Phosphate deprivation induces transfer of DGDG galactolipid from chloroplast to mitochondria

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In many soils plants have to grow in a shortage of phosphate, leading to development of phosphate-saving mechanisms. At the cellular level, these mechanisms include conversion of phospholipids into glycolipids, mainly digalactosyldiacylglycerol (DGDG). The lipid changes are not restricted to plastid membranes where DGDG is synthesized and resides under normal conditions. In plant cells deprived of phosphate, mitochondria contain a high concentration of DGDG, whereas mitochondria have no glycolipids in control cells. Mitochondria do not synthesize this pool of DGDG, which structure is shown to be characteristic of a DGD type enzyme present in plastid envelope. The transfer of DGDG between plastid and mitochondria is investigated and detected between mitochondria-closely associated envelope vesicles and mitochondria. This transfer does not apparently involve the endomembrane system and would rather be dependent upon contacts between plastids and mitochondria. Contacts sites are favored at early stages of phosphate deprivation when DGDG cell content is just starting to respond to phosphate deprivation.

Introduction

Phosphorus is an essential macro element for plant growth and development, but in most soils it is moderately available due to adsorption properties (Raghothama, 1999, 2000). Plant cells have developed safety mechanisms circumventing this shortage, including decrease of their P_i consumption and mobilization of their P_i reserve. Phospholipids are a main form of cellular P_i reserve and their content markedly declines in plants during P_i starvation (Essigmann et al., 1998; Härtel et al., 1998).

In leaves, the most abundant membrane glycerolipids are not phospholipids, but glycolipids such as galactolipids, i.e., monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG). They represent up to 80% of leaf lipids (Joyard et al., 1996). Galactolipids were reported to be localized specifically in plastids and trace amounts of these lipids, which have been detected in the past in other isolated fractions of the cell, such as tonoplast (Haschke et al., 1990), were cautiously considered as possible contamination by plastid membranes. MGDG is synthesized from DAG and UDP-galactose by MGDG synthases, and this enzyme activity is located in the plastid envelope (Douce, 1974). In *Arabidopsis thaliana*, there are two types of MGDG synthases differing in their NH_2-terminal portion: type A with MGD1 and type B with MGD2 and MGD3 (Awai et al., 2001). In MGDG produced by these enzymes, galactose is linked to DAG via a β-glycosidic bond (Carter et al., 1956). On the other hand, two different mechanisms have been reported for the formation of DGDG: either by addition of galactose from UDP-galactose on MGDG with DGD1 or DGD2 enzymes (Kelly and Dormann, 2002; Kelly et al., 2003) or by reaction of two MGDG to form one DGDG and one DAG by the galactolipid–galactolipid galactosyltransferase enzyme (van Besouw and Wintermans, 1978). With DGD1 and DGD2, the inserted galactose is linked by an α-glycosidic bond (Kelly and Dormann, 2002; Kelly et al., 2003), leading to the α-β DGDG structure reported by Carter et al. (1956). The galactolipid–galactolipid galactosyltransferase enzyme generates a β-β DGDG structure because this enzyme activity correlates with the presence of oligogalactolipids carrying several galactose residues with β-glycosidic bonds (Kojima et al., 1990; Xu et al., 2003).

During P_i deprivation, the cellular DGDG content increases (Essigmann et al., 1998; Härtel et al., 1998) and the expression of genes encoding type B MGDG synthases (*MGD2* and *MGD3*; Awai et al., 2001) and DGDG synthases (*DGD1* and *DGD2*; Kelly and Dormann, 2002; Kelly et al.,

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Abbreviations used in this paper: BCCP, biotin carboxyl carrier protein; DGDG, digalactosyldiacylglycerol; DPG, diphosphatidylglycerol; HPPK, dihydropterin pyrophosphokinase; MGDG, monogalactosyldiacylglycerol; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SQDG, sulfoquinovosyldiacylglycerol; TriGDG, trigalactosyldiacylglycerol.

