Plant Physiol. (1985) 78, 211-214
0032-0889/85/78/0211/04/$01.00/0

# Cyclic and Noncyclic Photophosphorylation in Isolated Guard Cell Chloroplasts from *Vicia faba* L.[1]

Received for publication September 21, 1984 and in revised form January 22, 1985

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## ABSTRACT

**High rates of both cyclic and noncyclic photophosphorylation were measured in chloroplast lamellae isolated from purified guard cell protoplasts from *Vicia faba* L. Typical rates of light-dependent incorporation of $^{32}P$ into ATP were 100 and 190 micromoles ATP per milligram chlorophyll per hour for noncyclic (water to ferricyanide) and cyclic (phenazine methosulfate) photophosphorylation, respectively. These rates were 50 to 80% of those observed with mesophyll chloroplasts. Noncyclic photophosphorylation in guard cell chloroplasts was completely inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea supporting the notion that photophosphorylation is coupled to linear electron flow from photosystem II to photosystem I. Several lines of evidence indicated that contamination by mesophyll chloroplasts cannot account for the observed photophosphorylation rates.**

**A comparison of the photon fluence dependence of noncyclic photophosphorylation in mesophyll and guard cell chloroplasts showed significant differences between the two preparations, with half saturation at 0.04 and 0.08 millimole per square meter per second, respectively.**

Chloroplasts are a constant feature of guard cells (26). Recent studies have shown that guard cell chloroplasts have light harvesting pigments for PSI and PSII (5, 15, 21, 23, 27) and the capacity for photosynthetic electron transport (13, 21, 27) and $O_2$ evolution (7, 21). Indirect evidence for photophosphorylation in guard cell chloroplasts includes the presence of the $\alpha$- and $\beta$-subunits of the coupling factor complex (5), the demonstration of typical Chl *a* fluorescence transients (10, 11, 13, 21), and the light-induced 518-nm electrochromic shift (6). A role of guard cell chloroplasts in stomatal movements is supported by the wavelength dependence of stomatal opening (12, 19, 20, 29), the inhibition of opening by the photosynthetic electron transport inhibitor, DCMU, particularly under red light (19, 20), and observations showing that achlorophyllous stomata of the orchid *Paphiopedilum* fail to open under red irradiation (28).

The hypothesis that guard cell chloroplasts supply ATP for active ion transport during stomatal movements is a classic concept in stomatal physiology (8, 16). Photophosphorylation has been measured in guard cell chloroplasts from epidermal peel preparations (9, 17), but these results have been difficult to interpret because of the possible contamination from mesophyll chloroplasts (27). Since the ratio of Chl content in guard cells to mesophyll cells is about 1 to 80 (22), a 1.3% mesophyll contamination would yield preparations containing equal amounts of Chl from the two cell types. Such levels of contamination are difficult to avoid in work with epidermal peels.

The availability of highly purified preparations of guard cell protoplasts (21, 22) as a source of isolated chloroplasts provided us with an improved approach to this question. We report here that isolated guard cell chloroplasts from *Vicia*, judged free of mesophyll contamination by several criteria, exhibited high rates of cyclic and noncyclic photophosphorylation. A comparison of the photon fluence dependency of photophosphorylation in guard cell and mesophyll chloroplasts showed significant differences which might have functional implications.

## MATERIALS AND METHODS

**Preparation of Guard Cell and Mesophyll Chloroplasts.** Plants of *Vicia faba* L. cv Long Pod (Atlee Burpee Co., Warminster, PA) were grown in a commercial potting soil under natural light in a greenhouse (19). Epidermal strips were obtained from the abaxial (lower) surface of young, fully expanded leaves (4- to 7-week-old plants) and accumulated in ice-cold distilled $H_2O$. The strips were cut into small pieces with scissors, sonicated for 30 s at 0°C (Ultrasonics Inc. model W-225), and washed with distilled $H_2O$. The sonication treatment breaks the epidermal cells and removes most adhering mesophyll contaminants from the strips (6, 12, 21).

