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CHLOROPHYLL FLUORESCENCE AT HIGH TEMPERATURE

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An increase in fluorescence emission attributed to PS I becomes apparent when leaves and chloroplasts enriched in stroma thylakoids are heated to high temperature (65–70°C). This conclusion is drawn from observations on sun and shade leaves, French press-fractionated chloroplasts and grana-deficient bundle sheath chloroplasts of C_4 plants.

When leaves and chloroplasts are illuminated with weak light and heated, the fluorescence yield from chlorophyll increases sharply at temperatures damaging to the functioning of the photosynthetic apparatus. This increase in fluorescence coincides with the onset of irreversible inhibition of light-limited and light-saturated CO_2 fixation [1,2]. Chlorophyll *a* fluorescence at room temperature arises mainly from PS II [3], and a heat-induced block of PS II reaction centres combined with a functional dissociation of light-harvesting chlorophyll *a/b* protein from PS II has been postulated to occur at the temperature of fluorescence increase [4]. This rise usually occurs in the range 40–50°C and with further heating of the sample, fluorescence yield increases and then declines [1]. We have heated leaves and chloroplasts beyond this usual range to study and observed a second rise in fluorescence which can be related to the PS I content of the preparation.

Samples were illuminated with weak exciting light (10^{-7} mol · m^{-2} · s^{-1}) of 430 nm or 480 nm (half-bandwidth around 7 nm). Leaf discs or chloroplast preparations were sealed between spaced microscope coverslips resting on a brass block and heated at a rate of 1 C deg./min. Temperature was recorded with a fine copper-constantan thermocouple at the surface from which fluorescence was detected. Fluorescence was measured at 690 nm through a narrow-bandpass interference filter in front of a Hamamatsu R928 photomultiplier. Temperature and fluorescence signals were amplified and displayed on an *X-Y* recorder. The electrical gain for the fluorescence signal was adjusted so that the difference between illuminated and darkened samples at room temperature (fluorescence from PS II) gave one unit on the chart recorder.

Fig. 1 shows the fluorescence profiles for heated leaves of the sun species *Atriplex triangularis* (*A. patula*) [5] grown in full sunlight (daily photon fluence rate of 45 mol · m^{-2} · day^{-1}, 400–700 nm; Stanford, CA; September) and grown at approx. one-seventh of this dose, and for the forest floor species *Asarum caudatum* collected from a deeply shaded site (less than 2 mol · m^{-2} · day^{-1}). With light of 430 nm, which preferentially excites chlorophyll *a*, leaves of

Abbreviations: PS, Photosystem; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate; Tricine, *N*-tris(hydroxymethyl)glycine.

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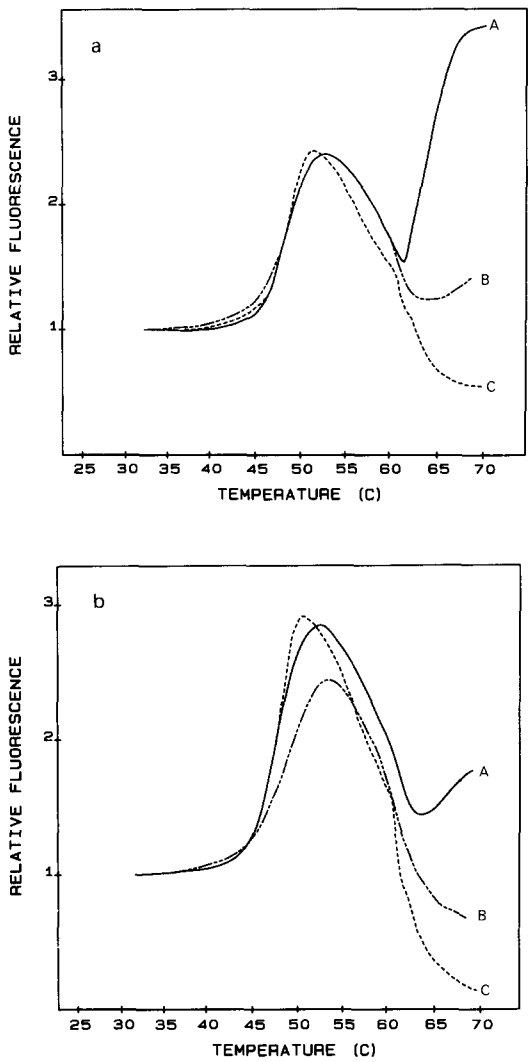

