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Ultrastructural Observations in the Suppression of Granal Development in Bundle Sheath Chloroplasts of NADP-ME Type C_4 Monocot and Dicot Species

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Abstract : We examined chloroplast development with respect to the stacking of grana in *Zea mays* L., an NADP-ME type C_4 monocot, and in *Portulaca grandiflora*, an NADP-ME type C_4 dicot species. In both species, mean numbers of thylakoids per granum in bundle sheath chloroplasts were suppressed less than 3 throughout chloroplast development while they were increased gradually in mesophyll chloroplasts. It was concluded that in both phylogenetically distinct NADP-ME type C_4 plants the stacking of grana was suppressed in bundle sheath chloroplasts from early developmental stages.
Key words : Bundle sheath chloroplast, C_4 plant, Grana, NADP-malic enzyme type, *Portulaca grandiflora*, *Zea mays* L.

NADP-ME型C_4単子葉および双子葉植物における維管束鞘葉緑体グラナの発達抑制に関する微細構造観察

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要　旨：NADP-ME 型 C_4 単子葉植物であるトウモロコシと，同型の C_4 双子葉植物であるマツバボタンについて，グラナの重なり（スタッキング）に注目して葉緑体の発達過程を観察した．両植物種において，維管束鞘葉緑体では1グラナを形成するチラコイド数の平均値は，葉緑体発達過程を通して3未満に抑えられていたのに対し，葉肉葉緑体では発達にともない徐々に増大した．したがって，系統発生的に異なる2種類の NADP-ME 型 C_4 植物において，維管束鞘緑体ではグラナの発達は共に葉緑体発達初期から抑えられていると考えられた．
キーワード：維管束鞘葉緑体，NADP-ME 型，グラナ，C_4 植物，トウモロコシ，マツバボタン．

It is known that the C_4 pathway of photosynthesis has evolved widely in many plant taxa, both in monocots and dicots[12]. Amongst C_4 plants there are three biochemical subgroups basically differing in the type of decarboxylating enzyme : NADP-malic enzyme (NADP-ME) type, NAD-malic enzyme (NAD-ME) type, and phosphoenolpyruvate carboxykinase (PEP-CK) type[5,6,7,8]. Among these, the NADP-ME type of C_4 plants have agranal chloroplasts in bundle sheath cells which are arranged centrifugally in monocots and centripetally in dicots[3,5,6,7]. Several researchers have observed ultrastructural changes in bundle sheath chloroplasts of NADP-ME type C_4 plants during leaf development[4,9,10,11,13]. Laetsch and Price[10] reported that the bundle sheath plastids of sugarcane initially show an increase in the number of thylakoids per granum, which decrease as the plastids approach maturity. Kirchanski[9] also observed a similar process in maize leaves. However, detailed quantitative analysis has not yet been made. In addition, no detailed observations have been made about the ultrastructural changes in chloroplasts of NADP-ME type dicots. It would be interesting and necessary to comparatively examine the developmental process of chloroplasts in both monocots and dicots of NADP-ME type C_4 plants, which have differentially located bundle sheath chloroplasts.

In this study we compared the ultrastructural changes observed in mesophyll and bundle sheath chloroplasts, especially those associated with the suppression of granal development in bundle sheath chloroplasts, during the process of leaf development in NADP-ME type C_4 plants. We examined two phylogenetically distinct species : *Zea mays*, a monocot with centrifugally arranged bundle sheath chloroplasts, and *Portulaca grandiflora*, a dicot with centripetally arranged bundle sheath chloroplasts. The positional regulation of chloroplast differentiaton with respect to the lack of grana in bundle sheath cells is discussed.

