A vesicle transport system inside chloroplasts

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Abstract Intracellular transport via membrane vesicle traffic is a well known feature of eukaryotic cells. Yet, no vesicle transport system has been described for prokaryotes or organelles of prokaryotic origin, such as chloroplasts and mitochondria. Here we show that chloroplasts possess a vesicle transport system with features similar to vesicle traffic in homotypic membrane fusion. Vesicle formation and fusion is affected by specific inhibitors, e.g. nucleotide analogues, protein phosphatase inhibitors and Ca^{2+} antagonists. This vesicle transfer is an ongoing process in mature chloroplasts indicating that it represents an important new pathway in the formation and maintenance of the thylakoid membranes.

Key words: Chloroplast; Vesicle transport; Lipid; Microcystin LR; Envelope

1. Introduction

A specific feature of eukaryotic cells is their high degree of compartmentalisation. Several different membrane-surrounded subcompartments can be found in a single cell. This makes communication with or transfer between different compartments an essential requirement for eukaryotic cells. Central to this organisation is the secretory pathway through which proteins travel by vesicle transport to their appropriate location [1–3]. Proteins are sorted into vesicles which are released from a donor compartment. These vesicles then fuse with the target membrane thus releasing their cargo. In a similar fashion, vesicle traffic is essential to synaptic transmission [4], endocytosis [5] and vacuole formation [6,7]. In the latter the lipid bilayer of the vesicle, and not any specific content, is the cargo of the vesicle transport.

Whereas vesicle traffic is a common process in eukaryotic cells it has not been described for prokaryotes. Higher plants and green algae perform photosynthesis in an organelle of prokaryotic origin, the chloroplast. The photosynthetic machinery is located on a special intraplastidal membrane system, the thylakoids, and the ability to build up and alter this membrane is an essential feature of oxygenic photosynthesis. Chloroplasts are separated from the cytosol by a double membrane and it is assumed that the thylakoid membranes which are formed during the maturation of chloroplasts derive from the inner envelope [8–10]. Many of the components required for the biogenesis and maintenance of thylakoids are synthesised on the inner envelope and have to be transferred to the thylakoid membrane even in mature chloroplasts, i.e. galactolipids and carotenoids [11,12]. Vesicular structures have been described inside chloroplasts by early electron microscopic studies [10,13] but a transport via vesicles has not been demonstrated. Here we present experimental data that a dynamic vesicular transport system exists inside chloroplasts.

2. Materials and methods

2.1. In organello analysis of vesicle formation

Chloroplasts were isolated from leaves of 8-day-old peas (*Pisum sativum* var. Golf) as previously described [14] in a buffer containing 330 mM sorbitol, 20 mM MOPS, 20 mM Tris, 5 mM $MgCl_2$, 10 mM KCl, 10 mM ascorbic acid, 0.2 mM $MnCl_2$, and 1% bovine serum albumin, pH 7.5. Inhibitors were used in the following final concentrations: 2 mM A23187; 5 mM NaF+0.05 mM $AlCl_3$ (AlF_4^-); 10 mM *o*-ATP; 100 ng/μl brefeldin A; 10 μM calyculin; 100 μM cantharidin; 10 mM GMP/PNP; 5 mM GTPγS; 10 mM *o*-GTP; 100 μM mastoparan; 100 μM microcystin LR; 10 mM *N*-ethylmaleimide; 100 μM ophiobolin A, and 100 μM W7. For electron microscopic analysis, chloroplasts were fixed at 4°C overnight in 0.1 M phosphate buffer (pH 7.2), containing 4% glutaraldehyde, followed by postfixation in 2% OsO_4 overnight. Samples were dehydrated in a graded acetone series and embedded in Epon 812. They were stained with 2% aqueous uranyl acetate for 10 min, followed by 5 min in lead citrate, and analysed by electron microscopy. To quantify the inhibition experiments, the number of vesicles in at least 150 different chloroplasts from several independent experiments were evaluated.

