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THE ENDOPLASMIC RETICULUM - GOLGI SYSTEM IS A MAJOR SITE OF PLASTOQUINONE SYNTHESIS IN SPINACH LEAVES

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Plastoquinone biosynthesis was recently found to be enriched in the microsomal fraction of spinach leaves. Since it is generally assumed that this lipid may predominantly be synthesized in the chloroplast envelope we have prepared total microsomes, containing both endoplasmic reticulum and Golgi membranes, and chloroplast envelopes. Marker enzymes and lipid content showed no significant cross-contamination. Nonaprenyl-2-methylquinol transferase, participating in plastoquinone synthesis, was found in the microsomes but was absent from the chloroplast envelopes and thylakoid membranes. Nonaprenyl-4-hydroxybenzoate transferase activity, an enzyme of ubiquinone biosynthetic system, in microsomes exceeded more than 10 times that found in chloroplast envelopes. The result indicates that in plants plastoquinone is synthesized in the endoplasmic reticulum-Golgi system and transported to the chloroplasts.

Both plastoquinone (PQ) and ubiquinone (UQ) are essential components of the plant cell, participating in electron transport in the chloroplast and mitochondria, respectively. Additionally, it is also established, that UQ has a broad distribution and is found in most of the cellular organelles and membranes, which indicates additional functions than participation in the respiration (1). Because of these basic roles in cellular functions, information on the biosynthesis of these lipids is of considerable interest.

The mevalonate pathway, which produces the isoprenoid side-chains of both PQ and UQ is highly active in plant cells, where it produces more lipid end-products than the corresponding

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Abbreviations:

FPP, farnesyl pyrophosphate; NPHB, nonaprenyl-4-hydroxybenzoate; NPMQ, nonaprenyl-2-methylquinol; PQ, plastoquinone; UQ, ubiquinone; ER, endoplasmic reticulum.

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pathway in animal cells (2). The initial portion of this anabolic sequence -converting acetyl-CoA to farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) - is localized in the cytosol, but the presence of various subsequent enzymes in different organelles of plant cells has not yet been studied in detail. In animal cells FPP-synthase is present in four different organelles, in addition to the cytosol and dolichol and cholesterol synthesis take place both in the ER and peroxisomes (3).

In previous studies, chloroplasts were isolated from the spinach and PQ synthesis was found in the envelope fraction but not in thylakoid membranes (4, 5). Experiments with isolated subfractions from spinach leaves have recently demonstrated that the synthesis of both PQ and UQ can occur in the ER-Golgi system (6). The substrate for plant *trans*-prenyltransferase is cytoplasmic GPP in contrast to many other systems, where FPP serves as the substrate for this enzyme (7).

If the ER-Golgi system is the only localisation for the synthesis of these lipids, then they must be subsequently transported to chloroplasts and mitochondria, respectively, where they participate in redox reactions. On the other hand, taking into consideration recent findings on distribution of the biosynthetic enzymes of mevalonate pathway (3), it is quite possible that PQ synthesis has also multiple localisation and is present both in the ER-Golgi system and in chloroplast envelope. This possibility was investigated by preparing and analyzing subfractions of high purity.

Materials and Methods

Chemicals - Solanesol was isolated from Virginia tobacco leaves (Tobacco nicotiana). Pyrophosphorylation was performed as described earlier (8). Labeling of solanesol with tritium was achieved using [^{3}H]sodium borohydride (57 Ci/mmol, Amersham Corp.) according to Keenan and Kruczek (9).

Plant material - Spinach (Spinacia oleracea) was grown under artificial light at 18^{o}C with a daily light period of 10 h. Fully expanded 6-week- old leaves, with the mid-vein removed, were used in this studies.

Subfractionation - Whole homogenate, chloroplasts and total microsomes were prepared as described earlier (5, 10). The microsomal fraction also contains the majority of the Golgi membranes. Chloroplasts were purified using a Percoll gradient and their envelopes were prepared according to the procedure described by Douce and Joyard (11). Intact chloroplasts were swollen in a hypotonic medium consisting of 10 mM tricine-4 mM $MgCl_2$, pH 7.8. This mixture was then layered onto a discontinuous sucrose gradient consisting of a bottom layer of 0.93 M sucrose with a layer of 0.6 M sucrose above this. After centrifugation in a SW 27 rotor (Beckman), chloroplast envelopes were recovered between the 0.93 M and 0.6 M sucrose layers and thylakoid membranes were at the bottom of the tube. All fractions were resuspended in 0.25 M sucrose.