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2003) is stimulated. The induced synthesis of DGDG involves several compartments of the cell. DAG backbone of DGDG was traced back from extraplastidial phosphatidylcholine (PC) (Roughan, 1970; Williams et al., 2000; Kelly et al., 2003), and lipid analyses during the first steps of P_i deprivation indicated that indeed PC was transformed into DGDG via DAG (Jouhet et al., 2003). Galactose insertion for synthesis of newly formed DGDG is expected to occur in plastids because enzymes encoded by *MGD2*, *MGD3*, *DGD1*, and *DGD2* can all be addressed to the plastid envelope, very likely to the outer envelope (Awai et al., 2001; Froehlich et al., 2001; Kelly et al., 2003). Consistently, newly synthesized DGDG was proposed to replace missing PC (Härtel and Benning, 2000; Härtel et al., 2000) because (1) PC content is highly reduced; and (2) PC and DGDG adopt similar bilayer conformation in the membranes. These lipid changes cannot be limited to plastid membranes because the bulk of PC is located outside plastids (Dorne et al., 1985). Indeed, a recent report has shown that DGDG accumulates in oat plasma membrane during P_i deprivation (Andersson et al., 2003). However, the plasma membrane represents a low proportion of the cellular membrane surface, and therefore the plasma membrane lipid change cannot solely explain the high amounts of cellular PC and DGDG being affected by P_i deprivation.

Mitochondria are organelles limited by a double membrane like plastids. In plant cells, they represent ~10% of cell membranes and contribute to 10–20% of total cellular PC (Douce, 1985). During P_i deprivation, mitochondria seem relatively protected from the induced stress. The cellular proportion of diphosphatidylglycerol (DPG), a specific mitochondrial phospholipid, remains relatively constant (Jouhet et al., 2003). Moreover, respiration is not affected except for the cyanide-resistant pathway that is enhanced (Rébeillé et al., 1984). A possible response of the plant cell to P_i deprivation is that DGDG could be transferred to mitochondria as well, and therefore contributes to adaptation of this organelle to the stress conditions. To probe this hypothesis, we cultivated *A. thaliana* cells with defined levels of P_i and set up a procedure to isolate the mitochondria. In this article, we report that under P_i deprivation, mitochondria contain a high concentration of DGDG in contrast with mitochondria from cells grown with sufficient supply of P_i. The α-β anomeric structure of DGDG present in mitochondria is characteristic of DGDG synthesized through a DGD type enzyme. We further point out that a transfer of DGDG occurs between plastid envelope and mitochondria. This transfer is apparently dependent on contact between plastids and mitochondria. The contacts seem to be favored at early stages of P_i deprivation when DGDG cell content is just starting to respond to P_i deprivation.

Results

DGDG is detected in plastids and in mitochondria of P_i-deprived cells

3 d after the beginning of P_i deprivation, *A. thaliana* cells grown as a suspension in liquid medium don't divide anymore. Their lipid composition has been modified compared with control cells. Particularly, the level of DGDG has increased from less than 10% up to 30–35% of total glycerolipids during the first day of deprivation, and this level is now stable (Jouhet et al., 2003). To understand where DGDG is localized at this stage, routinely grown *Arabidopsis* cell cultures were transferred for 3 d in a medium devoid of P_i ($-P_i$ medium) or containing 1 mM P_i (control). Subcellular localization of DGDG was then assayed using antibodies raised against this lipid. Specificity of anti-DGDG antibodies for DGDG was assessed based on absence of reaction with other lipids or proteins of the cell (Maréchal et al., 2002). Fig. 1 A and Fig. S1 (available at http://www.jcb.org/cgi/content/full/jcb.200407022/DC1) show that in control cells, DGDG was only detected in plastids because DGDG-coupled epifluorescence was associated with epifluorescence coupled with the biotin carboxyl carrier protein (BCCP) subunit of acetyl-CoA synthetase, a protein present in the plastid stroma (Alban et al., 1994). In P_i-deprived cells, the situation appears to be more complex because DGDG-associated fluorescence was no more restricted to plastids and was also visible in many small spots distinct from plastids (Fig. 1 B). Some of these spots were present at the periphery of the cell, likely associated with plasma membrane as reported by Andersson et al. (2003). In addition, by comparison with the

