L-Tyrosine as a Precursor of Flavonoids in Buckwheat Cotyledons

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Exogenous L-tyrosine was incorporated 13–14 times less effectively than exogenous L-phenylalanine into flavonoids in excised cotyledons of 3 days old buckwheat seedlings but proved to be an only 5 times poorer precursor when the experiments were carried out with 5 days old material. Simultaneous administration of L-phenylalanine markedly reduced incorporation of L-tyrosine into flavonoids. A similar decrease in the utilization of exogenous L-tyrosine for flavonoid biosynthesis occurred in kinetin-treated cotyledons. However, in cotyledons treated with high doses of glyphosate, an inhibitor of the shikimic acid pathway, an increase in the formation of flavonoids from exogenously supplied L-tyrosine was observed. Under all conditions the relative incorporation rate of exogenous L-tyrosine was highest for anthocyanins and lowest for C-glycosylflavones while within the latter class of compounds the luteolinic derivatives orientin and isoorientin incorporated more label than their apigeninic analogues vitexin and isovitexin. PAL and TAL activities were found to be present in the cotyledons in a ratio of 50:1. The possible role of L-tyrosine as an alternative natural precursor for the biosynthesis of flavonoids and other related polyphenols is discussed.

Introduction

L-phenylalanine is generally considered the common natural precursor of the majority of plant phenolics [1] but the possible role of L-tyrosine in the biosynthesis of these compounds has remained largely obscure. Of all plants, only grasses usually show an activity of L-tyrosine ammonia-lyase (TAL) comparable with that of L-phenylalanine ammonia-lyase (PAL) [2–5]. Thus L-tyrosine may be an additional true precursor of phenolics in the *Gramineae*, but not in other plants. In wheat, barley, and other cereals exogenous L-tyrosine was incorporated into polyphenols as, or nearly as effectively, as L-phenylalanine [3, 6–10] while in most other plants it was used rather poorly for phenolic biosynthesis [3, 8].

However, in several dicotyledonous plants and also in a species of algae [11], the utilization of exogenous L-tyrosine for polyphenol biosynthesis equalled or even exceeded that of exogenous L-phenylalanine [12–16]. This indicates that the role of L-phenylalanine as a unique precursor of polyphenols may be overestimated.

In order to shed new light into the problem a series of experiments with excised buckwheat cotyledons has been carried out in this laboratory comparing exogenous labelled L-tyrosine and L-phenylalanine as precursor for building flavonoids.

Abbreviations: PAL, L-phenylalanine ammonia-lyase; TAL, L-tyrosine ammonia-lyase.

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Materials and Methods

Plant material and treatment procedures

The experiments were carried out with isolated buckwheat (*Fagopyrum esculentum* Moench) cotyledons excised from 72–120 h old etiolated seedlings grown in water. The excised material was soaked for 3–5 min in a 2×10^{-3} M solution of [1-^{14}C]-L-tyrosine or [1-^{14}C]-L-phenylalanine (spec. activities 0.3–0.4 Ci/mol) and was then incubated for 40 h at 25 ± 1 °C either in the light (white fluorescent tubes, 28×10^3 erg · cm^{-2} · s^{-1}) or in the dark on filter paper moistened with the same solutions.

In the experiments with kinetin the excised cotyledons were first soaked for 15 min in a saturated solution of kinetin, then rinsed with water and transferred to the incubation medium containing L-tyrosine. Glyphosate (10^{-4} – 10^{-2} M), when used for an additional treatment, was applied continuously with tyrosine (10^{-3} M).

All experiments were run in triplicate, and the results were subjected to statistical evaluation by Student's significance test.

Flavonoid assay and radioactivity measurements

Anthocyanins were determined photometrically, rutin and individual C-glycosylflavones likewise after their separation by two-dimensional paper chromatography as described elsewhere [17]. Radioactivity of separate flavonoids was assayed in the ethanolic eluates with the use of a Beckman LS-100C liquid-scintillation counter [17].

PAL and TAL assay

Activities of the two ammonia lyases were determined by the amounts of cinnamic acid or *p*-coumaric acid, respectively, formed during incubation for 4 h at 35 °C [18].

