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CINNAMATE AND SHIKIMATE INCORPORATION INTO 3′,4′- AND 3′,4′,5′-HYDROXY SUBSTITUTED ANTHOCYANINS: ARE THERE ALTERNATIVE PATHWAYS?

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Key Word Index—*Petunia hybrida*; Solanaceae; anthocyanin accumulation; cinnamate-[^{14}C]-incorporation; shikimate-[^{14}C]-incorporation; flavonoid biosynthesis.

Abstract—The incorporation of shikimate-[^{14}C] and cinnamate-[^{14}C] into 3′,4′- and 3′,4′,5′-hydroxy substituted anthocyanins was studied in isolated petals of *Petunia hybrida*. According to the dilution values, the incorporation of shikimate-[^{14}C] was about 3–6 times better than that of cinnamate-[^{14}C]. However a comparison of the incorporation of the 2 precursors on a relative basis showed no significant differences in the relative proportions of the specific activities of the 3′,4′-dihydroxysubstituted cyanidin-3-monoglucoside and the 3′,4′,5′-trihydroxysubstituted delphinidin-3-monoglucoside. This result and the [^{14}C]-incorporation behaviour of the 3′-methoxy-4′-hydroxysubstituted peonidin-3-monoglucoside do not support the hypothesis that there are alternative pathways of flavonoid biosynthesis.

INTRODUCTION

Judged from dilution analysis, shikimate seems to be a better precursor for flavonoid biosynthesis than phenylalanine or cinnamate; hence a hypothesis of alternative pathways in the biosynthesis of phenylpropane derivatives has been put forward [1–5]. This hypothesis was corroborated by data concerning the biochemistry of cinnamic acids [6–9]. However, as was explicitly pointed out by Swain and Williams when reviewing these data [9], it has been assumed when interpreting the dilution analysis that uptake, transport, and poolsize were about equivalent for the different precursors tested although this was not investigated.

This prompted the following study for testing the hypothesis of alternative pathways. For the test an experimental setup was devised, with advantages compared to previous studies: (i) The precursors were fed directly to the isolated anthocyanin-bearing parts of petals of *Petunia hybrida*. This material has a large surface and a thickness of only a few cell layers. This gives an optimal contact for the uptake of the precursors and transport is practically eliminated. Moreover such application allows comparatively short incubation periods. (ii) In the petals of the genotype of *Petunia hybrida* used, the main anthocyanins are the 3-monoglucosides of cyanidin (=*cya*), delphinidin (=*del*), and peonidin (=*peo*). Thus in the same tissue the [^{14}C]-incorporation into a 3′,4′-dihydroxy-, a 3′-methoxy-4′,hydroxy-, and a 3′,4′,5′-trihydroxy-substituted anthocyanin could be simultaneously studied. Accordingly, on the one hand, differences in dilution could be attributed to processes other than uptake or transport while, on the other hand, if after shikimate-[^{14}C]- and cinnamate-[^{14}C]-feeding the relative proportion of labeling between the 3 anthocyanins should show no difference, then the interpretation of variation in dilution in favour of shikimate by alternative pathways could be considered as not being necessarily pertinent. Because, if according to hypothesis shikimate is a better precursor for

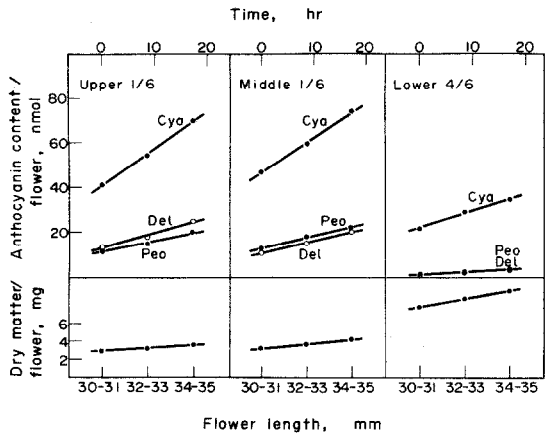

Fig. 1. The accumulation of cyanidin-, delphinidin-, and peonidin-3-monoglucoside and of dry matter in different parts of the petals of *Petunia hybrida* as a function of flower length and of time. The values are averages of two experiments with 25–40 petals per point.

