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Presence of Both Photosystems in Guard Cells of *Vicia faba* L.

IMPLICATIONS FOR ENVIRONMENTAL SIGNAL PROCESSING[1]

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ABSTRACT

A new procedure is reported for high-yield isolation of guard cell protoplasts from *Vicia faba* L. Delayed light emission and P_{700} content plus absorption and fluorescence emission spectra of these protoplast extracts are reported. It is concluded that both photosystems are present. The presence of photosystem II and the absence of the reductive-step enzyme of the Calvin-Benson Cycle (Outlaw WH Jr, J Manchester, CH DiCamelli, DD Randall, B Rapp, GM Veith 1979 Proc Natl Acad Sci USA 76: 6371-6375) in a cell has no precedent in the literature. It is speculated that noncyclic photosynthetic electron flow is an environmental sensor which causes stomata to remain open in light.

CO_2 and H_2O movement between a leaf and the atmosphere is controlled by stomatal aperture. Thus, to reduce water loss, it is important for stomata to process information concerning PAR. Sensing of PAR and CO_2 concentration can be independent (34). Despite the importance of these sensors to the physiology of the whole plant, almost no information concerning them has been reported.

We will show here that components of PSII and PSI are present in *Vicia faba* L. guard cells. These cells also lack the reductive step enzyme of the Calvin-Benson cycle [NADP-glyceraldehyde-P dehydrogenase (25)]. This unique situation is the basis of the hypothesis offered for how guard cells sense the continued presence of PAR.

MATERIALS AND METHODS

Chemicals were from Sigma except Cellulysin (Calbiochem) and DCMU (duPont).

PROTOPLAST ISOLATION

Procedure.

Step 1. The midrib and leaf edges were dissected away from fully expanded bifoliate leaves of greenhouse grown *V. faba* L. cv. Long Pod. Leaf material was collected on ice until 50 g accumulated (requiring about 40 plants).

Step 2. The leaf pieces were infiltrated under reduced pressure with enzymic digestion medium consisting of 150 ml 0.3 M mannitol, 10 mM sodium ascorbate, 10 mM $CaCl_2$, 4% (w/v) Cellulysin (pH 5.5). Incubation was for 1 h at 30 C in a 1-liter flask in a shaker bath (60 5-cm excursions/min).

Step 3. One hundred fifty ml 0.7 M mannitol was added and incubation was continued for 1 h.

Step 4. Digestion was interrupted by pouring the material over a 295-μm screen (Nitex) and washing with 0.7 M mannitol containing 10 mM sodium ascorbate. Epidermal strips (coming from both surfaces) and vein nets retained on the screen were transferred to 0.7 M mannitol containing 10 mM sodium ascorbate. Then the epidermal strips were manually separated from the vein nets and transferred to a separate container. The strips had few adhering mesophyll cells, but epidermal cells were intact.

In some experiments, mesophyll cells which passed through the screen were carried through steps similar to the remainder of the isolation procedure and used as controls.

Step 5. Digestion was continued for 1 h as in Step 2 except (*a*) mannitol was increased to 0.7 M, (*b*) volume was reduced to 100 ml, and (*c*) shaker-bath speed was reduced to 30 excursions/min.

Step 6. The strips were collected and washed as in Step 4. If microscopic examination showed contaminating cells, Steps 5 and 6 were repeated.

Step 7. Digestion was continued until guard cell protoplasts were released (about 3 h). These protoplasts were isolated by passage through a cascade of screens (nominally 295, 166, and 20 μm) and collected by centrifugation (1500*g*, 5 min). The protoplasts were washed three times with 0.7 M mannitol containing 10 mM sodium ascorbate and examined for purity.

Comments. Isolation of guard-cell protoplasts has been reported (25, 32, 43). The long preparation time required to make epidermal peels limits yield [*e.g.* Schnabl *et al.* (32) reported less than 1 μl packed cell volume/preparation[5]]. To some extent, the present method overcomes this limitation. Our yield has been 20 to 100 μg Chl (*i.e.* maximally 60 μl packed cell volume ≃ 50% of theoretical yield based on 0.2 m^2 of leaflet).

