Planta (1999) 208: 472–479

Planta
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Ultrastructure of chloroplast protuberances in rice leaves preserved by high-pressure freezing

Timothy M. Bourett[1], Kirk J. Czymmek[2], Richard J. Howard[1]

[1]DuPont Agricultural Products, P.O. Box 80402, Wilmington, DE 19880-0402, USA
[2]University of Delaware, Department of Biological Sciences, Newark, DE 19713, USA

Received: 21 December 1998 / Accepted: 29 January 1999

Abstract. High-pressure freezing and freeze substitution were used to prepare leaves of rice (*Oryza sativa* L.) for ultrastructural analysis. Under these preparative conditions, plastid-derived stroma-containing protuberances were preserved and described with the electron microscope for the first time. Similar protuberances were observed previously only in living cells examined with the light microscope. Infoldings of the chloroplast inner envelope were a prominent ultrastructural feature of protuberances. Infoldings were also observed in the main body of the chloroplasts and sometimes appeared contiguous with thylakoid membranes. Protuberances also contained infoldings in the form of bifurcated tubules. Apparent interconnection between protuberances of adjacent plastids was observed only in one instance. A distinct gradient in the staining density of thylakoid lumina appeared to be a function of grana position and orientation relative to the cell wall. Immunocytochemistry was used to determine that the stroma within protuberances contained 1,5-bisphosphate carboxylase/oxygenase enzyme.

Key words: Chloroplast development – Chloroplast protuberances – Freeze substitution – *Oryza* (chloroplast structure) – Stromule

Introduction

In living cells of vascular plants, individual plastids have been shown by light microscopy to possess a peripheral jacket, lacking thylakoids, from which protuberances arise (Heitz 1936; Spencer and Unt 1965 and references therein; Vesk et al. 1965). Hanson and colleagues coined the term *stromule* to describe these "stroma-filled" structures (World Wide Web page www.bio.cornell.edu/genetics/hanson/hanson.html). Visualizing chloroplasts by targeting green fluorescent protein to the stroma, they showed that these structures were highly dynamic, lacked thylakoids and intermittently appeared to interconnect plastids (Kohler et al. 1997a). In addition, Hanson's group suggested that these connections might function as a means of chemical communication between plastids, since an interplastid exchange of green fluorescent protein was readily observed in living cells.

In fixed specimens, the lack of data to support the existence of protuberances is remarkable, given the vast number of studies in which they could have been discovered. In discussing their findings of protuberances in living cells, Kohler et al. (1997a) cited an example of protuberance preservation in specimens prepared with potassium permanganate (Weier and Thomson 1962). However, it is highly unlikely that labile structures such as protuberances could be preserved by any such traditional chemical fixation procedure. Significant alteration of cellular structures is now considered implicit with any such protocol that requires seconds or minutes to immobilize intracellular components (Coetzee and Van der Merwe 1985; Mersey and McCully 1978). Undoubtedly, the Weier and Thomson (1962) structures that were purported as protuberances (Kohler et al. 1997a), represent artifact.

Among current strategies, methods which employ rapid freezing are generally accepted as preserving cells closer to their native state than do chemical fixation methods (see Ding et al. 1991a; Robards 1991; Samuels et al. 1995; Thijssen et al. 1997). With monolayers of cells less than 10 μm in thickness, plunge freezing in a liquid cryogen can yield well-frozen specimens. For samples larger than this, such as tissues, high-pressure freezing is the only means available to freeze samples routinely without the formation of structure-disrupting ice crystals (Kaeser et al. 1989; Kiss and Staehelin 1995).

Here we have used high-pressure freezing to identify and describe the ultrastructure of protuberances for the first time. Our results from mesophyll chloroplasts of rice

Correspondence to: R.J. Howard
E-mail: Richard.J.Howard@usa.dupont.com
Fax: 1(302) 695 4296

leaves suggest that much could be learned about plastid membrane dynamics from high-pressure-frozen samples.

