LhMYB12, Regulating Tepal Anthocyanin Pigmentation in Asiatic Hybrid Lilies, is Derived from Lilium dauricum and L. bulbiferum

Masumi Yamagishi* and Takashi Nakatsuka

1Research Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan
2Faculty of Agriculture, Shizuoka University, Shizuoka 422-8529, Japan

Genes encoding a MYB12 transcription factor, which regulates anthocyanin biosynthesis, are the key genes causing variations in the anthocyanin colors in lily flowers. However, the origin of the MYB12 in Asiatic hybrid lilies (Lilium spp.) is not completely known. In this study, we analyzed anthocyanin pigments in tepals of L. maculatum, L. lancifolium, L. callosum, L. leichtlinii, L. davidii, L. bulbiferum, and L. dauricum, and clarified that L. dauricum and L. bulbiferum accumulated a single anthocyanin, cyanidin 3-O-rutinoside, in entire tepals, although these wild species had orange-colored tepals. The sequencing of MYB12 genes revealed seven allelic sequences among ten L. dauricum plants and two allelic sequences in one L. bulbiferum plant. These MYB12 sequences were the same as or similar to the sequences isolated from pink Asiatic hybrid lily cultivars, indicating that L. dauricum and L. bulbiferum have contributed to the anthocyanin coloration of these cultivars.

Key Words: cyanidin 3-O-rutinoside, flower color, R2R3-MYB, section Daurolirion, section Sinomartagon.

Introduction

Lilies (Lilium spp.) are among the commercially most important and widely cultivated floriculture crops. Cut flower production of lilies has ranked second only to chrysanthemums in Japan in recent years (Ministry of Agriculture, Forestry and Fisheries, 2014, http://www.maff.go.jp/j/tokei/kikaku/nenji/index.html, December 10, 2016). Interspecific hybridization is the principal method for lily breeding. Asiatic hybrid lilies are derived from crosses among L. dauricum, L. bulbiferum, L. maculatum, L. lancifolium, L. callosum, L. leichtlinii, L. davidii, L. cernuum, and others, whereas Oriental hybrid lilies are derived from crosses among L. auratum, L. speciosum, L. rubellum, L. japonicum, L. nobilisimum, and others (belonging to the section Archerilion, Leslie, 1982).

Flower color is a crucial characteristic that determines the commercial value of floriculture crops. Much interest has been expressed in developing cultivars that bear flowers with novel hues, intensities, and patterns of color. A typical feature of Asiatic hybrid lilies is the huge variation in hues including yellows (because of carotenoids), oranges (because of carotenoids), pinks (because of anthocyanins), reds (because of the combined presence of anthocyanins and carotenoids), and white (Yamagishi, 2013; Yamagishi et al., 2010a, b). In addition to the hues, Asiatic hybrid lily cultivars often develop raised red spots on the interior surfaces of their tepals (Abe et al., 2002), and sometimes develop splatter-type spots, which are morphologically and genetically distinct from the raised spots (Yamagishi and Akagi, 2013; Yamagishi et al., 2014b). Cyanidin 3-rutinoside is a major anthocyanin that accumulates in the lily tepals and tepal spots (Nørbaek and Kondo, 1999).

The activity of anthocyanin biosynthesis enzymes is primarily regulated by complexes that consist of R2R3-MYB transcription factors, basic helix-loop-helix (bHLH) transcription factors, and WD40 proteins (Koes et al., 2005; Lai et al., 2013; Xu et al., 2015). In lilies, LhMYB12 (R2R3-MYB) determines tepal-specific anthocyanin biosynthesis together with LhbHLH2 (Nakatsuka et al., 2009) and LhWD40A (Suzuki et al., 2016). LhMYB12 often gives rise to full-pink-colored tepals in Asiatic and Oriental hybrid lilies (Lai et al., 2012; Yamagishi, 2011; Yamagishi et al., 2010b). An
indicate that LhMYB12 is a key factor that determines hues in tepals (Yamagishi et al., 2012). These results indicate that LhMYB12 is a key factor that determines hues, intensities, and patterns of anthocyanin colors in lily flowers. Thus, understanding of the natural variation in MYB12 among the wild species is important in lily breeding and for the introduction of novel genes or alleles of MYB12 that bring unnatural color phenotypes into new lily cultivars. For progress in such research, it is necessary to determine the wild species that donated MYB12 to the lily cultivars.

