Optical Manipulation Reveals Strong Attracting Forces at Membrane Contact Sites between Endoplasmic Reticulum and Chloroplasts

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Eukaryote cells depend on membrane lipid trafficking from biogenic membranes, like the endoplasmic reticulum (ER), to other membranes in the cell. Two major routes for membrane lipid transport are recognized: vesicular trafficking and lipid transfer at zones of close contact between membranes. Specific ER regions involved in such membrane contact sites (MCSs) have been isolated, and lipid transfer at MCSs as well as protein-protein interactions between the partaking membranes have been demonstrated (reviewed by Holthuis, J. C. M., and Levine, T. P. (2005) Nat. Rev. 6, 209–220). Here we present the first demonstration of the physical association between membranes involved in MCSs: by using optical imaging and manipulation, strong attractive forces between ER and chloroplasts are revealed. We used Arabidopsis thaliana expressing green fluorescent protein in the ER lumen and observed leaf protoplasts by confocal microscopy. The ER network was evident, with ER branch end points apparently localized at chloroplast surfaces. After rupture of a protoplast using a laser scalpel, the cell content was released. ER fragments remained attached to the released chloroplasts and could be stretched out by optical tweezers. The applied force, 400 pN, could not drag a chloroplast free from its attached ER, which could reflect protein-protein interactions at the ER-chloroplast MCSs. As chloroplasts rely on import of ER-synthesized lipids, we propose that lipid transfer occurs at these MCSs. We suggest that lipid transfer at the MCSs also occurs in the opposite direction, for example to channel plastid-synthesized acyl groups to supply substrates for ER-localized synthesis of membrane and storage lipids.

In eukaryote cells, the endoplasmic reticulum (ER) is a major site for the synthesis of membrane lipids. These lipids are transported to their target membranes in bulk form as vesicle constituents or as monomers at membrane contact sites (MCSs) between the ER and the target membrane. With target membranes outside the secretory pathway, lipid trafficking occurs exclusively via MCSs (reviewed by Holthuis and Levine (1)). In plants, it is well established that a major lipid trafficking route is from the ER to the chloroplast, to supply substrates for the synthesis of the chloroplast galacto- and sulfolipids in the chloroplast envelope (2–5). The molecular details of lipid transport from the ER to the chloroplast to date remain obscure, but earlier we reported biochemical evidence for ER-chloroplast associations: lipid synthesizing activities usually attributed to the ER remained associated with highly purified chloroplasts (6), and transfer of lipid precursor for chloroplast lipid synthesis could be reconstituted between isolated enriched ER and isolated chloroplasts (7).

It is well established that in plants, as in other eukaryotes, the ER forms an extended network throughout the cytosol (8). Close associations between ER and non-green plastids have been visualized in several plant tissues by electron microscopy (9–12), and associations between ER and chloroplasts have been visualized in cell cultures by laser scanning confocal microscopy (13). In the present study, we took advantage of the development of lasers for microscopic manipulation of biological samples to study MCSs between ER and chloroplasts. Using laser scalps and optical tweezers (14), we aimed to determine the force of attraction between chloroplasts and the attached ER. In addition, we isolated and characterized a chloroplast-associated ER fraction.

MATERIALS AND METHODS

Membrane Isolation and Characterization—Arabidopsis thaliana stably expressing green fluorescent protein (GFP) in the ER lumen (15) was grown for 4–5 weeks in a growth chamber at 60% relative humidity and an 8-h light (24 °C)/16-h dark (20 °C) regime. Protoplasts were isolated from the leaves (16) and were used directly for optical manipulation or as starting material for chloroplast isolation (17) after lysis by three strokes in a chilled hand homogenizer.

Garden pea (Pisum sativum L. cv. Kelvedon Wonder (Weibull, Sweden)) was grown for 7–8 days in a climate chamber (6). From harvest on, all steps in the membrane isolation

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The on-line version of this article (available at http://www.jbc.org) contains supplemental movies 1–3.
procedures were carried out at 4 °C. To isolate chloroplast-associated membranes of ER origin, a procedure based on the isolation of mitochondria-associated ER from purified yeast mitochondria was used (18) (see Fig. 1A). Intact chloroplasts were isolated from pea seedlings (17). The first chloroplast supernatant was centrifuged for 15 min at 10,000 × g_{max} and the resulting supernatant was centrifuged for 30 min at 100,000 × g_{max}. The resulting pellet is referred to as the microsome (MS) fraction. The MS pellet and the pellet of intact chloroplasts were each suspended in 10 mM MES/KOH, pH 6.0, and 0.33 M sucrose. After 15-min incubation on ice, the suspensions were top-loaded onto continuous sucrose gradients (20–50% w/v sucrose) in the same buffer. The gradients were centrifuged for 60 min in a swing out rotor at 100,000 × g_{max}. From each gradient, the top band was collected, diluted in 30 mM HEPES/KOH, pH 7.0, 10 mM KCl, and 2.5 mM MgCl₂, and pelleted at 100,000 × g_{max} for 30 min. The membranes were re-suspended in the same buffer as used for pelleting, frozen in liquid nitrogen, and stored at −80 °C until further analyses. Envelope membranes were isolated from chloroplasts isolated from 9- to 10-day-old pea seedlings as described (19). Analyses of membrane components and marker enzyme activities were assayed as described previously (20).

