montreux’, and transcription of *LhDFR* was detected only in the basal part where spots were present, i.e. the *LhDFR* expression at stage 2 correlated with anthocyanin biosynthesis in the spots. *LhMYB12* transcription was not detected at stage 2, indicating that *LhMYB12* does not contribute to *LhDFR* expression at this stage and to spot pigmentation. Instead, *LhMYB6* transcription was high at stages 1 and 2 only in the basal part, suggesting that *LhMYB6* stimulates *LhDFR* expression and regulates spot pigmentation.

Quantitative RT–PCR was done to clarify the spatial expression pattern of *LhMYB6* and *LhMYB12* genes in various organs of ‘Montreux’. Flower organs were collected from stage 4 flowers. Anthocyanin pigment was in the anthers, filaments, styles and tepals. *LhMYB6* transcription was detected in all organs and was relatively high in ovaries (Fig. 5D) that showed no anthocyanin pigmentation. The *LhMYB12* transcript accumulated in tepals, filaments and styles (Fig. 5E). Transcription of *LhMYB12* was not detected in anthers, but anthocyanin pigment accumulated in them. *LhDFR* transcription was detected in tepals, filaments and styles, but not in anthers, ovaries and leaves (Fig. 5F).

**Transcription of *LhMYB6* and *LhMYB12* in F1 plants**

The temporal transcriptional profile in tepals suggests that *LhMYB6* regulates spot pigmentation (Fig. 5A). To confirm this role of *LhMYB6*, transcription of *LhMYB6* was analyzed using F1 plants derived from the cross between ‘Montreux’ (pink tepals with spots) and ‘Connecticut King’ (yellow tepals without spots). Because tepal anthocyanin and tepal spots are inherited independently (Abe et al. 2002), pink tepal individuals without tepal spots and yellow tepal individuals with spots segregated in F1 plants. Stage 2 tepals were used for this analysis. Real-time RT–PCR showed that *LhMYB6* transcription in F1 plants with spots was higher than in F1 plants without spots (*t* = 7.87, *P* < 0.01%) (Fig. 6), indicating that *LhMYB6* regulates spot pigmentation. *LhMYB12* transcription was not detected in these F1 plants at this stage (data not shown), indicating that *LhMYB12* does not contribute to spot pigmentation.

To confirm the role of *LhMYB12* in regulating tepal pigmentation, its transcription in tepals of F1 plants was analyzed at tepal stage 5. F1 plants with yellow or white tepals (no anthocyanin) showed no transcription, but F1 plants with pink tepals (due to anthocyanin) showed *LhMYB12* transcription (Fig. 7), indicating that *LhMYB12* regulates tepal pigmentation.

**Effect of light on *LhMYB6* transcription in leaves**

Because juvenile lily shoots often accumulate anthocyanin at sprouting, we previously analyzed the effect of light on anthocyanin accumulation and transcription of *LhbHLH1* and *LhbHLH2* in juvenile leaves (Nakatsuka et al. 2009). When Asiatic hybrid lily cultivar ‘Vivaldi’ was grown in the dark, the anthocyanin content was very low in leaves, but the anthocyanin content rapidly increased during 2 d of light exposure and peaked at 4 d of light exposure. At 6 and 8 d of light treatment, the amount of anthocyanin pigment declined and was nearly the same level as that in the dark [Nakatsuka et al. (2009) showed temporal changes of anthocyanin content in leaves]. In this study, transcription of *LhMYB6*, *LhMYB12* and *LhDFR* was analyzed using the same leaf materials as in the previous study (Fig. 8). The expression of *LhMYB6* was not detected in the dark (0 d), but it increased rapidly during 2 d of light exposure. Then the transcription declined rapidly and was low at 6 and 8 d of light treatment. A similar transcription profile was detected for *LhDFR*, i.e. its transcription was not detected in the dark (0 d), increased rapidly during 2 d of light exposure and then declined rapidly. In contrast to *LhMYB6* expression, transcription of *LhDFR* was nearly zero at 8 d of light treatment. Transcription of *LhMYB12* was not detected during the 8 d
Discussion

