Distribution of Phenylalanine Transaminase and Phenylalanine Ammonia-Lyase Activities in Etiolated and Light Irradiated Radish Seedlings (*Raphanus sativus* L.)

Photoregulation of enzyme activity is an important control mechanism in plant growth[1]. In particular it has been demonstrated that the phenylalanine ammonia-lyase activities (PAL) of many dicotyledons are controlled by phytochrome or other blue light receptors[2]. Our present work aims to examine whether another enzyme playing a major rôle in phenylalanine metabolism – i.e. the phenylalanine transaminase (TR) – is also under photocontrol. This enzyme catalyzes transamination between phenylalanine-phenylpyruvate and other α-keto acid-amino acid systems. It is thus closely connected with amino acid synthesis, a process very active in the earlier phases of seedling formation[3]. An effect of light on TR activity level in peach apices has been reported by EREZ[4].

In this report we investigated the levels of both TR and PAL activities in the different parts of radish seedlings, and we compared the effects of light on the two activities. Moreover, owing to the probable errors indicated by EREZ[4] and regarding the suitability of the PAL assay used in previous experiments with radish[5], we discuss also the possibilities of interference of TR with the PAL assay.

Experimental. Raphanus sativus L. seeds cv. "tondo rosso quarantino" were obtained from Fratelli Ingegnoli, Milano. Sowing was as previously described[6]. Seedlings were grown in darkness in glass crystallizing dishes sealed with transparent plastic foil, in a temperature controlled cabinet at 23–24 °C. Then 48 h after sowing, the dishes were transferred to a thermostated (24 °C) irradiation field and the intact seedlings in the sealed containers were exposed to light. White light was obtained from two 14-W Gro-Lux fluorescent lamps; far red light was filtered through plastic filter (no transmission under 690 nm) from an incandescent source equipped with a heat filter. Red light was obtained with a Balzers interference filter (peak transmission at 655 nm, 40 nm half width).

Enzyme extraction and partial purification on Sephadex G-25 "fine" was carried out as reported previously[7], with the exception that 0.1 *M* borate buffer pH 8.8 was used. The spectrophotometric assay of both TR and PAL activities was carried out in parallel at 25 °C. For TR assay, 0.5 ml of enzyme solution (corresponding to the protein content of 2.5 organs), 100 μmoles of borate buffer pH 8.8, 50 μmoles of L-phenylalanine, 2 μmoles of α-keto glutarate (α-KG) and distilled water for a total volume of 2 ml were mixed in 1-cm path cuvette. Increase in absorbance at 290 nm was recorded against a control without phenylalanine and α-KG. For PAL assay, the same mixture was incubated without α-KG and the absorbance increase at 290 nm was recorded against a control without phenylalanine. The absorbance increase in both cases was linear for at least 2 h and proportional to the volume of extract used. An enzyme unit is that amount of enzyme producing an increase at 290 nm of 0.001 in 60 min under the conditions reported above.

Results. Figure 1 shows 2 typical kinetics of absorbance increase at 290 nm obtained with the procedure used by EREZ[4]. Briefly, the PAL activity has been determined with the classical method of ZUCKER[8], in a system containing radish extract purified with Sephadex G-25 (thus eliminating the low molecular weight substances as the α-keto acids) plus borate buffer and phenylalanine as substrate. In the parallel assay, a saturating quantity of α-KG (2 μmoles) was added at zero time. In the presence of the α-keto acid TR catalyzes the transamination of phenylalanine with the production of phenylpyruvate and the corresponding amino acids[9,10]. In borate buffer, an absorbance increase at 290 nm arose, due not only to the formation of cinnamate but also to the enol-borate com-

