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New approach of monitoring changes in chlorophyll *a* fluorescence of single guard cells and protoplasts in response to physiological stimuli

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ABSTRACT

A new type of microfluorometer was applied to assess photosynthesis at the single-cell level by chlorophyll fluorescence using the saturation pulse method. A microscopy–pulse amplitude modulation (PAM) chlorophyll fluorometer was combined with a Zeiss Axiovert 25 inverted epifluorescence microscope for high-resolution measurements on single mesophyll and guard cells and the respective protoplasts. Available information includes effective quantum yield of photosystem II, relative electron transport rate and energization of the thylakoid membrane due to the transthylakoidal proton gradient. Dark–light induction curves of guard cell (GCPs) and mesophyll cell protoplasts (MCPs) displayed very similar characteristics, indicating similar functional organization of thylakoid membranes in both types of chloroplasts. Light response curves, however, revealed much earlier saturation of photosynthetic electron flow in GCPs than in MCPs. Under anaerobiosis, photosynthetic electron flow and membrane energization were severely suppressed. A similar effect was observed in guard cells when epidermal peels were incubated with the fungal toxin fusicoccin which activates the plasma membrane H^+-ATPase and causes irreversible opening of stomata. The drop in electron transport rate was prevented by blocking ATP consumption of the H^+ pump or by glucose addition. These results show that chlorophyll fluorescence quenching analysis allows profound insights into stomatal physiology.

Key-words: *Vicia faba* L.; anaerobiosis; chlorophyll fluorescence; fluorescence microscopy; fusicoccin; guard cell; mesophyll cell; photosynthesis; stomatal opening.

INTRODUCTION

Plants control the exchange of carbon dioxide (CO_2) and water vapour between the leaf and the atmosphere through the regulation of stomatal aperture. A number of environmental factors including CO_2, humidity, drought, red and blue light have been shown to directly affect stomatal movement. In response to environmental stimuli, the two guard cells in the stomatal complex alter their turgor, which is followed by volume changes and finally stomatal aperture. The osmotic motor that drives these hydrodynamic valves is based on the accumulation and release of K^+ salts (Hedrich & Schroeder 1989; Assmann 1993; Willmer & Fricker 1996; MacRobbie 1987). Therefore, this cell type has been used to study the mechanisms which underlie stimulus–response coupling in higher plants.

Stomatal opening is induced by red and blue light, indicating that guard cells are equipped with at least two photoreceptors (Zeiger, Iino & Shimazaki 1987). The action spectrum of red light-induced stomatal opening strongly correlates with photosynthesis and thus is associated with the chloroplasts. Recent findings on a blue light photoreceptor in guard cells provide evidence that blue light perception is likely to occur in the chloroplasts as well [for a review see Zeiger & Zhu (1998)]. Guard cells contain only a few small chloroplasts (Nelson & Mayo 1975), which in contrast to mesophyll cells have poorly developed grana (Sack 1987; Willmer & Fricker 1996). Nevertheless, the photochemical and biochemical analysis of guard cell chloroplasts have provided evidence for both photosystems (PS) I and II (Zeiger, Armond & Melis 1980; Outlaw *et al*. 1981; Mawson *et al*. 1984) and the major Calvin cycle enzymes (Shimazaki & Zeiger 1985; Zemel & Gepstein 1985; Gotow, Taylor & Zeiger 1988; Tallman & Zeiger 1988; Shimazaki *et al*. 1989; Goh, Oku & Shimazaki 1997). Furthermore, this organelle is a site of synthesis of organic anions such as malate, ATP and NADPH [for a review see Assmann (1993) and Willmer & Fricker (1996)]. With respect to the energy metabolism of guard cells in the light, chloroplasts have been discussed as the potential energy source for H^+ pumping, as red (Serrano, Zeiger & Hagiwara 1988) and blue light (Assmann, Simoncini & Schroeder 1985; Shimazaki, Iino & Zeiger 1986) trigger the activity of electrogenic H^+ pumps in the plasma membrane of guard cells. Given the small volume of guard cells with respect to other cells of a leaf, data obtained from isolated protoplasts, however, did not gain broad acceptance and in view of possible contamination from mesophyll cells the role of guard

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cell photosynthesis in stomatal movement is still controversial.

To distinguish the properties of guard cells with respect to, for example mesophyll cells, single-cell techniques such as the patch-clamp technique have been used to specifically assess the electrical properties of this type of sensory cell. In this way, many plasma membrane transport processes, especially ion channel activities in guard cells, have been characterized in detail, and unequivocally linked to stomatal responses towards environmental stimuli (Hedrich & Schroeder 1989; Assmann 1993; Hedrich 1994, 1995; MacRobbie 1987). Intrinsic problems of such single-cell measurements are possible heterogeneities in the photosynthetic performance of individual cells, as well as possible changes of activity due to various manipulations involved with single-cell electrophysiological measurements. In order to overcome these problems, a method is required which allows photosynthetic activity to be monitored at the single-cell level, potentially simultaneously with electrophysiological measurements.