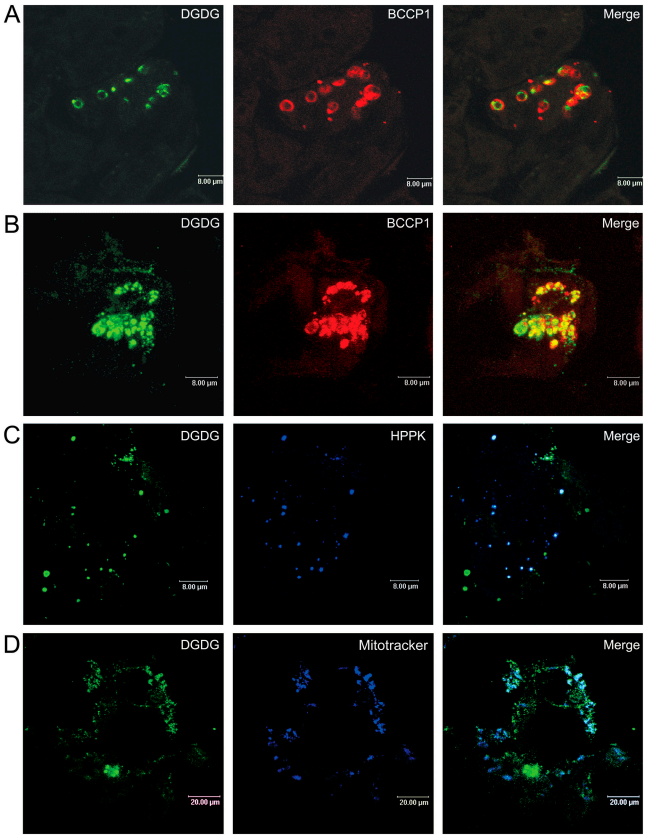

Figure 1. **Localization of DGDG in mitochondria of *A. thaliana* cells deprived of P_i for 3 d.** Cells (A, control; B–D, P_i-deprived) were processed for indirect immunofluorescence labeling using anti-DGDG with secondary antibodies coupled to BODIPY and either anti-BCCP1, for chloroplast detection (A and B), or anti-HPPK (C), for mitochondria detection, with secondary antibodies coupled to Alexa 594. In D, mitochondria were visualized by staining with Mitotracker orange CMTMRos. Cells were observed by confocal microscopy. Bars: A–C, 8 μm; D, 20 μm.

epifluorescence coupled with dihydropterin pyrophosphokinase (HPPK), a protein from mitochondria (Mouillon et al., 2002), we observed that DGDG-coupled fluorescence was also associated with that of mitochondrial markers (Fig. 1 C). Co-labeling with DGDG antibodies and MitoTracker orange CMTMRos (Molecular Probes, Inc.) confirmed this observation (Fig. 1 D). Therefore, these data indicate that in cells deprived of P_i, DGDG is present outside of plastids, and notably in mitochondria.

Isolation of mitochondria from *A. thaliana* cells grown without P_i

To further investigate their lipid composition, mitochondria were isolated and purified from *Arabidopsis* cells either deprived of P_i for 3 d or sufficiently provided with P_i (control cells). O_2 consumption of isolated mitochondria was analyzed on each purified fraction (Fig. 2 A) and according to Neuburger et al. (1982), data indicated that the preparations were highly enriched in functionally intact mitochondria. To obtain sufficient amounts of lipids for analysis, three mitochondrial preparations obtained in each condition were pooled. Mitochondria purity was further controlled by Western blot on this mix. We detected approximately a fivefold enrichment of the mitochondrial inner membrane protein NAD9 (Lamattina et al., 1993), the outer membrane proteins TOM20 and TOM40 (Werhahn et al., 2001), and of the HPPK matrix protein (Mouillon et al., 2002) in the mitochondrial fractions, as compared with the whole-cell fractions (Fig. 2 B). Taking into consideration that mitochondria may represent 15–20% of total cell protein, the enrichment in mitochondria markers indicated that the isolated mitochondria were rather pure. Nevertheless, in order to ascertain galactolipid content of mitochondria, we measured the cross-contamination of the isolated organelles by plastid membranes classically reported to be enriched in galactolipids. Contamination by chloroplast membranes was measured by following the thylakoid marker LHCII (Vallon et al., 1986), the inner envelope marker E37 (Teyssier et al., 1996), and the outer envelope marker OEP21 (Bolter et al., 1999). Contamination by thylakoids was negligible because mitochondrial fractions contained 40 times less LHCII than chloroplasts (Fig. 2 C). The envelope markers OEP21 and E37 were both five times less abundant in mitochondria than in chloroplasts, and taking into account that envelope proteins likely represent ~4% of chloroplast total proteins, this indicated that mitochondria (1 mg protein) contained <0.6% envelope proteins (6 μg protein) (Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200407022/DC1).