For experiments based on Chl, guard cell protoplast purity can be crucial. From guard cell pair dry mass [6 ng, (24)], protein/dry mass ratio [52 mg/$g_{dry\ weight}$, Outlaw, W.H. Jr and J. Manchester, unpublished], and protein/Chl ratio (35:1, reported here), we

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[5] It appears that Schnabl *et al.* (32) calculated the volume of their protoplast suspension to be $V_T = n4/3\ \pi\ (\bar{r})^3$ where n is number of protoplasts and $\bar{r}$ is average radius (their Table 2). This is not correct because $V\ \alpha\ r^3$. In one experiment, we calculated V_T [$= \Sigma 4/3\ \pi\ (r_i)^3$] and compared it to $n4/3\ \pi\ (\bar{r})^3$. Fortuitously, because of protoplast size distribution, (which looked symmetrical) the two results were not appreciably different ($\bar{r}$ = 17.4 ± 1.5 (SD) μm; $\bar{V}$ = 2798 ± 802 (SD) μm^3, n = 144). Thus, we conclude that the reported yield of Schnabl *et al.* (32) is about right.

calculate the Chl content of a single guard cell protoplast to be 4.5 pg. Similar calculations based on conversion factors for palisade cells (13, 26, unpublished work of Outlaw, W. H. Jr. and J. Manchester) show they contain 125 pg. Chl each. [This corresponds to 0.3 to 0.5 pg. Chl/guard cell chloroplast (chloroplast/cell ratio, refs. 27, 28)] and 2.1 pg Chl/palisade cell chloroplast [chloroplast/cell ratio (28)]. This means a guard cell protoplast preparation with only 3% contamination (cell basis) by palisade parenchyma will contain approximately equal amounts of Chl from both cell types. A less quantitative way of assessing the importance of contamination is to examine the ultrastructure. The total chloroplast volume of a guard cell is approximately 1/20 that of palisade cell (calculated from ref. 28) and for mesophyll, the number of thylakoids/granum is greater (2). Thus, our chemical data agree with structural studies. Contamination by spongy parenchyma is about 50% as bad as contamination by palisade parenchyma. Contamination by epidermal cells is relatively unimportant as they have few and underdeveloped chloroplasts. The purity of our preparations was estimated to be 95% or greater (cell basis), with almost none of the contamination coming from chlorenchyma (Fig. 1). There was some contamination by cell wall debris, but this was judged not to be important for the present study.

The arguments in the preceding paragraph make it unlikely that epidermal strips can be used as a useful proxy for guard cell chloroplast components; epidermal peel work, however, is included under "Discussion" for the sake of comparison.

PROTOPLAST EXTRACTION

For the Chl *a/b* determinations and the acetone absorption spectrum, protoplasts were extracted in 80% (v/v) acetone. The extracts was filtered before spectral analysis.

For protein determination (one experiment reported here, other comparisons to be published later), protoplasts were homogenized in 1 ml 0.7 M mannitol, 0 C. The homogenate was dialyzed twice for 4 h against 500 volumes H_2O. The homogenate was made 0.5 M in NaCl and then the protein was precipitated by making the solution 0.3 M in trichloroacetic acid. The resulting pellet was dissolved in H_2O and subsequently precipitated three times by trichloroacetic acid. Finally, the pellet was dissolved in 0.1 N NaOH and dialyzed against 500 volumes H_2O for 1 h.

For all other assays, protoplasts in 0.7 M mannitol were extracted by passing the suspension through a French press three times at 1,300 atm.

