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Isolation of Guard Cell Protoplasts from Mechanically Prepared Epidermis of *Vicia faba* Leaves[1]

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ABSTRACT

A method for isolating guard cell protoplasts (GCP) from mechanically prepared epidermis of *Vicia faba* is described. Epidermis was prepared by homogenizing leaves in a Waring blender in a solution of 10% Ficoll, 5 millimolar $CaCl_2$, and 0.1% polyvinylpyrrolidone 40 (PVP). Attached mesophyll and epidermal cells were removed by shaking epidermis in a solution of Cellulysin, mannitol, $CaCl_2$, PVP, and pepstatin A. Cleaned epidermis was transferred to a solution of mannitol, $CaCl_2$, PVP, pepstatin A, cellulase "Onozuka" RS, and pectolyase Y-23 for the isolation of GCP. Preparations made by this method included both adaxial and abaxial GCP and contained ≤0.017% mesophyll protoplasts, ≤0.6% mesophyll fragments, and no epidermal cell contaminants. Yields averaged 9×10^4 protoplasts/leaflet and 98 to 100% of the GCP excluded trypan blue, concentrated neutral red, and hydrolyzed fluorescein diacetate. Isolated GCP increased in diameter by 2.2 micrometers after incubation in darkness in 10 micromolar fusicoccin, 0.4 molar mannitol, 5 millimolar KCl, and 1 millimolar $CaCl_2$. Illumination of GCP with 800 micromoles per square meter per second of red light resulted in alkalinization of their suspension medium. When 10 micromolar per square meter per second of blue light was superimposed onto the red light background, the medium acidified. Measurements of chlorophyll *a* fast fluorescence transients from isolated GCP indicated that GCP were capable of electron transport, and slow transients contained the "M" peak usually associated with a functional photosynthetic carbon reduction pathway.

Guard cell protoplasts (GCP[3]) have become an increasingly important tool for studies of the osmotic properties (4, 9, 10, 20), physiology (2, 4, 14, 15), metabolism (5, 15), biochemistry (3, 5), and membrane characteristics (2, 11, 12) of stomatal guard cells (see ref. 18 for a review). In recent years, improvements in cellulolytic enzymes and refinement of digestion protocols have made possible the isolation of highly purified preparations of viable GCP in sufficient quantities for biochemical analysis (5, 18). On the other hand, methods for isolating GCP are limited to a small number of species from which leaf epidermic can be easily separated from underlying mesophyll tissue (17, 18). Contaminating epidermal and mesophyll cells adhering to detached epidermis are removed during the preparation of GCP by a differential enzymic digestion (13). Mesophyll contamination, however, can be kept to reasonably low levels only if epidermis is first detached from between leaf veins (17, 18). Such a laborious procedure extends the time needed to prepare large numbers of GCP to several hours, limiting protoplast yield and the number of experiments that can be conducted in 1 d (18). Attempts have been made to prepare GCP from whole leaf sections, but the purity of such preparations was unsatisfactory (6).

In this study we report on a method for isolating GCP from epidermis prepared by mechanical homogenization of *Vicia faba* leaves. This procedure eliminates the time-consuming manual dissection of leaf epidermis and yields large numbers of physiologically competent, highly purified GCP.

MATERIALS AND METHODS

Plant material

Plants of *Vicia faba* L., cv 'Long pod,' were grown from seeds (W. Atlee Burpee Co., Riverside, CA) in potting soil (Supersoil, Chino, CA) in a greenhouse under natural light conditions. Temperatures ranged between 15 and 25°C. Plants were watered daily. After the 3rd week following planting, plants were fertilized once a week with a solution of Spoonit (Plantsmith, Mountain View, CA) at a concentration of approximately 3 g L^{-1}.

Assessment of GCP Yield, Purity, and Viability

Protoplast yields were measured with a hemocytometer using the mean of three to four determinations. Contamination by mesophyll protoplasts, epidermal protoplasts, and small clusters of chloroplasts from broken mesophyll cells ("mesophyll fragments") was assessed by examining three microscopic fields of three samples from two GCP preparations made on separate days (total GCP counted = 5769). Small mesophyll protoplasts had smaller, more numerous chloroplasts that were a deeper shade of green than those of GCP, making it possible to distinguish readily such cells from GCP.