3. Results

3.1. Vesicle accumulation in isolated chloroplasts

A biochemical characterisation of the potential vesicular transport made it necessary to establish an in organello system to analyse vesicle formation and fusion. Chloroplasts from garden pea were isolated in a quick and gentle manner and incubated under various conditions which might lead to the accumulation of vesicles. In yeast, microcystin LR was shown to affect membrane fusion in vacuole formation by inhibition of protein phosphatase 1 [15,16]. Indeed the addition of microcystin LR resulted in an accumulation of vesicles in isolated chloroplasts (Fig. 1, middle panel). Without inhibitor chloroplasts do not display any vesicle accumulation under these conditions (Fig. 1, upper panel). In animal tissue vesicle transport, i.e. endoplasmic reticulum to Golgi or Golgi to plasma membrane, can be halted by low temperature [17]. Under these conditions vesicles accumulate because fusion of the vesicles with their target membrane is more sensitive to temperature than vesicle formation. A similar phenomenon of low-temperature vesicle accumulation has been observed in chloroplasts of intact leaf tissue [18,19]. When isolated chloroplasts were incubated at 4°C vesicles accumulated like in the

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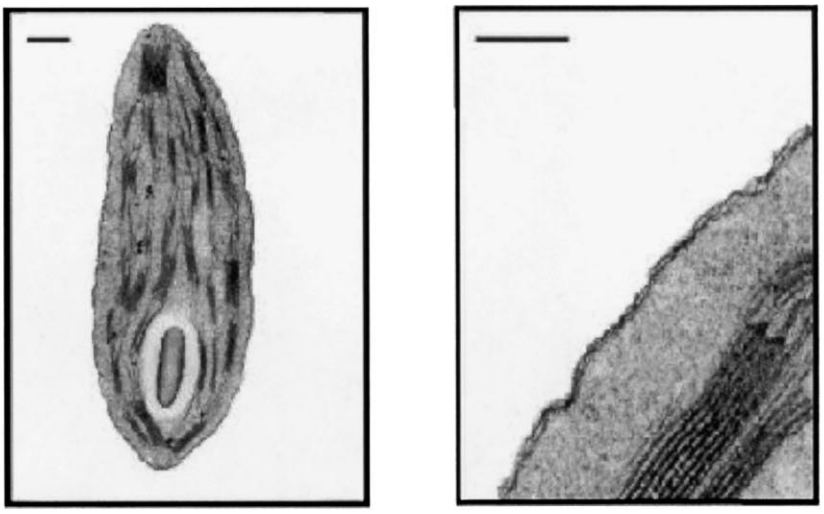
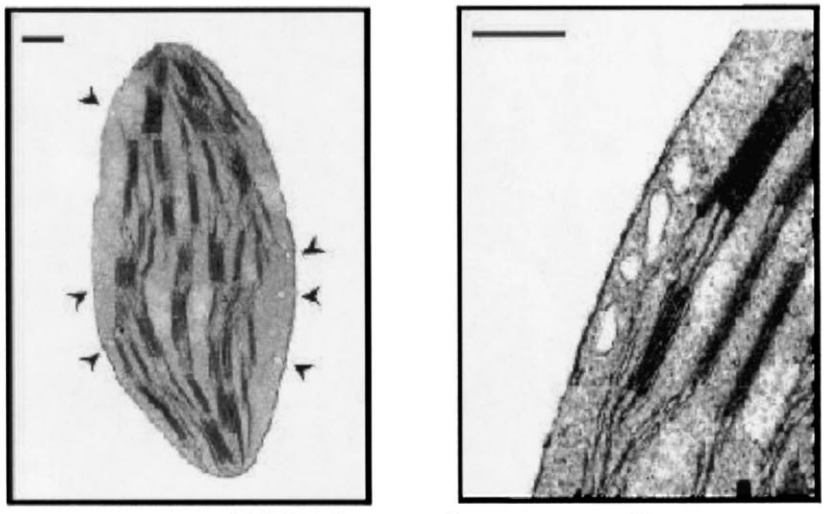
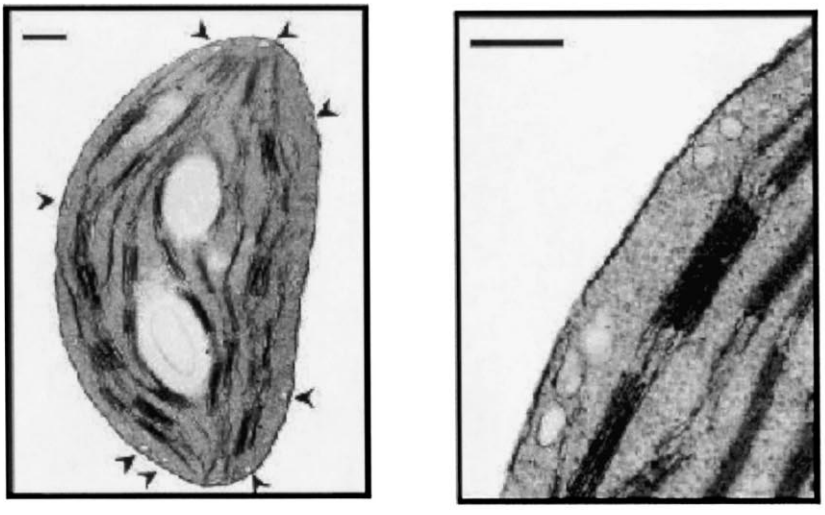


