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Biochemical, Spectral and Structural Study of Olive Necrotic 8147 Mutant of *Zea Mays* L.

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With 7 figures

Summary

Comparative study between Olive Necrotic (ON) 8147 mutant and normal *Zea mays* L. chloroplasts revealed that the mutant had: (1) a lowered ratio of pigments (chlorophylls (Chl) and carotenoids) to proteins, (2) a higher ratio of pigment system I to II (based on the ratios of Ch *a* to *b*, pigment system I to II emission at 298 and 77° K, and the variable to constant fluorescence), (3) a higher saturation rate of electron flow (dichlorophenol indophenol, DCPIP, Hill reaction (light reaction II) and methyl viologen reduction with reduced dichlorophenol indophenol in the presence of dichlorophenyl dimethylurea (light reaction I), (4) a smaller photosynthetic unit (based on the ratio of Chl to P700, the reaction center of system I), and (5) a preponderance of intergranal lamellae. The relationship of photochemical characteristics to ultrastructure is emphasized.

Introduction

The genetic control and plastid biochemistry of several chlorophyll (Chl) deficient mutants of higher plants and algae have been described (see review in KIRK and TILNEY-BASSETT, 1967). The majority of these mutants are lethal or their photosynthetic rates are lower than that of the wild type. However, several viable Chl-deficient mutants of barley, soybean, etc., are known (see e.g., BOARDMAN and HIGHKIN, 1966; KECK et al., 1970; SCHMID, 1967). We have investigated a mutant of maize (ON 8147) which is necrotic but has high photosynthetic ability. As compared to normal maize, ON 8147 has higher saturation rates, on Chl and protein basis, of photosynthesis in leaf discs and of the light reactions (I and II) in isolated chloroplasts. Furthermore, ON 8147 has increased proportions of pigment system I relative to pigment system II; this has been shown only for the barley mutant (GOODCHILD et al., 1966). If the cause(s) of necrosis can be overcome in this mutant, proper

crosses could lead to the production of hybrids with agricultural significance. In this paper, we describe biochemical, spectral and structural studies of this maize mutant.

Materials and Methods

ON 8147 provided by the Plant Genetics Laboratory, University of Illinois, has a spontaneous nuclear recessive mutation located on chromosome 1 (R. J. Lambert, personal communication). The plants were grown for 10–15 days in a controlled temperature greenhouse in 16 hours light (21° C) and 8 hours darkness (16° C). Normal maize plants (single cross hybrid GSC 50) were grown under similar conditions. The mutant is pale green in color and necrotic symptoms appear in 10 to 15 days depending on the growth conditions.

For the preparation of chloroplasts from mutant plants, non-necrotic portions of the leaves were used. Eight g fresh weight of the mutant and 5 g of normal plants were separately ground in a chilled mortar with 25 ml of 0.02 M (Tris (Hydroxymethyl) Aminomethane-HCl) buffer (pH 7.8), containing 0.4 M sorbitol, 0.01 M NaCl and 6 mg/ml Carbowax 4000. The brei was filtered through eight layers of cheese-clooth, centrifuged at 200 × g for 1 minute to remove cell debris, and centrifuged again at 1000 × g for 8 minutes to pellet the chloroplasts. The chloroplast fraction was washed once in the homogenizing mixture and finally the chloroplasts (mainly mesophyll) were suspended in various buffers depending on the type of assay performed.

Absorption measurements of chloroplast suspensions were made with a Bausch and Lomb (Spectronic 505) recording spetrophotometer equipped with an integrating sphere. Total Chl and the individual concentrations of Chl *a* and *b* were determined in 80% acetone using the method of Arnon (1949). Protein content of washed chloroplast fragments was determined as described by Warburg and Christian (1942).

Photosynthesis of 0.5 cm (diameter) leaf discs, impregnated with 0.1 M carbonate-bicarbonate buffer, was measured as O_2 exchange with differential manometer (Emerson and Chalmers, 1955) at 20° C in saturating white light (2.5×10^6 ergs $\times$ $cm^{-2} \times sec^{-1}$).