In experiments designed to optimize isolation conditions, viability of GCP was assessed initially by examining guard cell chloroplasts for damage caused by the isolation procedure.

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[3] Abbreviation: GCP, guard cell protoplasts.

Cells with fragmented, granular chloroplasts were considered to be damaged and unlikely to give normal physiological responses (8). Viability of GCP was further assessed by estimating exclusion of trypan blue (7), concentration of neutral red (7), and hydrolysis of fluorescein diacetate (19).

Osmotic responses of GCP were evaluated by incubating them for 45 min in darkness at room temperature in 10 μM fusicoccin, 0.45 M mannitol, 5 mM KCl, and 1 mM $CaCl_2$ (9, 10). Protoplast diameters were measured with a microscope equipped with a TV camera and monitor.

The physiological competence of GCP isolated from mechanically prepared epidermis was assessed by measuring red light-induced alkalinization and blue light-induced acidification under conditions described previously (14). Photosynthetic activity in chloroplasts from GCP was evaluated by measuring fluorescence transients associated with photosynthetic electron transport and photophosphorylation by microfluorospectrophotometry (22) (B Mawson, E Zeiger, unpublished data). Guard cell protoplasts were illuminated with green actinic light, and both the fast and slow variable fluorescence transients were recorded (22) (B Mawson, E Zeiger, unpublished data). Chl content of guard cell chloroplasts was measured by the method of Arnon (1).

RESULTS

Experiments designed to optimize various parameters affecting GCP yield, viability, and purity resulted in the following method. Leaflets were harvested from the second node from the top of 3 to 4 week old plants and stored in Petri dishes lined with moistened paper towels. Confining leaf selection for each experiment to a single node ensured uniform wall digestion and protoplast release. Midveins were removed from leaves with a razor blade and each half of the leaf was cut into eight pieces. Pieces collected from 10 leaves were transferred to a 250 mL stainless steel Waring blender jar and suspended in 100 mL of a homogenization solution containing 10% Ficoll (type 400), 5 mM $CaCl_2$, and 0.1% PVP 40. Leaves were homogenized with a Waring commercial blender (model 7011) at 20,000 to 22,000 rpm (measured with blender cup empty) at room temperature. Leaves from 3 week old plants were homogenized for 50 s; those from 4 week old plants were homogenized for 60 to 70 s. Epidermal fragments from successive homogenizations of each 10-leaf batch were collected and combined by filtering homogenates through a nylon net with a mesh size of 220 μm, and rinsed thoroughly on the net for 2 to 3 min under running tap water.

In order to remove epidermal and mesophyll cells, epidermis was transferred to 100 mL of solution containing 0.25 M D-mannitol, 0.7% Cellulysin (Behring Diagnostics, LaJolla, CA), 1 mM $CaCl_2$, 0.1% PVP 40, and 1 μg/ml of pepstatin A, pH 5.55. Epidermal fragments were incubated at 26 to 27°C in an reciprocating water bath at 135 excursions/min. After 45 min of incubation, fragments were examined microscopically for attached mesophyll. If little mesophyll remained, incubation was continued for an additional 15 to 30 min. If large amounts of mesophyll remained, epidermis was retrieved on the nylon net, rinsed with a solution of 0.25 M mannitol, 1 mM $CaCl_2$, and incubated in fresh enzyme solution as described above for another 45 min. Digestion with Cellulysin solution could be performed for 5 to 6 h without releasing GCP. After cleaning, epidermis was collected on the nylon net, rinsed thoroughly with 500 mL of 0.25 M mannitol, 1 mM $CaCl_2$, and transferred to 50 mL of a solution containing 0.35 M mannitol, 1 mM $CaCl_2$, 1% cellulase "Onozuka" RS (Yakult Honsha Co., Ltd., Tokyo, Japan), 0.01% pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd., Tokyo, Japan), and 1 μg/ml pepstatin A, pH 5.55. Epidermis was incubated at 26 to 27°C in an oscillating water bath at 44 excursions/min until GCP were released into the medium, usually after about 2 h. Optimal times for protoplast release were assessed by microscopic examination of digesting epidermal fragments. The solution was poured through a 30 μm nylon net into 50-mL conical centrifuge tubes, and GCP were collected by centrifugation at 60g for 7 min. Protoplasts were washed three times with 0.35 M mannitol, 1 mM $CaCl_2$ by centrifugation.

