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SUBCELLULAR LOCALIZATION OF ACYLTRANSFERASES FOR QUINOLIZIDINE ALKALOID BIOSYNTHESIS IN *LUPINUS*

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Key Word Index—*Lupinus*; Leguminosae; quinolizidine alkaloids; lupin alkaloids; tigloyltransferase; *p*-coumaroyltransferase; subcellular localization; biosynthesis.

Abstract—The subcellular localization of the two acyltransferases, tigloyl-CoA: (−)-13α-hydroxymultiflorine/(+)-13α-hydroxylupanine *O*-tigloyltransferase (HMT/HLTase) and *p*-coumaroyl-CoA: (+)-epilupinine *O*-*p*-coumaroyltransferase (ECTase), for quinolizidine alkaloid biosynthesis has been investigated using the isolated organelles from the hypocotyls of *Lupinus albus* and *L. hirsutus* green seedlings. The two acyltransferases were not located in chloroplasts, where *de novo* synthesis of the quinolizidine alkaloid skeleton is supposed to take place. The HMT/HLTase activity was associated with the mitochondrial fractions and localized in the matrix, judged by the marker enzyme assay. However, the ECTase activity was not associated with the mitochondrial fractions. Intracellular transport of quinolizidine alkaloids is discussed in relation to the biosynthetic pathway of ester alkaloids in *Lupinus* plants.

INTRODUCTION

Quinolizidine alkaloids are mainly distributed in the subfamily Papilionoideae of the family Leguminosae, as in the species of the genus *Lupinus*. Thus, the quinolizidine alkaloids are also referred to as lupin alkaloids. Some quinolizidine alkaloids exhibit interesting biological activities towards animals, insects and plants [1].

The *in vivo* tracer experiments revealed that quinolizidine alkaloids are formed from L-lysine *via* cadaverine as the first detectable intermediate [2, 3]. The biosynthesis of the quinolizidine ring is assumed to arise from the cyclization of cadaverine units *via* and enzyme-bound intermediate [4, 5]. Then, some alkaloids are modified by intracellular enzymes through dehydrogenation, hydroxylation, esterification, etc.

Quinolizidine alkaloid esters are mainly distributed in the plants of the genera *Lupinus* [6], *Cytisus* [7], Pearsonia [8], *Calpurinia* [9], and *Rothia* [10] as the esters of tiglic acid and *p*-coumaric acid. Some ester alkaloids are formed during seedling development [11]. Among these plants containing ester alkaloids, *Lupinus* plants accumulate two types of quinolizidine alkaloids, i.e. tetracyclic alkaloids (lupanine), and bicyclic quinolizidine alkaloids (lupinine). Although the biological significance of these ester alkaloids is not yet clarified, these alkaloids are assumed to be end products of alkaloid biosynthesis and represent the alkaloid accumulation pattern in particular plant species.

Biochemicals information about quinolizidine alkaloid biosynthesis is poor, compared with those for other-type alkaloid biosynthesis, i.e. tropane, isoquinoline and indole alkaloids [12]. Nevertheless, we have recently demonstrated the presence of two alkaloid acyltransferases, HMT/HLTase and ECTase, in *L. hirsutus* [13]. We have also first purified and characterized an alkaloid acyltransferase, HMT/HLTase, from *L. albus* (= *L. termis*) seedlings [6]. The HMT/HLTase catalyses the transfer of tigloyl group of tigloyl-CoA to the 13α(axial)-hydroxy residue of 13α-hydroxymultifrorine/13α-hydroxylupanine. This enzyme is widely distributed in *L. hirsutus*, *L. albus*, and Russell lupin (a hybrid of *L. polyphyllus* and *L. arboreus*). In contrast, the ECTase, which catalyses the transfer of *p*-coumaroyl group of *p*-coumaroyl-CoA to the hydroxy residue of (+)-epilupinine, and is distributed only in *L. hirsutus* [13].

Information on the cell physiology of quinolizidine alkaloid biosynthesis is also limited. Regarding intracellular localization of enzymes for quinolizidine alkaloids biosynthesis, only lysine decarboxylase (LDC) and the cyclizing enzyme responsible for quinolizidine skeleton formations were detected in chloroplasts of leaves of *L. polyphyllus* [14].

