ELSEVIER

Biochimica et Biophysica Acta 1463 (2000) 188–194

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

Subcellular localization of plastoquinone and ubiquinone synthesis in spinach cells

Malgorzata Wanke [a,*], Gustav Dallner [b], Ewa Swiezewska [a]

[a] *Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 02-106 Warsaw, Poland*
[b] *Department of Biochemistry, Stockholm University, S-10691 Stockholm, Sweden*

Received 23 July 1999; received in revised form 17 September 1999; accepted 29 September 1999

Abstract

In vivo labeling of spinach etiolated leaves with [^{3}H]mevalonate followed by rapid cell fractionation procedure showed that ER-Golgi membranes are involved in transport of plastoquinone (PQ) and ubiquinone (UQ) to plastids and mitochondria, respectively. Translocation of these lipids was inhibited by agents which affect protein and lipid intracellular transport causing structural and functional disintegration of the ER-Golgi system (monensin, brefeldin) and interfere with mitochondrial energy conservation (carbonyl cyanide *m*-chlorophenylhydrazone), but was not affected by colchicine which influences the organization of the cytoskeletal network. Colchicine treatment resulted in marked stimulation of PQ and UQ synthesis. Results of experiments with pre-exposure of etiolated seedlings to light suggest that translocation processes are dependent on the plastid developmental state and their capacity as acceptors of PQ. Thus, the experiments indicate that biosynthesis and transport of PQ and UQ involve multiple cellular compartments and that kinetics of the transport process is dependent on the actual physiological conditions. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Intracellular localization; Translocation process; Ubiquinone; Plastoquinone; Spinach leaf

1. Introduction

The major site for the synthesis of most lipids (sterols, phospholipids, dolichols, ubiquinone) in mammalian cells is the ER [1]. Consequently, the newly synthesized lipids have to be transported from the site of biosynthesis to various cellular organelles. The mechanisms of lipid traffic and sorting are highly selective since the lipid composition of different membranes is specific. Several possible routes for intracellular lipid movement have been considered: vesicular transport (from one organelle to another by vesicle budding and fusion) and spontaneous or protein mediated lipid translocation through the cytosol [1]. Lipid movement between intracellular organelles such as that of phosphatidylserine may occur between two types of membranes through close physical association. In this way, import of lipids from ER to mitochondria may occur via 'mitochondria associated ER membranes' (MAM) [2].

It is probable that lipid transport and sorting within cell organelles proceeds as a combination of the mechanisms noted above, thus the lipid traffic in the cell is a complex process. In addition, some lipids may be synthesized in more than one intracellular organelle, e.g. besides the ER ubiquinone is probably also synthesized in mitochondria [3], while both ER

* Corresponding author.

0005-2736/00/$ – see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0005-2736(99)00191-1

and peroxisomes biosynthesize cholesterol and dolichol [4]. Additionally, several compartments can be involved in the biosynthetic process of the same lipid, such as in the case of plasmalogens, where sequential reactions take place first in peroxisomes, followed by the ER. Some lipids are modified during their transport, PS is translocated from ER to mitochondria where it is modified to PE and the fatty acid composition of several phospholipids is remodeled [5].

PQ and UQ biosynthesis were found to be localized in the ER-Golgi membrane system of spinach cells [6,7]. Since PQ and UQ are accumulated and biologically active in chloroplasts and mitochondria, respectively, efficient and selective sorting mechanisms of these lipids within plant cells should exist. The multisite localization of biosynthesis is possibly also valid for these two isoprenoid lipids, since some synthetic steps were found to be associated with plastid envelopes [8] and mitochondria [9]. Additionally, the initial cytosolic system leading to farnesyl-PP includes two independent mechanisms, the mevalonate pathway and Rohmer's pathway, utilizing 1 deoxy-D-xylulose-5-phosphate [10].

In the present study, we have used in vivo labeling of spinach etiolated leaves with [^{3}H]mevalonate followed by preparation and analysis of subfractions to study the mechanisms of translocation UQ and PQ from the ER-Golgi system to mitochondria and chloroplasts, respectively. In order to interpret the mechanisms of quinone transport between cellular compartments, the effects of various compounds which affect protein and lipid intracellular transport and cause disintegration of ER-Golgi system, were also investigated.

