Plant Cell Physiol. 40(9): 986-992 (1999)
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Mass Exodus from Senescing Soybean Chloroplasts

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During leaf senescence, chloroplast proteins, lipids and pigments undergo massive degradation releasing large amounts of nutrients for reuse elsewhere. Understandably, the chloroplast-degrading machinery has been considered to operate within the chloroplast itself. However, most of those lipases and proteases that increase during senescence and have been localized occur in the vacuole or cytoplasm rather than in the chloroplast. In chloroplasts of senescing (monocarpic) soybean (*Glycine max*) leaves, numerous plastoglobuli (lipid-protein globules) protruded through the chloroplast envelope and emerged into the cytoplasm, where these globules acquired a polygonal coat and eventually disintegrated. The fluorescence characteristics of these cytoplasmic globules indicated that they contained chlorophyll or chlorophyll derivatives. The secreted globules were specific to senescing cells and were absent in old leaves of the "stay green" genotype $GGd_1d_1d_2d_2$ which shows a generalized inhibition of chloroplast degradation. These observations suggest that the globules secreted by the chloroplast carry photosynthetic components to the cytoplasm or vacuole where they are degraded. This blebbing from the chloroplast suggests the occurrence of a novel pathway for the degradation of photosynthetic components in senescing leaves, and it opens new approaches to the study of chloroplast breakdown and its regulation.

Key words: Chloroplast — Fluorescence — *Glycine max* — Plastoglobuli — Secretion — Senescence.

In leaves, senescence is characterized by massive breakdown of chloroplast proteins, lipids and photosynthetic pigments (Gepstein 1988, Noodén 1988). Since plants invest a major portion of their resources, e.g., nitrogen, in their chloroplasts, this breakdown releases large amounts of nutrients for reuse elsewhere. It also marks a very large loss of CO_2 assimilatory capacity. Despite this significance, relatively little is known about the breakdown of chloroplast components in senescing leaves.

Most of the degradative enzymes that are up-regulated in senescing leaves are spatially separated from the chloroplast components broken down. For example, key chlorophyll-degrading enzymes are localized in the chloroplast envelope membranes, not near the internal photosynthetic membranes (thylakoids) with the Chl (Matile et al. 1996). Also, the proteases and lipases whose expression increases during the breakdown of photosynthetic proteins and membranes appear to be targeted mostly to the vacuole and other sites outside the chloroplast (Drake et al. 1996, Buchanan-Wollaston 1997, Buchanan-Wollaston and Ainsworth 1997, Nam 1997, Xu et al. 1996). Likewise, components of the ubiquitin proteolytic pathway are expressed at higher levels in senescing leaves, but this pathway does not operate inside the chloroplast (Belknap and Garbarino 1996, Vierstra 1996). There are proteases in the chloroplast (Vierstra 1996), but the only one that has been characterized in relation to chloroplast breakdown, Clp protease, does not increase during senescence in leaves of the common bean which is closely related to the soybeans used here (Crafts-Brandner et al. 1996).

Except for the possible engulfment of chloroplasts in the vacuole (Gepstein 1988, Noodén 1988), the involvement of the vacuole or cytosol in chloroplast degradation has been overlooked, because there has been no reason to believe that large quantities of chloroplast components could be transported out of senescing chloroplasts. Osmiophilic (i.e., lipid) deposits have been reported in the cytoplasm of senescing leaves of various species (e.g., Mittelheuser and Van Steveninck 1971, Hurkman and Kennedy 1976), but understandably, it has been assumed that these lipids were derived from membranes outside the chloroplast. Here, we describe a new mode for the secretion of lipids, pigments and proteins from senescing chloroplasts of soybean, and we relate this secretion to the breakdown of photosynthetic membranes. This opens up some new perspectives on the breakdown of the chloroplasts.