22°C



22°C + microcystin



4°C

Fig. 1. Microcystin LR inhibits vesicle fusion in chloroplasts. Vesicle accumulation can be observed in chloroplasts incubated at room temperature in the presence but not in the absence of 100 μM microcystin LR. Incubation of chloroplasts at 4°C also results in vesicle accumulation. Scale bars represent 0.4 μm (left panels) and 0.1 μm (right panels).
←

presence of microcystin LR (Fig. 1, lower panel). At a given time the vesicles observed in a single plane of the thin-sectioned chloroplast would correspond to several hundred vesicles in an entire chloroplast. We conclude that vesicle formation is an ongoing and temperature-independent process in mature chloroplasts. Fusion, on the other hand, is inhibited by the protein phosphatase inhibitor microcystin LR and by low temperature.

In order to show that the membrane-encircled structures observed inside isolated chloroplasts are vesicles and not tubular extensions from the inner envelope we obtained serial thin sections from a number of samples. The vesicular structures persistently appeared and disappeared within one or two sections. Most often vesicles appeared only in a single section of a series (Fig. 2). The depth of the vesicles is estimated as 30–70 nm. Thus it is very similar to their width (30–70 nm, see scale bar in Fig. 1) giving them a globular shape expected for a membrane vesicle. While the vesicles are mainly observed in the stroma between the inner membrane of chloroplasts and the thylakoids, they can also be seen attached to these membranes indicating fission and fusion events (data not shown). We conclude that isolated chloroplasts can be used as a bona fide system to further study vesicle formation and fusion.

3.2. *Vesicle formation and fusion is inhibited by low-molecular-weight compounds*

A number of inhibitors are described for different proteins involved in cytoplasmic vesicle traffic. In order to examine inhibition of vesicle formation we incubated isolated chloroplasts at 4°C in the presence or absence of inhibitors. After 20 min of incubation the average number of vesicles was compared to a control experiment without inhibitor. In the control chloroplasts vesicles would accumulate since membrane fusion is inhibited by low temperature (Fig. 1). A reduction in the number of vesicles at 4°C indicated inhibition of vesicle formation. Addition of *o*-GTP, GTPγS and GMP/PNP, all of which are non-hydrolysable nucleotide analogues, resulted in a nearly complete loss of vesicle accumulation (Fig. 3). An even stronger inhibition was observed with AlF_4^-. No inhibition occurred by addition of several other substances including *o*-ATP, ophiobolin A or microcystin LR (Fig. 3 and Table 1). These results indicate a specific influence of certain effectors on vesicle formation.

