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Author(s)	許, 揚昕
Citation	北海道大学. 博士(農学) 甲第12704号
Issue Date	2017-03-23
Doc URL	http://hdl.handle.net/2115/65876
Type	theses (doctoral - abstract of entire text)
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学 位 論 文 の 要 約

Yang-hsin Hsu (許 揚昕)

Title

Analyses of anthocyanin modification pathway and glycosyltransferase genes involved in flower coloration in *Lobelia erinus*

(ロベリアの花色に関わるアントシアニン修飾経路および配糖化酵素の解析)

Introduction

The color plays important roles in the plant kingdom for attracting pollinators and seed dispersers, and it is also the most conspicuous horticultural trait for breeders as well as consumers. Anthocyanins, a subgroup of flavonoids, display the widest range of color from orange-red to violet-blue. From only six basic aglycones (pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin), anthocyanidins have diversified into more than 700 kinds in nature (Santos-Buelga *et al.*, 2013), through family- or species-specific modifications including glycosylations, acylations and methylation. The modifications of flavonoids are related to their characteristics, such as solubility, subcellular translocation, coloration, flavor and even the biomedical properties (Gachon *et al.*, 2005; Tanaka *et al.*, 2008; Zhao *et al.*, 2014); therefore, the studies on the catalytic enzymes would be applicable for engineering agriculturally beneficial traits of flavonoids in horticultural crops. Here, I used *Lobelia erinus*, which accumulates highly glucosylated and acylated anthocyanins in the petals to study the glycosyltransferases catalyze the modifications at different hydroxyl groups of anthocyanins and their involvement in flower coloration.

Results and Discussions

1. Modification pathway of Lobelinins in two cultivars, ‘Aqua Blue’ and ‘Aqua Lavender’

The anthocyanin compositions of two *Lobelia* cultivars, blue-flowered ‘Aqua Blue’ (AB) and mauve-flowered ‘Aqua Lavender’ (AL), were analyzed using HPLC and UPLC/TOF MS. The two major anthocyanins in AB extract were consistent with Lobelinin A [delphinidin

3-*O*-(6-*O*-(4-*O*-*p*-coumaroyl- α -L-rhamnosyl)- β -D-glucosyl)-5-*O*-(6-*O*-malonyl)- β -D-glucosyl-3'-*O*-(6-*O*-caffeoyl)- β -D-glucosyl-5'-*O*-(6-*O*-caffeoyl)- β -D-glucoside, molecular weight (mw) 1654.4] and Lobelinin B (delphinidin 3-*O*-(6-*O*-(4-*O*-*p*-coumaroyl- α -L-rhamnosyl)- β -D-glucosyl)-5-*O*-(6-*O*-malonyl)- β -D-glucosyl-3'-*O*-(6-*O*-caffeoyl)- β -D-glucosyl-5'-*O*-(6-*O*-feruloyl)- β -D-glucoside, mw 1668.4), respectively, which were previously identified as the major anthocyanins in the bluish violet petals of *L. erinus* (Kondo *et al.*, 1989). In contrast, the major anthocyanin in AL was consistent with authentic delphinidin 3-*O*-glucoside (Dp3G) (mw 465.4), and no peaks in the mass chromatograms corresponded to further glycosylated and/or acylated delphinidin intermediates of Lobelinins. Furthermore, neither Lobelinin A nor Lobelinin B was detected from AL. These results indicate that the difference in flower color in AL is due to the absence of rhamnosylation and acylation at the glucose moiety at the 3-hydroxy group on the C-ring as well as glucosylation and malonylation at the 5-hydroxy group on the A-ring and glucosylations and acylations at the 3'- and 5'-hydroxy groups on the B-ring of delphinidin.

