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Restoration of thylakoid appression by Δ^3-*trans*-hexadecenoic acid-containing phosphatidylglycerol in a mutant of *Chlamydomonas reinhardtii*. Relationships with the regulation of excitation energy distribution

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The *mf* 2 mutant of *Chlamydomonas reinhardtii* lacks both Photosystem II reaction centers (RC II) and Δ^3-*trans*-hexadecenoic fatty acid (C16:1-*trans*). Previous studies have shown that, by comparison with a simple mutant lacking only RC II, the additional deficiency in C16:1-*trans* results in: (1) a very low fluorescence yield; (2) a predominant and persistent energy transfer from the main light-harvesting antenna towards Photosystem I, which cannot be reversed by state II–state I transition; (3) absence of the oligomeric form of the chlorophyll *a* + *b*-protein complex CP II. These defects can be reversed by incorporating this fatty acid in vivo into the photosynthetic membranes of the *mf* 2 mutant with C16:1-*trans*-containing phosphatidylglycerol (PG-C16:1-*trans*) liposomes. In this paper, subsequent ultrastructural analyses show that: (1) by comparison with the simple RC-II-lacking mutant, the additional C16:1-*trans* deficiency in the *mf* 2 mutant results in extensive thylakoid destacking; (2) supplementation of the *mf* 2 mutant with PG-C16:1-*trans* liposomes during growth induces a significant re-appression of thylakoid membranes; (3) such restacking was not observed in the presence of cycloheximide or using PG-C16:0 (PG-palmitate) liposomes. A strong correlation therefore appears between the presence of C16:1-*trans* in the thylakoid membranes of *C. reinhardtii* and the ability of these membranes to stack, to present the oligomeric organization of CP II and to restore state II–state I transitions in excitation energy distribution between the two photosystems. The possible involvement of PG-C16:1-*trans* in the synthesis and/or in the stabilization of the CP II apoproteins during the biogenesis of thylakoids is considered.

Introduction

Extensive research in several laboratories has allowed the structure of stacked thylakoid membranes to be determined and tempting hypotheses to be proposed concerning the mechanisms involved in membrane appression and the function of the resulting thylakoid stacks [1–3]. It is now established that most of Photosystem II (PS II) and Photosystem I (PS I) are spatially segregated in the plane of thylakoid membranes and concentrated in appressed and non-appressed areas, respectively [4,5]. Grana stacks were observed to be related to the presence of the main light-harvesting chlorophyll *a* + *b*-protein complex (LHC) [6,7] through a proposed mechanism involving neutralization of negative electrical charges and subsequent suppression of electrostatic repulsive forces between adjacent thylakoids [8]. From a functional point of view, the spatial segregation of PS II and PS I in stacked and unstacked membranes now appears to be a

Abbreviations: CP II, main light-harvesting chlorophyll *a* + *b*-protein complex in *Chlamydomonas*; C16:1-*trans*, Δ^3-*trans*-hexadecenoic acid; LHC, main light-harvesting chlorophyll *a* + *b*-protein complex in higher plants; PG, phosphatidylglycerol; PG-C16:0, 16:0 (palmitate)-containing phosphatidylglycerol; PG-C16:1-*trans*, 16:1-*trans*-containing phosphatidylglycerol; PS I, Photosystem I; PS II, Photosystem II.

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prerequisite for the regulation of distribution of excitation energy between the two photosystems in higher plants and green algae. It has indeed been shown that such regulation involves lateral migration of LHC subunits between stacked and unstacked thylakoid areas, respectively containing segregated PS II and PS I [9,10]. The driving forces for these reversible movements of LHC subunits are probably related to negative charges [11,12] introduced by reversible phosphorylations, themselves controlled by the redox state of the plastoquinone pool [13,14].

Possible specific functions of lipids in membrane processes, and particularly in thylakoid appression and in the molecular architecture of the photosynthetic membrane [15–17], have generally been neglected, principally because little information highlighting their importance was available. Nevertheless, a very unusual fatty acid, Δ^3-*trans*-hexadecenoic acid (C16 : 1-*trans*), has been specifically found in all photosynthetic membranes of eukaryotic organisms. Possible roles of this fatty acid, which esterifies C2 of the glycerol of phosphatidylglycerol (PG), have received special attention [18]. A good correlation between its accumulation and the development of appressed membranes was observed in greening leaves of higher plants and it was proposed that PG-C16 : 1-*trans* could play a role in the stacking process [19–21]. It was later proposed that this lipid is implicated in the supramolecular organization of LHC monomers into oligomers [22–25].