Materials and methods

Plant material and growth conditions. Seedlings were generated as previously described (Valent et al. 1991) by sowing seeds of rice cultivar Yashiro-mochi (gift of Dr. Barbara Valent, DuPont Company), and maintaining under growth-chamber conditions for an initial 2 weeks. Plants were grown for another 48 h at room temperature and under light conditions that were not well controlled while the plants were transported to another laboratory for subsequent processing (see below). Samples were taken from the second true leaf.

Cryofixation. Rice leaves were frozen in either of two high-pressure-freezing instruments: a Leica EM HPF, with the assistance of Dr. Daniel Studer (University of Bern), and a Balzers HPM 010. The Balzers samples were pre-treated with either 1-hexadecene or dextrans (10,000 MW), with no apparent differential effect on protuberance preservation. All the data presented here were obtained from material prepared with the Leica instrument as follows. Immediately prior to high-pressure freezing, 1.7-mm-diameter leaf discs were taken from intact rice leaves using a stainless-steel punch (Grieshaber, Schaffhausen, Switzerland), and submerged in 1-hexadecene. Leaf discs were exposed to 1-hexadecene for less than 1 min prior to freezing.

For freezing, leaf discs were placed within the 0.2-mm cavity of an aluminum hat (3.0 mm diameter), a second hat was placed flat side down over the first, and this sandwich was loaded into the specimen holder of the HPF (Studer et al. 1995). Samples were frozen under high-pressure (2045 bar), and immediately transferred into liquid nitrogen where the sandwich was removed from the specimen holder.

Each sandwich was screened carefully to eliminate those samples where the quality of freezing was questionable. If either hat was deformed, or the leaf disc was no longer centered within the sandwich, the sample was discarded. If the sample appeared to be fully encapsulated in frozen 1-hexadecene, the two halves of the sandwich were separated since 1-hexadecene (m.p. 4.1 °C) remains solid at the substitution temperature and may impede substitution. Conformance to these guidelines was probably responsible in part for the consistently good quality freezing (see *Results*).

Samples were stored temporarily in liquid nitrogen until processing further. We used an automated freeze-substitution apparatus (model EM AFS; Leica, Deerfield, Ill., USA), where samples in 2% (w/v) osmium tetroxide in acetone (dried over calcium chloride) were held at −90 °C for 58 h, ramped from −90 °C to –60 °C over 6 h, held at −60 °C for 12 h, ramped from −60 °C to −30 °C over 6 h, held at −30 °C for 6 h, and finally transferred to and held at 4 °C for 30 min. Epon 812 resin (Studer et al. 1995) was used for embedment.

Some rice leaves without any pretreatment were cryofixed alternatively by plunging ca. 0.5-mm^2 leaf pieces into a liquid mixture of 90% (v/v) propane and 10% (v/v) ethane at –193 °C. These were then freeze-substituted in 2% osmium tetroxide in acetone and embedded in Quetol resin (Howard and O'Donnell 1987).

Electron microscopy and immunocytochemistry. Ultrathin sections were stained with either 2% (w/v) aqueous or saturated uranyl acetate in methanol, followed by lead citrate. Over 300 micrographs were recorded from a total of six sectioned leaves.

A rabbit polyclonal antiserum (diluted 1:500; gift from Dr. Steven Gutteridge, DuPont Company) was used to localize the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) on thin sections as described by Czymmek et al. (1996). Antibodies against the large subunit of Rubisco were raised in rabbits using the enzyme isolated from spinach. The large subunits were separated from the small subunits and purified as described by Newman and Gutteridge (1990). Specificity of the antiserum was characterized by immunoblot analysis of cell lysates from spinach and tobacco (Kanevski, et al. 1999). In the absence of an available pre-immune serum, the fidelity of localizations was tested by omission of the primary antibody as a control. Thin sections of osmium-treated, Epon 812-embedded samples proved adequate for immunological labeling, presumably because of the abundance of the Rubisco enzyme. For initial trials, sections were etched for 10 min in 10% (v/v) hydrogen peroxide. Subsequently, this step was eliminated as it was unnecessary for penetration of immunochemicals and shown to cause a decrease in the staining density of the stroma.

