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Chloroplast biogenesis. Cell-free transfer of envelope monogalactosylglycerides to thylakoids

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An ATP- and temperature-dependent transfer of monogalactosylglycerides from the chloroplast envelope to the chloroplast thylakoids was reconstituted in a cell-free system prepared from isolated chloroplasts of garden pea (*Pisum sativum*) or spinach (*Spinacia oleracea*). Isolated envelope membranes, in which the label was present exclusively in monogalactosylglycerides, were prepared radiolabeled *in vitro* with [^{14}C]galactose from UDP-[^{14}C]galactose to label galactolipids as the donor. ATP-dependent transfer of radioactivity from donor to unlabeled acceptor thylakoids, immobilized on nitrocellulose strips, was observed. In some experiments linear transfer for longer than 30 min of incubation was facilitated by the addition of stroma proteins but in other experiments stroma was without effect or inhibitory suggesting no absolute requirements for a soluble protein carrier. Transfer was donor specific. No membrane fraction tested (plasma membrane, tonoplast, endoplasmic reticulum, nuclei, Golgi apparatus, mitochondria or thylakoids) (isolated from tissue radiolabeled *in vivo* with [^{14}C]acetate) other than chloroplast envelopes demonstrated any significant ability to transfer labeled membrane lipids to immobilized thylakoids. Acceptor specificity, while not absolute, showed a 3–10-fold greater ATP-dependent transfer of labeled galactolipids from chloroplast envelopes to immobilized thylakoids than to other leaf membranes. The results provide independent confirmation of the potential for transfer of galactolipids between chloroplast envelopes and thylakoids suggested previously from ultrastructural studies and of the known location of thylakoid galactolipid biosynthetic activities in the chloroplast envelope.

Introduction

The majority of the thylakoid constituents are synthesized outside the plastid or in the plastid envelope. The dominant thylakoid lipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), are synthesized in the chloroplast envelope by step-wise galactosylation of diacylglycerol [1–4]. With isolated chloroplasts, newly synthesized galactolipids are transferred very rapidly from the envelope to the thylakoids [5,6].

From ultrastructural studies, transport of membrane material to the thylakoids has been suggested to be mediated by vesicles derived from the inner envelope membrane [7–11]. To investigate the mechanism of membrane transfer between chloroplast envelope and thylakoid, we have employed a completely cell-free system analogous to the system used previously to reconstitute membrane transfer between endoplasmic reticulum and Golgi apparatus with both plant [12,13] and animal [12,14] tissues. With this technique, radiolabeled donor membranes are activated using ATP and a cytosolic fraction to form transfer constituents to unlabeled donors [15,16] immobilized to nitrocellulose [14]. Results reported here demonstrate a cell-free reconstitution of time-, temperature- and ATP-dependent transfer of radiolabeled galactolipids from envelope membranes as donor in suspension to unlabeled thylakoids on nitrocellulose as acceptor.

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Materials and Methods

Plant material

Garden peas (*Pisum sativum* var. Kelvedon Wonder) were grown in soil in a climate chamber for approximately 3 to 4 weeks at 25°C and 12 h light. All leaves were harvested. Spinach (*Spinacia oleracea* var. Bloomsdale or Medania) was field (garden) grown, 4–6 weeks old, and was harvested and stored at 4°C overnight prior to homogenate preparation. Expanding leaves (4 to 8 cm long with petiole detached) were harvested.

Isolation of chloroplasts

The procedure, adapted from Walker [17], used a homogenization medium containing 50 mM Hepes, 0.01 M KCl, 3 mM EDTA, 0.01 M ascorbate, 0.1% bovine serum albumin and 5 mM dithiothreitol (pH 7.5) containing 0.33 M sucrose. The albumin, ascorbate, dithiothreitol and sucrose were added fresh to both homogenization and sucrose gradient solutions.

Approximately 25 g leaves were disrupted in 75 ml of medium for 5 s at full speed using a Waring Blender. The homogenate was filtered through Miracloth to remove cell walls, unbroken cells and debris and centrifuged for 90 s at $8000 \times g$ using a swing-out rotor (Sorvall HB-4). The supernatant was discarded and the pellet surface washed with homogenization medium to remove broken chloroplasts.

Isolation of chloroplast subfractions

To prepare chloroplast envelopes and thylakoids, the procedure of Douce et al. [18] was used. The chloroplasts were resuspended in 1.5 ml of 0.8 M sucrose containing 5 mM Tricine and 2 mM $MgCl_2$ (pH 7.6), and diluted with 36 ml 10 mM Tricine plus 4 mM $MgCl_2$ (pH 7.6), to swell and disrupt the plastids. The suspension of disrupted chloroplasts was loaded onto gradients consisting of 3 ml each of 1.5, 1.2, 0.93 and 0.6 M sucrose prepared in 5 mM Tricine and 2 mM $MgCl_2$ (pH 7.6). The gradients were centrifuged for 60 min at $53\,000 \times g_{av.}$ (20 000 rpm, Beckman SW-28 rotor). Material collecting at the 0.6/0.93 M sucrose interface was collected as the envelope fraction whereas thylakoids were collected from the 1.2/1.5 M sucrose interface.

