Detection of UDP–glucose:*cyclo*-DOPA 5-*O*-glucosyltransferase activity in four o'clocks (*Mirabilis jalapa* L.)

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Received 5 March 2004; revised 15 April 2004; accepted 15 April 2004

Available online 24 May 2004

Edited by Richard Cogdell

Abstract Although a pathway for betacyanin biosynthesis has been postulated, most of the catalytic steps have not yet been identified or demonstrated with biochemical evidence. In the postulated pathway, the glucose moiety of betanin is conjugated to the aglycone, betanidin, because the glucosyltransferase (GT) activity that produces betanin has been reported and its cDNA isolated. However, another pathway for betacyanin biosynthesis is proposed in which betanin is formed by GT acting at the 5,6-dihydroxyindoline-2-carboxylic acid (*cyclo*-DOPA) step, followed by condensation of the product with betalamic acid. Here, we show that GT activity acts upon *cyclo*-DOPA in the betacyanin synthetic pathway. A crude extract from the petals of four o'clocks (*Mirabilis jalapa* L.) was mixed with *cyclo*-DOPA and UDP–glucose. After the reaction was stopped with phosphoric acid, the product was chemically reacted with betalamic acid. In the final reaction mixture, betanin formation was confirmed by HPLC analysis, demonstrating *cyclo*-DOPA 5-*O*-glucosyltransferase activity. This activity was correlated with the accumulation of betanin during the development of four o'clock flowers and was detected in another five species of Centrospermae. These results indicate that the glucose moiety of betanin is introduced at the *cyclo*-DOPA step, which is followed by condensation with betalamic acid, and not at the betanidin aglycone step.

Keywords: Betacyanin; 5,6-Dihydroxyindoline-2-carboxylic acid; Glucosyltransferase; Centrospermae

1. Introduction

Recent advances in elucidating the synthetic pathways of plant pigments have been made in parallel with advances in biochemical and molecular biological techniques. In particular, the anthocyanin and carotenoid biosynthetic pathways have been well-characterized biochemically and at the molecular level [1,2]. One of the important red pigments in the plant kingdom, apart from anthocyanin, is betacyanin. The postulated betacyanin biosynthetic pathway is shown in Fig. 1. Although most enzymes involved at each catalytic step in the anthocyanin and carotenoid biosynthetic pathways have been identified at the molecular level, few of the enzymes and genes involved in betalain biosynthesis have been identified. The latest study identified the cDNA encoding the dihydroxyphenylalanine (DOPA) dioxygenase (AJ580598) involved in betalain biosynthesis.

Betalains are water-soluble nitrogenous pigments of the order Centrospermae, and include the red-violet betacyanins and yellow betaxanthins. They are considered to be iminium derivatives of betalamic acid with 5,6-dihydroxyindoline-2-carboxylic acid (*cyclo*-DOPA) and amino acids or amines. All betacyanins are composed of aglycone (betanidin) and glycosyl moieties, and some betacyanins are modified with acyl groups, whereas betaxanthins are not. In the betacyanin biosynthetic pathway, both *cyclo*-DOPA and betalamic acid are thought to be synthesized from DOPA, and then the aglycone, betanidin, is formed from the conjugation of these two moieties (Fig. 1, shown with thin arrows). The enzymes involved in the synthetic pathway of betanidin aglycone have not been identified, except for DOPA dioxygenase as mentioned above, whereas the activity of the glucosyltransferase (GT) that transfers the glucose moiety to betanidin aglycone has been reported [3]. Uridine diphosphate (UDP)–glucose:betanidin 5-*O*-GT activity was detected in the cells of a *Dorotheanthus bellidiformis* suspension culture and a cDNA encoding that enzyme was isolated [4]. However, a few reports have proposed another pathway, in which *cyclo*-DOPA might be glycosylated, followed by its condensation with betalamic acid (Fig. 1, shown with thick arrows). Accumulation of large amounts of *cyclo*-DOPA glucoside in young red beet has been verified [5] and was detected in beetroot peel [6]. The results of feeding experiments with *cyclo*-DOPA and *cyclo*-DOPA glucoside in *Celosia plumose* [7] showed that *cyclo*-DOPA glucoside is a more efficient precursor in amaranthin biosynthesis than betanin or betanidin. These reports imply that betanin (or amaranthin) is formed by GT acting upon *cyclo*-DOPA, followed by condensation of the product with betalamic acid.

In this study, we report *cyclo*-DOPA GT activity from four o'clocks and other plant species of the order Centrospermae, and discuss the betacyanin biosynthesis pathway via *cyclo*-DOPA glucoside.