An extensive study of the possible role of C16 : 1-*trans* in *Chlamydomonas reinhardtii* chloroplasts was undertaken in our laboratory. Two mutants lacking both PS II reaction centers and C16 : 1-*trans* were identified [26,27]. By comparison with a classical mutant lacking only PS II and with the wild type, it appeared that one of these mutants, designated *mf* 2: (1) lacks the oligomeric main light-harvesting antenna CP II; (2) displays permanent state II fluorescence characteristics and is unable to reverse to state I [27,28]. The crucial role of C16 : 1-*trans* in these two processes was demonstrated by supplementing growing cells with PG-C16 : 1-*trans* liposomes [28]: re-incorporation of this lipid into the thylakoid membranes of the *mf* 2 mutant resulted in the restoration of both the oligomeric CP II and the ability to perform state II–state I transition. It appeared that this effect is specific to C16 : 1-*trans*, and that it involves neosynthesized CP II apoproteins.

The present paper extends these functional and biochemical results with observations concerning the ultrastructural organization of thylakoids under same experimental conditions.

Materials and Methods

The photosynthesis mutants *Fl* 39 and *mf* 2 have been obtained in our laboratory from wild-type *C. reinhardtii*; their characteristics have been described in previous papers [26–28].

Algae were grown in Tris-acetate-phosphate medium [29] in the light and at 25°C. For supplementation experiments, PG-C16 : 1-*trans* or PG-C16 : 0 liposomes were aseptically added to the culture medium (0.1 mg/ml) and, when used, cycloheximide was added to a concentration (1.8 μM) just sufficient to stop cell division and growth, as indicated in Ref. 28.

For electron microscopy, cells were fixed with 1% glutaraldehyde in the growth medium for 20 min at room temperature, then with 2% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4) for 2 h at 4 °C. They were then washed extensively with the same buffer, post-fixed in 1% osmium tetroxide in the phosphate buffer for 2 h at 4°C and washed with distilled water. Cells were then dehydrated in increasing ethanol concentrations and in propylene oxide, and embedded in araldite resin for 60 h at 48°C. Ultrathin sections of cells were obtained with a LKB ultrotome III ultramicrotome, stained in 50%–50% saturated uranyl acetate/ethanol, poststained in lead citrate (Reynolds), and observed in a Hitachi HU 12 A microscope (75 kV).

For each experiment, samples were controlled for lipid and fatty acid composition, for the monomeric or oligomeric forms of CP II and for state transition characteristics, as described in Ref. 28.

Results

Ultrastructural organization of thylakoids

The general organization of thylakoid membranes in the chloroplast of the mutants *Fl* 39 and *mf* 2 and of the wild type can be compared in Figs. 1 and 2.

Although well-defined grana stacks cannot be observed as in higher plants, the wild type presents important stacks of about 4 to 8 thylakoids (Figs. 1A and 2A), as previously described by Goodenough and Levine [30]. It can be estimated that about 70% of photosynthetic membrane areas are involved in such appressions (Fig. 3).

The absence of PS II reaction center in the *Fl* 39 mutant does not dramatically alter the general organization of thylakoids in the chloroplast. Thylakoid membranes are still able to come in contact, however, forming thicker stacks of often more than 15 thylakoids (Figs. 1B and 2B). However, from quantitative measurements we could estimate that a somewhat reduced proportion of thylakoid areas ($\approx$ 38%) is involved in such membrane appressions.

