A Comparison Based on Delayed Light Emission and Fluorescence Induction of Intact Chloroplasts Isolated from Mesophyll Protoplasts and Bundle-Sheath Cells of Maize

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Chloroplasts or cells from maize (*Zea mays*) bundle sheath show a very low intensity of delayed light emission compared with mesophyll protoplasts or chloroplasts. The bundle-sheath chloroplasts retain only the fast (less than 1 ms) component of the emission. They also fail to show fluorescence induction in contrast to the mesophyll, which behaved normally. The mesophyll material could be made to resemble the bundle-sheath chloroplasts in respect of both phenomena by adding to it 1-(3',4'-dichlorophenyl)-3,3-dimethyl-urea and hydroxylamine, together. It is concluded that Photosystem II, that gives rise to both effects, is not active in the bundle sheath but may be present in an inhibited form.

Maize is a 'C₄' plant and shows Kranz anatomy; there are sheaths of cells containing agranal chloroplasts surrounding the vascular bundles of the leaf, and these in turn are surrounded by mesophyll tissue that contains granal chloroplasts. Our understanding of the C₄ photosynthetic process is based on the movement of carbon in the form of C₄ acids from the mesophyll to the bundle sheath. In the case of maize the C₄ acid is malate, which may supply not only CO₂ for assimilation, but also reducing equivalents, in which case the bundle sheath would be relieved of the need to carry out non-cyclic electron transport. This would avoid a high concentration of oxygen being produced at the point of CO₂ assimilation, where it would compete for the same enzyme. Whether in fact the bundle-sheath cells do possess the operational Photosystem II necessary for non-cyclic electron transport is in dispute.

There is an extensive literature, which shows some disagreement over the Photosystem-II capability of bundle-sheath chloroplasts. Woo *et al.* (1970) reported that NADP⁺ reduction did not take place in the bundle sheath, but did do so in the mesophyll chloroplasts. Smillie *et al.* (1972) presented evidence that both photosystems were in fact present, but that, in isolated chloroplasts, exogenous plastocyanin was required for their connection; also that Photosystem II required a higher light intensity. Anderson *et al.* (1972) showed that the photosynthetic unit (chlorophyll molecules per pigment P700) in

Sorghum (*Sorghum bicolor*) bundle-sheath chloroplasts was half the size of that in the mesophyll, and argued that the deficiency of Photosystem II in this plant and in maize was due to the lack of the pigment assembly. Photosystem-II photoreactions could be observed as reduction of indophenol dyes or ferricyanide, but at low activity.

Mesophyll protoplasts and bundle-sheath cells can now be prepared by a method using successively mechanical and enzymic disintegration (Rathnam & Edwards, 1976; Bialek *et al.*, 1977). Contamination of bundle sheath by mesophyll chloroplasts was estimated by electron microscopy at $3 \pm 2\%$, better than previous methods allowed. Horváth *et al.* (1978) found no O₂ evolution from bundle-sheath cells or chloroplasts, nor could they observe the absorbance change at 515nm related to energy-conserving Photosystem-II reactions. Mesophyll chloroplasts were normal in both respects. However, by using the older technique of differential grinding, these workers did observe oxygen evolution at rates of up to 23% of the mesophyll activity.

Delayed light emission has been used to investigate Photosystem II in bundle-sheath chloroplasts by Mayne *et al.* (1975), and they found that the ratio of the intensities of delayed light in mesophyll to bundle-sheath chloroplasts was 5.9:1 in maize, and varied from 2.9:1 to 15:1 in other C₄ plants of this group. They found no difference in the decay characteristics of the delayed light from the two sources,

however, and therefore the question of contamination must be considered.

It has been established [see Malkin (1977) for a review] that the millisecond-delayed light emission from chloroplasts originates from the back-reaction of the products of the primary reactions at the Photosystem-II reaction centre. The reaction centre involves a specific chlorophyll complex and an acceptor, termed 'Q' (after Duysens & Sweers, 1963), because the extent of oxidation of Q governs the extent of quenching of fluorescence. If the chlorophyll-Q complex is absent, there will be no delayed light emission, nor any variation in fluorescence. If the complex is intact and functioning, at least one component of the delayed light emission should be seen.