Guard cell protoplasts were isolated from the sonicated epidermal strips by a two-step enzymic digestion at 20 ± 1°C (21). In the first step, strips were digested in 4% cellulase (Onozuka R-10), 0.25 M mannitol, and 1 mM $CaCl_2$ for 1 h, with shaking at 60 strokes $min^{-1}$. After filtration through a 58-$\mu$m nylon mesh and washing in 0.4 M mannitol and 1 mM $CaCl_2$, the strips were subjected to a second digestion step for about 20 h, with the mannitol and $CaCl_2$ increased to 0.4 M and 5 mM, respectively, and the shaking reduced to about 40 strokes $min^{-1}$. Released guard cell protoplasts were separated from the epidermal peels by filtration through a 30-$\mu$m nylon mesh, collected by low-speed centrifugation (100*g*, 10 min) and stored in ice-cold 0.4 M mannitol and 1 mM $CaCl_2$. In a typical experiment, 150 leaves were peeled, yielding a total guard cell Chl of 10 to 15 $\mu$g.

For the isolation of chloroplasts, the guard cell protoplasts (300 $\mu$l) were poured onto a 5-$\mu$m nylon mesh which was placed at the top of a microfuge tube (E and K Scientific Products), filled with 0.8 ml of the chloroplast isolation medium (50 mM Tricine-NaOH buffer [pH 7.5], 0.4 M sucrose, 20 mM NaCl, and 5 mM $MgCl_2$). The protoplasts were ruptured by rapid centrifugation (16,000*g* for 10 s) in an Eppendorf microfuge (model 5412). The pellet containing the guard cell chloroplasts was gently homogenized in a Teflon homogenizer and suspended uniformly in the same medium at 0°C. For the isolation of mesophyll chloroplasts, 5 to 10 g (fresh weight) of *Vicia* leaf segments devoid of main veins were combined with 80 ml of ice-cold isolation medium and homogenized in an Ultra Turrax blender (Janke and Kunkel, West Germany) at 0°C. After filtration through four layers of Miracloth, the homogenate was centri-

[1] Supported by the National Science Foundation, the United States Department of Agriculture, and the United States Department of Energy.

fuged at 200*g* for 3 min. The chloroplasts were isolated from the supernatant by further centrifugation at 1500*g* for 7 min and suspended uniformly in the isolation medium. Chl concentrations were determined by the method of Arnon (1).

**Measurement of Photophosphorylation.** Light-dependent ATP formation was determined by measuring $^{32}P$ incorporation according to the method of Asada *et al.* (2). The basic reaction mixture (2.1 ml) contained 20 mM Tricine-NaOH buffer, pH 8.3, 0.1 M sucrose, 5 mM $MgCl_2$, 0.2 mM ADP, 0.91 mM $K_2HPO_4$ (0.4 $\mu$Ci $^{32}P$), 0.6 mM potassium ferricyanide, and chloroplasts containing 0.2 to 0.6 $\mu$g Chl. For the measurement of cyclic photophosphorylation, the potassium ferricyanide was replaced by 0.05 mM PMS[2]. Unreacted $^{32}P$-labeled orthophosphate was extracted as phosphomolybdic acid by organic solvents (2). Radioactivity of the final aqueous phase was measured as Cerenkov radiation (2). Typical counts for a sample with 0.55 $\mu$g Chl were 350, 580, and 70 cpm for the ferricyanide, PMS, and dark-ferricyanide treatments, respectively.

The guard cell chloroplast preparations were handled under dim green light at room temperature. Photophosphorylation was initiated by illumination with orange actinic light from the bottom of the test tube, and the reaction was terminated after 3 min by adding 5 ml of 10% $HClO_4$ to 2.1 ml of the reaction mixture. Actinic light was provided by a tungsten lamp (Sylvania, 150 w), filtered by one layer of orange gelatinous film (Cinemoid 5A) and a 4-cm layer of 0.2% copper sulfate solution. Photon fluence rates at the surface of the tube, measured with a Li Cor quantum sensor (model 185B), was 0.61 mmol $m^{-2}$. $s^{-1}$, unless otherwise specified.$^{32}P$ radioactivity was measured in a Beckmann liquid scintillation counter in a coincidence mode, at room temperature. The counting efficiency was corrected by internal standardization.