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Abbreviations: DOPA, dihydroxyphenylalanine; HPLC, high-performance liquid chromatography; *cyclo*-DOPA, 5,6-dihydroxyindoline-2-carboxylic acid

0014-5793/$22.00
doi:10.1016/j.febslet.2004.04.097

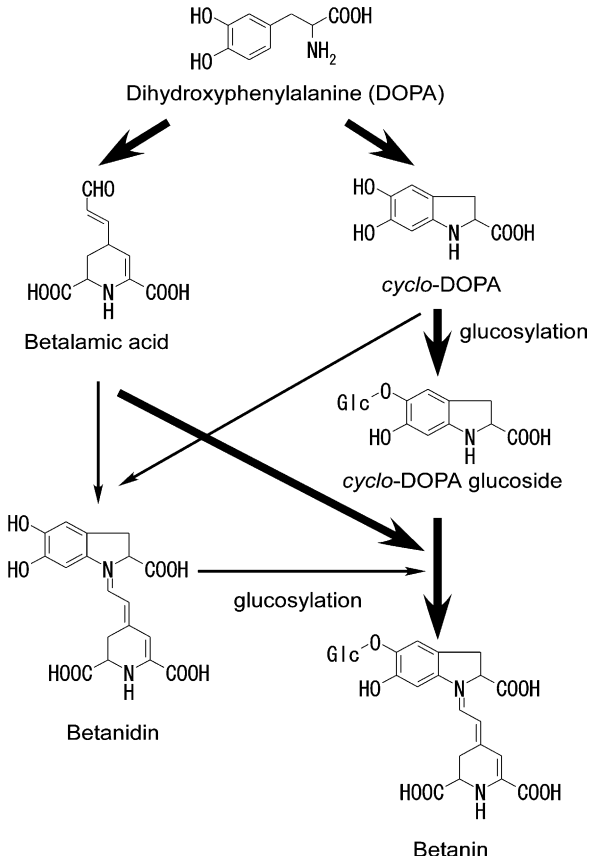

Fig. 1. Scheme of betanin biosynthesis. Thin lines represent the traditional pathway and thick lines represent the novel pathway proposed from the results presented in this paper.

2. Materials and methods

2.1. Plant materials

Petals of four o'clocks and stems of poke-weed (*Phytolacca americana*) were collected in the yard of Tokyo University of Agriculture and Technology (Koganei, Tokyo, Japan) and stored at −80 °C after freezing in liquid nitrogen. Other plants, *Portulaca grandiflora*, feather cockscomb (*Celosia cristata*), Christmas cactus (*Schlumbergera truncata* sp. "Eva"), carnation (*Dianthus caryophyllus*), and tulip (*Tulipa gesneriana* L.) were purchased from flower shops, and red beet (*Beta vulgaris*) was bought from a market.

2.2. Substrates and chemicals

Betanin (red beet extract), L-DOPA, and dopamine were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Sugar donors, adenosine diphosphate (ADP)–, guanosine diphosphate (GDP)–, thymidine diphosphate (TDP)–, and UDP–glucose, UDP–galactose, UDP–glucuronic acid, and UDP–*N*-acetylglucosamine were purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA). Cyanidin was obtained from the Funakoshi Co. Ltd. (Tokyo, Japan). Tyrosine was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

cyclo-DOPA was prepared by the enzymatic oxidation of DOPA to dopachrome using tyrosinase (Sigma–Aldrich) and subsequent reduction with ascorbic acid [8], followed by neutralization with the addition of 1 N NaOH for the enzymatic assay. Betalamic acid was obtained by hydrolyzation of betanin, as previously reported [8]. Betanidin and isobetanidin were synthesized from betanin and isobetanin, respectively, treated with almond β-glucosidase (Sigma–Aldrich).

2.3. Protein extract preparation from four o'clock plants and other plant materials

Petals (1 g) of four o'clocks or other plants were ground using a mortar and pestle in liquid nitrogen and then thawed in 10 ml of extraction buffer (0.1 M potassium phosphate [pH 7.5], 0.5 M Na-ascorbate and 7 mM 2-mercaptoethanol) containing 5% (w/v) polyvinylpolypyrrolidone (Sigma–Aldrich) on ice. Dowex 1 × 4 resin (1 g of the Cl^- form, equilibrated with extraction buffer) was added and the mixture was stirred for 2 min. It was then filtered through a cloth and centrifuged at 20 000 × *g* for 5 min. An aliquot of the supernatant (200 μl) was added to 800 μl of $(NH_4)_2SO_4$ (4.1 M) and the mixture was incubated on ice for 5 min, then centrifuged at 20 000 × *g* for 5 min. The precipitate was dissolved in 50 μl of assay buffer (0.1 M potassium phosphate [pH 7.5] and 7 mM 2-mercaptoethanol) and desalted using a MicroSpin G-25 column (Amersham Biosciences Corp., Piscataway, NJ).

