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Phosphorylation by intact bundle sheath chloroplasts from maize

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SUMMARY

A method is described for the isolation of intact bundle sheath chloroplasts and intact mesophyll chloroplasts from maize leaves. Bundle sheath chloroplasts gave rates of non-cyclic phosphorylation which were 11–19% of the rates obtained with mesophyll chloroplasts. Both types of chloroplasts gave high rates of cyclic phosphorylation with phenazine methosulphate as cofactor. Hill reaction rates of uncoupled bundle sheath chloroplasts were 15% of uncoupled mesophyll chloroplasts at 40 000 lux and 26% at 118 000 lux with 2,3′,6-trichlorophenolindophenol as oxidant. Using ferricyanide as oxidant the corresponding values were 9% and 12%, respectively.

Previously[1], a differential grind procedure was described for the separation of mesophyll chloroplasts and bundle sheath chloroplast fragments from the leaves of some plants with the C_4-dicarboxylic acid pathway of photosynthesis. The agranal bundle sheath chloroplast fragments of *Sorghum bicolor* and *Zea mays* were found to be deficient in Photosystem II as compared with their respective granal mesophyll chloroplasts[1–3].

A deficiency of reducing power normally generated by Photosystem II creates a problem for the reduction of 3-phosphoglycerate in the bundle sheath chloroplasts. It has been suggested that the reducing ability of these chloroplasts may come from the oxidative decarboxylation of malate transported from the mesophyll chloroplasts[4–6]. It thus seems a possibility that the prime function of the agranal bundle sheath chloroplasts of C_4 plants is the production of ATP by cyclic electron flow.

The possibility existed that in our earlier experiments[1–3] the harsh blending treatment used to liberate the bundle sheath chloroplast fragments from the bundle sheath cells may have selectively inactivated Photosystem II or removed some of its components. In the present work, we have therefore developed a method for the isolation of both intact bundle sheath and mesophyll chloroplasts from *Zea mays* and compared their capacity for Hill reaction and non-cyclic and cyclic photophosphorylation. The bundle sheath

Abbreviations: PMS, phenazine methosulphate; TCIP, 2,3′,6-trichlorophenolindophenol; DCMU, 3-(3-chlorophenyl)-1,1-dimethylurea.

chloroplasts gave high rates of cyclic phosphorylation with phenazine methosulphate (PMS), but they were relatively inactive in non-cyclic phosphorylation. This result also supports our earlier conclusion[1–3] that the agranal bundle sheath chloroplasts are deficient in Photosystem II as compared with the mesophyll chloroplasts. Since the activities of the intact maize bundle sheath chloroplasts are comparable with maize bundle sheath chloroplast fragments isolated by the Mill treatment used in previous studies[1–3], we feel that the latter isolation procedure has not led to an artifactual production of Photosystem II deficiencies in the agranal bundle sheath chloroplasts of maize and sorghum.

Seedlings of *Zea mays* L. (Var. NES 1002) were grown either in a controlled glasshouse (30° day/25° night) of CSIRO Phytotron or in an artificially lit cabinet (16 h day at 3000 ft candles and 30°; 8 h night at 25°) for 9–14 days. Pre-chilled leaves (10 g) were cut into strips (2–3 mm wide) in a small dish containing 100 ml of isolation medium [30 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane sulphonate buffer, pH 7.4 (TES buffer), 0.33 M sorbitol, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM dithiothreitol and 0.5% bovine serum albumin] and blended with 1 g of Polyclar AT (insoluble polyvinylpyrrolidone from General Aniline and Film Corp., Dyestuffs and Chemical Division, New York) in the Servall Omnimixer for 4 sec at 38% of line voltage. The brei was filtered through two layers of

