Distribution of Phenylalanine Ammonia-Lyase and 4-Coumarate: CoA Ligase in Oat Primary Leaf Tissues

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4-Coumarate: CoA ligase (CL) isolated from oat primary leaves shows two activity maxima in 5 and 7 day old seedlings, the first of which corresponds with the highest flavone accumulation rate. In this plant stage most of L-phenylalanine ammonia-lyase (PAL) is located in the epidermal tissues, whereas CL dominates in mesophyll protoplasts and in the vascular elements. In the older leaves, in which PAL is only detectable in the epidermis and in vascular elements, most of CL is located in the latter. The CL from different tissues varies significantly in substrate specificity. Investigations of intact chloroplasts from isolated protoplasts revealed that approximately half of the PAL activity can be extracted from this organelle, whereas there is no direct evidence for the existence of CL in plastids.

Introduction

Earlier studies on flavonoid metabolism of developing oat primary leaves demonstrated the characteristic and tissue specific accumulation of several C-glycosylflavone O-glycosides [1, 2]. When seedlings are grown under standard conditions in a phytotron, the lower and upper epidermis as well as the mesophyll of the leaf not only store similar amounts of flavonoid but also show different patterns of three major flavone components.

Comparative investigations of biosynthetic enzymes in the epidermis and the mesophyll of oat leaves revealed that the distribution of phenylalanine ammonia-lyase (PAL), the key enzyme of the common phenylpropanoid pathway, corresponds in its activity with flavonoid levels obtained from these tissues [3]. A second enzyme, "flavanone synthase" (FS), characteristic for specific flavonoid synthesis, although following a similar time course as that obtained for PAL, differs in being localised primarily in the mesophyll [4]. Chalcone isomerase (CI) is also localised in the mesophyll but varys from both, FS and PAL, in its developmental characteristics [3]. Thus biosynthetic potentials of the three enzymes behave in a different way in both epidermal and mesophyll tissues.

Abbreviations: PAL, L-phenylalanine ammonia-lyase; TAL, L-tyrosine ammonia-lyase; CL, 4-coumarate: CoA ligase; FS, "flavanone synthase"; CI, chalcone isomerase; pCA, *p*-coumaric acid; CA, caffeic acid; FA, ferulic acid; SA, sinapic acid; DTT, dithiothreitol; HPLC, high performance liquid chromatography.

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Flavonoid metabolism in oat leaves appears to differ from that occurring in leaf organs of other species. In mustard cotyledones [5, 6] or onion scales [7–9], enzymes PAL and CI were found to be induced and localised, together with accumulating flavonoids, principally in the epidermal tissues. In a recent study of green leaves of nine dicotyledon species, it was demonstrated [9] that their leaves contain flavonoids, mainly flavonol glycosides, which are located primarily in the epidermis. However, anthocyanins of foliage leaves are frequently restricted to either the epidermis or the mesophyll. This change may depend on the age of the organ or its physiological state [10].

The present paper gives a more detailed analysis of our previous investigations on tissue localisation and kinetics of flavonoid metabolism of oat seedlings. This includes a further look into the distribution patterns of the biochemical potential of 4-coumarate: CoA ligase (CL) in comparison to PAL in the upper and lower epidermis and in the mesophyll. The PAL reaction leads to the product *t*-cinnamic acid, whereas CL is responsible for the biosynthesis of hydroxycinnamate CoA thiol esters. In most of the cases reported, CL represents the principle reaction in the hydroxycinnamate pathway, the products of which are *e.g.* cinnamic acid esters, lignin and flavonoid compounds [11].

In addition, oat leaf mesophyll was enzymatically separated into two main components: 1. the photosynthetically active protoplasts in which the occurrence of characteristic flavone profiles [12] together with FS activity [4] has been recently demonstrated, and 2. the vascular strands in which lignification

takes place during leaf development. Furthermore, chloroplasts were isolated from protoplasts to check the localisation of PAL and CL activity in this organelle [*cf.* 13].