Table 1. Accumulation of the anthocyanin-3-monoglucosides in isolated petals of *Petunia hybrida*

Number of expt*	Anthocyanin content (nmol/petal) 0 hr incubation *peo*	*cya*	*del*	25 hr incubation *peo*	*cya*	*del*
1	11	108	10	11	154	20
2	10	106	8	10	103	19
3	10	114	8	13	152	27
4	13	134	13	13	166	33
5	12	112	10	18	228	48
6	11	105	10	10	115	23
7	11	106	11	13	128	35
Average	11	112	10	13	149	29
Relative proportion	10	100	9	9	100	20
Δ absolute				+2	+37	+19
Δ%				+18	+33	+190

*The two Petri dishes in each experiment contained the corresponding halves of symmetrically bisected petals of 80 flowers 25–28 mm in length in 10 ml of H_2O at 5000 lx fluorescent white light and 25°.

3′,4′,5′-substituted flavonoids than cinnamate, then after shikimate-[^{14}C]-feeding the specific activity of *del* on a relative basis should be higher than that of *cya* and *peo*. In order to define the experimental system used, the accumulation of the anthocyanins was examined in petals of intact flowers *in situ*, flowers *in vitro*, and in isolated petals of *Petunia hybrida*.

RESULTS

Anthocyanin accumulation. In petals of intact flowers with a length of 19–35 mm *in situ* in the greenhouse, the accumulation of the 3 anthocyanins and of the dry matter is linear with flower length and with time (Fig. 1). There are, however, gradients in the distribution of the single anthocyanins and about 80% of the total anthocyanin content is in the upper 1/3 of the petals. Petals of intact flowers kept *in vitro* under fluorescent white light (5000 lx, 25°) for 25 hr increase in length from 27 to 37 mm, in dry matter from 9·7 to 10·7 mg, in *cya* content from 141 to 216, *del* content from 11 to 32, and *peo* content from 11 to 23 nMol per flower ($\bar{x}$, $n = 5$). Compared with the situation *in situ* the relative proportions of the accumulation of the 3 anthocyanins change in favour of *peo* by 38% and *del* by 88%. In isolated petals in the light (Table 1) the relative proportions differ again as compared with those of petals *in situ* as also with those of petals of intact flowers *in vitro*. The dry matter increases by 4%. In isolated petals in the dark over a 25 hr period the *cya* and *del* content remains unchanged ($\Delta_{max} < 5\%$), the *peo* content increases by 100%, and the dry matter decreases by 7% ($\bar{x}$, $n = 7$). These data show that the relative proportions of the accumulation rates depend significantly ($p < 0{\cdot}05$) on the respective status of the petals. This was similar to the results reported for the other frequently used experimental systems, the petals of *Impatiens balsamina* [10] and *Salpiglossis sinuata* [11]. With regard to the experiments reported here it is important to note that regardless of the conditions, the same kinds of anthocyanins accumulate, they are formed in relatively large amounts, and under any given condition they accumulate with sufficient reproducibility for comparative conclusions to be drawn (Table 1).

Influence of cinnamate and shikimate on anthocyanin accumulation. In isolated petals, cinnamate has no influence on anthocyanin accumulation and dry matter content up to a concentration of 1 mM ($\Delta_{max} < 5\%$ for concentrations of 0, 0·3 and 1·0 mM; $\bar{x}$, $n = 5$). At a concentration of 1·0 mM, shikimate increases the *del* accumulation specifically by $\Delta 20 \pm 7\%$; at a concentration of 3 mM, the accumulation of all 3 anthocyanins is inhibited by about 10%. The dry matter remains unchanged. In subsequent feeding experiments the

Table 2. The incorporation of cinnamate-[^{14}C] and shikimate-[^{14}C] into the anthocyanin-3-monoglucosides in isolated petals of *Petunia hybrida*

Number of expt*	Anthocyanin content (μmol) *peo*	*cya*	*del*	Specific activity (dpm/nmol) *peo*	*cya*	*del*
Incubation with cinnamate-[^{14}C]						
1	1·13	12·60	1·24	205	152	963
2	0·89	7·33	0·89	171	133	712
3	1·33	12·50	1·23	205	157	843
4	0·68	8·87	0·79	270	187	911
5	1·32	12·67	1·48	196	160	602
6	1·13	12·87	1·46	193	165	741
Average	1·08	11·14	1·18	207	159	795
Incubation with shikimate-[^{14}C]						
1	1·21	12·17	1·12	425	135	102
2	0·91	9·43	0·94	386	188	107
3	1·07	13·27	1·12	363	168	101
4	0·64	7·97	0·70	355	135	96
5	1·11	10·30	1·68	347	194	111
6	0·93	10·93	1·18	595	246	141
Average	0·98	10·68	1·12	412	178	110

