Co-Association of Cytochrome *f* Catabolites and Plastid-Lipid-Associated Protein with Chloroplast Lipid Particles[1]

Matthew D. Smith[2], Donny D. Licatalosi[3], and John E. Thompson*

Department of Biology, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1

Distinguishable populations of lipid particles isolated from chloroplasts of yellow wax bean (*Phaseolus vulgaris* L. cv Kinghorn Wax) leaves have been found to contain plastid-lipid-associated protein (J. Pozueta-Romero, F. Rafia, G. Houlné, C. Cheniclet, J.P. Carde, M.-L. Schantz, R. Schantz [1997] Plant Physiol 115: 1185–1194). One population is comprised of plastoglobuli obtained from sonicated chloroplasts by flotation centrifugation. Higher density lipid-protein particles isolated from chloroplast stroma by ultrafiltration constitute a second population. Inasmuch as the stromal lipid-protein particles contain plastid-lipid-associated protein, but are distinguishable from plastoglobuli in terms of their lipid and protein composition, they appear to be plastoglobuli-like particles. Of particular interest is the finding that plastoglobuli and the higher density lipid-protein particles both contain catabolites of the thylakoid protein, cytochrome *f*. These observations support the view that there are distinguishable populations of plastoglobuli-like particles in chloroplasts. They further suggest that the formation of these particles may allow removal of protein catabolites from the thylakoid membrane that are destined for degradation as part of normal thylakoid turnover.

Plastoglobuli are lipid bodies found in all types of plastids. They have been extensively described, yet their exact structure and chemical composition are not known with certainty (Lichtenthaler, 1968; Hansmann and Sitte, 1982; Steinmüller and Tevini, 1985a; Pozueta-Romero et al., 1997). It is not clear, for example, whether plastoglobuli are circumscribed by a polar lipid monolayer analogous to the phospholipid monolayer that surrounds cytosolic oil bodies from seeds and other organs (Murphy, 1993; Huang, 1996). This uncertainty stems from conflicting reports about the presence of galactolipids in plastoglobuli and whether it is possible to visualize a one-half-unit membrane by electron microscopy (Greenwood et al., 1963; Simpson and Lee, 1976; Hansmann and Sitte, 1982; Steinmüller and Tevini, 1985a). A recent study of tapetal cell elaioplasts provides more definitive evidence for a monolayer of galactolipids surrounding the neutral lipid core of plastoglobuli and indicates that these lipid bodies originate from thylakoids (Hernández-Pinzón et al., 1999). This evidence is in agreement with a model for plastid lipid body organization proposed by Knoth et al. (1986).

There are also conflicting reports regarding the presence of proteins in plastoglobuli. Steinmüller and Tevini (1985a) have suggested that protein associated with isolated plastoglobuli is artifactual, whereas others have argued that numerous proteins are native constituents of plastoglobuli (Bailey and Whyborn, 1963; Hansmann and Sitte, 1982; Hernández-Pinzón et al., 1999; Kessler et al., 1999). Some proteins associated with plastoglobuli appear to be members of a family of proteins characterized as lipid-associated proteins. These include plastid-lipid associated-protein (PAP), fibrillin, plastoglobulin 1, carotenoid-associated protein, carotene globule protein, and the 32- and 34-kD chloroplast drought-induced stress proteins (Deruère et al., 1994; Katz et al., 1995; Vishnevetsky et al., 1996; Pozueta-Romero et al., 1997; Eymery and Rey, 1999; Hernández-Pinzón et al., 1999; Kessler et al., 1999). These proteins range in size from 30 to 38 kD and are thought to be involved in maintaining the structural stability of plastid lipid bodies (Ting et al., 1998). Members of this family may also play a role in plant responses to environmental stress (Rey et al., 2000). In light of accumulating evidence for their existence, it seems likely that these proteins are not only genuine components of plastoglobuli, but also serve as markers for plastid lipid bodies.

The functional role of plastoglobuli has not been conclusively established (Lichtenthaler, 1968; Tuquet and Newman, 1980). However, it is assumed, based on a reduction in their size and abundance during thylakoid biogenesis and their accumulation and increase in size during thylakoid degradation, that they store thylakoid components, especially those liberated during dissolution of the thylakoid membrane (Sprey and Lichtenthaler, 1966; Lichtenthaler, 1968; Lichtenthaler and Weinert, 1970). Indeed plastoglobuli isolated from senescing leaves are enriched in thylakoid lipid catabolites (Steinmüller and Tevini,

[1] This work was supported by the Natural Sciences and Engineering Research Council of Canada. M.D.S. received a PGS-B student scholarship from the Natural Sciences and Engineering Research Council of Canada.

[2] Present address: Department of Biological Sciences, Rutgers University, Newark, NJ 07102.

[3] Present address: Department of Biochemistry, University of Colorado Health Sciences Center, Denver, CO 80262.

* Corresponding author; e-mail jet@sciborg.uwaterloo.ca; fax 519–746–2543.

1985b). There is also evidence for an accumulation of triacylglycerols in the leaves of some species following ozone or drought stress that is coincident with an increase in the size and abundance of plastoglobuli (Sakaki et al., 1985, 1990; Pääkkönen et al., 1998). Plastoglobuli may also function as a depot for surplus lipids in general (Greenwood et al., 1963; Thomson and Platt, 1973). More recent evidence suggests that plastoglobuli of senescing chloroplasts are exuded through the chloroplast envelope into the cytoplasm and subsequently degraded (Guiamét et al., 1999).

Differences in lipid composition between plastoglobuli from chloroplasts and chromoplasts, and even among chloroplastic plastoglobuli have been interpreted as reflecting subpopulations of plastoglobuli (Simpson and Lee, 1976). In an earlier study Bailey and Whyborn (1963) characterized two classes of lipid particles in chloroplasts of sugarbeet leaves that were distinguishable on the basis of differences in density. More recently plastoglobuli of differing densities were isolated from chloroplasts of pea leaves using a Suc gradient (Kessler et al., 1999). It has also been proposed that differences in the electron density of plastoglobuli in the chloroplasts of some species, for example, peppers, reflect differences in chemical composition (Simpson and Lee, 1976).

Another class of lipid bodies, termed lipid-protein particles, has been isolated from the stroma of chloroplasts from mature yellow wax bean (*Phaseolus vulgaris* L. cv Kinghorn Wax) leaves (Ghosh et al., 1994; Smith et al., 1997). These particles contain thylakoid proteins and their metabolites as well as other chloroplast proteins, and are also enriched in thylakoid lipid catabolites, in particular free fatty acids (Ghosh et al., 1994; Smith et al., 1997). It has been proposed that these particles are formed from thylakoids and play an integral role in normal thylakoid turnover, allowing removal of thylakoid protein and lipid catabolites that would otherwise destabilize the bilayer (Ghosh et al., 1994; Thompson et al., 1998). Transmission electron microscopy has indicated that these stromal lipid-protein particles bear morphological resemblance to plastoglobuli. Specifically they are globular rather than microvesicular in nature (Ghosh et al., 1994; Thompson et al., 1998).