To unveil the modification pathway of Lobelinins and which modification reactions are responsible for the differences in flower color between AB and AL, the modification activities in flowers were investigated *in vitro*. Crude proteins were extracted from the flower buds at developmental stage 3 of AB and AL, and the catalytic reactions were analyzed by MALDI-TOF MS and HPLC. The glucosylation at the 3-hydroxy group on delphinidin (Dp) were first examined using UDP-glucose as a sugar donor. The crude proteins from AB and AL transformed Dp to Dp3G, indicating that both cultivars possess delphinidin 3-*O*-glucosyltransferase activities. In the MALDI-TOF MS and HPLC analysis, there were no signals corresponding to further glucosylated products of Dp3G: delphinidin diglucoside (i.e., delphinidin 3,5-*O*-diglucoside, delphinidin 3,3'-*O*-diglucoside), delphinidin triglucoside (i.e., delphinidin 3,5,3'-*O*-triglucoside, delphinidin 3,3',5'-*O*-triglucoside) or delphinidin 3,5,3',5'-*O*-tetraglucoside. These results suggest that no further glucosylation occurred toward Dp3G, and a rhamnosylation of Dp3G precedes other glucosylations for Lobelinin synthesis.

I next examined rhamnosyltransferase activities from Dp3G to delphinidin 3-*O*-(6-*O*- α -L-rhamnosyl)- β -D-glucoside (i.e., delphinidin 3-*O*-rutinoside, Dp3RG) in AB and AL. The sugar donor, UDP-rhamnose, for this reaction is not commercially available. I thus

used the native UDP-rhamnose synthase in *Lobelia*, presumably similar to RHM2/MUM4, the multidomain protein that converts UDP-glucose to UDP-rhamnose in *Arabidopsis* (Oka *et al.*, 2007). UDP-rhamnose was first synthesized by reacting UDP-glucose with NADPH, NAD⁺ and crude proteins isolated from *L. erinus* cultivar AB, and then performed phenol-chloroform extraction to remove proteins. The enzyme-free UDP-rhamnose preparation was used in the second reaction to detect rhamnosyltransferase activities in crude protein of AB or AL with Dp3G. Dp3RG was detected from the resulting reaction catalyzed by crude protein from AB in MALDI-TOF MS and HPLC analysis. However, Dp3RG was not detected from the reaction catalyzed by crude proteins from AL, indicating the lack of rhamnosyltransferase activity to produce Dp3RG in AL.

In addition to rhamnose residue, Dp3G (the dominant anthocyanin in AL) also lacks several glucose residues and acyl residues compared to Lobelinins, I thus further examined other enzymatic activities against Dp3RG in crude protein from AL. Using the crude protein from AL, reactions from Dp3RG to delphinidin 3-*O-p*-coumaroylrutinoside (Dp3CRG) were examined. In the presence of *p*-coumaroyl-CoA, Dp3RG was successfully converted to Dp3CRG. In addition, delphinidin 3-*O-p*-coumaroylrutinosyl-5-*O*-glucoside (Dp3CRG5G) was formed in the presence of *p*-coumaroyl-CoA and UDP-glucose. Next, by reacting with malonyl-CoA, Dp3CRG5G was malonylated into delphinidin 3-*O-p*-coumaroylrutinosyl-5-*O*-malonylglucoside (Dp3CRG5MG). Finally, UDP-glucose-dependent glucosylations of the B-ring were carried out to form delphinidin 3-*O-p*-coumaroylrutinosyl-5-*O*-malonylglucosyl-3'-*O*-glucoside (Dp3CRG5MG3'G) and Dp3CRG5MG3'G5'G *in vitro*. Same results were obtained with the use of crude protein from AB. Interestingly, 5-*O*-glucosylation activities of the crude proteins from AB and AL were specific toward Dp3RCG; Dp3G or Dp3RG did not react with UDP-glucose. In addition, 3',5'-*O*-glucosyltransferase also exhibited substrate specificity only toward Dp3CRG5MG. The results showed that Lobelinins were synthesized from Dp3G in a single pathway in AB; after the glucosylation at the 3-hydroxyl group of delphinidin, the first stable anthocyanin, Dp3G, was conjugated with rhamnosyl and coumaroyl residues at glucosyl moiety, followed by glucosylation and malonylation the 5-hydroxyl group, and ended with the two glucosylations and two acylations at the 3'- and 5'-hydroxyl groups. In AL, only the

rhamnosyltransferases to synthesize Dp3RG from Dp3G were deficient, while other modifications were also carried out *in vitro* using crude protein from AL. These results suggest that because the pathway is executed in a stepwise manner, the lack of only rhamnosyltransferase activity in AL would terminate the flow of the modifications, resulting in Dp3G accumulation and the flower color variation.