2.4. GT activity assay

The reaction mixture consisted of 50 μl of the *cyclo*-DOPA synthesis mixture described above, 1 mM sugar donor, and 20 μl of the protein solution, prepared as described above. For the other substrates, the reaction mixture contained 50 mM potassium phosphate buffer (pH 7.5), 1 mM UDP–glucose, 7 mM 2-mercaptoethanol, and 0.5 mM substrate (tyrosine, DOPA, or dopamine) dissolved in water. The mixture was incubated for 30 min at 30 °C, after which 4 μl of 20% phosphoric acid was added to terminate the enzymatic reaction. Then, the betalamic acid (50 μl) synthesized above was added to the reaction mixture for the chemical condensation reaction. For betanidin GT, the enzyme assay was performed according to Heuer et al. [9].

The reaction products were analyzed by reversed-phase, high-performance liquid chromatography (HPLC) using Beckman System Gold apparatus (128 diode array detector module, 126 pumps; Beckman Coulter Inc., Fullerton, CA). The HPLC was equipped with a Wakopak Handy ODS column (i.d. 4.6 mm × 250 mm; Wako Pure Chemical Industries Ltd.). Reaction products were separated by linear-gradient elution over 15 min from 10% to 50% solvent A (1.5% phosphoric acid in water) and isocratic elution with solvent B (80% methanol) for 5 min (flow rate, 1 ml/min).

The reaction mixture for cyanidin GT contained 50 mM potassium phosphate buffer (pH 7.5), 1 mM UDP–glucose, 7 mM 2-mercaptoethanol, 0.2 mM cyanidin dissolved in 2-methoxyethanol (Wako Pure Chemical Industries Ltd.), and the crude enzyme preparation in a final volume of 50 μl. The reaction mixture was incubated at 37 °C for 5 min and the reaction was terminated by the addition of 2.5 μl of 20% phosphoric acid. Reaction products were separated by linear-gradient elution in 20 min from 20% to 80% solvent A (1.5% phosphoric acid in water) and isocratic elution with solvent B (80% methanol) for 10 min (flow rate, 1 ml/min).

2.5. Quantification of the relative amounts of betacyanin in plant materials

Harvested tissues were frozen in liquid nitrogen and lyophilized. Betacyanins were extracted in 500 μl of extraction buffer (1.5% phosphoric acid and 80% methanol) from 5 mg of the tissues. The relative betanin concentrations were determined by HPLC analysis using the protocol described above.

3. Results

3.1. Detection of cyclo-DOPA GT activity in four o'clocks

The crude extract prepared from the petals of four o'clocks was incubated with both *cyclo*-DOPA and UDP–glucose for 30 min at 30 °C and the enzymatic reaction was terminated by the addition of phosphoric acid followed by chemical condensation with betalamic acid under acidic conditions. The HPLC elution profile showed that the reaction products contained betanin and isobetanin (Fig. 2A), which was confirmed by co-chromatography with betanin, isobetanin, betanidin, and isobetanidin. On the other hand, when UDP–glucose was omitted from the enzyme reaction mixture, only aglycones of betanin and isobetanin were detected, whereas betanin and isobetanin were not (Fig. 2B). These results suggest that *cyclo*-DOPA was glucosylated in the enzymatic reaction.

The relative betacyanin content and *cyclo*-DOPA GT activity in the petals of flowers during developmental stages, and

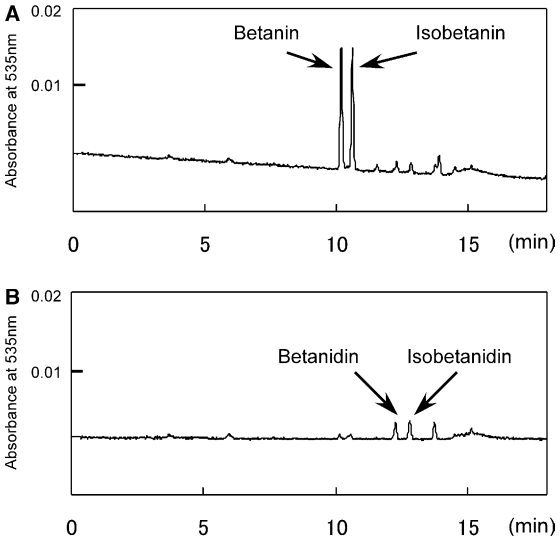

Fig. 2. HPLC analysis of the products of the *cyclo*-DOPA GT assay, which chemically reacted with betalamic acid. Chromatograms show the separated reaction products when the enzyme was mixed with UDP–glucose (A) or with no sugar donor (B).

in both leaves and stems, were measured (Fig. 3), revealing that *cyclo*-DOPA GT activity correlated with the accumulation of betanin in four o'clocks.