In the present paper, we investigate intracellular

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Table 1. Subcellular localization of HMT/HLTase in *L. albus*

Subcellular fraction		Enzyme activity (pkat mg protein^{-1}) HMT/HLTase	Photoreduction (chloroplasts)	Fumarase (mitochondria)	PFPase (cytosol)
Homogenate		3.06	14.1	2450	9.5
Chloroplasts	(Crude)	3.69	15.5	<1	<0.1
	(Pure)	<0.1	95.9	<1	<0.1
Mitochondria	(Crude)	6.75	1.1	405	3.5
	(Pure)	99.6	11.9	5760	<0.1
Microsomes		5.75	N.D.*	617	N.D.
Cytosol		3.75	42.8	1770	12.0

Hypocotyls of *L. albus* seedlings were used for preparation of a homogenate and for subsequent isolation of subcellular fractions as described in the Experimental. The activities of photoreduction, fumarse and pyrophosphate: fructose-6-phosphate 1-phosphotransferase (PTPase) were determined as the maker activities for chloroplasts, mitochondria and cytosol, respectively.

*N.D., not determined.

localization of two alkaloid acyltransferases, HMT/HLTase and ECTase in the green seedlings of *L. hirsutus* and *L. albus*. We discuss the putative metabolic and transport pathways of quinolizidine alkaloids in *Lupinus* plant cells.

RESULTS

The subcellular organelles were purified from the homogenates of hypocotyls of 10-day-old seedlings of *L. albus* by differential centrifugation and Percoll-density gradient sedimentation. Each separated organelle was subjected to determination of HMT/HLTase activity and organelle marker enzyme activities for chloroplast, mitochondrion and cytosol. Table 1 shows the distribution of HMT/HLTase and the marker enzymes in the fractions of homogenate, crude and pure chloroplasts, crude and pure mitochondria, microsomes, and cytosol. The highest specific activity of HMT/HLTase based on the protein concentration was obtained in the purified mitochondrial fraction. This mitochondrial fractions was found to contain also the highest activity of fumarase, the mitochondrion maker enzyme, and a slight activity of PFPase, the cytosol marker enzyme, but was free of the cytosolic contamination. These results suggested that HMT/HLTase was localized in mitochondria of *L. albus* cells.

To clarify the intra-organelle distribution of HMT/HLTase in mitochondria, the activity of HMT/HLTase was determined in the matrix and the membrane fractions of isolated mitochondria. The purified mitochondria were disintegrated by several cycles of freeze-thawing in a hypotonic buffer. The resulting solution was separated into supernatant (matrix) and pellets (membrane fractions) by centrifugation. Table 2 shows the results of distribution of the enzyme activities, HMT/HLTase and fumarase in the mitochondrial fractions. There was little difference in the total activity of HMT/HLTase in the matrix and the membrane fractions. However, the specific activity of HMT/HLTase in the matrix (640 pkat mg^{-1} protein) was higher than that of HMT/HLTase in the membrane fractions (190 pkat mg^{-1} protein). Since fumarase is known to be localized in the matrix, the higher specific activity of fumarase in the matrix was also exhibited. The results indicated that HMT/HLTase exists in the matrix of mitochondria of *L. albus* cells.

ECTase, which catalyses the formation of *p*-coumaroyl-ester of a bicyclic quinolizidine alkaloid, (+)-epilupinine, is present in *L. hirsutus*, but not in *L. albus*. To elucidate the subcellular localization of ECTase, the isolated organelles from green seedlings of *L. hirsutus* were used for enzyme assay. In addition to ECTase activity, HMT/HLTase was simultaneously assayed (Table 3). The data indicated that HMT/HLTase was associated with mitochondria as in the case of *L. albus*, but ECTase was not localized in mitochondria. These results indicated that HMT/