2. Identification of rhamnosyltransferase involved in Lobelinin modification

The anthocyanin rhamnosyltransferase genes in AB and AL were then cloned using the degenerate primers designed basing on sequences of other characterized rhamnosyltransferases, 3RT in *Petunia hybrida* (Kroon *et al.*, 1994; Brugliera *et al.*, 1994) and Cs1,6RhaT in *Citrus sinensis* (Frydman *et al.*, 2013). Two candidate genes were obtained from the cDNA library of AB, namely *ABRT2* and *ABRT4*. The 1419-bp open reading frames of *ABRT2* and *ABRT4* encoded 472 amino acids, and they shared 95% identity in cDNA sequences and 96% identity in amino acid sequences with each other. The phylogenetic tree constructed with other flavonoid glycosyltransferases showed that *ABRT2* and *ABRT4* clustered with the branch-forming GTs. In addition, *ABRT2* and *ABRT4* formed a subcluster with rhamnosyltransferases that catalyze the rhamnosylation of flavonoid 3-*O*-glucoside at the hydroxy group on C6 of glucose moiety. In this subcluster, *ABRT2* had 61% amino acid similarity with 3RT in *Petunia hybrida* (Kroon *et al.*, 1994; Brugliera *et al.*, 1994), 60% similarity with Cs1,6RhaT in *Citrus sinensis* (Frydman *et al.*, 2013) and 53% similarity with UDP- β -L-rhamnose: flavonol 3-*O*-glucoside α -1,6-L-rhamnosyltransferase in *Glycine max* (Rodas *et al.*, 2014). This result suggest that *ABRT2* and *ABRT4* would possibly function as the 1 \rightarrow 6 rhamnosyltransferases that responsible for Lobelinin synthesis.

Compared with *ABRT2* and *ABRT4*, a homologous clone obtained from AL (referred to as *ALRT1*) was 1414-bp long and had a five-base deletion at nucleotide position 740. A conserved motif in plant UGTs, the plant secondary product glycosyltransferase (PSPG) box, is involved in the binding of UDP-sugar and thus is essential for the enzymatic activity of GTs (Shao *et al.*, 2005). In *ABRT2* and *ABRT4*, the PSPG box was located at amino acid residues 343 to 386. The *ALRT1* with five-base deletion generated a truncated polypeptide that consisted of 280 amino acids without the PSPG box, suggesting functional loss of

ALRT1. The mutation in the rhamnosyltransferase in AL supports the assumption that *ABRT2* and *ABRT4* might be involved in color development in *L. erinus*.

The expression profile of *ABRT2* and *ABRT4* in stems, leaves and flowers at four developmental stages was examined by qRT-PCR. *ABRTs* transcripts accumulated at the highest level in the flower buds at an early stage, and then gradually decreased during flowering. In addition, *ABRT2* and *ABRT4* expressed substantially lower in vegetative tissues than in the floral tissues, implying the functional importance of *ABRTs* in flowers.