In an earlier study, we determined the MYB12 sequences in wild lily species that have been used to develop Asiatic and Oriental hybrid lilies and compared them to the MYB12 sequences in Asiatic and Oriental hybrid lily cultivars (Yamagishi et al., 2014a). The pink- and red-colored Oriental hybrid lily cultivars had the same or similar MYB12 sequences as L. rubellum and L. speciosum, indicating that LhMYB12 in Oriental hybrid lilies is mainly derived from these two species. Among the pink-colored Asiatic hybrid lily cultivars, ‘Vermeer’ had the same MYB12 sequence as L. cernuum, which shows full-pink tepal pigmentation, indicating that ‘Vermeer’ LhMYB12 is derived from L. cernuum. However, the MYB12 sequences in other Asiatic hybrid lily cultivars are comparatively distantly related to the L. cernuum sequence. Thus, it is assumed that in other wild species, MYB12 genes have contributed to the pink coloration in Asiatic hybrid lilies.

To screen the wild species that have potentially contributed to the pink coloration in Asiatic hybrid lilies, pigments were extracted from the tepals of seven Lilium species in this study. As pink water-soluble pigments were detected from L. dauricum and L. bulbiferum, the anthocyanin pigments in these two species were further analyzed in detail and their MYB12 genes were sequenced to understand the origin of the genes that regulate the pink color of flowers in Asiatic hybrid lilies.

Materials and Methods

Wild species of L. dauricum, L. bulbiferum var. bulbiferum, L. maculatum, L. lancifolium, L. callosum, L. leichtlinii, and L. davidii were used in this study. L. bulbiferum, L. callosum, and L. davidii plants were derived from the collections at the Yurigahara Park, Sapporo, Japan. L. lancifolium bulbs were purchased from a local market and L. leichtlinii plants were collected from natural habitats in Hokkaido, Japan. These plants were grown at the experimental farm of Hokkaido University, Sapporo, Japan. Flowers of L. maculatum were directly collected from a natural habitat at Izu-Oshima, Tokyo, Japan. Ten L. dauricum plants were obtained from six natural habitats in Japan and China (Table 1). The two L. dauricum plants from Higashidoori, Aomori, Japan, and the Greater Khingan, China, were derived from the collections at Yurigahara Park and grown at the experimental farm of Hokkaido University. The flowers of other L. dauricum plants were directly collected from their natural habitats in Hokkaido. One Asiatic hybrid lily ‘Sugar Love’, MYB12 sequence of which was additionally determined, was purchased from a local market.

Anthocyanin pigments in tepal backgrounds were analyzed. First, pigments were extracted in 5% formic acid overnight from the tepals of the seven wild species. Second, the pigments in the tepals of L. dauricum and L. bulbiferum were extracted with a mixture of methanol, acetic acid, and water (4:1.5, v/v/v) to analyze anthocyanins qualitatively. After filtration through ZORBAX Eclipse Plus C18 (Agilent Technologies, Tokyo, Japan), the extract was analyzed by high-performance liquid chromatography (HPLC), using an Agilent 1290 infinity system (Agilent Technologies), with a YMC-Triart C18 column (2.0 × 50 mm, 1.9 μm, YMC Co., Ltd, Kyoto, Japan). A linear gradient of 10–60% solvent B (1.5% H2PO4, 20% acetic acid, and 25% acetonitrile) in solvent A (1.5% H2PO4) was passed over a 9-min period. The anthocyanins were identified on the basis of absorption spectra obtained using a photodiode array detector. Finally, for quantitative analysis, the pigments were extracted in 5% formic acid and their absorbance was measured at 515 nm, as described previously (Yamagishi, 2016).