## RESULTS

**Evaluation of the Purity of Guard Cell Protoplasts.** Mesophyll protoplasts can be easily distinguished from guard cell protoplasts under bright field microscopy because of their larger size and higher chloroplast content. Extensive examination of purified guard cell protoplast preparations failed to reveal mesophyll cell contaminants. A typical optical field from a guard cell protoplast preparation is shown in Figure 1A. The same preparations were examined under fluorescence microscopy to test for the presence of free mesophyll chloroplasts, which are larger and fluoresce much more intensely than guard cell chloroplasts (30), also with negative results.

Further evaluation of this criterion of purity was undertaken by preparing defined mixtures of guard cell and mesophyll protoplasts. For that purpose, the cell densities of separate preparations of the two protoplast types were determined with a hemocytometer and sufficient mesophyll protoplasts to contribute 1 or 5% of the final cell number was added to purified preparations of guard cell protoplasts. In initial experiments, the differences in osmotic potential between standard mesophyll and guard cell protoplast media (0.6 and 0.4 M mannitol, respectively) was ignored, and the calculated aliquot of mesophyll protoplast suspension was added to the 0.4 M mannitol medium with the guard cell protoplasts. Mesophyll protoplasts burst in this medium, making it necessary to conduct the mixing experiments in 0.6 M mannitol. Guard cell protoplasts shrank in that solution but remained stable. Examination of these mixed preparations under bright field (Fig. 1B) and fluorescence microscopy readily revealed the presence of mesophyll protoplasts, at both levels of contamination. Thus, the maximum contamination level of the purified guard cell protoplast preparations was significantly lower than 1%.

