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The Kinetics of *in Vivo* State Transitions in Mesophyll and Guard Cell Chloroplasts Monitored by 77 K Fluorescence Emission Spectra[1]

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ABSTRACT

Fluorescence emission spectral peaks at 685, 695 and 730 nanometers (F685, F695, and F730) were recorded 77 K from diluted leaf tissue and epidermal powders prepared from *Saxifraga cernua*. The time course for state 1 to state 2 transitions was monitored as changes in the ratios of the three emission peaks. During illumination with light 2 (580 nm) the F730/F695 and F730/F685 ratios increased within minutes to establish a condition characteristic of state 2. A major difference between the two chloroplast types was the more rapid establishment of state 2 by mesophyll chloroplasts. An increase in light 2 intensity caused an increase in the magnitude of the F730/F695 ratio for both chloroplast types and, for guard cell chloroplasts, a decrease in the time required to establish the new ratio. The role of reversible phosphorylation of the light-harvesting chlorophyll *a/b* protein complex in regulating state transitions for both mesophyll and guard cell chloroplasts was assessed using DCMU and sodium fluoride, a specific phosphatase inhibitor. DCMU-treated mesophyll and epidermal tissues failed to show a state 1-state 2 transition. NaF-treated tissues attained state 2 but lacked the ability to revert back to state 1.

The presence of PSI and PSII in guard cell chloroplasts and their involvement in photophosphorylation represents a possible energy source for stomatal movements (12, 18, 24). This would be in addition to energy provided by oxidative phosphorylation (24). It has been proposed that the PAR-dependent photosystems in the guard cell chloroplast may serve as environmental sensors for changes in light quality and intensity and thereby participate in the regulation of stomatal conductance in coordination with mesophyll cell photosynthesis (12, 16). For this sensing to be most efficient, a property intrinsic to the guard cell chloroplast may be present to adjust to changes in light conditions in a manner similar to that found in mesophyll cell chloroplasts (5, 21). Changes in the amount of excitation associated with the three major Chl-protein complexes (LHC,[2] PSI, and PSII) are concomitant with the state transition process which is hypothetically necessary to optimize ATP and NADPH production in mesophyll chloroplasts during changes in light quality (21). Recently, it has been proposed that the physiological mechanism responsible for state transitions may be regulated by the redox potential of plastoquinone which, in turn, alters the proportion of LHC servicing each of the two photosystems (1, 3).

It has been proposed that state transitions can be followed by changes in the ratios of the fluorescence maxima emitted by PSI and PSII from leaf tissue which has been frozen to low (77 K) temperatures (9, 10, 14, 17). We have monitored the kinetics of *in vivo* state transitions in this way by analysis of 77 K fluorescence emission spectra for both guard cell and mesophyll cell chloroplasts during incubation of epidermal strips and leaf discs under different lighting conditions. We demonstrate qualitative similarities between the two chloroplast types during state 1 to state 2 transitions and the possible involvement of reversible phosphorylation of a molecule participating in such state transitions.

MATERIALS AND METHODS

Plant Material and Incubation Procedure. *Saxifraga cernua* was grown under conditions previously described (12). The 77 K fluorescence emission spectra from guard cell chloroplasts were measured from epidermal strips which were sonicated for a short period of time to remove adherent mesophyll chloroplasts (see Fig. 1D, Ref 12). Spectra from mesophyll cell chloroplasts were obtained from leaf discs (7 mm diameter). To measure the time course for the state transitions, epidermal strips (15 × 3 mm) were placed in small Petri plates (30 strips/plate) containing 10 ml of distilled H_2O, placed in a water bath, and incubated in the dark or light 1 for 60 min at 20°C in order to establish state 1. The entire contents of one plate, or 1 leaf disc, was quickly removed and prepared for spectofluorometric readings described below. The remaining plates containing epidermal strips, or leaf discs, were then illuminated from above with either white light (full spectrum, 400–700 nm) or light 2 at a photon flux density of 500 $\mu mol/m^2 \cdot s$ by three Westinghouse PAR lamps (300 W) fitted over a 7 cm thick water filter. The bottom of the water bath was lined with a mirror to allow illumination of the bottom layer of mesophyll cells. The contents of each plate, or 1 leaf disc, was then sampled at known intervals. Light filters were constructed from combinations of plastic filters (Rank Strand Electric Ltd, Canada). Light 1 was obtained by combining filters No. 20 and No. 33 which served as a far-red highpass filter (>700 nm). Light 2 was obtained using filters No. 6 and No. 33 (>580 nm). The spectral properties of the filters were determined by a dual beam Unicam SP1800 spectrophotometer using clear plastic (No. 30) as the reference.

