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ORGANIZATION OF THE PHOTOSYNTHETIC MEMBRANE IN MAIZE MESOPHYLL AND BUNDLE SHEATH CHLOROPLASTS

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SUMMARY

The freeze-fracturing technique has been used to investigate membrane architecture in the mesophyll and bundle sheath chloroplasts of *Zea mays*. The structural organization of mesophyll chloroplasts is virtually identical to that of other species of higher plants which have been investigated with this technique. Characteristic distributions of particles of various sizes are seen on each fracture face after membrane splitting during the fracturing process, and these distributions indicate the differentiation of the membrane system into stacked (grana) and unstacked (stroma) regions, typical of grana-containing chloroplasts. Bundle sheath chloroplasts contain very few grana, and the thylakoids of these plastids are therefore largely unstacked. Analysis of artificially unstacked mesophyll chloroplasts indicates that this difference is not merely related to the presence or absence of adhesion between adjacent thylakoids, but reflects a substantial difference in membrane substructure between mesophyll and bundle sheath photosynthetic membranes. Bundle sheath thylakoids contain virtually the same number of small (P fracture face) particles as mesophyll thylakoids, but contain only 40 % as many of the larger (E fracture face) tetrameric particles.

These differences, together with biochemical data indicating the comparative deficiency of bundle sheath chloroplasts in Photosystem II activity, suggest that the E face particles are related to the presence or absence of Photosystem II activity.

INTRODUCTION

The presence of two distinctly different classes of chloroplasts in *Zea mays* has been recognized since the first reports of Hodge et al. [1]. They observed that chloroplasts found in mesophyll tissue were similar to those reported in other species of higher plants, while those found in bundle sheath tissue, in contrast, appeared to lack grana. Subsequent studies showed that this absence of grana was not so complete as first suspected, and these bundle sheath chloroplasts are now understood to possess rudimentary grana [2–4]. The occurrence of this chloroplast dimorphism in a wide variety of species exhibiting the so-called C4 (Hatch-Slack) pathway of carbon fixation has recently been reviewed by Laetsch [5].

The first efforts to study photosynthetic reactions in bundle sheath and mesophyll chloroplasts suggested that while the bioenergetic characteristics of mesophyll chloroplasts were fairly typical of higher plants, the chloroplasts of bundle sheath cells were almost totally lacking in Photosystem II activity [6, 7]. This view was modified after later studies [8, 9], and it now seems clear that the bundle sheath chloroplasts of *Z. mays* contain significant and measurable amounts of Photosystem II activity. Nevertheless, in comparison to mesophyll chloroplasts from adjacent tissue, their measurable Photosystem II activity is clearly much reduced; however, Photosystem I activity at a high level can be measured in each type of chloroplast. An excellent review of the differences in structure and function between mesophyll and bundle sheath chloroplasts of C4 plants has recently been provided by Bishop [10].

The work reported here is a characterization of photosynthetic membrane substructure in mesophyll and bundle sheath chloroplasts of *Z. mays*. We have tried to take advantage of the reduced Photosystem II activity in bundle sheath chloroplasts to make some correlation between membrane substructure and the presence or absence of Photosystem II activity at high rates.

MATERIALS AND METHODS

Seedlings (*Z. mays*, var.) were grown in vermiculite in a growth chamber (22–25 °C) with a light period of 16 h for 2–3 weeks. 50 g of leaves were cut into small strips and homogenized in a Waring Blendor in 150 ml of a buffer solution containing 300 mM NaCl, 50 mM Tricine/NaOH, pH 7.5, and 2 mM $MgCl_2$. After 5 s of high speed homogenization, the suspension was removed, and filtered through eight layers of gauze. This filtrate was then centrifuged 2 min at $250 \times g$. The supernatant was then centrifuged for 10 min at $3000 \times g$. The resulting pellet contained approx. 95 % mesophyll chloroplasts.

Bundle sheath chloroplasts were isolated from the residue trapped in the gauze. This residue was suspended in more buffer, and thoroughly homogenized at high speed in the blender for 25 s. The material was again filtered through gauze, and the filtrate discarded. The residue was ground in a mortar and pestle with a small amount of buffer. After grinding, the suspension was again filtered through eight layers of gauze. The filtrate was centrifuged for 2 min at $250 \times g$, the pellet discarded, and the supernatant centrifuged at $3000 \times g$ for 10 min. The resultant pellet contained approx. 65 % bundle sheath chloroplasts.