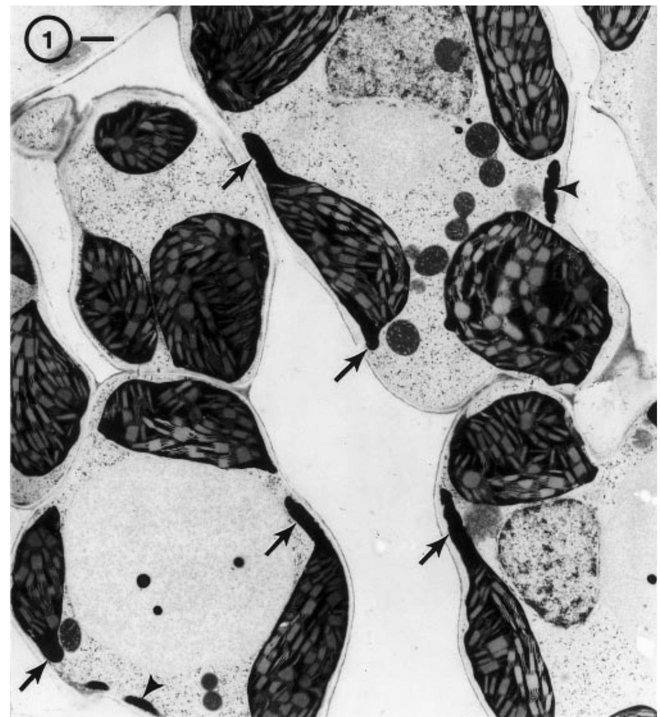


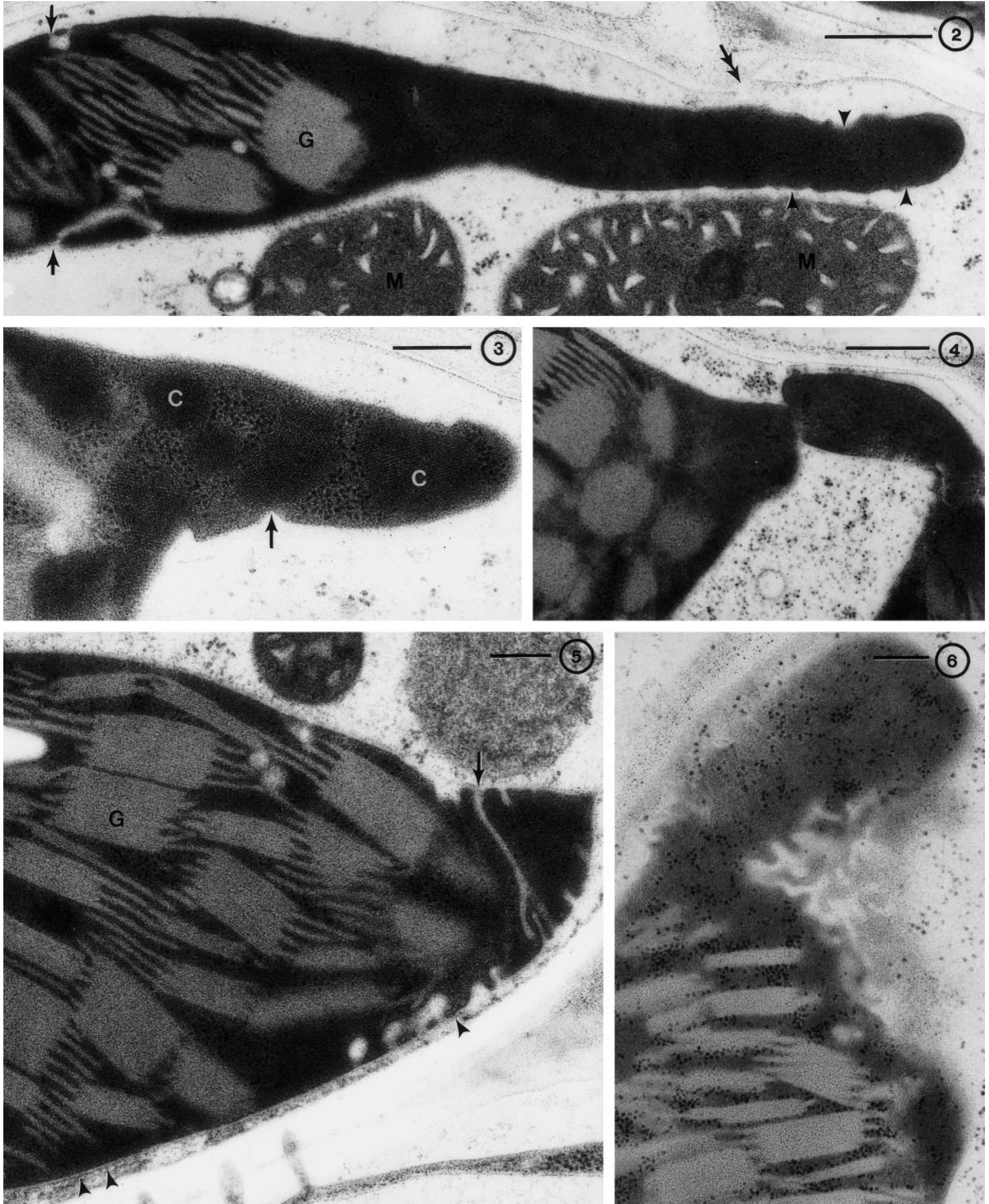
Fig. 1. Typical cross-sectioned rice leaf after high-pressure freezing illustrates the abundance of chloroplast-associated protuberances (*arrows*) in mesophyll cells. Protuberances do not necessarily appear in the same section plane with the main body of the chloroplast (*arrowheads*). ×4400; bar = 1.0 μm

