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Cytochemical and Cytofluorometric Evidence for Guard Cell Photosystems[1]

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ABSTRACT

Evidence for photosynthetic linear electron transport in guard cells was obtained with two sensitive methods of high spacial resolution. Light-dependent diaminobenzidine oxidation (an indicator of PSI) and DCMU-sensitive, light-dependent thiocarbamyl nitroblue tetrazolium reduction (an indicator of PSII) were observed in guard cell plastids of *Hordeum vulgare* L. cv Himalaya using electron microscopic cytochemical procedures. DCMU-sensitive Chl *a* fluorescence induction (an indicator of PSII) was detected in individual guard cell pairs of *Vicia faba* L. cv Longpod using an ultramicrofluorometer. At least for these species, we conclude these results are proof for the presence of PSII in guard cell chloroplasts, which until now has been somewhat controversial.

Chloroplasts are prominent organelles in guard cells of virtually all species. However, the ultrastructure and enzyme complement of these chloroplasts are markedly different from that of mesophyll chloroplasts. Guard cell chloroplasts are generally smaller and more spherical and have peripheral reticulum and fewer thylakoids per granum (1). In general, guard cells do not function in CO_2 reduction (17, 20). Thus, reports (19, 31) of the presence of both photosystems in 'sufficiently-pure' preparations of guard cells (16) raises the interesting possibility of a specific function for photosynthetic electron transport in these cells unrelated to CO_2 reduction (15, 19, 24). However, Schnabl and Hampp (23) reported the absence of PSII in guard cell protoplasts, which had been suggested by some earlier reports (6) because of low Chl *b* concentrations and the absence of light-dependent O_2 evolution in epidermal peels. Therefore, the question of whether guard cells have both photosystems has remained somewhat contentious. Because these differences may be technique-related, we have reinvestigated this question with specific, highly sensitive methods, which eliminate the possibility of false positive results from contamination and conclude that guard cell plastids of *Vicia faba* and *Hordeum vulgare* have both PSI and II.

MATERIALS AND METHODS

CYTOCHEMICAL EXPERIMENTS (STONEVILLE, MS)

Procedure. Seeds of *Hordeum vulgare* L. cv Himalaya were imbibed in excess 5 mM $CaSO_4$ (pH 5.7) in darkness at 22°C. After 24 h, the seeds were planted in moist vermiculite for growth under constant illumination (400 μE m^{-2} s^{-1} PAR) at the same temperature. Other samples were maintained in the dark for the same period to serve as an etiolated control.

About 10 1-mm^2 pieces from the terminal 5 to 10 mm of the primary leaf of 5-d-old seedlings were fixed for 1 h in 0.1 M phosphate buffer (ph 7.2) containing 4% (w/v) paraformaldehyde in darkness at 0 to 4°C (darkness and the low temperature were maintained in all steps prior to embedment except as noted). The samples were washed three times for 15 min in 0.1 M phosphate buffer (pH 7.2). Then, they were incubated for 1 h in one of three different specific reagent solutions [0.1 M phosphate buffer (pH 7.2) containing either 1 mg DAB[2]/ml or 1 mg TCNBT/ml in 0.5% v/v) dimethyl sulfoxide (required for solubilization of the tetrazolium) ± 1 μM DCMU]. For photosystem localization, the leaf pieces were incubated in fresh specific reagent solution at 22°C in darkness (for controls) or under 400 μE m^{-2} s^{-1} PAR. Guard cells in epidermal strips incubated in TCNBT in the light developed a strong purple coloration after a 1-h incubation and, thus, this length of incubation was chosen for the electron microscopic localization of the tetrazolium reduction. After 1 h, the leaf pieces were transferred to 0.1 M phosphate buffer (pH 7.2) containing 0.15 M sucrose (to reduce thylakoid dilation). After 30-min incubation, the leaf pieces were washed for 15 min in 0.1 M cacodylate buffer (pH 7.2) and postfixed for 1 h in similar buffer amended to 1% (w/v) OsO_4. The samples were dehydrated, embedded (27), and sectioned. The unstained sections were mounted on uncoated fine copper mesh grids and observed with a Hitachi HU-11C electron microscope.[3]

Comments. DAB photooxidation is a specific method for localizing PSI (2, 9, 13, 26, 30). To our knowledge, the only criticism of this method was made by Olah and Mueller (14), who found that DAB could be oxidized by plastidic polyphenoloxidase. This interference is not likely to have been important here because of the absence of polyphenoloxidase in guard cell plastids (25). Moreover, the activity of this enzyme in barley grown as described here is very low (D. E. Blume, unpublished) and dark control treatments did not oxidize DAB, although polyphenoloxidase is active in both light and dark.