Results

General aspects

When L-tyrosine or L-phenylalanine was administered at 2×10^{-3} M concentration, a pair of cotyledons absorbed up to 200 or 240 nmol, respectively, of the two amino acids during a 40 h period of incubation under continuous illumination. Uptake in the dark was reduced by 50 to 70% and also decreased with increasing age of the cotyledons. While the absorption of the two amino acids was similar, phenylalanine was preferentially incorporated into flavonoids (Table I). Simultaneous administration of L-phenylalanine markedly reduced the incorporation of L-tyrosine, but a reciprocal effect of L-tyrosine on the incorporation of L-phenylalanine was not observed.

Older cotyledons incorporated both amino acids at lower rates into flavonoids, the decrease being more than threefold in the case of L-phenylalanine, but not exceeding a 25 to 30 percent in the case of L-tyrosine. Consequently, the relative efficiency of L-tyrosine as a precursor for flavonoid biosynthesis increased with the age of the plant material. In 5 days old cotyledons, the total amount of flavonoids synthesized from exogenous L-tyrosine was only 5 times lower than the amount of flavonoids synthesized from exogenous L-phenylalanine (Table II).

In the dark the absolute production of flavonoids from exogenous L-tyrosine, as well as the relative incorporation of that precursor into flavonoids was substantially lower than in the light (Table III). In similar dark-incubation experiments with L-phenylalanine carried out by us earlier [17, 19] the absolute amount of flavonoids produced from exogenous precursor showed only a 2–2.5-fold decrease but the relative incorporation of exogenous L-phenylalanine into flavonoids even markedly increased in the absence of light.

Incorporation of L-tyrosine into individual flavonoids

It has been repeatedly shown by us that buckwheat cotyledons incorporate exogenous L-phenylalanine into individual flavonoids in ratios markedly

Table I. Incorporation of [1-^{14}C]-labelled L-phenylalanine (Phe) and L-tyrosine (Tyr) into flavonoids in 80 h old buckwheat cotyledons.

Incubation medium[a]	Total amount of flavonoids synthesized during incubation (40 h)[b], nmol/pair of cotyledons	Total radioactivity of flavonoids, dpm/pair of cotyledons	Specific activity of radioactive precursors, dpm/nmol	Flavonoids synthesized from exogenous precursors[c]	
				nmol/pair of cotyledons	%
Water	432	–	–	–	–
Phe-^{14}C	462	70 738	1092	64.8[a]	14.0[a]
Phe-^{14}C + Tyr	467	72 166	1050	68.7[a]	14.7[a]
Tyr-^{14}C	384	13 731	2808	4.89[b]	1.27[b]
Tyr-^{14}C + Phe	434	11 846	2991	3.96[c]	0.91[c]

[a] Incubation conditions: 40 h under continuous illumination.
[b] Calculated by subtracting zero-time content of flavonoids from their content in the cotyledons at the end of incubation.
[c] The values followed by different superscript letters are significantly different at 95% confidence level.

Table II. Age dependence of the incorporation of exogenous [1-^{14}C]-L-phenylalanine (Phe) and [1-^{14}C]-L-tyrosine (Tyr) into flavonoids in excised buckwheat cotyledons.

Age of cotyledons[a]	Radioactive precursor	Total amount of flavonoids synthesized during incubation (40 h), nmol/pair of cotyledons	Flavonoids synthesized from exogenous precursors[b]	
			nmol/pair of cotyledons	%
3 days	Phe	422	67.8[a]	16.1[a]
	Tyr	351	4.83[d]	1.4[d]
4 days	Phe	290	27.1[b]	9.3[b]
	Tyr	261	3.83[e]	1.5[d]
5 days	Phe	304	21.3[c]	7.0[c]
	Tyr	280	4.00[e]	1.4[d]

[a] Incubation conditions: 40 h under continuous illumination.
[b] The values followed by different superscript letters are significantly different at 95% confidence level.

Table III. Influence of light on the incorporation of [1-^{14}C]-L-tyrosine into flavonoids in excised buckwheat cotyledons.