Materials and Methods

Plant material: Avena sativa L. was grown under standard conditions in a phytotron [14]. For studying enzyme activities as a function of primary leaf development, 4–8 day old seedlings were used. Tissue distribution, protoplast and chloroplast experiments were done with 5 and 7 day old primary leaves, respectively.

Chemicals: Cinnamic acids were purchased from Roth (Karlsruhe); [2-^{14}C]-*p*-coumaric acid was synthesized via the Knoevenagel reaction [15] with [2-^{14}C]malonic acid (47.6 mCi/mmol) from NEN (Boston), [ring-^{3}H]-*p*-coumaric acid with the TAL reaction [16] by the use of oat leaves [17] from L-[2,3,5,6-^{3}H]tyrosine (104 Ci/mmol); the latter substrate and L-[U-^{14}C]phenylalanine (513 mCi/mmol) were purchased from Amersham Buchler (Braunschweig). Polyclar AT, Dowex 1X2 (Cl^-), 200–400 mesh, and dithiothreitol (DTT) were obtained from Serva (Heidelberg), all biochemicals from Boehringer (Mannheim), and all other chemicals (analytical grade) from Merck (Darmstadt).

Methods: Preparation of leaf sections was done as described elsewhere [3], the isolation of protoplasts was carried out according to [12], taking 3- and 5-cm pieces respectively, from 5 and 7 day old oat leaves for PAL measurements; for CL investigations whole leaves were used. The peeled epidermis and the vascular tissues remaining after digestion of the leaf pieces were extracted separately. Protoplast yield was based on chlorophyll recovery [*cf.* 12].

The preparation of enzyme crude extracts starting with the leaf sections was done as described earlier [4]. The extraction of protoplasts, epidermal and vascular tissues for CL activity was carried out at 0–4 °C as follows: the starting material (40–60 epidermis strips, protoplasts and vascular strands of the same number of leaves) was separately ground in a mortar with 2–4 ml of 0.1 M KH_2PO_4/K_2HPO_4 buffer pH 7.3 (40 mM DTT) mixed up with Polyclar AT, Dowex 1X2 (Cl^-) and quartz sand; after centrifugation (20 min, 48,000 × *g*), the clear supernatant served as enzyme crude extract. For the extraction and assay of PAL, 0.1 M Tris/HCl buffer pH 8.8 (2 mM 2-mercaptoethanol) was used instead of phosphate buffer. With protoplasts, comparable results were obtained by use of glycine/NaOH and borate buffer after pH control [18]. Ultrasonic treatment for 2–3 sec followed by sedimentation of the cellular residue led to the PAL crude extracts of protoplasts. Epidermis and vascular elements were treated as described for the CL extraction. Intact chloroplasts were isolated from protoplasts by use of a linear sucrose gradient according to [12] or by differential centrifugation [4].

Enzyme assays: CL was measured optically as described by Gross and Zenk [19] or in the corresponding radioactive test using 10^6 dpm [2-^{14}C]- or 5.5×10^6 dpm [ring-^{3}H]-*p*-coumaric acid in a total reaction volume of 0.05 ml, PAL optically [20] or in a radioactive test with 5 nmol L-[U-^{14}C]- and 60 nmol unlabelled phenylalanine in a total reaction volume of 0.06 ml (≙ 1.08 mM phenylalanine). All assays were performed at 30 °C.

Product identification and quantification was done either by HPLC or polyamide column chromatography in the case of the CL assay with pCA as substrate [21] or by two-dimensional TLC on microcristalline cellulose (Avicel; Macherey & Nagel, Düren) with I: *n*-butanol/NH_4OH/H_2O (75:10:65 by vol.; upper phase) and II: 2% formic acid as solvent systems in the case of the radioactive PAL assay. The formation of the CoA thiolesters of the other cinnamic acids was followed at their absorption maxima [15].