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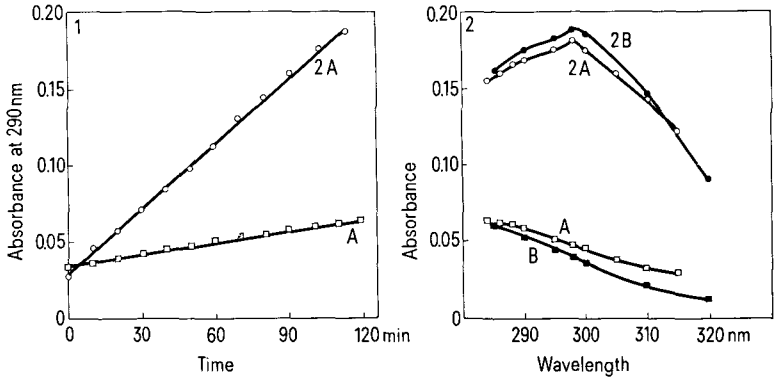

Fig. 1 (left). Time course of the increase of absorbance at 290 nm in the 2 reaction systems of the coupled assay. Curve A) represents the formation of cinnamate due to the PAL activity only, since the reaction mixture consisted of 0.5 ml enzyme, 100 μmoles of borate buffer and 50 μmoles of phenylalanine in a final volume of 2 ml. In 2A) at zero time, α-KG was added to obtain the final concentration of 1 m*M*. The absorbance increase in 2A) is due to the formation of both cinnamate and enol-borate complex of phenylpyruvate. The difference between the 2 rates of absorbance increase give the TR activity.

Fig. 2 (right). The enhancement of the absorbance of the reaction system by the presence of borate and α-KG. A) absorption spectra of the reaction system used for Figure 1 (in borate buffer) after 100 min of incubation. Curve 2A) shows the effect of adding α-KG to a final concentration of 1 m*M*. B) absorption spectra of the reaction mixture made with *Tris*-HCl instead of borate and containing α-KG. In 2B) boric acid was added at the end of the incubation at a final concentration of 0.1 *M*.

Table I. Distribution of PAL and TR activities in radish seedlings

Organ and treatment	Enzyme activity (Units / organ ± SE)		Fresh weight	Protein
	PAL	TR	(mg/organ ± SE)	(mg/organ)
Cotyledons, 3 h D	2.50 ± 0.42	21.25 ± 0.85	13.56 ± 0.16	1.00
Cotyledons, 3 h WL	5.86 ± 0.29	25.15 ± 1.35	13.55 ± 0.20	—
Cotyledons, 5 min R + 175 min D	3.08 ± 0.21	23.48 ± 1.87	—	—
Cotyledons, 5 min R + 5 min FR + 170 min D	2.72 ± 0.08	25.60 ± 0.92	—	—
Cotyledons, 3 h FR	8.90 ± 0.30	27.00 ± 1.16	—	—
Hypocotyl, 3 h D	2.00 ± 0.23	4.12 ± 0.60	6.87 ± 0.14	0.66
Hypocotyl, 3 h WL	5.60 ± 0.13	4.40 ± 0.47	6.83 ± 0.15	—
Root, 3 h D	12.72 ± 0.94	2.56 ± 1.40	9.11 ± 0.76	0.15
Root, 3 h WL	22.60 ± 2.84	3.68 ± 0.66	9.75 ± 0.74	—

Treatment started 48 h after sowing. Seedlings were dissected just before enzyme extraction. Each determination was made on extracts from 50 organs. Mean and standard errors are from 3 independent experiments (8 for cotyledons). The protein content was determined with the biuret reagent. D, R, FR and WL mean darkness, red, far red and white light respectively; total irradiance at the seedling level: 65, 140, and 560 $\mu W/cm^2$. Enzyme units as described in Experimental.

plex of phenylpyruvate, which strongly absorbs in the 300 nm region[10]. This formation of the borate complex is currently used to measure the activity of TR[11].

We can assume that the absorbance increase at 290 nm is due to both TR and PAL activities in the reaction mixture with phenylalanine and α-KG, while without α-KG, only PAL activity – i.e. cinnamate formation – can develop. Therefore the difference between the 2 rates of absorbance increase give the actual TR activity – i.e. formation of the enol-borate complex of phenylpyruvate.

Spectra of Figure 2 indicate that α-KG and borate are both necessary to increase the absorbance of the assay system in the 300 nm region. This fact is a proof of the presence of TR in the extract, as pointed out by Lin et al.[10], and the absorbance maximum in the region of 300 nm agrees well with the spectra of phenylpyruvate in borate reported by EREZ[4].