For preparation of stroma, intact chloroplasts prepared as above from 50 g of leaves were resuspended in 3 ml of 10 mM Tricine and 4 mM $MgCl_2$ (pH 7.6), without sucrose and disrupted using 10 up and down strokes with a teflon glass tissue homogenizer. Membranes were pelleted by centrifugation for 60 min at $64\,100 \times g_{av.}$ (22 000 rpm, Beckman SW-28 rotor). The supernatant was removed carefully to avoid dislodging any of the packed membrane pellet and was used as the source of stroma.

Labeling of isolated envelope as donor

Envelope membranes were resuspended in 100 μl of 10 mM Tricine, 0.4 mM $MgCl_2$ and 0.3 M sucrose (pH 7.6), to which were added either 50 μCi (to characterize individual lipids) or 5 μCi UDP-[^{14}C]galactose (Amersham, specific activity 19 μCi/mmol). The mixture was incubated for 1 h at room temperature and layered over 10 ml of incubation medium containing 0.33 M sucrose followed by centrifugation for 20 min at $64\,100 \times g_{av.}$ (22 000 rpm, Beckman SW-28 Rotor) to pellet the labeled membranes.

In vivo labeling and isolation of donor membranes

Acetate-labeled donors were prepared only from spinach. Primary leaves (15–20 g) were excised and the petioles immersed in deionized water containing 200 μCi [^{3}H]- or [^{14}C]acetate. To label, the leaves and radioactive solution were distributed equally among 4 1.8 ml Eppendorf cups and placed in the light. After the initial radiolabeled solution was taken up, an additional 1 ml of deionized water was added to each cup and the uptake process repeated. After a second 1 ml portion of water was taken up, the cups were transferred to a bath with water covering the tops of the cups where they remained, illuminated, overnight (12–15 h). Chloroplasts and chloroplast subfractions were isolated from the labeled leaves as described above. Other labeled donor membrane fractions (plasma membrane, Golgi apparatus, endoplasmic reticulum, nuclei, tonoplast, mitochondria) were prepared as described [13].

Isolation of extra-plastidic acceptor membranes

Spinach leaves (25–50 g) for preparation of acceptor fractions were harvested without petioles and stored refrigerated or chilled to ice bath temperature prior to homogenate preparation. Membranes were isolated as described [13].

The reconstituted membrane transfer system

Incubations were in 8 ml glass shell vials at 25°C (room temperature) or 4°C (ice bath temperature) with or without ATP plus stroma. The ATP/ATP regenerating system [19,20] contained 30 mM Hepes-KOH, 30 mM KCl, 2.5 mM Mg acetate, 50 μM ATP, 300 μM UTP, 2 mM creatine phosphate (rabbit muscle) and 10 IU/ml creatine phosphokinase (rabbit muscle), pH 7.0. The final transfer medium was freshly constituted by combining 100 μl of 30 mM Hepes-KOH containing 30 mM KCl and 2.5 mM Mg acetate (Hepes/$Mg(OAc)_2$/KCl), 100 μl of radiolabeled donor, 200 μl stroma and either 100 μl ATP + ATP regenerating system (+ATP) or Hepes/$Mg(OAc)_2$/KCl (−ATP). The reactions were started with the addition of ATP + ATP regenerating system immediately before addition of the immobilized acceptors.

Preparation of radiolabeled donors

Radiolabeled donors were held as pellets on ice. A very small wedge was removed from the center of the pellet using a pointed spatula and transferred to fixative for electron microscopy. Just prior to reconstituting the transfer system, the pellets were resuspended in the required volume of Hepes/$Mg(OAc)_2$/KCl, usually 400 to 450 μl for four assay vials, using a pasteur pipette and/or homogenization using a glass-teflon homogenizer. A 5-μl sample was removed for determination of radioactivity and samples of 1, 2 and 4 μl were removed for determination of protein. Sufficient membranes were resuspended to give a final protein concentration of between 2 mg and 6 mg/ml depending on membrane type. Since it was important to use freshly resuspended donor in the assays, appropriate protein concentrations were estimated from pellet size and turbidity to be in the correct range. Measured protein concentrations are given with the figures.

Preparation of immobilized unlabeled acceptor

Preparation of immobilized acceptor was begun 1 to 2 h in advance of reconstitution of the assay. Pellets were resuspended in Hepes/$Mg(OAc)_2$/KCl as described for donor fractions at a final concentration of 1 to 10 mg/ml depending on membrane source. Strips 0.5 × 1 cm were cut from nitrocellulose sheets (S.&S. Scientific, Keene, NY). The strips were added one at a time using a fine forceps to ensure uniform contact to the cold resuspended acceptor fractions and incubated 0.5 to 1 h at 4°C (usually 1 h although maximum protein was bound within 0.5 h). At this point, strips for electron microscopy and determination of protein bound were removed and rinsed through four changes of cold Hepes/$Mg(OAc)_2$/KCl. The strips remaining were then removed with forceps en masse and added to 5% bovine serum albumin prepared in Hepes/$Mg(OAc)_2$/KCl and incubated an additional 0.5 to 1 h (usually 1 h) at 4°C with shaking to block sites unoccupied by membranes on the nitrocellulose surface. The strips, each loaded with 10 to 40 μg acceptor membrane and blocked with albumin, were then rinsed through four changes of cold Hepes/$Mg(OAc)_2$/KCl, edge blotted to remove excess solution and added to the transfer system, folded slightly in the center and standing on edge, five per vial. In control experiments, replacement of albumin with other purified animal proteins such as carbonic anhydrase or crude plant proteins from coconut milk did not affect the results. All manipulations involving strips were with forceps. Care was taken to avoid contact with any protein containing surface prior to loading the strips.