A 'diluted tissue powder' was prepared for each sample ac-

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[2] Abbreviation: LHC, light-harvesting chlorophyll *a/b* protein complex.

cording to the method of Weis (22) with the following modifications. At the known sampling times, tissue was quickly transferred to a 5 ml beaker containing liquid N_2 and quickly ground to a fine powder with a small Teflon pestle. To the beaker was added 70% (v/v) aqueous glycerol, the volume of which was adjusted to give equal Chl concentrations between samples, and rapidly stirred to suspend the powder evenly throughout the glycerol solution. The suspension was then quickly transferred to a small glass cuvette and rapidly frozen and stored in liquid N_2 prior to spectral analysis. Total preparation time was approximately 15 s to final freezing. Final Chl concentration, determined by the method of Mackinney (13), was 2 to 3 μg/ml.

Prior to treatment of mesophyll and epidermal tissue with either DCMU or NaF, the tissue was floated on water and dark adapted for 30 min after which time the water was replaced with distilled H_2O containing either chemical and buffered with 50 mM Hepes (pH 7.25). The tissue was then further dark adapted or illuminated with light 1 for 60 min at 20°C.

Light intensities were measured with a Li-Cor quantum sensor (model LI-185).

77 K Spectra. Low temperature fluorescence emission spectra were measured with a fluorescence spectrofluorometer (SLM 4800, Urbana, IL) interfaced with a Tektronix 4051 desk-top computer and a Tektronix 4662 X-Y plotter. The spectrofluorometer cuvette was equipped with a custom-build liquid N_2 cooled cryostat. Several problems associated with 77 K measurements were given particular attention. Self-absorption of the shorter wavelengths associated with fluorescence emission from PSII is routinely observed in spectra obtained from intact leaf tissue. This was minimized by preparing dilute tissue powder suspensions with Chl concentrations reduced to the limit of detection by the spectrofluorometer.

A second problem, recently reported by Kyle *et al.* (11) is the light (excitation)-intensity dependent quenching of fluorescence during spectral measurements at low temperatures. We have also observed similar quenching, particularly of the F695 maximum which is a predominant peak from *S. cernua*. This quenching was eliminated by lowering the excitation intensity and reducing the excitation slit width to 4 nm.

RESULTS

77 K Fluorescence Emission Spectra. The fluorescence emission spectra from both frozen powdered epidermal and mesophyll tissue clearly resolved three maxima at 685, 695 and 730 nm (Fig. 1). These emission maxima have been assigned to LHC, PSII reaction center Chl *a* protein complex, and the PSI Chl *a* complex, respectively (15). Spectra from either light 1 or dark-adapted guard cell and mesophyll cell tissue showed a much lower F730 peak relative to either the F695 or the F685 maxima (Fig. 1, A and C). Incubation in light 2 for 2 min reversed this pattern, causing a greater proportion of fluorescence originating from F730 (assigned to the PSI complex) relative to F695 (PSII) or F685 (LHC) for both chloroplast types (Fig. 1, B and D).

Low temperature emission spectra from guard cell chloroplasts consistently showed a much greater relative proportion of fluorescence associated with F685 compared to low temperature emission spectra from their mesophyll cell chloroplasts.

Time-Dependent Changes in State 1-State 2 Transitions. The development of state 2 by mesophyll cell chloroplasts previously adapted to state 1 is indicated by an increase in the F730/F695 ratio as shown in Figure 2. Tissue exposed to light 1 or dark-adapted tissue consistently showed F730/F695 ratios of 0.84 ± 0.03 (mean ± SD, $n = 9$). Changes in the F730/F695 ratio (Fig. 2A) during illumination with light 2 were completed within 2 min which is similar to that reported for the state 1-state 2 transition by intact pea leaves (8). This time course was closely matched by the F730/F685 ratio (Fig. 2B). Changes in the F695/F685 ratio were minor (Fig. 2C).