Materials and Methods

Plant culture and sampling—Soybeans (*Glycine max* [L.] Merr.), cv. Clark, and a near-isogenic line of cv. Clark carrying the $GGd_1d_1d_2d_2$ genotype, were grown in environmental control chambers as described (Guiamét et al. 1991). Samples were taken from leaves on the main stem about 1/3 down from the tip, ca. leaf #7 up from the base. Days after flowering (DAF) and % Chl loss were used to characterize the developmental stage of the leaves. Pallisade mesophyll cells were examined with the confocal and electron microscopes.

Abbreviations: DAF, days after flowering; HEPES, (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]); LHC II, light-harvesting complex II.

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Transmission electron microscopy—Leaf sections (1 × 10 mm) were cut, fixed (2 h) in glutaraldehyde (2% w/v in 50 mM, pH 7.0 sodium phosphate buffer) and post-fixed (2 h) with osmium tetroxide (2% w/v) dissolved in phosphate buffer. The specimens were dehydrated (45 min each) through a series of acetone concentrations (5% v/v, then 10 to 100% in 10% steps) and embedded in Spurr's resin (Roland and Vian 1991). Thin sections were cut and stained with uranyl acetate and lead citrate (Roland and Vian 1991). For each stage, three leaves were sampled and one grid was prepared per leaf.

Confocal microscopy of live cells—In order to visualize the individual cells better, a cell suspension was prepared by gently homogenizing the leaves in buffer (50 mM HEPES, pH 7.5; 1 mM EDTA) with a hand-held Potter-type homogenizer. The homogenate was filtered through one layer of Miracloth and then through a 10-μm pore nylon mesh. Intact cells retained on the nylon mesh were suspended in buffer and collected for observation. Examination of these suspensions under a light microscope revealed that they contained mostly intact cells with negligible amounts of broken cells or isolated chloroplasts. Cell suspensions were observed with a confocal microscope (Bio-Rad MRC-600, Bio-Rad, Hercules, CA) with excitation at 647 nm and detection in the 680–740 nm range, which correspond respectively to the red absorption band and the room temperature fluorescence spectrum of Chl (Rudiger and Schoch 1988).

Isolation and analysis of plastoglobuli and thylakoids—The plastoglobuli have very different properties from the thylakoids, and this is refledcted in the purification procedures. Plastoglobuli were isolated and purified by floatation on a Ficoll cushion essentially as in Steinmuller and Tevini (1985). Leaves were ground in a buffer (50 mM HEPES, pH 7.5, 0.4 M sucrose, 1 mM $MgCl_2$, 1 mM EDTA, 35 mM NaCl, 0.005% v/v β-mercaptoethanol and 1% w/v insoluble polyvinylpolypyrrolidone), the homogenate was filtered through Miracloth and centrifuged at 2,000 × *g* for 5 min at 4°C. The chloroplast pellet was resuspended in buffer (10 mM HEPES, pH 7.5, 0.005% v/v β-mercaptoethanol) to rupture plastids, and the thylakoids were pelleted by centrifugation at 12,000 × *g* for 20 min. Then, the supernatant was centrifuged at 150,000 × *g* for 1 h. The yellowish, slightly turbid band floating on top of the supernatant was collected, poured into a centrifuge tube, mixed with an equal volume of HEPES buffer containing Ficoll (5% w/v) and over layered with 1 vol. Ficoll (2.5% w/v in HEPES buffer) and then with 0.15 vol. HEPES buffer. This was centrifuged at 250,000 × *g* for 1 h, and the yellowish band floating on top of the cushion containing the plastoglobuli was collected for further analysis. Chl was measured as described by Inskeep and Bloom (1985) and total carotenoids as in Thomas (1987). The thylakoid proteins CP47 and LHC II were run on standard SDS-polyacrylamide gel electrophoresis and detected by Western blot using mono-specific antibodies provided by Drs. R. Sayre (Ohio State University) and B.R. Green (University of British Columbia) respectively. The gels were blotted onto nitrocellulose membranes, and the blots were developed with an enhanced chemiluminescence detection kit (Rennaissance TM, DuPont) and quantified with a Bio-Rad GS 363 Molecular Imager System. The blots showed only one band with the antiCP47 antibody (ca. 45 kDa) and three bands (26.5, 27 and 29 kDa) with the LHCII antibodies (data not shown).