GCP isolated by the method described are shown in Figure 1. Typical preparations contained $\leq$0.017% mesophyll protoplasts and $\leq$0.6% mesophyll fragments; no epidermal protoplasts were found. Protoplasts were spherical and contained intact chloroplasts (Fig. 1A). In all preparations examined, 98 to 100% of GCP excluded trypan blue, concentrated neutral red, and hydrolyzed fluorescein diacetate (Fig. 1B). The average yield of GCP was 9×10^4/leaflet (n = 5, SE = 1.5×10^4), and yields from 50 leaflets ranged between 2.5 and 4.5 $\times 10^6$ protoplasts (n = 3). Chl content per GCP was 1.8 pg.

Fusicoccin treatment caused a statistically significant increase in GCP diameter to 21.5 $\pm$ 0.11 μm compared to controls containing only KCl (19.3 $\pm$ 0.09 μm) or fusicoccin but no KCl (19.7 $\pm$ 0.09 μm) (mean $\pm$ 1 SE of the sample mean; n = 300/treatment; Scheffe F-test, 0.05 level of significance). Diameters of GCP were normally distributed after fusicoccin treatment (Fig. 2).

Responses of GCP to illumination with red and blue light are shown in Figure 3. When GCP were illuminated with 800 μmol m^{-2} s^{-1} of red light, they alkalinized the suspension medium from an initial pH of 6.42 to a steady-state pH of 6.52 after about 30 min (Fig. 3). When 10 μmol m^{-2} s^{-1} of blue light was superimposed on the red light background, GCP acidified the medium to a pH of about 6.5 after 18 min (Fig. 3).

Fast fluorescence transients from both guard cell and mesophyll protoplasts showed the O-I-P transition associated with reduction of Q, the primary electron acceptor of PSII (22) (Fig. 4, C and D). Slow fluorescence transients from both types of protoplasts contained the P to S phase followed by an M peak(s) (Fig. 4, A and B). Both the induction and quenching patterns of variable fluorescence from chloroplasts of GCP were qualitatively similar to those recorded from mesophyll protoplasts (Fig. 4, A and B). No variable fluorescence could be detected in contaminating mesophyll fragments (Fig. 4C).

DISCUSSION

The newly developed method relies on mechanical separation of leaf epidermis to circumvent the more time consuming, manual detachment procedures (17, 18). The viscosity of the Ficoll solution appears to protect guard cells from damage during leaf homogenization. Attempts to use lower concen-

