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Pigment accumulation and transcription of *LhMYB12* and anthocyanin biosynthesis genes during flower development in the Asiatic hybrid lily (*Lilium* spp.)

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ABSTRACT

Anthocyanin biosynthesis is often regulated by MYB transcription factors that are classified into AN2 and C1 subgroups. The AN2 subgroup regulates the late genes in the anthocyanin biosynthesis pathway of eudicots, whereas the C1 subgroup controls both early and late genes in monocots. Anthocyanin is a major pigment in Asiatic hybrid lilies (*Lilium* spp.), with LhMYB12 being the first AN2 subgroup in monocots. In this study, the accumulation of pigments and gene transcripts during flower bud development was evaluated to determine the genes regulated by LhMYB12. LhMYB12 and anthocyanin biosynthesis genes showed the same transcription profiles, with LhMYB12 directly activating the promoters of chalcone synthase and dihydroflavonol 4-reductase. This indicates that LhMYB12 regulates both early and late genes, despite belonging to the AN2 subgroup. The cultivar Landini accumulated anthocyanin and flavonol. The contents of these pigments increased during the late stages of flower bud development; this might result from the coordinated expression of early and late genes. During the early stages of flower bud development, the tepals contained no flavonoids but accumulated cinnamic acid derivatives. These results indicate that the profiles of pigment accumulation and gene transcription in lily tepals are unique among angiosperm flowers.

1. Introduction

The genus *Lilium* comprises more than 90 species, which are classified into sections [1–3]. Species belonging to the Sino-martagon section are mainly distributed in East Asia, and Asiatic hybrid lilies (*Lilium* spp.) are derived from interspecific crosses of these species [4,5]. Asiatic hybrid lilies represent a significant breakthrough in lily breeding history, because the wide release of Asiatic hybrid lilies several decades ago led to the lily becoming one of the most important flower bulbs and cut flowers. Even today, Asiatic hybrid lilies have an important position among ornamental plants [6]. One major quality of Asiatic hybrid lilies is the availability of a much greater range of flower colours (orange, yellow, white, pink, red, and chocolate-brown) in comparison with the other hybrid lilies [6,7]. Tepals are pigmented pink and chocolate-brown by anthocyanins, yellow and orange by carotenoids, and red by the presence of both anthocyanins and orange carotenoids [7,8]. Asiatic hybrid lilies often have dark red spots on the interior surfaces of their tepals; within these spots, anthocyanins accumulate in the swelled epidermis and parenchyma cells [9]. Anthocyanins in lilies comprise cyanidin 3-*O*-β-rutinoside and cyanidin 3-*O*-β-rutinoside-7-*O*-β-glucoside; the former is the major anthocyanin, and the latter is the minor anthocyanin only accumulating in small amounts in a few cultivars [10].

Phenylalanine ammonia-lyase (PAL) catalyses the first reaction, and converts phenylalanine into *trans*-cinnamic acid in the phenylpropanoid biosynthesis pathway (Fig. 1). Cinnamic acid is the precursor for flavonoids and a wide range of metabolites, such as hydrocinnamic acids, coumarins, stilbenes, and lignins [11,12]. PAL plays a critical role in controlling the metabolite flux of the whole pathway [13], and often constitutes a small multigene family in higher plants [14–16]. The flavonoid biosynthetic pathway has been intensively studied [17,18], showing

Abbreviations: 3GT, UDP-glucose:flavonoid 3-*O*-glucosyltransferase; ANS, anthocyanidin synthase; bZIP, basic leucine zipper; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; dpa, day(s) post anthesis; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; FLUC, firefly luciferase; HPLC, high-performance liquid chromatography; ORF, open reading frames; PAL, phenylalanine ammonia-lyase; qRT-PCR, quantitative reverse transcription-PCR; RACE, rapid amplification of cDNA ends; RLUC, *Renilla* luciferase; RT-PCR, reverse transcription-PCR; St, stage.