Fig. 1. Fluorescence yield in 430 nm (a) and 480 nm (b) exciting light from heated leaf segments of *A. triangularis* and *A. caudatum*. *Atriplex* was grown at 30°C day/20°C night (16/8 h) in full sunlight (A) or approximately one-seventh of this (B) achieved with neutral density screens. *Asarum* leaves (C) were collected from a deeply shaded area near the laboratory. Chlorophyll *a/b* ratios were 3.1, 2.7 and 2.26 for A, B and C, respectively.

these plants showed a rise in fluorescence at a temperature near to 45°C which peaked at 50–55°C and then declined (Fig. 1a). A second peak of fluorescence was evident for *Atriplex* heated above 60°C, which exceeded the height of the first peak for plants grown in full sunlight, but not in shade grown *Atriplex*. The extreme shade plant *Asarum* displayed no second rise and fluorescence declined above 50°C, levelling off near 70°C. These observations suggest that the high temperature fluorescence component is more marked in sun plants grown at high light.

The ratio of the electrical gains necessary to produce one unit of fluorescence on the chart recorder at room temperature for the *Atriplex* and *Asarum* leaves when excited with 480 nm light compared with 430 nm light was close to 1.0, indicating that both wavelengths were equally effective at stimulating fluorescence either through chlorophyll *a* or chlorophyll *b*. The height of the first peak was also similar with light of either wavelength. By contrast, excitation with 480 nm light (Fig. 1b), which preferentially stimulates chlorophyll *b*, led to a substantial reduction in relative fluorescence at high temperature, indicating that this component of fluorescence probably originated from a chlorophyll *a*-containing pigment-protein.

Fluorescence-vs.-temperature profiles for maize (*Zea mays*) leaves represent emission from the outermost granal mesophyll chloroplasts (chlorophyll *a/b* ratio, 2.98), since fluorescence from the inner bundle sheath chloroplasts of this C_4 plant would have been reabsorbed by the surrounding mesophyll (Fig. 2). Excitation with 480 nm light (not shown) gave a similar profile except that fluorescence was reduced substantially above 55–60°C. The ratio of the electrical gains required to obtain equal fluorescence at room temperature with both wavelengths was 1.0 as was evident for the C_3 *Atriplex* and *Asarum* leaves. Leaves of maize and its close relative, teosinte (*Euchlaena mexicana*),were also ground in a mortar to give preparations of bundle sheath strands from which most of the outer mesophyll cells had abraded. Bundle sheath chloroplasts in these particular C_4 species are extremely deficient in grana lamellae [6,7] and exhibited high chlorophyll *a/b* ratios; viz. maize, 3.79; teosinte, 4.78. The chlorophyll *b*-depleted maize bundle sheath preparations (Fig. 2) measured in 480 nm light required a gain 1.5-times that in 430 nm light to obtain the same level of fluorescence at room temperature. As the temperature increased the fluorescence yield in 430 nm light for the maize bundle sheath preparation also increased, showing a pronounced rise above 60°C. In 480 nm light a small

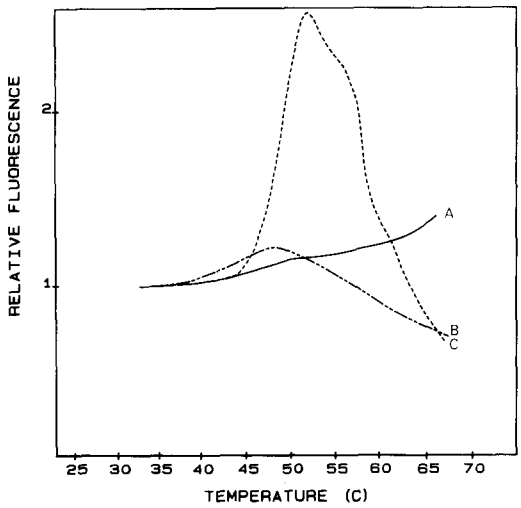

Fig. 2. Fluorescence yield from heated maize. Plants were grown at 28°C/21°C night (14/10 h) with light provided by high-intensity fluorescent lamps giving an average photon fluence rate of 12.6 mol · min^{-2} · day^{-1} (400–700 nm). Strands of bundle sheath cells were isolated by grinding leaf segments in a mortar with 0.1 M Hepes (pH 7.9)/10 mM $MgCl_2$/1 mM EDTA until only pale green fibers (vascular tissue and bundle sheath cells) were present when examined by the light microscope. These were collected on Miracloth, washed with the grinding medium and used directly. (A) bundle sheath preparation excited with 430 nm light; (B) bundle sheath preparation excited with 480 nm light; (C) leaf segment excited with 430 nm light. Chlorophyll *a/b* ratio was 2.98 for mesophyll chloroplasts (liberated by gently grinding leaf segments) and 3.79 for the bundle sheath preparation.