Flower pigmentation in Asiatic hybrid lily is temporally and spatially controlled by the expression of anthocyanin biosynthesis genes (Nakatsuka et al. 2003). Because transcription of anthocyanin biosynthesis genes is mainly regulated by interaction between R2R3-MYB and bHLH transcription factors (Schwinn and Davies 2004, Koes et al. 2005), we characterized LhbHLH1 (Jaf13 homolog) and LhbHLH2 (AN1 homolog) genes previously (Nakatsuka et al. 2009) and R2R3-MYB genes in this study. Partial cDNA sequences of R2R3-MYB were isolated using degenerate primers designed from conserved regions of R2R3 repeats. Such a PCR-based strategy was successfully applied to maize (Rabinowicz et al. 1999), Arabidopsis (Romero et al. 1998), orchid (Wu et al. 2003) and gentian (Nakatsuka et al. 2008). After RACE-PCR, full-length cDNA sequences of R2R3-MYB genes, LhMYB6 and LhMYB12, were isolated. Phylogenetic analysis (Fig. 2) and the presence of a conserved motif in the variable region (Supplementary Fig. S1) indicated that they belong to the AN2 subgroup. This is the first report of AN2 subgroup MYB genes isolated from monocot species. However, we do not know whether the AN2 subgroup MYB sequences are rare or common in monocots because R2R3-MYB genes controlling flavonoid biosynthesis have been isolated in only three families in monocots; family Liliflorae (the AN2 subgroup; this study), Orchidaceae (OgMYB1 in orchid, the C1 subgroup; Chiu and Yeh 2008) and Poaceae (ZmC1, ZmPL, OsC1, etc., the C1 subgroup; Cone et al. 1993, Reddy et al. 1998). Isolation of such R2R3-MYB genes from other families of monocots is necessary to answer this question. The rice genome has no R2R3-MYB sequence homologous with AN2 (Jiang et al. 2004, Matus et al. 2008), suggesting that during evolution the rice genome lost AN2 homologous genes after separation of rice–lily lineages.

The following evidence indicates that LhMYB6 and LhMYB12 are transcription factors regulating anthocyanin biosynthesis in Asiatic hybrid lilies. (i) In a transient assay, LhMYB6 and LhMYB12 genes together with the LhbHLH2 gene activated transcription of anthocyanin biosynthesis genes in lily tissues. (ii) LhMYB6 and LhMYB12 interacted with LhbHLH2 protein in a yeast two-hybrid system, in the same way that R2R3-MYB proteins that regulate anthocyanin biosynthesis in other species interact with bHLH proteins. (iii) LhMYB6 and LhMYB12 were expressed in organs where anthocyanin pigment accumulated, with some exceptions such as in anthers (we discuss this exception later).

LhbHLH2 protein was necessary when LhMYB12 activated the transcription of anthocyanin biosynthesis genes (Fig. 4). However, the interaction of LhMYB12 with LhbHLH2 was weaker than that between LhMYB6 and LhbHLH2 in yeast two-hybrid analysis (Fig. 3). Do these results suggest that another bHLH protein that specifically interacts with LhMYB12 is expressed in lily flowers, or does LhMYB12 make up for the weak interaction with LhbHLH2 protein by its high expression, which was about 10–100 times higher than that of LhMYB6? Further study is necessary to answer this question.

Although both LhMYB6 and LhMYB12 regulated anthocyanin biosynthesis, their transcriptional profiles differed in flower organs (Fig. 5). LhMYB12 transcript appeared in tepal stages 3, 4 and 5 in both the basal and upper parts of ‘Montreux’ tepals. This increase in LhMYB12 transcript was accompanied by accumulation of anthocyanin pigment and LhDFR expression in tepals. Similarly, LhMYB12 transcript was detected in filaments and styles where LhDFR transcript and anthocyanin accumulated. These results indicate that LhMYB12 regulates anthocyanin pigmentation in tepals, filaments and styles. However, transcription of LhMYB6 was high at tepal stages 1 and 2 in only the basal part of ‘Montreux’ that had spot pigmentation. Transcription of LhMYB6 in tepals was higher in F1 plants with tepal spots than in F1 plants without tepal spots (Fig. 6). These results indicate that LhMYB6 regulates pigmentation in tepal spots. That is, two R2R3-MYB genes regulate the anthocyanin pigmentation, one gene in tepals, filaments and styles, and the other gene in tepal spots. Although the same anthocyanin cyanidin 3-O-β-rutinoside is accumulated in tepal and tepal spots (Abe et al. 2002), its biosynthesis is regulated independently.
LhMYB12 transcripts accumulated in 'Montreux' tepals, but their expression was not detected in 'Connecticut King' tepals (Fig. 5). LhMYB12 transcript was detected in pink tepals of F₁ plants but not in yellow or white tepals of F₁ plants (Fig. 7), indicating that LhMYB12 determines whether tepals will be pigmented by anthocyanin or not. Because the presence or absence of tepal anthocyanin is controlled by the single dominant locus LILIIUM ANTHOCYANIN PIGMENTATION (LAP) (Abe et al. 2002), LhMYB12 should be on the LAP locus. To prove this, mapping LhMYB12 onto the linkage map of 'Montreux' (Abe et al. 2002) is necessary.