The present study is a re-examination of these two phenomena, making use of the very pure bundle-sheath and mesophyll chloroplasts now available.

Materials and Methods

Plant material

Seedlings of maize (*Zea mays* L. convar. KSC 360) were grown in a greenhouse for 10–12 days. Leaves were harvested in the morning in order to obtain chloroplasts depleted of starch.

Isolation of protoplasts and intact chloroplasts from the mesophyll tissue

The lower epidermis of the leaves was peeled off with a scalpel and floated on the surface of an enzyme solution containing 0.6M-D-sorbitol, 0.1M-KH₂PO₄, 0.25% Macerozyme R-10, 1.0% cellulase Onozuka R-10 and 0.5% potassium dextran sulphate, adjusted to pH 5.8, and incubated for 1.5 h at 37°C. The resulting protoplasts were washed three times in 0.6M-D-sorbitol and transferred into a suspending medium containing 0.4M-D-sorbitol, 10mM-NaCl, 5mM-MgCl₂, 1mM-MnCl₂, 2mM-EDTA, 0.4% bovine serum albumin and 50mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.5 (Reeves & Hall, 1973). The protoplasts were ruptured by forcing them by a gentle pressure through a needle of 0.5mm diameter. The cell debris and chloroplasts were separated by low-speed differential centrifugation (300g for 20s, and 500g for 2min respectively).

Isolation of bundle-sheath cells and intact bundle-sheath chloroplasts

The isolation started from bundle-sheath strands prepared in a medium of 0.4M-D-sorbitol with a VirTis homogenizer, operating at 10000rev./min for 40s. The strands were digested in an enzyme mixture containing 0.4M-D-sorbitol, 0.1M-KH₂PO₄, 0.5%

Macerozyme R-10, 1.0% helicase and 1.0% cellulase for 2h at pH 5.8 and 37°C. This enzyme treatment resulted in the release of the bundle-sheath cells surrounding the vascular bundles, and a virtually complete loss of the cellulosic and pectinic compounds of the cell wall. The cells were washed three times in 0.4M-D-sorbitol, suspended and broken by the same procedure as described for mesophyll protoplasts, but with more pressure. Macerozyme and cellulase preparations were purchased from Kinki Yakult MFG, Nishinomiya, Japan; the helicase was obtained from Industrie Biologique Française, Gennevilliers, France; potassium dextran sulphate was from Meito Sangyo, Nagoya, Japan.

Delayed light emission

This was observed by means of a Becquerel phosphoroscope, in essential detail as described by Bertsch *et al.* (1967). The oscilloscope trace was photographed after an induction period of approx. 1 min. The maximum useful speed of this instrument was 15000rev./min.

Fluorescence induction

This was performed with a simple modular fluorimeter. The sample was excited by a 60W tungsten/iodine lamp and a Corning 9592 (blue) filter, and the emission was observed by means of an EMI 9558 photomultiplier and the complementary filter (Schott RG10, red).

Results and Discussion

The mesophyll protoplasts and chloroplasts showed delayed light emission that was of the form expected and observed in many laboratories' studies on granate chloroplasts. An illustration is given in Fig. 1(a). The bundle-sheath cells and chloroplasts on the other hand showed a very low activity, indeed, at times longer than 1 ms and virtually unmeasurable (Fig. 1b). The form differed from that of the mesophyll material in that the emission at less than 1 ms was relatively much greater in the bundle sheath.

There have been several analyses of the decay of delayed light emission in granate chloroplasts, and it has been suggested that several discrete components are involved. Thus Itoh & Murata (1974) found three components, with half-decay times of 0.2, 0.8 and 35ms. Only the shortest component is noticeable in the bundle-sheath preparations in the present study. At the same time the low intensity of delayed light emission at say 1.5 ms, compared with the mesophyll cells, shows that the limit of contamination of the bundle-sheath material is better than 1%.

Itoh & Murata (1974) inferred from their analysis that the fastest component of delayed light emission (less than 1ms) originates from reaction centres

immediately after the trapping of quanta; later stages (1–10 ms, 10 ms and longer) were presumed to be related to electron-transport (thermal) reactions, more distant from the reaction centre. These later processes appear to be deficient in our bundle-sheath chloroplasts.