In contrast, the *mf* 2 mutant, lacking both PS II and C16 : 1-*trans*, presents a very altered organization of chloroplast membranes: despite a quite normal amount of photosynthetic membranes, most of them are not appressed (Fig. 1C), about 7% only of thylakoid areas

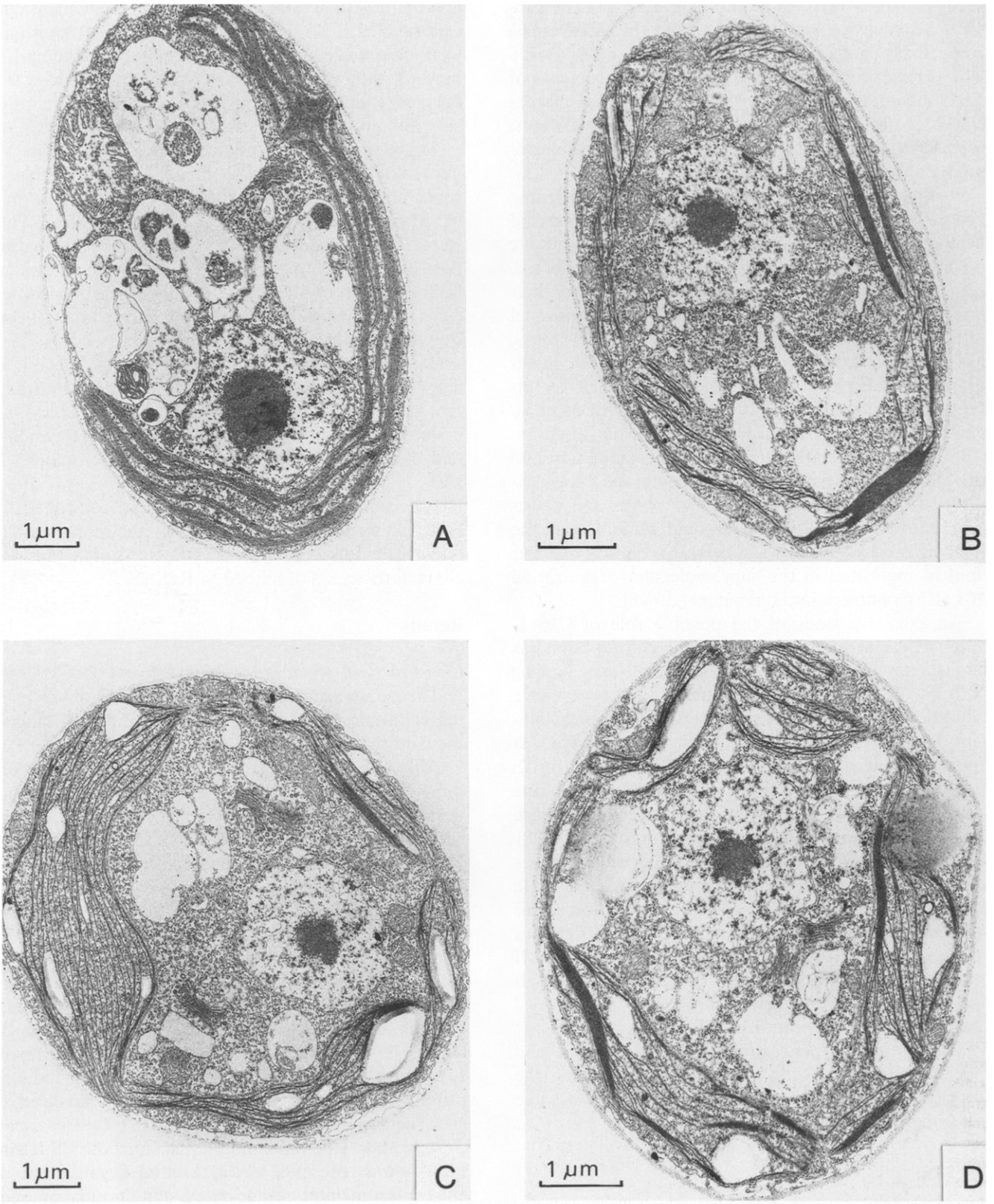

Fig. 1. General organization of *C. reinhardtii* cells. (A) wild type; (B) *Fl* 39 mutant devoid of PS II reaction centers; (C) *mf* 2 mutant devoid of both PS II reaction centers and the C16:1-*trans* fatty acid; (D) *mf* 2 mutant supplemented with PG-C16:1-*trans* liposomes.