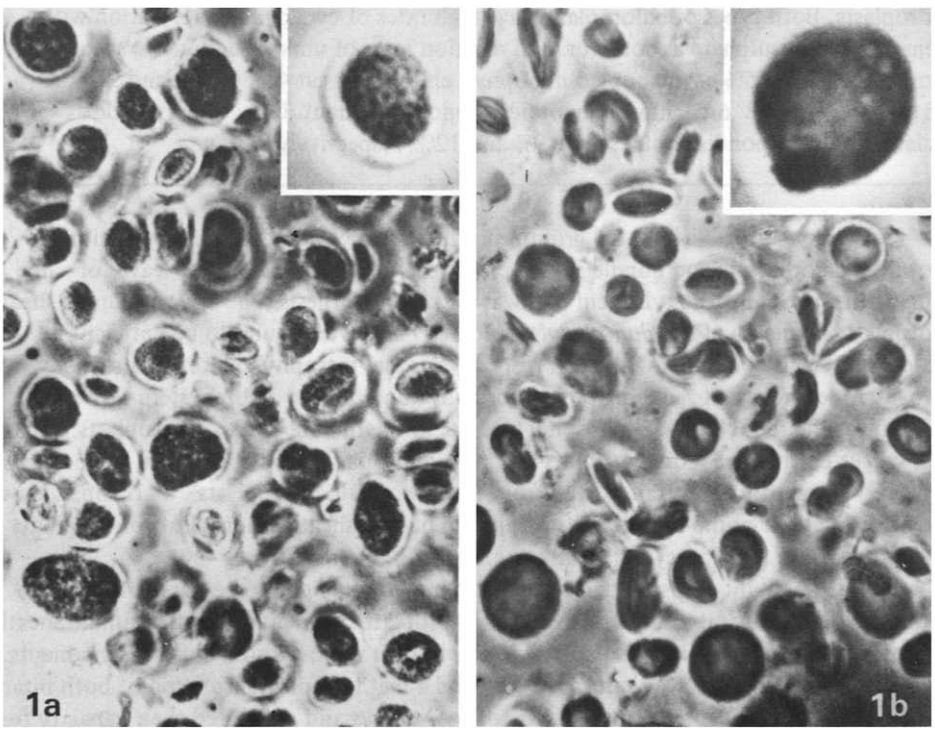

Fig. 1. Photomicrographs of maize mesophyll (1a) and bundle sheath (1b) chloroplast preparations taken under phase contrast. Each insert shows a more magnified view of a single chloroplast of each type viewed at right angles to the plane.

Miracloth, the filtrate centrifuged for 30 sec at 300 × *g* and the supernatant centrifuged for 10 min at 1000 × *g* to yield mesophyll chloroplasts.

The residue from the Miracloth was resuspended in 50 ml of isolation medium and blended for 1 min at 75% of line voltage. The brei was filtered through Miracloth, washed with isolation medium and the residue examined by light microscopy. At this stage the residue consists of long strands of bundle sheath cells attached to lengths of vascular tissue with some contaminating mesophyll cells. A second blend (0.25–1 min) was performed to remove these mesophyll cells, but it is essential that the blending time be kept to a minimum to ensure that the vascular strands are not broken into fragments which are too short for the subsequent chop procedure. The bundle sheath cell residue was carefully washed with isolation medium.

For the isolation of intact bundle sheath chloroplasts (Procedure A), the residue was placed in a cooled, flat-bottomed plastic dish, moistened with isolation medium and chopped with a single razor blade, driven mechanically in a reciprocating motion. The brei was rinsed with isolation medium, and the green liquid filtered through two layers of Miracloth, the residue was chopped once more and the combined filtrate centrifuged for 5 min at 1000 × *g*.

For the isolation of bundle sheath chloroplast fragments (Procedure B) the bundle sheath cell residue was ground for 1 min in a Janke Kunkle Mill and the fragments isolated as described previously[1].