Distribution of PAL and TR. We used the difference method described above to measure the activities of PAL and TR in the various parts of 51-h-old radish seedling, etiolated or irradiated with white light for 3 h just before extraction (Table I). At this age, the radish seedling is large enough to be handled and responds optimally to light[7]. Table I reports activities per organ, but fresh weight and protein content are also indicated. In the etiolated seedling, maximum PAL activity is found in the root, while the TR level is higher in the cotyledons. This relative distribution applies also on fresh weight basis or total protein content.

Table II. α-keto glutarate content of the various parts of the radish seedling

Organ	α-keto glutarate (μmoles per 1 g fresh weight)	
	Darkness	White light
Cotyledons	0.490	0.597
Hypocotyl	0.239	0.335
Root	0.235	0.270

Light treatment started 48 h after sowing. Seedlings were dissected after 3 h light or darkness, just before extraction; α-KG was determined in the neutralized acid extracts with $(NH_4)_2SO_4$, NADH and glutamate dehydrogenase, according to BERGMEYER and BERNT[13]. Figures are averages from 3 independent experiments.

The increase of PAL activity due to white light is clear in all the 3 parts of the seedling, while TR is weakly enhanced by light only in cotyledons. We have examined the effect of 3 h far red light and of short pulses of red and far red light only in cotyledons. This kind of investigation was suggested by previous work concerning the far red effect on PAL[5,12]. The data of Table I show a quite consistent increase of PAL activity (about 200%) together with a barely significant increase of TR activity. This lower sensibility of TR to far red irradiation agrees well with the low sensibility to white light. A brief irradiation with red light alone or followed by far red had no significant effect either on PAL or on TR as compared to dark controls. The intensity of the red light used was enough to saturate other photoresponses in the same material[6]. These results on cotyledons confirm the strong specific effect of far red light on the PAL activity of radish hypocotyls[5].

Remarks on TR-PAL interference. As a consequence of the presence of TR in radish extracts, an interference with PAL determinations of the kind suggested by EREZ[4] is possible. The degree of this interference depends on the presence of a suitable amount of endogenous α-keto acids. We determined α-KG with an enzymatic method[13] in the various parts of the radish seedling (Table II) and found a level of α-KG consistently higher than that reported by KRETOVICH and GEIKO[14] in various seedlings. From the data of Table II, we can expect in the cuvette about 2 to 20×10^{-3} μmoles, enough to form some phenylpyruvate during the PAL assay.

SAUNDERS and MCCLURE[15] found no significant difference attributable to the inclusion of α-KG in the PAL assay system of barley, pea and buckweath: the disagreement with the situation in radish may derive from the fact that in the former case the extracts were tested for TR activity without removing the endogenous α-keto acids[15]. If these were present in sufficient quantity to satu-

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rate the TR, no difference in absorbance at 290 nm would be expected between mixtures containing only phenylalanine or phenylalanine and α-KG.

In radish, the TR activity is relatively high in cotyledons and very low in roots; therefore one must expect a higher degree of interference with the PAL assay in the case of cotyledons. This is in agreement with the finding that the coefficient of variation for the PAL activity (determined in crude extracts and borate) is higher for cotyledons than for roots[16]. In hypocotyls, the TR activity is quite low and independent from light. This fact, and the relative constance of the α-KG content in darkness and in light, suggests that the data of BELLINI and HILLMAN[5], obtained with hypocotyls from 48-h-old seedlings, can be interpreted as the sum of two parts, the first one stable and inherent to TR, the second one variable in the different experimental conditions and due to the true PAL activity. Moreover, the confirmation of the non-efficiency of one single red light pulse – this is the crucial point in the paper cited[5] – has subsequently been reported using determinations made on purified extracts[6].