In the present study plastoglobuli and higher-density stromal lipid-protein particles have been isolated from chloroplasts of yellow wax bean leaves. They both contain the plastoglobuli-specific protein, PAP, indicating that the stromal lipid-protein particles are plastoglobuli-like particles, and they also contain catabolites of the thylakoid protein, cytochrome *f*. The results suggest that plastoglobuli and plastoglobuli-like lipid particles may be involved in thylakoid turnover, allowing removal of protein catabolites from the thylakoid membrane that are destined for degradation.

RESULTS

Polypeptide Composition of Plastoglobuli and Stromal Lipid-Protein Particles

Plastoglobuli were isolated from sonicated chloroplasts of yellow wax bean leaves by flotation centrifugation (Fig. 1A). Transmission electron microscopy confirmed that the purified floated plastoglobuli were about 300 nm in diameter and not contaminated with membranes or fibrils (data not shown). Fractionation of washed plastoglobuli by SDS-PAGE indicated that they contain several proteins including a 32-kD polypeptide that cross-reacts with antibody raised

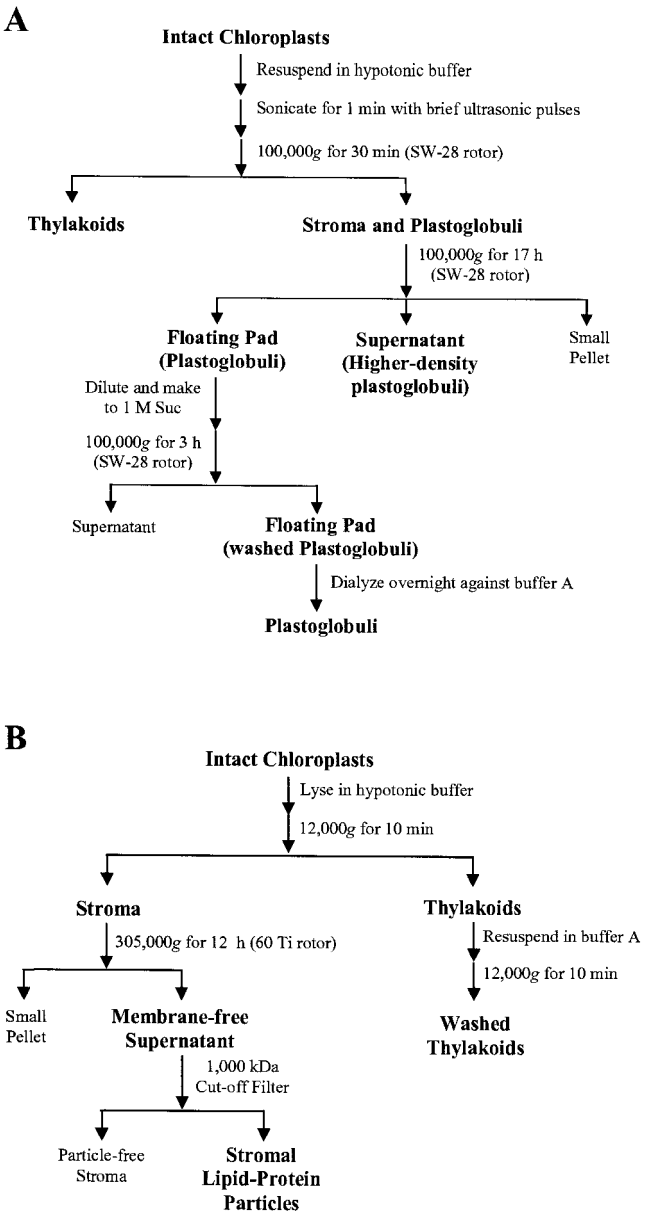

Figure 1. Flow chart illustrating the isolation of: A, floated plastoglobuli and higher-density plastoglobuli from sonicated chloroplasts; and B, lipid-protein particles from the stroma of non-sonicated chloroplasts.

against PAP from peppers (Fig. 2, A and B, lane 1). The polypeptide composition of the supernatant beneath the floated pad of plastoglobuli was also examined by SDS-PAGE. The supernatant was collected as four equal fractions (F1, F2, F3, and F4), and the protein compositions of these fractions proved to be closely similar to each other and to that of floated plastoglobuli (Fig. 2A, lanes 1–5). In addition, western-blot analysis of the supernatant revealed that each fraction contains the 32-kD PAP (Fig. 2B, lanes 2–5). These observations collectively indicate that the supernatant contains higher-density plastoglobuli, possibly a mixture of globular and fibrillar plastoglobules, that did not float during centrifugation (Fig. 1A). Rubisco was not detectable in gels of the supernatant fractions because the holoenzyme sediments during protracted high-speed centrifugation (data not shown).

The polypeptide composition of stromal lipid-protein particles, which were isolated from chloroplasts that had not been sonicated (Fig. 1B), was also examined by SDS-PAGE. The protein composition of these particles was clearly distinguishable from that of both floated plastoglobuli and the higher-density plastoglobuli (Fig. 2A, lanes 1–6) and from that of purified thylakoid membranes (Fig. 2A, lanes 6 and 7). Of particular interest, though, is the finding that these stromal lipid-protein particles contain the 32-kD PAP (Fig. 2B, lane 6), for this indicates that they are plastoglobuli-like particles. It is unlikely that the presence of PAP in this fraction reflects contamination by plastoglobuli inasmuch as its abundance relative to other proteins is comparable for floated plastoglobuli and purified stromal lipid-protein particles (Fig. 2B, lanes 1 and 6).

PAP antibodies also reacted with a 32-kD protein associated with thylakoids (Fig. 2B, lane 7). This protein is the same size as the PAP associated with plastoglobuli and stromal lipid-protein particles suggesting that it is a thylakoid-associated PAP (Fig. 2B, lanes 1–6). However, the 32-kD PAP is clearly more abundant relative to other proteins in plastoglobuli and stromal lipid-protein particles than in thylakoids (Fig. 2B). The PAP antibodies also cross-reacted with a 28-kD polypeptide associated with thylakoids (Fig. 2B, lane 7). It is likely that this 28-kD polypeptide represents another member of the PAP/fibrillin family, for all members of this protein family studied to date have very similar amino acid sequences and are likely to have common antigenic regions (Ting et al., 1998; Hernández-Pinzón et al., 1999; Kessler et al., 1999). Indeed it has been noted previously that two members of this protein family with slightly different molecular weights are both present in chloroplasts of potato (Eymery and Rey, 1999) and both associated with elaioplast lipid bodies (Hernández-Pinzón et al., 1999).