* In each of the expts, 140 petals of flowers 26–30 mm in length were equally distributed according to their individually measured length in 2 parallel lots. The lower not much anthocyanin bearing halves (see Fig. 1) were cut off and discarded and the upper halves symmetrically bisected before incubation in Petri dishes containing a 6 ml aq soln of 8 ± 10% μCi cinnamate-[^{14}C] in the one dish and 11 ± 16% μCi shikimate-[^{14}C] in the other. The incubation period was 5 hr at 5000 lx fluorescent white light and 25°. Values are given per Petri dish with 70 upper petal halves.

concentration of cinnamate was 23 μM and of shikimate 0·7 mM. As a consequence it is to be expected that after shikimate-[^{14}C]-feeding, the ^{14}C-incorporation in *del* will be relatively higher by about 20% than after cinnamate-[^{14}C]-feeding. This effect is probably due to the regulatory influence of substituted phenyl- and phenylpropane-derivatives on the biosynthesis of these metabolites [12,13].

Comparison of cinnamate-[^{14}C]*- and shikimate-*[^{14}C]*-incorporation.* The data of the incorporation comparison are given in Table 2. From the averages, the following dilution factors can be calculated: after cinnamate-[^{14}C]-feeding; for *cya* 760, *del* 150, *peo* 585: and after shikimate-[^{14}C]-feeding; for *cya* 230, *del* 38, *peo* 100. After shikimate-[^{14}C]-feeding the dilution values are lower by factors of 3·3, 4·0, and 5·9 for *cya*, *del*, and *peo* respectively. The ratios of the relative proportions of the specific activities *cya/del* are after cinnamate-[^{14}C]-feeding 100/502 ± 36 and after shikimate-[^{14}C]-feeding 100/630 ± 33. The difference with Δ26 ± 8% corresponds with the difference of 20 ± 7% to be expected. The ratios for *cya/peo* are 100/130 ± 4 and 100/236 ± 21 demonstrating a significantly higher specific activity for *peo* after shikimate-[^{14}C]-feeding of Δ82 ± 10%.

DISCUSSION

The difference in the dilution of cinnamate-[^{14}C] and shikimate-[^{14}C] in *Petunia* anthocyanins confirms in principle the reported results [1–5]. However, the differences in dilution by factors of 3·3–5·9 are not comparable with those hitherto reported. For example, in *Viola cornuta* petals shikimate-[^{14}C] was incorporated 140 times better into delphinidin than was cinnamate-[^{14}C] [3]. As in some other systems an interference of uptake and transport indeed cannot be excluded in *Viola*, because the precursors were fed through the pedicel over a period of several days. Furthermore, in *Petunia*, apart from the comparatively small differences in dilution, a comparison of the relative proportions of the specific activities does not indicate any significant preference of shikimate-[^{14}C]-incorporation into *del* compared with *cya* which should have occurred, provided there are alternate pathways as suggested. In contrast, in spite of the fact that a simple explanation cannot be given at present, the significantly higher [^{14}C]-incorporation into *peo* after shikimate-[^{14}C]-feeding is not consistent with the hypothesis. Therefore, in *Petunia* at least, this hypothesis does not seem to apply. Yet there are two other mechanisms offering a possible explanation for the preference of shikimate-[^{14}C]-incorporation over cinnamate-[^{14}C]-incorporation. On the one hand there could be metabolic channeling by compartmentation. It is known that cinnamic acids are rapidly converted to their glucose esters and accumulated in the vacuole [14,15]. In *Petunia* petals, only 2–4% of the cinnamic acids are not esterified [16]. Thus the metabolic availability of shikimate may be much greater than that of cinnamate. On the other hand, there could be an easier intake of shikimate into the biosynthetic pathway than of cinnamate by means of catalytic facilitation *via* multienzyme

complexes [15,17]. The results of the present study make it appear that also these mechanisms should be taken into consideration.

EXPERIMENTAL

Genetic constitution of the genotype of *Petunia hybrida* used (V 77 = 11mmffK) [18] and the anthocyanins of the petals [19] are known. The petals were taken from plants grown in a greenhouse. Hence, the petals differed in anthocyanin content and particularly in their physiological condition from experiment to experiment and, therefore, the single expts are listed in the tables. Culture of flowers *in vitro* in Knop's soln [20]. Quantitative anthocyanin determination, and the purification of the anthocyanins to constant sp. act. was carried out as described previously [21]. Details of experimental conditions are given with the Tables. Cinnamate-[^{14}C]-3, sp. act. 54·5 mCi mMol^{-1}, was purchased from the Département des Radioéléments, Gif-Sur-Yvette, shikimate-[^{14}C]-(U), sp. act. 1·86 mCi mMol^{-1}, from New England Nuclear GmbH. For statistical evaluation of differences the Wilcoxon matched pairs signed rank test was applied [22], in some cases the s.e. is given.

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