Photosystem (PS) II activity, at 20° C, was followed spectrophotometrically using water as reductant and DCPIP as oxidant as described by Braun and Govindjee (1972). Photosystem I activity, in the presence of dichlorophenyl dimethyl urea, was measured at 20° C using $DCPIPH_2$ as electron donor and methyl viologen as acceptor; the oxygen uptake by reduced methyl viologen was monitored by a Clark type concentration electrode.

Light induced absorbance changes for cytochrome f and P700 were measured by a split-beam differential spectrophotometer (Sybesma and Fowler, 1968). The measuring beam obtained by a monochromator had a half band width (1/2 B. W. of 6.6 nm and 16.5 nm for cytochrome f and P700 respectively. Light-induced absorbance changes due to cytochrome f were measured at 422 nm (photomultiplier, Amperex 56 AVP protected by Corning filters C.S. 4–96 and C.S. 4–94; actinic beam (696 nm, 6.5 nm 1/2 B.W.; 5.2×10^4 ergs $\times$ $cm^{-2} \times sec^{-1}$). The P700 was measured at 703 nm (photomultiplier, Amperex 56 CVP protected by a 703 nm interference filter (1/2 B.W., 12.5 nm); actinic beam (480 nm, 11 nm 1/2 B.W., or 436 nm, 8 nm, 1/2 B.W.); 2.0×10^4 ergs $\times$ $cm^{-2} \times sec^{-1}$).

Fluorescence measurements were made with an instrument described by Shimony et al. (1967), the time course of fluorescence as detailed by Munday and Govindjee (1969), and 77° K fluorescence as outlined by Cho and Govindjee (1970). Emission spectra were scanned with a 1/2 B.W. of 6.6 nm, and are presented after correction for the spectral sensitivity of the photomultiplier (EMI 9558B) and the transmission efficiency of the analyzing monochromator. Chloroplasts suspension medium and other details of measurements are described in the legends of figures.

Electron micrographs were prepared as published by Paolillo and Reighard (1968).

Results and Discussion

1. Pigments and protein content

a) Analysis of leaves

The amount of total Chl in the mutant, on fresh weight basis, is one third that in normal leaves (Table 1). The ratio of Chl a/b, in acetone extracts of leaves, is 3–4 fold higher in the mutant. Although the amount of total carotenoids is 1.5 times more in the normal than in mutant plant, the ratio of carotenoids to total chlorophyll is about 2 times more in the mutant because of a greater deficiency in the chlorophylls.

Table 1: Pigment content and rate of photosynthesis in leaves of ON 8147 and normal maize.

Leaf Material	mg Total Chlorophylls / g Leaf Fresh Wt.	mg Total Carotenoids*) / g Fresh Wt.	Chl a/b	Total Carotenoids / Total Chlorophylls	Rate of Photosynthesis μmole O_2 evolved $\times$ mg^{-1} Chl $\times$ hr^{-1}
Normal maize	1.85	0.27	3.5 ± 0.5	0.15	100
ON 8147	0.62	0.18	11.4 ± 1.4	0.29	540

*) Mainly an estimate of β-carotene and lutein.

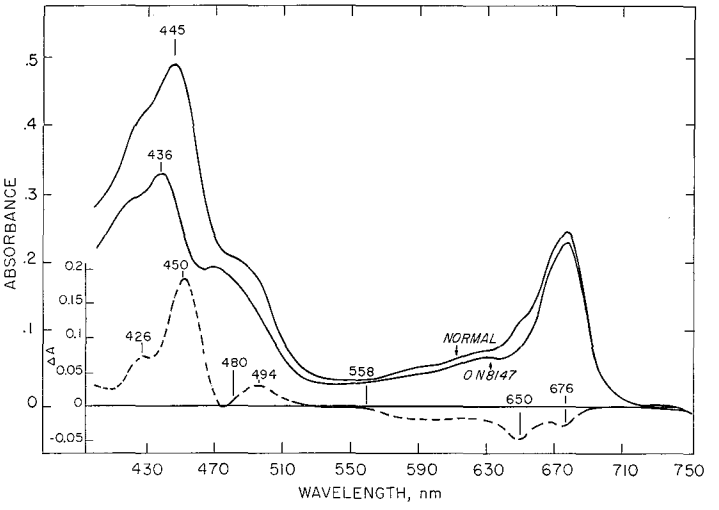

Fig. 1: Room temperature absorption spectra of chloroplast suspensions (normalized at 710 nm). Dashed curve depicts the difference absorption spectrum (mutant minus normal).