LhMYB6 regulated spot pigmentation. When spots developed on tepals of 'Montreux', cell division occurred on the inside of tepals to form small mounds on the tepal surfaces followed by pigment accumulation. Interestingly, pigment accumulated not only in epidermis cells but also in cells inside tepals (M. Yamagishi, unpublished results). However, the interior surfaces of 'Connecticut King' tepals were smooth with no mounds or projections. Thus, cell division to form spots may not occur in 'Connecticut King' tepals. Because a small amount of LhMYB6 transcripts was detected in 'Connecticut King' tepals (Fig. 5A), 'Connecticut King' should have the LhMYB6 gene. However, because cell division to form spots did not occur in this cultivar, LhMYB6 transcription was not activated in the basal part of tepals. That is, spot pigmentation regulated by LhMYB6 should be downstream of the event of cell division.

Species in genera such as Tricyrtis and Alstroemeria in monocots and Rhododendron in eudicots form spots (or blotches) on the interior surfaces of their tepals or petals. As no precise examination of spot formation in these species exists, we do not know whether these spots are anatomically the same. Further study of spot formation in lilies and other plant species is necessary because tepal spots are an important characteristic that causes variation in color pattern in flowers.

Many Lilium species and cultivars accumulate anthocyanin in their juvenile shoots, and this pigment usually disappears when leaves expand well. Such anthocyanin is thought to protect plants from strong sunlight until sufficient pigments necessary for photosynthesis accumulate (Gould 2004). Light-induced expression of LhMYB6 in leaves (Fig. 8) indicates that LhMYB6 is responsible for biosynthesis of such anthocyanin in vegetative organs. LhMYB12 showed no light-induced expression in leaves. Transcription of bHLH genes was also examined (Nakatsuka et al. 2009) using the same materials as those in this study. The LhbHLH1 transcript accumulated slightly in leaves at 0 d and was detected at high levels at 2, 4, 6 and 8 d of treatment. The expression of LhbHLH2 was not detected in the dark (at 0 d), but the LhbHLH2 transcript rapidly accumulated in response to light at 2 d and then declined to some extent. In contrast to LhMYB6 whose transcription was low at 6 and 8 d of light treatment (Fig. 8), transcription of LhbHLH1 and LhbHLH2 was detected at high levels at 6 and 8 d of light treatment even though the anthocyanin contents were nearly zero at 8 d of treatment (Nakatsuka et al. 2009), indicating that LhMYB6 limits light-induced anthocyanin pigmentation in leaves more closely than LhbHLH1 and LhbHLH2.

No transcription of LhMYB6 in leaves was detected in the dark (at 0 d), but a small amount (relative expression of about 0.01–0.02) of LhMYB6 transcripts was detected at 6 and 8 d of light exposure (Fig. 8). Such low levels of LhMYB6 transcription also appeared in flower organs, such as anthers, filaments and styles of 'Montreux' (Fig. 5D) and in the upper part of 'Montreux' tepals and the basal part of 'Connecticut King' tepals (Fig. 5A). These results indicate that the expression of LhMYB6 is constantly induced by light. However, LhMYB6 did not activate LhDFR expression when its relative transcription was about 0.01–0.02 because LhDFR expression was very low at 8 d of light exposure. When light exposure began, the level of LhMYB6 transcription was high enough to induce LhDFR expression. Light-induced expression of R2R3-MYB genes has been reported for MdMYB1 in apple (Takos et al. 2006) and PAP1 and PAP2 in Arabidopsis (Cominelli et al. 2008). The expression of these R2R3-MYB genes is induced by light exposure, but the expression keeps the levels high enough to induce expression of anthocyanin biosynthesis genes under light. That is, the response of LhMYB6 to light exposure is not completely the same as that of MdMYB1, PAP1 and PAP2.