Pioneering work of Zeiger and coworkers (Zeiger *et al.* 1980; Melis & Zeiger 1982; Mawson & Zeiger 1991) has shown that basic information on photosynthesis in single guard cells can be obtained by chlorophyll fluorescence measurements. This work revealed that guard cell chloroplasts display normal, PS II-dependent, linear electron transport (Zeiger *et al.* 1980), which responds to changes in CO_2 (Melis & Zeiger 1982) and is modulated by blue light (Mawson & Zeiger 1991). In this early work, chlorophyll fluorescence was excited by the same strong actinic light which also induced dark–light induction transients (Kautsky effect), similar to the original Kautsky method (Kautsky & Franck 1943). While this method provides large signals and has contributed considerably to the elucidation of basic photosynthetic mechanisms, its range of practical applications is limited by the existence of photochemical and non-photochemical fluorescence quenching mechanisms, between which it cannot distinguish. As non-photochemical quenching emerges within a few seconds of illumination, reliable interpretation of fluorescence induction curves was for a long time restricted to the rapid transients (Lavorel & Etienne 1977; Briantais *et al*. 1986). This limitation was overcome by the saturation pulse method of fluorescence quenching analysis (Bradbury & Baker 1981; Schreiber, Schliwa & Bilger 1986) and by pulse amplitude modulation (PAM) fluorimetry (Schreiber *et al*. 1986). In contrast to the original Kautsky approach, PAM fluorimetry employs a separate, high frequency-modulated measuring light, such that modulated fluorescence can be separated from any non-modulated background signal, including the fluorescence excited by continuous actinic light. In this way, chlorophyll fluorescence yield instead of fluorescence intensity is assessed, with the main advantage that the yield contrary to intensity does not vary by more than a factor of 5, being closely related to the quantum yield of photosynthetic energy conversion in PS II reaction centres (Genty, Briantais & Baker 1989). Since its introduction in 1985, PAM fluorimetry in conjunction with the saturation pulse method of quenching analysis has proven to be a powerful tool in probing the intrinsic function of the photosynthetic apparatus in higher plants and algae (Krause & Weis 1991; Schreiber, Bilger & Neubauer 1994). So far, however, this approach has not yet been applied to the study of single guard cell photosynthesis. Cardon & Berry (1992) assessed modulated fluorescence yield of single guard cell pairs in the albino parts of variegated leaves, but for technical reasons were unable to apply the saturation pulse method. Recently, Oxborough & Baker (1997) demonstrated that in principle assessment of photosynthetic performance at the level of single guard cells by fluorescence quenching analysis is possible using a highly sensitive fluorescence imaging system in conjunction with an epifluoresence microscope. While this imaging system does not employ pulse-modulated measuring light, it achieves assessment of fluorescence yield via computer-assisted changing of filters using reflected light images as reference.

Only very recently, a new type of PAM chlorophyll fluorometer was developed (Schreiber 1998) which does qualify for chlorophyll fluorescence quenching analysis at the level of single cells and chloroplasts. For the present study, this new fluorometer was combined with an inverted epifluorescence microscope, as usually applied in conjunction with electrophysiological measurements. Here the first measurements with the new microscopy–PAM fluorometer on single guard cells and guard cell protoplasts (GCP) are reported, with the main aim of demonstrating the principles of the new approach and the performance of the new measuring system. It will be shown that various physiological stimuli, known to be related to stomatal movement, cause distinct responses in photosynthetic electron transport of guard cell chloroplasts.

MATERIALS AND METHODS

The experimental system

A prototype of the very recently introduced microscopy–PAM chlorophyll fluorometer (Schreiber 1998) was adapted to an inverted epifluorescence microscope (model Axiovert 25, Zeiss GmbH, Göttingen, Germany). The essential components of the microscopy–PAM fluorometer are the PAM control unit (Heinz Walz GmbH, Effeltrich, Germany), a blue light-emitting diode (LED; type NSBG 500, Nichia, Tokyo, Japan) serving as a pulse-modulated measuring light source, and a miniature photomultiplier (type PC/PM-MC, Walz). The modulated blue LED substitutes for the usual xenon arc excitation lamp of the epifluorescence microscope. Its light is filtered through a blue filter (BG 39, Schott, Mainz, Germany). It is focused via the collimator optics and the objective lens of the microscope on the sample plane. The photomultiplier is mounted on top of the photoadaptor of the microscope where an ocular lens (10 ×) and an iris diaphragm are installed.

A block diagram of the measuring system is shown in Fig. 1. The pulse-modulated blue excitation light is reflected

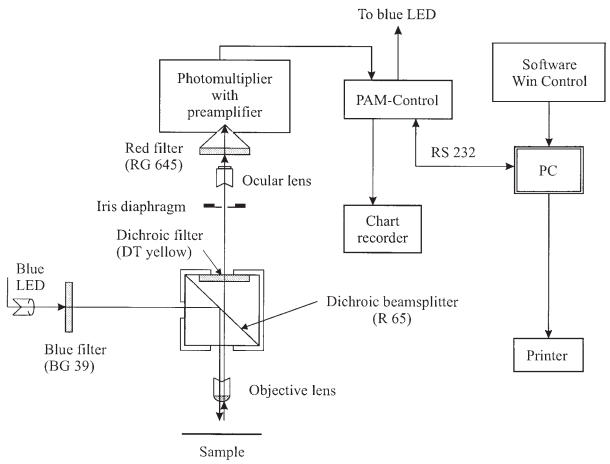

Figure 1. Block diagram of the microfluorometric set-up for measuring chlorophyll fluorescence at the single-cell level. The excitation light derived from a blue light-emitting diode (LED) is reflected by a dichroic beam splitter into the objective lens and focused in the sample plane. The red chlorophyll fluorescence collected by the same objective lens is directed via the ocular lens towards the photomultiplier detector. It passes a second dichroic filter and an iris diaphragm in the focal plane of the ocular as well as a filter in front of the photomultiplier. The preamplified signal is further processed by the pulse amplitude modulation (PAM) control unit which also controls the pulse program of the blue excitation beam. The system was operated and data were acquired/analysed under control of a Pentium personal computer and the WINCONTROL software (see text for further details).

by the dichroic beam splitter (R65, Balzers, Liechtenstein) into the objective. The red fluorescence is collected by the objective lens (20 ×, type Fluar, Zeiss) and directed with high efficiency via the dichroic beam splitter and a second dichroic filter (DT Yellow, Balzers) towards the detector. Both dichroic filters serve the purpose of reflecting scattered blue light. On its way to the ocular lens and the photomultiplier detector, the fluorescence passes an iris diaphragm in the focal plane of the ocular. Hence, the field of view can be narrowed down, such that specific sample areas can be selectively assessed. The photomultiplier is protected by a red-glass filter (RG 645, Schott) against remaining traces of scattered blue excitation light.

Light intensity was measured with a specially developed pin-hole detector, which connects to the PAM control unit. The carrier of this detector has the outer dimensions of a microscope slide, such that it can be installed in exactly the same way as a microscopic sample. The light-sensitive area of the detector is restricted by a 0·3 mm diameter pin-hole in a thin (0·3 mm) black-anodized aluminium plate covering a blue-enhanced silicon photodiode. The hole is filled with epoxy resin containing some titanium dioxide, such that any radiation hitting the pin-hole is highly scattered. The upper surface of the pin-hole is flat and polished with fine-grain (0·3 μm) abrasive. It is this scattering surface on which the measuring beam is focused, such that a sharp fluorescence image of the pin-hole entrance can be viewed via the ocular. Due to the strong scattering, the amount of direct light penetrating into deeper layers of the pin-hole is minimized, and the measured quantum flux density corresponds to that in the focal plane. Furthermore, by the scattering properties of the pin-hole surface, the intensity measurement is rendered insensitive to the angle with which the light enters the hole. The diffusing epoxy displays strong yellow fluorescence upon excitation by the blue measuring light. This feature facilitates focusing of the excitation beam in the entrance plane of the pin-hole which is equivalent to the sample plane in chlorophyll fluorescence measurements.

