Excitation-wavelength-dependence of laser-induced delayed light-emission from mesophyll and bundle-sheath cells of maize

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Mesophyll and bundle-sheath cells from maize (*Zea mays*) were examined for delayed light-emission. An enzymic procedure was used to isolate the mesophyll and bundle-sheath cells. A tunable dye laser was used at 695–720 nm to excite delayed light-emission. The mesophyll cells show a typical Photosystem-II dependence on excitation wavelength for delayed light-emission, and this emission was eliminated by hydroxylamine and 3-(3,4-dichlorophenyl)-1,1-dimethylurea. The bundle-sheath cells show no delayed light-emission with these excitation wavelengths. Photosystem-I emission was not observed from either cell type, there being no effect of Methyl Viologen on the observed signals.

Most delayed light-emission is believed to result from charge recombination at Photosystem-II reaction centres (Govindjee & Jursinic, 1979). It has not been clear whether or not Photosystem I contributes to room-temperature delayed light-emission. Bertsch *et al.* (1967), using a phosphoroscope, observed 250 times more delayed light-emission in the time range 0.75–4.2 ms from wild-type *Scenedesmus obliquus* than from a mutant containing only Photosystem I. Ruby & Brown (1971), using a flash-lamp and an electronically gated photomultiplier, observed significant delayed light-emission from this same *Scenedesmus obliquus* mutant at 500 μ s after the flash. The delayed light-emission relative to the wild-type was typically about 10%, and depended on growing conditions and whether whole cells or fractions were used.

Lurie *et al.* (1972) observed a 3-(3,4-dichlorophenyl)-1,1-dimethylurea-insensitive delayed light-emission from Photosystem-I particles. However, they concluded that this was due to emission from residual Photosystem-II centres in which the 3-(3,4-dichlorophenyl)-1,1-dimethylurea-binding site was altered by detergent solubilization of the chloroplast lamellae. Recently Sonneveld *et al.* (1981) measured very-short-lived delayed light-emission from Photosystem I. These Photosystem-I particles showed delayed light-emission with a lifetime of about 0.1 μ s in the presence of dithionite and strong pre-illumination.

From these previous studies it appears that any delayed light-emission from Photosystem I is much less than that from Photosystem II. These investigators have used mutants or disruptive techniques to

obtain their samples, in order to have systems with low amounts of background Photosystem-II delayed light-emission. In the present investigation we study non-mutant cells that are naturally enriched with Photosystem I.

In C_4 -dicarboxylic acid-pathway plants there are two distinct photosynthetic cell types: mesophyll and bundle sheath. The C_4 -dicarboxylic acid-pathway photosynthetic process is based on the movement of C_4 acids from the mesophyll to the bundle-sheath cells. The mesophyll cells contain both Photosystem I and Photosystem II; they produce O_2 and fix little or no CO_2 . The bundle-sheath cells contain Photosystem I, but are believed to have little or no Photosystem II present. The bundle-sheath cells fix CO_2 , but whether or not they evolve O_2 is in dispute (Horvath *et al.*, 1978; Chapman *et al.*, 1980).

Most of the previous workers who have examined delayed light-emission from bundle-sheath cells used differential grinding techniques to isolate the two different cell types. Using a phosphoroscope, Mayne *et al.* (1974) found the ratio of delayed light-emission of mesophyll to bundle-sheath chloroplasts to be 5.9:1. However, the decay kinetics for both signals were the same.

Gregory *et al.* (1979) used enzymic-digestion techniques to separate the two cell types. They reported a mesophyll/bundle-sheath ratio of delayed light-emission of about 20:1 at 200 μ s after the end of the phosphoroscope light-flash. The decay kinetics of the signals from the two types of cells were quite different. The authors interpreted their results as evidence that Photosystem II is present in the bundle-sheath cells in an inhibited form.

It seems possible that these authors may have been observing Photosystem-I delayed light-emission from their bundle-sheath samples. We consider that further investigation is necessary with the use of more precise optical techniques. The present study is designed to examine the delayed light-emission from the mesophyll cells and the Photosystem-I-enriched bundle-sheath cells. It is known that Photosystem II's absorption peaks at about 680 nm and falls off sharply at about 710 nm, whereas Photosystem I's absorption peaks near 690 nm and has a shoulder extending out past 720 nm (Goedheer, 1962). If there is a delayed light-emission response from Photosystem I, then we should be able to probe for it by exciting the system with far-red light past the absorption fall-off of Photosystem II.