The rhamnosylation velocities of *ABRT2* and *ABRT4* were examined to confirm whether *ABRTs* catalyze the transfer of rhamnosyl moiety to flavonoid substrates. The sugar donor, UDP-rhamnose was prepared using a soluble protein fraction derived from RHM2/MUM4-expressing *E. coli*. The UDP-rhamnose preparation mixture and purified recombinant *ABRTs* were reacting with various flavonoid substrates including delphinidin 3-*O*-glucoside. The resulting products were then identified and quantified using HPLC with authentic standards. *ABRT4* possessed UDP-rhamnose-dependent rhamnosyltransferase activities against flavonoid monoglucosides, cyanidin 3-*O*-glucoside, pelargonidin 3-*O*-glucoside, malvidin 3-*O*-glucoside, apigenin 7-*O*-glucoside and quercetin 3-*O*-glucoside, regardless of the positions of glucose moieties on aglycone (3- or 7-hydroxyl group). However, it did not use flavonoid diglucoside, delphinidin 3,5-*O*-diglucoside as a substrate. *ABRT2* exhibited similar substrate preference, except that activity with malvidin 3-*O*-glucoside was not detectable. In the sugar donor preference assay, no glucosylated product of Dp3G was detected from the reactions catalyzed by *ABRT2* or *ABRT4*, suggesting that *ABRTs* do not use UDP-glucose as sugar donor or that the activity toward UDP-glucose was lower than the detectable level. These results suggest that both *ABRT2* and *ABRT4* have UDP-rhamnose-dependent rhamnosyltransferase activities toward a variety of flavonoid substrates. In the sequence alignment of *ABRT2* and *ABRT4*, there were nineteen different amino acids, and the amino acids of *ABRT2* differed from that of *ABRT4* may result in the reduction of its' activity.

Because crystal structure analyses on the plant glycosyltransferases have been reported only for UDP-glucose-dependent glucosyltransferases (Shao *et al.*, 2005; Offen *et al.*, 2006; Li *et al.*, 2007; Brazier-Hicks *et al.*, 2007; Hiromoto *et al.*, 2013), the amino acid residues that

are essential for the function of UDP-rhamnose-dependent rhamnosyltransferases are still unknown. By sequence comparison and homology-based structure modeling, the amino acid residues essential for the enzyme functions in branch-forming rhamnosyltransferases would be presumed. VvGT1 is a flavonoid 3-*O*-glycosyltransferase exhibiting broad sugar-donor specificity except for UDP-rhamnose. The three-dimensional structure of VvGT1 revealed that the hydroxyl group at C4 and C6 of glucose interacts with Trp353 and Thr141 in VvGT1, respectively (Offen *et al.*, 2006). In a multiple sequence alignment of ABRT2/4 and other UGTs, I found that the corresponding sites of Trp353 and Thr141 were replaced with Phe (364) and Val (142), respectively, and even in all other branch-forming rhamnosyltransferases. It is likely that Phe364 can better accommodate the hydroxyl group at C4 of rhamnose that confers the configuration differed from glucose. The replacement of Thr (polar side-chained amino acid) at 141 with Val (hydrophobic side-chained amino acid) is conceivable because rhamnose lacks a hydroxyl group at C6. A similar replacement of Thr with Ala (hydrophobic side-chained amino acid) has also been described for *Arabidopsis* flavonol 3-*O*-rhamnosyltransferase (UGT78D1) (Offen *et al.*, 2006). For further confirmation, the homology-based structure model of ABRT4 was constructed using SWISS-MODEL (Biasini, *et al.*, 2014). Comparing a model structure of ABRT4 bound with β -L-rhamnose and the crystal structure of UGT71G1 bound with UDP-glucose, which has high specificity for UDP-glucose as sugar donor, revealed that in UGT71G1 the hydroxy group at C6 of glucose interacts with Thr143, while ABRT2/4 possess Val142 at the equivalent position, confirming the hypothesis that Val142, together with Phe143 appear to form hydrophobic environment for accommodating the methyl group of rhamnose. The model structure of ABRT4 also shows that Lys383 interacting with the axial hydroxy group at C2 of rhamnose might also participates in UDP-rhamnose recognition. Another characteristic amino residue distant from the sugar donor-binding domain in ABRT2/4 is Met15, which is also conserved among all the other characterized branch-forming GTs (Yonekura-Sakakibara *et al.* 2012); however, their functions remained unclear. Further experiments such as point mutagenesis and crystal structure determination of the branch-forming GTs will be needed to confirm and elucidate the amino acids essential for the enzyme functions.