The photomultiplier signal is preamplified and then processed in the PAM control unit, from which it can be directly recorded by analog devices (such as a chart recorder) or transferred via a RS 232 interface to a Pentium personal computer (PC). The system was operated in conjunction with special software provided for system control via the keyboard, data acquisition and analysis (WINCONTROL, Walz). A digital printer served for data output.

Plant materials

Broad bean (*Vicia faba* L. cv. Grünkernige Hangdown and Osnabrücker Markt, Gebag, Hannover, Germany) plants were grown in a greenhouse at 20–24 °C/14–16 °C (day/night) under sunlight as previously described (Schulz-Lessdorf, Lohse & Hedrich 1996). Fully expanded leaves from the third to fourth nodes of 3–4-week-old plants were used in the experiments. *Arabidopsis thaliana* L. C24 (cv. Columbia, Arabidopsis Stock Center, Ohio, USA) plants were grown in a greenhouse at 23 °C/16 °C (day/night) under sunlight as previously described (Brüggemann *et al*. 1999) and 5–7-week-old plants before flowering were used in the experiments. Leaves were harvested between 0900 and 0930 h for all experiments.

Protoplast preparations

GCPs from *Vicia* and *Arabidopsis* leaves were isolated from the abaxial epidermes by two-step enzymatic digestion (Goh, Oku & Shimazaki 1995) with slight modification. Briefly, the epidermal peels from *Vicia* leaves were digested in 1% (w/v) cellulase Onozuka RS (Yakult Pharmaceutical Co., Tokyo, Japan), 250 mol m^{-3} sorbitol, 0·5% (w/v) bovine serum albumin (BSA), 1 mol m^{-3} $CaCl_2$, 10 mol m^{-3} Na-ascorbate, pH 5·5 for 50 min at 28 °C. After the incubation, the peels were gathered on a Nylon net (200 μm mesh size) and thoroughly washed in 400 mol m^{-3} sorbitol containing 1 mol m^{-3} $CaCl_2$. The peels were then subjected to the second digestion for 2 h at 28 °C, using a medium containing 1% (w/v) cellulase Onozuka RS, 0·02% (w/v) pectolyase Y-23 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 400 mol m^{-3} sorbitol, 0·5% (w/v) BSA, 1 mol m^{-3} $CaCl_2$, and 10 mol m^{-3} Na-ascorbate at pH 5·5. The released protoplasts were harvested by filtration through a Nylon net (20 μm mesh size), collected by centrifugation

(110*g*, 7 min), and rinsed in 400 mol m^{-3} sorbitol containing 1 mol m^{-3} $CaCl_2$. GCPs from *Arabidopsis* leaves were isolated as above, but using 500 mol m^{-3} sorbitol in the second digestion step. Isolated protoplasts were suspended in 400 mol m^{-3} sorbitol containing 1 mol m^{-3} $CaCl_2$ for *Vicia* GCPs and 500 mol m^{-3} sorbitol containing 1 mol m^{-3} $CaCl_2$ for *Arabidopsis* GCPs and kept in the dark on ice until use.

For comparative measurements, also mesophyll cell protoplasts (MCPs) were isolated from *Vicia* leaves. The procedure was performed as described previously (Sakai & Kondo 1985) with slight modification. The enzymatic solution used here consisted of 0·5% (w/v) macerozyme R-10 (Yakult Pharmaceutical Co.), 1% (w/v) cellulase Onozuka RS, 0·5% (w/v) BSA, 600 mol m^{-3} sorbitol, and 1 mol m^{-3} $CaCl_2$ at pH 5·5. The incubation was carried out for 1 h at 28 °C. Isolated protoplasts were suspended in 600 mol m^{-3} sorbitol containing 1 mol m^{-3} $CaCl_2$ and kept in the dark on ice until use.

Preparation of epidermal peels

Epidermal peels were manually detached with forceps from the abaxial surfaces of *Vicia* leaves. To remove mesophyll contaminants from the peels, they were washed briefly in deionized water and then mechanically stirred at 300 r.p.m. min^{-1} in a solution of 10% (w/v) Ficoll, 0·2% (w/v) PVP K-40, 50 mol m^{-3} KCl, and 1 mol m^{-3} $CaCl_2$ for 15 min in the dark. The epidermes were rinsed thoroughly with deionized water, suspended in 10 mol m^{-3} MES/Tris (pH 6·1) containing 10 mol m^{-3} KCl and kept in the dark on ice until use. In a typical experiment, the epidermes had ≈ 85% viable guard cells, as estimated by chlorophyll fluorescence. The characteristic pattern of chlorophyll fluorescence from single guard cell pairs was clearly similar to that found in single protoplasts.

Experiments

Measurements of chlorophyll *a* fluorescence from single GCPs were performed in a solution of 10 mol m^{-3} MES/Tris (pH 6·1), 10 mol m^{-3} KCl, 1 mol m^{-3} $CaCl_2$, and 400 mol m^{-3} sorbitol containing 2·5 mol m^{-3} $NaHCO_3$. In the case of *Arabidopsis* GCPs, the measuring solution contained 500 mol m^{-3} sorbitol for adjustment of their osmolarity. The appropriate osmolarity for *Vicia* MCPs was adjusted by 600 mol m^{-3} sorbitol.

In order to investigate the influence of O_2 on guard cell photosynthesis, two experimental approaches were used to deplete samples from O_2. Full depletion of O_2 was achieved by an enzymatic trap consisting of 20 mol m^{-3} glucose/glucose oxidase (30 units cm^{-3})/catalase (2987 units cm^{-3}) in the protoplast suspension medium. The enzyme reaction was run for 10 min before measurement. Controls were kept under air equilibrated conditions. Alternatively, the epidermal peels were kept for 30 min in the dark underneath a microscope coverslip, with O_2 being depleted by respiration. Control experiments were performed with epidermal peels being kept for 30 min in the dark in contact with air.