Lipid analysis -Lipids were extracted with chloroform:methanol:water, 1:1:0.3 (v/v), at 37^{o}C for 30 min and these extracts adjusted to a final chloroform:methanol:water ratio of 3:2:1. The lipids of interest were then separated using reversed-phase HPLC. A linear gradient was employed from the initial methanol:water (9:1) in pump system A to methanol:2-propanol (4:1) in pump system B. The absorption of the elute was monitored at 210 nm and eluted radioactivity quantitated using a radioactivity flow detector.

Chemical and enzymatic measurements - Assays of NPHB- and NPMQ-transferase activities were performed as described earlier (6). The products were analysed by reversed-phase HPLC and monitored by their absorption at 210 nm. NADPH-cytochrome c reductase and UDP-galactosyl transferase activities were assayed as reported earlier (12, 13).Chlorophyll and β-carotene were quantitated according to previously described procedures (14, 15).

Protein was determined employing the Bio-Rad procedure with bovine serum albumin as standard (16).

Results

The aim of the present investigation was to examine the relative extents of PQ and UQ synthesis in microsomes and chloroplast envelopes. For this purpose microsomes, chloroplasts and chloroplast envelopes from spinach leaves were prepared and characterized.

The degree of cross-contamination of the subfractions employed was analyzed by measuring two marker enzymes, as well as chlorophyll, α-tocopherol and β-carotene (Table 1). NADPH-cytochrome c reductase is an enzyme associated with microsomes and was not found in detectable amounts in the isolated chloroplasts or in their envelopes. Galactolipid synthesis occurs both in the ER-Golgi system and in chloroplasts, but glycoprotein synthesis is localized exclusively to the first of these compartments. When the transfer of galactose from UDP-galactose to protein in lipid-extracted preparations was analyzed only the microsomal fraction exhibited activity. Since chlorophyll is a constitutive exclusive component of the thylakoid membranes, this compound was not found in appreciable amounts in the microsomes and chloroplast envelopes prepared here. The α–tocopherol content in chloroplasts was 1.2 μg/mg protein and the level of this antioxidant lipid was four times higher in isolated chloroplast envelopes. Most significantly, α-tocopherol was not present in detectable amounts in microsomes. This demonstrated the absence of contamination by chloroplast envelopes,

Table 1. *The distributions of chlorophyll, α–tocopherol, β–carotene and marker enzymes in subcellular fractions from spinach leaves.* The enzymes activities were determined using different and appriopate protein concentrations for the individual fractions. Chlorophyll and β–carotene were extracted and quantitated spectrophotometrically. α-Tocopherol was determined by HPLC. The values presented are the means + S.D. of 5 experiments.
ND = not detectable.

FRACTION	NADPH-cyt.c reductase (mmol cyt.c red/min/mg prot.)	UDP-Gal-transferase (nmol gal transfered/h/ mg prot.)	Chlorophyll (μg/mg prot.)	α-Tocopherol (μg/mg prot.)	β–Carotene (μg/mg prot.)
Homogenate	1.5 ± 0.13	0.015 ±0.002	205 ± 26	0.52 ± 0.06	18 ± 1.1
Microsomes	9.3 ± 0.97	0.084 ±0.01	17 ± 2	ND	3 ± 0.23
Chloroplast	ND	0.003 ±0.0003	453 ± 31	1.20 ± 0.09	46 ± 4.9
Chloroplast envelopes	ND	ND	14 ± 1.6	4.7 ± 0.3	15 ± 2.3

Table 2 *Quinone lipid contents of whole homogenate and subcellular fractions from spinach leaves.* The fractions were isolated as described under Materials and Methods. All lipids were isolated using a single HPLC program and identified using appropriate standards. The values presented are the means±S.D.of 5 experiments.
ND = not detectable

	QUINONE LIPID		
	PQ	UQ9	UQ10
FRACTION	(μg/mg protein)		
Homogenate	1.50 ±0.17	0.11 ±0.014	0.10 ±0.013
Microsomes	0.20 ±0.023	0.20 ±0.022	0.16 ±0.012
Chloroplasts	4.10 ±0.30	ND	ND
Chloroplast envelopes	1.00 ±0.09	ND	ND

which might have been liberated during homogenisation, in the microsomal fraction. β–Carotene is known to be present in thylakoids and, like chlorophyll, this compound was also absent from microsomes. These results demonstrate that both the microsomes and chloroplasts envelopes employed in the present study were of high purity.