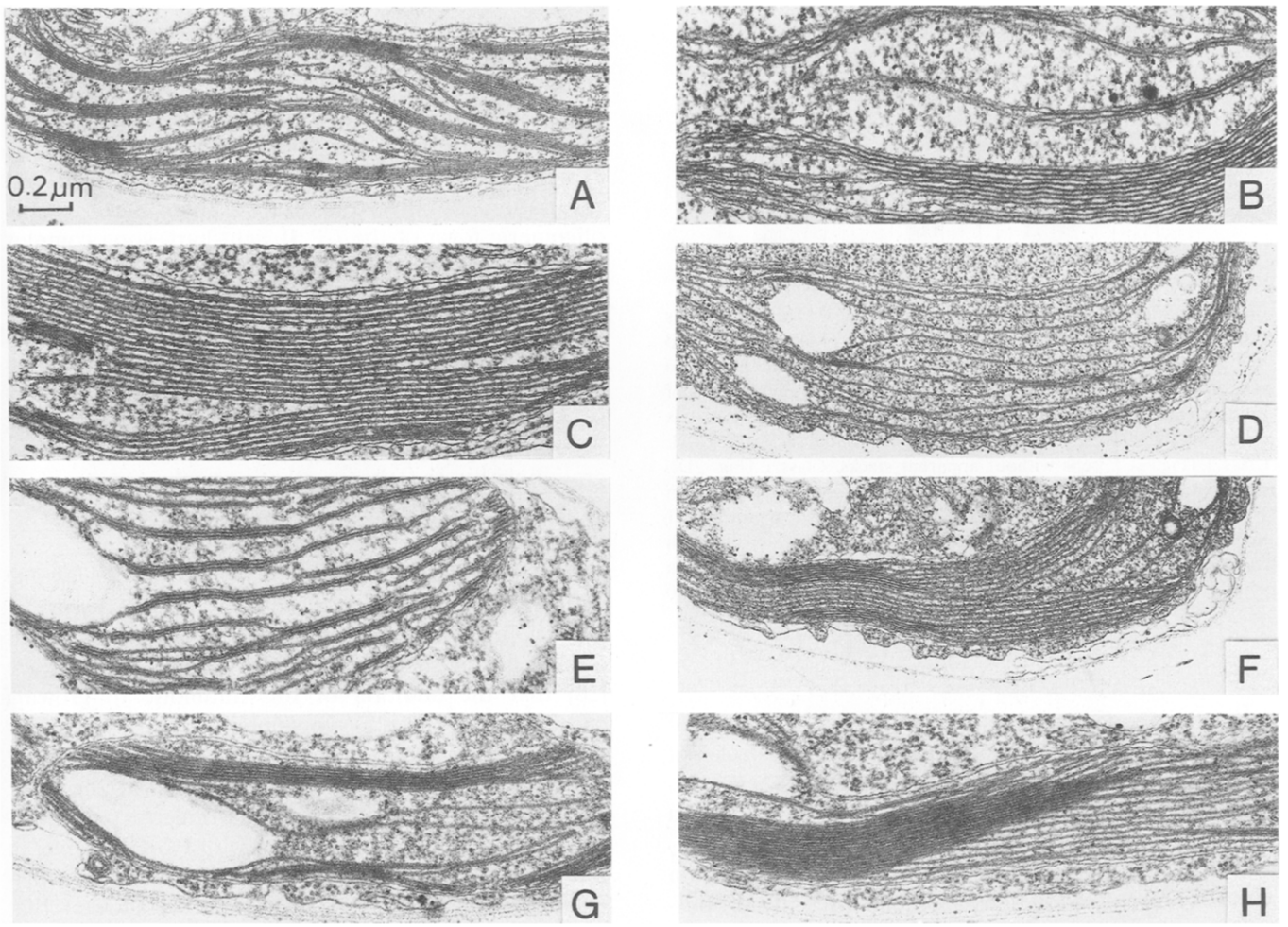

Fig. 2. Details of typical thylakoid stacks found in wild type (A); in *Fl* 39 (B) and in *mf* 2, untreated or supplemented with PG-C16:1-*trans* liposomes (C–H) (same magnification for all micrographs). Different types of stack can be distinguished by their length and by the number of appressed thylakoids; they were observable in all the analyzed mutant strains, but with different and specific frequencies (see Fig. 4).