To investigate the effect of fusicoccin (FC) on guard cell photosynthesis, stomatal opening in the dark was induced by submerging epidermal peels in a solution of 10 mol m^{-3} MES/Tris (pH 6·1) and 10 mol m^{-3} KCl containing 6·7 mmol m^{-3} FC. After different incubation times, FC-treated epidermes were transferred to the reaction medium, which was composed of 10 mol m^{-3} MES/Tris (pH 6·1), 10 mol m^{-3} KCl, and 2·5 mol m^{-3} $NaHCO_3$. For all experiments, a stock solution of $NaHCO_3$ was prepared just before use and the pH of the solution was adjusted to 6·1 under ambient conditions. All measurements were carried out at room temperature.

RESULTS AND DISCUSSION

System performance in measurements with single MCPs and GCPs

The measuring system allows suitable samples to be inspected first via the ocular of the microscope and then, for the actual fluorescence measurements, the field of view to be narrowed down with the help of an iris diaphragm. In this way, the background signal is minimized and it can be assured that the assessed fluorescence originates almost exclusively from the selected cells. Figure 2a shows typical recordings from single protoplasts, which differ in size and chloroplast number. The corresponding photographs of a *Vicia* MCP (top), GCP (centre) and *Arabidopsis* GCP (bottom) are displayed in Fig. 2b. Both GCP and MCP showed the well-known dark–light induction kinetics (Kautsky effect). Additional information on fluorescence quenching and, hence, on the status of the chloroplasts, was obtained by repetitive application of saturating light pulses. The signal/noise ratio (S/N, evaluated during a saturation pulse applied to a dark-adapted sample) was very satisfactory not only with a single MCP (S/N > 100), but also with a GCP of *Vicia* (S/N = 50). For the latter, however, in order to reach the same signal amplitude as with the MCP, amplification had to be increased by a factor of 27·4. Similar fluorescence information was obtained with an extraordinary small single GCP (diameter = 6·7 μm) of *Arabidopsis*. In this case the fluorescence intensity was ≈ 180 times lower than with a MCP of *Vicia* and the S/N was only ≈ 5. While this S/N is too low for quantitative assessment of fluorescence parameters by a single recording, it can be readily improved by averaging a number of such recordings, a feature provided by the WINCONTROL software which is distributed with the microscopy–PAM fluorometer (data not shown).

In the following, the fluorescence parameters measured by the new approach will be briefly explained and a comparison with earlier single guard cell fluorescence recordings will be made. When the weak measuring light is switched on (ML on), the minimal fluorescence yield, F_o, is