2. Materials and methods

2.1. Materials

Spinach seedlings (*Spinacia oleracea* cv Medania) were grown for 11–14 days in darkness at 22–25°C in vermiculite. (*R*,*S*)-[5-^{3}H]mevalonate (specific activity, 3.52 Ci/mmol) was prepared using [^{3}H]borohydride (Amersham) according to Keller [11]. Brefeldin A, monensin, colchicine and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were obtained from Sigma, HPLC solvents from Baker and Merck. Plastoquinone standard was prepared from spinach leaves [6].

2.2. [^{3}H]Mevalonate labeling of spinach leaves

The seedling leaves (0.5 g fresh weight/dish) were cut into small pieces and floated in Petri dishes (diameter 2 cm) in a culture medium [12] supplemented with 0.05% Tween 20. Labeling was performed for 12 or 16 h at room temperature under continuous laboratory light with 0.5 mCi [^{3}H]mevalonate in 0.5 ml medium. Colchicine (0.2%) treatment was continued for 16 h while CCCP (20 μM), brefeldin A (7.5 μg/ml) and monensin (50 μM) treatment for 12 h. After incubation the leaves were washed five times with ice-cold 0.25 M sucrose.

2.3. Greening experiments

Roots were excised from the seedlings and solutions were fed in through the cut stem. Tubes (1 ml) were used for the [^{3}H]mevalonate labeling, each containing 0.5 ml of spinach growth medium as described earlier [9] for about 0.5 g of plants under continuous laboratory light. For these experiments we used green seedlings (grown in the growth chamber at 22°C with a 10-h photoperiod (700 W m^{-2}, OSRAM Power Star HQ1-E 400 W/DV and Sylvania F66W/D-RS), etiolated and etiolated seedlings exposed to the light in a growth chamber for 4 and 20 h prior to labeling. Labeling with [^{3}H]mevalonate was carried out as described above at a room temperature for 16 h under continuous laboratory light.

2.4. Cellular fractionation of spinach tissue

For preparations of cellular subfractions spinach seedlings were homogenized using a mortal and pestle in 0.25 M sucrose at 4°C. The homogenate was filtered through two layers of nylon net (mesh size 20 μm) and centrifuged at 1000 rpm for 3 min. The supernatant was centrifuged for 10 min at 6000 rpm to pellet the mitochondrial and chloroplast fraction. The remaining supernatant was centrifuged at 80 000 rpm for 15 min to separate microsomal and cytosolic fractions. All pellets were resuspended in 0.9% NaCl.

Purity of subfractions was analyzed with marker

enzymes as described earlier [6]. Cross-contamination was below 20%.

2.5. Lipid analysis

2.5.1. Lipid extraction

Lipids from subfractions were extracted according to Bligh and Dyer [13] with minor modifications. Extracts were purified on a Silica gel columns (230–400 mesh, Merck) and the lipid samples were dissolved in 50 μl of chloroform:methanol (2:1).

2.5.2. HPLC analysis

Analysis of lipids was performed by reversed-phase HPLC using a C-18 column (Hewlett-Packard Hypersil ODS 3-μm). A linear gradient was employed from the initial methanol:water (9:1) in pump system A to methanol:2-propanol (8:2) in pump system B at flow rate 1.5 ml/min, with a program time of 45 min. The absorbance at 210 nm and radioactivity of the eluate were monitored using a UV-detector connected to a radioactivity flow detector (Radiomatic Instruments, Tampa, FL) with a cell volume of 0.5 ml.

2.6. Chemical measurement

Protein was determined using the Lowry method [14] with bovine serum albumin as standard.

3. Results

During the initial 0.5 h of incubation of the etiolated spinach leaves with [^{3}H]mevalonate, a low and equal labeling of mitochondrial and microsomal ubiquinone-9 and ubiquinone-10 was observed (Fig. 1A,B). After 3 h, incorporation of [^{3}H]mevalonate into mitochondrial UQ-9 and UQ-10 was increased and significantly exceeded that in microsomes. During the next 21 h of incubation, there was a further, slow increase in the mitochondrial UQ-9 labeling, while the mitochondrial [^{3}H]ubiquinone-10 reached a plateau value. These results are in line with the concept that the newly synthesized UQ is rapidly translocated from the ER-Golgi to mitochondria.