Results and Discussion

Earlier investigations demonstrated the correspondence of the highest flavone accumulation rate with the maxima of PAL [17] and FS [4] activities in 5 day old seedlings. Most of the PAL activity is located in the middle section of the leaf with a maximum in 5 and 6 day old plants, whereas in the other leaf region with relatively high quantities of PAL, the 1-cm basal section, the activity is maximal in 4 and 5 day old plants; the 1-cm top region looses its small activity during leaf growth [3]. The specific activity of PAL remains at a constantly high level in the basal section and deminishes during growth in the middle and upper part of the leaf. The FS activity follows a very similar time course.

CL activity as a function of leaf development

Fig. 1 a shows the distribution of CL activity in the three sections of the developing oat primary leaf.

The relation is similar to those of PAL and FS [3, 4] with the exception of a second peak in 7 day old plants. The specific activity (Fig. 1 b) increases in the basal region up to 6 day old leaves, whereas it decreases, similar to PAL and FS, in the other leaf sections. These findings together with our earlier results indicate a highly coordinated development of three important enzymes of flavone metabolism (PAL, CL, and FS) and its correlation with the accumulation of biosynthetic end products, the C-glucosylflavone O-glycosides, in growing oat primary leaves. The CL activity, however, reaches a second maximum in 7 day old plants at the time when the flavone synthesis already decreases. This seems to be an indication for the participation of CL in other metabolic pathways at this stage of leaf development.

Occurrence of PAL and CL activities in different leaf tissues

In the next step of investigation the localisation of PAL and CL activities in the different tissues of the oat leaf was analysed: upper and lower epidermis, mesophyll cells (protoplasts), and vascular elements. Total flavone is distributed in similar amounts to the two epidermal and the mesophyll tissues [*cf.* 1, 2]. Earlier investigations, in which it was not discriminated between protoplasts and vascular tissue, showed a nearly equal distribution of PAL activity in the upper and lower epidermis and in the mesophyll of 5 day old plants and a dominance in the lower epidermis of 7 day old plants [3]. FS activity, however, is located in the mesophyll cells (protoplasts) to nearly 90% [4].

As it is shown in Fig. 2, most of the PAL activity (about 80%) of 5 day old leaves is located in the upper and lower epidermis and only small amounts are found in the mesophyll (protoplasts and vascular elements). The CL activity, however, could be recovered from the mesophyll of these plants to approximately 70% (Fig. 3 a). As further leaf development shows, there is a tendency towards increasing CL activity in the vascular tissue: 7 day old plants do