Table 2. Intra-organelle distribution of HMT/HLTase in mitochondria

Subcellular fraction	HMT/HLTase Specific activity (pkat mg protein^{-1})	HMT/HLTase Total activity (pkat)	Fumarase Specific activity (nkat mg protein^{-1})	Fumarase Total activity (nkat)	Total protein recovery (%)
Pure mitochondria	333	1116	36.6	122.4	100
Supernatant (matrix)	640	768	41.4	49.6	36
Pellet (membrane fractions)	190	584	5.5	16.9	92

Isolated mitochondria were disrupted by repeated freeze–thawing in the buffer (20 mM Mops–KOH, pH 7.2, 1 mM EDTA and 0.2% [w/v] BSA). After centrifugation of 10 000 *g* for 10 min, supernatant and pellet were separated and designated as matrix and membrane fractions, respectively.

Table 3. Subcellular localization of HMT/HLTase and ECTase in *L. hirsutus*

Subcellular fraction		Enzyme Activity (pkat mg protein^{-1}) HMT/HLTase	ECTase
Homogenaste		0.08	0.3
Chloroplasts	(Crude)	3.72	1.2
	(Pure)	<0.01	<0.1
Mitochondria	(Crude)	5.49	0.7
	(Pure)	7.30	<0.1
Microsomes		2.23	2.2
Cytosol		0.24	4.6

Subcellular fractions were obtained from hypocotyls of 14-day-old *L. hirsutus* seedlings as described in the Experimental. The activities of HMT/HLTase and ECTase were determined.

HLTase and ECTase were distributed in different subcellular compartments.

DISCUSSION

The quinolizidine alkaloid esters are widely distributed as major components of plant secondary metabolites in plants of the genus *Lupinus* [1]. However, information of subcellular localization of enzymes for quinolizidine alkaloid biosynthesis is limited. Only the activities of LDC and of the forming enzyme of quinolizidine skeleton were detected in chloroplasts of *L. polyphyllus* [14]. The production of quinolizidine alkaloids in *Sophora* and *Thermopsis* plant tissue culture was correlated with cell differentiation, in particular with the amounts of chloropyll [15, 16], suggesting a close relationship between alkaloid-biosynthetic ability and chloroplast differentiation. Our present study demonstrated that two alkaloid acyltransferases, HMT/HLTase and ECTase, are localized in different subcellular compartments of *Lupinus* plants. HMT/HLTase is localized in the matrix of mitochondria; and ECTase is assumed to be present in another organelle (but not in mitochondria).

By combining all data available so far, we propose the hypothesis of a putative intracellular transport system in quinolizidine alkaloid biosynthesis as shown in Fig. 1, and discussed as follows.