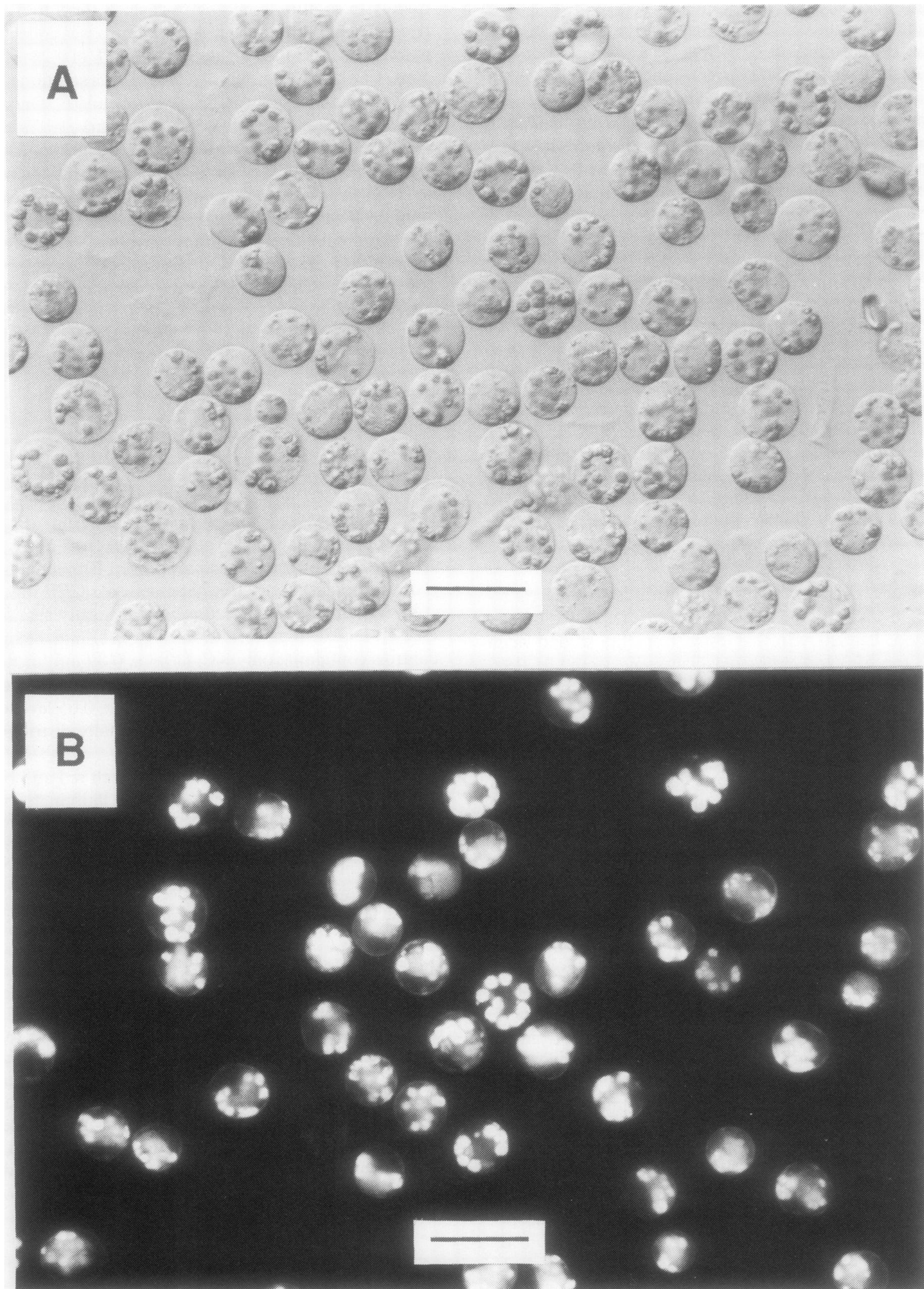

Figure 1. Isolated GCP from *V. faba*. Cells were suspended in 0.35 M mannitol, 1 mM $CaCl_2$. A, Differential interference contrast optics; large organelles in protoplasts are chloroplasts; bar = 30 μm. B, GCP stained with fluorescein diacetate; bar = 30 μm.