Age of cotyledons	Total amount of flavonoids synthesized during incubation (40 h), nmol/pair of cotyledons	Flavonoids synthesized from exogenous L-tyrosine	
		nmol/pair of cotyledons	%
Incubation in the light			
3 days	474	5.01	1.1
4 days	209	3.31	1.6
5 days	347	3.42	1.0
Incubation in the dark			
3 days	130	0.51	0.4
4 days	53.5	0.36	0.7
5 days	51.5	0.22	0.4

Table IV. Incorporation of [1-^{14}C]-L-tyrosine into individual flavonoids in excised buckwheat cotyledons[a].

Flavonoid	Total amount synthesized during incubation, nmol/pair of cotyledons	The amount synthesized from exogenous L-tyrosine	
		nmol/pair of cotyledons	%
A comparison between major groups of flavonoids			
Anthocyanins	16.7	0.51	3.05
Rutin	73.0	1.32	1.81
C-glycosylflavones	234.5	2.39	1.02
A comparison between individual C-glycosylflavones			
Orientin	25.7	0.41	1.60
Vitexin	46.1	0.46	1.00
Isoorientin	72.3	0.73	1.01
Isovitexin	90.4	0.79	0.87

[a] Results of an experiment using 80 h old cotyledons. Incubation conditions: 40 h under continuous illumination.

different from the ratio of their accumulation, with the relative labelling always being highest in anthocyanins and, within the C-glycosylflavones, being higher in the luteolinic C-glycosylflavones orientin and isoorientin than in their apigeninic analogues vitexin and isovitexin, respectively [17, 19, 20]. In the present study, label from exogenous L-tyrosine, at a background of much lower general labelling, showed essentially the same pattern of distribution between individual flavonoids (Table IV), even though the relative incorporation rates of tyrosine and phenylalanine into separate flavonoids did not fully coincide. In a number of parallel experiments the incorporation ratio Phe:Tyr was markedly lower in the case of anthocyanins and rutin than in the case of C-glycosylflavones being, for example, 9:1 against 16:1 for 3 days old cotyledons, or 6:1 against 9:1, respectively, for 4 days old material.

Experiments with glyphosate

The suppression of L-tyrosine incorporation into flavonoids in the presence of L-phenylalanine (Table I; see also [10]) prompted us to study biosynthetic use of exogenous L-tyrosine in the cotyledons treated with glyphosate (N-phosphonomethylglycine), a herbicide which was shown to interfere with the shikimic acid pathway and to reduce the formation of aromatic amino acids [21 – 23]. The idea was that under conditions of a

reduced endogenous supply of L-phenylalanine label from exogenous L-tyrosine might be more effectively channelled into flavonoids.

However, at lower concentrations of the herbicide these expectations were not fulfilled. Although glyphosate inhibited total production of flavonoids at all concentrations it also reduced their synthesis from exogenous L-tyrosine, and the relative labelling of flavonoids remained approximately at the same level as in the control material. Only at the highest glyphosate concentration (10^{-2} M), which produced an about 30-fold decrease in the accumulation of flavonoids, the synthesis of these compounds from the exogenous L-tyrosine did show an absolute increase, and a significant rise in the relative labelling of flavonoids, as expected, occurred in the treated cotyledons (Table V).

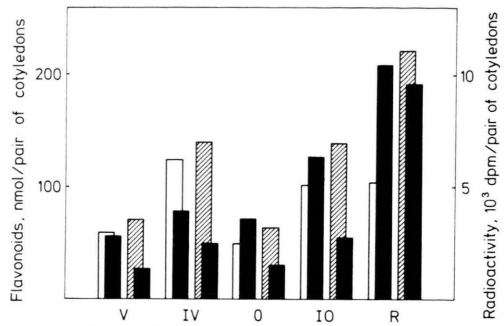

Fig. Influence of kinetin on the incorporation of exogenous [1-^{14}C]-L-tyrosine into flavonoids in 80 h old excised buckwheat cotyledons incubated for 40 h under continuous illumination. Synthesis of flavonoids in untreated cotyledons – white bars, synthesis of flavonoids in cotyledons treated with kinetin – shaded bars, radioactivity of flavonoids in the respective material reflecting incorporation of exogenous L-tyrosine – black bars. Vitexin (V), isovitexin (IV), orientin (O), isoorientin (IO), rutin (R).