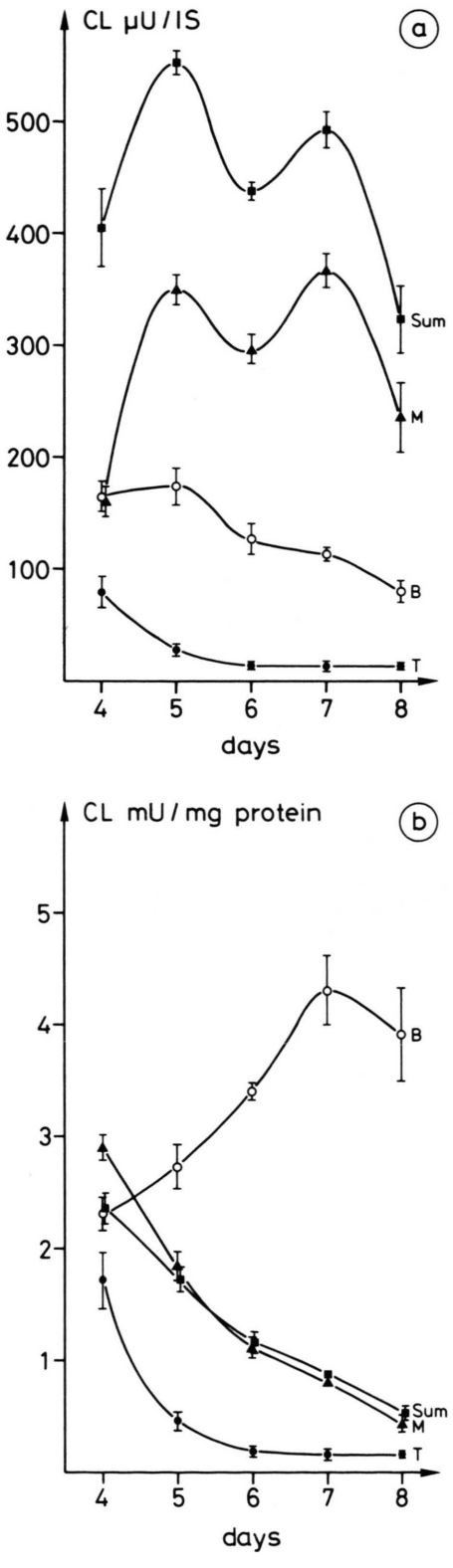

Fig. 1. Kinetics of CL activity in oat primary leaves; a) activity per individual section, (●–●) top section, (▲–▲) middle section, (○–○) basal section, (■–■) sum; one unit (U) is defined as 1 µmol pCA-CoA thiolester formed per minute under the reaction conditions; data represent means of six experiments; standard deviations are indicated; b) specific activities of CL in different leaf sections.

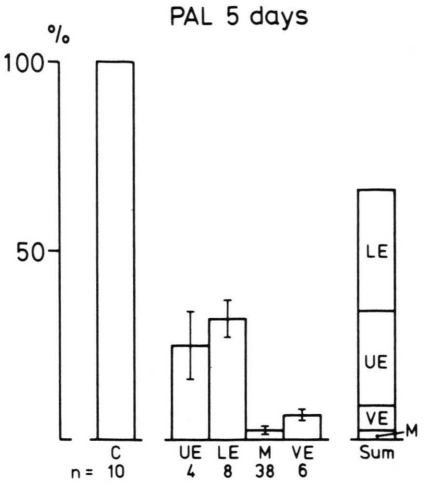

Fig. 2. Relative distribution of PAL activity in different leaf tissues; (C) control with unpeeled leaf, (UE/LE) upper/lower epidermis, (M) mesophyll protoplasts, (VE) vascular elements, (n) number of experiments; 100% PAL ≙ 803 ± 18 μU/individual section (IS). 1 U ≙ 1 μmol of product formed per min under the reaction conditions.

not contain any CL activity in the epidermal tissues, 65% of the extractable activity are located in the vascular elements, 35% in the mesophyll cells (protoplasts) (Fig. 3b).

In 5 day old plants 34% of PAL activity of the whole leaf are lost during protoplast isolation; in the older leaves PAL activity can be measured only in the epidermis [3] and in the vascular elements (data not shown) while nearly 90% of the total enzyme activity are lost during protoplast preparation. The reason for this loss has remained unknown up to now. In contrast to PAL, nearly 100% of the CL activity of the whole leaf can be found in the separated tissues.

A comparison of these data with the distribution of PAL in 6 day old barley shoots [22] reveals significant differences; in barley, only 10–20% of PAL activity are located in the epidermal tissues, whereas up to 90% are found in the mesophyll (protoplasts and vascular elements). Furthermore, the location of CI is similar to PAL with the exception of vascular elements which lack extractable CI activity.

Table I shows the specific activities of CL as obtained for the three different tissues of 5 and 7 day old oat leaves. In both stages the highest values are measured in vascular elements. In the case of PAL of

Table I. Specific PAL and CL activities from different leaf tissues.

Plant age [d]	Enzyme	Tissue	Specific activity [mU/mg protein]	*n*
5	PAL	Upper epidermis	22.0 ± 4.7	4
		Lower epidermis	29.1 ± 8.6	8
		Mesophyll protoplasts	0.22 ± 0.1	38
		Vascular elements	13.5 ± 4.5	6
5	CL	Lower epidermis	4.0 ± 1.4	8
		Mesophyll protoplasts	1.6 ± 0.1	8
		Vascular elements	37.4 ± 5.6	8
7	CL	Lower epidermis	–	3
		Mesophyll protoplasts	0.9 ± 0.3	3
		Vascular elements	29.3 ± 1.3	3