To study inhibition of vesicle fusion, isolated chloroplasts were preincubated at 4°C (Fig. 3B). Vesicles started to accumulate after 5 min of incubation at 4°C and the number increased for 20 min when the maximum of vesicle accumula-

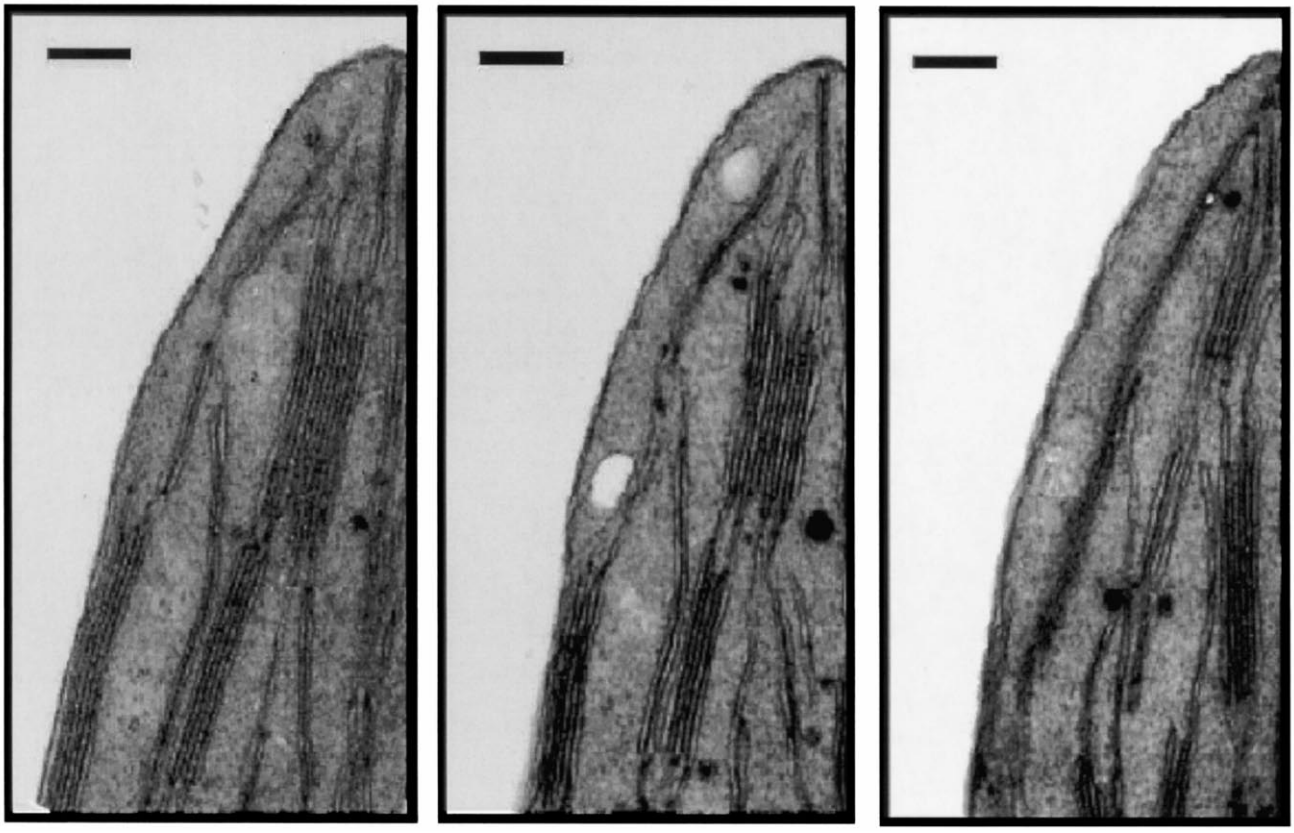


Fig. 2. Serial ultra-thin sections of a chloroplast incubated at 4°C. Most often vesicles can be observed in a single section of a set. Scale bars represent 1 μm.