Maize bundle sheath chloroplasts isolated by the chopping Procedure A are readily distinguishable from mesophyll chloroplasts by phase contrast microscopy (Fig. 1). Identification of both types of chloroplasts is most conveniently made when they are free of starch. Under these conditions, the bundle sheath chloroplasts have regular outlines and are characterized by a fairly uniform, pale green internal matrix lacking any resolvable substructure when viewed at right angles to the plane. In contrast mesophyll chloroplasts contain many grana which are easily resolved under phase contrast. Both types of chloroplasts if allowed to swell on the microscope slide reveal a completely enclosing membrane. This is taken as evidence that at the time of isolation such chloroplasts were intact at least to the extent that their outer membrane has been retained. Thus, they would correspond to the Class I category of Spencer and Wildman[7]. Visual examination of bundle sheath chloroplasts indicated contamination with mesophyll chloroplasts in the range from 2 to 10%. The mesophyll chloroplast preparations contained up to 15% bundle sheath chloroplasts. The latter arise from the inevitable rupture of some bundle sheath cells during the first brief blending (4 sec).

Intact bundle sheath chloroplasts from *Zea mays* had a chlorophyll *a*/*b* ratio of 5.39 (average of 7 experiments) which did not differ significantly from the chlorophyll *a*/*b* ratio of 5.26 of bundle sheath chloroplast fragments isolated in the Janke Kunkle Mill. Maize mesophyll chloroplasts had an average chlorophyll *a*/*b* ratio of 3.20 and leaf extracts gave a ratio of 3.63.

Hill reaction activities are shown in Table I. High rates of photoreduction of 2,3′,6-trichlorophenolindophenol (TCIP) and ferricyanide were obtained with mesophyll chloroplasts, and these were increased 2–3-fold in the presence of the uncoupler, methylamine. Coupled rates were already saturated at 20 000 lux and uncoupled rates at 40 000–50 000 lux[8]. Hill activities of the bundle sheath chloroplasts were low in

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TABLE I

HILL REACTION ACTIVITIES OF MAIZE CHLOROPLASTS

The chloroplasts were washed once in suspension medium (10 mM phosphate buffer, pH 7.4, 0.33 M sorbitol, 1 mM $MgCl_2$, 5 mM dithiothreitol and 0.5% bovine serum albumin) and resuspended in suspension medium *minus* dithiothreitol. For TCIP reduction, the reaction mixture (3 ml) contained chloroplasts (10 μg chlorophyll) and (in μmoles): Tris–HCl buffer, pH 7.8, 40; NaCl, 70; TCIP, 0.06; and methylamine hydrochloride (MA), 30. For ferricyanide reduction, the reaction mixture (3 ml) contained chloroplasts (20–25 μg chlorophyll) and (in μmoles): Tris–HCl buffer, pH 8.0, 40; NaCl, 70; $MgCl_2$,10; $K_3Fe(CN)_6$, 1; and MA, 150. Hill reactions were assayed as described previously with white light illumination[2].

Chloroplasts	*Light intensity (lux × 10^{-3})*	*TCIP reduction (μmoles/mg chlorophyll per h)*		*Ferricyanide reduction (μmoles/mg chlorophyll per h)*	
		–MA	*+MA*	*–MA*	*+MA*
Mesophyll	40	190	417	198	619
Intact bundle sheath	40	63	63	55	55
Bundle sheath fragments	40	65	66	93	93
Intact bundle sheath	118	109	111	70	76
Bundle sheath fragments	118	130	142	147	166

comparison with mesophyll chloroplasts and were not influenced by methylamine (Table I). The Hill reaction in bundle sheath chloroplasts required higher intensities for saturation (Table I). As reported elsewhere[8], intact bundle sheath chloroplasts were saturated at 50 000–90 000 lux and the bundle sheath fragments at 90 000–118 000 lux. Activities of the bundle sheath chloroplast fragments prepared by the harsher treatment in the Mill (Procedure B) were higher than those of the intact bundle sheath chloroplasts. The activities of maize bundle sheath chloroplasts were almost double those observed previously with sorghum bundle sheath chloroplast fragments, whereas the activities of the maize mesophyll chloroplasts were about 20% lower than those of sorghum mesophyll chloroplasts[2]. It seems that maize bundle sheath chloroplasts which are agranal except for occasional regions of appressed lamellae contain more Photosystem II than do the more completely agranal sorghum bundle sheath chloroplasts. On present evidence, however, there is no direct correlation between Photosystem II activity and the extent of appressed lamellae.