Isolated chloroplast membranes were infiltrated with glycerol to a final concentration of 25 % (v/v) over the course of 1 h, then rapidly frozen in liquid Freon 12 and transferred for storage to liquid nitrogen. Replicas were prepared without etching at −100 °C according to the method of Moor and Mühlethaler [11] on a Balzers freeze-etching apparatus. Replicas were cleaned in liquid bleach and chromic acid, and were examined in a Philips 300 electron microscope.

Counts of particle density were prepared from micrographs enlarged to a final magnification of 100 000. Measurements of particle diameters were prepared from micrographs enlarged to 200 000 diameters, and actual measurements were made through a seven times magnifier fitted with a micrometer scale calibrated to 0.1 mm.

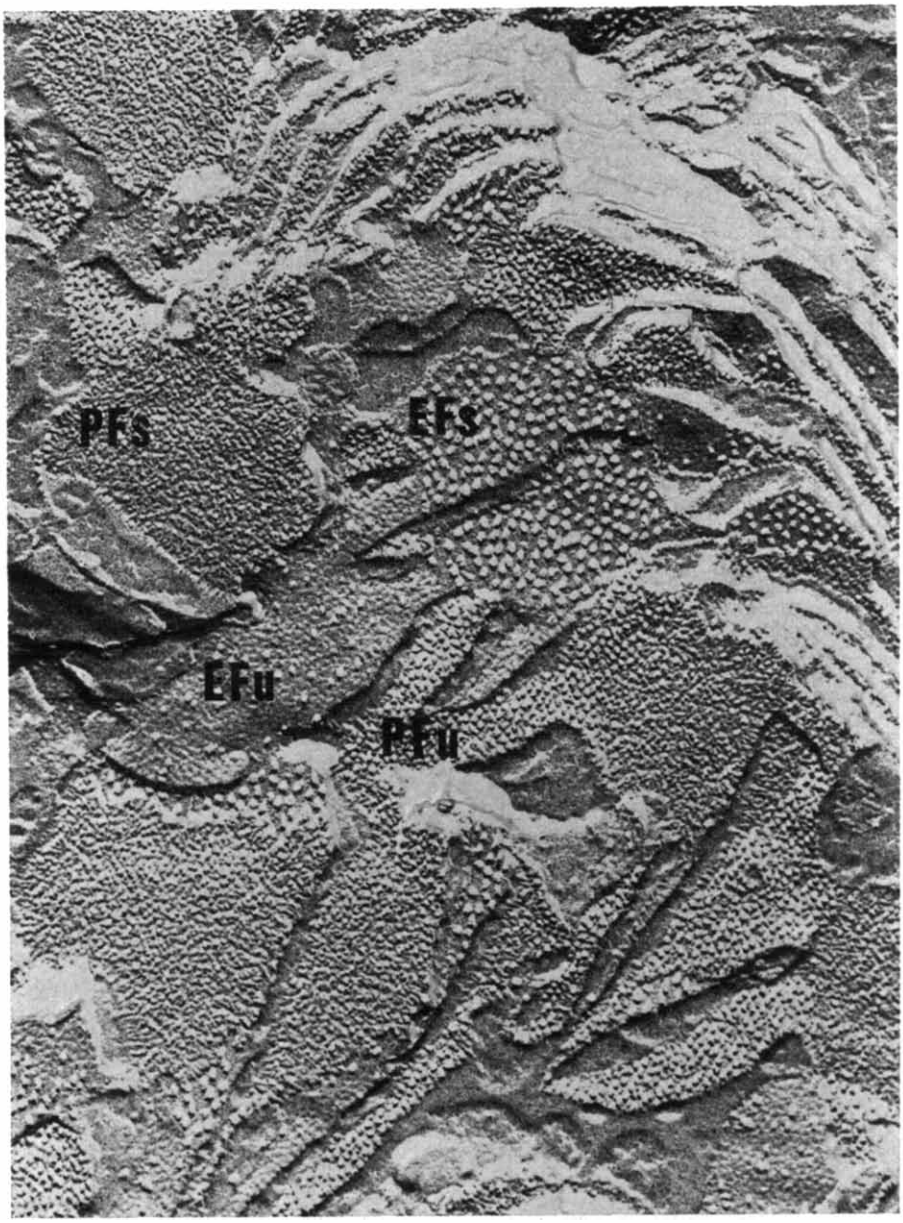

Fig. 1. Replica of an isolated, freeze-fractured mesophyll chloroplast. Four types of fracture faces are apparent. EFs and PFs faces are derived from membrane splitting in stacked (grana) regions of the membrane system, and EFu and PFu faces from splitting in unstacked (stroma) regions of the system. The membrane substructure is typical of the appearance of photosynthetic membranes of other higher plants. Magnification, 75 000.