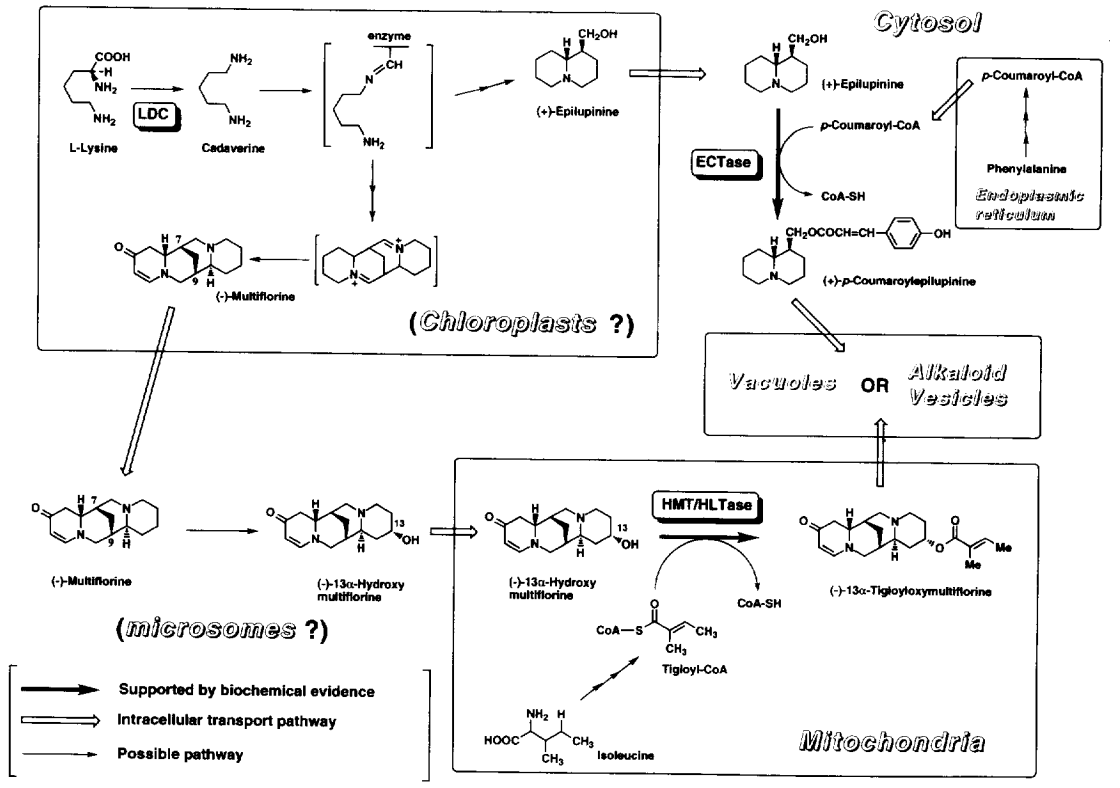

Fig. 1. Putative intracellular transport system for quinolizidine alkaloid biosynthesis in *Lupinus* plant cell.

(i) Chloroplasts may be responsible of the formation of the quinolizidine skeleton, such as (−)-multiflorine/(+)-lupanine and (+)-epilupinine, from L-lysine via cadaverine. Subsequently only tetracyclic alkaloids, (−)-multiflorine/(+)-lupanine, are transported to microsomes and are hydroxylated at the 13α-position of alkaloids by microsomal oxidation. However, the participation of these organelles (chloroplasts and microsomes) for quinolizidine alkaloid biosynthesis needs to be clarified experimentally in further detail.

(ii) (−)-13α-Hydroxymultiflorine and (+)-13α-hydroxylupanine are then transported into mitochondria. The tigloyl group from tigloyl-CoA is transferred to the 13α-hydroxyl residue of the alkaloids by the function of HMT/HLTase. In the literature, tigloyl esters of alkaloids are found not only in the quinolizidine alkaloids but also in the tropane alkaloids. In *Datura* plants, the biosynthetic pathway of the tigloyl esters of tropane alkaloids, *e.g.* tigloidine and meteloidine, was investigated by tracer experiments [17, 18]. The tigloyl group of these alkaloids was proved to be derived from tigloyl-CoA originated from isoleucine via three conversion steps. Regarding subcellular localization of converting enzymes from isoleucine to tigloyl-CoA, mitochondria are responsible for tigloyl-CoA biosynthesis in rat liver and kidney [19, 20]. Suppose this pathway is also present in plant mitochondria, tigloyl-CoA is readily available in mitochondria for tigloyl transfer to the alkaloids.

(iii) (+)-Epilupinine, a bicyclic alkaloid, can probably be directly transported in cytosol from chloroplasts and is conjugated with the *p*-coumaroyl group from *p*-coumaroyl-CoA to the hydroxy residue of the alkaloid by the function of ECTase. *p*-Coumaroyl-CoA is known to be synthesized from L-phenylalanine via cinnamic acid and *p*-coumaric acid through the phenylpropanoid pathway [21]. Regarding subcellular localization of phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C4H), enzymes of phenylpropanoid pathway, these enzymes are associated with the endoplasmic reticulum (ER) membranes appeared in the microsomal fractions in *Hippeastrum* [22]. However, some other enzymes of the flavonoid pathway were shown to be present in various organelles, e.g. chalcone synthase in cytosol or endoplasmic reticulum membranes, and chalcone isomerase in cytosol [23]. Thus, it was assumed that *p*-coumaroyl-CoA can be transported from endoplasmic reticulum membranes into cytosol and is readily available in cytosol for *p*-coumaroyl transfer to the (+)-epilupinine in cytosol.