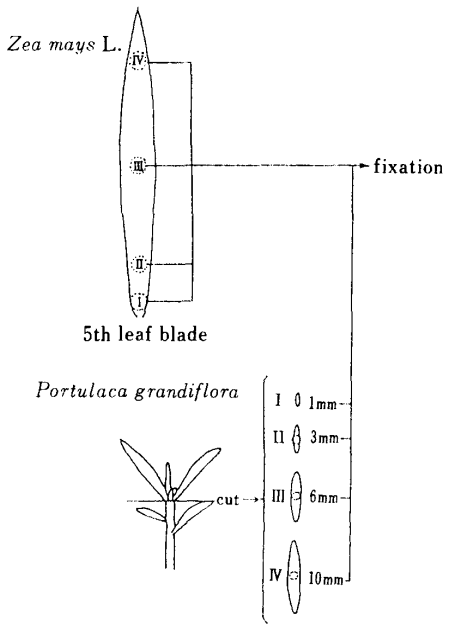
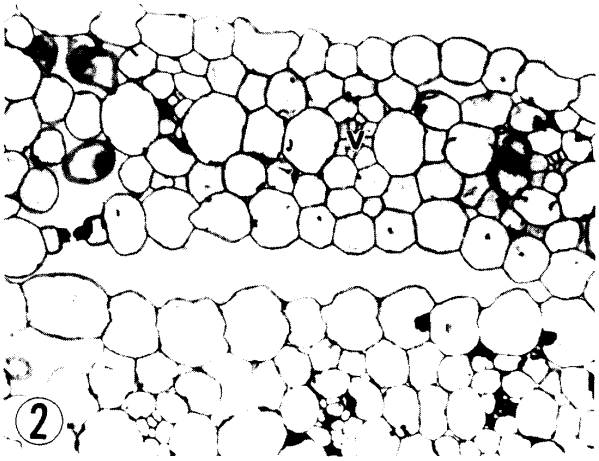


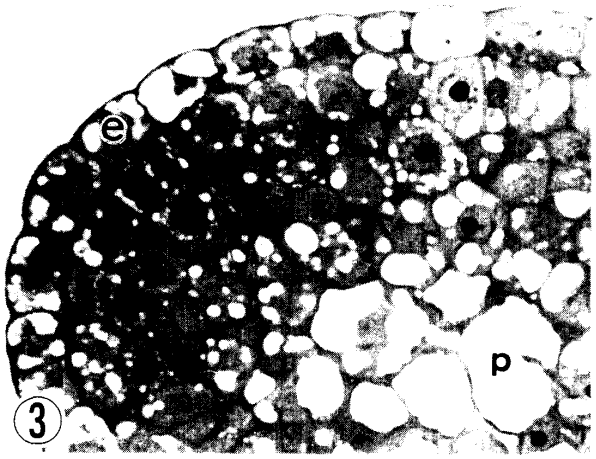
Fig. 1. Diagram of material preparation used for light and electron microscopy. I, II, III and IV indicate developmental stages.

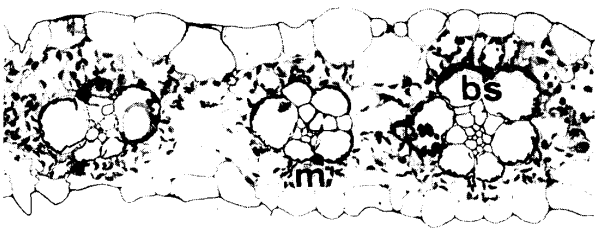
Materials and Methods

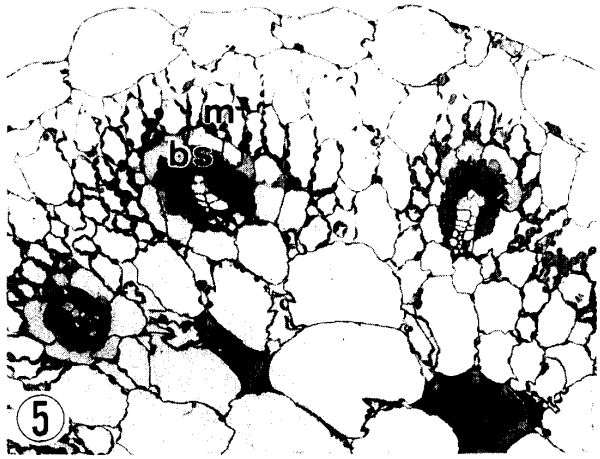
Seeds of *Zea mays* and *P. grandiflora* were sown in pots and grown in the green house. Illumination and temperature were naturally controlled.