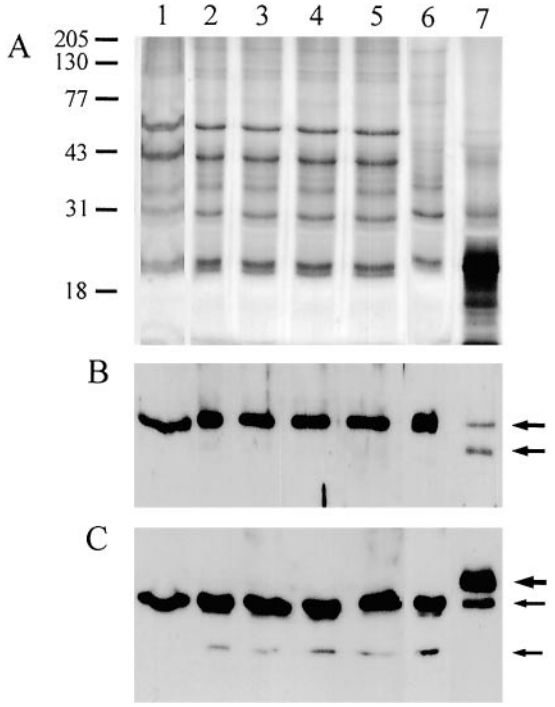

Figure 2. SDS-PAGE and western blots of floated plastoglobuli, higher density plastoglobuli, stromal lipid-protein particles, and thylakoids. A, Silver-stained SDS-PAGE gel. Lane 1, Floated plastoglobuli; lane 2, higher-density plastoglobuli (fraction F1); lane 3, higher-density plastoglobuli (fraction F2); lane 4, higher-density plastoglobuli (fraction F3); lane 5, higher-density plastoglobuli (fraction F4); lane 6, stromal lipid-protein particles; lane 7, thylakoids. Lanes were loaded with equal protein (1.2 μg). Molecular mass markers (kD) are indicated. B, Western blot probed with PAP antibody. Lanes are as in A. The upper arrow indicates the position of a 32-kD protein that cross-reacts with PAP antibody. The lower arrow indicates the position of a 28-kD protein that cross-reacts with PAP antibody. C, Western blot probed with polyclonal antibody raised against SDS-PAGE-purified cytochrome *f*. Lanes are as in A. The thick arrow indicates the position of mature, full-length cytochrome *f*. Two lower M_r catabolites are indicated by thin arrows.

Association of Cytochrome *f* with Plastoglobuli and Stromal Lipid-Protein Particles

One of the characteristic features of stromal lipid-protein particles is that they contain proteolytic catabolites of certain thylakoid proteins (Ghosh et al., 1994). In light of the finding that these particles also contain PAP and, to this degree, resemble plastoglobuli, the possibility that plastoglobuli might contain thylakoid protein catabolites as well was examined. Specifically, western blots were probed for proteolytic fragments of cytochrome *f* with antibody raised against the full-length protein. Native cytochrome *f* in thylakoids was clearly recognized by the antibody (Fig. 2C, lane 7). Stromal lipid-protein particles contain two lower M_r polypeptides that also cross-react with cytochrome *f* antibody and hence can be presumed to be proteolytic catabolites of the native protein (Fig. 2C, lane 6). The largest and most abundant of these is also present in thylakoid membranes, although at a much lower level (Fig. 2C, lane

7), and in some cases is resolvable as two components (Fig. 2C, lane 6). The same catabolites of cytochrome *f* were also detectable in western blots of the higher-density plastoglobuli (Fig. 2C, lanes 2–5), and the larger most abundant catabolite was discernible in floated plastoglobuli as well (Fig. 2C, lane 1). It is unlikely that this reflects contamination by stromal lipid-protein particles inasmuch as the abundance of the cytochrome *f* catabolite relative to other proteins is comparable for stromal lipid-protein particles and floated plastoglobuli (Fig. 2C, lanes 1 and 6).

In other experiments stromal lipid-protein particles were fractionated by gel-filtration chromatography on a Sephacryl column and the proteins of the eluted fractions were separated by SDS-PAGE and probed for cytochrome *f* and PAP by western blotting. The eluted fractions were also analyzed for lipid. The finding that cytochrome *f* catabolites and PAP co-elute from the column with each other and with lipid (Fig. 3) is consistent with the contention that they are all associated with the lipid-protein particles. In some of the eluted fractions only the larger catabolite of cytochrome *f* was detectable (Fig. 3A, lanes 1 and 2), and in others small amounts of full-length cytochrome *f* were discernible (Fig. 3A, lanes 4 and 5).

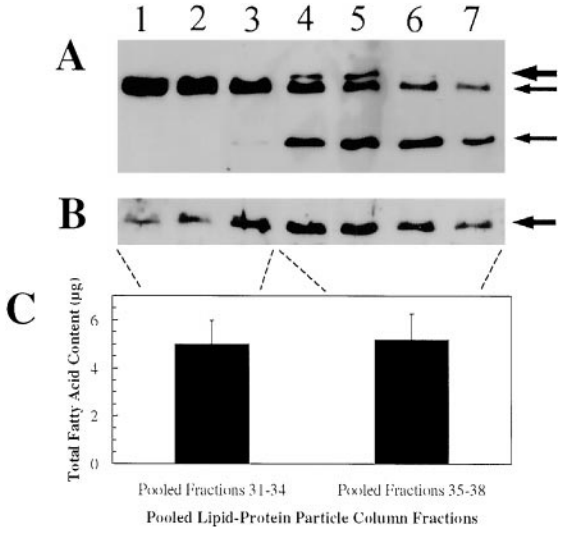

Figure 3. Immunodetection of cytochrome *f* and PAP and quantitation of fatty acid co-associated with stromal lipid-protein particles fractionated on a Sephacryl size-exclusion column. A, Western blot probed with polyclonal antibody raised against SDS-PAGE-purified mature, full-length cytochrome *f*. Lane 1, Fraction 32; lane 2, fraction 33; lane 3, fraction 34; lane 4, fraction 35; lane 5, fraction 36; lane 6, fraction 37; lane 7, fraction 38. Lanes were loaded with equal volume. The thick arrow indicates the position of full-length cytochrome *f*. Lower M_r catabolites are indicated by thin arrows. B, Western blot from A stripped of antibodies used for detection of cytochrome *f* and reprobed with PAP antibodies. The arrow indicates the position of the 32-kD PAP. Lanes are as in A. C, Total fatty acid content of pooled column fractions.

Fatty Acid Composition of Plastoglobuli and Stromal Lipid-Protein Particles

The finding that floated plastoglobuli, higher-density plastoglobuli and stromal lipid-protein particles contain the same fatty acids that are found in thylakoids (Fig. 4) lends further support to the contention that they are derived from thylakoids. Of particular note is the fact that floated plastoglobuli and the higher-density plastoglobuli share with thylakoids the trait of having high levels (>60% of the total fatty acid complement) of linolenic acid. Indeed the fatty acid compositions of these two plastoglobuli fractions are closely similar to each other and to that of thylakoids (Fig. 4). However, in keeping with a previous report (Hansmann and Sitte, 1982), the plastoglobuli fractions contain higher levels of the shorter-chain fatty acid, myristic acid, than are found in thylakoids (Fig. 4). The fatty acid composition of stromal lipid-protein particles is clearly distinguishable from those of both plastoglobuli and thylakoids in that linolenic acid comprises only approximately 28% of the total fatty acid complement (Fig. 4).