Although anthocyanin accumulated in anthers, LhMYB12 transcripts were not detected, and transcription of LhMYB6 was not high in anthers (Fig. 5). The expression pattern of structural genes and other regulatory genes is unique in lily anthers. Among three CHS genes, LhCHSα and LhCHSβ, which are major CHS genes expressed in tepals, are not expressed in anthers, and only LhCHSγ is expressed in anthers (Nakatsuka et al. 2003). Transcription of the LhDFR gene that was high in tepals, filaments and styles was not detected in anthers (Fig. 5), suggesting that another DFR gene acts in anthers. Few incidences of transcription of both LhbHLH1 and LhbHLH2 are detected in anthers (Nakatsuka et al. 2009). Thus, different regulatory genes and structural genes should exist to biosynthesize anthocyanin in lily anthers. The LhDFR transcript in anthers was detected by Northern blot analysis by Nakatsuka et al. (2003) but it was not detected in this study by RT–PCR, indicating that a putative DFR gene that acts in lily anthers has high homology with LhDFR but does not have the sequences of the primers used in this study. In other species, regulation of anthocyanin biosynthesis is also complicated in anthers. For example, PhAN4 controls anthocyanin production in petunia anthers instead of PhAN2 (Spelt et al. 2000) by regulating the expression of PhCH5J, but not of PhCH5A (Quattrocchio et al. 1993).

The expression of LhMYB6 was relatively high in ovaries (Fig. 5). Accumulation of anthocyanin was not detected in ovaries, but flavonols accumulated in them (M. Yamagishi, unpublished result). Flavonols are believed to be essential for pollen germination and tube growth (Napoli et al. 1999), and flavonols and the transcripts of flavonol biosynthesis genes accumulate in developing ovules and placentas of ovaries.
in *Antirrhinum majus* (Moyano et al. 1996). However, R2R3-MYB transcription factors different from those regulating anthocyanin biosynthesis usually regulate flavonol accumulation (Moyano et al. 1996, Stracke et al. 2007). Thus, LhMYB6 may not contribute directly to flavonol biosynthesis in lily ovaries. The function of LhMYB6 in ovaries remains to be determined.

In conclusion, this study showed that newly identified AN2 subgroup MYB transcription factors, LhMYB6 and LhMYB12, definitely participate in regulating anthocyanin biosynthesis in Asiatic hybrid lily. LhMYB6 is multifunctional, regulating both spot pigmentation in tepals and light-induced pigmentation in leaves. LhMYB12 regulates anthocyanin biosynthesis in flower tepals, filaments and styles. That is, each R2R3-MYB regulates anthocyanin biosyntheses in organs and tissues. In the AN2 subgroup R2R3-MYB, two or more genes are often included in a single plant species, such as *PAP1*, *PAP2*, *AtMYB113* and *AtMYB114* in Arabidopsis (Gonzalez et al. 2008), *AmROSEA1* and *AmROSEA2* in snapdragon (Schwinn et al. 2006), and *VlMYBA1* and *VlMYBA2* in grape (Walker et al. 2007). These sets of genes usually show functional redundancy, acting in the same organs for a similar period. One exception is *AmVENOSA* in snapdragon, which pigments the epidermal layer only in regions overlying the vascular tissues of petals, whereas *AmROSEA1* and *AmROSEA2* pigment the epidermal layer in wider regions of petals (Schwinn et al. 2006). Thus, compared with these genes, LhMYB6 and LhMYB12 have diverse functions. A more precise examination of LhMYB6 and LhMYB12 should be valuable to determine how they gained such diverse functions.