b) Absorption spectra of chloroplast suspensions

The ratio of absorbance at 678 nm (Chl *a*) and 650 nm (Chl *b*) is 2.3 and 3.1 in normal and mutant chloroplasts respectively (Fig. 1). A higher Chl *a*/*b* ratio in the mutant, shown here, confirms the data of Table 1. The difference absorption spectrum of mutant minus normal samples (dashed curve, Fig. 1) shows clearly that the mutant contains less Chl *b* and Chl *a* 673 (mainly system II pigments) relative to the long wave length forms of Chl *a* (mainly system I pigments). In addition, there appears to be more carotenoids in the mutant (represented by difference bands with peaks at 426, 450 and 494 nm) relative to Chl *a* and Chl *b*. This is in agreement with the higher ratio of carotenoids to chlorophylls (Table 1).

c) Protein content

The lamaellar proteins in chloroplast fragments of normal chloroplasts are 22 mg/mg Chl compared to 57 in ON 8147, the ratio of the latter to the former being 2.6.

2. Photochemical reactions

a) Photosynthesis of leaf discs

The rates of O_2 evolved $\times$ mg^{-1} Chl $\times$ hr^{-1} for mutant and normal leaf discs are 540 and 100, respectively (Table 1, last column). These rates are equivalent to 4.5 and 9.5 μmoles $\times$ mg^{-1} protein $\times$ hr^{-1} in normal and mutant leaf discs respectively. This suggests that the high saturation rates of photosynthesis in the mutant may be due to the presence of about twice as many (or twice as efficient) dark limiting enzymes. If the maximum photosynthetic rate in continuous light is equivalent to the inverse of the turn over time (k) times the concentration (C) of the limiting enzyme (see Kok and Cheniae, 1966), one has to postulate either a high concentration of C (*i.e.*, a smaller photosynthetic unit, PSU) or a shorter turnover time in the mutant. Data presented later in this paper suggest that the mutant contains a smaller PSU.

b) Photosystem II activities of isolated chloroplasts

The rate of reduction of DCPIP saturated around 20×10^5 and 6×10^5 ergs $\times$ cm^{-2} $\times$ sec^{-1} (incident white light) in mutant and normal chloroplasts respectively (Fig. 2). At 30×10^5 ergs $\times$ cm^{-2} $\times$ sec^{-1} of incident white light, the rate of DCPIP reduction was 412 and 186 μmoles $\times$ mg^{-1} Chl $\times$ hr^{-1} for mutant and normal chloroplasts, respectively. The ratio of the rate of DCPIP reduced in mutant to that in normal chloroplasts is 2.2; this ratio, however, decreases to one at 1×10^5 ergs $\times$ cm^{-2} $\times$ sec^{-1}. At intensities below 1×10^5 ergs $\times$ cm^{-2} $\times$ sec^{-1}, the quantum efficiency of the DCPIP Hill reaction in the mutant should be lower than in normal chloroplasts because of the presence of less chlorophyll per photosynthetic unit (i.e., the probability of absorption within one unit is lower; see section 3 below) in the former. The saturation rates of pigment system (PS) II activities

of mutant and normal maize chloroplasts, expressed on the basis of chlorophyll in PS II, are calculated to be 1442 and 372 μmoles $\times$ Chl $a_{II}^{-1} \times hr^{-1}$ respectively. The ratio of these rates is about 4.4. However, the saturation rates of PS II per mg total protein per hour were calculated to be 7.2 for ON 8147 and 7.0 for normal maize. If we assume that the proteins are distributed in the two systems in the same way as chlorophylls, then the system II activity per mg protein of system II would be about twice as high in the mutant compared to normal.