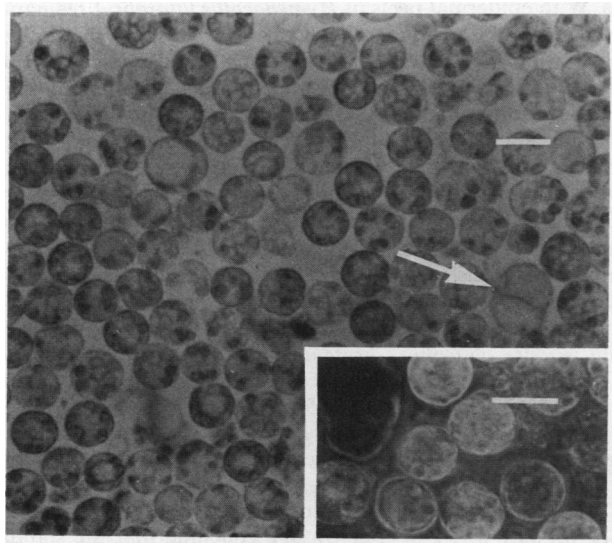

FIG. 1. Guard cell protoplasts isolated from *V. faba* leaflets. The bar is 20 μm. Guard cell protoplasts have numerous chloroplasts, which makes it easy to distinguish them from larger epidermal cell protoplasts (marked by arrow). The inset shows a mesophyll cell protoplast with guard cell protoplasts.

SPECTRAL ANALYSIS

Total Chl (for protein/Chl ratio) and Chl *a/b* (three experiments) was determined by the method of Strain *et al.* (37). Total Chl (for delayed light emission, P_{700} content, and low temperature fluorescence emission spectrum) was obtained by our previous procedure (18), which was a modified extraction procedure of Seely and Jensen (33) and the assay of Arnon (3).

ASSAYS

Protein was assayed according to Lowry *et al.* (15).

The low-temperature (77 K) fluorescence emission spectra (three experiments for guard cells and whole leaf, one experiment for mesophyll) were obtained as reported by Mayne *et al.* (18, 19).

Delayed light emission (three experiments) was measured with a Becquerel phosphoroscope (8). Guard cell extracts were illuminated for approximately 0.6 ms and the delayed light was measured 0.3 ms later. (The signal on an oscilloscope was photographed for Fig. 4).

Photoinduced P_{700} oxidation (three experiments for guard cells and whole leaf, two experiments for mesophyll cells) was measured with a single-beam instrument similar to that of Maxwell and Biggins (17). The P_{700} was reduced with an excess of ascorbate mediated by pyocyanin before illumination.

RESULTS

The Chl *a/b* in guard cell protoplast extracts was 2.76 ± 0.17 ($\bar{x}$ ± SE, $n = 3$) and 2.26 ± 0.03 ($n = 4$) for whole leaflet extracts. The protein/Chl ratio was 35:1 for guard cells ($n = 1$). The 80% acetone absorption spectrum for whole leaf extract was similar to that for guard cells (Fig. 2); both extracts absorbed maximally near 430 and 680 nm. A higher resolution spectrum between 630 and 700 nm was obtained for aqueous extracts of guard cells (Fig. 2, inset). The absorption maximum was near 680 nm; in addition, the 650-nm band due to Chl *b* is seen.

The 77 K fluorescence emission spectra of whole leaf, mesophyll, and guard cell extracts had maxima near 685 and 735 nm

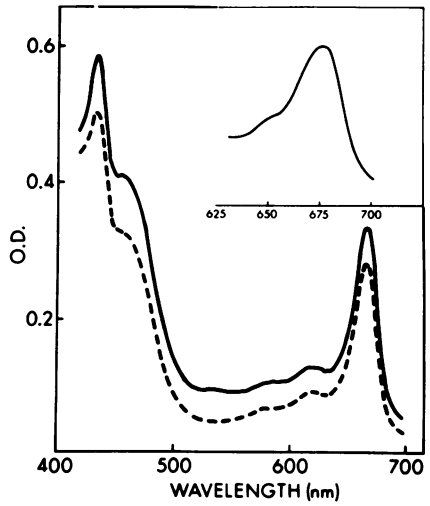

FIG. 2. Absorption spectrum of an 80% acetone extract of guard cell protoplasts (—) and whole leaf (- - -). Inset, higher resolution absorption spectrum of an aqueous guard cell extract.

(Fig. 3). The ratios of fluorescence at 685 to that at 735 nm were 0.56, 0.41, and 1.52 for extracts of whole-leaf, mesophyll, and guard-cell extracts, respectively.

Delayed light emission was observed from guard-cell extract and was diminished by inclusion of 35 μM DCMU (Fig. 4).