Optical Imaging and Manipulation—Specimens obtained from GFP-expressing A. thaliana were imaged with a Zeiss 510LSM confocal microscope. A plan apochromat 63 × 1.4 objective was used. Both GFP and chlorophyll were excited with a 488-nm laser; GFP emission was monitored at 505–530 and chlorophyll through a 650-nm long pass filter.

The technical platform for simultaneous optical manipulation and confocal imaging is described elsewhere (14). In this study the optical tweezers made use of a 1064-nm diode-pumped Nd:YVO₄ laser, and the power in the optical trap typically ranged from 500 milliwatt to 2 watts. The nitrogen laser used as the optical scalpel had a wavelength of 337 nm, a pulse width less than 4 ns, and maximum pulse energy of 300 J. The pulse energy of the laser scalpel was optimized with respect to the rupture level of the outer boundary of the cell by two neutral density filters combined with crossed polarizers. Calibration of the linearity of the trapping potential was performed using the drag force method (21).

RESULTS

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Imaging of Chloroplast-associated ER—To study the spatial relationship between the ER and the chloroplasts, we used transgenic A. thaliana expressing GFP in the ER lumen (15). Protoplasts isolated from leaves of 4- to 5-week-old plants were observed by confocal microscopy. The ER appeared as an extensive network enclosing the chloroplasts, with ER branch end points apparently localized at the chloroplast surfaces (Fig. 2A). This observation is in accordance with previous studies (8, 13), but neither these studies nor the present observation demonstrate actual physical connections between the ER and the chloroplast surface. We then proceeded to isolate chloroplasts from the GFP-expressing leaf protoplasts. The majority of the isolated chloroplasts were associated with GFP-fluorescing bodies (Fig. 2B). This observation suggests the occurrence of specific areas of association between the ER and the chloroplast strong enough to survive the chloroplast isolation procedure. The presence of the luminal fluorescent ER protein also suggests that the attached ER had been part of the ER continuum of the cell.

Optical Manipulation of Chloroplast-associated ER—To further study the association between ER and chloroplasts, we uti-
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lized optical manipulation. A confocal microscope equipped with optical tweezers and a laser scalpel was used for simultaneous imaging and optical manipulation (14). Leaf protoplasts were isolated from the GFP-expressing *A. thaliana*. One protoplast at a time was observed and subjected to a single pulse from the laser scalpel focused at its outer boundary (Fig. 3A). This treatment ruptured the cell and released its contents, which settled onto the coverslip (Fig. 3, B and C). The pulse energy needed to rupture the cell boundary ranged from 50 to 75 μJ, measured at the entrance aperture of the microscope objective. We were able to capture a single chloroplast with the optical tweezers just after the disruption of the cell (Fig. 3D). When this chloroplast was pulled free of the other chloroplasts and cellular constituents, a strand of GFP-fluorescent ER was stretched out between this chloroplast and other chloroplasts (Fig. 3, E and F). This course of events clearly demonstrates genuine physical connections between chloroplasts and the ER in *A. thaliana* (also see movies 1 and 2 in the supplemental material, from which the panels in Fig. 3 originate). We repeated this procedure several times, with independently isolated protoplasts, and obtained the same results each time.

Our next step was to determine the extent of the associative forces between an isolated chloroplast and its associated ER. Here, it was evident that the MCS constituted a small distinct area of attachment. The fluorescent ER attached to a chloroplast could be captured in the optical trap (Fig. 4A). When the chloroplast attached to the ER was moved, the captured ER strand was stretched out (Fig. 4B). The ER strand did not separate from the chloroplast nor did it break. This was demonstrated by releasing the ER strand from the optical trap, which instantly resulted in that the ER relaxed back toward the chloroplast surface (Fig. 4, C and D; see movie 3 in the supplemental material). The experiment was repeated several times with independently isolated chloroplasts. We were always able to deform and stretch the ER to a significant degree. However, we were never able to pull the ER free of the chloroplast or to break it with the 400 pN optical force applied nor could we detect any deformation of chloroplasts during these experiments.