The time course for the changes in the fluorescence emission ratios for guard cell chloroplasts during illumination is shown in Figure 3. Dark- or light 1-adapted tissue showed F730/F695 ratios similar to those found for mesophyll chloroplasts (0.83 ± 0.04; mean ± SD, $n = 9$) which increased over a 10 min period to 1.20 when illuminated with either white light or light 2 adjusted to equal fluence rates (Fig. 3A). Changes in the height of the LHC peak normalized to 730 nm (F730/F685 ratio) resembled the F730/F695 ratio changes (Fig. 3B) except that the time course appeared to be approximately twice as long. These changes in both the F730/F695 and F730/F685 ratios were similar to those observed for mesophyll chloroplasts. Similar also was a lack of any significant change in the F695/F685 ratio (Fig. 3C). The major difference between the two chloroplast types was the more rapid establishment of state 2 by mesophyll cell chloroplasts when illuminated with the same photon fluence rate.

The response of the state 1-state 2 transition by guard cell chloroplasts to light intensity was compared to that of mesophyll chloroplasts to confirm the different sensitivities of the two chloroplast types described earlier (12). The changes in the F730/F695 ratios for mesophyll cell chloroplasts illuminated at three different intensities are shown in Figure 4. The time required for the establishment of state 2 was relatively independent of the light intensity, the increase in the F730/F695 ratios reaching a maximum value within 2 min. The major effect of higher photon fluence rates was to increase the F730/F695 ratio.

Similar results were obtained from guard cell chloroplasts illuminated at two different intensities (Fig. 5); a higher proton fluence rate increased the magnitude of the F730/F695 ratio. In all cases the changes in the F730/F685 ratios mimicked the changes in F730/F695 reported here. However, several important differences were observed when the time courses for the establishment of state 2 for the two chloroplast types were compared. At the low light intensity (100 μmol/m^2·s, lower curves in Figs. 4 and 5) guard cell chloroplasts showed a 5.7-fold greater final F730/F695 ratio than that for mesophyll cell chloroplasts. However the time required to establish the final ratio was much more rapid in mesophyll cell chloroplasts. Indeed, the time required to establish state 2 in mesophyll chloroplasts appeared relatively independent of the light 2 intensity. This was not observed for guard cell chloroplasts which showed a more rapid development to maximum F730/F695 values with increasing light 2 intensity (Fig. 5). Thus, light intensity determines not only the magnitude of the state 2 condition in guard cells but also the time required for the establishment of state 2.

To test whether the establishment of state 2 in guard cell chloroplasts was dependent on the blue photoreceptor, dark-adapted tissue was illuminated with light of wavelength 580 nm. The time course for state 2 transition (Fig. 6) was identical to that obtained for white light. If a nonchlorophyllous blue light receptor were involved in the state 1-state 2 transition, one may have expected a reduction in rate of change of F730/F695 or in the final ratio.

Chemical Inhibitor Studies. It has been proposed that in mesophyll cell chloroplasts redistribution of absorbed light between the two photosystems during a state 1-state 2 transition involves a change in the degree of phosphorylation of thylakoid membrane proteins, specifically LHC, which becomes associated with PSI (1, 3) and may result in an increase in α transfer from phosphorylated LHC to PSI (7).

According to several reports (1, 4), incubation of mesophyll cell chloroplasts with DCMU prevents the development of state 2 due to the inhibition of kinase activity which is activated by the reduction of plastoquinone. For both mesophyll cell chloroplasts (Fig. 7) and guard cell chloroplasts (Fig. 8) DCMU prevented the effective establishment of state 2.