The P_{700} content was slightly lower in guard cells than in whole leaf on a Chl basis (*e.g.* Fig. 5 shows photoinduced oxidation in this system was 0.14 and 0.21 ΔT/transmittance/light flash/mg Chl, respectively).

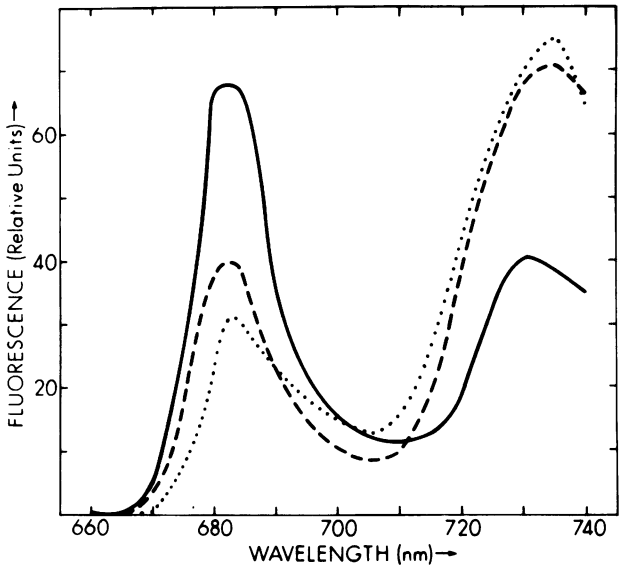

FIG. 3. Low-temperature fluorescence emission spectra of guard cell extract (——), whole leaflet chloroplast extract (– – –), and extract of mesophyll (·····).

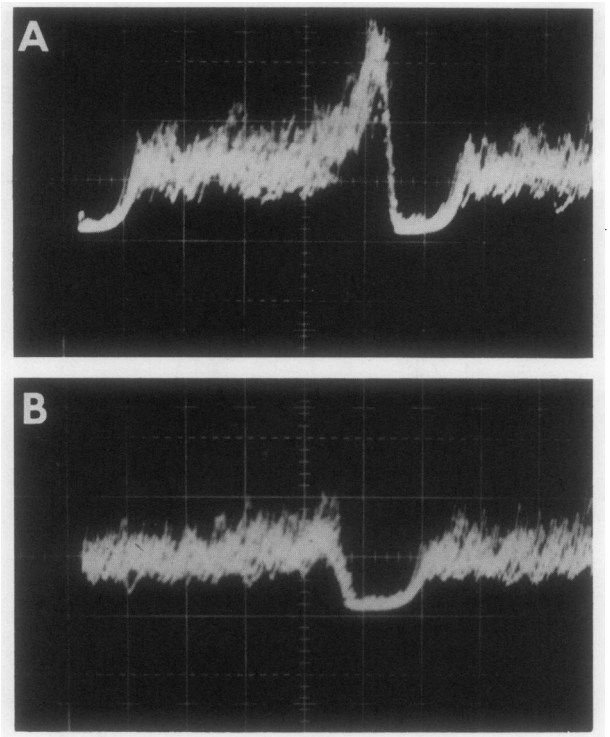

FIG. 4. Delayed light emission of guard cell extract without DCMU (A) and with DCMU (B). Light emission was measured by opening an emission shutter after blocking excitation light.