(iv) The quinolizidine alkaloid esters, i.e. (−)-13α-tigloyloxymultiflorine/(+)-13α-tigloyloxylupanine and (+)-*p*-coumaroylepilupinine, are possibly stored in vacuoles or alkaloid vesicles. Some alkaloids, such as tropane and isoquinoline alkaloids, are known to be stored in vacuoles [24, 25] and in alkaloid vesicles [12, 26]. The quinolizidine alkaloid esters in subcellular organelles are predominantly distributed in cytosolic fractions, rather than in chloroplasts, mitochondria and microsomes (unpublished results). Since vacuoles or alkaloid vesicles could be broken and fractionalized into the cytosol by our present method, we assumed that quinolizidine alkaloid esters were stored in vacuoles or alkaloid vesicles.

EXPERIMENTAL

Plant material. The seeds of the *L. albus* and *L. hirsutus* were germinated in moistened vermiculite in the greenhouse at 25°. Hypocotyls were harvested from 10–14-day-old green seedlings.

Chemicals. All standard alkaloids used in this study were from our laboratory stock. Tigloyl-CoA, fructose-2,6-biphosphate, fructose-6-phosphate, triose phosphate isomerase, glycerol-3-phosphate dehydrogenase and aldolase were from Sigma. *p*-Coumaroyl-CoA was prepared as described previously [27]. Other chemicals were of the highest grade available.

Preparation of subcellular fractions. All operations for the isolation of organelles were performed at 4° unless otherwise stated. Chloroplasts were isolated by the method of ref. [28] with modifications. Freshly harvested hypocotyls (150 g) of 10-day-old *L. albus* seedlings were cut into small pieces and then placed in 450 ml of homogenizing buffer (50 mM Mes–KOH, pH 6.1, containing 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 2 mM Na isoascorbate and 0.1% [w/v] BSA). After homogenization with a Waring blender for 30 sec, the homogenate was filtered through 2 layers of cotton cloth. The filtrate, designated as the homogenate, was centrifuged at 1000 *g* for 100 sec. The supernatant was used for the isolation of mitochondria (see below). The pellet, designated as crude chloroplasts, was suspended in resuspension buffer A (50 mM Hepes–KOH, pH 7.9, containing 330 mM sorbitol and 1 mM EDTA) and then overlaid on a step-gradient of Percoll® (Pharmacia, Uppsala, Sweden), with concns of 40% (v/v) and 80% (v/v), in buffer A. After centrifugation at 1000 *g* for 15 min, the purified chloroplasts were collected from the interface between the 40 and 80% Percoll layers and washed once with buffer A.

Mitochondria were isolated according to the method of ref. [29] with modifications. The supernatant obtained on centrifugation at 1000 *g* for 100 sec as described above was then sepd by centrifugation at 3000 g for 5 min. The resulting supernatant was further sepd by centrifugation at 12 000 *g* for 20 min. The 12 000 *g* supernatant was used for isolation of the cytosolic fr. (see below); the ppt, designated as crude mitochondria, was resuspended in buffer B (20 mM Mops–KOH, pH7.2, containing 300 mM mannitol, 1 mM EDTA and 0.2% [w/v] BSA), followed by centrifugation at 1500 *g* for 10 min. The resulting supernatant was centrifuged again at 12 000 *g* for 20 min. The pellet was resuspended in buffer B and then fractionated on a step-gradient of Percoll, with concns of 13.5% (v/v), 21% (v/v) and 45% (v/v), in buffer B by centrifugation at 7500 *g* for 30 min. The pure mitochondria were collected from the interface between the 21 and 45% Percoll layers and washed once with buffer B.

The supernatant obtained on centrifugation at 12 000 *g* for 20 min was further sepd by centrifugation at 100 000 *g* for 30 min. The pellet was resuspended 0.02% (v/v) Triton X-100 in buffer B and used as the microsomal fr. The resulting supernatant was used as the cytosolic fr.

Subcellular frs from freshly harvested hypocotyls (100 g) of 14-day-old *L. hirsutus* seedlings were prepared by the same method as described above.