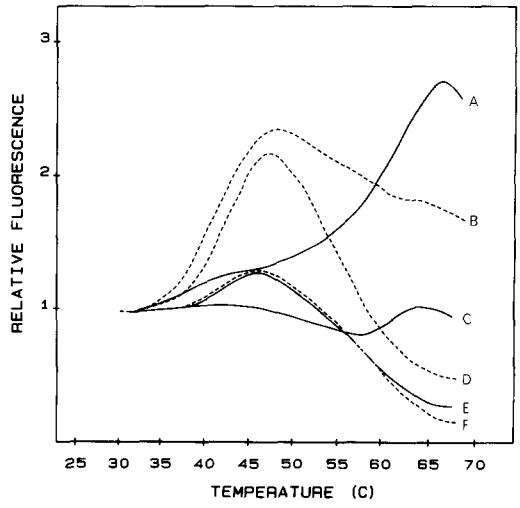

Fig. 3. Fluorescence yield from heated spinach chloroplast fractions. Chloroplasts were prepared from market spinach by briefly homogenizing leaves in a Waring Blender with 50 mM Tricine (pH 7.8)/0.4 M sucrose/5 mM $MgCl_2$/10 mM NaCl. The homogenate was filtered through Miracloth and centrifuged at 5000 × *g* for 5 min. Some of the pellet was resuspended in the isolation medium to provide samples of chloroplasts. The rest of the pellet was resuspended in 25 mM Tricine (pH 7.8)/0.2 M sucrose/5 mM $MgCl_2$/10 mM NaCl and passed twice through an Aminco French pressure cell at 65 MPa to disrupt the photosynthetic lamellae. The mixture of membrane fragments was centrifuged at 1000 × *g* for 5 min to remove unbroken chloroplasts and the pellet discarded. The supernatant was centrifuged at 40 000 × *g* for 30 min which resulted in the sedimentation of a heavy fraction (grana lamellae; chlorophyll *a/b* ratio, 2.48). The supernatant fraction (enriched in stroma lamellae; chlorophyll *a/b* ratio, 4.24) was used directly in this experiment. (A) 40 000 × *g* supernatant excited with 430 nm light; (B) chloroplasts (chlorophyll *a/b* ratio, 2.69) excited with 430 nm light; (C) 40 000 × *g* supernatant excited with 480 nm light; (D) chloroplasts excited with 480 nm light; (E) 40 000 × *g* precipitate excited with 430 nm light; (F) 40 000 × *g* precipitate excited with 480 nm light.

peak was observed at 50°C, but then fluorescence yield declined with further heating. Teosinte bundle sheath preparations (not shown) gave similar responses to maize, except that no peak was observed near 50°C in 480 nm light. These data indicate that increases in fluorescence yield at high temperature arise from stroma lamellae.

This hypothesis was further tested by fractionating spinach (*Spinacia oleracea*) chloroplasts into grana and stroma fractions with a French pressure cell [8,9]. The fluorescence response of isolated chloroplasts to heating (Fig. 3) was similar to that in the case of intact spinach leaf (not shown) except that the threshold temperature to the initial fluorescence rise was lowered by about 5°C. Chloroplasts in 430 nm light displayed an initial peak of fluorescence at 45–50°C which gradually declined to a shoulder near 65°C. With 480 nm light, however, chloroplasts showed a peak of fluorescence at 45–50°C which then declined continually above this temperature. The 40 000 × *g* precipitate from French-pressed chloroplasts, which is enriched in grana [8,9] and low in chlorophyll *a/b* ratio, displayed the first peak seen in unfractionated chloroplasts, but lacked the shoulder at high temperature. The 40 000 × *g* supernatant fraction, which was high in chlorophyll *a/b* ratio and

enriched in stroma membranes [8,9] showed a small rise in fluorescence in the region of the first peak evident in chloroplast preparations, followed by a very steep increase which peaked at 65°C and coincided with the shoulder observed for chloroplasts in 430 nm light. Unlike the 40 000 × *g* precipiate where gain ratios of 0.9–1.0 gave equal relative fluorescence at room temperature, gain ratios of 1.7–1.8 (480 nm vs. 430 nm) were required to normalize fluorescence of the 40 000 × *g* supernatant fraction at room temperature. This reflects the depleted level of chlorophyll *b* in the supernatant fraction and the relative ineffectiveness of 480 nm light in exciting chlorophyll *a*. Centrifugation of the 40 000 × *g* supernatant at 160 000 × *g* for 1 h [8,9] gave a precipitate of similar chlorophyll *a/b* ratio and fluorescence characteristics to the 40 000 × *g* supernatant. These observations support the conclusion drawn from the grana-deficient bundle sheath chloroplasts that stroma thylakoids are the source of increased fluorescence yield at high temperature.