Results

Secretion of osmiophilic globules from senescing chloroplasts—In mature, non-senescent leaves at midday, chloroplasts contained a well-organized network of photosynthetic thylakoid membranes (Fig. 1) and only a few small plastoglobuli, i.e., osmiophilic droplets rich in lipids (Tevini and Steinmuller 1985). At this stage, there were very few globules in the cytoplasm, and none of these were associated with the chloroplasts. As leaves senesced and the thylakoid membranes broke down, numerous large plastoglobuli accumulated in the chloroplasts (Fig. 2). These plastoglobuli contain lipids derived from the disassembly of thylakoids (Tevini and Steinmuller 1985) as well as Chl, carotenoids and photosynthetic proteins (Table 1). The amounts of Chl, CP47 (a photosystem II reaction center protein) and the 26.5+27 kDa LHC II polypeptides (LHCB3 and LHCB2 respectively) relative to total protein were lower in plastoglobuli than in thylakoids, while the 29 kDa LHC II polypeptide (LHCB1) relative to total protein was similar in both. Compared to thylakoids, plastoglobuli contained less CP47 relative to LHC II. The Chl *a/b* ratio and the Chl/carotenoid ratio were also lower in plastoglobuli than in thylakoids. Thus, photosynthetic components are present in different relative ratios in plastoglobuli compared with thylakoids, so they do not simply represent contamination of the plastoglobule preparation with thylakoids. Using a similar protocol (i.e., rupture of isolated chloroplasts and removal of thylakoid membranes by repeated ultracentrifugation), Ghosh et al. (1994) isolated lipid-protein globules from young chloroplasts of *Phaseolus*. These lipid-protein globules are derived from thylakoid membranes, but, as with the plastoglobuli we isolated from soybeans, their lipid and protein composition differs strikingly from thylakoids. Moreover, they contain protein catabolites not present in thylakoids, which indicates that photosynthetic molecules present in lipid-protein

Fig. 1 Non-senescent leaf mesophyll cells (26 DAF) containing active chloroplasts with a well-developed network of photosynthetic membranes and no osmiophilic globules in the cytoplasm. These cells were stained with OsO_4. The bar represents 1 μm.

Table 1 Composition of plastoglobuli and thylakoid membranes isolated from mid-senescent leaves (about 50% of chlorophyll degraded) of soybeans cv. Clark

	Chlorophyll	Carotenoids	Chl *a/b* ratio	Chl/ carotenoid	CP47	LHCB1	LHCB3+ LHCB2
	mg (mg protein)$^{-1}$				units[a] (mg protein)$^{-1}$		
Thylakoids	261.9 (5.1)	51.1 (12.9)	2.08	5.13	3.36 (0.33)	6.95 (0.35)	6.58 (0.63)
Plastoglobuli	60.3 (2.7)	34.6 (0.7)	1.05	1.74	0.59 (0.09)	4.92 (0.38)	2.59 (0.08)

The amounts of the LHCB1 and LHCB2+LHCB3 polypeptides of LHC II (the main Chl *a/b* binding-proteins of photosystem II) are given separately. The numbers between parentheses represent the standard error of the mean.
[a] These are relative units of integrated signal on the gel read by the scanner.

globules isolated from chloroplasts are not due to contamination by thylakoid membranes.