RESULTS

The appearance of a typical mesophyll chloroplast, prepared by freeze-

Fig. 2. A bundle sheath chloroplast. In contrast to mesophyll chloroplasts (Fig. 1) only a few small regions of membrane stacking (EFs and PFs) are present. The membrane consists of largely unstacked thylakoid sheets, and the paucity of particles on the E fracture faces in comparison to the E faces of mesophyll chloroplasts is evident. Magnification, 75 000.

fracturing, is shown in Fig. 1. The fact that freeze-fracturing splits biological membranes along a roughly central plane is by now well established [12]. The structures visible in Fig. 1, therefore, represent internal components of the photosynthetic mem-

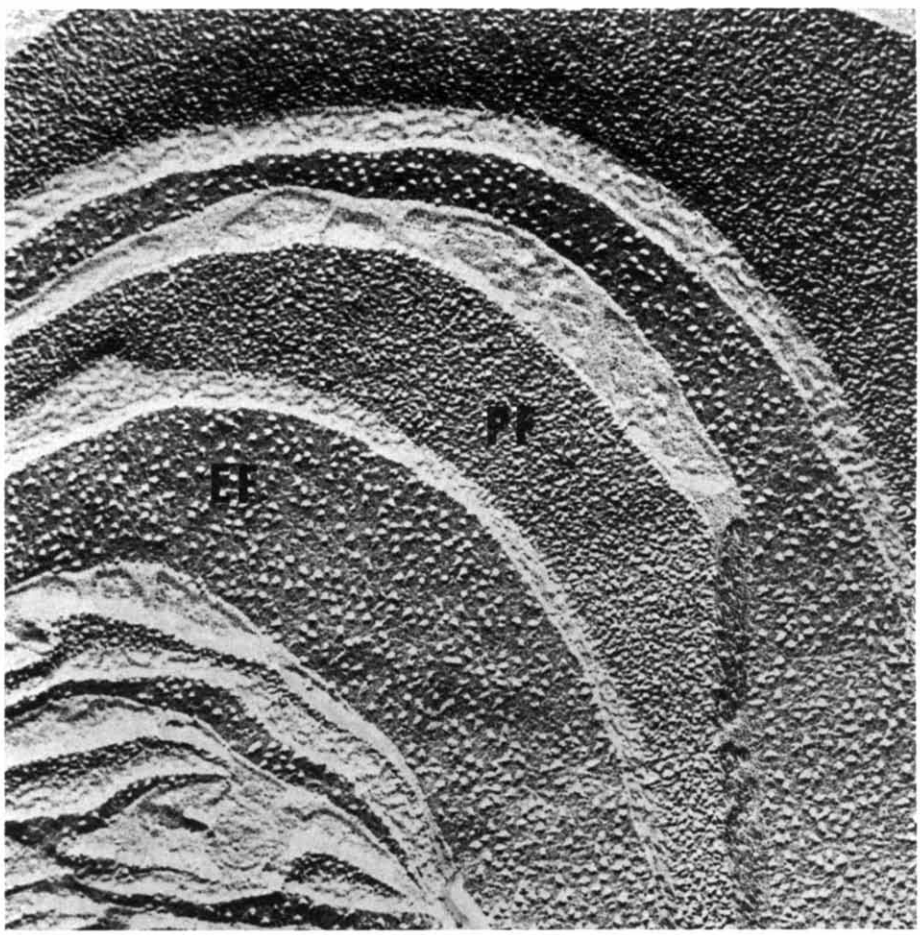

Fig. 3. An artificially unstacked maize mesophyll chloroplast. Only two fracture faces are present, because the membrane is no longer differentiated into stacked and unstacked regions. Note however, that particle density of the E fracture face (EF) is still much higher than that seen on the E fracture faces of bundle sheath chloroplast membranes. Magnification, 75 000.

brane. The appearance of maize mesophyll chloroplast membranes is quite similar to that of other thylakoids which have been examined by this process [13–16]. Four distinct types of fracture faces are present, which are labeled EFs, EFu, PFs, and PFu in accordance with the nomenclature of Branton et al. [17] as modified by Staehelin et al. [16]. The EFs and PFs images of the membrane are derived from the splitting of membranes in stacked (grana) regions of the chloroplast, while EFu and PFu images are derived from the splitting of thylakoids in unstacked (stroma) regions of the membrane system. E fracture faces are formed from the lumenal half of the membrane, while P faces are formed from the stromal half of the membrane.