Results

Excellent freezing, as indicated by lack of ice crystal damage (Figs. 1–13) in all rice leaves processed, resulted from use of the high-pressure freezer. Plunge-frozen samples were generally preserved poorly, but in many cases well enough to resolve gross chloroplast morphology. Examination of leaf cross-section ultrastructure revealed that many mesophyll chloroplasts contained thylakoid-lacking protuberances emanating from the main body of the plastid (Fig. 1). When measured from median longitudinal sections, protuberances had a mean diameter of 475 nm (SD ± 74, n = 20) and a maximum observed length of 4.1 μm. Protuberances were observed also in plunge-frozen samples.

Protuberances appeared to consist mainly of stroma, continuous with that of the main body of the chloroplast (Figs. 2–4). Vesicles were not observed within protuberances, including the apex (Fig. 2). The stroma within

both protuberances and the main body of the plastid contained regions of ribosome exclusion (Fig. 3). When sectioned in the proper orientation, these ribosome-less regions were sometimes shown to contain crystalline bodies (Fig. 3). One leaf sample had appreciably higher numbers of these crystalline bodies than other leaves. In the hundreds of chloroplasts examined in thin sections, evidence suggesting that two independent plastids were



connected via protuberances was noted in only one instance (Fig. 4).

A prominent feature of protuberances was numerous surface infoldings (Figs. 2, 3, 5–9). Infoldings were also associated with the surface of the main body of the plastid (Figs. 2, 5), but were observed more frequently in protuberances (Figs. 2, 3, 5–9). Lumen staining of these infoldings was generally less dense than that of thylakoids (Figs. 5–9). In sections tangential to the plastid surface, some infoldings were more tubular and bifurcated (Fig. 6). Despite the poor staining of membranes in this freeze-substitution preparation, the chloroplast outer envelope was occasionally discernable. In these instances, the infoldings appeared to be contiguous with the plastid inner envelope (Fig. 5), and sometimes with the thylakoid system (Figs. 7–9).

Another consistent feature of chloroplasts was a distinct gradient in the staining density of thylakoid lumina as a function of grana position and orientation relative to the cell wall (Figs. 1, 10). In grana nearer to the cell wall, where thylakoid stacks were oriented more or less perpendicular to the plastid envelope, thylakoid lumina were stained more densely than in grana closer to the central vacuole of the mesophyll cell. The plane of the flattened thylakoids in grana closer to the central vacuole was parallel to the surface of the chloroplast (Fig. 10). Gradients of luminal staining were most apparent in median longitudinal sections through chloroplasts.