Reduction of tetrazoliums by PSII is an accepted cytochemical assay procedure (5). However, of the tetrazoliums tested, only TNCBT (9) and DS-NBT (K. C. Vaughn, unpublished) were suitable for electron microscopic cytochemistry of PSII (7).

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[2] Abbreviations: DAB, diaminobenzidene; TCNBT, thiocarbamyl nitroblue tetrazolium.

[3] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

CYTOFLUOROMETRIC EXPERIMENTS (TALLAHASSEE, FL)

Procedure. *Vicia faba* L. cv Longpod was grown in a growth cabinet (600 μE m^{-2} s^{-1}; 60% RH; 14-h photoperiod; 25/20°C). Epidermal strips (50 mm^2) from the abaxial surface of fully expanded bifoliate leaflets were peeled at an obtuse angle and rinsed under running tap water for 5 to 10 s. Then, each strip was rinsed with 1 to 2 ml of water or water + 10 μM DCMU. (Both rinses also contained 0.1% [v/v] ethanol, which was the stock-DCMU solvent.) Individual strips were mounted leaf-side down on a microscope slide in about 50 μl of a solution like that used for rinsing. After placing a coverslip over the mounted tissue, the slide was inverted and secured on a microfluorometer stage.

An epidermal peel was briefly examined with dim transmitted light (100 w, 12-v tungsten-halogen lamp supplied with 6 v) on a Leitz Diavert microscope, which was the microfluorometer stand. A guard cell pair, apparently free of contaminating chloroplasts, was centered in the field. By use of blacklight illumination, a variable aperture diaphragm was adjusted to ensure that subsequently measured fluorescence originated from the immediate area of the centered guard cell pair. Two pieces of red plastic (%T < 1 at 400–695 nm; % transmittance = −513 + 0.73 [wavelength] for 700–750 nm; determined on a Hg line-calibrated Zeiss PMQII/M4QII spectrophotometer) were placed between the lamp and sample. This far-red illumination for 1 min (to oxidize PSII) slowed the F_0/F_{max} rise. Fluorescence kinetics were measured after concomitantly closing an electronic shutter controlling transmitted light and opening an electronic shutter (measured response time = 2.5 ms) controlling excitation light. The excitation light was from a 100-w Hg bulb and was delivered by epi-illumination. Before impinging on the guard cell pair, the excitation light sequentially (a) passed through a 1% neutral density filter (without diminution of excitation light; F_{max} did not recover fully on subsequent measurement); (b) passed through a Bausch and Lomb Hg 436 interference filter; (c) passed through a Phloempak condenser; (d) was reflected by a 510-nm dichroic mirror (Leitz RKP 510); and (e) passed through the objective lens (Leitz dry bright-field 63×/0.83). Emission light collected by the objective lens passed through (a) the 510-nm mirror, (b) the variable-aperture diaphragm, (c) a Turner 25 filter (a sharp cut red filter, 50% T at 610 nm), and (d) an interference wedge housing set at the neutral position. The fluorescence was detected by a Leitz MPV compact photometer. The photomultiplier was a Hamamasuta R928, which has a rise time of 2.6 ns; the slowest electronic component was the impedance converter (National Semiconductor LM 302), which operates at 100 kHz. These values are included to show that fluorescence induction was not an instrumental artifact. The instantaneous photometer signal was displayed on a Tektronix 5441 storage oscilloscope. This instrument was triggered by the signal opening the excitation light shutter. All measurements were made in a darkened room at 22 to 25°C. Total time between harvesting the epidermal peel and recording fluorescence was less than 10 min. At the end of each experiment, a sham measurement (with excitation light blocked) was made to establish baseline and signal pick-up. Then, the neutral density filter (in the excitation pathway) was removed and the area measured was examined by fluorescence microscopy to ensure the absence of contaminating chloroplasts.