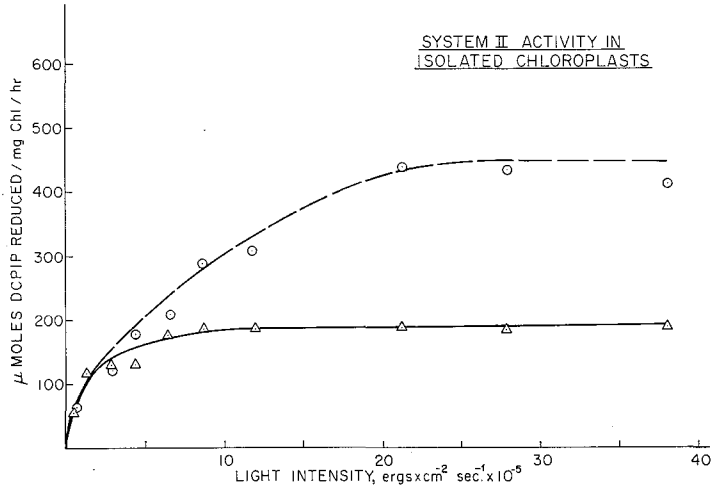

Fig. 2: Light intensity curves for the reduction of DCPIP by isolated chloroplasts (i.e., PS II activities) of mutant (–⊙–) and normal (–△–) plants. The reaction mixture contained (in 3 ml) chloroplasts containing 10 μg chlorophyll and (in μmoles): phosphate buffer (pH 7.2), 50; NaCl, 10; $MgCl_2$, 0.003; DCPIP, 0.048. The reaction rates were measured by the decrease in absorbance at 603 nm after illumination for 30 sec.

c) Photosystem I activities of isolated chloroplasts

At light saturation (incident intensity, 4×10^5 ergs $\times$ $cm^{-2} \times sec^{-1}$ of white light), the rate of oxygen consumption by photoreduced methyl viologen in mutant chloroplasts was 486 μeq $\times$ mg^{-1} Chl $\times$ hr^{-1} compared to 80 μeq $\times$ mg^{-1} Chl $\times$ hr^{-1} for normal chloroplasts (Fig. 3). The ratio of these rates of PS I of the mutant and of normal chloroplasts is about 6.0. However, this ratio decreases to 1 at about 1×10^5 ergs $\times$ $cm^{-2} \times sec^{-1}$. The decrease in ratio, as the light intensity was lowered, suggests that the quantum efficiency may be lower in the mutant than in normal chloroplasts, at intensities below 1×10^5 ergs $\times$ $cm^{-2} \times sec^{-1}$.

The ratio of the saturation rates of PS II expressed as DCPIP reduced per total chlorophyll of mutant and normal chloroplasts was 2.2 compared to a ratio of 6.0 for rates of PS I of mutant and normal maize. The discrepancy between the values of these ratios disappears if rates of PS I and PS II activities are expressed per

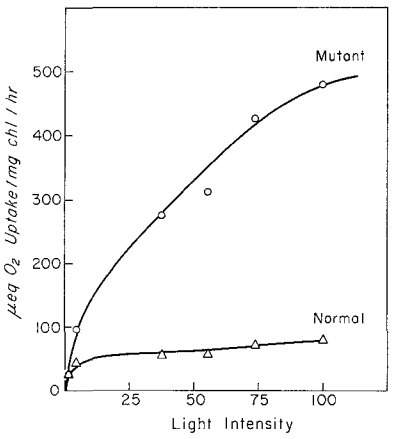

Fig. 3: Light intensity curves for the reduction of methyl viologen (i.e., PS I activity) in isolated chloroplasts. The reaction mixture contained (in 4 ml) chloroplasts containing 40 μg chlorophyll, and (in mM): TES buffer (pH 7.3), 50; Sucrose, 0.2; Methyl viologen, 0.01; DCPIP, 0.1; Ascorbate, 3; DCMU, 0.01. The reaction rates were determined by measuring O_2 uptake by reduced methyl viologen. The 100 % intensity corresponds to 4×10^5 ergs $\times$ $cm^{-2} \times sec^{-1}$.