**Materials and Methods**

**Plant materials**

Asiatic hybrid lily (*Lilium* spp.) cultivars ‘Montreux’, ‘Connecticut King’ and ‘Vivaldi’ were used. For the analysis of temporal gene expression, tepals in field-grown ‘Montreux’ and ‘Connecticut King’ were collected at each tepal developmental stage. Tepal segments about 7 mm long and 5–15 mm wide that did not include midribs were cut from basal or upper parts of tepals and were used for RNA isolation. The basal part segments in ‘Montreux’ contained tepal spots but the upper part segments did not. Similarly, tepals of field-grown *F*₁ plants derived from crosses between ‘Montreux’ and ‘Connecticut King’ were collected at stage 2 or 5. To analyze organ-specific gene expression, anthers, filaments, styles, ovaries and tepals at tepal developmental stage 4 and leaves in field-grown ‘Montreux’ were collected.

To analyze the effect of light on gene expression in leaves, ‘Vivaldi’ bulbs were germinated in complete darkness for 2 weeks and then were grown in a 16 h light/8 h dark photoperiod at 23°C. Each leaf of light-treated plants was collected at 0, 2, 4, 6 and 8 d of light exposure and was used to isolate RNA (details in Nakatsuka et al. 2009).

**RNA isolation and cDNA synthesis**

Total RNA was isolated from 50–100 mg FW materials and was purified using the RNeasy Plant Mini Kit combined with RNase-free DNase (Qiagen, Tokyo, Japan). First-strand cDNA was synthesized using Super Script III reverse transcriptase (Invitrogen, Tokyo, Japan) and oligo(dT) or poly(T) adaptor primers (Supplementary Table S1 lists all primer sequences).

**Isolation of R2R3-MYB homologs from lily tepals**

DNA fragments that encode R2R3-MYB genes were amplified by PCR with cDNA from ‘Montreux’ tepals (stage 4) and degenerate primers dMYBF and dMYBR that were originally designed from the conserved DNA-binding domain of R2R3-MYB proteins that control anthocyanin biosynthesis in other plant species. Amplified fragments were inserted into the pGEM-T-easy vector using the TA cloning method (Promega, Tokyo, Japan) and were sequenced using an ABI DNA sequencer and a BigDye Terminator Sequencing system (Applied Biosystems, Tokyo, Japan). Full-length cDNA sequences were determined by RACE-PCR. 3’ RACE-PCR was carried out between an adaptor-1 primer and each gene-specific primer. Single fragments were amplified by nested PCR between an adaptor-1 primer and each nested primer. 5’ RACE-PCR was performed as follows (Masuda et al. 1997). First-strand cDNA of ‘Montreux’ tepals transcribed by oligo(dT) primer was treated with RNase H (TAKARA BIO. INC., Ohtsu, Japan) and was purified using Microcon YM-100 centrifugal filter devices (Millipore, Tokyo, Japan). A poly(G) tail was added to the 3’ terminus of the first-strand cDNA using terminal deoxynucleotidyl transferase and dGTP (Roche, Basel, Switzerland). The plus strands were amplified using PrimeSTAR HD DNA polymerase (TAKARA BIO. INC.) and a poly(C) adaptor primer. After purifying the product using a High Pure PCR Product Purification kit (Roche), the 5’ end sequence was amplified by PCR with an adaptor-2 primer and each gene-specific primer. Single fragments were amplified using an adaptor-2 primer and each nested primer. After purifying the RACE-PCR products using a High Pure PCR Product Purification kit (Roche), they were sequenced directly using an ABI DNA sequencer and a BigDye Terminator Sequencing system (Applied Biosystems, Japan).

The cDNA sequences were translated into amino acid sequences using Four Peaks software (http://mekentosj.com/4peaks/). Deduced amino acid sequences in lily genes and appropriate genes in other plant species (Fig. 2) were aligned using CLUSTALW (Thompson et al. 1994) at gap open penalty 10 and gap extension penalty 0.05. The genetic distances were calculated using the Kimura 2-parameter (Kimura 1980), and a phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987). One hundred bootstrap replicates were analyzed. The software PROTDIST, NEIGHBOR, DRAWGRAM, SEQBOOT and CONSENSE from a PHYLIP 3.6 package (http://evolution.genetics.washington.edu/phylip.html) were used for the calculations.
Yeast two-hybrid analysis