In contrast to UQ-9 and UQ-10 the subcellular distribution of newly synthesized [^{3}H]plastoquinone

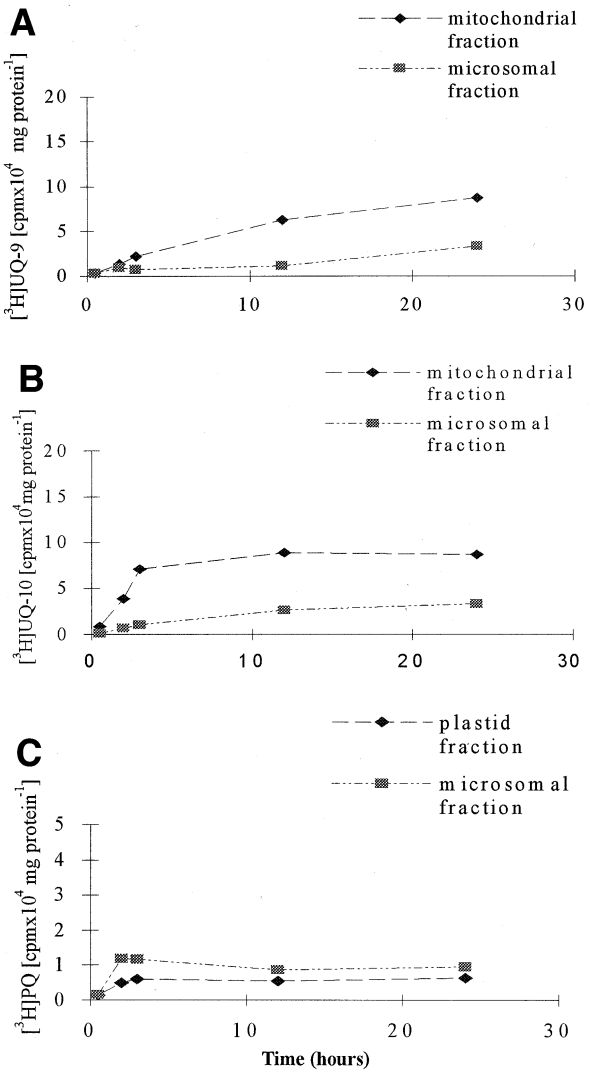

Fig. 1. Labeling of newly synthesized UQ-9 (A), UQ-10 (B) and PQ (C) with [^{3}H]mevalonate. Etiolated leaves were incubated with [^{3}H]mevalonate under laboratory light. Mitochondrial, plastid and microsomal fractions were isolated after various times of incubation. The lipids were analysed by reversed phase HPLC and radioactivity (cpm$\times 10^4$ mg protein^{-1}) of UQ-9, UQ-10 and PQ was determined in the isolated subfractions.

was totally different. After 0.5 h, a low and equal level of labeling of plastid and microsomal PQ was observed (Fig. 1C). After 3 h, the amount of labeled PQ in the microsomal fraction increased and exceeded that of the plastid fraction. It appears that at the early stages of seedling greening, translocation processes from ER to plastids are slow, leading to accumulation of [^{3}H]PQ at the site of biosynthesis.