The four fracture faces formed from maize mesophyll chloroplasts are virtually identical with those found in other higher plant chloroplasts.

Bundle sheath chloroplasts present quite a different picture, however (Fig. 2).

Bundle sheath chloroplasts are extensively unstacked [1–4], and EFu and PFu fracture faces predominate in the freeze-fractured replica. Small stacked (EFs and PFs) regions are nonetheless present, corresponding to the rudimentary grana which are known to be present in these membranes. A careful comparison of Figs. 1 and 2 shows the comparative paucity of large E fracture face particles in bundle sheath chloroplast membranes. The actual number of particles on each fracture face is difficult to quantitate in mesophyll chloroplasts (where extensive stacking exists) because the percentage of membrane area involved in each of the four fracture faces must be carefully calculated, introducing another possibility for error into the final result.

In order to facilitate a direct comparison of membrane substructure between mesophyll and bundle sheath chloroplast membranes, we have unstacked mesophyll chloroplasts in vitro after the method of Izawa and Good [18]. This procedure eliminates stacked regions and, as shown by Goodenough and Staehelin [14], changes the membrane image in freeze-fracture to one containing only the two (E and P) fracture faces of the now apparently homogeneous membrane system. This procedure, in effect, allows us to answer a basic question about the bundle sheath photosynthetic membrane: Is it merely an "unstacked" version of the mesophyll membrane, or do the photosynthetic membranes of bundle sheath chloroplasts possess an important difference in membrane composition which might be either the cause or effect of their primarily unstacked organization?

The two fracture faces which are present in maize mesophyll chloroplasts after artificial unstacking are shown in Fig. 3. These are now much more like bundle sheath chloroplasts (Fig. 2) in appearance, since the image is no longer complicated by the presence of large amounts of stacked membrane (with its EFs and PFs faces). However, although the P fracture faces in Figs. 2 and 3 seem quite similar, a cursory examination of this figure also seems to show that the concentration of large particles seen on the EF fracture face is much higher in mesophyll photosynthetic membranes than it is in bundle sheath ones.

By measuring particle sizes and distributions on each of the fracture faces of these membranes, we have attempted to quantitate what differences, if any, may exist between the two types of photosynthetic membranes in *Z. mays*. Although it is simpler to express the sizes of particles on each fracture face with a single mean or median value, we believe that the actual nature of each membrane face is most accurately considered by the analysis of a histogram of particle sizes as shown in Fig. 4. Figs. 4 and 5 show particle sizes for each of the four fracture faces found in *Z. mays* mesophyll and bundle sheath chloroplasts. Despite the great differences in appearance of each chloroplast type, it can be seen that particle size measurements indicate a surprising degree of consistency between the two types of chloroplasts.

The process of unstacking does not result in a simple elimination of the EFs and PFs faces, as shown in Fig. 6. Rather, as has been shown in a careful study of particle sizes and concentrations during the unstacking process performed by Ojakian and Satir [19], particles of different sizes on E fracture faces in stacked and unstacked regions become randomly intermixed in the resulting E fracture face of the unstacked preparation. Thus, in Fig. 6, we see a particle size distribution indicative of the intermixing of relatively large (Fig. 4) EFs and relatively small (Fig. 5) EFu particles which occurs during unstacking. Similar changes are observed on the P fracture faces, although the smaller particle sizes on these faces make the changes less apparent.