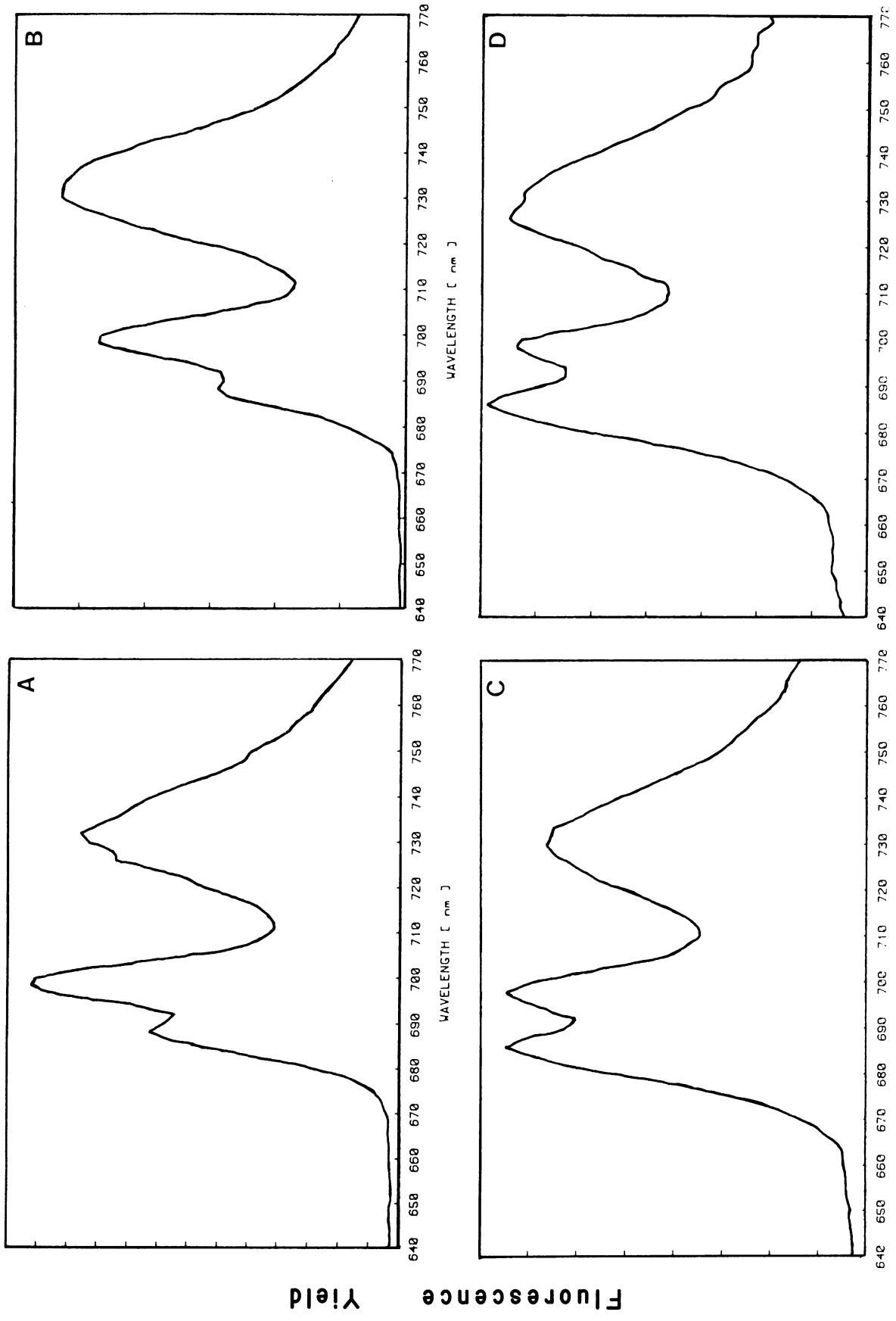

FIG. 1. Low temperature (77 K) fluorescence emission spectra of diluted mesophyll cell powder after exposure of leaf discs to (A) 60 min of light 1 followed by (B) 2 min of light 2. Similar spectra for diluted guard cell powder from epidermal peels after exposure to (C) 60 min of light 1 followed by (D) 2 min of light 2.