Greening experiments were also performed in or-

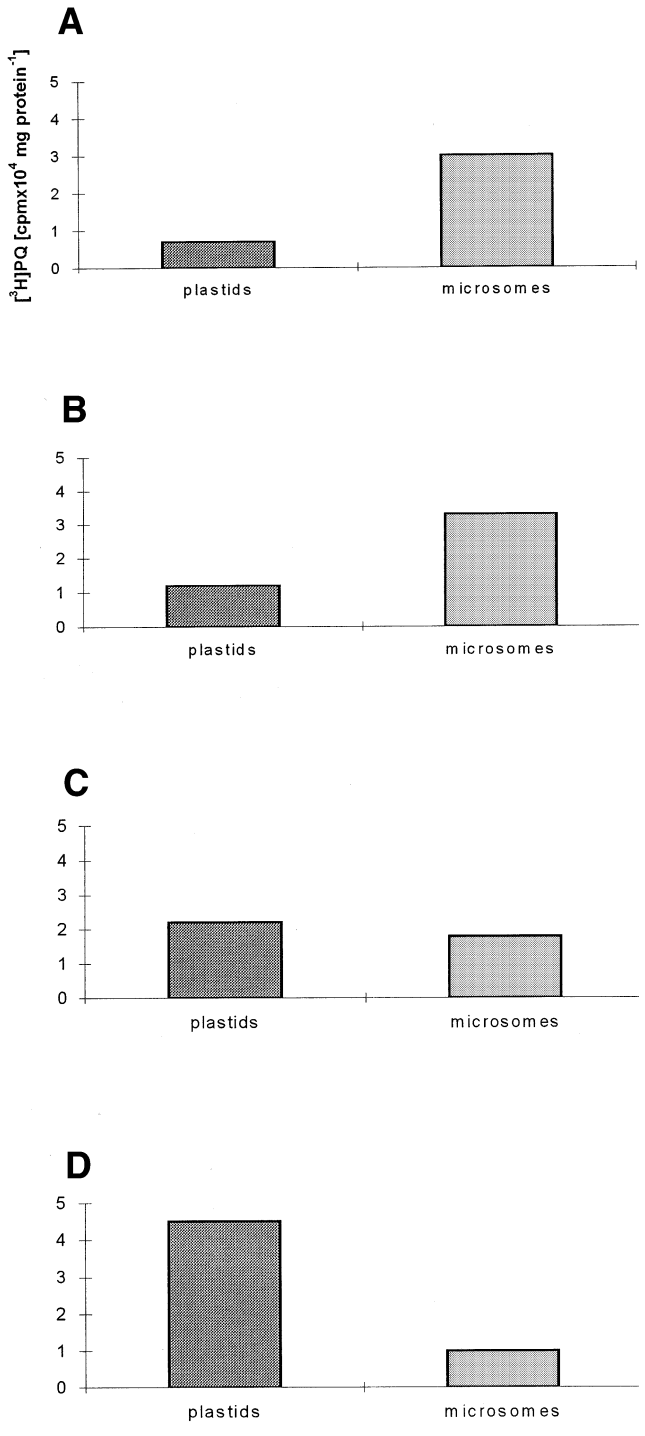

Fig. 2. Intracellular shift of newly labeled [^{3}H]PQ as a result of light pretreatment. Etiolated seedlings (A), etiolated seedlings pre-exposed to light for 4 h (B), etiolated seedlings pre-exposed to light for 20 h prior to labeling (C) and seedlings grown under regular light conditions (D). Labeling with [^{3}H]mevalonate was performed for 16 h under light conditions.

der to investigate the subcellular distribution of PQ, not only in etiolated, but also in greening and green seedlings. In etiolated seedlings, the amount of labeled PQ was four times higher in microsomes in comparison to plastids (Fig. 2A). This distribution pattern, however, is considerably modified upon illumination. After 4 h of light pre-exposure the level of [^{3}H]PQ in the plastid fraction was increased and the ratio plastid:microsomes was around 1:2 (Fig. 2B). A 20-h-long light pre-exposure further increased the ratio, and PQ content in plastids was somewhat higher that in the microsomal fraction (Fig. 2C). Finally, in green seedlings, plants grown in regular light conditions from the beginning of the experiments, the amount of [^{3}H]PQ in chloroplast fraction was 4.5 times higher than that in the microsomal fraction (Fig. 2D). Thus, the developmental changes elicited by illumination markedly modified the cellular distribution pattern of PQ. Total labeling of PQ is higher in this group of experiments than in the others. It could be explained by different in vivo labeling technique applied in this case (see Section 2).