For light and electron microscopy, the expanding fifth leaf of maize and various sizes of leaves of *P. grandiflora* were used as shown in Fig. 1. Small segments of leaves were fixed in 5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) and post-fixed in 2% osmium tetroxide in the same buffer. Samples were dehydrated in a series of graded acetone and propylene oxide and embedded in Epon 812 or Spurr's resin (TAAB). Semithin and ultrathin sections were cut with a diamond knife on Ultracut-N microtome (Reichert Nissei). Semithin sections were stained with toluidine blue O and observed under a light microscope (Nikon OPTIPHOTO). Ultrathin sections were mounted on grids and stained









with uranyl acetate followed by lead citrate and examined under a transmission electron microscope (Hitachi H-600).

The number of thylakoids per granum and the percentage of bundle sheath cells in which all chloroplasts were completely located centrifugally (*Zea mays*) or centripetally (*P. grandiflora*) were determined on light or electron micrographs.

Results

1. Monocotyledonous species

At the base of the leaf blade (stage I) of *Zea mays*, as shown in Fig. 2, veins were already initiated while the Kranz anatomy was not yet obvious. Some small chloroplasts which had undeveloped grana containing 2-3 thylakoids were observed in the cells adjacent to the pro-vascular tissues (Fig. 6). Most of these chloroplasts were arranged at random in the cells (Table 1). Chloroplasts in outer meristematic cells contained grana with more thylakoids (Fig. 7).

In stage II, mesophyll and bundle sheath cells were completely differentiated and Kranz anatomy was apparent. However, the centrifugal localization of bundle sheath chloroplasts was not formed at all (Table 1). Although some of the grana in bundle sheath chloroplasts appeared relatively developed, many of them contained only 2-3 thylakoids (Fig. 8), and the mean number of thylakoids per granum was less than 3 (Table 1). Mesophyll chloroplasts at this stage possessed many developed grana and were distinguished from those of the bundle sheath chloroplasts (Fig. 9).

Bundle sheath chloroplasts at developmental stage III and IV were morphologically similar (compare Fig. 10 and 12). Although some of the bundle sheath cells had randomly located chloroplasts at these stages (Fig. 4 and Table 1), however, ultrastructural differences between these and centrifugally located chloroplasts were not observed. Their grana were inconspicuous, containing less than 3 thylakoids per granum (Table 1). In contrast, mesophyll chloroplasts at these stages had adequately developed grana (Fig. 11 and 13). While they were also morphologically similar, mean number of thylakoids per granum at stage IV were more than those at stage III (Table 1).

2. Dicotyledonous species

In cross sections of young leaves (stage I) of *P. grandiflora*, procambial cells could not be observed. However, epidermal and parenchymatous tissues were differentiated (Fig. 3). In meristematic cells small chloroplasts with grana containing up to 5 thylakoids were observed (Fig. 14).

In stage II, many procambial cells were observed while Kranz anatomy was not completely formed (data not shown). Although most chloroplasts in the cells adjacent to the provascular tissues were not located