Characterization of the Cytochrome *f* Catabolites Associated with Stromal Lipid-Protein Particles

The cytochrome *f* catabolites associated with stromal lipid-protein particles were further characterized by western-blot analysis with antibodies prepared against different regions of mature cytochrome *f*. For this purpose polyclonal antibodies were raised against SDS-PAGE-purified mature full-length cytochrome *f* and against synthetic peptides corresponding to the C terminus and the N terminus of the protein. A diagrammatic representation of the topography of cytochrome *f* in the thylakoid membrane is shown in Figure 5A, and the portions of the protein corresponding to the synthetic peptides that were used to generate polyclonal antibodies are indicated.

The polyclonal antibody raised against SDS-PAGE-purified mature full-length cytochrome *f* cross-reacted with native cytochrome *f* in thylakoids (Fig. 5B, lane 2). This antibody also recognized the larger of the lower M_r cytochrome *f* catabolites present in both thylakoids and stromal lipid-protein particles, as well as the smaller catabolite in the lipid-protein particles (Fig. 5B, lanes 1 and 2). The larger catabolite, indicated by the upper thin arrow in Figure 5B, is approximately 3.5 kD smaller than the full-length thylakoid protein, and the smaller catabolite indicated by the lower thin arrow in Figure 5B is approximately 8.5 kD smaller than the native full-length cytochrome *f*. Both of the terminus-specific antibodies reacted strongly with the mature full-length protein associated with thylakoids (Fig. 5, C and D, lane 2). However, neither of the terminus-specific antibodies reacted with the lower M_r forms of cytochrome *f* associated with thylakoids or lipid-protein particles (Fig. 5, C and D). These findings

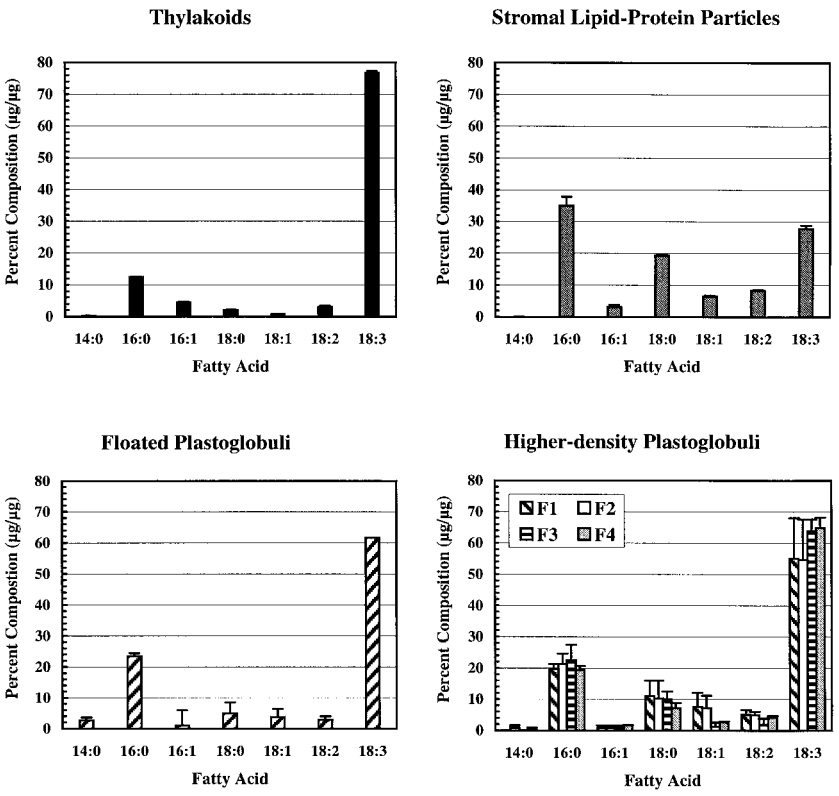

Figure 4. Fatty acid composition of total lipid extracts from thylakoids, stromal lipid-protein particles, floated plastoglobuli, and higher-density plastoglobuli. Values are expressed as means ± SE. 14:0, Myristic acid; 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; and 18:3, linolenic acid. F1, F2, F3, and F4 are higher-density plastoglobuli supernatant fractions collected sequentially from beneath the floated plastoglobuli.

support the contention that the lower M_r forms of cytochrome *f* detectable in thylakoids and lipid-protein particles are catabolites of the mature protein. Specifically, these catabolites lack portions of the N terminus and the C terminus. N-terminal microsequencing confirmed that the larger, more abundant catabolite is derived from cytochrome *f* (Fig. 6). Moreover, alignment of the eight-amino acid microsequence with cytochrome *f* sequence for broad bean indicated that this catabolite is lacking the first 26 amino acids (approximately 2.8 kD) of the mature N terminus (Fig. 6). Given that this catabolite is approximately 3.5 kD smaller than the mature form of the protein associated with thylakoids, it follows that ap-

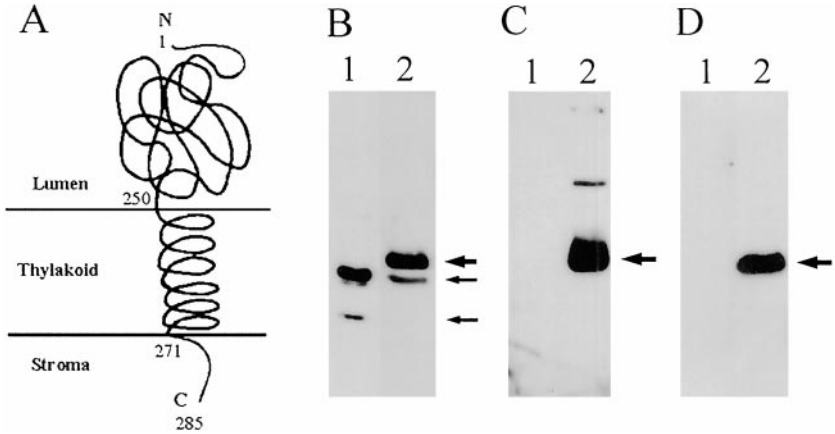

Figure 5. Western blots demonstrating that the lower M_r forms of cytochrome *f* associated with stromal lipid-protein particles and thylakoids are catabolites of the mature protein. A, Schematic representation showing the localization of cytochrome *f* in the thylakoid membrane (adapted from Gray, 1992). Numbers refer to amino acid residues and the portions of the protein against which the terminus-specific antibodies were raised are indicated by lighter shading. B, Western blot probed with antibody raised against SDS-PAGE-purified full-length cytochrome *f*. Lane 1, Stromal lipid-protein particles; lane 2, thylakoids. The thick arrow indicates the position of mature cytochrome *f*. Thin arrows indicate the positions of two lower M_r catabolites. C, Western blot probed with N terminus-specific antibody. Lanes are as in B. The arrow indicates the position of mature cytochrome *f*. D, Western blot probed with C terminus-specific antibody. Lanes are as in B. The arrow indicates the position of mature cytochrome *f*. All lanes were loaded with equal protein (5 μg).

```
Pv    1                          ANKP -DIXV                                                    100
Vf    YPIFAQQGYE NPREATGRIV CANCHLANKP VDIEVPQAIL PDTVFEAVVR IPYDMQVKQV LANGKKGALN VGAVLILPEG FELAPPDRLS PEIKEKIGNL

Pv    101                                                                                   200
Vf    SPQSYRPTKK NIIVIGPVPG KKYSEITFPI LSPDPATKRD VYFLKYPIYV GGTRGRGQIY PDGSKSNNNV YNATATGVVN KKIRKEKGGY EITIVDGSDG

Pv    201                                                                           285
Vf    REVIDIIPPG PELLVSEGES IKLDQPLTSN PNVGGFGQGD AEIVLQDPLR VQGLLLFLAS IILAQIFLVL KKKQFEKVQL SEMNF
```