The results of labeling in the presence of CCCP, monensin, brefeldin (BFA) and colchicine indicated that [^{3}H]ubiquinone-9 and -10 biosynthesis (total level in homogenate) is reduced by monensin and BFA, whereas CCCP had no effect on this process. Interestingly, colchicine stimulated biosynthesis of both: [^{3}H]UQ-9 and UQ-10 (data not shown).

When BFA and monensin were present, relatively higher level of [^{3}H]UQ in microsomal fractions of drug-treated plants versus non-treated plants was observed. The ratio of content of [^{3}H]UQ-9 in mitochondrial fraction toward microsomal was decreased from 3 to about 1.7 upon treatment (Fig. 3A). In the case of UQ-10, the same trend was also observed (Fig. 3B), however the results were less pronounced. Since labeling of UQ-9 and UQ-10 upon monensin treatment was at low level the scale for these experiments in Fig. 3A and B was expanded 10-fold. These findings indicate that in the presence of drug, the [^{3}H]UQs were unable to leave the ER as a consequence of disorganization of structure and function of the ER-Golgi network. We also examined the influence of CCCP on UQ transport. No inhibitory effect on translocation of UQ-9 and UQ-10 from the ER to mitochondria was observed (Fig. 3A,B). Colchicine treatment did not cause modification of