Following incubation with acceptor, the strips were removed with forceps, washed through four changes of cold Hepes/$Mg(OAc)_2$/KCl, edge blotted to remove excess solution and placed on edge on metal foil to air dry. Radioactivity was determined by scintillation methods.

Protein determinations

Proteins were determined using the BCA procedure [21] with bovine serum albumin as standard.

Electron microscopy

Fractions and nitrocellulose acceptor strips were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate (pH 7.2) at room temperature followed by post-fixation in 1% osmium tetroxide in the same buffer. Dehydration was through an acetone series, with embedment in Epon [22]. Thin sections were examined and photographed with a Philips EM 200 electron microscope.

Results

In a cell-free system reconstituted with pea chloroplast membranes, transfer of radioactivity from labeled donor envelope membranes to unlabeled acceptor thylakoids was time-dependent over 1 h at both 23°C and 4°C both with or without ATP (Fig. 1). The isolated donor envelope fraction had been labeled in vitro with UDP-[^{14}C]galactose. Analyses of lipid extracts by thin-layer chromatography of lipid extracts showed that most of the donor label as well as most of the label transferred was MGDG. After 1 h of incubation, the total transfer of label from donor envelope to acceptor

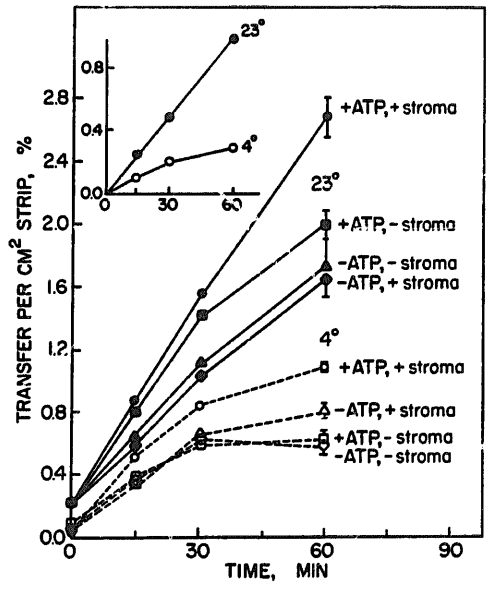

Fig. 1. Transfer per cm^2 strip as percent of total donor radioactivity for pea chloroplast envelope fractions in solution to chloroplast thylakoids immobilized on nitrocellulose strips (24 μg protein) at 23°C (solid symbols/lines) and 4°C (open symbols/dashed lines). The stroma concentration was 3.3 mg/ml and the total donor radioactivity was 520000 dpm (120 μg protein). Inset shows only the ATP-dependent transfer.

thylakoids at 23 °C was approximately double that at 4°C (Fig. 1), whereas the ATP-dependent increment of transfer increased 3-fold comparing 23°C and 4°C (Fig. 1, inset). Transfer at 23°C occurred in the presence or absence of ATP during the first 30 min without a requirement for stroma while the stromal fraction appeared stimulatory for sustained transfer (Fig. 1). At 4 °C, stroma was stimulatory for ATP-requiring transfer, while only the sustained transfer (after 30 min) was stimulated by stroma when ATP was absent (Fig. 1). Boiled donor or acceptor (85 °C, 30 min) failed to support ATP-dependent transfer.

Corresponding experiments with spinach chloroplast fractions yielded results qualitatively similar to those obtained with pea chloroplast fractions (Fig. 2). Transfer of labeled lipids from envelope donor to thylakoid acceptor was both time- and temperature-dependent and stimulated by ATP (Figs. 2 and 3). The ATP-dependent transfer at 23°C was stimulated by 3 mg/ml of stromal proteins (Fig. 2), while a higher concentration of stroma (10 mg protein/ml) was frequently without effect or even inhibitory (Fig. 3). The inconsistent effects of stroma on reconstituted transfer with spinach membranes remained even though the source and quality of the stromal fractions were systematically varied. Stromal fractions isolated from various sources, including different spinach varieties grown hydroponically or in a greenhouse, and field-grown as well as market-bought spinach and from either expanding or mature leaves yielded similar results.