In addition to plastoglobuli in the chloroplast, we found similar osmiophilic globules in the cytoplasm of senescing cells near the chloroplasts (Fig. 2). These lipid globules in the cytoplasm appeared to be produced by secretion of the plastoglobuli from the chloroplasts. Electron micrographs of senescing cells showed plastoglobuli pressing against (Fig. 3A) and even protruding through the chloroplast envelope (Fig. 3B). These globules emerge on all sides of the chloroplast, not just the gravitational "topside". Some plastoglobuli were connected by a strand of electron-dense material with the same texture as most of those outside the chloroplast (Fig. 3A, B), and these appear to be globules in the process of emerging from the chloroplast. The protrusions in the chloroplast surface suggest that the globules are squeezing out through the chloroplast envelope. Fibrous material appeared to radiate out from the globules inside the chloroplast and could be associated with this secretion process. Aside from the emerging globules, these chloroplasts showed no other irregularities in their surfaces. Moreover, the cytoplasmic globules have a very different texture from the chloroplast components other than the globules, so they cannot be accounted for by convolutions in or protrusions from the chloroplast surface.

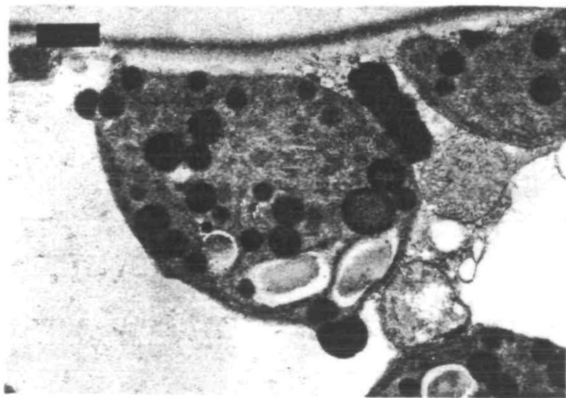

Fig. 2 Chloroplasts in mesophyll cells of senescing leaves (46 DAF, 50% Chl loss) showing "blebs" and numerous osmiophilic globules in the cytoplasm. Prepared the same way as in Fig. 1. The bar represents 1 μm.

Confocal microscopy of the blebbing chloroplasts—To ensure that the cytoplasmic globules were not an artifact of preparation for electron microscopy and to determine if they contained Chl, we observed live, senescing cells through a confocal microscope with the excitation wavelength set to match the red absorption band of Chl and the detection set at the Chl fluorescence emission spectrum respectively at room temperature (Rudiger and Schoch 1988). Under these conditions, cytoplasmic globules and even the emerging blebs in senescing cells fluoresced strongly (Fig. 4), indicating the presence of Chl or some of its colored metabolites (Matile et al. 1996). Significantly, there was no diffuse fluorescence elsewhere in the cytoplasm. In contrast to senescing cells, no fluorescent globules were found in nonsenescing leaf cells (data not shown).

Association of production of the cytoplasmic globules with senescence—The cytoplasmic globules associated with the chloroplast occurred only in senescing leaves (Table 2). In the mesophyll of senescing leaves (at 50% Chl loss), 21% of all chloroplasts in a section showed emerging globules, and 53% of all cells contained at least one chloroplast with associated cytoplasmic globules.

To further check the connection between thylakoid membrane degradation and the secretion of lipid globules from chloroplasts, we examined mesophyll cells in the leaves of the "stay green" mutant $GGd_1d_1d_2d_2$ at a stage when the wild-type cells were senescing. This gene combination in soybean causes generalized preservation of thylakoid membranes and of the major Chl-protein complexes, although it does not delay leaf abscission (Guiamét et al. 1990, Guiamét and Giannibelli 1996). In $GGd_1d_1d_2d_2$, even chloroplasts at a late stage in leaf senescence (only a