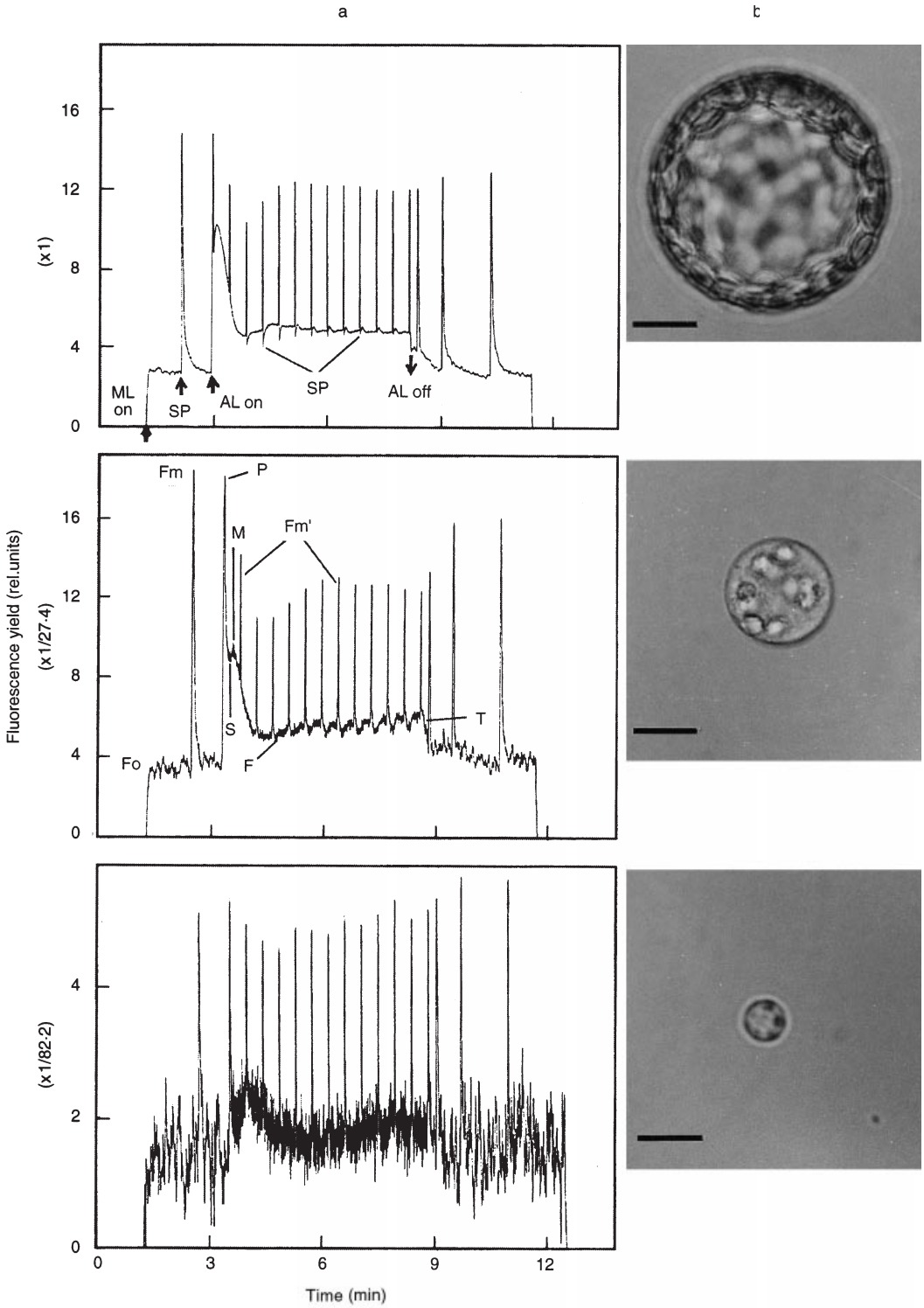

Figure 2. Typical recordings of dark–light induction transients of single protoplasts with fluorescence quenching analysis by the saturation pulse method and corresponding micrographs of the investigated samples. (a) Fluorescence responses of *Vicia* mesophyll cell protoplasts (MCP; top), *Vicia* guard cell protoplasts (GCP; centre) and *Arabidopsis* GCP (bottom). The measuring light (ML) intensity was 0·8 μmol m^{-2} s^{-1} of photosynthetically active radiation. Saturating light pulses (SP) inducing maximal fluorescence yields, F_m and F_m', were applied at 40 s following the onset of ML and every 20 s after onset of actinic light (AL). The intensity of saturation pulses was 4280 and 1550 μmol m^{-2} s^{-1} for *Vicia* MCP/GCP and *Arabidopsis* GCP, respectively (see text for further details). (b) The attached micrographs show typical sizes and chloroplast contents of *Vicia* MCP (top), *Vicia* GCP (centre) and *Arabidopsis* GCP (bottom) as seen under a light microscope. The bar represents 10 μm.

assessed which is characteristic for the dark-adapted state with all PS II reaction centres being open (see Fig. 2a). It is important to note that the measuring beam intensity is sufficiently low to allow continuous recording of F_o, i.e. there is no accumulation of reduced acceptors at PS II, which would lead to an induction phenomenon, as in the original work of Zeiger and coworkers (Zeiger *et al.* 1980; Melis & Zeiger 1982). Despite such low measuring light, the F_o information is obtained within fractions of a second, which compares favourably with the 15 min time required for obtaining an F_o image by the technique of Oxborough & Baker (1997). Upon application of a brief (0·8 s) saturating light pulse, the fluorescence yield of the dark-adapted sample is transiently increased to its maximal level, F_m. The increase of fluorescence yield from F_o to F_m is called variable fluorescence, F_v, and the ratio F_v/F_m has been shown to correspond to the potential maximum quantum yield of light energy conversion in PS II (Butler 1978; Björkman 1987). After fluorescence yield has returned close to the F_o level, actinic light is turned on (AL on) and repetitive saturation pulses are applied (at 20 s intervals) to assess the change of maximal fluorescence yield during illumination (F_m'). The Kautsky effect observed in the GCP followed the pattern of a typical dark–light induction curve well known from numerous studies on whole leaves (Briantais *et al.* 1986; Krause & Weis 1991; Schreiber *et al.* 1994). There were no major differences between the curves of GCP (centre panel) and MCP (top panel), contrary to earlier single-cell measurements of Melis & Zeiger (1982) and Mawson & Zeiger (1991). While the definite cause of this discrepancy is not clear, it may be relevant that in the study of Melis & Zeiger (1982) leaf segments were enclosed in a 1 mm thick cuvette, and in the study of Mawson & Zeiger (1991) epidermal peels were enclosed between coverslips. In view of the high dark respiratory O_2 uptake rate and the pronounced O_2 requirement for photosynthetic electron transport in guard cells (Mawson 1993), it appears possible that in these earlier studies the induction curves of guard cells were influenced by local, partial anaerobiosis (see also the section on guard cells under anaerobiosis below).

In the PAM measurements of Fig. 2a, upon the onset of continuous actinic light, fluorescence yield first rapidly rises to a peak, P, and then more slowly declines to a quasi-stationary level, S, before it briefly rises to a secondary maximum, M, and then finally reaches the terminal steady-state level, T. It may be mentioned that before the advent of PAM fluorimetry the M-peak phenomenon and the slow fluorescence decline from M to T were interpreted in terms of Calvin cycle activity and proton gradient formation, respectively. Such a phenomenological approach, however, has been replaced by a more quantitative assessment using the saturation pulse quenching analysis (Walker 1992). The repetitive fluorescence increases ($F_m' - F$) induced by each saturation pulse contain the essential information for quenching analysis, resulting in assessment of the PS II quantum yield and energy status of the chloroplast (Genty *et al.* 1989). Any decrease of F_m' with respect to the original F_m reflects non-photochemical quenching, whereas the difference $F_m' - F$ is caused by photochemical quenching, which is a relative measure of open PS II reaction centres.

The so-called quenching coefficients, *qN* and *qP*, were defined (Schreiber *et al.* 1986; Bilger & Schreiber 1986), the calculation of which, however, requires knowledge of minimal fluorescence yield, F_o', in a given state of preillumination. In order to assess F_o', the actinic light must be turned off and the electron pool at the acceptor side of PS II must be quickly reoxidized with the help of far-red light, before relaxation of non-photochemical quenching sets in. In the present study, no use of far-red light could be made because of excessive disturbance of the photomultiplier. Therefore, instead of *qN* and *qP*, the fluorescence parameters NPQ (Bilger & Björkman 1990) and $\Delta F/F_m'$ (Genty *et al.* 1989) are presented, the calculation of which does not require knowledge of F_o'.

In intact leaves and isolated chloroplasts, a major component of non-photochemical quenching is energy-dependent quenching, *qE*, which is caused by acidification of the thylakoid internal space. It can be distinguished from other forms of non-photochemical quenching by its rapid reversal upon light-off. In the measurements of Fig. 2a, in order to gain information on the reversal of non-photochemical quenching, saturation pulses were also applied during the dark period following light-off. The dark intervals between the pulses were stepwise increased in order to minimize the illumination effect. It is apparent that *Vicia* GCP displayed substantial light-induced non-photochemical quenching, most of which was reversed within 1 min following light-off (Fig. 2a, centre). Unless guard cell fluorescence is governed by different quenching mechanisms than mesophyll fluorescence, which appears highly unlikely in view of the very similar phenomenology of fluorescence induction, these data argue for substantial energy-dependent quenching in guard cells. Notably, also in guard cells during the first minute of illumination there is a pronounced decrease of F_m', with partial recovery during prolonged illumination. In leaves, the recovery of high F_m' values depends on the presence of CO_2 and Calvin cycle activity (Schreiber *et al.* 1986). Hence, this phenomenon is believed to reflect the onset of the Calvin cycle following light activation, when ATP consumption is initiated and consequently there is a drop in transthylakoidal ΔpH. If this rationale is also applied to guard cell chloroplasts, they would appear to display very similar membrane energization by photosynthetic electron flow coupled to vectorial proton translocation, activation of Calvin cycle enzymes and steady-state CO_2-dependent electron flow. However, more extensive work will be required to validate this conclusion.