We earlier proposed the concept of chloroplast-associated ER in pea, based on biochemical studies (6, 7). To investigate whether chloroplast-ER associations could also be visually demonstrated in pea, we incubated leaves with the fluorescent probe dihexaoxacarbocyanine (DiOC₆), which labels the endomembrane system (22). Isolated chloroplasts were observed by confocal microscopy and small fluorescent bodies were again observed associated with the chloroplasts. The chloroplast envelope also became labeled with DiOC₆ but to a markedly lower extent. The putative ER-derived membranes on the pea chloroplasts could also be captured with the optical tweezers and stretched but not pulled free from the chloroplast when applying an optical force of ~425 pN (Fig. 4, E and F). However, after treatment with 0.3 mg·ml⁻¹ trypsin, the putative ER-derived membrane could be pulled free from a chloroplast using an optical force of 130 ± 7 pN.

Isolation of an ER Fraction Associated to Intact Chloroplasts—We thus had evidence suggesting chloroplast-associated ER also in pea and, furthermore, that this putative ER could be separated from the chloroplasts. To isolate this fraction a method used to isolate an ER frac-
tion associated with yeast mitochondria (18) was adapted to pea chloroplasts (Fig. 1A). Chloroplasts were isolated and the associated putative ER was released from the chloroplasts by incubation at a lowered pH (18). After sucrose gradient centrifugation, a membrane fraction was collected from the top of the gradient. We denote this fraction plastid associated membranes (PLAMs), analogous to the ER associations reported for mitochondria, MAMs (mitochondria associated membranes) (18, 23), and plasma membrane, PAMs (plasma membrane associated membranes) (24).

Typically 150–300 μg of PLAM protein was obtained from intact chloroplasts corresponding to 3–5 μg of chlorophyll. Membrane composition and marker enzyme activities were measured in this fraction and, for comparison, also in a chloroplast envelope fraction and in a light microscope fraction (Light MS) obtained by treating a bulk leaf membrane fraction identically to the treatment of the intact chloroplasts (Fig. 1A).

Analyses of polypeptide patterns (Fig. 1B), lipid compositions (Table 1) and enzyme activities (results not shown) clearly demonstrate that the pea PLAM fraction was neither of chloroplast envelope origin nor representative of a general light membrane fraction. It did not contain inner mitochondrial membrane nor was the thylakoid membrane present to any significant extent. The PLAM fraction contained the ER-associated activities NADH-dependent cytochrome c reductase and phosphatidylcholine synthase, whereas the chloroplast envelope localized synthesis of the major thylakoid lipid monogalactosyldiacylglycerol (MGDG) was about 15 times higher in an isolated chloroplast envelope fraction than in the PLAM fraction. The lipid composition of the PLAM was compared with those of the Light MS and the published lipid composition of an outer envelope fraction isolated from pea chloroplasts (25) (Table 1). Phospholipids constituted more than 80% of the PLAM and Light MS lipids but less than 60% of the chloroplast outer envelope lipids. The phospholipid compositions of the three fractions differed markedly. The low proportion of glucosylceramides and the barely detectable proportion of free sterols in the PLAM fraction indicate that tonoplast and plasma membrane (20) were largely absent from this fraction. In the PLAM fraction, MGDG and digalactosyldiacylglycerol (DGDG) together constituted 10% of the lipids, with approximately equal contributions by each lipid. The PLAM thus clearly differed from the outer chloroplast envelope, where galactolipids were far more abundant due to a high DGDG content. The polypeptide patterns of the PLAM fraction distinctly differed from those of isolated chloroplast envelope (Env) and the light MS (Light MS) fraction (Fig. 1B).

**DISCUSSION**

Membranes of eukaryotic cells receive their lipid constituents as vesicles and/or by lipid transfer at MCSs. Membranes involved in the vesicular secretory pathway have been shown to receive lipids by both pathways (1), whereas lipid transfer between the ER and mitochondria both in yeast (18) and animal cells (23) has been demonstrated to occur at MCSs between a specific domain of the ER and the mitochondria. In plants, a corresponding lipid trafficking route, between ER and plastids, is assumed to provide the plastids with membrane lipid precursors (4–7). However, the association at MCSs between ER and its various target membranes has not been demonstrated previously, neither with plant, animal, nor yeast cells.

We here provide the first dynamic demonstration of strong attracting forces at MCS. The investigation used confocal microscopy in combination with a laser scalpel and optical tweezers to visualize and optically manipulate ER-chloroplast MCSs. When the laser scalpel disrupted a protoplast, the ER network broke up and ER fragments remained associated with the chloroplasts, thus demonstrating ER-chloroplast MCSs. The chloroplast-ER associations withstood a force exceeding 400 pN, as applied by optical tweezers. Rupture strengths between 160 and 300 pN of specific protein-protein interactions have been reported for the biotin-streptavidin interaction (26). It is thus feasible that protein-protein interaction mediates the association at the ER-chloroplast MCSs. This conclusion is supported by that protease treatment allowed the separation of ER and chloroplasts when a much lower optical force was applied.