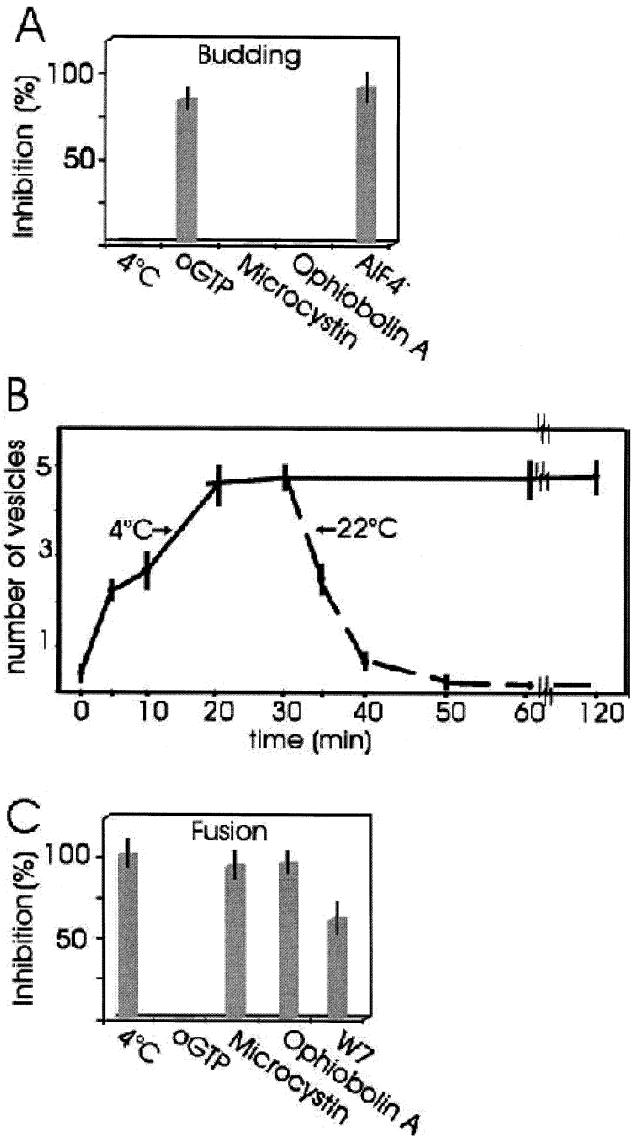


Fig. 3. Inhibition of vesicle formation and fusion. A: To analyse inhibition of vesicle formation isolated chloroplasts were incubated at 4°C with and without addition of inhibitors. Inhibition of vesicle formation resulted in a decrease of the number of accumulated vesicles compared to an inhibitor-free control. B: Vesicle accumulation is reversible. Chloroplasts were incubated at 4°C until the maximum of vesicle accumulation was reached. Upon a shift to 22°C the vesicles disappeared within 10 min. C: To analyse fusion inhibition isolated chloroplasts were preincubated at 4°C for 20 min to allow vesicle accumulation to occur. Inhibitors were added and after 10 min further incubation at 4°C the chloroplasts were moved to 22°C. The numbers in A–C represent an average of three independent experiments and >150 different chloroplasts.

tion was reached (Fig. 3B). Potential inhibitors were added and the chloroplasts were incubated for another 10 min at 4°C before being moved to room temperature. Without inhibitor the transfer to room temperature resulted in a complete disappearance of vesicles within 10 min because vesicle fusion is no longer inhibited by low temperature (Fig. 3B). The inhibition of fusion is indicated by the number of vesicles compared to chloroplasts left at 4°C. The most significant effect on vesicle fusion was observed with microcystin LR and ophiobolin A (Fig. 3C). W7 and calyculin caused a lesser but still significant inhibition. No inhibition was observed with mastoparan, *N*-ethylmaleimide, brefeldin A, A23187 or any of the nucleotide analogues (Fig. 3C and Table 1). These results show that also vesicle fusion is affected by specific low-molecular-weight components and we conclude from our data that chloroplasts contain a protein-mediated vesicle formation and fusion machinery.

4. Discussion

One of the most elusive aspects of thylakoid formation is the exact mechanism by which the membrane itself is formed. It is believed that the membrane material originates from the chloroplast inner envelope [8–10,19]. In young, not yet fully differentiated plastids a continuum is observed between the inner envelope and developing internal membranes. In mature chloroplasts no connection can be seen between the inner envelope and the thylakoids and it was speculated that thylakoids might be maintained by a flux of membrane vesicles. Here we give strong experimental evidence that a protein-mediated vesicle transport system actually exists inside chloroplasts. Surprisingly the features of this vesicle transport system resemble those of typical eukaryotic systems. From our results obtained with the low-molecular-weight effectors we can deduce several components which might be involved in this plastidal vesicle transport system.