In the yeast two-hybrid assay, the Matchmaker Two-Hybrid System 3 (Clontech, Mountain View, CA, USA) was employed to investigate protein interactions between LhbHLH2 (accession No. AB222076) and LhMYB6, and between LhbHLH2 and LhMYB12. The open reading frame (ORF) sequences of LhMYB6, LhMYB12 and LhbHLH2 were amplified by PCR and were cloned into the pGAD-T7 (LhMYB6 and LhMYB12) or pGBK-T7 (LhbHLH2) vectors (Clontech). All constructs were transformed into Saccharomyces cerevisiae strain AH109 (Clontech) using the S.c. easyComp transformation kit (Invitrogen, Japan). Yeast transformants were grown at 30°C for 2 or 4 d on a selective medium without leucine (–LEU) and tryptophan (–TRP), on a selective medium without leucine, tryptophan, histidine (–HIS) and adenine (–ADE) or on a selective medium without leucine, tryptophan, histidine and adenine with 10 mM 3-AT.

Transient expression assay of LhMYB6 and LhMYB12

To construct vectors for a transient expression assay, LhMYB6, LhMYB12 and LhbHLH2 ORF fragments were amplified. Vectors p35Spro-LhMYB6, p35Spro-LhMYB12 and p35Spro-LhbHLH2 were constructed to replace the enhanced β-glucoronidase (EGUS) gene of pKD0330 with each ORF by using Stul and Smal restriction sites. pKD0330 is a pBluescript vector containing the EGUS gene under the control of the cauliflower mosaic virus 35S promoter and a nopaline synthase terminator from Agrobacterium tumefaciens (K. Fujino, unpublished).

To evaluate whether LhMYB6, LhMYB12 and LhbHLH2 were responsible for regulation of anthocyanin biosynthesis in lily, transient expression assays were done using bulb scales of ‘Montreux’ and particle bombardment system PDS-1000/He (Bio-Rad, Hercules, CA, USA). A 1 mg aliquot of gold particles (1 μm in diameter) was precipitated together with 7.5 μg of p35Spro-LhMYB6 or p35Spro-LhMYB12 plasmids and 15 μg of p35Spro-LhbHLH2 plasmid, using CaCl2 and spermidine, and was finally suspended in 22 μl of ethanol. Pieces of sterilized bulb scales were placed on plates containing 20 ml of Linsmaier and Skoog nutrient solution supplemented with 1% agar (Wako Pure Chemical, Osaka, Japan) and were bombarded using 10 μl particles for each shot and 650 p.s.i. pressure plates. After 48 h of bombardment, RNA was isolated from the bulb scales and RT–PCR was carried out. Primer sets LhMYB12cf–LhMYB12jr for LhMYB12 and LhbHLH2cf–LhbHLH2dr for LhbHLH2, and primers for RT–PCR for LhMYB6, LhDFR (accession No. AB058641), LhCHSa (AB058638) and LhCHSb (AB058639) were used. Reaction conditions consisted of pre-heating at 94°C for 5 min, 32 cycles (LhMYB6 and LhCHSsa) or 35 cycles (LhMYB12, LhbHLH2, LhDFR and LhCHSb) at 94°C for 30 s and at 60°C for 80 s, and final extension at 72°C for 5 min.

Expression analysis of LhMYB6 and LhMYB12 in Asiatic hybrid lily

To investigate the temporal and spatial expression of lily MYB genes and LhDFR, cDNA from flower organs and leaves and primer pairs for RT–PCR were used for real-time PCR. SYBR Premix Ex Taq (TAKARA BIO, INC.) was used to intercalate SYBR Green I in amplified products. Signals were monitored using the Chromo4 real-time PCR system (Bio-Rad). Reaction conditions consisted of pre-heating at 94°C for 5 min, 40 cycles at 94°C for 30 s and at 63°C for 60 s (LhMYB6) or at 60°C for 90 s (LhMYB12, LhbHLH2 and LhDFR), and final extension at 72°C for 5 min. The amount of LhActin mRNA (accession No. AB438963) in each sample was determined to normalize the differences in the amount of mRNA of other genes.

Supplementary data

Supplementary data are available at PCP online.

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References