Rates of photophosphorylation by maize chloroplasts are shown in Table II. The mesophyll chloroplasts gave good rates of non-cyclic phosphorylation with ferricyanide as oxidant; P/e_2 ratios were in the range of 0.7 to 1.0 and the phosphorylation was completely inhibited by 3-(3-chlorophenyl)-1,1-dimethylurea (DCMU). Both intact bundle sheath chloroplasts and fragments gave much lower rates of non-cyclic phosphorylation, which were 11–19% of the rates with mesophyll chloroplasts. In contrast, the bundle sheath chloroplasts were capable of high rates of cyclic phosphorylation with PMS as cofactor and the intact chloroplasts were slightly more active than mesophyll chloroplasts. The bundle sheath chloroplasts fragments were less active in cyclic phosphorylation than the intact chloroplasts.

In view of the postulated role of cyclic phosphorylation for agranal bundle sheath chloroplasts of C_4 plants, we tested the ability of maize bundle sheath chloroplasts to perform endogenous cyclic phosphorylation. Although the rates were very low compared with PMS-mediated phosphorylation it does seem that the isolated bundle sheath chloroplasts are capable of an endogenous phosphorylation, which is insensitive to DCMU (5–25 μmoles ATP per mg chlorophyll per h). At present, we are investigating conditions

TABLE II

RATES OF PHOTOPHOSPHORYLATION BY MAIZE CHLOROPLASTS

The reaction mixture (3 ml) contained (in μmoles): Tricine buffer, pH 8.0, 100; NaCl, 40; $MgCl_2$, 12; sodium, potassium phosphate, pH 7.8 (containing 10^5–10^6 counts/min of $^{32}P_i$), 12; ADP, 12; and the cofactors PMS, 0.1 or $K_3Fe(CN)_6$, 3; and chloroplasts containing 30–50 μg chlorophyll. Standard reaction conditions were: illumination time, 4 min; gas phase, air; temp., 20°; light intensity, 80 000 lux. Reactions were terminated by the addition of trichloroacetic acid to a final concn. of 3% and $^{32}P_i$ incorporation into ATP was measured by the method of Avron[9].

Chloroplasts	*μmoles ATP per mg chlorophyll per h*	
	PMS	*$K_3Fe(CN)_6$*
Mesophyll	520	96
Intact bundle sheath	537	14
Bundle sheath fragments	443	15

of incubation in an attempt to improve these rates.

The method described here for the isolation of intact bundle sheath chloroplasts from maize is not suitable for *Sorghum bicolor*. A longer blending time is needed to release the mesophyll chloroplasts from sorghum, and this disrupts the vascular strands into fragments which are too small for the chop procedure. However, Polya and Osmond (in preparation) have shown that bundle sheath chloroplast fragments prepared from *Sorghum bicolor* by the Mill procedure are active in cyclic phosphorylation with PMS as cofactor, but not in non-cyclic phosphorylation with ferricyanide as oxidant.

The present studies with intact bundle sheath chloroplasts from maize support our earlier conclusion, based on work with chloroplast fragments[1–3], that the bundle sheath chloroplasts of some C_4 plants are deficient in Photosystem II, as compared with mesophyll chloroplasts.

The apparent discrepancies between our photochemical data and those of Bishop *et al.*[10] and Smillie *et al.*[11] arise from the much lower absolute rates for Hill reaction activities reported by them for maize mesophyll chloroplasts with TCIP and ferricyanide as oxidants and the consequent higher *relative* rates in the bundle sheath chloroplasts.

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