The quinone lipid contents of the fractions were very different (Table 2). PQ was found to be present in chloroplasts in an amount of about 4 μg/mg protein, whereas the level of this redox active lipid in the envelopes was four times less and this specific content in the microsomes was only 5% that in whole chloroplasts. Spinach leaves contain both UQ9 and UQ10, redox lipids known to be associated with the mitochondrial respiratory chain. Both this forms of UQ were recovered in microsomes in accordance with the earlier findings (6), but completely absent from chloroplasts and their envelopes.

Utilizing a rapid extraction - partition - HPLC procedure (17), the *in vivo* redox state of these lipids was determined (Table 3). After the commonly employed extraction procedure, these lipids are completely oxidized, but during the procedure employed here, which takes only 15 min, these compounds retain their *in vivo* redox state. PQ and UQ9 and UQ10 appeared to be largely in the reduced form in spinach leaves since upon HPLC analysis with an electrochemical detector as much as 80% of these compounds were in their quinol state.

The enzymes involved in PQ and UQ synthesis, which transfer the isoprenoid side-chain to the precursor ring of homogentisic acid and 4-hydroxybenzoate, respectively, have been characterized and are probably rate-limiting steps in the biosynthesis of these lipids (6, 18). The products of these condensation reactions between the isoprenoid side-chains and the precursor rings can be quantitated by reversed-phase HPLC (19). The product of the NPHB-transferase activity, nonaprenyl-4-hydroxybenzoate, eluted early, after 2 min. (Fig. 1A). If the incubation was performed in the presence of high concentrations of microsomal protein, the

Table 3. *Redox state of plastoquinone (PQ) and ubiquinone (UQ) in homogenates of spinach leaves.* PQ and UQ were extracted with petroleum ether - methanol. Aliquots of petroleum extract were evaporated, the residue was dissolved in chloroform:methanol 2:1 and subjected to HPLC. The reduced lipids were quantitated using an electrochemical detector. The values are the means ±S.D. of 5 experiments.

	Reduced form	
	(μg/mg protein)	(% of total)
PQ	1.24 ±0.140	81
UQ9	0.12 ±0.011	83
UQ10	0.08 ±0.007	80

intermediate in PQ synthesis, nonaprenyl-2-methylquinol, was also visible, eluting after 17 min. At the much lower protein concentrations, sufficient for assays of UQ synthesis, no product of NPMQ-transferase activity could be detected (Fig.1B). When chloroplasts were