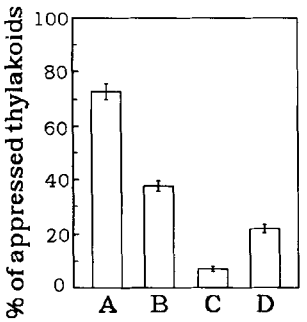

Fig. 3. Proportions of appressed thylakoid membrane areas in: (A) wild type; (B) *Fl* 39; (C) *mf* 2; (D) *mf* 2+PG-C16:1-*trans* liposomes. For each class of (A), (B), (C) and (D) cells, several micrographs were measured for the total length of thylakoid membranes and for the length of those involved in membrane stacks. The mean percent of stacked membrane areas in each class was calculated and multiplied by the frequency of the cell class. Results of each class were added in order to obtain the average value of the proportion of stacked thylakoid membrane length, which reflects the proportion of thylakoid membrane areas involved in stacks in each analyzed cell population.

being involved in stacks, the aspects and frequency of which are presented in Figs. 2C–2H and in Fig. 4, respectively.

By comparison with the *Fl* 39 mutant, it therefore can be assumed that in the *mf* 2 mutant, the additional deficiency of C16:1-*trans* and the significantly lower content in PG [26] could be related to the inability of thylakoid membranes to come in contact and to form appressed membrane stacks.

Effects of PG-C16:1-trans liposomes on the ultrastructural organization of mf 2 thylakoids

It was previously shown that *mf* 2 cells grown for 39 h in the presence of PG-C16:1-*trans* liposomes incorporate significant amount of this lipid in their thylakoid membranes [28]. Comparison of Figs. 1C and 1D shows that supplementation of *mf* 2 cells by such liposomes results in a significant modification of the general organization of thylakoids. Most of the supple-

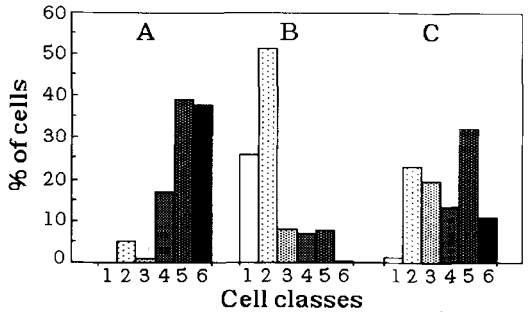

Fig. 4. Histograms showing the distribution of *C. reinhardtii* cells exhibiting different types of thylakoid stack. (A) *Fl* 39; (B) *mf* 2 and (C) *mf* 2 supplemented with PG-C16:1-*trans*. For each of two separate experiments, 200 to 300 cells were classified on the basis of predominant types of thylakoid stack observed on thin sections. Six classes of stacks were defined, which are illustrated in Fig. 2, C–H. Class 1 (Fig. 2C): cells without apparent stacks. Class 2 (Fig. 2D): cells mainly exhibiting stacks of 2–3 thylakoids on a length less than 1 μm. Class 3 (Fig. 2E): stacks of 2–3 thylakoids on a length from 1 to 3 μm. Class 4 (Fig. 2F): stacks of 4–7 thylakoids on a length shorter than 1 μm. Class 5 (Fig. 2G): stacks of 4–7 thylakoids on a length from 1 μm to 3 μm. Class 6 (Fig. 2H): stacks of 8–20 thylakoids on a length from 1 to 3 μm.

mented cells now resemble those of the *Fl* 39 mutant (Fig. 1B and 1D) and present membrane stacks of similar aspect to those already observed in some non-supplemented *mf* 2 cells (Figs. 2C to 2H), but with a significantly higher frequency (Fig. 4). From quantitative measurements we estimated that supplementation with PG-C16:1-*trans* liposomes increases the proportion of appressed thylakoid area from about 7% in non-treated *mf* 2 cells to about 22% in supplemented *mf* 2 cells (Fig. 3).

It was verified for each experiment that such a membrane reorganization was correlative to the aforementioned restoration of both the ability to form oligomeric CP II light-harvesting complex and the capacity to perform state II–state I transition as shown by low-temperature fluorescence emission spectra.

Moreover, the specificity of PG-C16:1-*trans* liposomes for such restoration processes was verified with similar experiments using PG-C16:0 liposomes. Such experimental conditions did not result in significant restoration of thylakoid stacks (Fig. 5A), of the oligomeric form of the CP II light-harvesting complex or of the state II–state I transition.