When thin sections were probed with an antibody against the large subunit of the Rubisco enzyme (Figs. 11–13), binding to the stroma within both the main body of the chloroplast (Fig. 11) and the protuberance (Fig. 13) was observed. The crystalline entities described above were also recognized by the antibody (Fig. 12). No labeling was found when the primary antibody was omitted.

Specimens prepared by high-pressure freezing and freeze substitution were not entirely ideal. Artifacts included breaks in the plasma membrane and cell wall, and low-level membrane contrast. There was also apparent plasmolysis in some instances (Figs. 2, 10).

Discussion

Many reports can be found in the literature concerning the positioning, transport or streaming of various organelles within cytoplasm (e.g. see reviews by Schliwa 1984; Williamson 1993). However, little attention has been given to the ostensibly autonomous movements of individual organelles that one might describe as pleomorphism. Studies of such intrinsic motility of organelles observed in living cells by phase-contrast light microscopy have been reported for mitochondria and chloroplasts (see Bereiter-Hahn and Vöth 1994; Spencer and Unt 1965; and references therein). The peripheral jacket of chloroplasts has been recorded on film giving frequent rise to highly dynamic protuberances that extend into the cytoplasm ("Organelles in Living Cells," 1962, Film no. 37431, Univ. CA Extension Center for Media and Independent Learning, Berkeley, CA 94704; see also video references in Kohler et al. 1997b). This and other studies, cited above, support the belief that such protuberances are a normal and important feature in living cells.

This report is the first electron-microscope characterization of protuberances in plant plastids, a success we attribute directly to the application of high-pressure freezing. In addition to preservation of the protuberances themselves, high-pressure freezing permitted identification of novel infoldings associated with the protuberance inner envelope. High-pressure freezing has been shown previously to preserve a variety of highly dynamic and presumably labile structures in plant tissues. Examples include actin microfilaments associated with preprophase bands in root cells (Ding et al. 1991b), tubular-vesicular complexes associated with fungal pathogenesis (Welter et al. 1988) and plant cell plate formation (Samuels et al. 1995), multivesicular bodies and coated vesicles in reproductive tissue (Thijssen et al. 1997), exocytotic events during secretion (Samuels et al. 1995) and unique endoplasmic reticulum affiliations with other endomembrane components (Craig and Staehelin 1988).

Various examples of artifacts have been attributed, here and elsewhere (Craig and Staehelin 1988; Ding et al. 1992; Hyde et al. 1991; Kiss and Staehelin 1995; Semmler et al. 1998), to high-pressure freezing but it is unlikely that protuberances were *created* by this technique since they can be observed in living tissue. With regard to the infoldings in protuberances reported here, again artifact seems unlikely. Recently, high-pressure freezing was reported to have caused structural modifications, in the form of infoldings, to artificial liposomes (Semmler et al. 1998), but we doubt that cellular membranes would be differentially affected, and infoldings were not observed in association with any other organelle. Protuberances were not a result of the 1-hexadecene pre-treatment because they were found also in plastids of rice leaves preserved by plunge-freezing (i.e. without any pre-treat-