The fact that vesicle formation is inhibited by *o*-GTP but not *o*-ATP indicates that budding is controlled via a GTPase. Several low-molecular-weight GTPases are involved in distinct steps of vesicle traffic [20]. Also high-molecular-weight GTPases such as dynamin have been shown to function in vesicle budding [21,22]. Several dynamin-like proteins have been found in the genome of *Arabidopsis thaliana* [23,24] and one of them, Adl2, has been localised inside the chloroplast [24]. Therefore dynamin would be a likely candidate for a GTPase involved in plastidal vesicle transport.

On the site of the target membrane our results indicate a protein phosphatase–calmodulin complex similar to the one involved in homotypic membrane fusion [7,16]. It was shown for yeast vacuole formation that protein phosphatase 1 is involved in the final step of lipid bilayer fusion [15,16] and that this event could be inhibited by microcystin LR, a well known inhibitor of protein phosphatase 1 and 2a [25]. Since our experiments showed that fusion of vesicles in chloroplasts is inhibited by microcystin LR (and other inhibitors of protein phosphatases), a protein phosphatase might be involved in this process. Fusion was also inhibited by ophiobolin A,

Table 1
Inhibition of vesicle formation and fusion in isolated chloroplasts

	Vesicle formation	Vesicle fusion
A23187	−	−
Brefeldin A	−	−
Calyculin	n.d.	+
Cantharidin	n.d.	+
GMP/PNP	++	−
GTPγS	++	−
Mastoparan	−	−
N-Ethylmaleimide	−	−

Inhibition was analysed qualitatively by estimating the numbers of vesicles in chloroplasts from three independent preparations. ++ inhibition was greater than 50%, + inhibition was less than 50%, − no inhibition was observed, n.d. not determined.

which is an inhibitor of calmodulin, and by the Ca^{2+} antagonist W7. Both Ca^{2+} and calmodulin have been identified as part of the fusion complex in homotypic membrane fusion [26,27]. In the genome of *A. thaliana* several calmodulin isoforms can be detected with a potential chloroplast localisation. Mastoparan, another inhibitor of protein phosphatase 1, nevertheless failed to show any effect on vesicle fusion in our system. The reason might be that vesicle fusion in chloroplasts is not sensitive to this compound. On the other hand, any inhibitor used in this in organello system is required to cross the inner and outer envelopes of the chloroplasts, a process that is tightly controlled [28,29].

So far our data do not implicate any factors in the steps between vesicle formation and fusion. Yet, many of these factors are likely required for the specificity of membrane traffic, ensuring that a vesicle fuses with its appropriate target membrane. In contrast to the cytosol, the chloroplast stroma is only exposed to two membrane surfaces, i.e. the inner envelope and the thylakoids. Thus the vesicle transport system inside the chloroplast might be much simpler than the vesicle traffic systems of the cytosol.

What might be the function of this plastidal vesicle transport system? At the moment there is very little evidence on the cargo transported by these vesicles. Like in vacuole formation the lipid bilayer itself might be the means to bring polar lipids from the inner envelope to the thylakoids [11]. A vesicle accumulation can be observed in a number of plant mutants that are affected in thylakoid development [30,31]. Alternatively, other components of the thylakoid membrane, e.g. carotenoids, tocopherol, might be transported by means of vesicle traffic [31,32].

While vesicle transport is a common phenomenon in eukaryotic cells, such a system has not been described in prokaryotes. Chloroplasts are organelles derived from a prokaryotic organism by endosymbiosis. Having a vesicle transport system functioning inside chloroplasts is surprising and raises the question whether this system was already established in the prokaryotic ancestor before the endosymbiotic event took place or whether it was introduced to the chloroplast by the eukaryotic host. Only recently a protein, Vipp1, was identified that is essential for thylakoid formation in both cyanobacteria and *Arabidopsis* [18,33]. Disruption of the *vipp1* gene locus in *Arabidopsis* not only resulted in chloroplasts depleted of thylakoid membranes but they also no longer exhibited vesicle formation at low temperature. This implies that both eukaryotic and prokaryotic components might be involved in vesicle transport in chloroplasts.

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