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Fluorescence Properties of Guard Cell Chloroplasts

EVIDENCE FOR LINEAR ELECTRON TRANSPORT AND LIGHT-HARVESTING PIGMENTS OF PHOTOSYSTEMS I AND II[1]

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ABSTRACT

The presence of chloroplasts in guard cells from leaf epidermis, coleoptile, flowers, and albino portions of variegated leaves was established by incident fluorescence microscopy, thus confirming the notion that guard cell chloroplasts are remarkably conserved. Room temperature emission spectra from a few chloroplasts in a single guard cell of *Vicia faba* showed one major peak at around 683 nanometers. Low-temperature (77 K) emission spectra from peels of albino portions of *Chlorophytum comosum* leaves and from mesophyll chloroplasts of green parts of the same leaves showed major peaks at around 687 and 733 nanometers, peaks usually attributed to photosystem II and photosystem I pigment systems, respectively. Spectra of peels of *V. faba* leaves showed similar peaks. However, fluorescence microscopy revealed that the *Vicia* peels, as well as those from *Allium cepa* and *Tulipa* sp., were contaminated with non-guard cell chloroplasts which were practically undetectable under bright field illumination. These observations pose restrictions on the use of epidermal peels as a source of isolated guard cell chloroplasts. Studies on the 3-(3,4-dichlorophenyl)-1,1-dimethylurea-sensitive variable fluorescence kinetics of uncontaminated epidermal peels of *C. comosum* indicated that guard cell chloroplasts operate a normal, photosystem II-dependent, linear electron transport. The above properties in combination with their reported inability to fix CO_2 photosynthetically may render the guard cell chloroplasts optimally suited to supply the reducing and high-energy phosphate equivalents needed to sustain active ion transport during stomatal opening in daylight.

Chloroplasts are a central structural feature of stomatal guard cells (14). Many investigators have suggested ways in which these chloroplasts might be crucial for stomatal functioning (7, 39), but most hypotheses have proven untenable in the face of new discoveries or have not received conclusive experimental support. A better understanding of the physiological and biochemical properties of guard-cell chloroplasts is crucial for a definition of their role in stomatal movements. Further progress depends largely on our ability to obtain pure preparations of guard cells and their chloroplasts.

Here, we used albino portions of variegated leaves from *Chlorophytum comosum* as a source of uncontaminated guard cell chloroplasts and studied their pigment content by fluorescence spectroscopy. Our findings indicate that guard cell chloroplasts contain light-harvesting pigments of both PSI and PSII. The fluorescence induction kinetics of guard-cell and mesophyll chloroplasts is also presented. Our results support the notion that guard-cell chloroplasts operate a linear electron transport system. We postulate that a central role of guard cell chloroplasts is to provide light-dependent energy to sustain the active ion transport required for stomatal opening during the day.

MATERIALS AND METHODS

Plant Material. Seeds of *Allium cepa* (Keystone Co., CA), *Hordeum vulgare* cv. Early Bonus, *Vicia faba* (W. Atlee Burpee Co., CA), and *Zea mays* cv. Bear Hybrid were germinated in pots and grown in a greenhouse. Mature plants of *Paphiopedilum harrisianum*, *Paphiopedilum insignes*, *C. comosum* ("spider plant"), *Cymbidium* sp., and *Saintpaulia* sp. were purchased from local nurseries; bulbs of *Tulipa* sp. were grown in the greenhouse. Flowers of *Zantedeschia aethiopica* were collected from ornamental plants growing in the vicinity of the laboratory.

Fluorescence Microscopy. The fluorescence of single cells was observed in an American Optical H10TU-VF4 microscope equipped with an AO 2070C vertical illuminator for incident fluorescence microscopy and a 50-w Hg lamp. Incident fluorescence microscopy provides strong excitation at high magnifications and, because of the geometrical configuration of the system, it is independent of the thickness of the specimen, thus allowing observations of intact tissue as well as conventional thin preparations. The fluorescence exciting light passed through a BG12 Schott filter, a 500 nm cutoff dichroic beam splitter, and an OG515 cutoff Schott filter. Emission spectra of single cells were measured with a Nanospec/10 microfluorospectrophotometer (38).