Figure 6. N-terminal microsequence (shown in boldface) of the larger, more abundant catabolite of cytochrome *f* in stromal lipid-protein particles from yellow wax bean (P*v*; see Fig. 5) aligned with the amino acid sequence of mature cytochrome *f* protein from broad bean (V*f*). A gap (represented by a dash) has been introduced into the microsequence to align it with the sequence of the mature protein. The X in the microsequence represents an ambiguous residue. The numbers correspond to amino acid residues beginning with the mature N terminus. The sequences were aligned using MultAlin version 5.3.3 (Corpet, 1988), a multiple sequence alignment program available on the World Wide Web (www.toulouse.inra.fr).

proximately 0.7 kD (six amino acids) is missing from the C terminus of the protein.

To determine whether the larger, abundant catabolite contains the transmembrane α-helix of cytochrome *f*, a recombinant fragment of the protein was expressed in *Escherichia coli*. This truncated cytochrome *f* lacked the 35 C-terminal residues of the protein, including the stromal domain (15 amino acids) and the transmembrane α-helix (20 amino acids), and thus corresponded to the large hydrophilic globular N terminus (250 amino acids; Fig. 5A). The relative sizes of this truncated cytochrome *f*, the mature protein and the larger, abundant catabolite were then compared by SDS-PAGE. Of particular interest is the finding that the truncated cytochrome *f* is smaller than either the native protein or the catabolite (Fig. 7). Since the catabolite is missing portions of both the N terminus and the C terminus, yet is still larger than the truncated cytochrome *f*, it must also include all or part of the transmembrane α-helix. This contention is consistent with the fact that the cytochrome *f* catabolite is associated with thylakoid membranes as well as stromal lipid-protein particles, for it is presumably anchored in the thylakoid through its transmembrane α-helix. It seems likely that the catabolite is also anchored in the lipid-protein particles through its transmembrane α-helix.

DISCUSSION

Two classes of plastoglobuli, those that float during protracted high-speed centrifugation and those with a higher buoyant density that remain suspended in the supernatant formed during this centrifugation have been isolated. Both were obtained from chloroplasts that had been sonicated, a strategy designed to release plastoglobuli from thylakoids. The two classes of plastoglobuli have the same major proteins and they also both contain the plastoglobuli-specific protein, PAP. As well, the fatty acid compositions of both types of plastoglobuli are closely similar to each other and to that for thylakoids, which is consistent with the contention that they originate from thylakoids. It has been noted previously that plastoglobuli, like thylakoids, are enriched in linolenic acid (Hernández-Pinzón et al., 1999). The different buoyant densities of the two classes of plastoglobuli presumably arise from differences in protein-to-lipid ratios, possibly reflecting formation at different sites along the plane of the thylakoid membrane.

A second population of lipid particles previously termed stromal lipid-protein particles (Ghosh et al., 1994) was isolated from chloroplasts that had not been sonicated. Earlier studies have indicated that these stromal lipid-protein particles contain galactolipids, are enriched in free fatty acids by comparison with thylakoid membranes, and also contain proteolytic catabolites of thylakoid photosynthetic proteins (Ghosh et al., 1994). Indeed it has been proposed that these particles are formed by blebbing from thylakoid membranes in much the same manner that oil bodies are formed from the endoplasmic reticulum, and that their formation allows removal of lipid and protein metabolites from the thylakoid membrane that would otherwise accumulate and destabilize its structure (Ghosh et al., 1994; Thompson et al., 1998). It is apparent from the present study that these stromal lipid-protein particles also contain the plastoglobuli-specific protein, PAP, indicating that they are generically related to plastoglobuli. Like plastoglobuli they appear to originate from thylakoids as judged from the finding that they contain galactolipids, as well as catabolites of thylakoid proteins (Ghosh et al., 1994; Thompson et al., 1998). The stromal lipid-protein particles are also, however, clearly distinguishable from plastoglobuli in that they have a distinct protein composition, as well as distinct lipid and fatty acid compositions and thus they appear to be a unique class of plastoglobuli-like

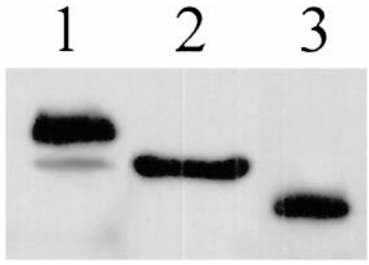

Figure 7. Western blot of a truncated recombinant form of cytochrome *f*, thylakoids, and stromal lipid-protein particles. The blot was probed with antibody raised against SDS-PAGE-purified mature, full-length cytochrome *f*. Lane 1, Full-length cytochrome *f* of thylakoids (5 μg of protein); lane 2, the larger more abundant cytochrome *f* catabolite of stromal lipid-protein particles (5 μg of protein); and lane 3, truncated recombinant cytochrome *f* (10 μg of protein).

particles. Previous studies have demonstrated that plastoglobuli are enriched in triacylglycerol and plastoquinone, and also contain galactolipids and carotenoids (Steinmüller and Tevini, 1985b; Hernández-Pinzón et al., 1999). By contrast, pigments and triacylglycerol are not detectable in stromal lipid-protein particles, but these particles do contain galactolipids and are enriched in free fatty acids (Ghosh et al., 1994; Smith et al., 1997). The finding in the present study that PAP is also present in thylakoid membranes, albeit at a low relative concentration, is consistent with the contention that plastoglobuli and stromal lipid-protein particles, which both contain this protein, are formed from thylakoid membranes. Although it is conceivable that the PAP associated with stromal lipid-protein particles could have originated from pre-existing plastoglobuli, this appears unlikely because PAP has a large hydrophobic domain that would strongly anchor it in plastoglobuli and stromal lipid-protein particles, respectively.

It has been suggested that PAP may be involved in maintaining the structural integrity of chloroplastic lipid particles in much the same way that the oleosin appears to be a structural protein of seed oil bodies (Murphy, 1993; Pozueta-Romero et al., 1997; Ting et al., 1998; Eymery and Rey, 1999; Rey et al., 2000). Oleosin is anchored in oil bodies through a central hydrophobic domain that is embedded in the hydrophobic core of the lipid particle (Murphy, 1993). The hydropathy plot for PAP from peppers reveals hydrophobic domains that could also penetrate into the hydrophobic interior of a lipid particle (data not shown). Similar analyses for other members of the PAP/fibrillin family have also revealed domains that could potentially anchor the protein in a lipid matrix (Vishnevetsky et al., 1996; Ting et al., 1998). Indeed, in an earlier modeling study Knoth et al. (1986) predicted that there is a 30-kD protein component embedded in the neutral lipid matrix of plastid lipid particles.