3.2. Substrate specificity of cyclo-DOPA GT

The substrate specificity of *cyclo*-DOPA GT was determined for four kinds of UDP–sugars, and several sugar donors, including ADP–, TDP–, and GDP–glucose (Table 1). UDP–glucose was the sugar donor most strongly preferred by *cyclo*-DOPA GT, although it also used UDP–galactose as substrate, whereas *cyclo*-DOPA GT did not use ADP–, CDP–, or GDP–glucose as substrate. When betanidin, tyrosine, DOPA, dopamine, or cyanidin was incubated in the GT reaction mixture as the sugar acceptor instead of *cyclo*-DOPA, no glycosylated products were detected (Table 1).

3.3. cyclo-DOPA GT activity in other plant species

Proteins were extracted from six other plants, all of which belong to the order Centrospermae: *P. grandiflora* (stem), feather cockscomb (flower), poke-weed (stem), and red beet (beetroot), which produce betacyanin, and carnation and tulip, which do not produce betacyanin. *cyclo*-DOPA GT activity was detected in all the betacyanin-producing plants, but was not detected in either of the non-betacyanin-producing plants, although cyanidin 3GT activity was detected in the latter (data not shown).

4. Discussion

Many GTs involved in flavonoid biosynthesis have been investigated biochemically and with molecular biological techniques in numerous previous studies (reviewed in [1]). Betanidin 5GT activity was detected in the cells of a *D. bellidiformis* suspension culture and in bracts of *Bougainvillea glabra* [10], but no betanidin 5GT activity has been found in other species. We did not detect betanidin 5GT activity in the flowers of *D. bellidiformis* (data not shown).

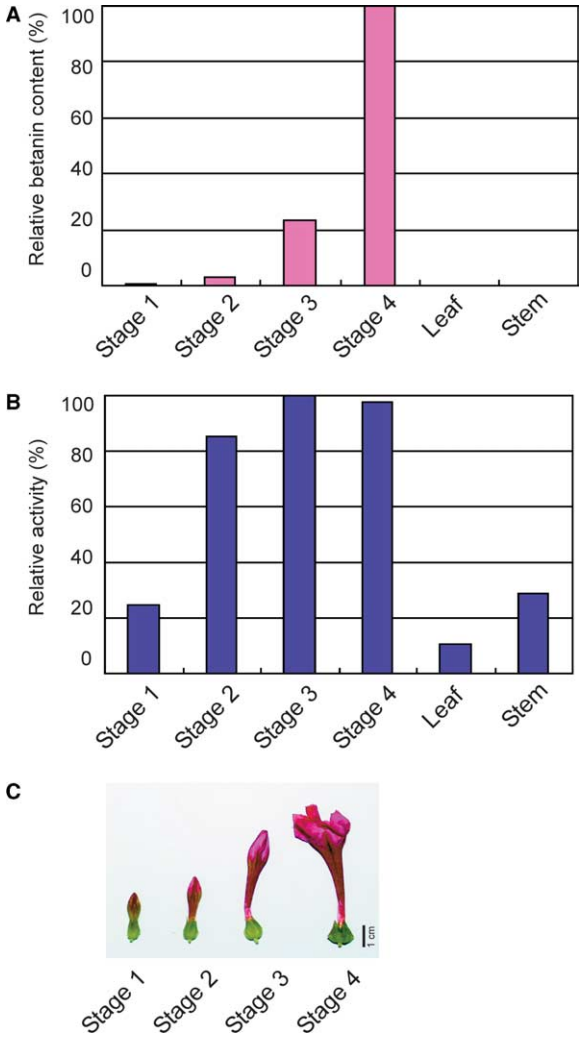

Fig. 3. Relative *cyclo*-DOPA GT activities (A) and betanin content (B) in flower, leaf, and stem of four o'clocks. Developmental stages 1–4 are shown in (C).

Table 1
Relative activity of the *cyclo*-DOPA 5-GT with various sugar donors and sugar acceptors

Sugar donor	Relative activity (%)
UDP-Glc	100.0
ADP-Glc	2.3
GDP-Glc	–
TDP-Glc	2.7
UDP-Gal	24.8
UDP-GlcUA	–
UDP-AG	1.4
Tyrosine	–
DOPA	–
Dopamine	–
Cyanidin	–
Betanidin	–

– below the limits of detection.