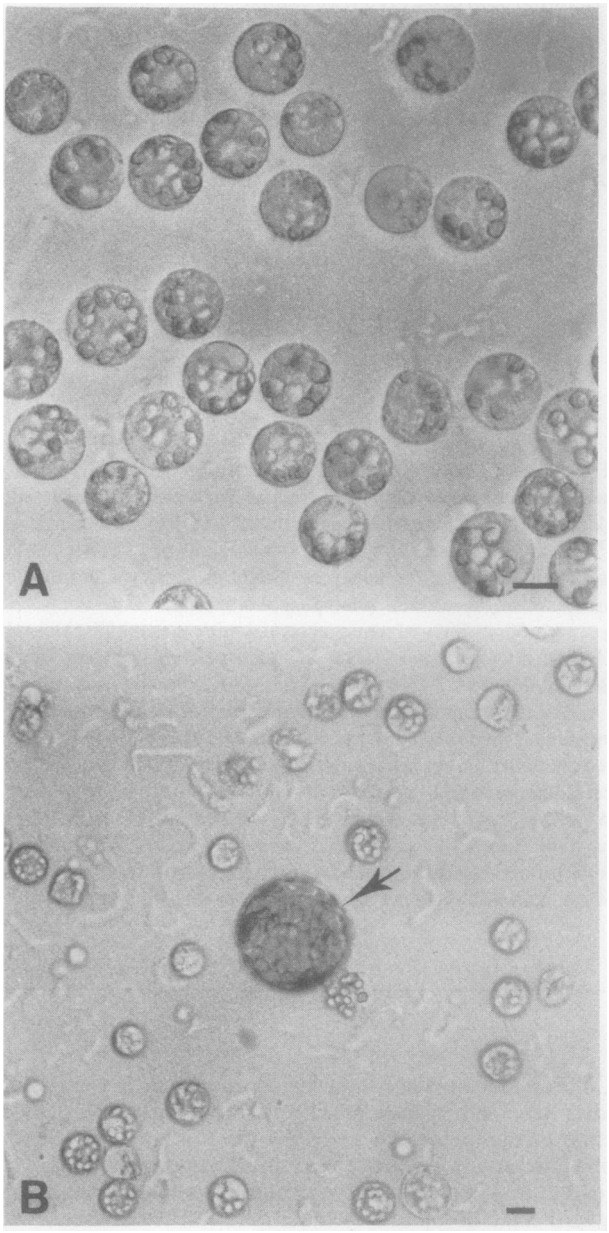


FIG. 1. A, Isolated guard cell protoplasts from *Vicia faba* in 0.4 M mannitol. The guard cell chloroplasts can be clearly seen. B, Micrograph from a preparation in which 1% (on a cell number basis) mesophyll protoplasts from *Vicia* were added to a suspension of guard cell protoplasts in 0.6 M mannitol. Arrow, mesophyll protoplast. Scale bars, 10 $\mu$m.

**Photophosphorylation in Guard Cell and Mesophyll Chloroplasts.** As measured by light-dependent (0.61 mmol $m^{-2}$ $s^{-1}$) $^{32}P$ incorporation into ATP, chloroplasts isolated from *Vicia* guard cell protoplasts exhibited significant rates of photophosphorylation. Typical rates were 100 and 190 $\mu$mol ATP $mg^{-1}$ Chl·$h^{-1}$ for noncyclic (water to ferricyanide) and PSI (PMS) dependent photophosphorylation, respectively (Table I), representing 66 and 53% of the rates measured with mesophyll chloroplasts. Noncyclic photophosphorylation in guard cell chloroplasts was as high as 80% of the mesophyll values in some experiments (Fig. 2). Addition of DCMU to the reaction mixture completely inhibited noncyclic photophosphorylation, supporting the notion that coupling of photophosphorylation to electron flow from

[2] Abbreviations: PMS, phenazine methosulfate; RuBP, ribulose 1,5-biphosphate.

Table I. *Typical Rates of Noncyclic and Cyclic Photophosphorylation in Guard Cell and Mesophyll Chloroplasts*

Basic reaction mixture contained 0.1 M sucrose, 20 mM Tricine-NaOH (pH 8.3), 5 mM $MgCl_2$, 0.2 mM ADP, 0.91 mM $K_2HPO_4$ (0.4 $\mu$Ci $^{32}P$), 0.6 mM potassium ferricyanide, and chloroplasts. The amount of Chl in the reaction mixture for guard cell and mesophyll chloroplasts was 0.55 and 0.51 $\mu$g, respectively. For the measurement of cyclic photophosphorylation, the ferricyanide was replaced with 0.05 mM PMS. DCMU was added at 10 $\mu$M.

| Reaction | Rate of ATP Formed in | |
|---|---|---|
| | Guard cell chloroplasts | Mesophyll chloroplasts |
| | $\mu mol\ mg^{-1}\ Chl \cdot h^{-1}$ | |
| $H_2O \rightarrow Fe(CN)_6^{3-}$ | 103 | 155 |
| $H_2O \rightarrow Fe(CN)_6^{3-}$ (+DCMU) | 0 | 0 |
| PMS | 193 | 365 |

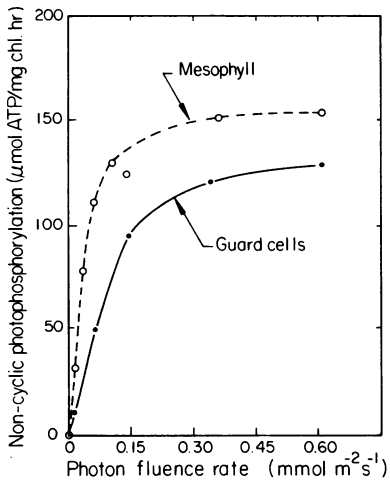


FIG. 2. Rate of noncyclic photophosphorylation as a function of incident photon fluence rate of orange light in guard cell (●) and mesophyll (○) chloroplast preparations. Basic reaction mixture as in Table I. Total Chl in the reaction mixture was 0.38 and 0.51 $\mu$g for guard cell and mesophyll chloroplasts, respectively. Light intensity was reduced with neutral density filters.