Comments. Induction of Chl *a* fluorescence ("Kautsky effect") is reviewed in References 9 and 22. DCMU-sensitive fluorescence rise as reported here is indicative of reduction of PSII electron acceptor and intersystem electron carriers.

RESULTS

Cytochemical Experiments. Deposition of DAB photooxidation product was present exclusively along the lumen side of thylakoids of guard cell plastids in light-incubated tissue (Fig. 1A). No reaction was observed in dark-incubated tissue (not shown) or etioplasts (Fig. 1B) although mitochondria stained due to the action of Cyt *c* oxidase. These data suggest that the oxidation of DAB is dependent upon the presence of both light and Chl, neither of which is required for polyphenoloxidase-related oxidation of DAB (14).

Guard cell chloroplast staining by TCNBT was observed in light-incubated tissue (Fig. 1C), but not in dark-incubated tissue (not shown). This staining was mostly localized over granal regions of the plastid which are concentrated around the starch grains. Inclusion of DCMU in the specific reagent solution prevented TCNBT staining (Fig. 1D). Small electron-dense particles of unreacted TCNBT are found free in the stoma of these plastids, which indicates that the staining differences noted are not due to uptake differences of the cytochemical reagent. Similar cytochemical results have been obtained in mesophyll cell plastids of barley (26).

Cytofluorometric Experiments. $F_0 \rightarrow$ P fluorescence rise of individual guard cell pairs (Fig. 2A) was speeded by inclusion of DCMU (Fig. 2B). Fluorescence induction with the same guard cell pair was reproducible at light intensities used, but required a dark recovery period (usually 5 min) or illumination with far-red light between fluorescence measurements. At higher light intensities, the fluorescence rise was more rapid. However, at light intensities only seven times greater than indicated in the protocol, F_{max} values were not completely restored after dark adaptation. Kinetic measurements made over a longer time period (10–50 s) exhibited approximately linear decay (33–40% decline from the F_{max} value at 50 s). Additional measurements on the same guard cell pair after dark adaptation showed the F_{max} to have been restored. These subsequent measurements showed similar kinetics to the first made. The level of fluorescence varied somewhat from guard cell pair to guard cell pair, which prevents a direct comparison of F_{max} values obtained in the presence or absence of DCMU.

Fluorescence from an equal area of ordinary epidermal cells, which by eye were confirmed also to have small fluorescing chloroplasts, was about 25% as high as that from a guard cell pair. At the sensitivity of these measurements, epidermal cells exhibited little, if any, fluorescence change.

Fluorescence transients from mesophyll contaminants on the epidermal peels were also measured (with less signal amplification). Although the resolution was somewhat better than that obtained with guard cells, fine detail was lacking. Nevertheless, these tracings clearly exhibited fluorescence induction and decline.

DISCUSSION

Whether guard cells have PSII has been the subject of numerous investigations. Unfortunately, opposite results have been obtained. The reasons for the conflicting data include the technical challenge of studying the biochemistry of a few cells dispersed in the heterogeneous tissue comprising a leaf. The nature of these conflicting reports is briefly illustrated by contrasting data for *Vicia faba* published in the past decade. Lurie (8) obtained positive results for PSII (delayed light emission, O_2 evolution, noncyclic photophosphorylation) with epidermal peels. The Chl content of her epidermal peels was ~0.6 $\mu g/cm^2$, which, if restricted to guard cells, is calculated to be ~4 pg Chl/guard cell chloroplast. Other values based on sonicated epidermal peels (21) and guard cells protoplasts (19) are about 0.3 pg Chl/guard cell chloroplast. Thus, it appears that about 90% of the Chl in Lurie's samples was from contamination. Indeed, this risk of contamination was the reason Willmer *et al.* (29) rejected ordinary epidermal peels of *Vicia* as suitable material for guard cell Chl determinations. Both Lurie (8) and Pallas and Dilley (21) reported higher Chl *a/b* in extracts of epidermal tissue than in those of leaf. However, Outlaw *et al.* (19) reported similar Chl *a/b* for leaf and guard cell protoplasts. Using "sufficiently-pure" guard cell preparations (see 16), Outlaw *et al.* (19) and Ogawa *et al.* (15) reported the presence of PSII