Experiments with kinetin

Kinetin has been shown to increase the availability of endogenous L-phenylalanine for flavonoid biosynthesis in buckwheat cotyledons and to reduce the relative incorporation of exogenous phenylalanine [17]. Likewise, kinetin reduced the incorporation of exogenous tyrosine into flavonoids, especially the C-glycosylflavones (Fig.). The total absolute amount of flavonoids synthesized from exogenous L-tyrosine proved to be about 1.5 times lower in the cotyledons treated with kinetin than in the untreated material (5.86 and 8.97 nmols per pair of cotyledons, respectively) while their relative content in the total flavonoids decreased in that material even more than twice (2.01% in control, 0.92% in kinetin-treated cotyledons).

Table V. Influence of glyphosate on the incorporation of [1-^{14}C]-L-tyrosine into flavonoids in buckwheat cotyledons[a].

Incubation solution	Total amount of flavonoids synthesized during incubation, nmol/pair of cotyledons	Flavonoids synthesized from exogenous L-tyrosine[b]	
		nmol/pair of cotyledons	%
Tyr alone	438	1.98[a]	0.45[a]
Tyr + glyphosate 10^{-4} M	117	0.609[b]	0.52[a]
Tyr + glyphosate 10^{-3} M	91.5	0.640[b]	0.70[a]
Tyr + glyphosate 10^{-2} M	14.7	0.785[c]	5.34[b]

[a] Experiments were carried out with 80 h old cotyledons. Incubation conditions: 40 h under continuous illumination. Concentration of L-tyrosine in the incubation solutions – 10^{-3} M.
[b] The values followed by different superscript letters significantly different at 95% confidence level.

A comparison of PAL and TAL activities

Contrary to results obtained with buckwheat hypocotyls [24, 25], extracts from buckwheat cotyledons contained both PAL and TAL activities their ratio being strongly in favour of PAL. The average activity levels of PAL and TAL in the illuminated cotyledons were found to be 48.7 and 0.91 nmol/h/pair of cotyledons, respectively, with only little variation of these values in the material of different age. The high PAL/TAL ratio may explain the low capacity of tyrosine to compete with L-phenylalanine. It is still necessary to emphasize that during 40-h-incubation the TAL activity recorded here could theoretically account for the deamination of 30–35 nmol of tyrosine per pair of cotyledons. The amount of flavonoids formed from exogenous L-tyrosine was, in fact, about 9–10 times lower (*cf.* Tables I–III).

Discussion

If one assumes that the biochemical conversions of plant metabolites are identical irrespective of whether the metabolites are produced endogenously or supplied exogenously then the results of the

present study may indicate L-tyrosine as an alternative though 13–14 times less effective than L-phenylalanine precursor of flavonoids in buckwheat cotyledons. L-tyrosine might thus contribute 7–8% of the total flavonoids in 3 days old cotyledons, and presumably up to 15–16% in older material. As the cotyledons of young buckwheat seedlings synthesize about 300–400 nmol of flavonoids during a 40 h period of incubation under continuous illumination, the absolute amount of these compounds originating from L-tyrosine could reach 30–35 nmol per seedling, on the average. That amount seems to be in a good accord with the potential for tyrosine deamination of the cotyledons.

However, it is far from being clear to which extent the rates of incorporation of exogenous metabolites, as well as measurements of extractable activities of enzymes reflect cell metabolism *in vivo*. Metabolic reactions in a living cell are generally organized into a rigid and well coordinated system in which sequentially acting enzymes of particulate pathways are often bound to membrane structures and act as multienzyme complexes. Convincing evidence is accumulating that similar multienzyme complexes or conglomerates of coordinatively functioning enzymes consuming L-phenylalanine as the initial substrate are operating also in phenolic biosynthesis [26–28]. Therefore, despite the fact that L-tyrosine may serve as a substrate for PAL *in vitro*, it may nevertheless not be available for flavonoid biosynthesis *in vivo* and incorporation of exogenous tyrosine into flavonoids may simply be a consequence of the breakdown of barriers protecting natural enzymic complexes involved in flavonoid biosynthesis.

Of course, these considerations remain purely speculative at present. It still deserves notice that even under the most favourable competition situation arising in cotyledons after severe inhibition of the production of endogenous L-phenylalanine and L-tyrosine by glyphosate, incorporation of exogenous L-tyrosine into flavonoids was only slightly enhanced. If L-tyrosine really were a true alternative precursor of flavonoids and other related polyphenols, a much higher increase in the utilization of exogenous L-tyrosine could be expected to occur under these conditions.