Electron microscopy verified that the isolated chloroplast fractions consisted almost exclusively of intact chloroplasts with both envelope membranes intact and a uniform, electron-dense stroma for both pea

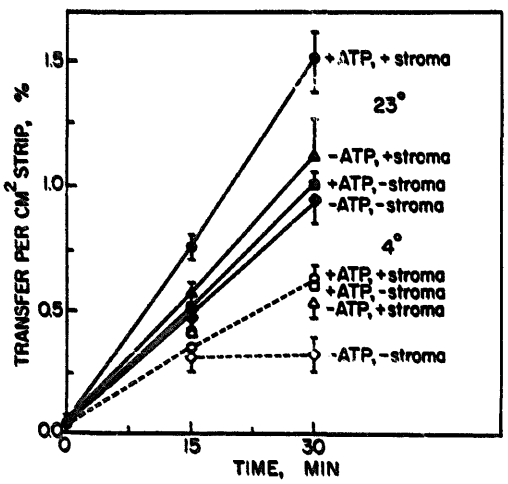

Fig. 2. Transfer per cm² strip as percent of total donor radioactivity for spinach chloroplast envelope fractions in solution to chloroplast thylakoids immobilized on nitrocellulose strips (26 μg protein) at 23°C (solid symbols/lines) and 4 °C (open symbols/dashed lines). The stroma concentration was 2.8 mg/ml and the total donor radioactivity was 210000 dpm (70 μg protein).

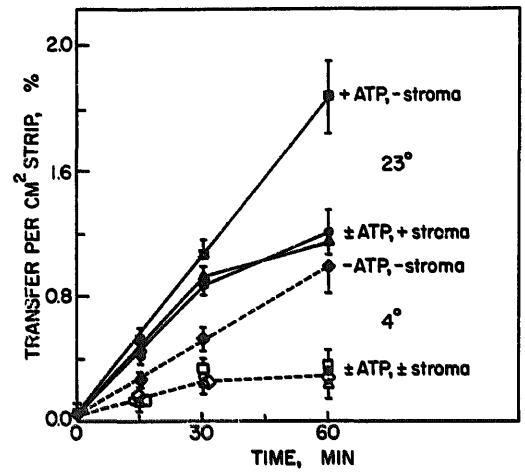

Fig. 3. Transfer per cm² strip as percent of total donor radioactivity for spinach chloroplast envelope fractions in solution to chloroplast thylakoids immobilized on nitrocellulose strips (22 μg protein) at 23 °C (solid symbols/lines) and 4 °C (open symbols/dashed lines). The stroma concentration was 10 mg/ml and the total donor radioactivity was 300000 dpm (125 μg protein).

(Fig. 4A) and spinach (Fig. 5A). The isolated envelope fractions consisted of rounded vesicles with bounding membranes of thickness 5.5 nm, comparable to that of the separate plastid envelopes in situ (Figs. 4B and 5B). Contaminating thylakoids accounted for no more than 10% of the envelope fraction isolated from pea chloroplasts and 5% of the spinach envelope fraction, based on morphometry. The thylakoid fractions consisted of stacked or single lamellae together with lamellar fragments (Figs. 4C and 5C). When absorbed to nitrocellulose, the thylakoids formed a layer of stacked membranes interspersed by single vesicles on the strip (Figs. 4D and 5D). When isolated envelope fractions, radiolabeled in vitro with UDP-[^{14}C]galactose, were incubated in the presence of ATP, the appearance of the fractions was altered from containing vesicles of a fairly uniform size (Figs. 4B and 5B) to also containing smaller vesicles (Fig. 6A). Vesicles of approx. 50–70 nm diameter constituted by morphometry 31 ± 15 intercepts per 100 total membrane intercepts. Small vesicles appeared less frequently in envelope fractions incubated without ATP (Fig. 6B) and constituted 12 ± 6 intercepts per 100 intercepts.

Specificity of transfer was determined for both donor (Table I) and acceptor (Table II) membranes. When different donor membranes were compared, they were isolated from spinach leaves labeled *in vivo* with [^{3}H]- or [^{14}C]acetate. All the isolated membrane fractions contained radiolabeled acyl lipids, with MGDG as the dominant labeled lipid of chloroplast fractions while phospholipids carried most of the label of other membrane fractions [13]. The composition and purities of each of the donor and acceptor fractions utilized in making these measurements has been determined from morphology and analyses of marker enzymes [13]. With

thylakoid membranes immobilized on nitrocellulose as acceptor, significant ATP-dependent transfer at 23°C occurred only with chloroplast envelopes as donor (Table I). With plasma membrane fractions, tonoplast fractions, nuclei, mitochondria or thylakoids or with membrane fractions enriched in endoplasmic reticulum or Golgi apparatus, ATP-dependent transfer was small (< 0.05%).