Quenching analysis, effective quantum yield and relative electron transport rate (ETR)

The microscopy–PAM is equipped with an extensive data acquisition system (PAM control EPROM in conjunction with WINCONTROL software) which allows automated assessment of the relevant fluorescence levels and on-line calculation of the essential fluorescence-derived parame-

ters. These include the effective quantum yield of PS II, the so-called Genty parameter $\Delta F/F_m' = (F_m' - F)/F_m'$ (Genty *et al*. 1989), the apparent relative ETR = $\Delta F/F_m' \times PAR \times c$ (where PAR is the photon flux density of incident photosynthetically active radiation, and the constant c corresponds to the absorption factor) (Schreiber *et al*. 1994) and the NPQ parameter of non-photochemical quenching, as defined by $(F_m - F_m')/F_m'$ (Bilger & Björkman 1990). As to the fluorescence-derived ETR parameter, it should be emphasized that it provides a relative measure of ETR only. The calculation of absolute rates would not only require knowledge of the fraction of incident quanta absorbed by the guard cell chloroplasts, but also of the energy distributed to PS II. The various fluorescence-derived parameters were determined repetitively during the course of a dark–light induction curve or after each of a series of consecutive illumination steps at increasing light intensities (so-called light curves). Such dark–light induction curves and light curves were recorded several times using the same computer-controlled illumination program and then the data were averaged. In this way, the relatively large variability observed between different protoplasts of the same preparation was removed and systematic differences could be assessed. In Fig. 3, averaged values of $\Delta F/F_m'$, ETR, and NPQ measured during dark–light induction are presented for *Vicia* GCP and MCP. It should be pointed out that the data variability, which is expressed in the mean ± standard deviation (SD), was mainly due to actual differences in the photosynthetic activity of the individual protoplasts, rather than to recording noise or errors in the quenching analysis. This may be concluded from the fact that ± SD amounted to 10–50%, particularly large in the case of NPQ, whereas the S/N ratio was in the order of 50 (corresponding to ≈ 2% noise; see original data in Fig. 2a and Figs 4–6). Actually, when, for example NPQ was measured repetitively with the same sample with 1 min dark intervals between consecutive measurements, ± SD was only 1·5% for $n = 9$ (data not shown).

A major finding, which in principle was already apparent from the single recordings in Fig. 2a, was that the observed fluorescence parameters and their general induction patterns were quite similar for MCP and GCP. While $\Delta F/F_m$ (maximal variable fluorescence of dark-adapted sample, also referred to as F_v/F_m) was close to 0·8 (the maximal potential quantum yield of charge separation in PS II) in MCP as well as in GCP, the effective quantum yield of PS II during illumination, $\Delta F/F_m'$, was systematically lower in GCP than in MCP (Fig. 3a). Corresponding differences were also apparent in the ETR data (Fig. 3b). During the course of an induction curve, the difference was most pronounced ≈ 40 s following the onset of illumination. The light-induced rise of NPQ was almost identical in MCP and GCP (Fig. 3c). However, relaxation of NPQ during illumination (reflecting Calvin cycle activation) was retarded and less pronounced in GCP. Furthermore, differences were observed in the kinetics of dark relaxation of NPQ, with the rapid phase (presumably reflecting the decay of energy-dependent quenching) showing a larger amplitude in GCP.

In Fig. 7, so-called rapid light curves (RLC) of *Vicia* GCP and MCP are compared. Such RLC, which involve relatively short illumination periods at increasing light intensities, give insight into the light saturation properties of a sample (Schreiber *et al*. 1997; White & Critchley 1999). When recorded with a light-adapted sample, RLC data reflect the dependence of the relative photosynthetic ETR on quantum flux density in a given state of light adaptation. These data showed even more clearly than the ETR data of Fig. 3b that the electron transport capacity of GCP is distinctly lower than that of MCP, with half saturation at 58 μmol quanta m^{-2} s^{-1} PAR in GCP and at 132 μmol quanta m^{-2} s^{-1} PAR in MCP. Such measurements involving the determination of effective PS II quantum yield and the

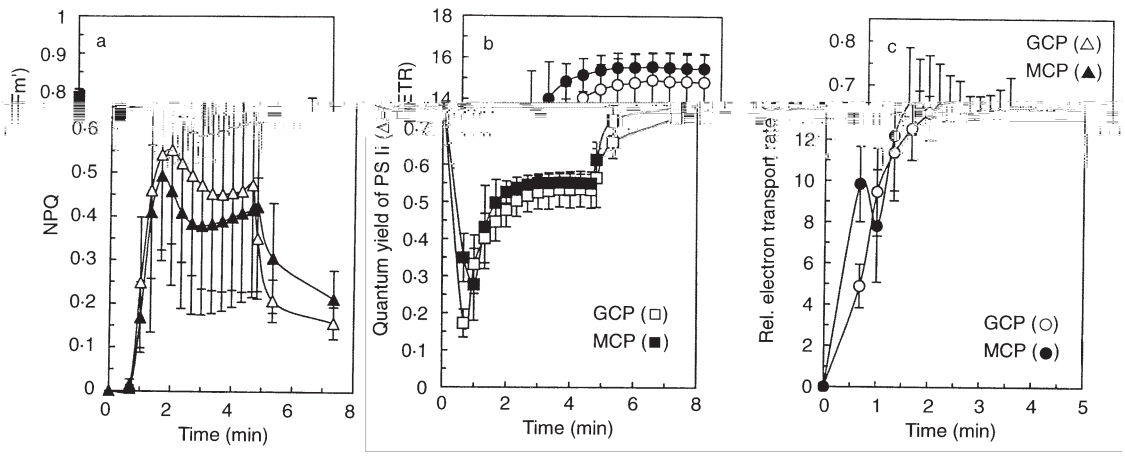

Figure 3. Characteristic fluorescence parameters of *Vicia* guard cell protoplasts (GCP) and mesophyll cell protoplasts (MCP) as assessed during dark–light induction by quenching analysis. (a) Effective photosystem II quantum yield, $\Delta F/F_m'$ (Genty parameter). (b) Relative electron transport rate, ETR. (c) Parameter of non-photochemical quenching, NPQ. The constant c in the equation for the ETR calculation (see text) was assumed to be 0·42. Actinic intensity was 67 μmol m^{-2} s^{-1}. The displayed data are the means ± standard deviation (SD) for MCP ($n = 13$) and GCP ($n = 16$). For other conditions, see legend to Fig. 2 and Materials and methods.