MYB12 sequences in L. dauricum, L. bulbiferum, and ‘Sugar Love’ were determined. The methods used for RNA isolation, cDNA synthesis, PCR amplification of MYB12, and sequencing of the amplified products were same as those described in an earlier report (Yamagishi et al., 2014a). Briefly, nearly full-length MYB12 sequences were amplified by PCR using the primers (forward; 5′-ATGTCCCACAGTTATGCTCCGC-3′ and reverse; 5′-CAGACTGTCACTCTCAAAG-3′ or 5′-TGTTACATGGACCTTGCTAT-3′) and tepal cDNA. The purified PCR products were sequenced from both sides. When double peaks were observed in the sequences, the alleles in the samples were interpreted as being heterozygous. Subsequently, PCR products were cloned into pGEM-T Easy vector (Promega, Tokyo, Japan) and sequenced. The nucleotide sequences were aligned, a neighbor-joining (NJ) tree was constructed, and bootstrap analysis (1000 replicates) was performed using the default parameters in Clustal X version 2.0 (Larkin et al., 2007).

Results

Tepal pigments were analyzed in seven wild species, which could contribute to develop Asiatic hybrid lilies. These species exhibited an orange tepal color and species other than L. bulbiferum and L. callosum had raised spots with dark red color on the tepals (Fig. 1). Because anthocyanins accumulate in the raised spots, pigments were extracted from the upper parts of the tepals, ex-
including the raised spots, to distinguish the pigments accumulated in the raised spots and tepal background. The extracts from *L. dauricum* and *L. bulbiferum* were pink but those from other species were clear (Fig. 2). Thus, the anthocyanin pigments in the tepals of *L. dauricum* and *L. bulbiferum* were subsequently qualitatively characterized. A single anthocyanin pigment was detected in the HPLC analysis; the absorption spectrum of this pigment was the same as that of cyanidin 3-O-rutinoside (Fig. 3). Quantitative analysis revealed an anthocyanin concentration of 0.40–1.39 μmol·g⁻¹ in the upper parts of tepals from six *L. dauricum* and one *L. bulbiferum* plants. These results indicate that *L. dauricum* and *L. bulbiferum* accumulate cyanidin 3-O-rutinoside in the tepal background.

Nearly full-length cDNA sequences of *MYB12* were Table 1. Sequence names and accession numbers of *MYB12*.

| Species, cultivars | Collected place         | No of plants | MYB12 sequence | Note                  |
|--------------------|-------------------------|--------------|----------------|-----------------------|
| *L. dauricum*      | Kitami, Hokkaido, Japan | 2            | *L. dauricum*-1| AB534586             |
|                    | Abashiri, Hokkaido, Japan| 1            | *L. dauricum*-2| LC157998             |
|                    | Koshimizu, Hokkaido, Japan| 3            | *L. dauricum*-3, -4, -5 | LC157995, LC157996, LC157997 |
|                    | Shiranuka, Hokkaido, Japan| 2            | *L. dauricum*-1| AB534586             |
|                    | Higashidori, Aomori, Japan| 1           | *L. dauricum*-7| LC157994             |
|                    | Greater Khingan, China  | 1            | *L. dauricum*-6| LC157993             |
| *L. bulbiferum*    | 1  | *L. bulbiferum*-1, -2 | AB188995, AB188996 |
| *L. cernuum*       | 3  | *L. cernuum* | AB904749 | Yamagishi et al. (2014a) |

Asiatic hybrid lilies

- ‘Vermeer’
- ‘Dalila’
- ‘Montreux’, ‘Toronto’, ‘Côte d’Azur’, ‘Blackout’
- ‘Renoir’, ‘Vivaldi’, ‘Panorama’
- ‘Landini’
- ‘Sugar Love’

section Archelirion (out-group)

* L. japonicum
* L. rubellum
* L. auratum
* L. speciosum

* Collections in Yurigahara Park, Sapporo, Japan.

† Three *L. dauricum* plants possessed three *MYB12* sequences heterozygously.

‡ A *L. bulbiferum* plant possessed two *MYB12* sequences heterozygously.

§ These cultivars possessed two *MYB12* sequences heterozygously.