Because the aglycones of flavonoid/anthocyanin are extremely hydrophobic compounds, the glycosylation of flavonoid/anthocyanin is absolutely necessary to produce a hydrophilic moiety for transfer into and compartmentalization

within the vacuole. Because both betanidin and betanin are hydrophilic compounds, the moiety composed of betacyanin can be glucosylated at any step of the biosynthetic pathway.

Here, we identified *cyclo*-DOPA GT activity in four o'clock flowers and propose another route of betacyanin biosynthesis (Fig. 1, shown with thick arrows) that differs from the conventional pathway (Fig. 1, shown with thin arrows). This activity correlates with the accumulation of betanin during the development of petals (Fig. 3). The activity was also detected in leaves and stems, in which little betacyanin accumulates. Compared with the relationship between GT activity and the accumulation of betacyanin in petals, the activity in leaves and stems seems to be high, relative to the accumulated betacyanin. The synthesis and accumulation of betacyanin in leaves and stems may be regulated at other catalytic steps, e.g., at betalamic acid synthesis or *cyclo*-DOPA synthesis, which may dictate the content of betanin.

cyclo-DOPA GT activity was detected in another five species of the order Centrospermae that produce betacyanin, but GT activity for neither cyanidin nor betanidin was detectable. Furthermore, we also detected *cyclo*-DOPA 6-*O*-GT activity in a crude extract prepared from the epidermis of *Gomphrena grobosa* in which gomphrenin (betanidin-6-glucoside) accumulates (data not shown). Betanidin 5-*O*- and 6-*O*-GT activities were identified in proteins extracted from cell-suspension cultures of *D. bellidiformis* [3,9], together with cDNA encoding betanidin GTs [4]. Only these two reports of betanidin 5-*O*- and 6-*O*-GT activity in betacyanin-producing plants have been published [11], although many homologs of betanidin 5-*O*- and 6-*O*-GT have been deposited in the nucleotide databases. All of these show similarity in their nucleotide sequences to those of *D. bellidiformis* 5-*O*- and 6-*O*-GT cDNAs, although with no verification of their enzymatic activities. The most important and crucial point is that while it is undoubtedly true that the cells of a *D. bellidiformis* suspension culture express enzymes that transfer glucose to betanidin, it has not been demonstrated that these enzymes are involved in betacyanin synthesis in betacyanin-accumulating cells, especially in the cells of intact plants.

Carnation is classified in the order Centrospermae but does not synthesize betacyanin, although it does synthesize anthocyanin. *cyclo*-DOPA GT activity was not detected in carnation, but cyanidin 3GT activity was, which was also the case for tulip (Table 2). The apparent mutual exclusivity of anthocyanins and betalains in the Centrospermae has generated considerable taxonomic debate, but is still an important and unresolved question in plant science [12]. The fact that *cyclo*-DOPA GT activity is detected exclusively in the families of the order Centrospermae, except for Caryophyllales, may support their taxonomic classification on the basis of molecular data. Whereas betanidin GT is considered to be related to flavonoid GT [10], *cyclo*-DOPA does not resemble, in terms of chemical structure, any substances contained in plants, so that the molecular evolution of *cyclo*-DOPA GT may have proceeded via a completely different route from that of the flavonoid GTs. When a cDNA encoding *cyclo*-DOPA GT is identified, the information in the nucleotide and amino acid sequences will contribute to an elucidation of its molecular evolution.

Table 2
Detection of *cyclo*-DOPA GT activity in several species

	cyclo-DOPA 5GT	Cyanidin 3GT
Four o'clocks (Nyctaginaceae)	+	–
Portulaca (Portulacaceae)	+	–
Poke-weed (Phytolaccaceae)	+	n.d.
Feather Cockscomb (Amaranthaceae)	+	–
Bougainvillea (Nyctaginaceae)	+	n.d.
Christmas Cactus (Cactaceae)	+	n.d.
Carnation (Caryophyllaceae)	+	–
Chrysanthemum (Compositae)	+	–

– below the limits of detection.
n.d. not determined.

This is the first report demonstrating UDP–glucose: *cyclo*-DOPA 5-*O*-GT activity. We propose that betacyanin is synthesized via *cyclo*-DOPA glucoside, and not in a pathway via the aglycone, betanidin.

Acknowledgements: This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science (15657008), Sports and Culture of Japan and by the National Institute of Floricultural Science (NIFS), the National Agricultural Research Organization (NARO), and the Japan Food Chemical Research Foundation.

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