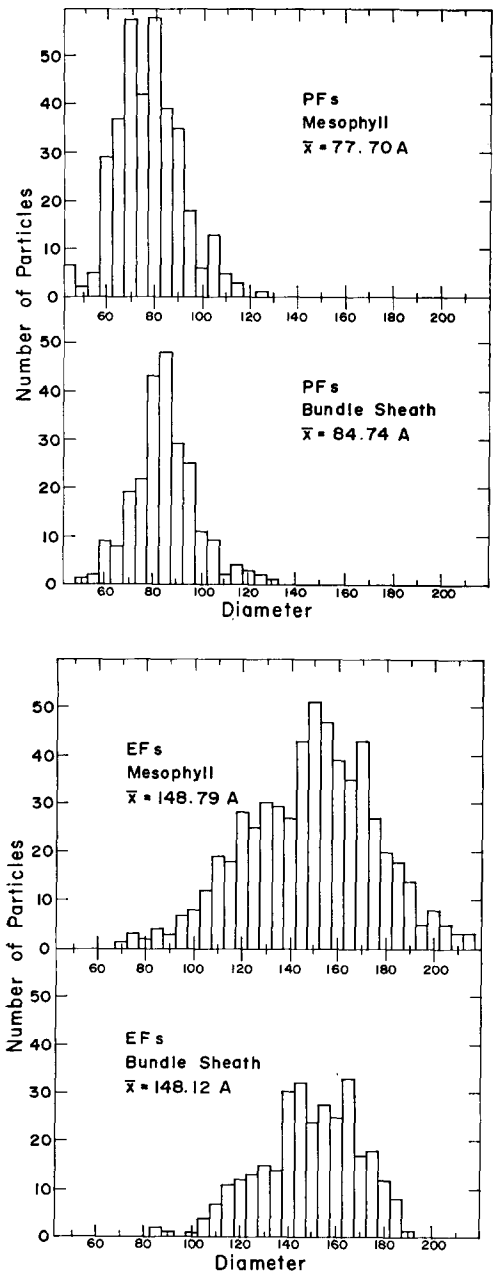

Fig. 4. Histograms of particle sizes from stacked membrane regions in mesophyll and bundle sheath chloroplasts. Despite the rarity of stacked regions in bundle sheath thylakoids, the distribution of particle sizes in these regions is virtually indistinguishable from measurements made on the much more extensive grana of mesophyll chloroplasts.

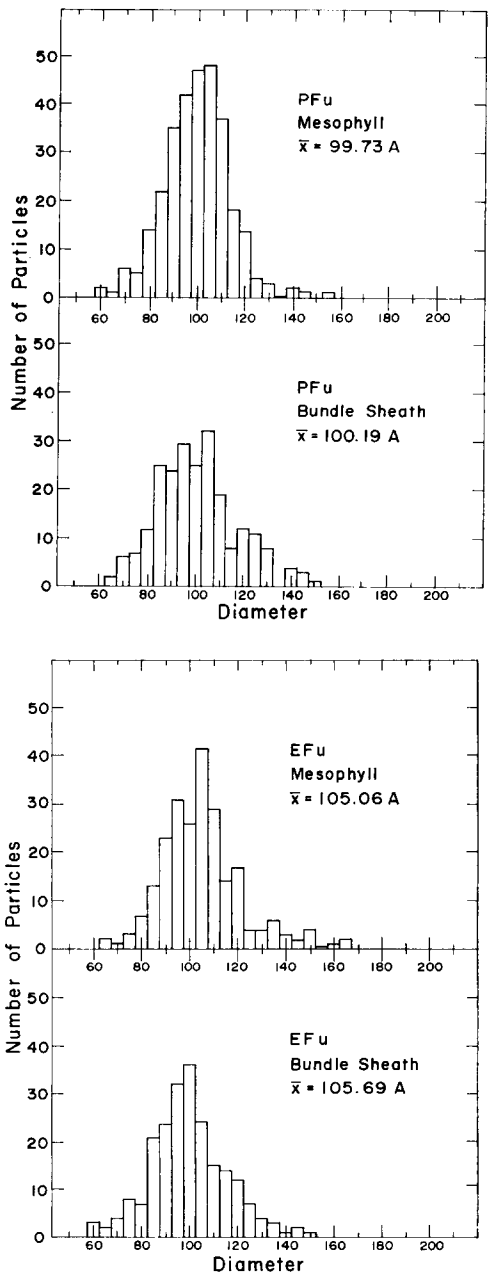

Fig. 5. Histograms similar to those of Fig. 4, but prepared from unstacked (stromal) regions of the membrane system. Again, the similarity of particle sizes between bundle sheath and mesophyll chloroplasts is apparent.