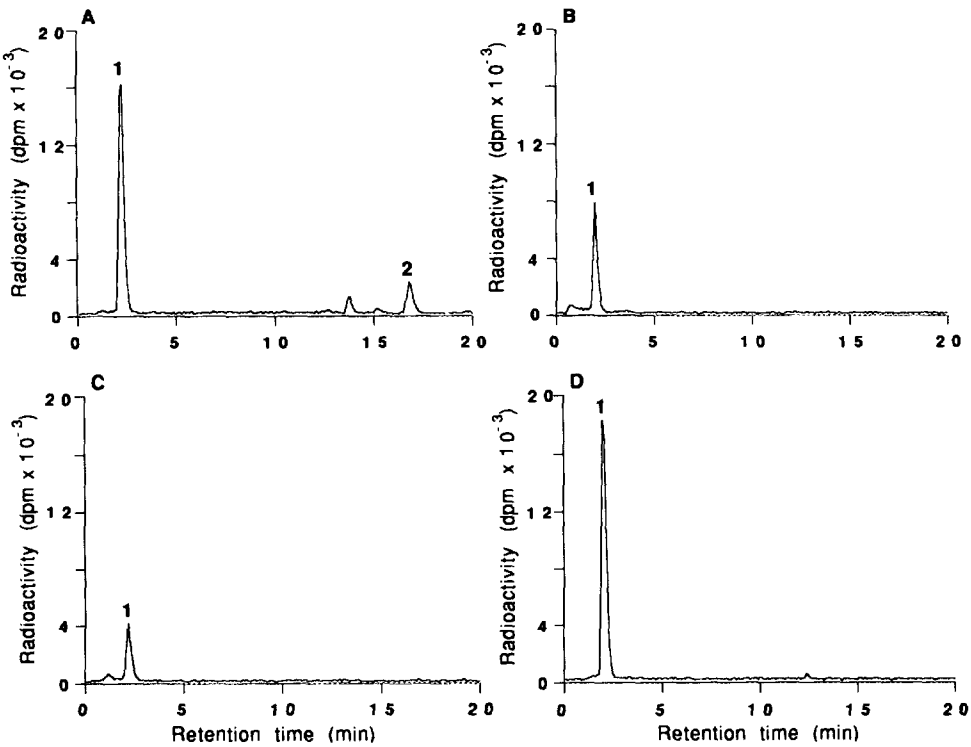

Fig.1. *HPLC analysis of the products of the NPHB- and NPMQ-transferases reactions.* Incubations were performed using [^{3}H]solanesyl-PP and 4-hydroxybenzoate and homogentisic acid, respectively, as substrates. A. Incubation with microsomes (50 μg protein). B. Microsomes (0.55 μg protein) as the enzyme source. C. Chloroplasts (125 μg protein) in the incubation mixture. D. Incubation with isolated chloroplast envelopes (36μg protein). 1= NPHB; 2= NPMQ.

Table 4. *Subcellular distributions of nonaprenyl - 4 - hydroxybenzoate and nonaprenyl- 2 -methylquinol transferase in spinach leaves.* The transferase activities were measured using [^{3}H]solanesyl-PP and excess amounts of 4-hydroxybenzoate and homogentisic acid, respectively, as substrates. The products were analyzed by HPLC and the radioactivity in the eluate quantitated using a radioactivity flow detector. The values presented are the means ± S.D. of 5 experiments.

	NPMQ - transferase	NPHB - transferase
	(dpm/mg protein/h)	
FRACTION		
Homogenate	19 ±0.80	4150 ±290
Microsomes	70 ±3.50	7230 ±650
Chloroplasts	3 ±0.32	60 ±6.8
Chloroplast envelopes	5 ±0.80	605 ±43
Thylakoid membranes	2±0.18	34±4.1

incubated in the presence of both precursor rings no NPMQ could be observed (Fig.1C). Upon incubating chloroplast envelopes with the same substrates, even with high concentrations of protein, only residual amount of PQ synthesis were detected (Fig. 1D).

The NPMQ-transferase activity, participating in PQ synthesis, was quantified for the isolated fractions (Table 4). The highest activity was detected in the microsomes, but only a very low activity could be found in the chloroplasts. Also, when the chloroplast envelopes were analysed, the transferase activity was very low. NPHB-transferase activity, involved in UQ synthesis, was heavily enriched in the microsomal fraction. Some minor activity was detected in the various chloroplast membranes, which may be a result of some unspecific reaction (6, 20).

Discussion

The organisation of the mevalonate pathway is complex and many of its biosynthetic enzymes are localized in several different cellular compartments. This has been established in detail in animal cells, where some enzymes of the pathway are recovered not only in the cytosol and/or microsomes, but also in peroxisomes, mitochondria and the Golgi system (21-23). However, the corresponding distribution pattern in plant cells has not been investigated in detail earlier.

Previously, it was found that the synthesis of both PQ and UQ can occur in the ER-Golgi system, which would require a transport mechanism(s) directing PQ to chloroplasts and UQ to mitochondria (6). It was originally suggested that PQ may be synthesized in the chloroplast envelope (4, 5), a reasonable suggestion in light of the proximity of this membrane to the

photosynthetic system. However, this appears from our present findings not to be the major site of the synthesis. Isolated chloroplast envelopes do not transfer solanesyl-PP to homogentisic acid and, consequently, do not appear to play a significant role in PQ synthesis. Nor are thylakoid membranes capable of synthesizing this lipid.

The spinach leaf cell is interesting not only from the point of view of the biosynthesis of PQ and UQ, but also as a system for studies on lipid transport and sorting. Both PQ and UQ can be synthesized in the ER-Golgi system and this localisation indicates the presence of specific targeting systems. PQ is exclusively transported to the chloroplasts, while UQ to mitochondria and to other cellular membrane compartments. The broad localisation of UQ in the cell is considered to be necessary for its antioxidant function. It was also demonstrated that PQ plays a role in plant cells as an antioxidant, too (24, 25). It will be of importance to characterize the specific receptor sites for these lipids on their respective organelles. For several phospholipids it has been established that after their synthesis on the ER, they are transported to other cellular membranes by cytoplasmic transport systems (26).

PQ is present at a low concentration at the site of its synthesis in the ER-Golgi system, while the level of this lipid is higher in chloroplast envelopes and it accumulates in thylakoid membranes. This pattern indicates sequential transport involving several compartments of the spinach cell, a process which can probably be analyzed in the future employing *in vivo* labeling and chase experiments.

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