Low-temperature Fluorescence Spectroscopy. Emission spectra of samples frozen in liquid N_2 (at 77 K) were obtained with a Perkin-Elmer MPF-3L fluorospectrophotometer equipped with a low-temperature accessory. Sample excitation was at 440 nm by a CS 4-96 Corning filter (half-band width, 10 nm). Fluorescence emission was detected through a CS 2-58 Corning filter with a half-band width of 10 nm. Specimens were attached to a holder which allowed direct contact of the sample with the liquid N_2 during the measurements.

Large-scale Preparation of Epidermal Peels. Large quantities of epidermal peels were mechanically separated from the rest of the leaf tissue by treating small (about 1× 2 cm) leaf segments in an Ultra Turrax blender (Janke and Kunkel, West Germany). Gentle grinding in the Ultra Turrax produces large numbers of intact epidermal peels, most of which are collected in the shaft of

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the instrument. This method was efficient in preparing epidermal peels from *Chlorophytum* and *Paphiopedilum*, thus eliminating time-consuming peeling by hand.

Variable Fluorescence Studies. Chloroplast fluorescence kinetics were measured with an apparatus described by Melis and Hart (16). Excitation light (0.7 mw cm^{-2}) was provided by a broadband blue CS 4-96 Corning filter and the fluorescence emission was separated at a right angle from the exciting beam by a combination of red cutoff CS 2-64 Corning and RG 5 Schott filters and detected by a Hamamatsu R562 phototube. The signal was finally displayed on a storage oscilloscope (Tektronix 5103N). To improve the resolution of the apparatus for the measurement of the fluorescence kinetics, the base line was suppressed at the photomultiplier output with a DC-offset compensation circuit. Since no attempt was made to separate light leak artifacts from the nonvariable fluorescence emission (F_o) of the chloroplasts, the variable fluorescence kinetics reported have been normalized between the values $F_o = 0$ and $F_{max} = 1.0$. PSI illumination was provided at a right angle to the phototube from a second light source by 700-nm Balzers interference and RG 8 Schott red cutoff filters. Guard-cell preparations of *C. comosum* were obtained from epidermal peel suspensions or from an intact, albino portion of a leaf with its abaxial surface facing the incident light beam. Mesophyll chloroplasts were obtained from green portions of leaves ground in the Ultra Turrax. DCMU, where indicated, was at a concentration of approximately 20 μM.

RESULTS

Survey of Chloroplasts in Guard Cells from Different Sources. Fluorescence microscopy has been used to detect chloroplasts in guard cells since the classic studies of Linsbauer (11). Using the sensitive technique of incident fluorescent microscopy, we confirmed the long-established notion that chloroplasts are a very persistent feature of stomatal guard cells. We found red fluorescing chloroplasts in guard cells from the leaf epiderms of many gymnosperms and angiosperms, including both monocots and dicots. The exceptions were *P. harrisianum* and *P. insignes*, thus confirming previous reports of the lack of chloroplasts in their guard cells (17, 22). The other orchidacean tested, *Cymbidium* sp., did contain chloroplasts in its guard cells. Guard cells from *Paphiopedilum* are reportedly functional (17), indicating that, in this genus, stomatal movement is not obligatorily coupled to chloroplast function in the guard cells. However, this alternative mechanism appears to be the exception rather than the rule.

Guard cells from coleoptiles of *Z. mays* and *H. vulgare* and from flowers of *Z. aethiopica* and *Saintpaulia* sp. also contained chloroplasts. The result was also positive in guard cells from the albino portions of variegated leaves from *C. comosum*. These findings are in agreement with previous reports on variegated leaves (24). Because *C. comosum* was our source of uncontaminated preparations of guard cell chloroplasts, albino portions of variegated leaves were extensively examined. In all cases, the abaxial epidermis of the albino portions showed a seemingly normal frequency of stomata, and all of the thousands of guard cells observed exhibited red fluorescing chloroplasts. The albino portions were completely devoid of mesophyll fluorescence.

Contamination of Epidermal Peels by Mesophyll Chloroplasts. Epidermal peels have frequently been used in studies of guard-cell chloroplasts and they are usually screened under bright field microscopy to eliminate those with significant levels of contaminating mesophyll cells. Using incident fluorescence microscopy, we found that another important source of contamination is mesophyll chloroplasts occurring outside the guard cells proper. These are practically undetectable under bright field microscopy. Large numbers of these chloroplasts were detected by fluorescence in epidermal peels of *V. faba*, *A. cepa* and *Tulipa* sp., regardless of the method used to make the peels (34). Treating suspensions of epidermal peels of *V. faba* with 4% (w/v) Cellulysin (Calbiochem) at pH 5.0 at room temperature for 2 to 3 h reduced the contamination significantly, but some contaminated areas remained, presumably in the folds of the epidermal peels inaccessible to the cellulolytic enzymes. Micrographs illustrating the contamination have been published elsewhere (34).