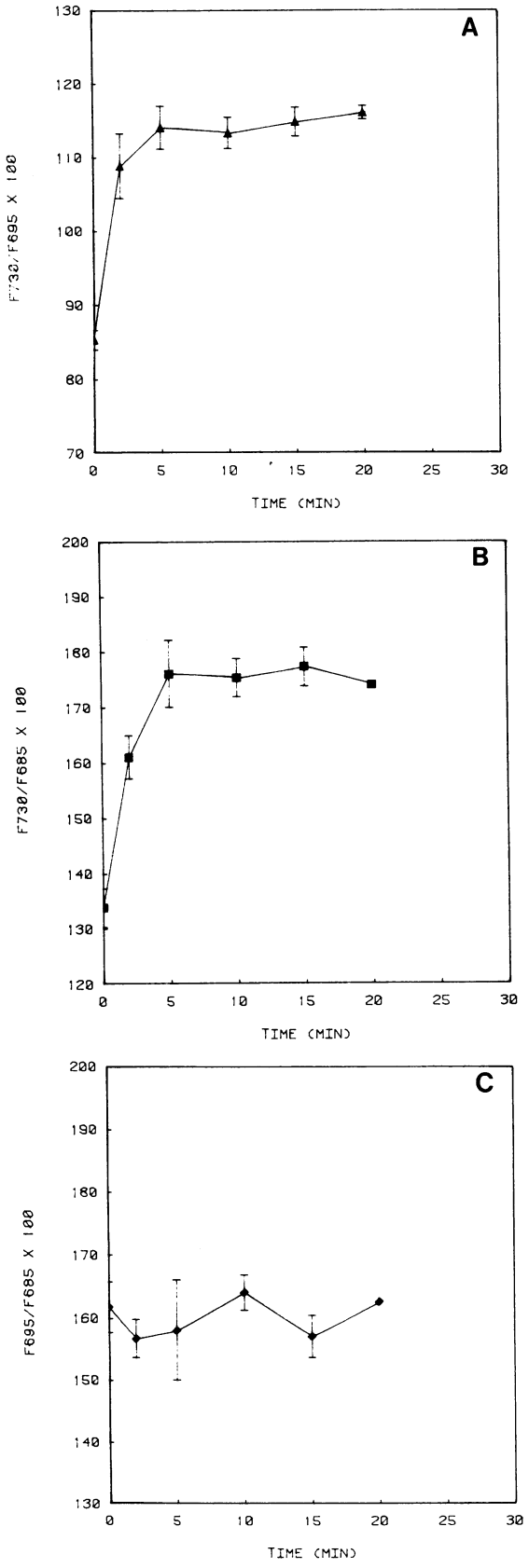

FIG. 2. Kinetics of changes in ratios of low temperature fluorescence maxima following illumination of mesophyll cell preparations to light 2. Changes in (A) F730/F695, (B) F730/685, and (C) F695/F685 ratios. Each point is the mean ± SD where $n = 4$.

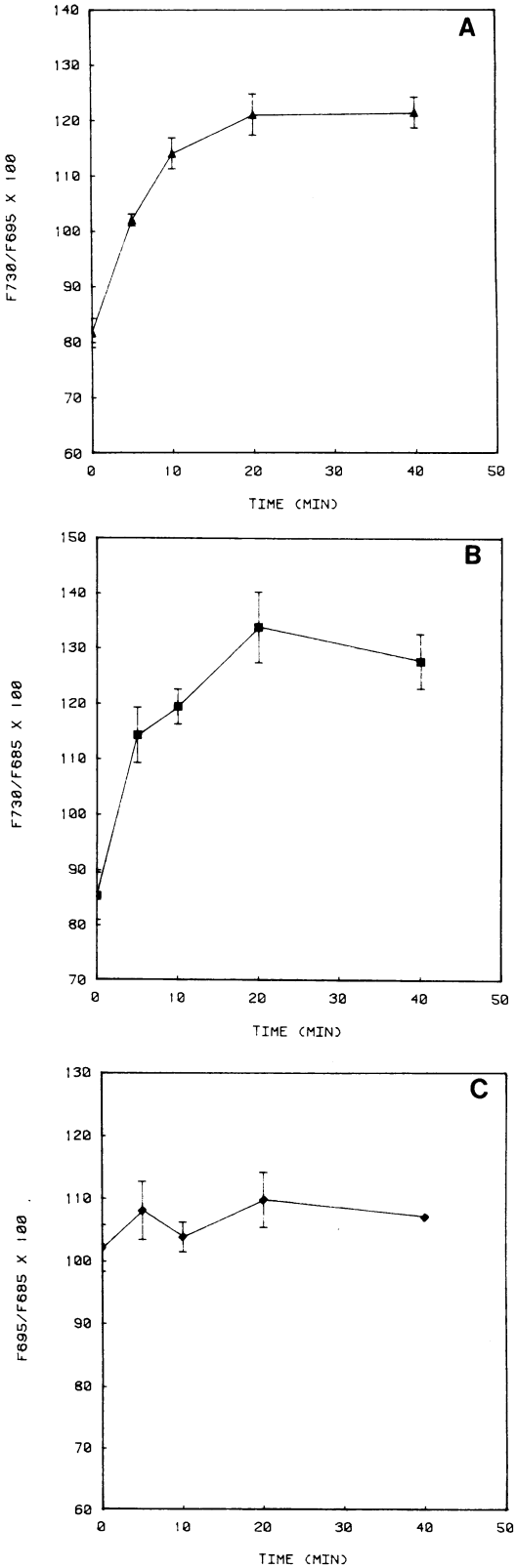

FIG. 3. Kinetics of changes in the ratios of low temperature fluorescence maxima following illumination of guard cell preparations to light 2. Changes in (A) F730/F695, (B) F730/F685, and (C) F695/F685 ratios. Each point is the mean ± SD, where $n = 4$.