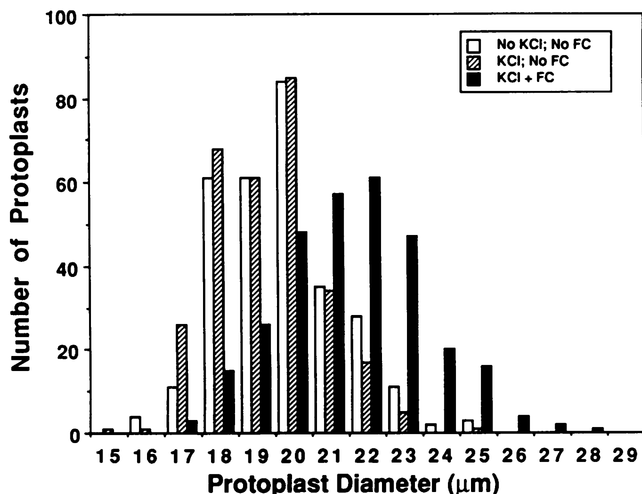

Figure 2. Distribution of diameters of *Vicia* GCP after incubation for 45 min in darkness in 0.45 M mannitol, 1 mM $CaCl_2$ (*open bars*), or 0.45 M mannitol, 1 mM $CaCl_2$ containing either 5 mM KCl (*hatched bars*), or 5 mM KCl and 10 μM fusicoccin (FC; *solid bars*). Diameters of 300 protoplasts per treatment were measured with a TV camera and monitor attached to a microscope.

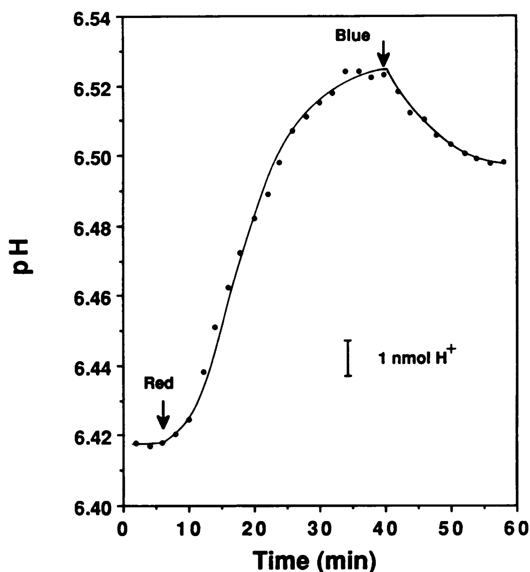

Figure 3. Red light-induced medium alkalinization by guard cell protoplasts of *V. faba* in response to continuous illumination with 800 μmol m^{-2} s^{-1} of red light, and blue light-induced acidification in response to superimposition of 10 μmol m^{-2} s^{-1} of continuous blue light onto the same red light background. GCP (10^6) were suspended in 1.2 ml of 0.5 mM Mes-NaOH buffer (pH 6.2) containing 0.35 M mannitol, 1 mM $CaCl_2$, and 10 mM KCl at 25°C. Medium pH was measured with a pH glass electrode (Beckman 39522) connected to a Beckman model 071 pH meter. Amounts of acid equivalents (*inset bar*) were determined by calibration with 10 nmol H^+ at the end of the experiment.