When [^{14}C]galactose-labeled envelope membranes were used as donor, thylakoids were the most efficient acceptor for ATP-dependent transfer at 23°C (Table II). Donor envelope and acceptor thylakoid fractions isolated from pea and spinach were interchangeable in the transfer system. Significant transfer also occurred from donor envelopes to acceptor envelopes in the absence of stroma. Other membranes or organelles isolated from spinach also exhibited acceptor activities with efficiencies varying between 1/2 and 1/20 that of

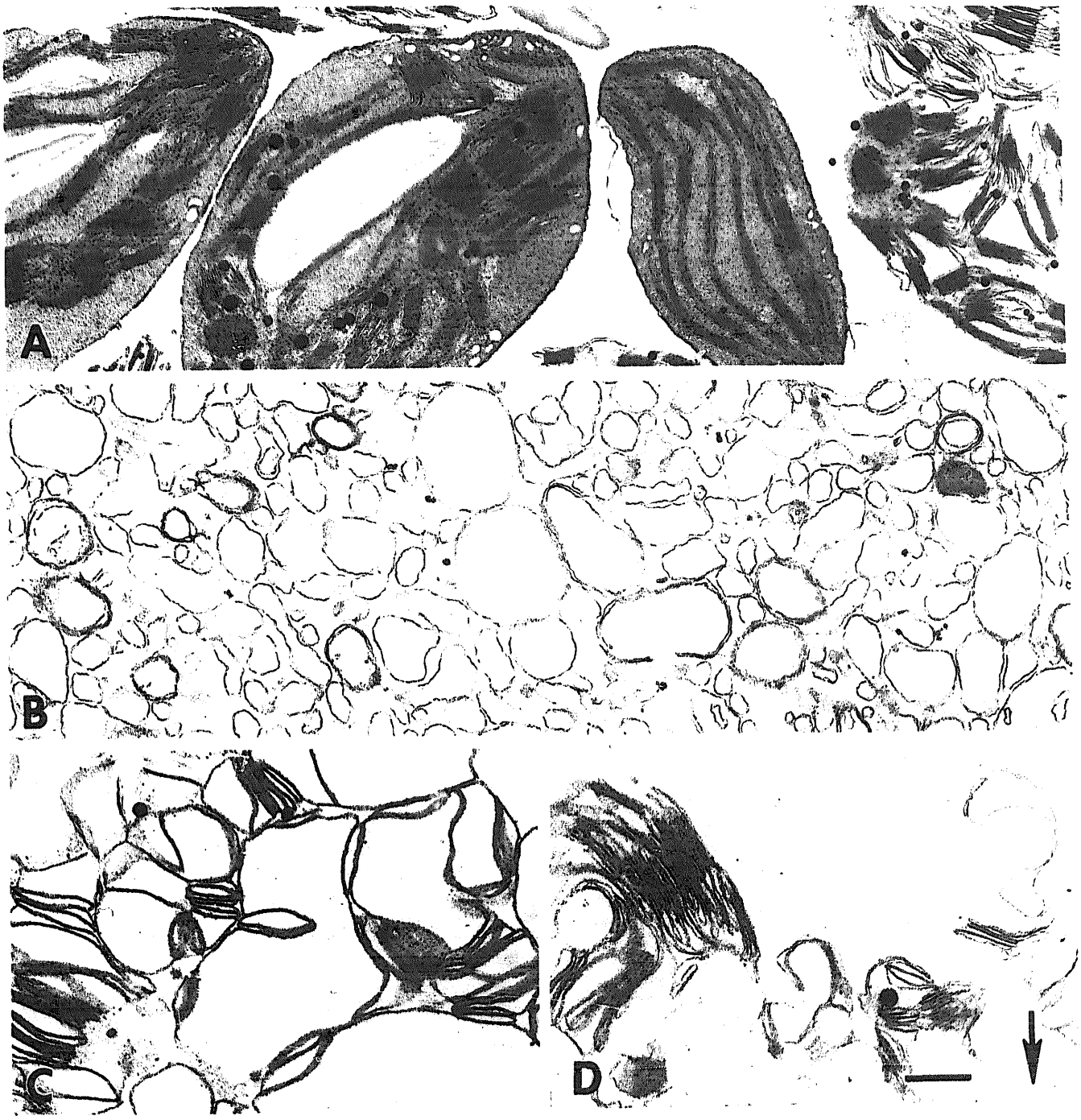

Fig. 4. Electron micrographs of the isolated pea fractions. (A) Intact chloroplasts. (B) Purified envelope fraction. (C) Thylakoid fraction (D) Thylakoid fraction immobilized on nitrocellulose. Arrow indicates the position of the nitrocellulose. Scale bar = 0.5 μm.

thylakoids (plasma membrane = chloroplast envelope > Golgi apparatus = tonoplast > mitochondria = endoplasmic reticulum = nuclei) (Table II).

Discussion

We have reconstituted transfer of galactose-containing lipids from donor envelope to acceptor thylakoid membranes using isolated envelope and thylakoid membranes. ATP stimulated the transfer.