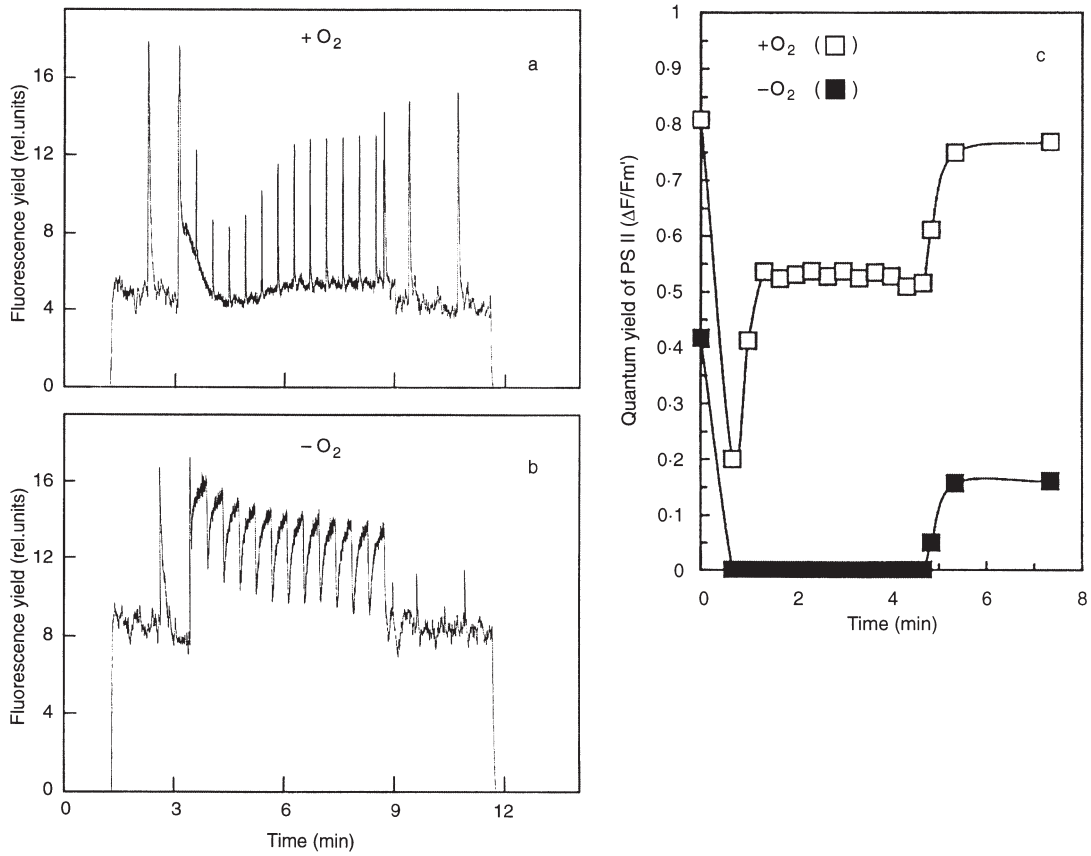

Figure 4. Effect of anaerobiosis on the fluorescence characteristics of *Vicia* guard cell protoplasts (GCP) during dark–light induction with repetitive application of saturation pulses. (a) Original recording, control sample. (b) Original recording, anaerobic sample after treatment with glucose/glucose oxidase (see Materials and methods). (c) On-line calculated values of effective quantum yield, $\Delta F/F_m'$, for control (open squares) and anaerobic sample (closed squares). Other conditions, see legend to Fig. 2.

calculation of relative ETR in single guard cells were not possible using the non-modulated fluorescence technique of earlier work (e.g. Mawson & Zeiger 1991). While similar information under quasi-steady-state conditions could well be obtained with the help of the special fluorescence imaging system of Oxborough & Baker (1997), such measurements so far have not been reported. However, it has already been noted by Shimazaki & Zeiger (1985) that non-cyclic photophosphorylation saturates at lower light intensities in guard cells than in mesophyll cells.

It is known that on a chlorophyll basis the O_2 evolution rate in GCP exceeds that of MCP by a factor of 3–4 (Shimazaki, Gotow & Kondo 1982; Shimazaki 1989). This difference, however, cannot be reflected by the present ETR measurements, as without knowledge of the absolute fraction of incident light reaching PS II, ETR is just a relative measure of electron transport (see above).

Fluorescence characteristics of single guard cells under anaerobiosis

The fluorescence-derived ETR values which reflect the relative rate of energy conversion in PS II (Genty *et al*. 1989; Schreiber *et al*. 1994), do not necessarily correspond to the rate of CO_2 fixation. There are other forms of electron transport passing PS II, such as the Mehler ascorbate peroxidase (MAP) cycle which depends on O_2 instead of CO_2 as the electron acceptor [for reviews see Schreiber *et al*. (1995) and Asada *et al*. (1998)]. Actually, Gotow *et al*. (1988) reported that the rate of CO_2 fixation was only 8% of photosynthetic O_2 evolution in GCP. And Mawson (1993) showed that the net rate of photosynthetic O_2 evolution in *V. faba* GCP was strongly O_2 dependent. As shown in Fig. 4, this earlier finding is impressively confirmed by chlorophyll fluorescence quenching analysis in single-cell measurements (compare traces in Fig. 4a & b). After removal of O_2 using the glucose/glucose oxidase trap (Fig. 4b), part of the PS II reaction centres are already closed in the dark, as revealed by a doubling of the apparent F_o and a corresponding decrease of the apparent F_v/F_m. Photochemical quenching is rapidly eliminated upon onset of actinic illumination. Saturation pulses induce fluorescence quenching instead of increases of fluorescence. The latter feature suggests that the primary acceptor of PS II, Q_A, is fully reduced by the actinic light and that there is transient accumulation of reduced pheophytin during saturating light pulses