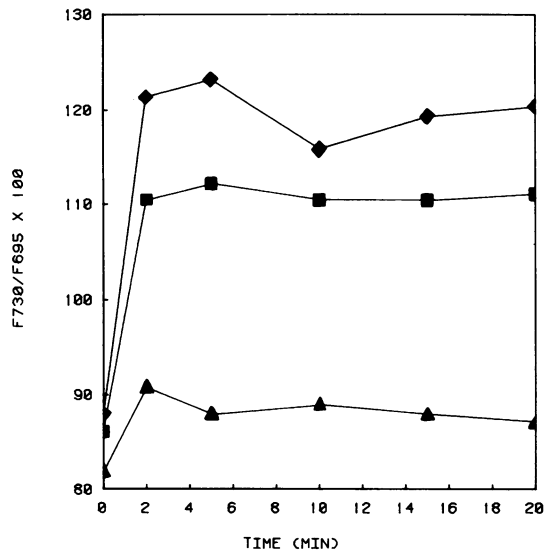

FIG. 4. Time courses for the changes in F730/F695 ratios following illumination of leaf discs with light 2 having a photon fluence rate of (▲) 100, (■) 250, and (♦) 1000 μmol/m^2·s.

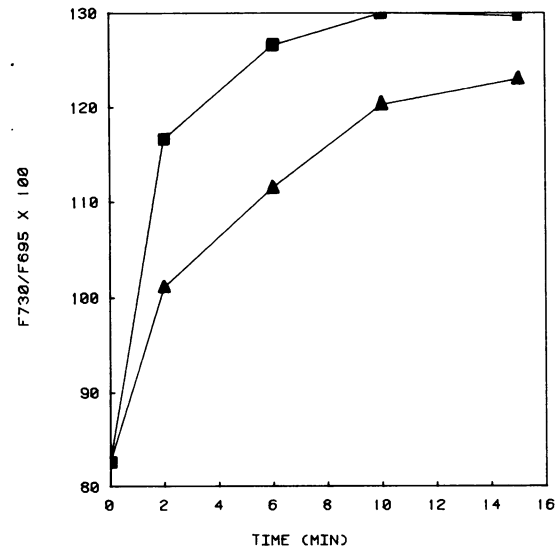

FIG. 5. Time courses for the changes in F730/F695 ratios following illumination of epidermal peels with light 2 having a photon fluence rate of (▲) 100 and (■) 500 μmol/m^2·s.

Canaani *et al.* (6) have reported that NaF specifically inhibited the back reaction (state 2-state 1 transition) in NaF-treated leaf discs of tobacco, apparently due to the inhibition of dephosphorylation of LHC by phosphatase. Both mesophyll chloroplasts (Fig. 7) and guard cell chloroplasts (Fig. 8) treated with NaF were capable of developing state 2. Indeed, for mesophyll cells the F730/F695 ratio was greater in the treated than in the untreated preparation. This increase is consistent with the results from tobacco leaf discs (6). Extinguishing light 2 (times shown by the arrow in Figs. 7 and 8) resulted in a reduction in the F730/F695 ratio in untreated tissue but this reduction was inhibited in both tissues by NaF. NaF appears then to inhibit the establishment of state 1 which normally follows dark treatment or substituting light 1 for light 2. Thus, guard cell chloroplasts may possess a mechanism for state transitions which is similar to that proposed for mesophyll cell chloroplasts involving reversible phosphorylation of LHC (21).

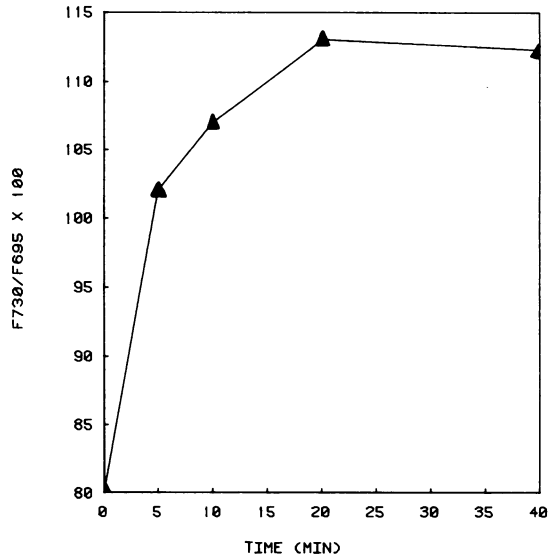

FIG. 6. The time course for changes in the F730/F695 ratios for epidermal peels upon illumination with light of wavelengths greater than 580 nm at a photon fluence rate of 560 μmol/m^2·s.