Explanation of Figures 2 to 20

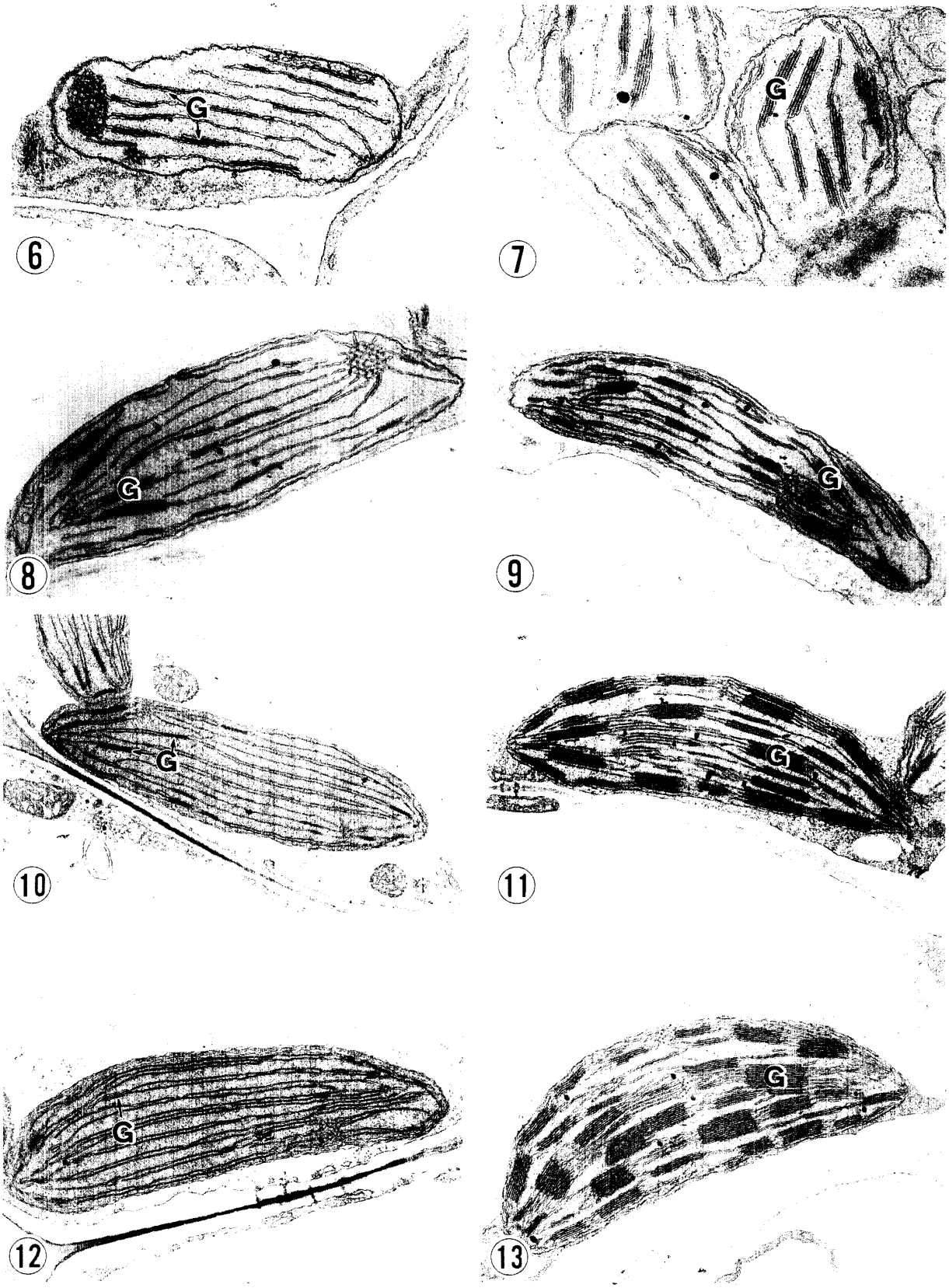
Figs. 2-5. Light micrographs of transections of *Zea mays* L. and *Portulaca grandiflora* leaves. Fig. 2 : *Zea mays* at stage I (×345), indicating Kranz anatomy is not yet obvious. Fig. 3 : *P. grandiflora* at stage I (×670). Fig. 4 : *Zea mays* at stage IV (×160), indicating Kranz anatomy and centrifugally located bundle sheath chloroplasts. Fig. 5 : *P. grandiflora* at stage IV (×170), indicating centripetal localization of bundle sheath chloroplasts is complete.

Figs. 6-13. Electron micrographs of chloroplasts in *Zea mays* L. Fig. 6 (×18000), 8 (×21000), 10 (×11000) and 12 (×14000) show bundle sheath chloroplasts at stage I, II, III and IV, and Fig. 7 (×23000), 9 (×15000), 11 (×13000) and 13 (×14000) show mesophyll chloroplasts at stage I, II, III and IV, respectively. Note at each stage bundle sheath chloroplasts are distinguishable from those of mesophyll.

Figs. 14-20. Chloroplasts in *Portulaca grandiflora*. Fig. 14 : At stage I, bundle sheath and mesophyll cells are not differentiated (×27000). Fig. 15-20 : Bundle sheath chloroplasts at stage II (Fig. 15, ×13000), III (Fig. 17, ×20000) and IV (Fig. 19, ×9000) are distinguishable from those of mesophyll at stage II (Fig. 16, ×18000), III (Fig. 18, ×19000) and IV (Fig. 20, ×13000), respectively.