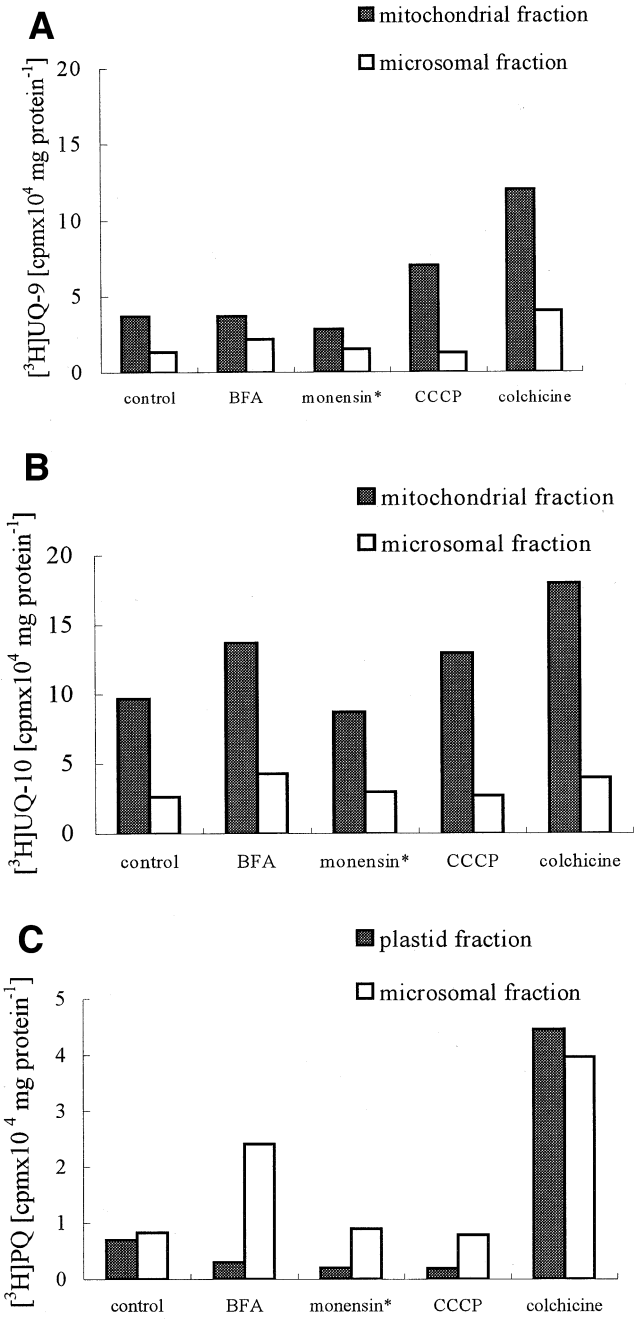

Fig. 3. Effect of BFA, monensin, CCCP and colchicine on intracellular lipid distribution. BFA (7.5 μg/ml), monensin (50 μM), CCCP (20 μM) and colchicine (0.2%) were included in the incubation medium, control plants were incubated in medium without any drugs. Etiolated seedlings were labeled with [^{3}H]mevalonate for 16 h under light conditions. After subfractionation, the lipids were isolated by HPLC and the radioactivity was determined with a Radiomatic flow detector. (A) [^{3}H]UQ-9 (cpm$\times 10^4$ mg protein^{-1}). (B) [^{3}H]UQ-10 (cpm$\times 10^4$ mg protein^{-1}). (C) [^{3}H]PQ (cpm$\times 10^4$ mg protein^{-1}). The scale for data of monensin treatment was expanded 10-fold.

the distribution pattern of the labeled UQ-9 and UQ-10.

Biosynthesis of PQ (total level of incorporation in homogenate) was significantly reduced by CCCP while no significant differences were observed after treatment with BFA and monensin (data not shown). Colchicine had the same stimulatory effect on PQ synthesis as in the case of UQ.

In control plants (i.e. labeled with mevalonate for 16 h under continuous light) in plastid and microsomal fractions [^{3}H]PQ content was similar (Fig. 3C). When BFA, monensin or CCCP were applied we observed significantly increased content of newly synthesized [^{3}H]PQ in the microsomal fraction. The ratio of [^{3}H]PQ content in plastids to microsomes was 1:1.2 in control plants, while in BFA treated plants it was 1:7, in monensin-1:3 (again the scale was 10-fold expanded) and in CCCP-treated plants 1:5. Like UQ, transport of the PQ was not influenced by colchicine treatment.

Thus, in the case of all lipids examined, i.e. UQ-9, UQ-10 and PQ, BFA and monensin treatments resulted in accumulation of the newly synthesized lipids at their site of biosynthesis, i.e. in the microsomes. CCCP had an inhibitory effect observed for PQ transport, but no change in distribution of [^{3}H]UQ could be found.

4. Discussion

The cellular functions and localization of plastoquinone and ubiquinone are studied in great detail. PQ participates in the electron transport of the photosynthetic process in chloroplasts and is also involved in a chlororespiratory pathway [15]. Recent studies have also shown antioxidant activity of the reduced form (PQH_2) of this lipid [16]. UQ is a component of a respiratory chain in mitochondria in all eucaryotic cells. It is also well established that UQH_2 plays a role as an antioxidant in animal cells both by inhibiting the initiation of lipid peroxidation and by regeneration of tocopheroxyl radicals [17]. This has also been observed in plants (A.M. Wagner, personal communication). Function of both PQ and UQ found in Golgi fraction of spinach cell [6] is not known.

The ER-Golgi localization of UQ and PQ synthe-

sis [6] does not exclude the possibility that, at least to some extent, other cellular organelles participate in this process. In *Arabidopsis*, the product of the gene encoding a 3,4-di-hydroxy-5-hexaprenylbenzoate (DHHB) methyltransferase which catalyzes the fourth step in the biosynthesis of UQ from *p*-hydroxybenzoic acid was found to be localized within mitochondrial membranes [18]. Additionally, it was also reported that some steps in PQ synthesis were found in chloroplast envelopes [19] and in UQ synthesis in mitochondria [20]. In animal cells, the major part of UQ synthesis is associated with the ER-Golgi system, but several steps of this process have also been identified in the mitochondria [3,21].

In the present study, we have employed in vivo labeling and a rapid cell fractionation procedure to investigate PQ and UQ distribution in plant cells. This method was useful for monitoring the appearance of newly synthesized lipids in organelles. [^{3}H]Mevalonate was found to be a substrate to study the biosynthesis of UQ and PQ. In our experimental conditions, the amount of [^{3}H]UQ formed de novo exceeded that of [^{3}H]PQ. This could be explained by the high PQ content in the plant material. Another possible explanation of lower tritium incorporation from mevalonate to PQ in comparison with UQ is the existence of a second metabolic pathway described for PQ [22]. According to this observation, PQ is formed via Rohmer's pathway. However, as indicated in that paper, the experiments were carried out under dim light conditions using a heterotrophically grown cell culture system devoid of functional chloroplasts. Consequently, the lack of acceptor organelles in this system makes it improbable that mevalonate pathway contributes to PQ synthesis. It will be a future task to study the contribution of these two systems to PQ synthesis under various physiological conditions. Relatively high ratio of amounts of newly labeled e.g. UQ-10 versus total sterol fraction (1:10) obtained in our experiments also confirm the contribution of mevalonate pathway in synthesis of quinones.