Transfer from the endoplasmic reticulum to the Golgi apparatus had previously been reconstituted with plant membranes in cell-free systems to study transfer of proteins [12] and of phospholipids [13]. In both animals and plants, transfer from the endoplasmic reticulum to the Golgi apparatus involved small membrane vesicles, usually referred to as transition vesicles, which originate from the transitional endoplasmic reticulum [12,14]. Reconstituted transfer between adjacent Golgi apparatus compartments [23] and between

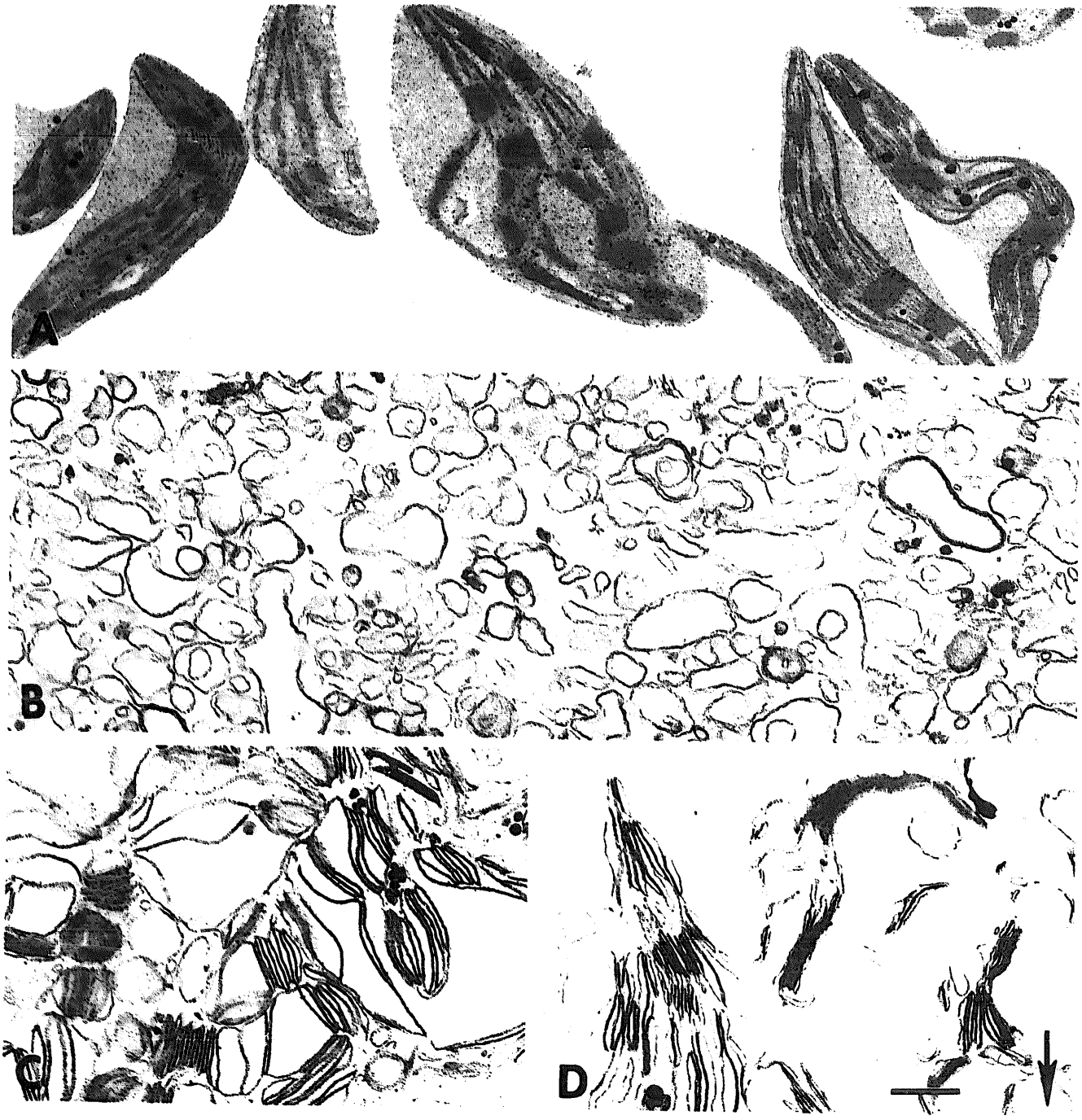

Fig. 5. Electron micrographs of the isolated spinach fractions. (A) Intact chloroplasts. (B) Purified envelope fraction. (C) Thylakoid fraction. (D) Thylakoid fraction immobilized on nitrocellulose. Arrow indicates the position of the nitrocellulose. Scale bar = 0.5 μm.