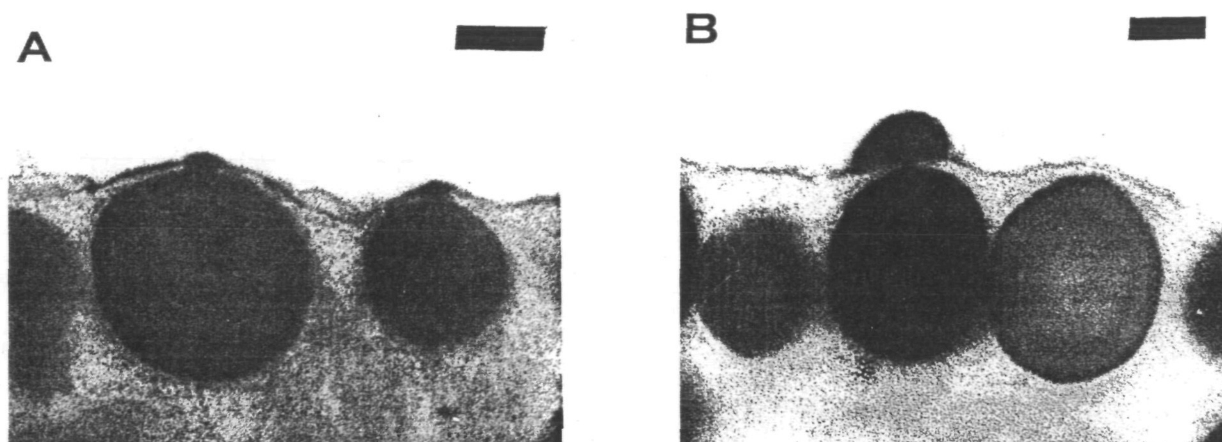

Fig. 3 Plastoglobuli in the process of emerging from a senescing chloroplast (46 DAF, 50% Chl loss). (A) A senescing chloroplast with an osmiophilic globule starting to penetrate the chloroplast envelope. (B) A plastoglobule and associated cytoplasmic globule connected by a strand of lipid material. Prepared the same way as in Fig. 1. Both bars represent 0.2 μm.

few days before abscission) showed an intact thylakoid network, only a few small plastoglobuli and no cytoplasmic globules associated with the chloroplast (Fig. 5, Table 2). Therefore, secretion of thylakoid components from the chloroplast was related to the breakdown of photosynthetic membranes during senescence.

Development of coats around the cytoplasmic globules—The cytoplasmic globules appeared to be transient. They did not accumulate, and most of them had disappeared before abscission of the leaf blade (data not shown). The emerging globules and most of the cytoplasmic globules lacked coats. However, some (ca. 20%) of the senescence-associated cytoplasmic globules were surrounded by a coat with a polygonal pattern characteristic of clathrin (Kreis et al. 1995). The interior of these coated globules was granular and less electron dense than the globules emerging from a chloroplast (Fig. 6). The coated globules were also larger, as if they had swollen. We expect that these coated globules represent a late step in the disintegration of the secreted lipid globules, and they are very transitory.

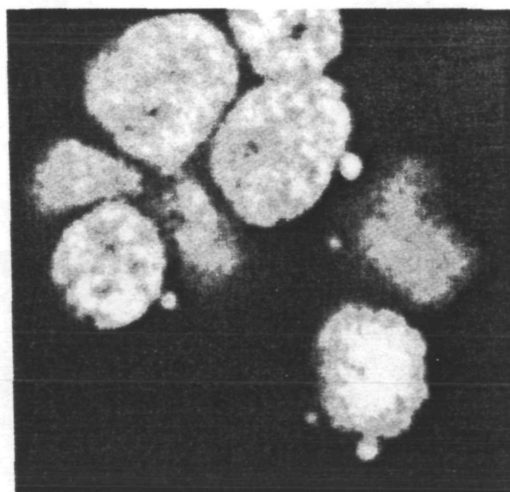

Fig. 4 Chlorophyll fluorescence in chloroplast and cytoplasmic globules of senescing cells (46 DAF, 50% Chl loss) of soybean cv. Clark. Note the brightly fluorescent cytoplasmic globules outside the chloroplast. For these observations, excitation was with a laser beam at 647 nm and detection in the range of 680 to 740 nm.