To examine the *in vivo* activity of ABRT2 and ABRT4 and verify that only the deficiency

of ALRT1 causes the flower color variation, I performed the complementation tests. The full-length open reading frames of *ABRT2* and *ABRT4* were cloned into the pIG121-Hm vector and introduced into AL through *Agrobacterium*-mediated transformation procedure. AL was transformed with pIG121-Hm-GFP as a control. Six *ABRT2*-expressing lines, five *ABRT4*-expressing lines and eight GFP-expressing lines were obtained. The expression of *ABRT2* or *ABRT4* in each transgenic plant was confirmed using allele-specific PCR to discriminate transcripts between endogenous *ALRT1* and transformed *ABRT2* or *ABRT4*. The transgenic AL plants that expressed *ABRT2* or *ABRT4* displayed blue flowers, while the control plants transformed with GFP remained mauve similar to the non-transformed AL. Both the HPLC chromatograms of the flower anthocyanins extracted from *ABRT2* and *ABRT4* transgenic plants were similar to AB, and Lobelinin A and Lobelinin B were detected at the similar level. These results indicate that both *ABRT2* and *ABRT4* function as rhamnosyltransferase *in vivo* and contribute to the blue color of flowers in *L. erinus*.

The subcellular localization of *ABRT2* and *ABRT4* was investigated using AL transformed with constructs of GFP fusion *ABRT2* or *ABRT4* at C or N terminus. The flowers of the transgenic plants expressing fusion proteins displayed blue color, suggesting that GFP tag does not interfere the *in vivo* function of *ABRTs* neither at C nor N terminus. The flower petals were sliced and observed using confocal microscope. Anthocyanins emitting autofluorescence localized at vacuole and at anthocyanin vacuolar inclusions (AVI)-like structures in the petal epidermal cells of GFP-expressing AL. AVIs, 3- to 10- μ m diameter densely packed anthocyanin deposits, have been observed in many plant species, and they effect the flower coloration by enhancing both intensity and blueness. This AVI-like structure was not observed in AB or *ABRT*-complemented lines, indicating that the formation of AVI-like vesicles would be related to the modifications of anthocyanins. GFP fusion *ABRTs* mainly localized to the cytosol. These results consisted with other studies which suggested that anthocyanidin aglycones were synthesized on the cytosolic side of endoplasmic reticulum (ER) membrane (Bowles *et al.*, 2006), underwent modifications at cytoplasm, and were transported to vacuole for accumulation, while exceptions utilizing acyl-glucose instead of UDP-glucose as sugar donor have been reported to be carried out in vacuoles in some species (Nishizaki *et al.*, 2013). Interestingly, the GFP fluorescence of *ABRT* fusion proteins was

also observed in some small vesicles probably locating in the vacuole. Anthocyanins were transported to vacuole by multiple routes, and one of the process reminiscent of microautophagy has been reported by Chanoca *et al.* (2015). They suggested that the cytoplasmic anthocyanin aggregates are directly engulfed by the vacuolar membrane resulting in the presence of single-membrane vesicles in vacuole like autophagic bodies. It is possible that the ABRT-contained vesicle would be formed by the autophagic process. Further studies on the subcellular localization of the vesicles using marker proteins will provide more information about how ABRT involved in Lobelinin biosynthesis and their transport.

Conclusion

Although a few studies have reported that rhamnosylation has a substantial effect on flower color, there had been no reports on the effect of rhamnosylation on blue coloration. Here, I demonstrated the significance of rhamnosyltransferases in the molecular mechanism for blue coloration in *L. erinus*; rhamnosylation is prerequisite for other modifications in the Lobelinin synthetic pathway and thus essential for the blue pigmentation. This study could be possibly applied to molecular breeding for novel flower colors.

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