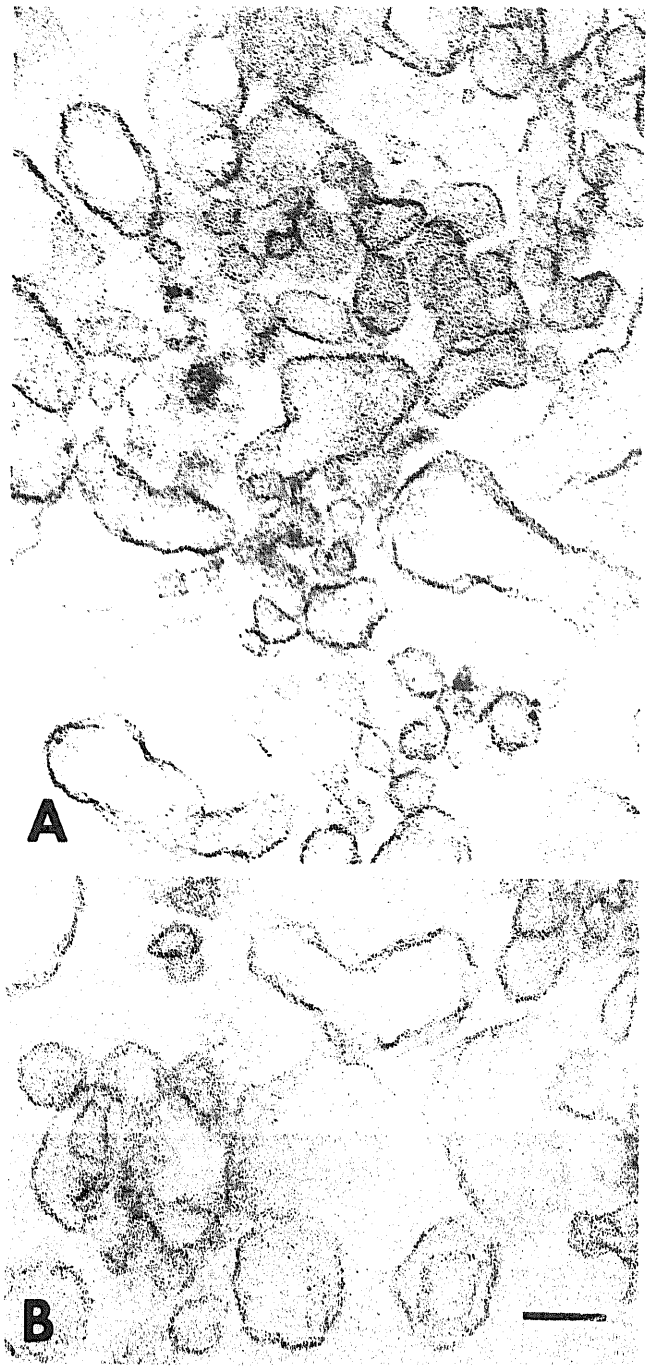

Fig. 6. Electron micrographs of the purified envelope fraction from spinach incubated for 30 min in the presence (A) of ATP + stroma or in the absence (B) of ATP + stroma. The stroma concentration was 3 mg/ml. In the presence of ATP, numerous small vesicular profiles were present (A). These vesicles were largely absent form the starting envelope fractions prior to incubation (Fig. 5B). In the absence of ATP + stroma, the appearance of the fractions was similar, except that the small vesicles were less evident. Scale bar = 0.1 μm.

the endoplasmic reticulum and the Golgi apparatus with both animal and plant membranes was dependent upon ATP and facilitated by cytosol [12–14]. For transfer of lipids, a contribution of soluble proteins transporting phospholipids [24,25], although not completely ruled out, was regarded as unlikely [26].

TABLE I

Donor specificity of transfer

Results are the increment of cell-free transfer (30 min) from spinach membranes to chloroplast thylakoids stimulated by ATP. The averages are of two or three determinations ± mean average or standard deviations. Donor membranes were from leaves excised and radiolabeled with [^{14}C]acetate. Donor protein and dpm are given per reaction mixture.

Donor protein			ATP-stimulated transfer per cm^2 strip (%)
Donor	μg	dpm	
Chloroplast envelope	100	35000	0.66 ± 0.28
Chloroplast thylakoid	600	225000	0.015 ± 0.01
Plasma membrane	560	250000	0.022 ± 0.008
Tonoplast	400	310000	0.018 ± 0.003
Nuclei	180	302000	0.028 ± 0.013
Endoplasmic reticulum	100	144000	0.05 ± 0.01
Golgi apparatus	230	75000	0.035 ± 0.015
Mitochondria	450	80000	< 0.01

Theoretically, transfer of lipids from envelope to thylakoid membranes could be mediated either by membrane vesicles derived from the inner envelope or by galactolipid-transporting proteins. Our results suggest that galactolipid transfer within the chloroplast does not require soluble lipid-transporting proteins since initial rates of ATP-dependent transfer were similar both in the presence and absence of stroma.

TABLE II

Acceptor specificity of transfer

Results are the increment of cell-free transfer (30 min) from the plastid envelope of pea or spinach to spinach membranes stimulated by ATP. The averages are of three determinations ± standard deviations. Acceptor membranes were unlabeled. Donor membranes were radiolabeled post isolation with [^{14}C]galactose (100 μg protein and 400000 dpm/reaction mixture). Values in parentheses are micrograms of protein per cm^2 of nitrocellulose.

Acceptor	ATP-stimulated transfer per cm^2 strip (%)	
	pea envelope	spinach envelope
Spinach		
Chloroplast thylakoid (25)	0.5 ± 0.3	0.66 ± 0.28
Plasma membrane (35)	0.16 ± 0.16	0.06 ± 0.05
Tonoplast (20)	0.10 ± 0.04	0.05 ± 0.04
Nuclei (50)	0.08 ± 0.08	0.04 ± 0.02
Endoplasmic reticulum (18)	0.08 ± 0.04	0.04 ± 0.02
Golgi apparatus (18)	0.08 ± 0.04	0.10 ± 0.05
Mitochondria (30)	0.10 ± 0.02	0.03 ± 0.02
Chloroplast envelope (12)		0.12 ± 0.05 [a]
Pea		
Chloroplast thylakoid (25)	0.56 ± 0.12	
Chloroplast envelope (10)		0.04 ± 0.01

[a] Transfer in the presence of stroma. In the absence of stroma, transfer stimulated by ATP with spinach chloroplast envelope as both donor and acceptor was 0.42 ± 0.26% per cm^2 strip. With other acceptors, the ATP-stimulated transfer in the presence or absence of cytosol were similar.