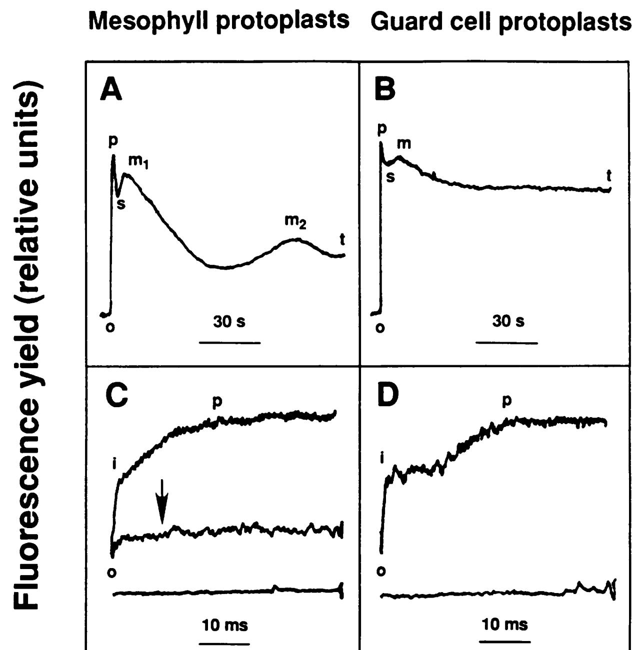

Figure 4. Slow fluorescence transients from mesophyll protoplasts (A) and from GCP (B) from *V. faba*. Panels C and D are fast fluorescence transients of the same preparations used to obtain the data shown in panels A and B. Panel C also contains a fast fluorescence transient of a mesophyll fragment (note *arrow*). Protoplasts were suspended in 0.35 M mannitol, 1 mM $CaCl_2$, 10 mM KCl, and 5 mM Mes buffer, pH 6.5. Chl *a* fluorescence was measured with an AO microscope equipped with a Nanospec/10 microfluorospectrophotometer modified by (a) removing the monochromator and (b) inserting a Corning 2-61 red filter and one layer of Cinemoid 5A orange film between the sample and the photomultiplier tube. Three to four protoplasts were selected visually and isolated by use of a variable slit in the area of the optical field visible to the photomultiplier. Protoplasts were illuminated by epi-fluorescence with 130 μmol m^{-2} s^{-1} of green actinic light provided by filtering light from a 12 V, 100 W tungsten halogen lamp through an interference filter with a bandpass of 546 nm. Traces were recorded by routing the signal from the photomultiplier tube to a storage oscilloscope (Tektronix 5103N) to measure fast transients and to a chart recorder (Kipp and Zonen BD41) to measure slow transients (BT Mawson, E Zeiger, unpublished results).

trations of Ficoll or shorter homogenization times decreased the size of epidermal fragments or increased the amount of mesophyll contaminating the fragments, respectively. Omission of Ficoll substantially increased damage to guard cell chloroplasts. Depending on leaf age, it was necessary to adjust blending times to minimize mesophyll tissue adhering to peels. If needed, epidermal fragments with large amounts of adhering mesophyll were removed with forceps prior to enzymatic treatment. Substitution of several dilutions of cellulase "Onozuka" RS for Cellulysin in the first digestion step were unsuccessful. Cellulase is more effective than Cellulysin, and it hydrolyzed guard cell walls too rapidly to allow an effective removal of epidermal and mesophyll cells without loss of GCP.

The purity of GCP prepared by the method described was comparable to that of conventional preparations (4, 15). There was some contamination with mesophyll fragments, but chloroplasts in such fragments appeared to be damaged and did not exhibit PSII activity when tested for variable fluorescence transients (Fig. 4C). These fragments, however,

could pose contamination problems for some types of studies such as Chl analysis. No attempts to separate these fragments were made, although fractionation of preparations should be feasible by the use of appropriate gradients (18). Yields were generally higher than those obtained with conventional methods, but it should be noted that the procedure described here yields a mixture of protoplasts from both adaxial and abaxial leaf surfaces. Upon elimination of the constraint of manual detachment of epidermis, yields became limited only by the availability of leaflets. Under the growth conditions described, 40 pots with 3 plants each yielded approximately 150 leaflets/week.

Results of experiments with vital stains and with fusicoccin indicated that GCP had intact membranes with functional proton-translocating ATPases (12). GCP isolated from mechanically prepared epidermis also showed the red light-induced alkalinization and blue light-induced acidification of their suspension medium characteristic of GCP isolated from manually detached epidermis (14, 15). The former has been attributed to operation of the photosynthetic carbon reduction pathway in GCP (4, 5, 15), while the latter has been associated with a plasmalemma, proton-translocating ATPase that is activated by blue light (2, 14). Patterns of fast variable fluorescence transients indicated that chloroplasts of GCP isolated from mechanically prepared epidermis were capable of photosynthetic electron transport and photophosphorylation (6, 21, 22). The presence of the "M" peak in slow fluorescence transients (22) also suggested that GCP contained a functional photosynthetic carbon reduction pathway, an observation consistent with recent results with *Vicia* epidermal peels (16) and with GCP prepared from manually detached epidermis (4, 5, 15).

Mechanical homogenization of leaves has been used previously to isolate epidermis from leaves of *Chlorophytum* (21). Homogenization of leaf pieces of *Nicotiana glauca* and *Commelina communis* under the conditions described above produced epidermal fragments similar to those from *Vicia*, indicating that the method can be adapted to other species, including some from which epidermis is not easily detached. Elimination of the time-consuming process of manually detaching leaf epidermis should be useful for for a variety of experimental applications requiring large-scale isolation of GCP.

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