chlorophyll in each system. In the mutant, there is about two and a half-fold more pigments in PS I relative to PS II. The saturation rates of PS I per chlorophyll of PS I are 680 μeq of O_2 consumed per hr, and 160 μeq O_2 consumed per hr for ON 8147 and normal maize respectively. The ratio of these rates (mutant/normal) is about four, similar to the ratio of rates of PS II, expressed on the basis of chlorophyll in PS II. The saturation rates of PS I per mg total protein are calculated to be 8.0 μeq $\times$ mg^{-1} protein $\times$ hr^{-1} and about 3.6 μeq $\times$ mg^{-1} protein $\times$ hr^{-1} for mutant and normal chloroplasts respectively. If we make the same assumption, as we made earlier, that the distribution of proteins between the two systems follows that of chlorophyll, then the system I activity per mg protein is about 1.5 times higher in the mutant than in the normal.

3. Estimation of the size of photosynthetic unit (PSU)

It was suggested earlier that in order to account for high photosynthetic saturation rates per mg Chl in the mutant, a smaller PSU, or faster turnover rate of dark enzymes (or both) compared to normal maize had to be postulated. To estimate the size of the PSU in mutant and normal chloroplasts, the concentrations of Chl/P 700 and Chl/Cyt f were measured (Table 2). In normal chloroplasts, the Chl/P 700 is 490 and in the mutant 250. Thus, PSU in the mutant is about half as large as in normal chloroplasts calculated on the basis of P 700. If, however, the size of PSU is expressed on the basis of Chl *a* of system I only, we estimate it to be 250 for normal

Table 2: Number of chlorophyll molecules per P700 and Cyt f in chloroplasts of ON 8147 and normal maize.

Chloroplasts	Chl/P700	Chl/Cyt f
Normal	490	450
ON 8147	250	113

and 160 for the mutant. The Chl/Cyt f content is 450 and 113 in normal and mutant chloroplasts respectively. Here, again, the estimate of the size of PSU based on chlorophylls of system I is 225 for normal and 75 for the mutant. The ratio of Chl/Cyt f in normal to that in mutant chloroplasts is about 3–4. Since the size of PSU in the mutant, measured on the basis of Cyt f, is smaller than that measured on a P 700 basis, there must be either a larger pool of Cyt f, or the rate of turnover of Cyt f (reoxidation by PS I) is faster than in normal maize. Thus, a higher light induced change of Cyt f relative to P 700, per Chl, would be observed. No distinction could be made between the two cases at this stage.

4. Fluorescence studies

a) Time courses of chlorophyll fluorescence

The time course of Chl *a* fluorescence yield of normal chloroplasts shows the typical fluorescence induction upon excitation with broad-band blue light (absorbed in both pigment systems): an initial F_0 followed by a biphasic rise to F_∞ (Fig. 4). In the mutant, fluorescence rises to a very low F_∞ level. The percent of variable to total fluorescence $[(F_\infty - F_0/F_{tot})]$ is 63 in normal compared to 31 in mutant chloroplasts, whereas the relative quantum yield of variable to constant fluorescence $[(F_\infty - F_0)/F_0)]$ is 1.69 and 0.46 in normal and mutant respectively. The ratio (F_∞/F_0) is 2.7 in normal compared to 1.45 in mutant chloroplasts.