PSII to PSI occurs in guard cell chloroplasts (11). The DCMU effect and the observed light dependency indicate that mitochondrial phosphorylation did not occur in these experimental conditions. $^{32}P$ incorporation was nil in darkness and in the absence of exogenous ADP. A total of six experiments performed with different protoplast isolates yielded comparable results (rates for noncyclic photophosphorylation in the six experiments were: 127, 98, 106, 129, 103, and 103 $\mu$mol ATP $mg^{-1}$ Chl·$h^{-1}$).

**Photon Fluence Dependency of Photophosphorylation in Guard Cell and Mesophyll Chloroplasts.** A comparison of the photon fluence dependency of noncyclic photophosphorylation in mesophyll and guard cell chloroplasts is shown in Figure 2. At low photon fluences, rates increased linearly with fluences in both preparations but the initial slope was about 60% less with guard cell chloroplasts. Half saturation occurred at about 0.04 mmol $m^{-2}$ $s^{-1}$ in mesophyll and 0.08 mmol $m^{-2}$ $s^{-1}$ in guard cell chloroplast preparations. Identical results were obtained in two separate experiments conducted with different protoplast isolates.

## DISCUSSION

A conclusive demonstration of photophosphorylation in guard cell chloroplast preparations critically depends on the lack of mesophyll contamination. Several lines of evidence indicate that this condition has been achieved in the experiments described here. Sonication of the *Vicia* epidermal peels removed most of the nonstomatal chloroplasts (6, 12, 21), and any remaining epidermal and mesophyll cells were eliminated by the first digestion step in 0.25 M mannitol. Furthermore, any residual mesophyll protoplasts would lyse during the extended digestion in 0.4 M mannitol. An independent criterion of purity arises from a previous study in which protoplasts were isolated with the same method used in the present work, showing clear-cut differences in the fluorescence transients from guard cell and mesophyll protoplast preparations (21). Contaminated guard cell protoplast preparations would be expected to show the characteristic M peak of the mesophyll (11, 21). Finally, guard cell chloroplasts exhibited rates of noncyclic photophosphorylation approaching 80% of the mesophyll rates, on a Chl basis. Given a ratio of Chl content in guard cells to mesophyll cells of about 1:80, it can be calculated that a minimum of 1% mesophyll contamination would be necessary to account for all the noncyclic photophosphorylation in a hypothetically inactive guard cell chloroplast preparation. The mixing experiments described in "Results" showed that in guard cell protoplast preparations known to be contaminated with 1% mesophyll protoplasts, the contaminant was readily detectable under bright field or fluorescence microscopy, whereas purified guard cell protoplast preparations showed virtually no mesophyll contaminants. The level of contamination of the purified preparations can therefore be estimated to be 0.1% or less, ruling out the possibility that contaminant mesophyll could account for the observed photophosphorylation activity.

The reported method for the isolation of purified guard cell chloroplasts opens previously unavailable opportunities for comparative biochemistry of mesophyll and guard cell chloroplasts. The *in vitro* photophosphorylation observed with isolated guard cell chloroplasts shows that a significant portion of their photosynthetic apparatus remains functional after the isolation. Further improvement of the isolation procedure might render intact chloroplasts, facilitating studies of stromal reactions.

Measurements of cyclic and noncyclic photophosphorylation in guard cell chloroplasts confirm previous inferences about their photophosphorylation capacity based on fluorescence spectroscopy (6, 10, 11, 13, 21). The measured photophosphorylation rates were similar, although somewhat lower than in the mesophyll. It is unclear if the observed lower rates were a result of an intrinsically low photosynthetic capacity or a consequence of some damage from the long enzymic digestion required for guard cell protoplast isolation, a step not used with the mesophyll material. A strict comparison under the same experimental conditions was not possible because of the basic differences between the isolation procedures (21) and the instability of the mesophyll protoplasts when subjected to long incubation in the cellulolytic enzymes.