![Fig. 1. Flowers of *Lilium* species (upper row) and Asiatic hybrid lily cultivars (lower row).](image-url)
amplified and sequenced from ten *L. dauricum* plants and one *L. bulbiferum* plant. Seven and two *MYB12* sequences were detected in *L. dauricum* and *L. bulbiferum*, respectively (Table 1). Four of the ten *L. dauricum* plants (two in Kitami and two in Shiranuka) had the same *MYB12* sequences (sequence name *L. dauricum*-1). Single sequences were amplified in the plants collected at Abashiri (*L. dauricum*-2), Higashidoori (*L. dauricum*-7), and the Greater Khingan (*L. dauricum*-6), and three sequences (*L. dauricum*-3, -4, and -5) were obtained in the three plants grown at Koshimizu. One *L. bulbiferum* plant possessed two *MYB12* sequences (*L. bulbiferum*-1 and *L. bulbiferum*-2).

The *MYB12* sequences of *L. dauricum* and *L. bulbiferum* were compared with those from *L. cernuum*, 11 Asiatic hybrid lily cultivars, and four Archerillion species (out-group). These Asiatic hybrid lily cultivars had pink or chocolate brown tepal colors (Fig. 1). Of the 11 Asiatic hybrid lily cultivars, the sequence from ‘Sugar Love’ was determined in this study, while those from other cultivars were analyzed in previous studies (Table 1). The nucleotide sequences were aligned (data not shown) and an NJ tree was constructed (Fig. 4). The seven sequences from *L. dauricum* and two sequences from *L. bulbiferum* clustered together with four of the five sequences detected in the Asiatic hybrid lily cultivars. Among them, *L. dauricum*-1 was identical to the Montreux sequence, which was detected in six Asiatic hybrid lily cultivars (Table 1). The *L. dauricum*-2 sequence differed from the *Sugar Love* sequence by one nucleotide. Two sequences from *L. bulbiferum* were closely related to the sequences detected in ‘Renoir’ and ‘Dalila’. However, *L. cernuum* and ‘Vermeer’ shared the same sequence (Yamagishi et al., 2014a), which was comparatively distantly related to the seven *L. dauricum* and two *L. bulbiferum* sequences. These results indicate that the *MYB12* sequences in *L. dauricum* and *L. bulbiferum* are closely related to the four Asiatic hybrid lily sequences other than the ‘Vermeer’ sequence.

**Discussion**

Because *MYB12* is the key gene that determines flower color characteristics among lily cultivars, it is valuable to clarify which wild species have donated *MYB12* genes in Asiatic hybrid lily cultivars. This study showed that *L. dauricum* and *L. bulbiferum* accumulated a single anthocyanin, cyanidin 3-O-rutinoside, in entire tepals and expressed *MYB12* genes in the tepals. The NJ tree of the *MYB12* sequences revealed that one *MYB12* sequence of *L. dauricum* was the same as the
Montreux sequence of \textit{LhMYB12}. In addition, the \textit{MYB12} sequences of \textit{L. dauricum} and \textit{L. bulbiferum} were closely related to those of the Asiatic hybrid lily cultivars other than ‘Vermeer’, which has the \textit{MYB12} sequence of \textit{L. cernuum}. Thus, in addition to \textit{L. cernuum}, \textit{L. dauricum}, and \textit{L. bulbiferum} have strongly contributed to the pink coloration of Asiatic hybrid lilies. Therefore, to search for natural variations in the \textit{MYB12} genes of \textit{L. dauricum}, \textit{L. bulbiferum}, and \textit{L. cernuum} will be crucial for producing novel color phenotypes in Asiatic hybrid lilies. In Oriental hybrid lilies, a spontaneous loss-of-function mutation in the \textit{MYB12} gene of \textit{L. speciesum} has been used to breed white tepal cultivars (Suzuki et al., 2015).