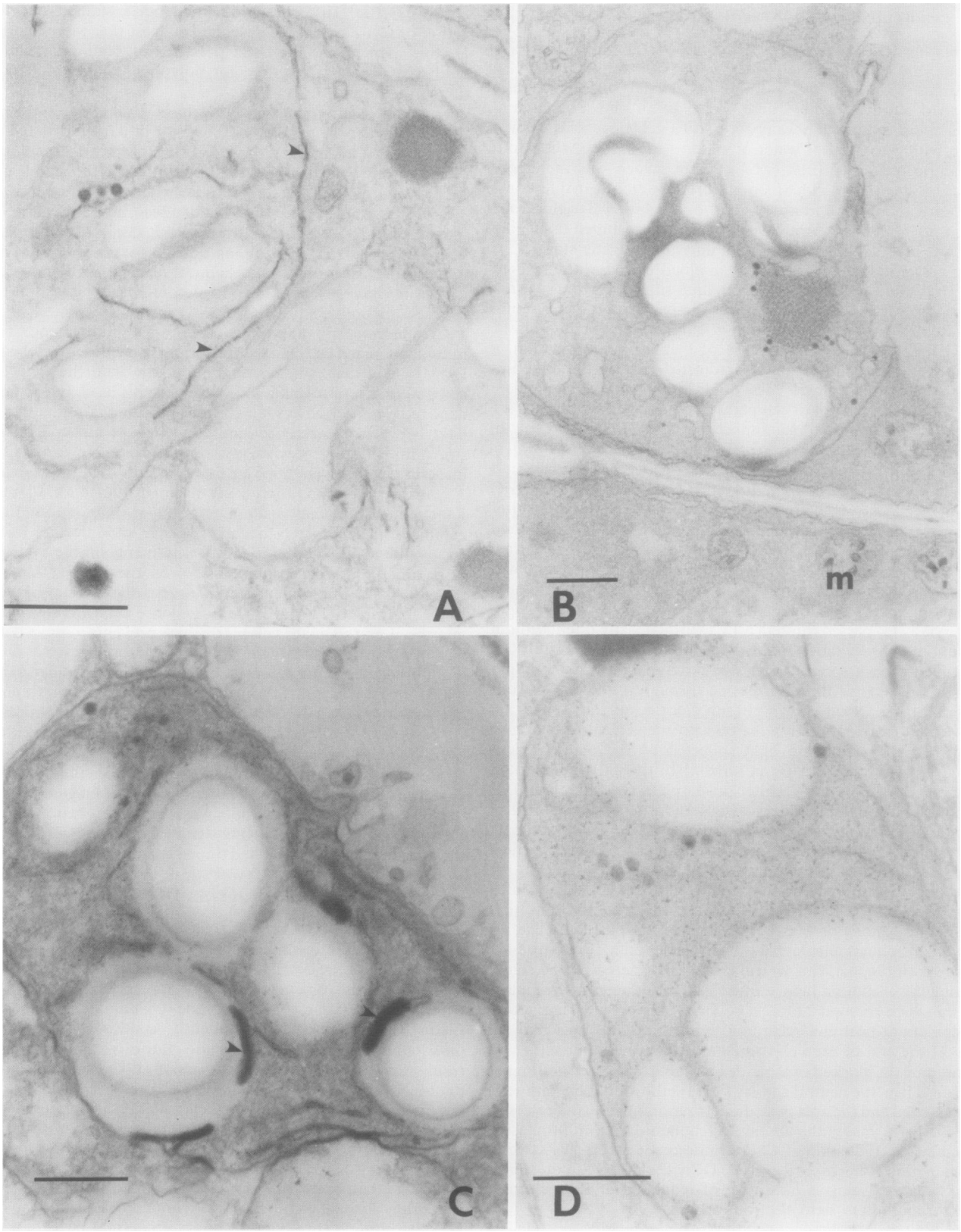

FIG. 1. Cytochemistry of barley guard cell plastids. A, DAB photooxidation in guard cell plastids occurs along the length of the lamellae (arrow). B, DAB oxidation in etioplasts. No plastid reactions are noted although considerable mitochondrial (m) depositions due to cytochrome oxidase are noted. C, TCNBT photoreduction in guard cell plastids occurs in the grana lamellae along the starch grains (arrows). D, Guard cell plastid incubated in the TCNBT media with 1 μM DCMU. No thylakoid reactions are evident although unreacted TCNBT is noted throughout the stroma. Bar, 1.0 μm in A and B and 0.5 μm in C and D.