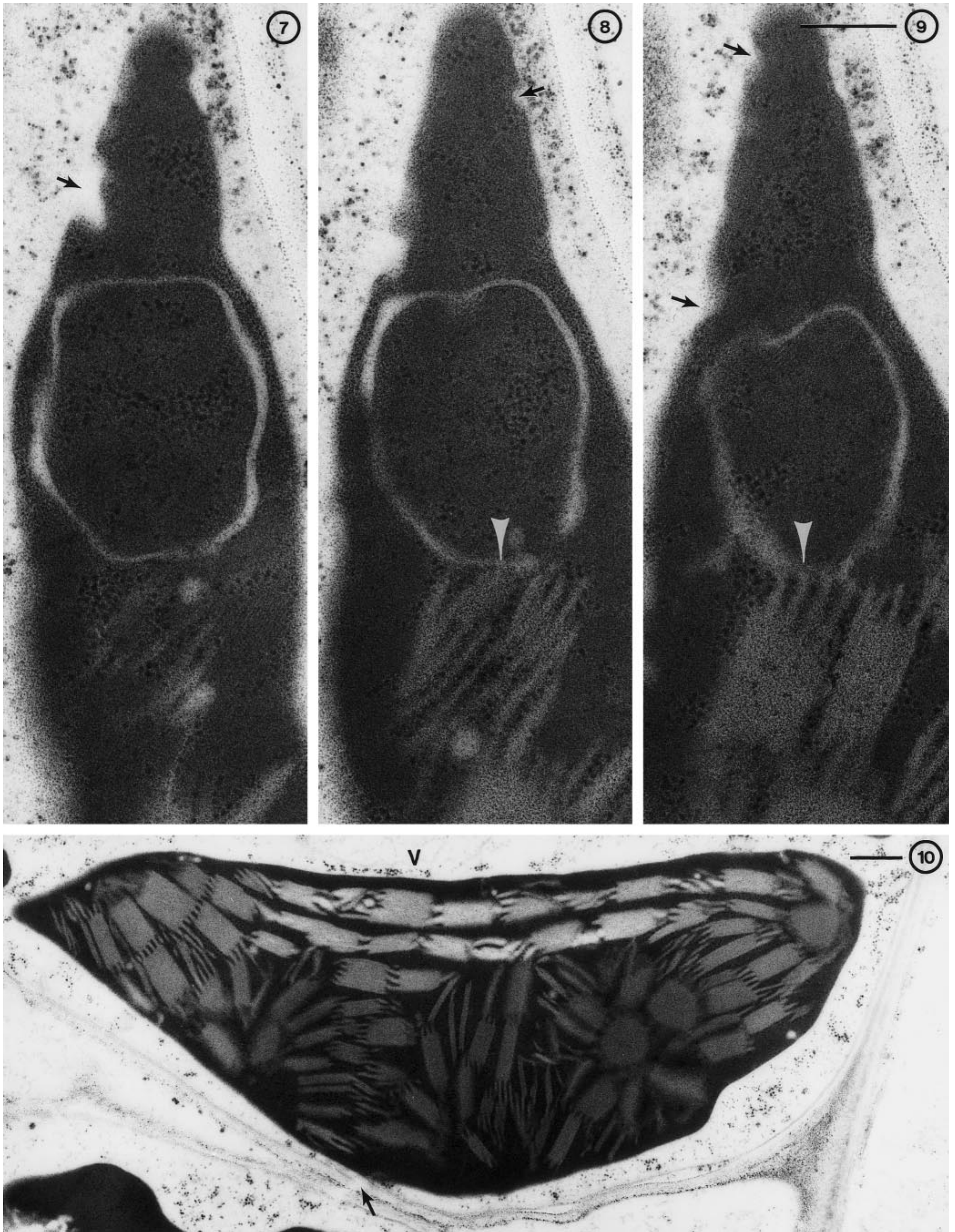
Fig. 2. A median longitudinal section through an elongated protuberance. Note the irregular profile of the protuberance surface (*arrowheads*), the lack of thylakoids within the protuberance, and surface infoldings associated with the main body of the plastid (*single arrows*). Occasional breaks in the plasma membrane (*stacked arrow*) represent an artifact of high-pressure freezing. *G*, granum; *M*, mitochondrion. ×38500; bar = 500 nm

Fig. 3. This protuberance contains many crystalline bodies (*C*). The irregular outline of the protuberance appears, in part, due to the envelope conforming to a crystalline body (*arrow*). ×54300; bar = 250 nm

Fig. 4. The proximity of these two protuberances appears to represent a physical connection. ×29500; bar = 500 nm

Fig. 5. Many infoldings appear at the base of this protuberance. One appears branched and traverses the entire protuberance (*arrow*). The chloroplast outer envelope is visible here, albeit poorly contrasted (*arrowheads*). The lumen of the infoldings is consistently less densely stained than that of thylakoids. *G*, granum. ×44500; bar = 250 nm