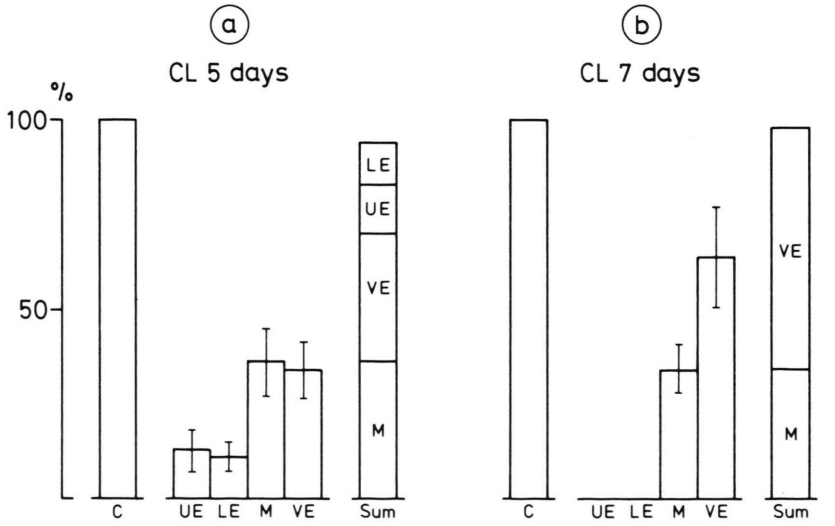

Fig. 3. Relative distribution of CL activity in different leaf tissues (for legend see Fig. 2); a) 5 day old seedlings, 100% CL ≙ 553 ± 12 μU/IS; data represent means of 7 experiments; b) 7 day old seedlings, 100% CL ≙ 493 ± 25 μU/IS; data represent means of 3 experiments.

Table II. Substrate specificity of CL from different leaf tissues. Comparison of relative initial rates, as measured with equimolar substrate concentrations.

Plant age [d]	Tissue	Reaction rate (%) with the substrate			
		pCA	CA	FA	SA
5	Lower epidermis	100	66	34	0
	Mesophyll protoplasts	100	41	35	0
	Vascular elements	100	8	33	0
7	Lower epidermis	0	0	0	0
	Mesophyll protoplasts	100	52	62	0
	Vascular elements	100	6	44	0

5 day old plants, however, the values of epidermal and vascular tissues clearly exceed those of mesophyll cells (protoplasts).

It can be seen from Table II that the CL activities from the different tissues vary significantly in substrate specificity. The enzyme preparation of vascular tissue converts caffeic acid to the correponding CoA thiolester to a very low extent, whereas the epidermal enzyme (5 day stage) shows an efficient reaction rate with this substrate. Besides *p*-coumaric acid the vascular enzyme mainly accepts ferulic acid as substrate. In all cases *p*-coumaric acid leads to the highest reaction rates, whereas sinapic acid is not converted at all.

Investigation of PAL and CL activities of chloroplasts

Complementary to earlier experiments with chloroplasts isolated from whole leaves with classical methods [13], we tried to demonstrate the activities of PAL and CL in this organelle isolated from protoplasts. Table III shows that about 50% of the PAL activity of protoplasts are located in intact chloroplasts. Whereas Nishizawa *et al.* found an activity increase of PAL in spinach chloroplasts caused by the addition of 15 mM DTT [23], this does not apply to PAL from oat chloroplasts.

In contrast to PAL, it has not been possible to directly demonstrate any CL activity in isolated chloroplasts [*cf.* 24]. After incubation of intact plastids (70 to 90% intactness according to the ferrycyanide test [25]) with the substrates of the CL by use of [2-^{14}C]- or [ring-^{3}H]-*p*-coumaric acid, five products could be separated by TLC, but none of these cochromatographed with authentical pCA-CoA thiolester [15]. Alkaline hydrolysis of the ^{14}C compounds led to free pCA while *p*-hydroxybenzoic acid appeared besides pCA and a third unidentified substance when the ^{3}H derivatives were used. It has not been possible up to now to discriminate between β-oxidation which would demand the CoA thiolester of pCA as a substrate (for review see ref. [11]) and a non-oxidative side chain shortening [26], which both lead to the same product, *p*-hydroxybenzoic acid.