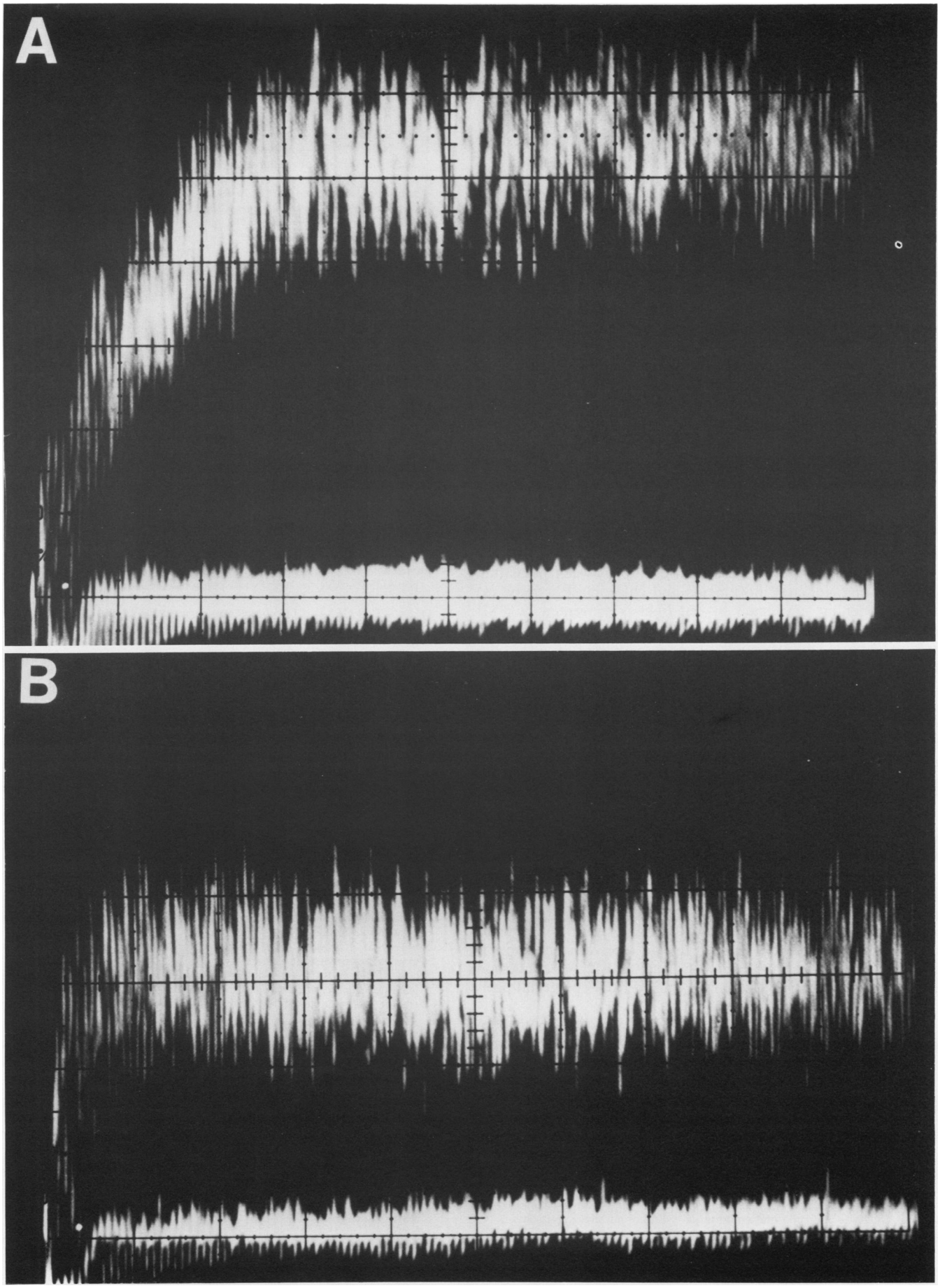

FIG. 2. Fluorescence induction of individual guard cells of *Vicia faba* by blue light after 1-min pre-illumination with weak far-red light. A, Control; B, +10 μm DCMU. For both, top trace: guard cell fluorescence; bottom trace: baseline and signal pick-up. Vertical axis: 10 ms/square; horizontal axis: 50 ms/square.

(delayed light emission, low temperature fluorescence emission spectrum, variable fluorescence) in guard cell chloroplasts. By contrast, Schnabl and Hampp (23) failed to detect PSII in extracts of pure preparations of guard cell protoplasts. The experiments reported in this paper are based on methods of high sensitivity and morphological resolution, which enabled us to avoid positive artifactual results from contaminating plastids of mesophyll of ordinary epidermal cells. Our data indicate guard cells of *H. vulgare* and *V. faba* conduct linear electron transport. To our knowledge, the only other experimental tests for PSII and PSI in guard cells conducted at high morphological resolution were by Das and Raghavendra (3), who did not display data.

Several potential functions of electron transport in guard cells must be considered. Net reduction of inorganic carbon may be excluded because guard cells (at least of C_3 and C_4 plants) lack significant levels of enzymes of the photosynthetic carbon reduction cycle (17, 20). However, effects of CO_2 on guard cell photochemistry have been reported. Epidermal peels from albino regions of variegated *Chlorophytum* leaves have normal guard cells, but lack contamination by functional mesophyll chloroplasts. The Chl *a* fluorescence kinetics of these *Chlorophytum* epidermal peels were affected by CO_2 (10), which is known to cause stomatal movements. Although qualitatively similar results were obtained with normal mesophyll, Melis and Zeiger (10) concluded that the CO_2 effect on guard cells was specific because these cells lack the ability to photosynthetically reduce CO_2. The validity of this conclusion is uncertain because of other direct "bicarbonate effects" on photosynthetic electron transport (28).