Because plasma membrane contains DGDG under P_i-deprived conditions (Andersson et al., 2003), we questioned the possible contamination of mitochondria by plasma membrane. The plasma membrane is characterized by content of sterols, e.g., acylated steryl-glycosides (Norberg and Liljenberg, 1991). We could not detect any acylated steryl-glycosides in mitochondria lipid extracts from P_i-deprived or control cells. Altogether, our results indicate that the mitochondrial fractions we prepared from *A. thaliana* cells were highly enriched in intact mitochondria and contained little cross-contamination by extramitochondrial membranes.

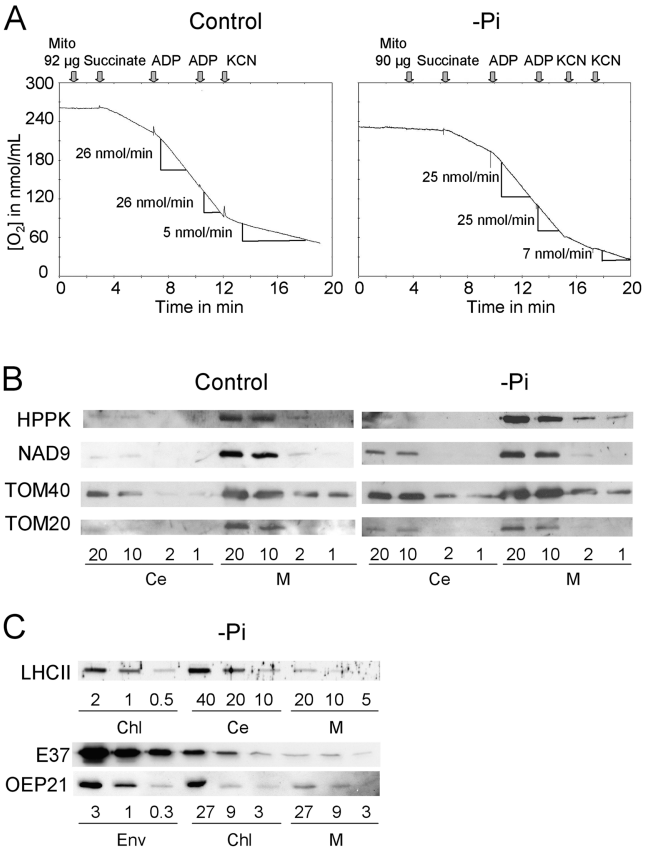

Figure 2. **Characterization of mitochondria fractions isolated from either control or 3 d P_i-deprived *A. thaliana* cells.** (A) To check purity and intactness of isolated mitochondria, succinate oxidation was followed by measuring O_2 consumption. On average, each purified fraction consumed ~280 nmol $O_2.min^{-1}.mg^{-1}$ protein in the presence of succinate and ADP, and O_2 consumption was stimulated 2.4 times by addition of ADP. Therefore, fractions were considered to be highly enriched in functionally intact mitochondria. Cyanide resistant pathway was slightly enhanced in P_i-deprived conditions as expected according to Rébeillé et al. (1984). (B) Comparative Western blot analysis of mitochondrial (M) and total cell extract (Ce) using antibodies specific for mitochondrial proteins, HPPK, a matrix protein, NAD9, an inner membrane protein, and TOM20 and TOM40 outer membrane proteins. (C) Western blot analysis of mitochondrial (M), chloroplast (Chl), and total cell extract (Ce) of P_i-deprived cells and of chloroplast envelope (Env) prepared from *Arabidopsis* plants as in Awai et al. (2001) using antibodies specific for chloroplast membrane proteins, LHCII for thylakoid, E37 for inner envelope membrane, and OEP21 for outer envelope membrane.