Discussion

The osmiophilic globules in the cytoplasm of senescing cells are derived from the chloroplasts—Osmiophilic globules occur in the cytoplasm of a variety of nonsenescent cells, and these may serve many different functions, e.g., secretion and lipid storage (Robards 1974, Gunning and Steer 1996). In several species, osmiophilic droplets accumulate in the cytoplasm of senescing cells; however, the origin and function of these deposits has not been established (Mittelheuser and Van Steveninck 1971, Hurkman and Kennedy 1976). Osmiophilic globules possibly derived from the chloroplast also accumulate at night in the cytosol of mature potato leaves (Semenova 1985) and in cells injured by ozone treatment (Mikkelsen and Heide-Jorgensen 1996). In mature leaves of common bean, osmiophilic globules containing thylakoid proteins and their degradation fragments have been shown to "bleb" from the thylakoid membranes and accumulate within the chloroplast (Ghosh et al. 1994), but it is not known if these lipid globules move out of the chloroplast.

The chloroplasts in senescing soybean leaves apparently form cytoplasmic globules by blebbing globules

Table 2 Frequency of chloroplasts with protruding lipid globules and cells with chloroplast-associated cytoplasmic globules in leaves of wild-type soybeans (cv. Clark) and of the "stay green" mutant $GGd_1d_1d_2d_2$ at different stages of senescence

	Percentage of chloroplasts with emerging globules		Percentage of cells with chloroplast-associated cytoplasmic globules	
	Wild type	$GGd_1d_1d_2d_2$	Wild type	$GGd_1d_1d_2d_2$
26 DAF (maximum Chl)	0 aA	n.d.	0 aA	n.d.
46 DAF (50% Chl)	21 bA	n.d.	53 bA	n.d.
52 DAF (25% Chl left)	28 bA	0 B	69 bA	0 B

DAF: days after flowering. n. d.: not determined. Means followed by the same letter are not significantly different at 5% level. Lower case letters indicate differences significant within a genotype across senescence stages; upper case letters indicate significant differences between genotypes at a given stage.

through their envelope, and these seem to be specifically associated with senescence. Moreover, confocal microscopy indicates that the blebs in the chloroplast surface and the senescence-related cytoplasmic globules are distinguished by their high concentrations of Chl (or a close metabolite). Some other species produce globules like soybean does, but in many species, the osmiophilic secretions are not globular and they spread out over the surface of the chloroplast (J.J. Guiamét unpublished data). The latter pattern is relatively inconspicuous and easily overlooked. This may be one of several reasons why this phenomenon has not been reported before. Judging from the protrusions in the chloroplast envelope, the plastoglobuli are actually pressing against and squeezing through the chloroplast outer membrane of the chloroplast. Thus, the globule emergence may represent a secretory process.

The osmiophilic globules in the cytoplasm of senescing cells contain chloroplast components—Several lines of evidence indicate that the cytoplasmic globules contain secreted chloroplast materials. Given that the plastoglobuli are known to contain chloroplast lipids and proteins (Tevini and Steinmuller 1985) and these plastoglobuli move out into the cytoplasm, the cytoplasmic globules can be expected to contain these chloroplast components. The osmiophilic nature of both the plastoglobules and the cytoplasmic globules certainly indicates lipid movement. Confocal microscopy shows the presence of Chl or close metabolites in the cytoplasmic globules. Preliminary studies on CP47 immunostaining suggest that the thylakoid protein CP47 may be associated with the blebs forming at the chloroplast surface, and it may occur in the cytoplasm

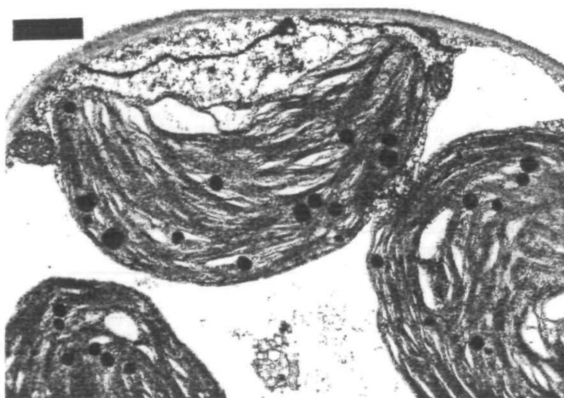

Fig. 5 Chloroplasts of the stay-green mutant genotype $GGd_1d_1d_2d_2$ at 52 DAF showing an intact, organized thylakoid network and a lack of chloroplast-associated cytoplasmic globules. Wild-type chloroplasts of the same age were at an advanced stage of deterioration, 75% Chl loss). Prepared the same way as in Fig. 1. The bar represents 1 μm.