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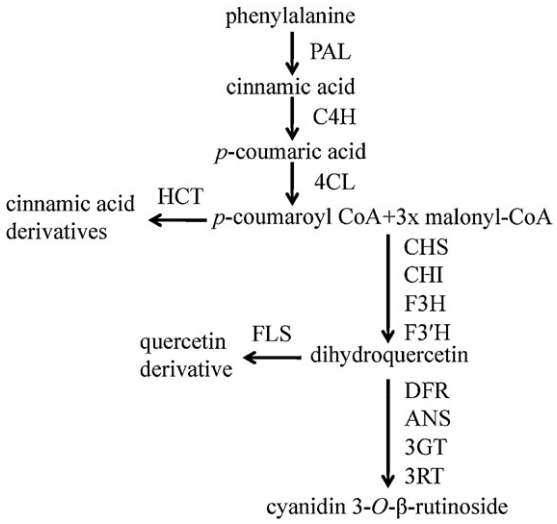

Fig. 1. The putative biosynthesis pathway leading to cinnamic acid derivatives, quercetin, and cyanidin detected in lily tepals. PAL: phenylalanine ammonia-lyase, C4H: cinnamate 4-hydroxylase, 4CL: 4-coumarate:CoA ligase, HCT: hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase [66,67], FLS: flavonol synthase, CHS: chalcone synthase, CHI: chalcone isomerase, F3H: flavanone 3-hydroxylase, F3′H: flavonoid 3′-hydroxylase, DFR: dihydroflavonol 4-reductase, ANS: anthocyanidin synthase, 3GT: UDP-glucose:flavonoid 3-*O*-glucosyltransferase, 3RT: UDP-rhamnose:anthocyanidin 3-*O*-glucoside-6″-*O*-rhamnosyltransferase.

that in the pathway leading to cyanidin 3-*O*-β-rutinoside, chalcone synthase (CHS) catalyses the first reaction. This is followed by chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3′-hydroxylase (F3′H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), UDP-glucose:flavonoid 3-*O*-glucosyltransferase (3GT), and UDP-rhamnose:anthocyanidin 3-*O*-glucoside-6″-*O*-rhamnosyltransferase.

The activity of anthocyanin biosynthesis enzymes is mainly regulated at the transcriptional level; thus, R2R3-MYB and basic helix-loop-helix (bHLH) transcription factors usually regulate the transcription of anthocyanin biosynthesis genes in flower organs [19–21]. Genes encoding R2R3-MYB transcription factors that regulate anthocyanin pigmentation are often divided into 2 subgroups due to their homology in amino acid sequences. One subgroup includes *PhAN2* in petunia (*Petunia* × *hybrida*) and *AtPAP1* in *Arabidopsis* (hereinafter referred to as the *AN2* subgroup), while the other subgroup includes *ZmC1* and *ZmPl* in maize (hereafter referred to as the *C1* subgroup) [22,23]. The *AN2* and *C1* subgroups are respectively categorised as subgroups 6 and 5 in the *Arabidopsis R2R3-MYB* gene family [22,24], and as subgroups N09 and N08 in the *R2R3-MYB* gene family of *Arabidopsis* and rice [25]. Most *R2R3-MYB* genes that regulate anthocyanin biosynthesis in flowers or fruits of eudicot species are in the *AN2* subgroup. In comparison, in monocots, the *R2R3-MYB* genes that regulate anthocyanin pigmentation in vegetative and reproductive organs of Poaceae (Gramineae) species and orchid *Oncidium* flowers all belong to the *C1* subgroup [23,26].