Materials and methods

Plant material

Seeds of maize (*Zea mays*; Ferry Morse, Golden Cross Bantam T-51) were grown for 10–14 days in a greenhouse. Harvesting of the leaves was done in the morning to obtain starch-depleted chloroplasts.

Isolation of mesophyll protoplasts and chloroplasts

Our methods for preparing mesophyll cells are based on enzymic techniques described by Horvath *et al.* (1978). Our procedures for enzymic mesophyll isolation are similar to theirs. In addition to the enzymic preparation, we also concentrated broken chloroplasts of both cell types by centrifuging the fluid left in the homogenizer during the isolation of the bundle sheath. We centrifuged these broken chloroplasts as we had done in the past for spinach chloroplasts, by using a procedure done by Avron (1974).

Isolation of bundle-sheath cells and chloroplasts

The methods for preparing our bundle-sheath samples were modified from those described by Horvath *et al.* (1978).

The isolation began by chopping the leaves in approx. 1 cm × 1 cm pieces, suspending them in a 0.4 M-D-sorbitol solution containing 5 mM-MgCl₂ at pH 7.5, and homogenizing them for 40 s at 10000 rev./min in a VirTis homogenizer. The resulting strands were filtered out with cheese-cloth, rinsed with the 0.4 M-D-sorbitol solution and digested for 3 h at 37°C. The enzyme solution contained 0.4 M-D-sorbitol, 0.1 M-KH₂PO₄, 1.0% (w/v) Macer-ase, 2.0% (w/v) helicase and 2.0% (w/v) cellulase, and was adjusted to pH 5.8. After incubation the cells were lightly shaken off the strands by manually swirling the enzyme solution. The strands were then filtered out with cheese-cloth, and the remaining solution was centrifuged. The first centrifugation was

at 188 g for 90 s. The precipitate from this was suspended in 0.4 M-D-sorbitol and centrifuged at 37 g for 90 s. The supernatant from the second centrifugation was then centrifuged at 188 g for 90 s. The precipitate from the final centrifugation, consisting of bundle-sheath cells, was suspended in the Hepes [4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid] solution (Horvath *et al.*, 1978), and forced through a 0.5 mm-diam. needle. The chloroplasts were then separated from the cell debris by first centrifuging at 300 g for 20 s and centrifuging the supernatant from this at 500 g for 2 min. To minimize scattering, the chloroplasts were put through a tissue homogenizer. Macer-ase (macero-zyne) and cellulase were purchased from Calbiochem, San Diego, CA, U.S.A.; the helicase was obtained from Industrie Biologique Française, Gennevilliers, France; potassium dextran sulphate was from Meito Sangyo, Nagoya, Japan.

Delayed light-emission

The delayed light-emission was measured in an apparatus similar to one previously described (Ruby, 1971). The excitation light was supplied by a xenon flash-lamp or a Chromatix CMX-4 flash-lamp pumped-pulse dye laser. We used Oxazine 720 as the dye at a repetition rate of 8 Hz. The dye was obtained from Exciton, Dayton, OH, U.S.A. The energy output of the laser was about 2.5 nanoeinsteins/pulse at 695 nm, about 7.0 nanoeinsteins/pulse at 710 nm and about 6.5 nanoeinsteins/pulse at 720 nm. The power output varied randomly from pulse to pulse by about 10%. The laser had a full width at half maximum of about 1 μs. There was a blue block filter in front of the laser to block light-leak from the flash tube. The photomultiplier had a horizontal polarizer and Corning far-red pass filters nos. 7–69 and 2–64 in front of it to minimize scattered-light artifacts.

Just before measurement, the sample was suspended in a solution of 0.1 M-KCl and 20 mM-Tricine {N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine} at pH 7.8. The chlorophyll concentration was between 1 and 2 μg/ml for both cell suspensions. The sample was in a cuvette with dimensions 1.0 cm × 1.0 cm × 1.5 cm.