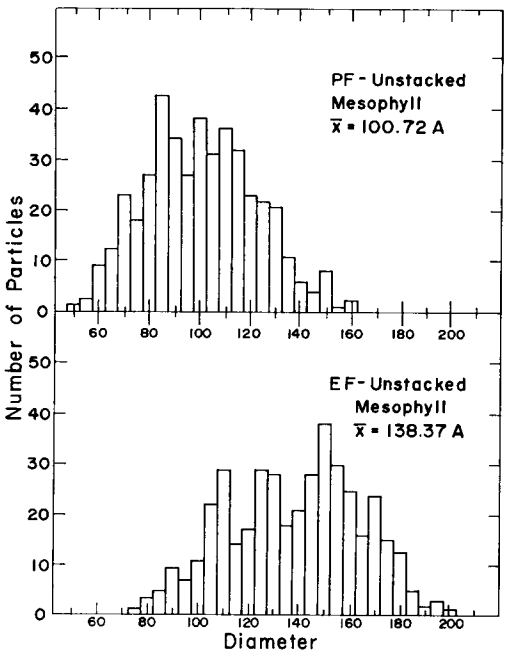

Fig. 6. Histograms of particle sizes of the two fracture faces seen in artificially unstacked mesophyll chloroplasts. Note that the range of particle sizes from EF faces, for example, may indicate an intermixing of particles from the EFs and EFu faces of mesophyll chloroplasts.

Therefore, particle size measurements indicate that bundle sheath chloroplasts are not identical structurally with artificially unstacked mesophyll chloroplast membranes.

Measurements of the number of particles per unit area in the photosynthetic membrane are complicated by problems in estimating the percentage of membrane surface area involved in stacking. Therefore, we have not attempted to determine these values for mesophyll chloroplast membranes in the stacked configuration. However, bundle sheath chloroplasts of maize, in which the percentage of membrane area involved in stacking is so small as to be negligible (2–4 %), and artificially unstacked mesophyll chloroplasts do not present this complication, and can be readily used for a comparison of membrane structure between the two chloroplast types.

Table I shows the results of a series of particle distribution measurements on

TABLE I

Results are expressed in number of particles/μm^2 $\pm$S.E.

	Particle densities	
	EF	PF
Mesophyll unstacked	949.5$\pm$171	3011$\pm$286
Bundle sheath	394$\pm$61	3019$\pm$479

P and F fracture faces of bundle sheath and unstacked mesophyll chloroplasts. The numbers of small particles visible on the P fracture faces is almost identical in the two types of membranes. However, the number of large particles visible on the E fracture faces of bundle sheath chloroplasts is much lower than the number of similar particles counted in the photosynthetic membrane of mesophyll chloroplasts. This is a significant difference which may be related to energetic and biochemical differences between the two membrane types.

Finally, it seems important to emphasize that the small grana present in bundle sheath chloroplasts are virtually identical, in terms of membrane substructure, with the much larger and more extensive grana in mesophyll chloroplasts. Fig. 7 shows a small circular granum from a bundle sheath chloroplast, and the characteristic types of membrane differentiation which are found in grana regions (larger E fracture face particles, concentration of E fracture face particles relative to the numbers found on EFu regions, smoother background matrix between particles on the EFs region relative to EFu, much rougher background and smaller particles on the PFs surface relative to PFu) are all clearly present. So, although the percentage of membrane surface area forming grana contacts in bundle sheath chloroplasts in maize is so small that in some respects it may be overlooked, it seems important to stress that those contacts which do exist are not trivial, but contain the same types of submembrane differentiation which mark the much larger grana of mesophyll chloroplasts.