In any event, new experimental approaches are obviously needed to solve the problem of the possible precursor role of L-tyrosine in the phenolic biosynthesis.

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[1] J. B. Harborne, Plant phenolics. In: Encyclopedia of Plant Physiology. New Series, Vol. 8: Secondary Plant Products (E. A. Bell and B. V. Charlwood, eds.), p. 329–402, Springer-Verlag, Berlin and Heidelberg 1980.
[2] A. C. Neish, Phytochemistry **1,** 1–24 (1961).
[3] S. A. Brown, Can. J. Bot. **39,** 253–258 (1961).
[4] M. R. Young, G. H. N. Towers, and A. C. Neish, Can. J. Bot. **44,** 341–349 (1966).
[5] N. O. Jangaard, Phytochemistry **13,** 1765–1768 (1974).
[6] S. Z. El-Basyouni, A. C. Neish, and G. H. N. Towers, Phytochemistry **3,** 627–639 (1964).
[7] T. Higuchi and S. A. Brown, Can. J. Biochem. Physiol. **41,** 621–628 (1963).
[8] S. A. Brown and A. C. Neish, Can. J. Biochem. Physiol. **33,** 948–962 (1955).
[9] M. M. Burrell and T. Ap Rees, Physiol. Plant Pathol. **4,** 497–508 (1974).
[10] L. E. Laanest, Plant Physiol. (Moscow) **28,** 103–110 (1981).
[11] W. Löffelhardt and H. Kindl, Z. Naturforsch. **31c,** 693–699 (1976).
[12] N. Ishikura and K. Hayashi, Bot. Mag. (Tokyo) **79,** 156–164 (1966).
[13] M. N. Zaprometov and V. Y. Bukhlaeva, Biokhimia (Moscow) **36,** 270–276 (1971).
[14] A. Razzaque and B. E. Ellis, Planta **137,** 287–291 (1977).
[15] M. H. Zenk and G. Müller, Z. Naturforsch. **19b,** 398–405 (1964).
[16] M. N. Zaprometov and V. Y. Bukhlaeva, Plant Physiol. (Moscow) **18,** 787–795 (1971).
[17] U. Margna and T. Vainjärv, Z. Naturforsch. **38c,** 711–718 (1983).
[18] M. Zucker, Plant Physiol. **40,** 779–784 (1965).
[19] U. V. Margna, L. E. Laanest, E. R. Margna, and T. R. Vainjärv, Formation of flavonoids in dark-incubated buckwheat seedlings fed with exogenous precursors. In: Regulation of Plant Growth and Metabolism (U. V. Margna, ed.), p. 169–184, Tallinn 1983 (in Russian).
[20] U. Margna and E. Margna, Biochem. Physiol. Pflanzen **173,** 2–10 (1978).
[21] N. Amrhein, J. Schab, and H. C. Steinrücken, Naturwissenschaften **67,** 356 (1980).
[22] H. Holländer and N. Amrhein, Plant Physiol. **66,** 823–829 (1980).
[23] N. Amrhein, B. Deus, P. Gehrke, and H. C. Steinrücken, Plant Physiol. **66,** 830–834 (1980).

[24] N. Amrhein and M. H. Zenk, Z. Pflanzenphysiol. **64,** 145–168 (1971).
[25] H. Holländer and N. Amrhein, Planta **152,** 374–378 (1981).
[26] H. A. Stafford, Possible multienzyme complexes regulating the formation of C_6-C_3 phenolic compounds and lignins in higher plants. In: Recent Advances in Phytochemistry, Vol. 8 (V. C. Runeckles and E. E. Conn, eds.), p. 53–79, Academic Press, New York 1974.
[27] U. Czichi and H. Kindl, Planta **134,** 133–143 (1977).
[28] H. Kindl, Compartments and microcompartments channelling intermediates in phenylpropanoid metabolism. In: Regulation of Secondary Product and Plant Hormone Metabolism. Proc. 12th FEBS Meeting, Vol. 55 (M. Luckner and K. Schreiber, eds.), p. 49–61, Pergamon Press, Oxford 1979.