Results and discussion

The delayed light-emission from both the enzymically prepared mesophyll cells and the broken chloroplasts prepared from the homogenate (Figs. 1a and 1b) is similar to what we have observed in the past with whole algal cells and spinach chloroplasts (Ruby, 1976; McCauley & Ruby, 1981). The plot of delayed light-emission signal versus excitation wavelength is typical of what would be expected for a Photosystem-II process (Fig. 2a). 3-(3,4-Dichloro-

Fig. 1. Delayed light-emission from maize mesophyll and bundle-sheath chloroplasts

(a) Delayed light-emission from enzymically prepared mesophyll cells. Excitation light is supplied by a xenon flash-lamp. (b) Delayed light-emission from a mixed population of broken mesophyll and bundle-sheath chloroplasts. Excitation light is supplied by the laser at a wavelength of 695 nm. (c) Delayed light-emission from broken bundle-sheath chloroplasts. Excitation light is the same as in (b). The sharp rise centred at 100 μ s after the flash in this photograph is a transient artifact produced when the photomultiplier is gated on. We see a response nearly identical with (c) with distilled water in the sample cuvette. Note the differences in scales.

phenyl)-1,1-dimethylurea and hydroxylamine are known to affect primarily Photosystem II (Izawa & Good, 1972; Den Haan *et al.*, 1974). The addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea and hydroxylamine to the samples containing mesophyll chloroplasts diminishes the signal to a small fraction of its initial value (Fig. 2a). Methyl Viologen is thought to accept electrons from Photosystem I (Avron, 1975). Any delayed light-emission produced from Photosystem I should be affected by

Fig. 2. Delayed light-emission from maize mesophyll and bundle-sheath chloroplasts as a function of wavelength

(a) Delayed light-emission from a mixed population of broken mesophyll and bundle-sheath chloroplasts 200 μ s after the laser pulse. ●, Control, no additions; □, steady-state response after incubation in the light with 10 μ M-Methyl Viologen; △, steady-state response after incubation in the light with 1 μ M-hydroxylamine and 10 μ M-3-(3,4-dichlorophenyl)-1,1-dimethylurea; ○, steady-state response after incubation in the light with Methyl Viologen, 3-(3,4-dichlorophenyl)-1,1-dimethylurea and hydroxylamine at the same concentrations as previously used. (b) Delayed light-emission from broken bundle-sheath chloroplasts 200 μ s after the laser pulse. Symbols have the same meaning as in (a). Signals for both (a) and (b) were only recorded at the wavelengths shown and were not corrected for differences in laser power at the different wavelengths. Signals are normalized to the maximum value of the control in (a).

Methyl Viologen. Our results show no significant change after the addition of this agent to the sample.

To the limits of our sensitivity, the bundle-sheath samples show no delayed light-emission, the trace in Fig. 1(c) being attributable to instrumental background response. At longer wavelengths there appears to be a slight increase in the response (Fig.

2b). We attribute this to the fact that more scattered light goes through our filters at longer wavelengths. The addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea and hydroxylamine produced no effect on the bundle-sheath response, indicating that there is little or no contamination by mesophyll (Fig. 2b). When Methyl Viologen is added there is no change, further indicating that Photosystem I is not producing a delayed light-emission response.

The wavelength-dependence and their response to the exogenous agents show that the mesophyll-chloroplast-containing samples were giving Photosystem-II responses. The negative results from the bundle-sheath samples indicate that the delayed light-emission 200 μ s after the laser pulse could not be more than 2% of that from the mesophyll.

These results may not be inconsistent with the millisecond-flash-width phosphoroscope studies reported by Gregory *et al.* (1979). We have shown in a previous study that microsecond-width flashes can produce significantly different delayed light-emission results from those obtained with millisecond-width phosphoroscope flashes (McCauley & Ruby, 1981). It is possible that the bundle-sheath signal reported by Gregory *et al.* (1979) could be enhanced as a consequence of the millisecond duration of the excitation pulse.

Our results may indicate that Photosystem II is not present in maize bundle-sheath cells. If this is true, then the reductant must be provided by malate. If Photosystem II is present in bundle-sheath cells, then some mechanism must be acting to quench delayed light-emission.

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