We may summarize our observations on the organization of bundle sheath and

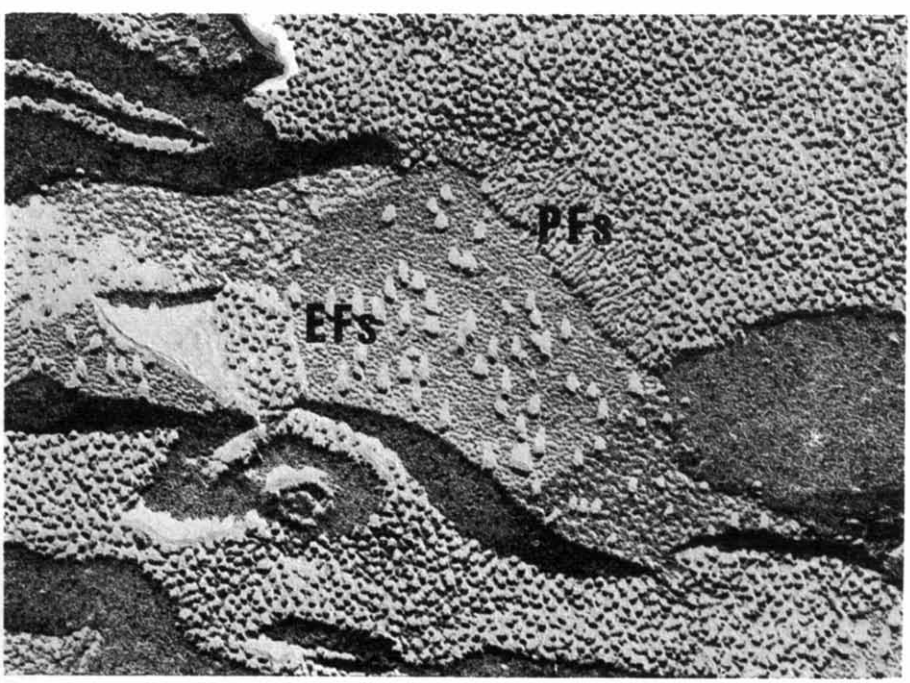

Fig. 7. Small grana region from bundle sheath chloroplast. Although these grana are very few and very small in comparison to grana from mesophyll chloroplasts, their structure is nonetheless quite similar to mesophyll grana. Magnification, 100 000.

mesophyll chloroplasts of *Z. mays* as follows: The mesophyll chloroplasts of *Z. mays* are very similar to other types of higher plant chloroplasts which have been studied with the freeze-fracture technique. Bundle sheath chloroplasts of *Zea mays* display a membrane substructure which, in its organization in stacked and unstacked regions, is very similar to the organization seen in mesophyll chloroplast membranes in those respective regions. Whether the cause or the effect of reduced thylakoid stacking, bundle sheath chloroplasts contain approx. 60 % fewer large particles on the E fracture face than do mesophyll chloroplasts. The number of small particles on the P fracture face is similar in each type.

DISCUSSION

A number of studies [6–10] have indicated that the bundle sheath chloroplasts of *Z. mays* contain reduced amounts of Photosystem II activity relative to mesophyll chloroplasts from the same plant. This characteristic makes them a good model system with which to test the localization of Photosystem II activity and its relationship to membrane substructure. Previous studies [20–22] have emphasized a correlation between the occurrence of Photosystem II activity and the large particles found primarily in thylakoid grana. These studies, however, have all involved fragmentation of the thylakoid membrane with detergent or by mechanical means in order to achieve a separation of grana and stroma membranes. As such, they are open to the criticism that the destructive effects of the fragmentation procedure may account in part for differences in photosynthetic activity which are found between the large grana fragments, and the smaller (possibly more fragile) stroma fragments.

As we have shown above, the major difference between maize bundle sheath and mesophyll chloroplasts in terms of membrane substructure is the dramatic reduction in the number of large EF particles in the former. Perhaps significantly, no change is apparent in the number of smaller particles seen on the P fracture faces of bundle sheath thylakoids. These results suggest that Photosystem II activity may be correlated with the presence of EF particles, a conclusion similar to that reached [20–22] with membrane fractionation techniques. The persistence of these particles in the bundle sheath membranes (although at reduced concentrations) seems quite consistent with recent observations concerning the presence of low levels of Photosystem II activity in these membranes. We suggest, as have others before us [21–23], that these particles may represent macromolecular complexes of many components in which the Photosystem II reactions take place.

These data are also consistent with recent reports by Armond, P. A., Staehelin, L. A. and Arntzen, C. J. (unpublished) and Miller et al. [24] suggesting that the light-harvesting chlorophyll-protein complex associated with Photosystem II is bound to a membrane-spanning tetramer [25] which, after freeze-fracturing, is visible as the large EF particle.

The comparative paucity of thylakoid stacking into grana in maize bundle sheath chloroplasts also suggests that the presence of a critical concentration of these tetramers (EFs particles) may be required for the membranes to be able to stack over large membrane areas.

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