Our data show clearly that variable fluorescence in the mutant is one-third that in normal chloroplasts. The rise in Chl *a* fluorescence yield of isolated chloroplasts on illumination has been attributed to the photoreduction of an electron acceptor Q by system II (DUYSENS and SWEERS, 1963). A decreased variable fluorescence in mutant chloroplasts could be explained as follows: In the mutant, less pigments are present in PS II relative to PS I (see Figs. 1 and 5, and Table 3); this causes an unequal distribution of quanta between PS I and PS II and the rate of QH reoxidation by PS I is faster than its formation, thus keeping a considerable part of the primary pool of oxidants (Q) in its oxidized state.

b) Relative chlorophyll fluorescence yields of chloroplasts

The ratio of the relative fluorescence yields at 685 nm (mainly system II) of normal to mutant chloroplasts was 1.54 (λ excitation, 430 nm; 1/2 B.W., 5 nm) (see Table 3). However, the same ratio measured at 710 nm (mainly system I) was 1.0.

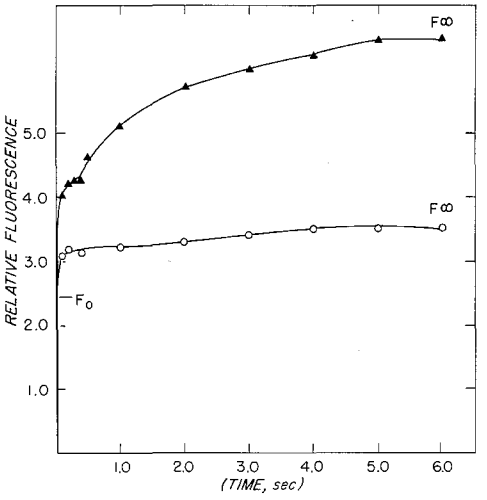

Fig. 4: Time course of chlorophyll fluorescence of chloroplasts from ON 8147 (–○–) and normal (–▲–) plants (normalized at Fo). Chloroplasts were suspended in 0.02 M tris-HCl (pH 7.2); 0.4 M sorbitol and 10 mM NaCl to give a chlorophyll concentration of 1.5 μM. Incident light intensity (broad-band blue light; 1.2 B.W., 120 nm), 2×10^5 ergs $\times$ cm^{-2} $\times$ sec^{-1}.

Table 3: Relative fluorescence yield of Chl at room temperature.

Source of Chloroplasts	Exciting wavelength, nm	Relative fluorescence yield at 685 nm	Relative fluorescence yield at 710 nm	F685/F710
Normal	430*)	57	10	5.7
ON 8147	430	37	10	3.7

*) Half band width, 5 nm.

The ratio of the relative fluorescence yields at 685 nm and 710 nm is 3.7 in mutant compared to 5.7 in normal chloroplasts. These results are also explained by assuming that in the mutant, there are relatively more pigments of PS I relative to PS II in agreement with the interpretation given above for lower variable fluorescence in the mutant than in normal chloroplasts.

c) Emission spectra of chlorophyll at at 77° K

The ratio of fluorescence at 735 nm (mainly system I) to 685 nm (mainly system II) at 77° K was 1.5 in normal and 3.2 in mutant chloroplasts (Fig. 5). The differences confirm that there are more pigments of PS I relative to pigments of PS II in the mutant than in normal maize. (Due to the use of wider band widths here, the F 696 band was not resolved; its presence was confirmed by using narrower band

widths in the analyzing monochromator. For a discussion of the assignment of fluorescence bands to pigment systems, see MOHANTY et al., 1972.)