Mitochondria isolated from P_i-deprived cells contain DGDG

Lipids were extracted from both types of mitochondria and their composition was compared with that of cells. Fig. 3 A shows results of glycerolipid analyses normalized to the total amount of glycerolipid in each fraction. The composition of cells grown with or without P_i was consistent with data published earlier (Essigmann et al., 1998; Härtel et al., 1998; Jouhet et al., 2003). DPG was found at a relatively high level in both types of cells, indicating that mitochondria lipids represent a fair proportion of total cell lipids. In P_i-deprived cells, we mainly observed a decrease in phospholipids and a high increase in DGDG and sulfoquinovosyldiacylglycerol (SQDG). Lipid composition of mitochondria isolated from control cells

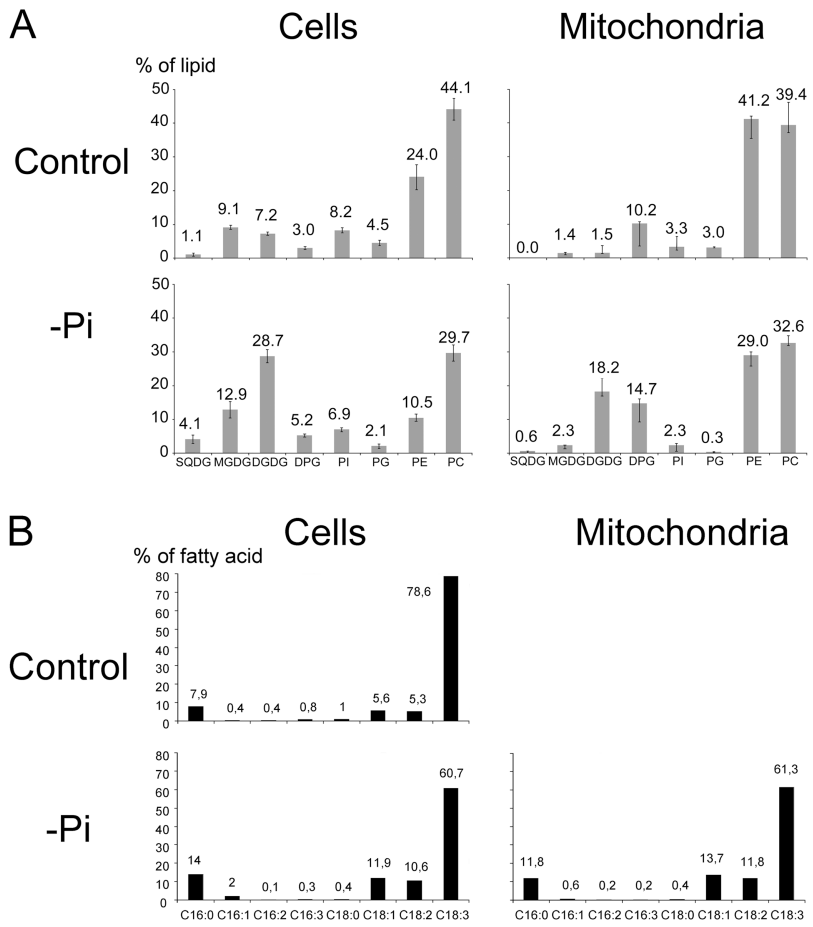

Figure 3. **Glycerolipid analysis of total cell and mitochondria fractions from control and 3 d P_i-deprived *A. thaliana* cells.** (A) Glycerolipid composition. SD was calculated on four independent measurements in each case. (B) Fatty acid composition of DGDG isolated either from total cell extracts from 3 d P_i-deprived cells and control cells or from mitochondria fraction from 3 d P_i-deprived cells.

was similar to that reported earlier, containing mostly phosphatidylethanolamine (PE) and PC (Douce, 1985; Harwood, 1987). Only traces of MGDG and DGDG were detected. The mol percentage of DPG was three times higher in isolated mitochondria than in whole cells. In mitochondria isolated from P_i-deprived cells, the levels of phospholipids (i.e., PC, PE, and PG) were all lower except for DPG, which was present in higher proportion. Contents in MGDG and SQDG were slightly higher than in mitochondria from control cells, but both remained at a low level. By contrast, the level of DGDG was remarkably higher, representing >18% of the $-P_i$ mitochondria lipids. By analyzing the lipid contamination attributable to chloroplast envelope with mitochondrial lipid data, we calculated that the amount of DGDG issued from envelope contamination is much lower than the amount of DGDG measured in the mitochondria fraction (Table S1), indicating that most of the DGDG detected in mitochondria upon 3 d of P_i deprivation was indeed located in mitochondria.