Thylakoid proteins are thought to be voided from the membrane bilayer in association with lipid during the process of normal thylakoid turnover (Thomas and Hilditch, 1987). The finding that plastoglobuli and stromal lipid-protein particles contain lower M_r catabolites of cytochrome *f* raises the possibility that their formation is part of normal thylakoid turnover. Cytochrome *f* is a major protein of the thylakoid membrane and a central component of the photosynthetic electron transport chain. It is highly conserved among higher plants and is anchored in the thylakoid through a single transmembrane α-helix near the C terminus (Gray, 1992). The 15-amino acid C terminus (residues 271–285) extends into the stroma and the large globular N terminus (residues 1–250) protrudes into the thylakoid lumen (Gray, 1992). N-terminal microsequencing of the larger, more abundant catabolite confirmed that it is derived from cytochrome *f*. This contention is further supported by an earlier report indicating that stromal lipid-protein particles containing the larger, more abundant catabolite of cytochrome *f* can be generated in vitro by illumination of isolated thylakoids. More specifically, decreased levels of native cytochrome *f* in the illuminated thylakoids are commensurate with the appearance of the larger, more abundant catabolite of cytochrome *f* in the in vitro-generated particles (Ghosh et al., 1994). In the present study the lower M_r catabolites of cytochrome *f* were not recognized by antibodies raised against synthetic peptides corresponding to the C terminus and N terminus of the full-length protein. This indicates that the catabolites are formed as a result of proteolytic cleavage at both ends of the protein, events that presumably render the protein non-functional and cause it to be voided from the thylakoid. The contention that the N terminus is cleaved is supported by the microsequencing data indicating that the larger catabolite lacks the first N-terminal 26 amino acids of the native cytochrome *f*. There would appear to be a protease capable of cleaving cytochrome *f* associated with thylakoids (Gray, 1992). Indeed the larger cytochrome *f* catabolite is actually discernible in thylakoids, albeit at a lower concentration than in the lipid particles, supporting the contention that at least this catabolite is formed on the thylakoid membrane and subsequently voided.

Several lines of evidence indicate that the lower M_r catabolites of cytochrome *f* detectable in isolated plastoglobuli and stromal lipid-protein particles are native components of the particles and are not simply free polypeptides. First, the cytochrome *f* catabolites associated with lipid-protein particles co-elute stoichiometrically during size-exclusion column chromatography of the particles, indicating that they are co-associated rather than free polypeptides. Second, the catabolites co-elute with lipid, which indicates that they are eluting as elements of lipid particles rather than as free polypeptides. Third, the cytochrome *f* catabolites were only detectable in a subset of the lipid-protein particles eluted from the size-exclusion column. This indicates that they are not simply free polypeptides adhering to the surface of the particles as contaminants, for if this were the case they would either be randomly associated with eluted particles or present in all of the eluted fractions. Fourth, at least one of the cytochrome *f* catabolites, the larger one, contains the transmembrane α-helix of the native protein, which is presumably embedded in the interior of the lipid particle. Indeed when lipid-protein particles were fractionated by size-exclusion chromatography, some of the separated particles were found to contain small amounts of the full-length cytochrome *f* protein, as well as its catabolites, and they all co-eluted indicating that they were co-associated. The full-length cytochrome *f* is presumably also anchored to the lipid-protein particles through its transmembrane α-helix. Full-length versions of thylakoid photosynthetic proteins have

been detected previously in stromal lipid-protein particles and are thought to be denatured proteins that are no longer functional and, accordingly, have been voided from the membrane bilayer (Ghosh et al., 1994). Finally the cytochrome *f* catabolites also co-elute with PAP during size-exclusion chromatography of lipid-protein particles indicating that they are co-associated with this protein as well. Since PAP and other members of the PAP/fibrillin protein family are known to be associated with plastid lipid particles, this also supports the contention that the cytochrome *f* catabolites are native elements of plastoglobuli and stromal lipid-protein particles.

It is possible, therefore, that the genesis of both plastoglobuli and stromal lipid-protein particles is an inherent feature of thylakoid turnover, allowing removal of cytochrome *f* and perhaps other photosynthetic proteins that have been proteolytically cleaved or otherwise damaged. The ultimate fate of the lipid particles remains unknown. However, presumably they are destined for degradation either within the stroma or following secretion into the cytosol as suggested by Guiamét et al. (1999). It has also been suggested that PAP may serve to target plastoglobuli for further metabolism within the stroma (Pozueta-Romero et al., 1997). The two distinct populations of chloroplast lipid particles appear to be generically related in that they both contain the plastoglobuli-specific protein, PAP. Indeed it seems reasonable to classify stromal lipid-protein particles as plastoglobuli-like particles. However, plastoglobuli and stromal lipid-protein particles are also distinguishable. In particular, they have different polypeptide and fatty acid compositions. Yet both are clearly derived from the thylakoid membrane and these differences may simply reflect different points of origin along the plane of the thylakoid membrane. Distinguishable populations of plastoglobuli have also been isolated recently from chloroplasts of pea on a Suc gradient (Kessler et al., 1999). Given their prospective role in thylakoid turnover, it is conceivable that the genesis of plastoglobuli and plastoglobuli-like particles is also involved in thylakoid repair, and even the dismantling of thylakoids, following episodes of environmental stress or during certain stages of development by facilitating the removal of damaged molecules. It has been reported, for example, that members of the PAP/fibrillin family are up-regulated in response to drought (Chen et al., 1998; Eymery and Rey, 1999) and in embryos of mid-cotyledonary stage oilseed rape when thylakoids are being dismantled (Hernández-Pinzón et al., 1999).

MATERIALS AND METHODS

Isolation of Chloroplasts

Yellow wax beans (*Phaseolus vulgaris* L. cv Kinghorn Wax) were grown in flats of Pro-mix BX (Premier Brands, Brampton, ON, Canada) under greenhouse conditions with a supplementary 16-h photoperiod of fluorescent light. Intact chloroplasts were isolated from the primary leaves of 14-d-old seedlings as previously described (Smith et al., 1997).

Isolation of Plastoglobuli and Stromal Lipid-Protein Particles

Plastoglobuli were isolated as illustrated in Figure 1A using an established protocol (Bailey and Whyborn, 1963). Briefly, intact chloroplasts were suspended in hypotonic lysis buffer containing 10 mM Epps (*N*-[hydroxyethyl]-piperazine-*N*′-[3-propane sulfonic acid])-KOH (pH 7.8), 10 mM $MgCl_2$, and 10 mM $NaHCO_3$, and the plastoglobuli were released from thylakoids by short pulses of sonication for 1 min using a sonifier (model 450, Branson Ultrasonics, Danbury, CT) operating at 20 kHz. The sonicated suspension was centrifuged at 100,000*g* for 30 min in an SW-28 rotor (Beckman, Fullerton, CA) to pellet the thylakoid membranes and the resulting supernatant containing the stroma and plastoglobuli was centrifuged again in an SW-28 rotor at 100,000*g* for 17 h. A floating pad of plastoglobuli formed during this centrifugation. The supernatant, which contained plastoglobuli of higher density that did not float, was collected (from top to bottom) as four sequential fractions of equal volume termed F1, F2, F3, and F4, respectively, and retained for analysis. The floated plastoglobuli were washed by dilution in buffer A (50 mM Epps-KOH, pH 7.8, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 250 mM D-sorbitol, and 1% [v/v] glycerol), and Suc was added to a final concentration of 1 M. The suspension was then centrifuged at 100,000*g* for 3 h in an SW-28 rotor. The resulting floating pad of washed plastoglobuli was collected and dialyzed against buffer A.