Recent evidence by Andersson and Anderson [10] on the distribution of chlorophyll-protein complexes within chloroplasts indicates that PS I is largely, if not exclusively, confined to stroma-exposed thylakoids and that 80–90% of PS II is located in appressed (grana) membranes. Their data are also consistent with optical difference spectroscopic measurements which show a differing stoichiometry of PS II to PS I between grana and stroma lamellae derived from fractionated chloroplasts [9], and with observations of greatly reduced PS II activity in grana-deficient bundle sheath chloroplasts of certain C_4 species such as maize [11–15]. These data lead us to conclude that the increased fluorescence yield we have observed at high temperature arises specifically from PS I.

Chlorophyll *b*, which is attached to light-harvesting chlorophyll *a/b* protein [16], is stoichiometrically linked to PS II and therefore largely confined to appressed membranes [10]. Consequently, the relative abundance of the two photosystems in mature and non-mutant chloroplasts is closely related to chlorophyll *a/b* ratio [9]. A similar relationship with chlorophyll *a/b* ratio can also be established when relative fluorescence associated with PS II (maximum fluorescence at 45–55°C) in 480 nm (or 430 nm) exciting light is compared with maximum fluorescence from PS I at high temperature (65–70°C) in 430 nm exciting light (Fig. 4). From the above it would also be expected that chloroplasts of sun-exposed leaves would have a greater proportion of thylakoid membranes exposed to the stroma than would shaded leaves. Electron micrographs of sun- and shade-grown *Atriplex* and of *Asarum* and *Alocasia* grown under extremely shaded conditions, indicate this [5,17,18].

Fluorescence at room temperature is predominantly from PS II, since the reaction centre of PS I, *P*-700, is apparently an efficient quencher of fluorescence from PS I [19]. The increase and decline in fluorescence seen near 50–55°C presumably occurs as the pigment-protein complexes associated with PS II of the grana are first dissociated [4] and then destroyed, or as fluorescence from them is quenched. Beyond this temperature range 430 nm light is much more effective at exciting a second fluorescence increase which is clearly associated with stroma membrane samples enriched in PS I. The enhancement of fluorescence at high temperature may arise from PS I as *P*-700 becomes ineffective as a quencher.

Temperature profiles of fluorescence from leaves

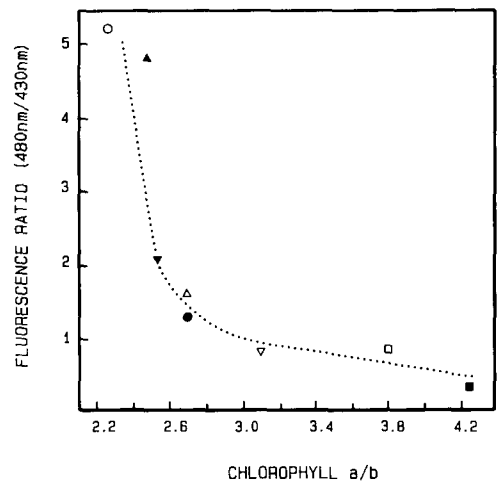

Fig. 4. Relative fluorescence from Photosystems II and I during heating as a function of chlorophyll *a/b* ratio. Fluorescence ratio was computed from maximum fluorescence at 45–55°C in 480 nm exciting light and maximum fluorescence at 65–70°C in 430 nm exciting light. Data taken from Figs. 1–3, except for ▾. ○, *Asarum*; △, *Atriplex*, shade grown; ▽, *Atriplex*, sun grown; □, maize, bundle sheath; ●, ▼, spinach chloroplasts; ▲, spinach, 40 000 × *g* precipitate; ■, spinach, 40 000 × *g* supernatant.

have been previously used to determine the threshold of irreversible inhibition of CO_2 fixation and to monitor photosynthetic acclimation to growth temperature [1,2,20,21]. Our observations show that by heating leaves well beyond the physiological range, information can be generated about the relative abundance of the two photosystems. It is usual for leaves exposed to full sunlight to be gradually shaded as growth progresses and canopies develop. Deeply shaded plants on forest floors, on the other hand, may suddenly experience enormous increases in photon fluence rate if deforestation occurs through natural catastrophe or man's activities. The reaction and adaptation of leaves to light quantity and quality can have profound consequences for photosynthetic function [5,18,22,23], yet little attention has been given to this. The fluorescence measurements described here provide a basis for rapidly detecting compositional changes in thylakoid membranes in response to altered light conditions.

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