DGDG is present on the surface of mitochondria in P_i-deprived cells

Antibodies raised against DGDG were used to test a possible agglutination of purified mitochondria (Fig. 4). In absence of antibodies, control and $-P_i$ purified mitochondria were visible under light microscope as nonaggregated, whereas they agglutinated in presence of antibodies against mitochondria outer membrane proteins TOM20 and TOM40. When antibodies raised against plastid proteins, like E37 and OEP21, were added to mitochondria, no agglutination was visible whenever OEP21 antibodies did induce chloroplast agglutination. With antibodies raised against DGDG, no agglutination of control mitochondria could be detected, but a strong agglutination was observed with mitochondria prepared from P_i-deprived cells. In conclusion, immunoagglutination assays indicated that in P_i-starved cells, DGDG is accessible on the mitochondrial outer surface to specific antibodies.

Structure of DGDG present in mitochondria

To characterize the overall structure of the mitochondria-associated DGDG, we analyzed its fatty acid composition and its polar head structure. Fig. 3 B shows that fatty acid composition of mitochondrial DGDG was fairly similar to that of P_i-deprived cell DGDG. Compared with DGDG present in control cells, the main characteristic of fatty acid composition of mitochondrial DGDG was an increase in 16C/18C ratio (0.09–0.13) with more 16:0 and an increase in more saturated species of C18, but globally DGDG remained highly enriched in 18:3. The anomeric structure of the polar head of mitochondrial DGDG was resolved by nuclear magnetic resonance (NMR). In ^{1}H-NMR, the anomeric proton in α- or β-glycosidic configuration gives characteristic doublet signals respectively at high