Lipid transfer proteins may function in the cell-free system. However, stromal contamination would be an unlikely source since the envelope membranes were exhaustively washed. The thylakoids immobilized on nitrocellulose were incubated for 1 h in 5% serum albumin and then washed four times with buffer.

For sustained ATP-dependent transfer, stroma promoted at lower concentrations but inhibited at higher concentrations. The effects of stroma were proportionally greater at 4 °C than at 23°C, suggesting some role for stromal constituents in the regulation of transfer and perhaps in its temperature dependency as well. Future experiments will be necessary using purified stromal constituents to clarify the roles of stromal proteins in the transfer process and if any galactolipid-transporting proteins similar to those isolated by Nishida and Yamata [27] are active in the reconstituted system.

Lipid transfer in cell-free systems from animal cells show both ATP- and temperature-dependent and ATP- and temperature-independent components [26]. The ATP- and temperature-dependent transfer was associated with vesicular transfer. The ATP- and temperature-independent transfer was via a non-vesicular mechanism [26]. In the present study, only the ATP-dependent transfer was strongly temperature dependent as with cell-free lipid transfer for liver fractions [26]. Comparing ATP-dependent transfer at 4 °C and 23 °C (Fig. 1), the Q_{10} was approx. 2 as expected for an ATP-dependent process.

In our experiments, we have used mixed envelope fractions radiolabeled predominantly in MGDG. Synthesis of MGDG is restricted in spinach chloroplasts to the inner envelope membranes [28,29]. From Block et al. [28,29], the inner envelope membrane contains about 500 nmol MGDG/mg protein while thylakoids contain half this amount of MGDG on a protein basis. If the envelope fractions used in our experiments contained equal amounts of inner and outer envelope membranes, each incubation with 100 μg donor envelope contained 50 μg inner envelope protein and 25 nmol inner envelope MGDG. Assuming that all label resided in MGDG of the inner membrane, a specific transfer of 0.7% of the label during 30 min of incubation would mean that 0.2 nmol MGDG was transferred to each strip. Each strip held about 25 μg of thylakoid proteins, and according to calculations, about 6 nmol of MGDG. Thus, the transfer rates observed in the cell-free system would be sufficient to replace all of the thylakoid MGDG in approximately 16 h. The rate of MGDG synthesis by isolated inner envelope membranes of spinach chloroplasts has been reported to be 880 nmol/mg protein/h [28,29], although up to 2700 nmol MGDG formed/mg protein/h has been reported for mixed envelope fractions [3]. These rates imply that the MGDG of the inner envelope membrane would turn over in less than an hour. By morphometry, the inner envelope membrane represents about 6% of the total membrane surface of the mature chloroplast (counting thylakoids as double membranes). If the MGDG content of the thylakoids were about half that of the outer envelope, the rate of turnover would be sufficient to replace all of the thylakoid MGDG once in about 8 h as compared to 16 h as calculated for the observed *in vitro* replacement rate.

In contrast to the situation for spinach, MGDG synthesis has been reported to occur as well in the outer envelope membrane of pea chloroplasts [30]. The mechanisms for transferring MGDG from the outer to the inner envelope membrane may have functioned in the reconstituted system. The results with reconstituted transfer would be consistent with such an inter-envelope transfer of lipids. In the absence of stroma, transfer from donor envelope to acceptor envelope was, after a 30 min incubation, approx. 65% of the transfer from envelope to thylakoids, and interestingly, stroma was strongly inhibitory to this activity.

To investigate donor specificity, donor membranes were isolated from spinach leaves incubated with [^{14}C]acetate to permit labeling of acyl lipids of all membrane fractions. In chloroplast membranes, the label resided predominantly in galactolipids. In non-plastidic membrane fractions, the label was associated mainly with phospholipids. The capacities of these fractions to function as donors to transfer label to acceptor thylakoids were only 3–4% of that from envelope to thylakoids. Acceptor specificity was investigated with envelope donor isolated from either pea or spinach containing radiolabeled galactolipids. Although transfer was most efficient to acceptor thylakoids, non-plastidic membranes, isolated from spinach, accepted envelope label at rates of up to 15% of those of thylakoids.

Whether or not proteins are transferred as well in the cell free system and if proteins and lipids follow parallel routes as they apparently do between the endoplasmic reticulum and the Golgi apparatus [26] are questions not yet addressed. One approach would be to utilize heterologous transfer systems lacking immunological cross-reactivity between donor and acceptor. Specific proteins transferred from the envelope preparations to the thylakoids could then be identified and quantitated. Possibilities for development of such systems are under investigation.

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