Emission Spectra of Guard Cell Chloroplasts. Fluorescence spectroscopy is a highly sensitive method for studying some biophysical aspects of chloroplasts (1, 10). Using the microfluorospectrophotometer, we obtained a room-temperature fluorescence emission spectrum from a few chloroplasts in a single guard cell of *V. faba*. The spectrum showed a distinct peak at around 683 nm and a broad shoulder in the 720- to 750-nm region (Fig. 1). Although limited in fine structure, room-temperature emission studies of single cells should be useful for kinetic analysis (10), under the same rationale employed in our experiments of variable fluorescence from epidermal peels described below.

Low-temperature (77 K) emission spectra were obtained in a fluorospectrophotometer from samples frozen in liquid N_2. Because of the high sensitivity of this technique, it was crucial to use preparations of guard cell chloroplasts free from contaminating mesophyll. The albino portions of variegated leaves from *C. comosum* provided an adequate source. An albino leaf segment, previously examined under fluorescence microscopy to ensure the absence of mesophyll chloroplasts, was mounted in a metal holder so that its abaxial surface faced the incident light beam and then was immersed in liquid N_2. The emission spectrum (Fig. 2) shows peaks at around 686 and 740 nm, peaks usually attributed to

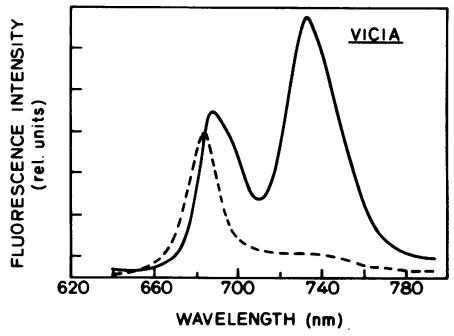

FIG. 1. Fluorescence emission spectra of guard-cell chloroplasts from *V. faba*. (– – –): room temperature emission spectrum of a few chloroplasts from a single guard cell obtained with a Nanospec/10 microfluorospectrophotometer, BG12 exciting filter, 500-nm cutoff dichroic beam splitter, 40× objective. (——), 77 K emission spectrum from a suspension of epidermal peels of *V. faba* obtained with a fluorospectrophotometer.

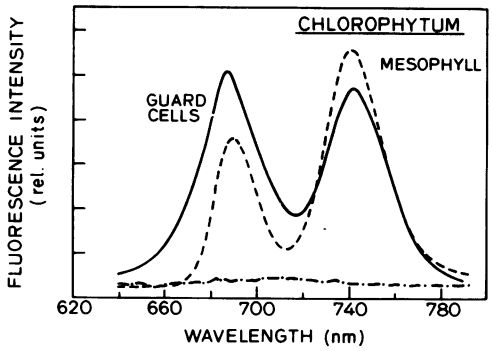

FIG. 2. The 77 K fluorescence emission spectra of chloroplasts from *C. comosum* obtained with a fluorospectrophotometer. (——), albino portion of a variegated leaf; (– – –), isolated mesophyll chloroplasts from a green portion of the same leaf; (–·–·–), spectrum from an albino portion devoid of the abaxial epidermis.

pigments from PSII and PSI, respectively (23). The spectrum from a suspension of chloroplasts isolated from a green portion of the same leaf shows identical peaks (Fig. 2).

Low-temperature emission spectra from a suspension of isolated peels from *V. faba* also exhibit peaks indicative of pigments from both photosystems (Fig. 1). Although these results are inconclusive because of the contamination described above, other investigators have obtained identical data from a preparation of isolated guard-cell protoplasts (19). We suggest that guard cell chloroplasts of both *Chlorophytum* and *Vicia* have pigments of both PSI and PSII.

We also obtained low-temperature emission spectra from abaxial epidermal peels of *P. harrisianum* prepared in the Ultra Turrax and confirmed to be free of contamination under fluorescence microscopy. The epidermis had no detectable fluorescence between 640 and 780 nm.