The contribution of photophosphorylation by guard cell chloroplasts to the energy supply during stomatal opening can be evaluated on the basis of data showing that, under saturating red irradiation, *Vicia* stomata in epidermal peels exhibit a net increase in aperture of about 4 $\mu$m over a 2-h period (19). Considering noncyclic photophosphorylation alone (Table I) and a Chl content of 2 pg guard $cell^{-1}$, irradiated guard cell chloroplasts would supply 0.41 pmol ATP guard $cell^{-1}$ over a 2-h period. Estimates of the ATP required to drive the ion uptake occurring during such an aperture increment (4) indicate that this calculated supply of ATP would suffice. Much more information is needed to validate these estimates for *in vivo* conditions, but the calculations strengthen previous conclusions based on physiological evidence, suggesting that guard cell photophosphorylation is the source of energy for stomatal opening in the leaf under

moderate to high irradiances (16, 26, 29).

Possible damage from the isolation procedure also constrains the interpretation of the differential light sensitivity of guard cell chloroplasts as compared to their mesophyll counterparts (Fig. 2). The *in vitro* findings, however, are validated by observations of the higher photon fluence requirement for stomatal opening in the red, which primarily depends on photosynthesis from guard cell chloroplasts, as compared with the response of mesophyll photosynthesis, which shows no similar threshold (29). Furthermore, a comparison of fluorescence transients from guard cell and mesophyll chloroplasts (10) showed a photon fluence dependency similar to that of *in vitro* photophosphorylation, indicating that the lower light sensitivity of guard cell chloroplasts might reflect an intrinsic property of their photosynthetic apparatus.

An evaluation of the rates of cyclic and noncyclic photophosphorylation in guard cell and mesophyll chloroplasts fails to reveal an unusually high PSI activity in guard cell chloroplasts, as postulated in previous studies (9, 16, 18). Comparable, high noncyclic photophosphorylation capacity in both preparations also substantiates electron flow from PSII to PSI in guard cell chloroplasts (13, 21, 27), implicating the reduction of $NADP^+$, as the physiological electron acceptor. The fate of the NADPH generated by guard cell chloroplasts remains an intriguing aspect of guard cell biology.

Demonstration of photophosphorylation capacity in guard cell chloroplasts adds to the growing number of features of these organelles found to match the properties of mesophyll chloroplasts, including light harvesting pigments of PSI and II, electron transport, and a $CO_2$ sensitivity of the slow fluorescence transients associated with the high energy state (11). Although the later feature is inconsistent with the reported lack of RuBP carboxylase in guard cell chloroplasts (14), a recent immunological study showing reactivity of these organelles to antibodies against RuBP carboxylase (5), may reopen the question of a complete absence of RuBP carboxylase in guard cells. Regardless of the definitive answer to that problem, the bulk of the available evidence indicates that guard cell chloroplasts do not fix significant amounts of $CO_2$ under conditions typical for mesophyll chloroplasts (14, 24, 25). In the absence of $CO_2$ fixation, the ATP generated in illuminated guard cell chloroplasts would be available for export to the cytoplasm, for use by a plasmalemma ATPase (26). Simultaneous responses of guard cell and mesophyll chloroplasts to light would therefore constitute one component of the parallel responses coupling mesophyll photosynthesis with stomatal conductance (3, 29). The emerging picture strengthens the notion of an important role of guard cell chloroplasts in the light response of stomata and the photocontrol of photosynthesis and stomatal conductance in the intact leaf.

*Acknowledgments*—We thank Ms. Judy Nishimoto for skillful assistance; Drs. P. Ray, H. Mooney, C. Yanofsky, and R. Simoni from the Department of Biological Sciences of Stanford University, and W. Briggs and J. Berry from the Carnegie Institution, for generous loan of equipment; and Dr. D. Grantz and Ms. Sally Assmann for valuable discussions. K. S. gratefully acknowledges a McKnight postdoctoral fellowship.

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