The observation that ER fragments associated with chloroplasts remained attached during a chloroplast isolation procedure fits with our earlier findings that lipid metabolizing activities attributed to the ER co-isolated with chloroplasts (6). In addition to visualize the association, we were able to isolate and biochemically characterize the associated ER, a putative PLAM fraction. Both its polypeptide pattern and membrane lipid composition differed substantially from the chloroplast envelope. The PLAM polypeptide composition also clearly differed from that of a light membrane fraction, which should rule out the possibility that the PLAM fraction represented membranes that randomly associated with the chloroplasts during the isolation procedure. The associated enzyme activities as well as the lipid composition of the PLAM fraction showed a resemblance to the ER, with a small degree of envelope membrane contamination. We suggest that the PLAM fraction largely represents the observed chloroplast-associated ER.

There is a quite extensive list of different identified, and suggested, structural and/or functional domains of the plant ER (8). Based on our findings, we propose that PLAMs should be added to the list of plant ER domains. Although the function(s) of this domain remains unidentified, by analogy with the MAMs, it appears reasonable to suggest that plant PLAMs are

**TABLE 1**

| Lipid              | Light MS | PLAM | OEM |
|--------------------|----------|------|-----|
| MGDG               | 7 ± 4    | 6 ± 1 | 6   |
| DGDG               | 1 ± 1    | 4 ± 1 | 33  |
| GlcCer             | 12 ± 4   | 3 ± 1 | NR  |
| PC                 | 19 ± 2   | 34 ± 4| 44  |
| PE                 | 35 ± 1   | 34 ± 5| 2   |
| FA                 | 1 ± 1    | 4 ± 2 | NR  |
| PG                 | 13 ± 3   | 6 ± 1 | 6   |
| PI                 | 13 ± 1   | 8 ± 1 | 5   |
| Sum PL             | 81 ± 8   | 86 ± 13| 57  |

*a* GlcCer, glucosylceramides; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, phospholipids. Free sterols were not detected in the PLAM or light MS fractions.

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involved in the trafficking of chloroplast membrane lipid precursors from the ER to chloroplasts and other plastids.

In addition to mediating transport of acyl lipids from the ER to plastids, PLAMs could also participate in lipid trafficking in the opposite direction. One instance could be during phosphate limitation, when significant proportions of phospholipids are replaced with the galactolipid DGDG (27) in the plasma membrane (20, 28), the mitochondria (29), and the tonoplast (20). This DGDG is most probably synthesized in the outer chloroplast envelope (30), and we suggest the possibility that PLAMs could partake in lipid trafficking from the chloroplast envelope to the ER for further transport to the other membranes. For DGDG transfer to mitochondria, however, a direct transfer between the organelles was suggested (29). The PLAM could also partake in the export of plastid-synthesized fatty acids to the ER. In a mutant of A. thaliana lacking ER-localized phospholipid acyl desaturases, non-plastid membrane acyl lipids nevertheless contained polyunsaturated acyl groups, indicating that the plastid-localized desaturases supplied the rest of the cell with polyunsaturated acyl chains (31). Yet another role for PLAMs would be an involvement in the trafficking of plastid-synthesized acyl chains to supply substrates for the ER-localized synthesis of triacylglycerols. A close connection between plastids and ER may be necessary for efficient channeling of acyl groups and is consistent with labeling kinetics observed during lipid metabolic studies of seed and leaf glycerolipid formation (32, 33). The recent report that α-carboxy anhydride is imported to the chloroplast after glycosylation in compartments of the secretory pathway (34) opens up speculation that PLAMs and PLAM-chloroplast MCSs also partake in protein trafficking. Clearly, the possible involvement of PLAMs and PLAM-chloroplast MCSs in bidirectional lipid trafficking, as well as their possible involvement in protein trafficking deserves further attention.

To summarize, we have presented the first visual evidence for strong attracting forces between two separate membrane compartments of a cell. By using optical manipulation techniques, strong physical associations between ER and chloroplasts were evident and the force of attraction may suggest protein-protein interactions. We also isolated a fraction of chloroplast-associated ER, the PLAM fraction, and an initial biochemical characterization revealed its relationship with the ER. We propose that trafficking of membrane lipids and membrane lipid precursors between the ER and the chloroplast envelope occurs at MCSs between the envelope and a specific subdomain of the ER, the PLAM.

Acknowledgments—The transformed Arabidopsis seeds were kindly provided by Prof. I. Hara-Nishimura (see Ref. 15), and we thank Prof. J. Ohlrogge for valuable input. The still images were obtained using equipment at the Center for Cellular Imaging whereas the optical manipulation experiments were performed at the Centre for Biophysical Imaging. Both centers, at Gothenburg University, are financed by Swegene.

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