Wild species that have been used to breed Asiatic hybrid lilies often have orange tepals, although \textit{L. cernuum} is an exception with pink tepals. From the orange tepals of \textit{L. lancifolium} (formerly \textit{L. tigrinum}), \textit{L. amabile}, \textit{L. davidi}, \textit{L. leichtlinii}, and \textit{L. pumilum}, the orange carotenoids, capsanthin and/or capsorubin, have been detected (Deli et al., 1998; Jeknić et al., 2012). This study showed that \textit{L. dauricum} and \textit{L. bulbiferum} accumulated anthocyanin in their entire tepals, although the tepals in these species were orange. The orange tepal color may have resulted from the combined presence of anthocyanins and carotenoids, because after the extraction of anthocyanin pigments from the tepals with formic acid, a yellow to orange color was left on the surface of the tepals (data not shown). Further analysis will be required to determine whether these two species accumulate capsanthin and capsorubin.

The genus \textit{Lilium} comprises more than 90 wild species, which are further classified into several sections (Comber, 1949). \textit{L. dauricum}, which dispersed to the east side of Asia, including Siberia, Northeast China, and Japan (Hokkaido and Aomori), is classified in the section Daurolirion. In contrast, \textit{L. bulbiferum} is native to Europe and is classified in the section Liriotypus, which consists of European species (Comber, 1949). However, a recent molecular phylogenetic analysis using internal transcribed spacer sequences of ribosomal DNA indicates that \textit{L. bulbiferum} is closely related to the species belonging to the section Daurolirion, and the authors insist that \textit{L. bulbiferum} should be classified in this section (Nishikawa et al., 1999). Our study showed that these two species possessed similar \textit{MYB12} sequences, which supports the contention of Nishikawa et al. (1999).

When were \textit{MYB12} genes in \textit{L. dauricum} and \textit{L. bulbiferum} introduced into Asiatic hybrid lily cultivars? It is believed that breeding of Asiatic hybrid lilies started in the 1600s using interspecific hybridization between \textit{L. dauricum} and \textit{L. maculatum} (orange tepals without anthocyanins, native to Japan), because the name of the hybrid (“Sukashiyuri” in Japanese) and its cultivars appear in Kadan-koumoku and Kadan-jikinsyou, which are classical horticultural books published in Japan in 1681 and 1694, respectively (Shimizu, 1987). In Sukashiyuri-baiyouhou, another classical horticultural book written by Isujiin Hanabishi and published in Japan in 1847, the traits of some of the “Sukashiyuri” cultivars are precisely introduced with color illustrations, and cultivars with red, orange, yellow, and pale yellow flowers are shown (these Japanese classical books can be accessed on the web site of the National Diet Library Digital Collections; http://dl.ndl.go.jp/). Because red tepals in lilies is produced by the combined presence of anthocyanins and orange carotenoids (Yamagishi, 2013), these texts suggest that some “Sukashiyuri” cultivars accumulated anthocyanins in their tepal background and that the \textit{MYB12} gene was introduced from \textit{L. dauricum} into Asiatic hybrid lilies in the 1600s. Subsequently, in the 1900s and 2000s, these old Japanese Asiatic hybrid lily cultivars (often named \textit{L. × elegans}) were hybridized with other wild species, such as \textit{L. lancifolium}, \textit{L. leichtlinii}, \textit{L. amabile}, \textit{L. davidi}, \textit{L. pumilum}, and \textit{L. cernuum}, belonging to the section Sinomartagon, to breed modern Asiatic hybrid lilies with diverse flower colors (McRae, 1998; Shimizu, 1987).

\textit{L. bulbiferum} was crossed with \textit{L. × elegans} in Holland to produce \textit{L. × hollandicum} (1879), which was the first Asiatic hybrid lily to be released in a country other than Japan (Shimizu, 1987). \textit{L. × hollandicum} is an important material that contributed to the production of Mid-Century hybrids, including “Enchantment”, which were used as elite parents during the crossbreeding of Asiatic hybrid lilies. In other cases, \textit{L. bulbiferum} was hybridized with other species to produce \textit{L. × cromottiae} and \textit{L. × falkovaja} in the first half of the 1900s (McRae, 1998). Thus, it is possible that \textit{MYB12} in \textit{L. bulbiferum} was introduced during these periods.

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