Fig. 6 A cytoplasmic globule with a disintegrating interior and a decorated surface in a leaf at 46 DAF and 50% Chl loss. The inset is an enlargement showing a polygonal pattern typical for the surface of these coated globules. Prepared the same way as in Fig. 1. The bar represents 0.2 μm.

(J.J. Guiamét unpublished data). Although the antiCP47 antibody also shows some signal over the cell walls and starch grains, the pre-immune serum also shows this binding but not that over the thylakoids, blebs or the cytoplasm, indicating that the latter signals with the antiCP47 are specific. Because CP47 is encoded in the plastid genome and synthesized by chloroplast ribosomes (Vermas and Ikeuchi 1991), its presence outside the chloroplast can be explained only by secretion from senescing plastids.

Some chloroplast components are broken down outside the chloroplast—Since the secreted globules contain chloroplast components and they do not accumulate in the cytoplasm, they must break down outside the chloroplast. The secretion of components of deteriorating thylakoids from the chloroplasts explains how cytosolic and vacuolar degradative enzymes expressed in senescing cells might be involved in the degradation of chloroplast components. Thus, the osmiophilic materials secreted in senescing leaves may represent, at least in part, chloroplast lipids including fatty acids in transit to their final site of degradation, i.e., peroxisomes (Gerhardt 1993). Since the plastoglobuli and presumably also the blebs contain thylakoid proteins, these proteins could be degraded by cytosolic or vacuolar proteases (Drake et al. 1996, Vierstra 1996, Buchanan-Wollaston 1997, Buchanan-Wollaston and Ainsworth 1997, Nam 1997).

The finding that Chl and/or some similar fluorescing catabolites occur in the emerging blebs and in the cytoplasmic globules suggests that at least some Chl breakdown takes place outside of the chloroplast. Thus, plastoglobuli may also function to carry Chl to the chloroplast envelope, where the Chl could be united with the enzymes that perform the first steps in Chl-degradation (Matile et al. 1996) and then moved out of the chloroplast. Indeed, the final colorless catabolites of Chl accumulate in the vacuole of senescing cells (Matile et al. 1996). Compartmentation of Chl (which is photodynamic) into globules and its degradation outside the chloroplast might help to limit photodamage.

The role of the coats around the globules?—Whether or not the coats around the cytoplasmic globules are clathrin remains to be determined, but the coat does have the characteristic polygonal pattern (Kreis et al. 1995). While clathrin and other coats may cause budding in membranes and form coats around the vesicles produced (Schmid 1993, Low and Chandra 1994, Staehelin and Moore 1995), here, there is no sign of any coating at the sites of emergence from the chloroplast or on the newly emerged globules, so the coating is not involved in the initial steps of this blebbing process.

Summary: proposal for a novel secretory pathway—Our observations, particularly the sequence of electron micrographs, indicate a massive export of photosynthetic components from deteriorating thylakoids to the cytoplasm and/or vacuole. This may constitute an important pathway involved in the breakdown of senescing chloroplasts and reclaiming these nutrients. Our observations reconcile the cytosolic or vacuolar location of many of the degradative enzymes up-regulated in senescing leaves with the massive loss of proteins and lipids in senescing chloroplasts. They also open new approaches to the important problem of chloroplast breakdown during senescence.

J.J.G. was supported in part by a Rockefeller Foundation Biotechnology Career Fellowship. J.J.G. is a researcher at CIC, Pcia de Buenos Aires, Argentina. Thanks are due to Drs. B.R. Green and R. Sayre for providing the antibodies used in this work.

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(Received February 15, 1999; Accepted July 13, 1999)