Conclusions. Although our data do not represent a critical kinetic analysis of the development of PAL and TR activities, they are sufficient to indicate a completely different distribution pattern of TR and PAL in the various parts of the radish seedling. This may be connected with the different rôle of the 2 enzymes: PAL is a key enzyme in the lignification process and this may explain its higher level in roots, while TR activity seems involved in amino acid synthesis, which is very active in the cotyledons of germinating seeds[3]. The effect of light is clearly more evident on PAL than on TR level, and this agrees with other data suggesting a higher sensitivity of the development of PAL activity to light, as compared to other enzymes, also of the transcinnamic acid pathway. The present investigation also confirms that the presence of TR activity in plant extracts interferes with the determination of PAL by spectrophotometric methods. This interference can be eliminated by removal of α-keto acids present, for example by passing crude extracts through Sephadex.

Summary. In dark-grown *Raphanus sativus* seedlings the level of phenylalanine transaminase is higher in cotyledons than in root and hypocotyl. The maximum activity of phenylalanine ammonia-lyase (PAL) is found in the root. Only PAL is significantly increased by light.

FRANCA TOMÈ, L. CAMPEDELLI and E. BELLINI

Istituto di Scienze Botaniche dell'Università e Centro di Studio del CNR per la Biologia Cellulare e Molecolare delle Piante, Via G. Colombo 60, I-20133 Milano (Italy),
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In vitro Lipid Synthesis by Lathyrogen-Treated L-929 Fibroblasts

A previous in vitro study has shown that the lathyrogen, B-aminopropionitrile fumarate (BAPN), in a concentration of 5 m*M* increased the secretion of collagen[1]. It has been suggested that certain lipids are present in close proximity to collagen fibres[2,3] and that the lipids play an important role in the calcification process[2]. The purpose of this study was to determine whether the rate of lipid synthesis paralleled the increased rate of collagen synthesis seen in 5 m*M* BAPN-treated cell cultures.

Materials and methods. Strain L-929 fibroblasts were grown in Eagle's minimum essential medium containing 10% calf serum. 5-day-old cultures were trypsinized and resuspended into a common suspension having approximately 100,000 cells per ml. The suspension was divided equally: one designated as the experimental to which was added 5 m*M* of BAPN, and the other as control to which was added an equal amount of sodium fumarate. Replicate cultures were prepared and grown for a 5-day period. On the 5th day, the medium was decanted and replaced with fresh control and experimental medium containing 1.25 μCi/ml ^{14}C-acetate. After 4 h incubation, the cells were washed and harvested.

For lipid analysis, the cells were extracted with chloroform/methanol (2/1); the lipid extracts were purified by washing with 0.9% sodium chloride[4]. Lipid classes were separated on silicic acid papers according to the chromatographic procedures of MARINETTI[5] and WUTHIER[6]. Radioactive lipid spots were located by autoradiography, cut from the papers, and counted in a Packard Tri-Carb Scintillation Spectrometer, model 3375, using BRAY's solution[7]. Lipids were identified on chroma-

Effect of 5 m*M* BAPN on lipid synthesis by L-929 fibroblasts from ^{14}C-acetate

Lipid class	Control		BAPN	
	CPM[a]	SD[a]	CPM[a]	SD[a]
Triglycerides	17,297	921	4,384	314
Lecithin	26.687	927	6,960	189
Mono- and diglycerides	5,335	644	2,089	338
Cholesterol	5,578	749	4,258	397
Free fatty acids	13,951	183	3,665	388
Cholesterol esters	877	558	582	249
Unknown	1,943	413	822	356
Phosphatidyl ethanolamine	2,996	168	1,187	10
Phosphatidyl inositol	4,032	229	1,266	83
Sphingomyelin	2,775	286	1,240	133
Lyso-lecithin	741	280	842	314
Phosphatidyl serine	1,464	59	707	55

[a] Mean and standard deviation of three samples; $p < 0.01$ in all cases. The samples analyzed were made by pooling 3 plates of cells. Each value represents the mean of 3 samples. The 3 control samples contained 15.49, 13.84 and 14.25 mg of phospholipid phosphorus, while the BAPN group contained 11.68 ,9.72 and 9.31 mg phospholipid phosphorus, respectively.

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