This specific role of the C16:1-*trans* fatty acid in the restoration of these structural, biochemical and functional parameters could be exerted either through some reorganization process of preexisting membrane components such as CP II monomers, or through some event occurring during their biosynthesis or their integration within the photosynthetic membrane.

Effect of cycloheximide on the restoration of membrane stacking in PG-C16:1-trans supplemented mf 2 cells

Cycloheximide is known to inhibit protein syntheses on 80 S cytoplasmic ribosomes. It was previously shown [28] that the addition of this antibiotic to growing cultures of *mf* 2 cells supplemented with PG-C16:1-*trans* prevents the restoration of both the oligomeric form of CP II and the ability to perform the state II–state I transition. As expected, ultrastructural analyses of *mf* 2 cells supplemented with PG-C16:*trans* in the presence of cycloheximide did not reveal any thylakoid reappression (Fig. 5B). Controls showed that cycloheximide alone does not exert by itself any important destacking effect on thylakoids of the wild-type and of the *Fl* 39 mutant. These results therefore suggest that the C16:1-*trans* fatty acid could be involved in some event associated with the organization state of some thylakoid component during its biosynthesis and/or macromolecular organization.

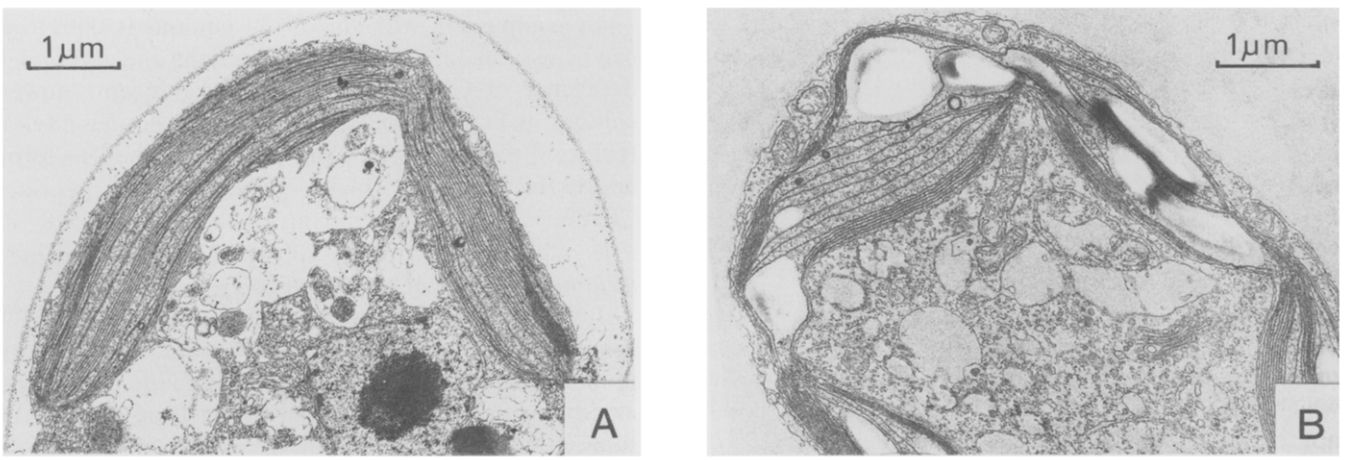

Fig. 5. General organization of *mf* 2 cells. (A) supplemented with PG-C16:0; (B) supplemented with PG-C16:1-*trans* liposomes in the presence of cycloheximide.

Discussion

In the preceding paper [28], it was hypothesized that PG-C16 : 1-*trans* is directly involved in the formation and/or the stabilization of the oligomeric CP II. Moreover, indirect participation of this lipid in state transitions was proposed through a mechanism by which CP II monomers only would be able to establish tight association with PS I, contrary to the oligomeric CP II, which would be confined in PS II membrane areas. In this paper, the additional information that thylakoids of the *mf* 2 mutant are largely deappressed extends these interpretations.