Higher density stromal lipid-protein particles were isolated as previously described (Ghosh et al., 1994) from chloroplasts that had not been sonicated to minimize the release of plastoglobuli from thylakoids (Fig. 1B). Briefly, intact chloroplasts were suspended in hypotonic lysis buffer and incubated for 30 min on ice in the dark. Lysis was stopped by the addition of an equal volume of double-strength buffer A. The suspension was centrifuged at 12,000*g* for 10 min to pellet thylakoids and associated plastoglobuli. The supernatant containing the stroma was centrifuged at 305,000*g* for 12 h in a 60-Ti rotor (Beckman) to remove any residual membranes. The stromal lipid-protein particles remained in suspension and were concentrated by passing the supernatant through a 1,000-kD cut-off filter (Fig. 1B). That most of the plastoglobuli remain associated with the pelleted thylakoids in this protocol is evident from the fact that the protracted centrifugation of the stroma to remove any residual membranes did not yield a visible pad of floated plastoglobules.

Antisera

Antibodies raised in rabbit against mature, full-length, SDS-PAGE-purified cytochrome *f* from spinach thylakoids

were kindly provided by Dr. Shimon Gepstein (Technion-Israel Institute of Technology). Antibodies against PAP from peppers were a generous gift from Dr. Rudolphe Schantz (Centre National de la Recherche Scientifique, France), and were raised in rabbit against a PAP-glutathione *S*-transferase fusion protein expressed in *Escherichia coli* (Pozueta-Romero et al., 1997).

Antibodies specific for both termini of cytochrome *f* were raised in rabbits against synthetic peptides conjugated to the carrier protein, Keyhole Limpet Hemocyanin, through a terminal Cys residue using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Drenckhahn et al., 1993; Collawn and Patterson, 1999). The N-terminal-specific antiserum was generated against a 21-amino acid peptide (YPIFAQQGYENPREATGRIVC) corresponding to the highly conserved N terminus of the mature cytochrome *f* protein. The C-terminal-specific antiserum was generated against a 16-amino acid peptide (CKKKQFEKVQLSEMNF) corresponding to the highly conserved 15 C-terminal residues of the mature cytochrome *f* protein plus an additional Cys residue added to the N terminus of the peptide to enable coupling to the carrier protein.

Protein Analysis and Western Blotting

Proteins were fractionated by SDS-PAGE in 12% (w/v) gels (Laemmli, 1970) and either stained with silver (Wray et al., 1981) or transferred to polyvinyldiene difluoride (PVDF) membranes using the semi-dry transfer method (semi-dry transfer cell, Bio-Rad, Hercules, CA). The blots were blocked by treatment for 30 s with 1 μg/mL polyvinyl alcohol (Miranda et al., 1993) and subsequent incubation for 30 min in phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween 20 and 5% (w/v) powdered milk before being probed with primary antibody. Antigens were visualized using secondary antibody coupled to horseradish peroxidase (DAKO, Carpenteria, CA) and a chemiluminescence detection system (Boehringer Mannheim/Roche, Basel). Some blots were stripped and reprobed with another primary antibody. This was achieved by soaking the PVDF membrane in PBS containing 2% (w/v) SDS and 100 mM β-mercaptoethanol for 15 min at 55°C. After rinsing with a large volume of water, the blot was washed for 15 min with PBS, blocked using 5% (w/v) powdered milk in PBS, and reprobed with new primary antibody. For N-terminal microsequencing, the polypeptide was cut out of the PVDF membrane and sequenced by Commonwealth Biotechnologies (Richmond, VA) using automated Edman degradation.

Expression of a Recombinant Fragment of Cytochrome *f* in *E. coli*

A 741-bp fragment of the cytochrome *f* gene (*petA*) from broad bean (Ko and Straus, 1987) corresponding to the globular N-terminal domain of the mature protein was expressed in *E. coli*. The gene fragment was amplified by PCR using an upstream primer (5′-CCC ATT TCC ATG GCA TAT CCT ATT TTT GCC C-3′) containing an *Nco*I restriction site extension, a downstream primer (5′-G GAC ACG AAG CTT ATC TTG AAG CAC TAT TTC-3′) containing a *Hin*dIII restriction site extension, and the full-length *petA* gene from broad bean as a template. The upstream primer allowed incorporation of an ATG start codon into the truncated *petA* gene. This was necessary since the 5′ end of the truncated gene corresponds to the N terminus of the mature protein formed by cleavage of the 35-amino acid thylakoid targeting sequence of apocytochrome *f* (Gray, 1992) and therefore lacks a transcription initiation codon. The amplified PCR product was digested with *Nco*I/*Hin*dIII and cloned into the expression vector, p*Trc* 99A (Amersham-Pharmacia, Buckinghamshire, UK), creating p*Trc* 99A-*petA*1. *E. coli* DH5-α cells transformed with p*Trc* 99A-*petA*1 were grown to $A_{600} = 0.6$, and expression of the truncated *petA* gene was induced by treatment with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 30°C. Cell lysate containing the cytochrome *f* recombinant protein was isolated as described by Sambrook et al. (1989).

Biochemical Analyses and Gel Filtration Chromatography

Protein measurements were performed according to Ghosh et al. (1988). The fatty acid content of total lipid extracts was determined by gas-liquid chromatography after transmethylation (Ghosh et al., 1994). Size-exclusion chromatography was carried out using a column (1.6 × 95 cm) of Sephacryl S-300 HR (Amersham-Pharmacia) as described (Smith et al., 1997).

ACKNOWLEDGMENTS

We gratefully acknowledge the donation of antisera from Drs. R. Schantz and S. Gepstein. Peptide synthesis was conducted in the laboratory of Dr. G. Lajoie, Department of Chemistry, University of Waterloo.

Received January 3, 2000; accepted May 31, 2000.