Product identification and quantification

The identification and quantification of the reaction products of the CL was done as earlier described by HPLC and polyamide column chromatography [21]. Cochromatography of aliquots from the radioactive PAL assay with some possible resultant and side reaction products (benzoic acid, phenylacetate, phenylpyruvate) [27] in a two-dimensional chromatography system in most cases led to

Table III. PAL activity of protoplasts and chloroplasts, isolated from protoplasts.

Fraction	Activity			
	[mU/leaf section]	[mU/mg protein]	[mU/mg chlorophyll]	*n*
Chloroplasts (total homogenate, 1000 × *g* sediment) [a]	0.009 ± 0.005	0.038 ± 0.015	0.85 ± 0.47	5–8
Protoplast supernatant (after ultrasonic treatment and sedimentation of membranes)	0.018 ± 0.005	0.220 ± 0.1	1.76 ± 0.54 [b]	38
Protoplasts (total homogenate) [c]	0.020 ± 0.015	0.067 ± 0.048	1.95 ± 1.5	5
Chloroplasts (from sucrose gradient)		0.10 ± 0.03 [d]	1.73 ± 0.59 [d]	4

[a] Addition of 15 mM DTT leads to a 22–66% inhibition.
[b] per mg chlorophyll of protoplasts before sedimentation of membranes.
[c] Addition of 15 mM DTT leads to a 40% inhibition.
[d] Measurement with 15 mM DTT; no significant differences were obtained without DTT.

the single radioactively labelled spot of cinnamic acid, the direct product of PAL reaction. Sometimes, up to 20% of the radioactivity could be found in the benzoic acid, a resultant product of cinnamic acid. The R_f-values of cinnamic and benzoic acid, phenylacetate and phenylpyruvate, respectively, in the two solvent systems are: I 0.47, 0.42, 0.42 and 0.80; II 0.62, 0.84, 0.93 and 0.

Conclusion

From the results of the present paper together with those obtained for FS [4] and CI [3] we suggest that in 5 day old oat primary leaves PAL, CL and FS serve in the biosynthesis of flavonoids in a coordinated manner. The findings indicate that phenylalanine is deaminated to cinnamic acid mainly in both epidermal tissues. The thiolester formation takes place both in the epidermis and in the mesophyll, whereas almost all of the FS and CI activities are located in the latter [4, 3].

The substrate specificity of the vascular CL together with its strong increase in activity in older primary leaves may be interpreted by its participation in lignin biosynthesis. This seems to be consistent with the results obtained with barley shoots, in which high quantities of PAL, but no CI could be measured in the vascular elements. Furthermore, these results apparently support the findings of Kamsteeg *et al.* [28], recently presented, which show that ring substitution can occur at the hydroxycinnamoyl CoA ester stage. As reported for other plants (for review see [29]), in oat sinapic acid cannot be esterified with CoA directly in the same manner as the other cinnamic acids. It has not been possible to synthesize the sinapic acid-CoA thiolester *in vitro* with any enzyme preparation of oat leaves, even with partially purified ones [30], so that the synthesis of the sinapic acid itself does not seem to take place on the free acid stage.

In the case of the epidermal tissue and the mesophyll cells (protoplasts) the relatively high rates of caffeic acid-CoA thiolester formation and the lack of flavones of the corresponding luteoline type [31] seem to point to the existence of some additional phenylpropanoid biosynthesis, i.e. of caffeic acid esters or other cinnamic acid derivatives.

Enzyme distibution studies *in vitro*, as presented, together with radioactive tracer experiments as performed *in situ* [32], may be promising towards answering questions on the autonomy of individual leaf tissues or cell compartments, with respect to phenylpropanoid metabolism. Regulatory interrelationships, possibly occurring between these tissues, may also be taken into consideration.

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