The transcription profiles of anthocyanin biosynthesis genes during flower bud development have been clearly identified in several floral crops [27–30]. Because of the transcription profiles, biosynthesis genes are divided into 2 groups: early genes and late genes. Early genes include *CHS* and *CHI*, which are necessary for anthocyanin biosynthesis and flavone or flavonol biosynthesis, while late genes include *DFR* and *ANS*, which are necessary for anthocyanin biosynthesis. Early genes are usually transcribed coordinately during the early bud development stages to produce flavones or flavonols, whereas both early and late genes are transcribed coordinately during late stages to produce anthocyanins [27–30]. In eudicots, the transcription of late genes is controlled by the R2R3-MYBs of AN2 subgroup [31]. For example, AN2 in petunia [32,33], ROSEA1 in snapdragon (*Antirrhinum majus*) [34], and GtMYB3 in gentian (*Gentiana triflora*) [35] mainly regulate the transcription of late genes. In contrast, in monocots, the R2R3-MYB proteins of the C1 subgroup, such as OgMYB1 in *Oncidium* [26] and ZmC1 in maize [36–38], regulate the transcription of both early and late genes [31]. However, it is not clear whether this difference in the regulation of early and late genes is due to the MYB subgroups or divergence between monocots and eudicots.

In Asiatic hybrid lilies, LhMYB12 and LhbHLH2 control anthocyanin biosynthesis in flower tepals [39,40]. LhMYB12 is the only R2R3-MYB of the AN2 subgroup that has been isolated in monocots, until now. The transient expression of LhMYB12 and LhbHLH2 induced the transcription of *LhCHS* and *LhDFR* after bombardment into non-pigmented bulb scales of lilies [40], suggesting that LhMYB12 regulates the transcription of both early and late genes. However, it is not clear whether LhMYB12 and LhbHLH2 directly bind to the promoter region of these anthocyanin biosynthesis genes to activate transcription. In addition, only *CHS* and *DFR* genes have been isolated in Asiatic hybrid lilies [41]; hence, more precise examination using more biosynthesis genes is necessary to determine the coordinated expression of LhMYB12 and anthocyanin biosynthesis genes.

In this study, we examined the accumulation of anthocyanin-related compounds in flowers of Asiatic hybrid lilies. cDNA for *PAL*, *F3H*, *F3′H*, and *ANS* genes was newly isolated, and the transcription profiles of *LhMYB12*, *PALs*, and anthocyanin biosynthesis genes were investigated during flower development. In addition, the effects of LhMYB12 on the activation of *PAL*, *CHS*, *F3H*, and *DFR* promoters were examined using a dual luciferase assay. Our results exhibit that (1) pigment accumulation in lily tepals is unique compared to flowers of other species and (2) LhMYB12 regulates the transcript accumulation of both early and late genes for anthocyanin biosynthesis.

2. Materials and methods

2.1. Plant materials

Asiatic hybrid lily cultivars 'Montreux' (pink-flowered), 'Landini' (chocolate brown-flowered), 'Silver Stone' (white-flowered), 'Navona' (white-flowered), 'Mirella' (orange-flowered), and 'Connecticut King' (yellow-flowered) were used. Orange and yellow pigments in 'Mirella' and 'Connecticut King', respectively, are caused by carotenoids. The tepals of 'Montreux' and 'Landini' accumulate anthocyanin pigments, with the dark red spots in the basal parts also containing anthocyanin pigments. The inner tepal segments used in this study did not include anthocyanin spots. 'Silver Stone', 'Navona', 'Mirella', and 'Connecticut King' do not accumulate anthocyanins in the tepals, and do not have spots. The flowers were divided into 9 developmental stages: stage (St) 1–St 5 and 1–4 days post anthesis (dpa). Stages 1–5 were determined on the basis of tepal pigmentation or flower shape: St 1, anthocyanin pigments were invisible; St 2, spots became visible; St 3, tepal pigmentation began; St 4, one day before anthesis; and St 5, the flower opened [41]. The plants were grown in the experimental farm of Hokkaido University, Sapporo, Japan.

2.2. Analysis of anthocyanin and related compounds in tepals

Anthocyanins and related compounds were extracted by washing the inner tepal segments twice with 10 ml of 10% acetic acid per gram fresh weight (FW). The extract solution of each sample was analysed by high-performance liquid chromatography (HPLC),