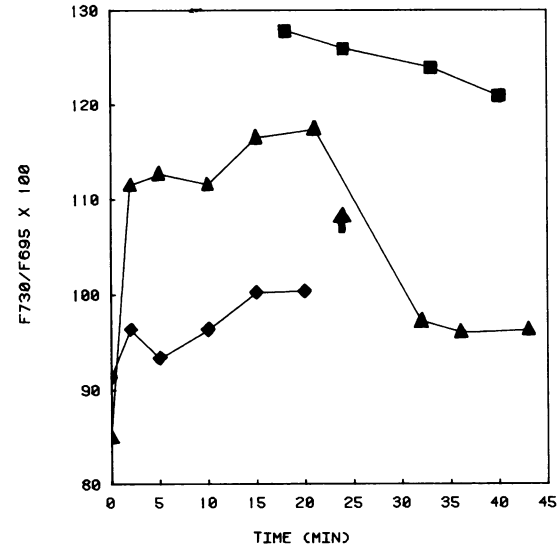

FIG. 7. The effect of NaF and DCMU on F730/F695 ratios in leaf discs during State transitions. Prior to time zero the tissue was exposed to light 1 for 60 min with (■) and without (▲) 40 mM NaF or 50 μM DCMU (♦). At time zero samples were exposed to light 2. After 24 min (arrow) samples were exposed to light 1.

DISCUSSION

Light-dependent stomatal responses, such as changes in diffusive conductance of intact leaf tissue (26) and stomatal opening in isolated epidermal peels (18) have been shown to be sensitive to both changes in intensity and quality of irradiation. These responses in guard cells appear to be primarily dependent on two photoreceptor systems: a blue light receptor which exhibits a low light intensity threshold and a PAR-dependent system which requires higher intensities of irradiation to initiate stomatal activity (24). The second system is associated with the guard cell chloroplast photosystems, PSI and PSII (12, 25) which have been implicated as possible 'sensors' for changes in PAR, thereby assisting in the regulation of stomatal conductance (12, 16). For guard cell chloroplasts to be effective sensors, some intrinsic mechanism may be present in order to adjust for changes in the

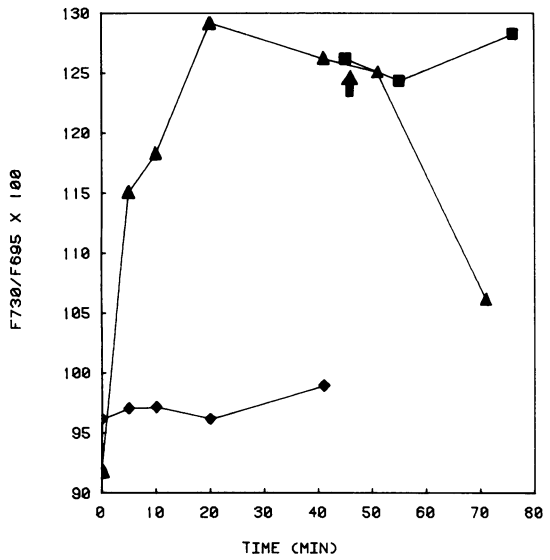

FIG. 8. The effect of NaF and DCMU on F730/F695 ratios in epidermal peels containing chloroplasts only in guard cells during State transitions. Prior to time zero the tissue was dark-adapted for 60 min with (■) and without (▲) 20 mM NaF or 50 μM DCMU (♦). After 47 min (arrow) samples were placed into darkness.

amount of light absorbed by the major Chl protein complexes (LHC, PSI, and PSII) as a result of fluctuations in either the intensity or quality of PAR (20) and thereby maintain maximum quantum efficiency in a manner analogous to that proposed for mesophyll chloroplasts (5). Such a mechanism exists in algae and mesophyll cell chloroplasts and serves to accomplish what are referred to as State transitions (23). State 1 results from overexcitation of PSI (or prolonged dark treatment), while state 2 results from excess excitation of PSII. The underlying molecular events which are thought to be associated with the redistribution of excitation between the two PAR photosystems have been reviewed (21). Currently, it is thought that state transitions involve the movement of LHC between the two photosystems (3). The identification of LHC, PSI, and PSII in guard cell chloroplasts by low temperature fluorescence emission spectra (12) suggests that a similar mechanism operates in stomatal guard cell chloroplasts.