The fact that thylakoids are mainly unstacked in the *mf* 2 mutant is in itself sufficient to explain, in the framework of generally accepted concepts on chloroplast molecular organization, the permanent state II fluorescence characteristics of this mutant and its inability to revert to state I. As shown by the *Fl* 39 mutant and according to other *Chlamydomonas* mutants [31] and to *Euglena* [32], destacking of thylakoid membranes is not related to the absence of PS II reaction centers. Moreover, it was shown that in such cases the main light-harvesting antenna (CP II or LHC) remains largely concentrated in stacked areas and therefore spatially segregated from PS I [31,32]. Unstacking of thylakoids in the *mf* 2 mutant would therefore result in the intermixing of these light-harvesting antennae and PS I in the plane of the membrane, in a way similar to that observed for PS II and PS I in thylakoids artificially unstacked by Mg^{2+}-depleted buffers [33–35]. Such intermixing of membrane components would increase energy transfers from the CP II to PS I, therefore mimicking a permanent state II. Partial restoration of thylakoid stacks by PG-C16 : 1-*trans* supplementation would reduce energy transfers to PS I by concentrating part of the main light-harvesting antenna in restacked thylakoids, and would also restore partial state II–state I transition by allowing migration of LHC subunits from unstacked to restacked thylakoid areas.

Concerning the involvement of PG-C16 : 1-*trans* in the thylakoid stacking process, the largely destacked thylakoids of the *mf* 2 mutant and the restacking effect of supplementation by this lipid is consistent with the observed correlation between thylakoid appressions and PG-C16 : 1-*trans* content during the light-induced development of etiolated chloroplasts of higher plants [19–21]. However, it could be argued that during greening, the development of grana is rather more directly related to the accumulation of the PG-C16 : 1-*trans*-containing chlorophyll *a* + *b*-protein complex, which was shown in most cases to mediate thylakoid appressions [6,7]. The extensive unstacking of chloroplast membranes in the *mf* 2 mutant, which contains quite normal amount of CP II in the monomeric form, suggests more strongly that only the oligomeric form of this complex is able to mediate thylakoid appression. PG-C16 : 1-*trans* would then be indirectly related to thylakoid appression through its involvement in the oligomerization of CP II.

The PG-C16 : 1-*trans*-induced oligomerization of CP II appears to involve neosynthesized monomers rather than preexisting ones, since it is prevented by cycloheximide. It therefore becomes questionable whether preexisting and neosynthesized monomers are identical in polypeptide composition, since it has been shown that different types of LHC subunit can be assembled from different sets of polypeptides [36,37]. Preliminary experiments have shown that the polypeptide composition of the CP II light-harvesting complex of the *mf* 2 mutant largely differs from that of the wild type (unpublished). This observation then would favor the hypothesis of a possible role of PG-C16 : 1-*trans* in post-translational modification of some CP II apoprotein(s), allowing their insertion in the photosynthetic membrane or their full integration in the CP II complex, in a way similar to the palmitoylation of the 32 kDa protein described by Mattoo and Edelman [38]. The resulting modification of the apoprotein composition of the CP II complex then would impair its oligomerization in the PG-C16 : 1-*trans*-lacking *mf* 2 mutant.

The mechanism by which PG-C16 : 1-*trans* would be involved in membrane stacking and in subsequent regulation of the distribution of excitation energy then could be considered as follows. It was proposed that thylakoids appress when a decrease in electrostatic repulsions between negatively charged adjacent membranes allows hydrophobic and/or Van der Waals attractions to predominate [8]. The involvement of LHC in the decrease of surface charge density was proposed to result (1) from the presence in this complex of a polypeptide bearing an NH_2 terminal, stromal exposed and positively charged peptide [39,40] and (2) from interactions of the LHC complex with Mg^{2+} cations which would screen residual negative charges [41]. In this context, the absence of thylakoid stacks in the *mf* 2 mutant, despite the presence of a quite normal amount of CP II, could be interpreted as resulting from the absence in the CP II complex of those apoproteins bearing this positively charged peptide, therefore impairing the oligomerization of CP II monomers and the reduction of surface charge density required for membrane stacking. In this model, C16 : 1-*trans* would play a direct role in the polypeptide composition of CP II and would therefore indirectly affect the oligomerization of CP II, membrane stacking and, subsequently, the regulation of the distribution of excitation energy.

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