LITERATURE CITED

Bailey JL, Whyborn AG (1963) The osmiophilic globules of chloroplasts: II. Globules of the spinach-beet chloroplast. Biochim Biophys Acta **78:** 163–174

Chen H-C, Klein A, Xiang M, Backhaus RA, Kuntz M (1998) Drought- and wound-induced expression in leaves of a gene encoding a chromoplast carotenoid-associated protein. Plant J **14:** 317–326

Collawn JF, Patterson Y (1999) Production of antipeptide antibodies. *In* FM Ausubel, R Brent, RE Kingston, DD Moore, JA Smith, JG Seidman, K Struhl, eds, Current Protocols in Molecular Biology on CD. John Wiley & Sons, New York

Corpet F (1988) Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res **16:** 10881–10890

Deruère J, Römer S, d'Harlingue A, Backhaus RA, Kuntz M, Camara B (1994) Fibril assembly and carotenoid overaccumulation in chromoplasts: a model for supramolecular lipoprotein structures. Plant Cell **6:** 119–133

Drenckhahn D, Jöns T, Schmitz F (1993) Production of polyclonal antibodies against proteins and peptides. *In* DJ Asai, ed, Methods in Cell Biology, Vol 37. Academic Press, New York, pp 7–56

Eymery F, Rey P (1999) Immunocytolocalization of CDSP 32 and CDSP 34, two chloroplastic drought-induced stress proteins in *Solanum tuberosum* plants. Plant Physiol Biochem **37:** 305–312

Ghosh S, Gepstein S, Heikkila JJ, Dumbroff EB (1988) Use of a scanning densitometer or an ELISA plate reader for measurement of nanogram amounts of protein in crude extracts from biological tissues. Anal Biochem **169:** 227–233

Ghosh S, Hudak KA, Dumbroff EB, Thompson JE (1994) Release of photosynthetic protein catabolites by blebbing from thylakoids. Plant Physiol **106:** 1547–1553

Gray JC (1992) Cytochrome *f*: structure, function and biosynthesis. Photosynth Res **34:** 359–374

Greenwood AD, Leech RM, Williams JP (1963) The osmiophilic globules of chloroplasts: I. Osmiophilic globules as a normal component of chloroplasts and their isolation and composition in *Vicia faba* L. Biochim Biophys Acta **78:** 148–162

Guiamét JJ, Pichersky E, Noodén LD (1999) Mass exodus from senescing soybean chloroplasts. Plant Cell Physiol **40:** 986–992

Hansmann P, Sitte P (1982) Composition and molecular structure of chromoplast globules of *Viola tricolor*. Plant Cell Rep **1:** 111–114

Hernández-Pinzón I, Ross JHE, Barnes KA, Damant AP, Murphy DJ (1999) Composition and role of tapetal lipid bodies in the biogenesis of the pollen coat of *Brassica napus*. Planta **208:** 588–598

Huang AHC (1996) Oleosins and oil bodies in seeds and other organs. Plant Physiol **110:** 1055–1061

Katz A, Jiménez C, Pick U (1995) Isolation and characterization of a protein associated with carotene globules in the alga *Dunaliella bardawil*. Plant Physiol **108:** 1657–1664

Kessler F, Schnell D, Blobel G (1999) Identification of proteins associated with plastoglobules isolated from pea (*Pisum sativum* L.) chloroplasts. Planta **208:** 107–113

Knoth R, Hansmann P, Sitte P (1986) Chromoplasts of *Palisota barteri*, and the molecular structure of chromoplast tubules. Planta **168:** 167–174

Ko K, Straus NA (1987) Sequence of the apocytochrome *f* gene encoded by the *Vicia faba* chloroplast genome. Nucleic Acids Res **15:** 2391

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Lichtenthaler HK (1968) Plastoglobuli and the fine structure of plastids. Endeavor **26:** 144–149

Lichtenthaler HK, Weinert H (1970) The correlation between lipoquinone accumulation and plastoglobuli formation in the chloroplasts of *Ficus elastica* Roxb. Z Naturforsch **25b:** 619–623

Miranda PV, Brandelli A, Tezon JG (1993) Instantaneous blocking for immunoblots. Anal Biochem **209:** 376–377

Murphy DJ (1993) Structure, function and biogenesis of storage lipid bodies and oleosins in plants. Prog Lipid Res **32:** 247–280

Pääkkönen E, Vahala J, Pohjolai M, Holopainen T, Kärenlampi L (1998) Physiological, stomatal and ultrastructural ozone responses in birch (*Betula pendula* Roth.) are modified by water stress. Plant Cell Environ **21:** 671–684

Pozueta-Romero J, Rafia F, Houlné G, Cheniclet C, Carde J-P, Schantz M-L, Schantz R (1997) A ubiquitous plant housekeeping gene, PAP, encodes a major protein component of bell pepper chromoplasts. Plant Physiol **115:** 1185–1194

Rey P, Gillet B, Romer S, Eymery F, Massimino J, Peltier G, Kuntz M (2000) Over-expression of a pepper plastid lipid-associated protein in tobacco leads to changes in plastid ultrastructure and plant development upon stress. Plant J **21:** 483–494

Sakaki T, Ohnishi J, Kondo N, Yamada M (1985) Polar and neutral lipid changes in spinach leaves with ozone fumigation: triacylglycerol synthesis from polar lipids. Plant Cell Physiol **26:** 253–262

Sakaki T, Saito K, Kawaguchi A, Kondo N, Yamada M (1990) Conversion of monogalactosyldiacylglycerols to triacylglycerols in ozone-fumigated spinach leaves. Plant Physiol **94:** 766–772

Sambrook J, Frisch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 17.37–17.39

Simpson DJ, Lee TH (1976) Plastoglobules of leaf chloroplasts of two cultivars of *Capsicum annuum*. Cytobios **15:** 139–147

Smith MD, Ghosh S, Dumbroff EB, Thompson JE (1997) Characterization of thylakoid-derived lipid-protein particles bearing the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. Plant Physiol **115:** 1073–1082

Sprey B, Lichtenthaler HK (1966) Zur frage der beziehungen zwischen plastoglobuli und thylakoidgense in gerstenkeimlingen. Z Naturforsch **21b:** 697–699

Steinmüller D, Tevini M (1985a) Composition and function of plastoglobuli: I. Isolation and purification from chloroplasts and chromoplasts. Planta **163:** 201–207

Steinmüller D, Tevini M (1985b) Composition and function of plastoglobuli: II. Lipid composition of leaves and plastoglobuli during beech leaf senescence. Planta **163:** 91–96

Thomas H, Hilditch P (1987) Metabolism of thylakoid membrane proteins during foliar senescence. *In* WW Thompson, EA Nothnagel, RC Huffaker, eds, Plant Senescence: Its Biochemistry and Physiology. The American Society of Plant Physiologists, Rockville, MD, pp 114–122

Thompson JE, Froese CD, Madey E, Smith MD, Hong Y (1998) Lipid metabolism during plant senescence. Prog Lipid Res **37:** 119–141

Thomson WW, Platt K (1973) Plastid ultrastructure in the

barrel cactus, *Echinocactus acanthodes*. New Phytol **72:** 791–797

Ting JTL, Wu SSH, Ratnayake C, Huang AHC (1998) Constituents of the tapetosomes and elaioplasts in *Brassica campestris* tapetum and their degradation and retention during microsporogenesis. Plant J **16:** 541–551

Tuquet C, Newman DW (1980) Aging and regreening in soybean cotyledons: 1. Ultrastructural changes in plastids and plastoglobuli. Cytobios **29:** 43–59

Vishnevetsky M, Ovadis M, Itzhaki H, Levy M, Libal-Weksler Y, Adam Z, Vainstein A (1996) Molecular cloning of a carotenoid-associated protein from *Cucumis sativus* corollas: homologous genes involved in carotenoid sequestration in chromoplasts. Plant J **10:** 1111–1118

Wray W, Bovikas T, Wray VP, Hancock R (1981) Silver staining of proteins in polyacrylamide gels. Anal Biochem **118:** 197–203