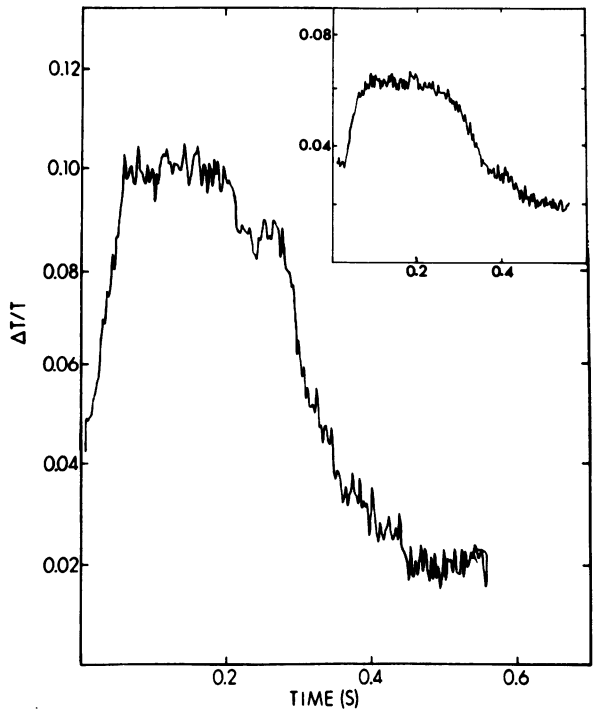

FIG. 5. Light-induced oxidation of P_{700} from guard cell extracts and whole-leaflet extract (inset). Chl concentrations were 4 and 5 μg/ml in the guard cell and whole leaflet extract, respectively. P_{700} oxidation in guard cell extract is integrated for 140 flashes of light; that for whole leaf, for 40 flashes.

DISCUSSION

The Chl *a/b* ratio found for *Vicia* guard cell protoplasts is typical of C_3 plants (7). However, high Chl *a/b* ratios (generally suggestive of high PSI/PSII) have been reported for *Vicia* epidermal peels [4.5 (27); 3.8 (16)]. We do not know why our value is so different from that of Pallas and Dilley (27), but the Lurie report (16) can be discounted because the Chl content of her samples (0.6 μg/cm^2) indicates that about 90% of it was not from guard cells. With epidermal peels of *Hymenocallis littoralis*, Freeman (9) found mostly Chl *a*, but other work indicates C_3-type Chl *a/b* ratios [*Commelina* and *Tulipa* (40); *Tulipa* (36)]. We caution that Chl *a/b* ratios are obtained by determining two measurements only a few nm apart; thus, Chl *a/b* ratios are highly dependent on resolution and accuracy of the spectrophotometers being used in comparisons. Altogether, we conclude guard cell chloroplasts have C_3-type Chl *a/b* ratios.

The absorption spectra for guard cell protoplast and whole leaf extracts were similar (Fig. 2). This matches the findings for extracts of epidermal peels by Willmer *et al.* [*Commelina* and *Tulipa* (40)] and Shaw and Maclachlan [*Tulipa* (36)], as well as the microspectrophotometric analysis of single guard-cell plastids by Yemm and Willis [*Rumex patientia*, *Chrysanthemum maximum*, *V. faba* (41)].

The low temperature fluorescence peak at 735 nm is mainly from PSI (6, 33), whereas the 685-nm peak originates in PSII and the light-harvesting Chl *a* + *b* complex (31). Our fluorescence data (Fig. 3) suggest the presence of PSII and constitute evidence for PSI. Delayed light emission (4), which could be blocked by DCMU, provided specific evidence for PSII (Fig. 4). Other evidence for PSI was P_{700} (Fig. 5). We conclude that the standard biophysical correlates of PSII and PSI are present in guard cells of *Vicia* and that the photosystems themselves, therefore, are present and functioning. However, we were unable to show light stimulation of O_2 evolution from guard cell protoplasts (unpublished), which requires the entire electron transport scheme to

remain coupled during isolation. We do not think this detracts from our conclusion, however, because mesophyll protoplasts subjected to the lengthy procedure required for guard-cell isolation also did not show light-stimulated O_2 evolution (unpublished). Our conclusion that PSII and PSI are present in *V. faba* guard cells is the same as that of Zeiger *et al.* (42) who report their findings for *Chlorophytum commosum* in a companion paper.