The rate of biosynthesis of PQ and UQ was examined as a function of time. Comparison of quinone content in subfractions has shown that in our experimental conditions de novo synthesized UQ is effectively transported to mitochondria, in contrast to PQ which is accumulated in the ER-Golgi system. In order to explain the reason for the microsomal PQ retardation, greening experiments were performed. Light pre-exposure of etiolated seedlings resulted in partial reconstitution of the transport of de novo labeled PQ from microsomal to plastid fraction. A shift of [^{3}H]PQ from microsomes to plastids was time-dependent concerning length of illumination and finally a higher amount of [^{3}H]PQ was found in plastids than in microsomes, approaching the distribution found in the intact green seedlings. Similar observations were made for native PQ content in spinach leaf subfractions. Independently of the age of the green leaf, PQ content in chloroplasts highly exceeded that in microsomes and the situation was reversed in etiolated seedlings (data not shown). These results suggest that translocation processes in the plant cell are dependent on the plastid developmental state. It is feasible that the chloroplast has to attain a certain level of structural development in order to serve as an acceptor for lipid synthesized outside of the organelle. If maturation of transport machinery is also light dependent, the cytosolic translocation system could be deficient in etiolated seedlings.

In order to further confirm that ER contributes to the biosynthesis of all quinones, we have tried to disintegrate the Golgi structure in vivo and analyze whether this situation causes some disturbances of quinone translocation from ER to mitochondria and chloroplasts. The employed compounds: BFA, monensin (which are Golgi system disintegrators) and CCCP (which is both an uncoupler and disintegrator of the ultrastructure of the Golgi compartment) are known to influence protein [23,24] and lipid [25,26] transport in animal cells. The experiments in these studies have demonstrated that BFA caused great changes in intracellular PQ distribution and also, to some extent, UQ distribution in spinach cells. The higher content of quinones in the microsomal fraction of treated plants shows that microsomes are the site of biosynthesis and simultaneously, that the Golgi system is involved in the translocation process of these quinones from the site of biosynthesis to the site of biological activity in plant cells. The other chemicals used, monensin and CCCP, had a similar inhibitory effect on the transport process, but the results were less pronounced.

Additionally, observed stimulation of UQ and PQ synthesis in experiments with colchicine treatment is difficult to explain at the moment. However, there are some literature data describing diverse effect of colchicine on animal cells. Colchicine treatment resulted in marked, 10-fold stimulation of prostaglandin synthesis [27], whereas in the case of hepatic proteins, this reagent caused accumulation of plasma proteins in Golgi-derived secretory vesicles [28].

From our results it is evident that Golgi membranes are involved in transport of PQ and UQ from ER to other organelles. Transport of these lipids was inhibited by monensin and brefeldin, drugs which are shown to affect vesicle-mediated processes also in the case of sphingolipids [29] and cholesterol in mammalian cells [30]. It appears that the vesicular mechanism plays a substantial role in the intracellular PQ and UQ trafficking in spinach cells. This vesicular transport seems to be independent of the organization of the cytoskeletal network since colchicine had no effect either on PQ or UQ distribution. Our previous studies on the influence of metabolic energy on PQ and UQ translocation demonstrated that UQ transport from the ER-Golgi system to mitochondria was energy dependent, but not completely blocked by energy poisons, whereas PQ transport was not influenced by these reagents [31]. It could suggest that also non-vesicular, ATP-independent process may operate in quinone translocation.

Summarizing a multiple pathway model of PQ and UQ intracellular transport, depending on lipid type and the membrane under consideration, should be discussed. Similarly, more than one transport pathway for export of integral membrane proteins from ER is recently suggested [32].