Variable Fluorescence of Guard Cell Chloroplasts. The fluorescence induction curve of higher plant chloroplasts reflects the light-dependent transition of PSII from a weekly fluorescent condition (F_o), when all photochemical centers of PSII are open and capable of performing a charge separation, to a more strongly fluorescent state (F_{max}) as these centers become closed. In terms of the quencher theory (3), the fluorescence rise is due to the photoreduction of the primary electron acceptor Q of PSII to its Q^- form (15, 29). According to the linear electron-transport scheme, electrons accumulate on Q after the reduction of the plastoquinone pool and the other electron acceptors by PSII. Consequently, in dark-adapted chloroplasts, the kinetics of the fluorescence induction is an indicator of the accumulation of electrons in a number of carriers located between the two photosystems. Figure 3A shows the light-induced fluorescence rise curve in dark-adapted isolated

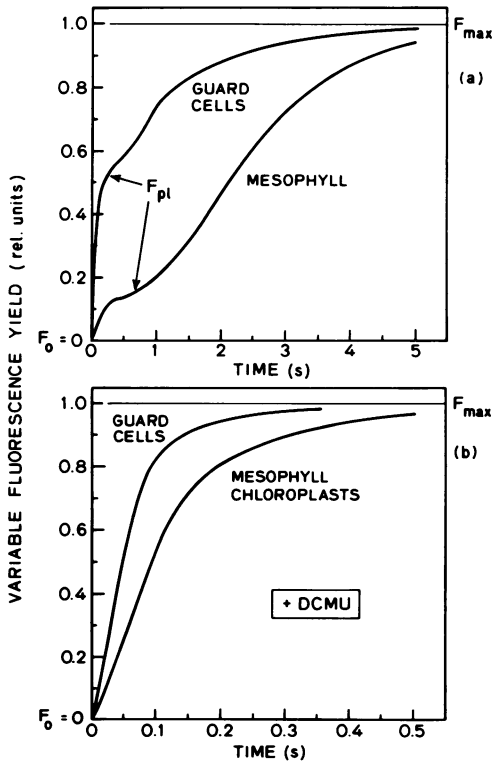

FIG. 3. Kinetics of the variable fluorescence yield from isolated mesophyll and guard cell chloroplasts from *C. comosum* in the absence of any artificial cofactors (a) and in the presence of the electron-transport inhibitor DCMU (b). Note the different time scales. The kinetic data have been normalized to the values of the initial fluorescence yield F_o ($F_o = 0$) and the maximum fluorescence yield F_{max} ($F_{max} = 1.0$). F_{pl}, DCMU-insensitive intermediary fluorescence yield level (4).

mesophyll chloroplasts and guard cells from *C. comosum*. Both mesophyll and guard-cell chloroplasts showed the typical kinetic transition from F_o to F_{max} through the intermediary fluorescence level (F_{pl}) (4, 8, 13). The two time courses were similar, suggesting that PSII mediated the reduction of a pool of electron acceptors in both cases. With both preparations, irradiation at 700 nm was capable of restoring the fluorescence levels to their dark-adapted state (data not shown), indicating that PSI was acting as a sink for electrons accumulated by PSII.

The presence of a functional PSII in guard-cell chloroplasts is further demonstrated by measurements of the variable fluorescence induction kinetics in the presence of the PSII inhibitor DCMU. Inasmuch as DCMU prevents the reoxidation of Q^- by the plastoquinone pool (3), fluorescence induction in its presence occurs in a much shorter period of time. Figure 3B shows that chloroplasts from both the mesophyll and guard cells of *C. comosum* respond to the inhibitor as predicted. Under these experimental conditions, the variable fluorescence induction reflects the reduction of the primary electron acceptor of PSII only.

The time courses of variable fluorescence induction also indicate some differences between the guard cell and mesophyll chloroplasts. At the beginning of the induction, the variable fluorescence of guard cells showed a F_o to F_{pl} transient (4) proportionately higher than that of mesophyll chloroplasts (Fig. 3A). This could be due to the somewhat reducing conditions prevailing in the guard cells, as opposed to the buffer surrounding the isolated mesophyll chloroplasts. A second difference is that the fluorescence rise of the guard cell chloroplasts in the presence of DCMU occurred twice as fast as that of the mesophyll chloroplasts, perhaps indicating a larger PSII unit size for the guard cell chloroplasts.