Fig. 6. A tangential section through the base of a protuberance showing branched tubular infoldings. ×41600; bar = 250 nm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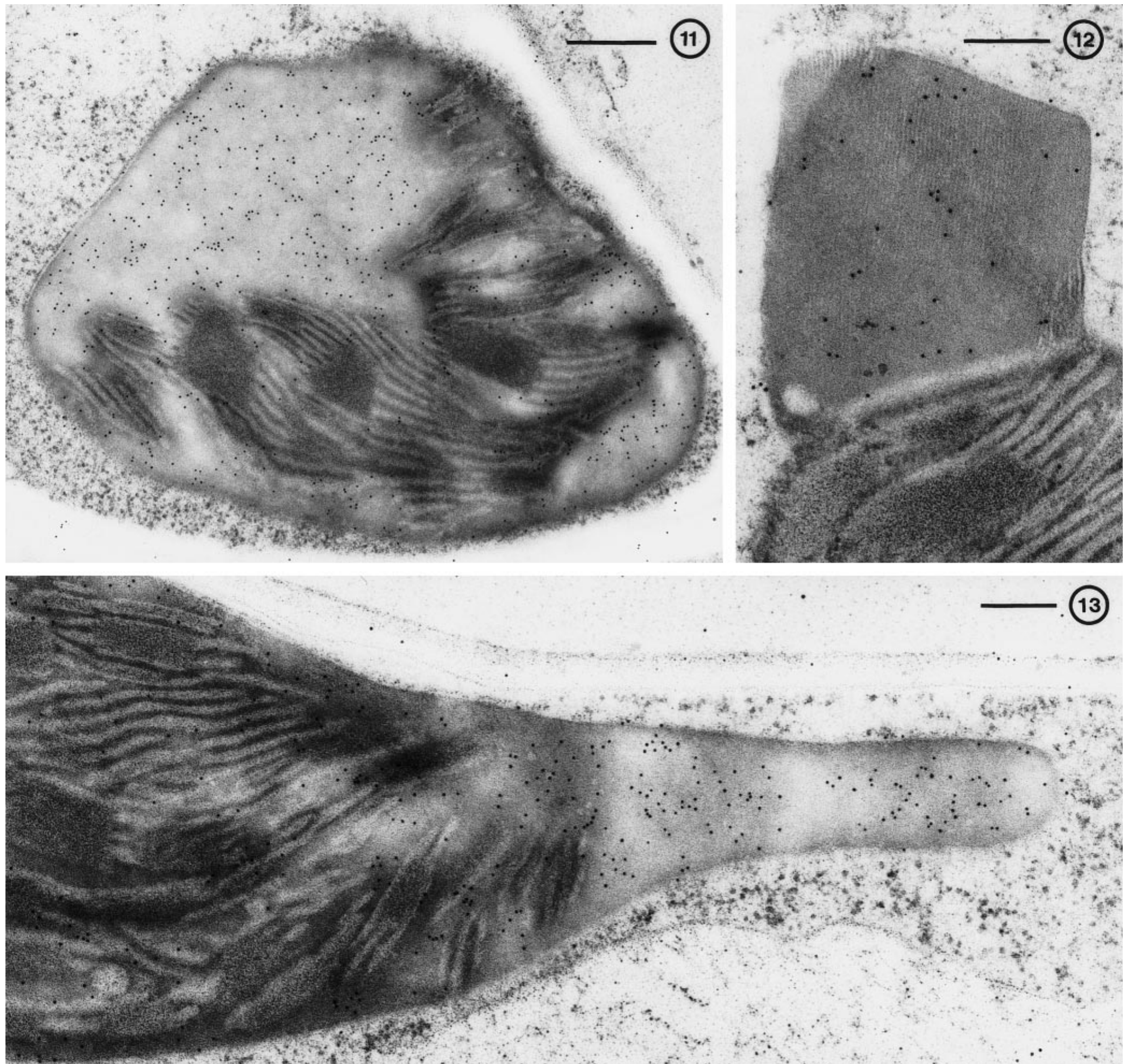
V

Figs. 7–9. Three serial sections through a transition region between the base of a protuberance and the thylakoid-containing main body of the chloroplast. Note the irregular profile of the protuberance surface (*arrows*) and apparent continuity (*arrowheads*) between a surface infolding and thylakoid membranes. *G*, granum. ×70300; bar = 250 nm

Fig. 10. Dramatic variation is seen in the staining density of thylakoid lumina within a single chloroplast. Lumen density is highest in the more randomly arranged granal stacks positioned nearest the cell wall. In granal stacks nearest the central vacuole (V), the thylakoid lumina are less densely stained and their orientation is parallel with the surface of the chloroplast. Apparent gaps at the cell periphery (*arrow*) represent an artifact of high-pressure freezing. ×20900; bar = 500 nm

ment), albeit poorly frozen (not illustrated), and in tissues pre-treated with dextrans.