Enzyme assays of alkaloid acyltransferase. The alkaloid acyltransferase activities (HMT/HLTase and ECTase) were assayed by the methods reported in ref. [16]. The standard reaction mixt. comprised 100 mM K-Pi, pH 8.0, 0.5 mM EDTA, 1 mM DTT, 0.15 mM alkaloid substrate [(−)-13α-hydroxymultiflorine or (+)-epilupinine], 0.15 mM acyl-CoA (tigloyl-CoA or *p*-coumaroyl-CoA) and the enzyme protein in a total vol. of 240 μl. After incubation for 60 min at 30°, the reaction was terminated by the addition of 20 μl of 6 M HCl. After adding 1 μg of *N*-methylcytisine (lupin alkaloid) dissolved in 10 μl of H_2O as an int. standard, the resulting acidified soln was extracted twice with 500 μl of EtOAc. The aq. soln was made alkaline by the addition of powdered K_2CO_3 (150 mg). The alkalized soln was then extracted twice with 500 μl EtOAc; then, the organic layers were combined and evapd *in vacuo*. The resulting alkaloidal residue was dissolved in 20 μl of MeOH. Analysis of ester alkaloids enzymatically formed were carried out by HPLC or GC-MS as described in ref. [6]. HPLC analysis was performed on a LiChrosorb Si 60 (5 μm) column (0.46 × 30 cm, Merck, Darmstadt, Germany), with monitoring at 327 nm for (−)-13α-tigloyloxymultiflorine and at 313 nm for (+)-*p*-coumaroylepilupinine, respectively. GC-MS analysis was carried out with a combined GC-MS system (Hewlett Packard, 5980II/5971A) equipped with a fused silica capillary column, DB-1 (J&W Scientific, CA, U.S.A.; 0.25 mm × 30 m), with a programmed temp. increase (100–300°, 15° min^{-1}).

Marker enzyme assays of subcellular compartments. The chloroplast marker was assayed as the photoreduction of ferricyanide measured colorimetrically [30, 31]. The reaction mixture contained 50 mM Hepes–KOH buffer, pH 7.9, 10 mM NH_4Cl, 0.40 mM $K_3Fe(CN)_6$ and 50 μl of the chloroplast preparation in a final vol. of 2.55 ml. The reaction mixture was illuminated for 2 min with artificial light (20 000 lux). The reaction mixture was terminated by the addition of Na_3 citrate (8.0 mM), 1,10-phenanthroline (2.0 mM) and $FeCl_3$ (0.4 mM). After leaving the reaction mixture for 3 min at room temp, the soln was filtered through a 0.45 μm-membrane (cellulose acetate filter DISMIC-25; Toyo, Tokyo, Japan). The photo-reduced ferrocyanide was determined spectrometrically at 510 nm as a Fe^{2+}-orthophenanthroline complex.

Fumarase activity was used as the marker for mitochondria [32]. The reaction mixt. comprised 80 mM NaPi buffer, pH 7.5, 4.0 mM DTT, 8.0 mM Na malate and 100 μl of the mitochondria preparation in a final vol. of 1.3 ml. The reaction was started by the addition of Na malate. After incubation at 25° for 5 min, fumarate formed was quantified spectrophotometrically at 240 nm.

Phyrophosphate: fructose-6-phosphate 1-phosphotransferase (PFPase) activity was used as the marker for the cytosol [33]. The reaction mixt. comprised 50 mM Hepes–KOH buffer, pH 7.8, 0.5 mM $MgCl_2$, 10 μM fructose-2, 6-bisphosphate, 5 mM fructose-6-phosphate, 10 units of triose phosphate isomerase, 1 unit of glycerol-3-phosphate dehydrogenase, 0.1 units of aldolase, 0.15 mM NADH, 0.6 mM $Na_4P_2O_7$ and 100 μl of the cytosol prepn in a final vol. of 1.0 ml. The reaction was started by the addition of $Na_4P_2O_7$. Oxidation of NADH was determined spectometrically at 340 nm at 25°.

Protein determination. Protein was determined by the method of ref. [34] using a commercial protein assay reagent (Bio-Rad Laboratories GmbH, Munich, Germany) was BSA as standard.

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