The fluorescence induction of mesophyll cells and chloroplasts is shown in Fig. 2(a), and is to be compared with the bundle-sheath protoplasts in Fig. 2(b). It is clear that the latter show no perceptible induction even at the greater scanning speed (Fig. 2c), and the fluorescence remains at the initial level. This is a further indication that there is no change in the redox state of a quencher during illumination of bundle-sheath chloroplasts.

The conclusion drawn from Figs. 1 and 2, as well as from the lack of oxygen production observed by Horváth *et al.* (1978), is that Photosystem II is not functioning in bundle-sheath cells. Nevertheless the fast component of delayed light (Fig. 1b) is evidence that Photosystem II is present in some form. When mesophyll protoplasts were treated with 40 μM -3-(3',4'-dichlorophenyl)-1,1-dimethylurea there was some decrease in the fluorescence-induction period (Fig. 3c), and when 0.25 μM -NH₂OH was added as well, the induction kinetics were more similar to

Fig. 1. Delayed light emission of maize mesophyll protoplasts and bundle-sheath cells

These are oscillograms of light emission from (a) mesophyll protoplasts containing 88 μg of chlorophyll in 1.5 ml of isolation medium (see the text), in a cuvette of 0.5 cm light-path, and (b) bundle-sheath cells containing 0.80 μg of chlorophyll as above. The arrow indicates a transient signal caused by a light leak, which marks the centre of the exciting flash. The ordinate (mV) is proportional to light intensity, increasing upward. Note the 20-fold difference in scales.

Fig. 2. Fluorescence induction in mesophyll protoplasts and bundle-sheath cells

These are oscillograms of fluorescent light emission from (a) mesophyll protoplasts containing 64 μg of chlorophyll in 2.6 ml of medium (see the text) in a cuvette of 1 cm light-path, or (b) and (c) bundle-sheath cells containing 48 μg of chlorophyll in 2.6 ml, as above. Note the difference in the time scales. In this equipment the trace is deflected downwards with increasing light intensity. The arrows indicate the onset of the exciting illumination.

those of the bundle-sheath cells (Fig. 3d). The treatment by the two reagents together (Fig. 3b), but not dichlorophenyldimethylurea alone (Fig. 3a) caused the delayed light emission to resemble that of the bundle sheath in so far as the intensity was greatly decreased, whereas the fastest component was relatively enhanced. Dichlorophenyldimethylurea is known (see the summary by Malkin, 1977) to inhibit electron transfer from Q to the plastoquinone pool, so that reduced Q is not reoxidized by Photosystem I, and charge separation by Photosystem II ceases. The intermediates on the oxidizing side of Photosystem II are then predominantly in their lower oxidation states and generate only the long-

Fig. 3. Effect of hydroxylamine and dichlorophenyl-dimethylurea on delayed light emission and fluorescence induction in maize mesophyll protoplasts

Delayed light emission (as in Fig. 1a): (a) with the addition of $40\text{ }\mu\text{M}$ -3-(3',4'-dichlorophenyl)-1,1-dimethylurea, (b) with the addition of $40\text{ }\mu\text{M}$ -dichlorodimethylurea and $0.25\text{ }\mu\text{M}$ -hydroxylamine. Fluorescence induction (as in Fig. 2a); (c) with $40\text{ }\mu\text{M}$ -dichlorodimethylurea and (d) with $40\text{ }\mu\text{M}$ -dichlorodimethylurea and $0.25\text{ }\mu\text{M}$ -hydroxylamine. Note the difference in the vertical scales. The arrows indicate the onset of the exciting illumination.

lived delayed light. Hydroxylamine on the other hand is an electron donor on the oxidizing side of Photosystem II, so that when it is used alone, the effect is

to eliminate the long-lived delayed light. The combination of the two reagents was first applied by Bennoun & Bouges (1972), who showed that the fast components of delayed light remained relatively uninhibited, as in the recent paper. Malkin (1977), after Lavorel (1973), found the mechanistic interpretation of this result to be a problem. However, the purpose of the present paper is to point out that lesions in Photosystem II of the type produced by the above reagents would account for the present finding that Photosystem II exists in maize bundle-sheath chloroplasts, but does not produce oxygen.

We would expect this result to apply to the group of C_4 plants in which malic acid is the C_4 carrier, but not for others, where aspartate is carrier.

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