These data indicate that guard cell chloroplasts have a functional PSII connected through an intermediate plastoquinone pool to PSI. A comparison of the areas confined by the ordinate, the F_{max} level, and the induction curve, in the presence and absence of DCMU (Fig. 3B) provides an estimate of the relative size of the plastoquinone pool (28). Our estimates (data not shown) indicate that the pool sizes in both types of chloroplasts are similar.

DISCUSSION

The remarkable conservation of chloroplasts in guard cells, most striking in albino portions of variegated leaves and in flowers, indicates to us that chloroplasts play a central role in stomatal function. The nature of this role, however, remains elusive.

The application of high-resolution techniques to the small samples of guard cell chloroplasts requires the use of homogeneous preparations. The high levels of contaminating chloroplasts we found in epidermal peels indicate that this widely used preparative method (2, 12, 20, 21, 26, 30–32) has some serious drawbacks, and some of the conclusions made under the assumption that these preparations were pure might need reinterpretation. With that limitation in mind, three systems are currently available to study guard cell chloroplasts: single guard cells analyzed by microfluorospectrophotometry, albino portions of variegated leaves (24), and isolated guard-cell protoplasts (19, 35).

The low-temperature emission spectra from mesophyll and guard-cell chloroplasts from *Chlorophytum* are very similar (Fig. 2), indicating the presence of pigments from both photosystems (23). Guard cells from albino portions of leaves have been reported to be capabe of opening, yet somewhat sluggishly (24). It is most likely, however, that their limited opening is due to the lack of a photosynthetic CO_2 sink in the underlying albino mesophyll tissue and does not reflect a deficiency in the guard cells proper (25). The emission characteristics of guard cell chloroplasts from *V. faba* reported here and the similar spectra found in extracts of *Vicia* guard cell protoplasts by Outlaw *et al.* (19) suggest that the presence of both photosynthetic pigments is a common feature of

guard cell chloroplasts.

Our evidence for a functional PSII connected to PSI through a large plastoquinone pool in guard cell chloroplasts indicates that these organelles operate a linear electron transport mechanism. In mesophyll chloroplasts, the final electron donor in the transport chain is H_2O, and the final electron acceptor is $NADP^+$. Thus, it seem reasonable to assume that guard cell chloroplasts oxidize H_2O and generate reducing equivalents in a pyridine nucleotide. That notion is consistent with findings of Höfler (6) and Shaw (27) that guard cell chloroplasts from *Allium*, *Vicia*, *Helianthus*, *Tradescantia*, *Galium*, and *Pelargonium* reduce silver nitrate after a few minutes of illumination. Recent evidence indicates that plastoquinone is the specific reducing agent in that reaction (9). Indirect evidence also suggests that guard cell chloroplasts are capable of photophosphorylation (7, 12) (A. Melis and E. Zeiger, unpublished data). Guard cell chloroplasts, therefore, seem capable of generating photochemical energy but incapable of reducing CO_2 photosynthetically (18, 21). What, then, is their function? An appraisal of the ionic content of open guard cells reveals a large ionic gradient (7) which has to be maintained for the stomata to remain open. Hence, during the day, with abundant solar radiation available, guard cell chloroplasts would constitute a source of energy for active ion transport. Such a function of guard cell chloroplasts has been advanced by several investigators (7, 39), and it is a main element of our recent postulation of a chemiosmotic mechanism as the basic means of sustaining active ion uptake in the guard cells (36, 37). Chloroplasts with active PSI and PSII but devoid of Calvin cycle enzymes would be optimally suited to fulfill that function. If this were the case, ATP and/or NADPH produced within the chloroplasts would have to become available at the presumed pump sites in the plasmalemma. Energy transfer between the chloroplast and the cytoplasm has been studied in several systems (5), but little is known about this aspect of the physiology of the guard cells.

A better understanding of the properties of guard cell chloroplasts has also a bearing on the important question of communication between stomata and the mesophyll tissue. There is convincing evidence that stomatal conductance and the photosynthetic rate of the mesophyll are tightly coupled (33). The apparent inability of guard cell chloroplasts to fix CO_2 photosynthetically and their lack of ribulose bisP carboxylase (18) rule out the likelihood of CO_2 modulation of guard-cell activity via their chloroplasts. The possibility that the coupling is accomplished by a mechanism sensing the rates of electron transport in guard cell and mesophyll chloroplasts deserves further investigation.

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