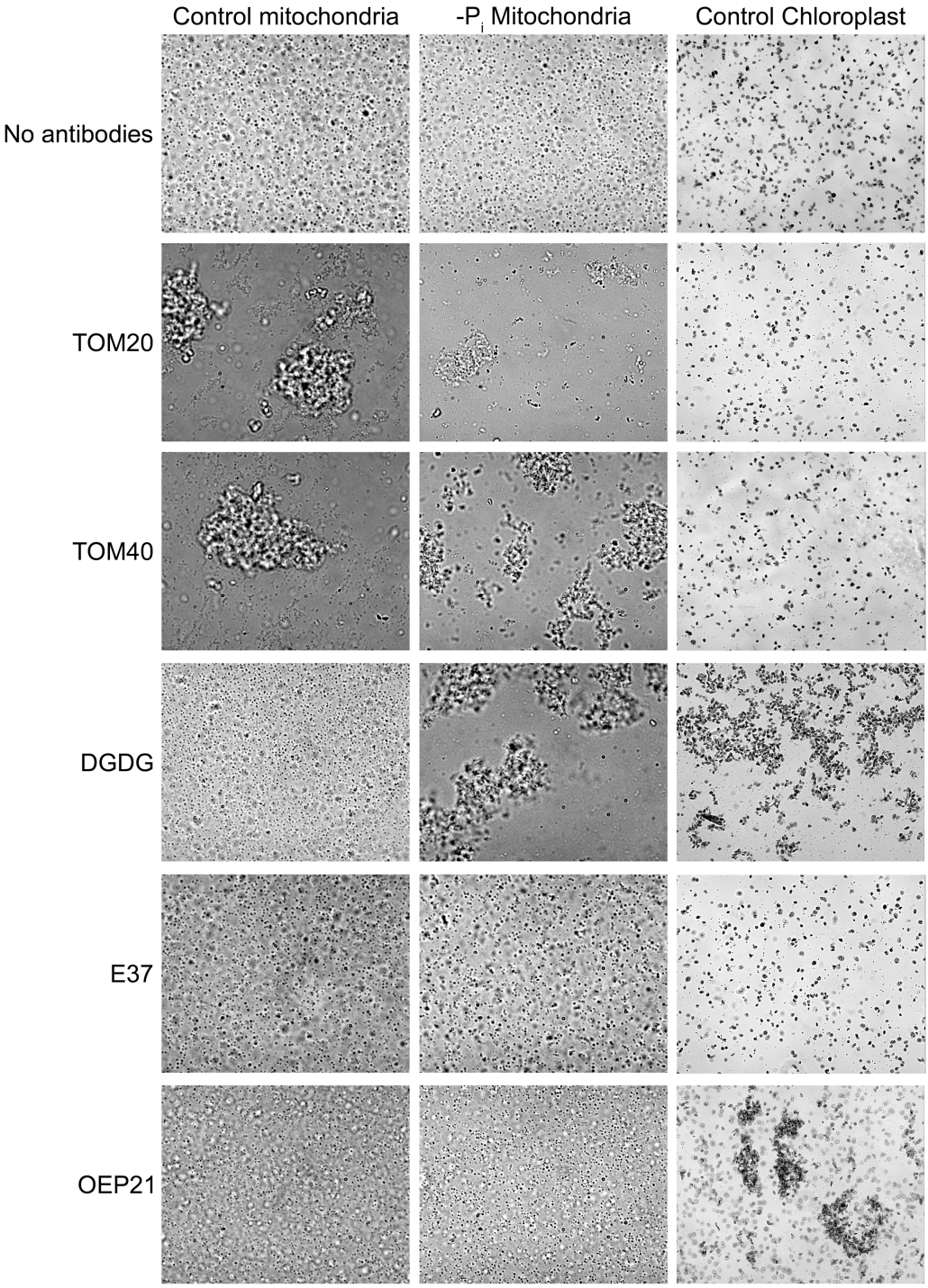

Figure 4. **Immunoagglutination assays of isolated mitochondria prepared from either control or 3 d P_i-deprived *A. thaliana* cells.** Mitochondria from P_i-deprived cells, control cells, and chloroplasts were incubated with antibodies as specified. Addition of anti-DGDG lead to agglutination of chloroplasts and of mitochondria of P_i-deprived cells only. Control antibodies were directed against E37 and OEP21 chloroplast inner and outer membrane protein, respectively, and TOM20 and TOM40 outer membrane mitochondrial proteins.

chemical shift (~5.0 ppm) or at low chemical shift (~4.0 ppm). The exact position of these signals depends on solvent, temperature, and molecular environment. To identify the precise position of these α- and β-doublet signals, several galactolipid molecules were analyzed by NMR and compared. Higher plant MGDG was reported to contain only a β-glycosidic bond (Carter et al., 1956), and indeed *Arabidopsis* cell MGDG gave a doublet signal at 4.0 ppm (Fig. 5 A). Two forms of DGDG have previously been reported in plants (Kojima et al., 1990; Xu et al., 2003); the main form containing a β-glycosidic bond on the first galactose and an α-glycosidic bond on the second galactose (Carter et al., 1956). DGDG extracted from either control or P_i-deprived *Arabidopsis* cells corresponded to this main form, with a doublet signal at 4.0 ppm for the β-bond and

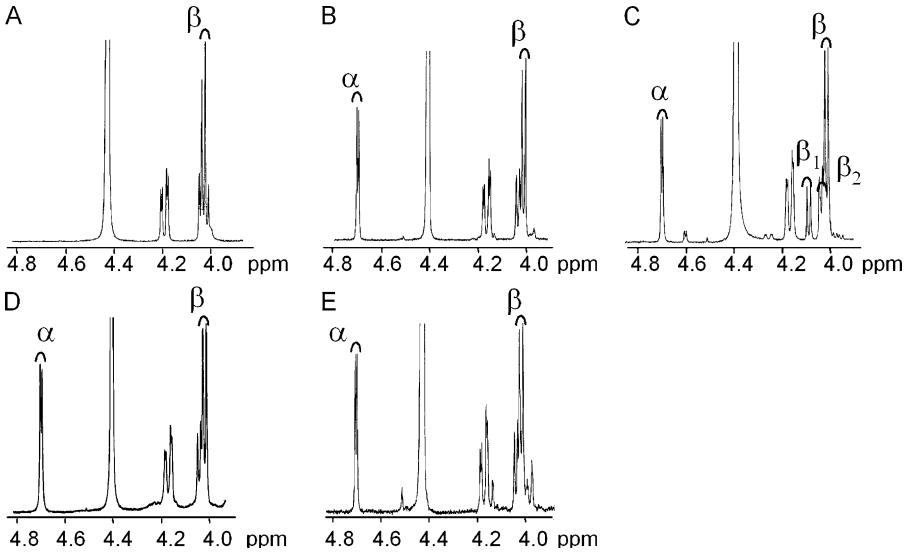

Figure 5. **^{1}H-NMR galactolipid analysis.** The α-peak is characterized by a doublet at 4.7 ppm and the β-peak by a doublet at 4.0 ppm. (A) β-MGDG from *A. thaliana* control cells. (B) α-β DGDG from *A. thaliana* control cells. (C) DGDG from *Spinacia oleracea* purified chloroplast envelope. In envelope fraction, two DGDG types are visible: α-β and β_1-β_2. (D) α-β DGDG from *A. thaliana* P_i-deprived cells. (E) Mitochondrial α-β DGDG from *A. thaliana* P_i-deprived cells.