Acknowledgements

We are thankful to Prof. Michel Rohmer for helpful discussions. This study was supported by the Polish State Committee for Scientific Research Project 6PO4A 040 12 and the Swedish Council for Agricultural and Forestry Research.

References

[1] E.A. Dawidowicz, Annu. Rev. Biochem. 56 (1987) 43–61.
[2] J.E. Vance, J. Biol. Chem. 265 (1990) 7248–7256.
[3] R.E. Olson, H. Rudney, Vitam. Horm. 40 (1983) 1–43.
[4] J. Grünler, J. Ericsson, G. Dallner, Biochim. Biophys. Acta 1212 (1994) 259–277.
[5] R.E. Pagano, Curr. Opin. Cell Biol. 2 (1990) 652–663.
[6] E. Swiezewska, G. Dallner, B. Andersson, L. Ernster, J. Biol. Chem. 268 (1993) 1494–1499.
[7] S. Osowska-Rogers, E. Swiezewska, B. Andersson, G. Dallner, Biochem. Biophys. Res. Commun. 205 (1994) 714–721.
[8] J.F. Pennock, Methods Enzymol. 110 (1985) 313–319.
[9] F. Lütke-Brinkhaus, H. Kleing, Methods Enzymol. 148 (1987) 486–490.
[10] H. Lichtenthaler, Fett. Lipid 100 (1998) 128–138.
[11] R.K. Keller, J. Biol. Chem. 261 (1986) 12053–12059.
[12] C.A. Shipton, I. Parmryd, E. Swiezewska, B. Andersson, G. Dallner, J. Biol. Chem. 270 (1995) 566–572.
[13] E.G. Bligh, W.J. Dyer, Can. J. Biochem. Physiol. 37 (1959) 911–913.
[14] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.
[15] T.S. Feild, L. Nedbal, D.R. Ort, Plant. Physiol. 116 (1998) 1209–1218.
[16] T. Hundall, P. Forsmark-Andrèe, L. Ernster, B. Andersson, Arch. Biochem. Biophys. 324 (1995) 117–122.
[17] L. Ernster, G. Dallner, Biochim. Biophys. Acta 1271 (1995) 195–204.
[18] M.H. Avelange-Macherel, J. Joyard, Plant J. 14 (1998) 203–213.
[19] J. Soll, G. Schultz, J. Joyard, R. Douce, M. Block, Arch. Biochem. Biophys. 238 (1985) 290–299.
[20] F. Lütke-Brinkhaus, B. Liedvogel, H. Kleining, Eur. J. Biochem. 141 (1984) 537–541.
[21] A. Kalén, B. Norling, E.L. Appelkvist, G. Dallner, Biochim. Biophys. Acta 926 (1987) 70–78.
[22] A. Dish, A. Hemmerlin, T.J. Bach, M. Rohmer, Biochem. J. 331 (1998) 615–621.
[23] Y. Misumi, K. Miki, A. Takatsuki, G. Tamura, Y. Ikehara, J. Biol. Chem. 261 (1986) 11398–11403.
[24] J.K. Burkhardt, S. Hester, Y. Argon, J. Cell Sci. 92 (1989) 643–654.
[25] M.R. Kaplan, R.D. Simoni, J. Cell Biol. 101 (1985) 441–445.
[26] L. Urbani, R.D. Simoni, J. Biol. Chem. 265 (1990) 1919–1923.
[27] D.R. Robinson, H. Smith, M.B. McGuire, L. Levine, Prostaglandins 10 (1975) 67–85.
[28] C.M. Redman, D. Banerjee, K. Howell, G.E. Palade, J. Cell Biol. 66 (1975) 42–59.
[29] T. Kobayashi, R.E. Pagano, J. Biol. Chem. 264 (1989) 5966–5973.
[30] M.R. Kaplan, R.D. Simoni, J. Cell Biol. 101 (1985) 446–453.
[31] M. Wanke, E. Swiezewska, G. Dallner, Adv. Plant Lipid Res. (1998) 450–453.
[32] L. Gomez, M.J. Chrispeels, Plant Cell 5 (1993) 1113–1124.