The 77 K spectra from *S. cernua* mesophyll and guard cell chloroplasts, prepared as 'diluted powders' clearly resolve three maxima originating from LHC, PSI, and PSII. Monitoring changes in the fluorescence emission maxima over time further characterizes the state transition events for both chloroplast types. Sample preparation by the diluted powder procedure described by Weis (22) is well suited for kinetic studies since the short preparation time allows rapid sampling and greatly reduces self-absorption. Furthermore, the technique permits the transitions to be followed *in vivo*.

Expressing the state transitions as changes in ratios of the F730/F695 fluorescence maxima does not discriminate between changes due to altered amounts of fluorescence from only one photosystem and changes due to altered fluorescence from both photosystems. Addition of an internal standard, such as fluorescein, to *in vitro* chloroplast preparations indicates that state 1-state 2 transitions result in an increase in fluorescence from PSI and a decrease in PSII (10). The use of *in vivo* tissue preparations, however, restricts the application of an internal standard for determining absolute changes in the fluorescence maxima. That reciprocal changes in the absorptive cross-sections of the two photosystems occur *in vivo* in leaves has recently been reported by Canaani and Malkin (7). Whether or not the relative increase in the amount of fluorescence from PSI (F730) occurring over a time course of several minutes during a state 1-state 2 transition in guard cell chloroplasts is due to similar changes in the photosystem cross-sections as that reported for mesophyll cell chloroplasts (7) requires further experimentation.

A major difference between the mesophyll and guard cell chloroplasts is the time required to establish state 2 by guard cell chloroplasts. This feature is consistent with results from our earlier report (12) which described differences in the fluorescence induction curves from the two chloroplast types. We suggested that ATP production by photophosphorylation in guard cell chloroplasts would be less than that produced by mesophyll chloroplasts (12). This suggestion has recently been verified (19). Furthermore, if indeed state transitions reflect a change in the amount of LHC associated with either photosystem (3, 21), it would appear that LHC movement in guard cell chloroplasts may be significantly slower than that in their mesophyll counterpart (at least at moderate light intensities). The slower establishment of state 2 by guard cell chloroplasts may, in part, be responsible for the differential time response between an increase in photosynthesis (rapid) and stomatal opening (slow) observed in whole leaves when illuminated with PAR (26).

Our experimental results involving the role of PAR intensity in state changes suggests that changes in PAR can be detected by guard cell chloroplasts and also emphasizes the different light responses of the two chloroplast types. The change in the F730/F695 ratio by mesophyll cell chloroplasts at low light intensities (100 $\mu mol/m^2 \cdot s$) is minimal. However, a 2.5-fold increase in intensity leads to a substantial increase in the F730/F695 ratio which we have interpreted as a transition to state 2.

Low light intensities cause much greater F730/F695 ratios in guard cells than occurs in mesophyll cells. This suggests that guard cells initially absorb a greater percentage of light into their PSII-LHC complex than do mesophyll chloroplasts. To account for this difference, we suggest that the larger F685 emission from guard cell chloroplasts may indicate a much larger LHC population compared to mesophyll cell chloroplasts. Therefore, there may exist a larger mobile pool of LHC (21) in guard cell chloroplasts which may serve either PSI or PSII depending on the lighting conditions.

In this respect, a high LHC content in guard cell chloroplasts is similar to that found in the mesophyll chloroplasts of shade plants. Stomata on the abaxial surface develop under lighting conditions which are (a) greatly attenuated compared to the adaxial surface and (b) rich in light 1 due to shading by mesophyll cells above. This lighting environment which is analogous to that found for shade plants, appears to alter the stoichiometries of the major Chl-protein complexes. Hence the number of PSII and LHC complexes increases while the number of PSI complexes decreases (2). A larger PSII-LHC content found in shade adapted plants and apparently in guard cell chloroplasts, may be required to absorb what little red light (light 2) penetrates the canopy. Thus when guard cell chloroplasts are exposed to our experimental lighting conditions (which, like direct sunlight, is rich in light 2) a considerable transition to state 2 most probably occurs. Comparative fluorescence emission spectral analysis from adaxial and abaxial guard cells is warranted to test this hypothesis.

Current models for the mechanism of state transitions envision reversible phosphorylation of LHC which causes migration of this Chl-protein complex between the two photosystems (1, 21). The responses of guard cell chloroplasts to both DCMU and NaF are similar to those shown by mesophyll cell chloroplasts. These suggest that both chloroplast types share a common mechanism for state transitions via reversible phosphorylation of LHC.

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