In C_3 plants such as rice, infoldings of the plastid inner envelope are common only in proplastids and during early stages of chloroplast development (Staehelin 1986). In C_4 plants, on the other hand, mature chloroplasts possess a complex of tubules and vesicles known as the peripheral reticulum (Sprey and Laetsch 1978). An exception in C_3 plants are the anastomosing tubules found in the periph-



Figs. 11–13. Immunogold localization of Rubisco enzyme in etched (**Figs. 11, 13**) and non-etched (**Fig. 12**) thin sections of rice chloroplasts. The enzyme is observed in the stroma of both the main body of the chloroplast (**Fig. 11**), and the protuberance (**Fig. 13**). Crystalline bodies observed in some chloroplasts are also labeled (**Fig. 12**). ×30400, bar = 500 nm (**Fig. 11**); ×57300, bar = 250 nm (**Fig. 12**); ×46500, bar = 250 nm (**Fig. 13**)

ery of guard-cell chloroplasts of *Vicia* (Pallas and Mollenhauer 1972). These structures appear very similar to the tubules observed in this study (see Fig. 6).

Although often presumed otherwise (Wellburn 1982), there is no conclusive evidence that thylakoids derive from infoldings/vesicles of the chloroplast inner envelope (Staehelin 1986). Here, some of the larger infoldings were contiguous with thylakoid membranes (see Figs. 7–9). Freeze-fracture analysis of high-pressure-frozen leaves could aid interpretation of these observations, as each chloroplast-associated membrane – viz. outer envelope, inner envelope, thylakoids, and stromal thylakoids – carries a distinctive complement of intramembrane particles (Sprey and Laetsch 1978; Staehelin 1986). Staehelin (1986) has suggested that such infoldings could reflect high levels of molecular transport between chloroplast and cytoplasm, and thus serve a similar function to the plasma-membrane-associated modifications in transfer cells (Pate and Gunning 1972). While fatty acids are known to be synthesized in plastids, phospholipid maturation requires the involvement of other cytoplasmic compartments. Likewise, the bulk of chloroplast proteins must be targeted to the chloroplast. Therefore, one would expect substantial lipid and protein trafficking into and out of plastids, trafficking that would be facilitated by increased surface area to volume ratios at the plastid-cytoplasm interface.

A previously reported artifact of high-pressure freezing, i.e. breaks in the plasma membrane and cell wall (Kiss et al. 1990), was observed here as well. In addition, cellular membranes were poorly stained, a problem often associated with freeze substitution (Hoch 1986). These preparative limitations represent minor drawbacks in light of the new findings that were obtained.

Hanson's group reported frequent interstromal contact in living cells and isolated chloroplasts (Kohler et al. 1997a). Why weren't more interconnecting protuberances observed here? Possible causes include our use of different plant material – taxonomically and physiologically different from that used by Kohler et al. (1997a) – and, especially, the small sample size afforded by thin-section analysis. One might also question why earlier studies, that employed cryofixation, didn't discover protuberances? Among plant organ studies with high-pressure freezing, leaves have received little attention. To our knowledge the literature contains no in-depth investigation of chloroplast morphogenesis using cryo-fixed higher-plant leaves.

Our results should provide sufficient incentive for using high-pressure freezing combined with freeze substitution to re-examine thylakoid/grana morphogenesis at the ultrastructural level. Such an approach could be especially rewarding for comparisons of wild-type plastids and those in mutants impaired in normal chloroplast differentiation.

We thank Dr. Daniel Studer (University of Bern) for his helpful guidance in freezing and processing our samples, and Ian Lamswood and Anne Korsen (Leica Company) for facilitating use of their automated freeze-substitution apparatus.

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