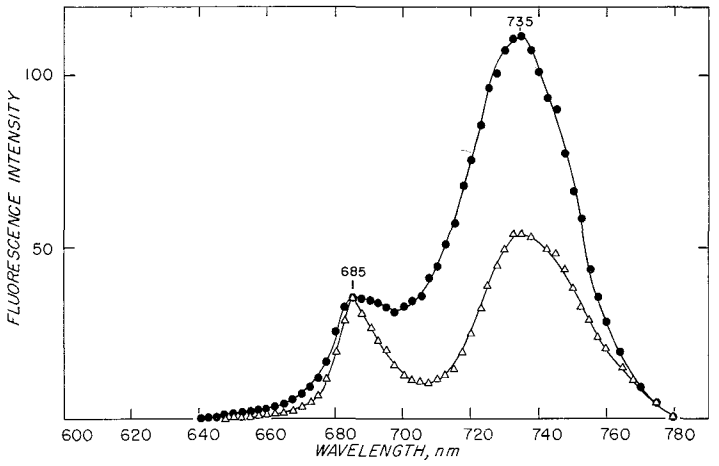

Fig. 5: Fluorescence emission spectra at 77° K of chloroplasts from ON 8147 (–●–) and normal (–△–) plants. Excitation wavelength, 440 nm; light intensity, 6×10^5 ergs$\times$cm$^{-2}\times$ sec^{-1}; 0.01 absorbance cm^{-1} at 678 nm.

5. Fine Structure of Plastids

The fine structure of plastids in the ON 8147 mutant was studied in an attempt to correlate the structural and the functional aspects observed in this study. The phenotype of mesophyll plastids in the ON 8147 mutant approaches normal, immature maize plastids in its most normal extreme (Fig. 6) while it is clearly abnormal in its most affected extreme (Fig. 7). Bundle sheath plastids in ON 8147 resemble normal bundle sheath plastids.

The mesophyll plastids of the primary leaves of ON 8147 in the most normal extreme (Fig. 6) have widely spaced grana stacks of ca. 2 to 6 compartements. The grana are loosely connected by intergranal membranes, or frets, and the compartements of a granum are interconnected at the margins. In the more abnormal plastids, small lamellar profiles are numerous. Grana are more widely spaced and diverse in size and shape. In the profiles, one observes regions where the lamellae seem to come together at a focus. In the extreme cases, there are few grana of any significance (Fig. 7). Other peculiarities in plastids of ON 8147 include a wide spacing of grana of normal size joined by numerous parallel frets, and accumulations of tubular or spherical vesicles that are not prolamellar bodies.

The mesophyll plastids of ON 8147 seem to have some resemblances to plastids in the Chl *b*-less mutant of barley (GOODCHILD et al., 1966), Su/su mutant of tobacco (SCHMID and GAFFRON, 1966), Chl-defficient mutant of pea (HIGHKIN et al., 1969)

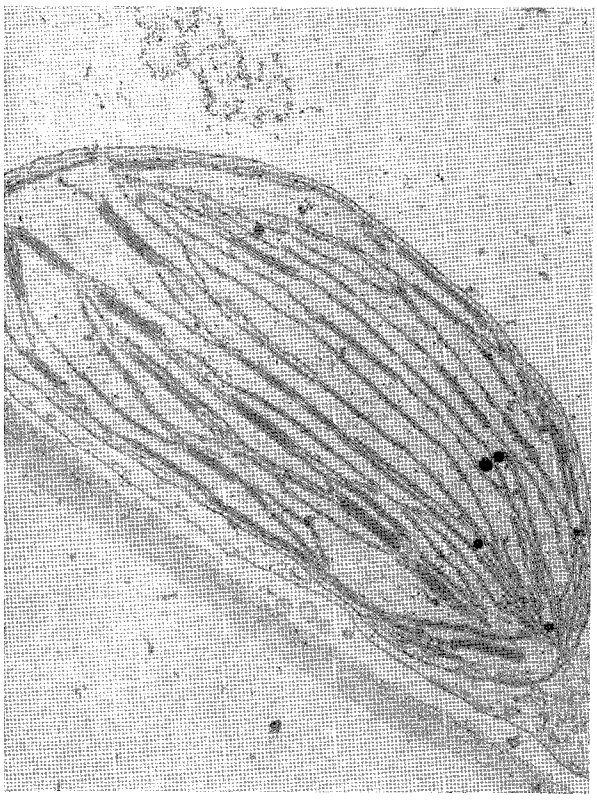

Fig. 6: Electron micrograph of the most nearly normal extreme in mesophyll chloroplasts of ON 8147 of maize. ×16,500.

and the heterozygous light green (LG) mutant of soybean (Keck and Dilley, 1970). Compared to the wild types, the chloroplasts of these mutants, in general, exhibit a reduced number of lamellae per grana, and a significant increase in single, unstacked lamellae. Associated with such structural changes these mutants also show higher Chl a/b ratios than wild types. However, only in the ON 8147 mutant of maize and the barley mutant the two photosystems are altered with an increase in pigments of PS I relative to pigments of PS II.