Guard cells, at least of *Vicia*, are extraordinary. They are the only cells known which lack the reductive step enzyme of the Calvin-Benson cycle (25) while having PSII (reported here). Two other green cells lack the Calvin-Benson cycle: mesophyll cells of C_4 plants (5) and heterocysts of Cyanobacteria (10). C_4 mesophyll cells have noncyclic photosynthetic electron flow but lack P-ribulokinase and ribulose bisP carboxylase. However, they have high levels of NADP-glyceraldehyde-P dehydrogenase and P-glyceric acid acts as a Hill oxidant (30). It is believed that part of the P-glyceric acid formed in bundle sheath cells (from CO_2 fixation) is shuttled to the mesophyll for reduction. Thus, an accurate picture of biochemical partitioning in C_4 plants is that CO_2 entry into the Calvin-Benson cycle occurs only in bundle sheath cells, but reduction occurs in bundle sheath cells and mesophyll cells. Heterocysts are specialized for dinitrogen fixation, which is extremely sensitive to O_2. Thus, these cells lack PSII and, therefore, do not evolve O_2. Without the electron from H_2O, it is not possible to have a net reduction of C. Perhaps it is best to look at heterocysts, not as green cells which lack the Calvin-Benson cycle, but as cells which have only PSI. Thus, guard cells are unique because the fate of the e^- donor generated by noncyclic photosynthetic electron flow is not to reduce C as it is in other cells. (We do not know if SO_4^{2-} or NO_3^- are reduced by guard cells but suspect this is not a major pathway because guard cells are such a small part of the whole plant.)

What are other functions of noncyclic photosynthetic electron flow? One result is maintainance of the light-sensitive membrane potential hyperpolarization (29). In *Vallisneria* leaf cells, Prins *et al.* (29) showed that PSI alone was sufficient for the initial hyperpolarization, but DCMU caused a slow depolarization. This is reminiscent of and, we speculate, directly parallel to the effect of DCMU on stomatal opening, where K^+ uptake correlates with light-sensitive membrane potential hyperpolarization[6] (20). On epidermal peels (where the effect is not via $\Delta[CO_2]$; see ref. 1), DCMU has little effect when closed stomata are caused to open by light [*Vicia* (12)]; however, already-open stomata close if DCMU is added to the medium containing the strips [*Senecio* (14)]. [There are also other data (39) showing that conditions required for stomatal opening are different from those required to keep stomata open.] We hypothesize that noncyclic photosynthetic electron transport results in a sensitive detection of continued presence of PAR [although PSI alone may be sufficient for the initial phase of K^+ uptake (12)]. The photosystems are eminently suited for this role: the absorption spectrum for the sensor regulating CO_2 entry into the leaf would be identical to the energy-harvesting system of photosynthetic cells. This hypothesis is consistent with the action spectrum required to keep stomata open (14) being like that for photosynthesis [and unlike the action spectrum causing stomata to open (34 and references therein)]. Moreover, two indirect lines of evidence suggest potassium-specific stomatal opening in light involves guard cell Chl: (*a*) *Paphiopedilum* spp. lacks chloroplasts in guard cells (21) and no K^+ uptake into guard cells occurs during stomatal opening in this species (22); and (*b*) the stomata of etiolated wheat (23) and albino barley (35) lack the normal light response of opening. We caution that this hypothesis is based on data from different species and cells; there is a need for more complete information on guard cells of one species. Moreover, H_2O oxidation by photosynthetic electron transport results in other physiological changes (*e.g.* enzyme activation), which may be important to stomatal opening. The caveats raised are particularly relevant in view of the picture emerging which shows guard cells to be so different biochemically from other cell types.

Note added in proof: H. Schnabl and H. Hampp have a paper in press ("Chloroplasts Derived from *Vicia* Guard Cell Protoplasts Lack Photosystem II and Ferredoxin-NADP:Oxidoreductase Activity", Naturwissenschaften) reporting the absence of PSII in *Vicia faba* L. guard cell chloroplasts. Klaus Raschke indicated in a footnote to a paper (*Plant Physiol.* 65: 88–93, 1980) that he observed changes in guard cells K^+ contents which correlated with changes in stomatal aperture in *Paphiopedilum leeanum*. C. Wilmer and J. Rutter (unpublished) observed more K^+ in guard cells of open stomata than those of closed stomata of *Paphiopedilum venustum* using histochemical techniques.

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[6] The discussion is restricted to light-induced (or -mediated or -maintained) stomatal opening. Cation specificity is different in light and dark (11, 38); therefore, dark opening may be by a different mechanism.

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