Some observations indicate a relationship between potassium uptake during stomatal movements and chloroplast activity: (a) stomatal opening stimulated by red light is prevented by PSII inhibitors (24); (b) guard cells of *Paphiopedilum* are unique in lacking Chl (11) and exceptional in lacking detectable accumulation of potassium in guard cells during stomatal opening (12, 18); and (c) Ogawa *et al.* (15) reported the P → S fluorescence decline in sonicated epidermal peels of *V. faba* was accelerated if the peels were incubated with KCl. They found even greater acceleration of the P → S decline if the potassium was supplied as the relatively impermeable phosphate salt and suggested chloroplast activity may be involved in malate formation.

At least two mechanisms for this interaction between photosynthetic electron transport and ion accumulation in guard cells are feasible. Pallas and Dilley (21) have calculated that guard cell photophosphorylation can provide sufficient ATP to drive ion uptake. Indeed, as indicated above (15), utilization of photosynthetically derived energy during ion accumulation does seem to occur. Another nonexclusive mechanism has been suggested by Outlaw *et al.* (19). Guard cells may "sense" PAR through regulation of enzyme activity. This suggestion receives some support from the recent findings of I. M. Rao and L. C. Anderson (unpublished) that some enzymes extracted from epidermal peels are modulated by SH reagents.

In summary, we have provided definitive proof for both photosystems in these guard cells. Probably, there is a specific function of photosynthetic electron transport during stomatal movements. The mechanism(s) remains to be elucidated. The photosystems in guard cells appear to be organized differently in guard cells than in mesophyll cells (10, 15). Moreover, the relationship between potassium uptake and chloroplast activity is not a general, obligatory one; potassium accumulates in guard cells during normal stomatal opening in darkness (4).

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