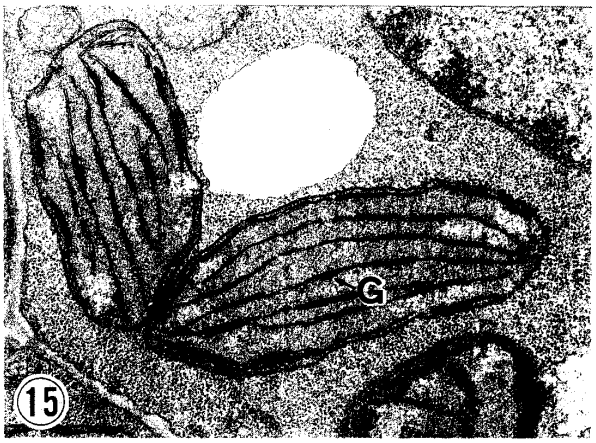
Abbreviations

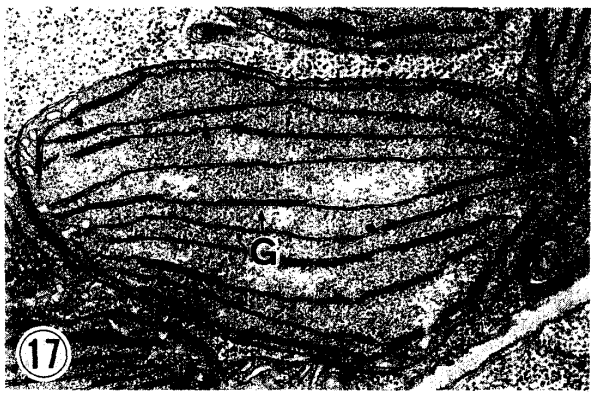
bs : bundle sheath cell, e : epidermal cell, G : granum, m : mesophyll cell, p : parenchymatous cell, v : vascular bundle.

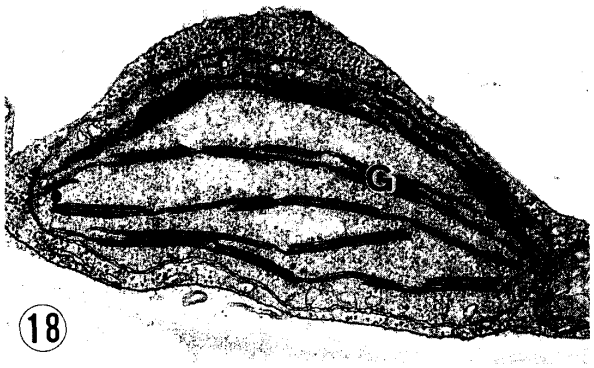


centripetally, as typically seen in bundle sheath cells of NADP-ME type C_4 dicots (Table 1), they were ultrastructurally characteristic of bundle sheath chloroplasts (Fig. 15). They possessed undeveloped grana and could be distinguished from those in outer meristematic cells (compare Fig. 15 and 16).

In cross sections of leaves at stage III, the







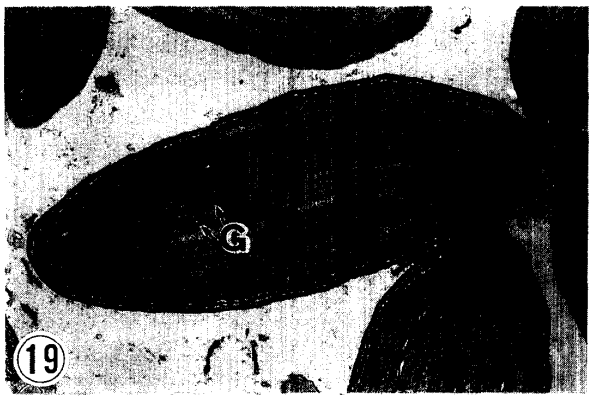


Table 1. Mean number of thylakoids per granum at different stages of leaf development in *Zea mays* and *Portulaca grandiflora*.

		Mean number of thylakoids/granum			
		Developmental stage			
		I	II	III	IV
Zea mays L.	MC[1]	3.0±0.1[3]	5.0±0.3	8.0±0.6	15.1±1.1
	BSC[2] (location)[4]	2.4±0.1 (0%)	2.5±0.1 (20%)	2.9±0.2 (25%)	2.8±0.1 (69%)
P. grandiflora	MC	2.7±0.5[5]	3.1±0.2	3.7±0.2	5.0±0.3
	BSC (location)		2.3±0.1 (9%)	2.1±0.1 (40%)	2.2±0.1 (98%)

1) MC=Mesophyll chloroplast.
2) BSC=Bundle sheath chloroplast.
3) S. E. of the mean.
4) The percentage of bundle sheath cells in which all chloroplasts observed were completely located centrifugally (*Zea mays*) or centripetally (*P. grandiflora*).
5) MC and BSC could not be determined.