Recently, several lines of evidence support the idea of PS I (not PS II) being associated with stroma lamellae of chloroplasts (see Park and Sane, 1971). In ON 8147, the high Chl a/b ratio, the lower fluorescence yield at 685 nm relative to 710 nm, the low level of variable fluorescence, and the high F735/F685 at 77° K – all indicative of high PS I activities – are associated with a structure of plastid with a greater proportion of stroma to grana lamellae. Thus, our data further support the idea that PS I (not PS II) is associated with stroma lamellae of chloroplasts.

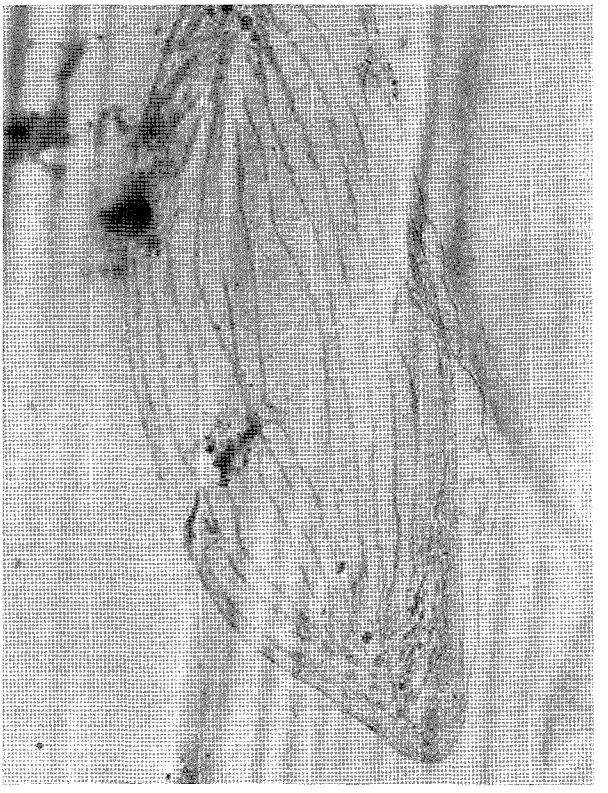

Fig. 7: Electron micrograph of a representative abnormal extreme in mesophyll chloroplasts of ON 8147 of maize. ×19,000.

6. *Concluding remarks*

The mutant ON 8147 of maize, having a spontaneous nuclear recessive mutation on chromosome 1 (R. J. LAMBERT, personal communication), is affected in its photochemical apparatus. This includes changes in the (1) *pigment composition:* 70 % lower chlorophyll content, 30 % lower carotenoid content, a twice greater ratio of carotenoids to chlorophylls, a three fold higher ratio of chlorophyll *a* to chlorophyll *b*, and preponderance of the weakly fluorescent pigment system I (as evidenced by lower ratio of variable to constant fluorescence, higher ratio of the far-red emission (F710–F735) to red emission (F685) both at 298 and 77° K, and lower relative fluorescence yield); (2) *photosynthetic saturation rates:* a five fold higher rate of O_2 evolved, a two fold higher rate of system II ($H_2O \rightarrow$ DCPIP) reaction, and a six fold higher rate of system I ($DCPIPH_2 \rightarrow$ Methyl viologen) reaction – all on Chl basis (3) *photosynthetic unit:* a two-fold smaller photosynthetic unit (number

of chlorophyll molecules per reaction center P700); and (4) *ultrastructure:* widely spaced grana stacks of ca. 2–6 compartments with abundant intergranal lamellae. A comparison of compositional and functional characteristics of ON 8147 shows that (a) the smaller photosynthetic units are associated with high saturation rates of photosynthetic activity, and (b) preponderance of stroma lamellae is associated with preponderance of pigment system I.

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