at 4.7 ppm for the α-bond (Fig. 5, B and D). In addition to the 4.7 ppm α-doublet, DGDG extracted from an isolated fraction of spinach chloroplast envelope contained three doublet signals in the range of the β signal (exact position at 4.0, 4.05, and 4.1 ppm; Fig. 5 C) that were indicative of presence of both α-β and β-β DGDG structures, the later structure very likely resulting from the activation of the galactolipid–galactolipid galactosyltransferase during the course of envelope isolation (Xu et al., 2003). In NMR spectra of mitochondrial DGDG, we observed only doublet signals characteristic for an α-glycosidic bond at 4.7 ppm and for a β-glycosidic bond at 4.0 ppm, with no signal at 4.1 ppm (Fig. 5 E). We concluded from these results that mitochondrial DGDG structure is 1,2-diacyl-3-*O*-(α-D-galactopyranosyl-(1→6)-*O*-β-D-galactopyranosyl)-*sn*-glycerol.

Galactolipid synthesis is not localized in mitochondria

Because DGD1 and DGD2 (that have been located in the chloroplast envelope; Froehlich et al., 2001; Kelly et al., 2003) have been reported to synthesize DGDG with an α-β structure and because up to date, galactolipid synthesis has not been found in another organelle but plastids, one might expect that DGDG is formed in plastids before transfer to mitochondria. We analyzed the galactolipid synthesis capability of mitochondria isolated from P_i-deprived cells. Table I shows that [C^{14}]galactose incorporation into galactolipids was in the range of 3–17 nmol.mg $prot^{-1}.h^{-1}$ in mitochondria, lower than in chloroplasts (~25 nmol.mg $prot^{-1}.h^{-1}$) and far much lower than those previously reported for isolated chloroplast envelope (~1 μmol.mg $prot^{-1}.h^{-1}$ as in Block et al. [1983]). Furthermore, the activity in mitochondria decreased when mitochondria were further purified. When reported to the amount of either E37 or OEP21, galactosyltransferase activity correlated with the level of both OEP21 or E37 (Table I), pointing to an association of inner and outer envelope membranes in the mitochondria preparation and indicating that the galactolipid synthesis activity found in the mitochondria fraction was attributable to cross-contamination by plastid envelope membranes.

Physical contact between mitochondria and chloroplasts

Lipid transfer between organelles and/or membrane vesicles can be activated by contact between membranes (Achleitner et al., 1999; Voelker, 2003). To investigate a possible increase of contact sites between plastids and mitochondria during P_i deprivation, we performed an EM survey of *Arabidopsis* cell suspensions. In cells that were subcultured into a standard medium for 3 d, we observed numerous round or elongated mitochondria and plastids containing a big starch grain and scarce thylakoid membranes with limited grana stacks. Cells are indeed photosynthetic (Axelos et al., 1992) but not fully autotrophic, and their growth is dependent on presence of sugar. In cells

Table I. **Comparison of galactolipid synthesis activity and chloroplast envelope contamination of various fractions isolated from *Arabidopsis* cells deprived for P_i for 12 h**

	E37	OEP21	Incorporated galactose	
	Relative units	*Relative units*	*nmol.mg^{-1}.h^{-1}*	*Relative units*
Chloroplasts	100	100	25.9	100
Cells	9.6	9.5	1.8	6.9
Crude mitochondria	67.2	64.5	16.6	64.1
Purified mitochondria	13.4	14.4	2.8	10.8

Mitochondria fractions were prepared at different stages of purification as described in Materials and methods. Proteins were analyzed by SDS-PAGE and Western blot, and chloroplast envelope contamination was defined by comparing anti-E37 or OEP21 ECL signal intensity of 10, 5, and 2.5 μg protein of a unique chloroplast fraction and 20, 10, and 5 μg of each mitochondria fraction.