Kranz anatomy was almost complete, and mesophyll and bundle sheath cells were distinct. At this stage bundle sheath cells were cytoplasmically dense and the chloroplasts were not centripetally located (Table 1). These chloroplasts had undeveloped grana as shown in Fig. 17, in contrast to mesophyll chloroplasts which contained some relatively well-developed grana (Fig. 18).

In mature leaves which were longer than 10 mm (stage IV), complete Kranz anatomy was observed and bundle sheath chloroplasts were arranged centripetally, which is characteristic of NADP-ME type C_4 dicots (Fig. 5 and Table 1). In bundle sheath chloroplasts, a few small grana composed of 2-3 thylakoids were observed (Fig. 19), while there were numerous well-developed grana in the mesophyll chloroplasts (Fig. 20).

Discussion

Our observations summarized in Table 1 indicate that the grana in bundle sheath chloroplasts were regulated to remain suppressed and bundle sheath chloroplasts were structurally distinguished from those of mesophyll in both *Zea mays* and *P. grandiflora* leaves from early in leaf development, when Kranz anatomy was not obvious.

With respect to cell ontogeny, it is known that the differentiation of bundle sheath cells correlates with biochemical sub-types of C_4 plants. In NADP-ME type of C_4 grasses, which generally have a single layer of sheath cells (no mestome sheath), it seems that bundle sheath cells are derived from the procambium, while mesophyll cells are derived from ground tissues[1,2]. In this respect, therefore, it is expected that bundle sheath and mesophyll cells will distinctively develop, and their chloroplasts also go through morphologically distinct processes. This seems to be consistent with our observations in *Zea mays*.

In addition, recent studies on immunolocalization of C_4 photosynthetic enzymes, or *in situ* hybridization of mRNA, indicate that the position-specific regulation of the photosynthetic roles of bundle sheath and mesophyll cells seems to occur early in leaf development, when the veins are already initiated and while the Kranz anatomy is not yet apparent[12]. Since it is assumed that the suppression of granal development in bundle sheath chloroplasts of the NADP-ME type is one of the position-specific expressions, our observations in *Zea mays* confirm these recent studies.

In NADP-ME type C_4 dicots, the ontogeny of bundle sheath cells and positional controls of C_4 gene expressions have not been studied in detail. As shown in Fig. 5, mestome sheath

cells were not observed in cross sections of *P. grandiflora* leaves. This may suggest that bundle sheath cells are derived from the procambium, a conclusion equivalent to that made in *Zea mays* above.

In general, it is known that grana have high photosystem II activity and generate NADPH as the reductive power. On the other hand, NADPH is generated from the process of decarboxylation of C_4 dicarboxylic acid in bundle sheath chloroplasts of NADP-ME type species[7]. Consequently, if bundle sheath chloroplast had well-developed grana, decarboxylation would be decreased by competetion for $NADP^+$ with grana generating NADPH. In this respect, the suppression of granal development in the bundle sheath chloroplast is essential for an NADP-ME type C_4 cycle, and therefore it could be speculated that the regulation of granal development must occur concurrently, with, or just before, the position-specific expression of C_4 photosynthetic enzymes.

It is assumed that the formation of Kranz anatomy, suppression of granal development and localization of bundle sheath chloroplast are important for the process of C_4 pathway, and hence a direct relationship among them is expected. In our observations, the suppression of granal development occurred when the Kranz anatomy was not completed and the localization of bundle sheath chloroplasts was not completed. From this, it is obvious that they have no relationship.

We are not certain if our observations reported here could be equated to those of Kirchanski[9] or Laetsch and Price[10]. In the present study, we could observe some relatively developed grana containing 4-5 thylakoids in bundle sheath chloroplasts at each developmental stage. However, the mean number of thylakoids per granum of bundle sheath chloroplasts were all less than 3 (Table 1), and it was easy to distinguish bundle sheath chloroplasts from those of the mesophyll. Therefore, it was concluded that the suppression of granal development in bundle sheath chloroplasts of NADP-ME type C_4 plants seems to occur from early in leaf development, when the Kranz anatomy is not yet apparent.

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