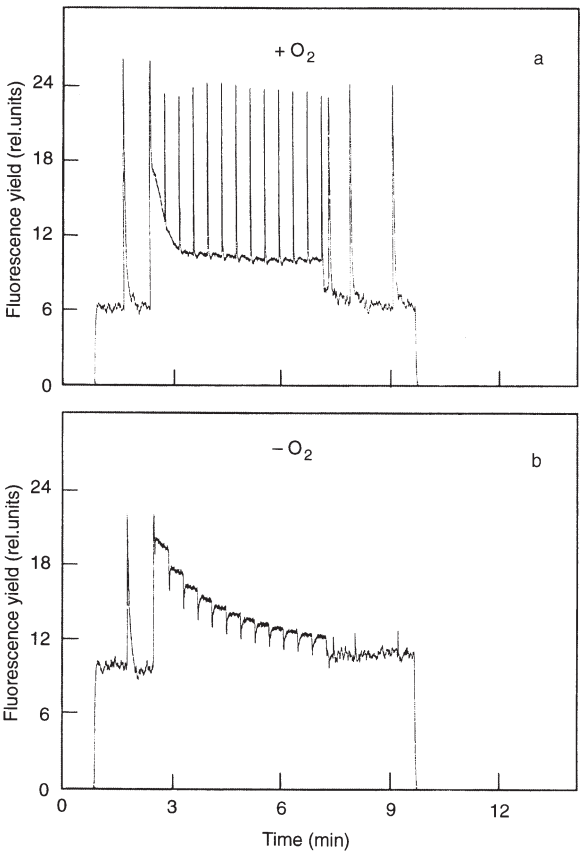

Figure 5. Effect of dark incubation of a *Vicia* epidermal peel underneath a microscope coverslip. (a) Control in the open system for 30 min. (b) After 30 min incubation in the closed system. The temperature was ≈ 25 °C. For other conditions, see legend to Fig. 2 and Materials and methods.

(Heber *et al*. 1985). A total block of PS II activity in the absence of O_2 is also reflected by the calculated values of effective PS II quantum yield ($\Delta F/F_m'$) as shown in Fig. 4c.

A central role of O_2-dependent electron flow was also apparent when epidermal peels were submersed underneath a microscope coverslip. Due to strong O_2 uptake by respiration in guard cells (Mawson 1993) and limited O_2 diffusion from the surrounding air, within 10–30 min (depending on the temperature and the size of the epidermal fragments) the fluorescence responses of the submersed guard cells indicated strong inhibition of photosynthetic electron flow (Fig. 5). It is conceivable that intermediate stages of anaerobiosis will yield fluorescence responses intermediate between those shown in Fig. 5a and b. While the severe inhibition of photosynthetic electron flow is clearly revealed by the saturation pulse technique, the continuous fluorescence response, which essentially was measured in earlier work on single guard cells (Melis & Zeiger 1982; Mawson & Zeiger 1991), just shows a slow down of the fluorescence decline from P to T.

These data suggest that O_2 is a competent electron acceptor in guard cell chloroplasts, as has been previously shown for intact leaves and spinach chloroplasts. In the latter case, an important role of the MAP cycle in ΔpH generation and light protection has been demonstrated (Schreiber *et al*. 1995). It can be readily shown that there is ascorbate peroxidase activity in *Vicia* GCP, as addition of H_2O_2 (0·5 mol m^{-3}) to an O_2-limited sample in the presence of ascorbate (5 mol m^{-3}) restored photochemical quenching (data not shown), similar to previous observations with spinach chloroplasts (Neubauer & Schreiber 1989). However, an alternative explanation for the pronounced O_2 requirement of guard cell photosynthetic electron flow must be considered on the basis of the work of Mawson (1993). The latter showed that in GCP the decrease of photosynthetic O_2 evolution with decreasing O_2 concentration is closely correlated with a decrease of respiratory O_2 uptake, suggesting close metabolic coupling between the two activities. Hence, it appears feasible that photosynthetic electron flow in guard cells depends on the export of reducing equivalents from the chloroplasts to the cytosol via a

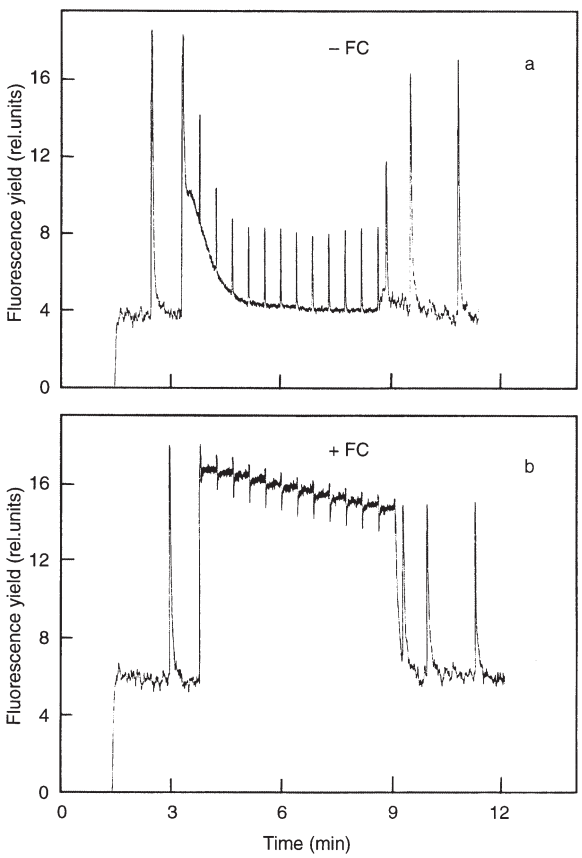

Figure 6. Effect of fusicoccin (FC) on the fluorescence characteristics of single *Vicia* guard cell pairs in an epidermal peel. (a) Control; (b) presence of FC. The peels were incubated in a buffered KCl solution for 3·5 h in the dark, with and without 6·7 mmol m^{-3} FC being present (see Materials and methods). In both cases, the medium contained 0·27% dimethylsulphoxide (DMSO). Actinic intensity was 67 μmol m^{-2} s^{-1} and saturation pulses had an intensity of 2904 μmol m^{-2} s^{-1}.

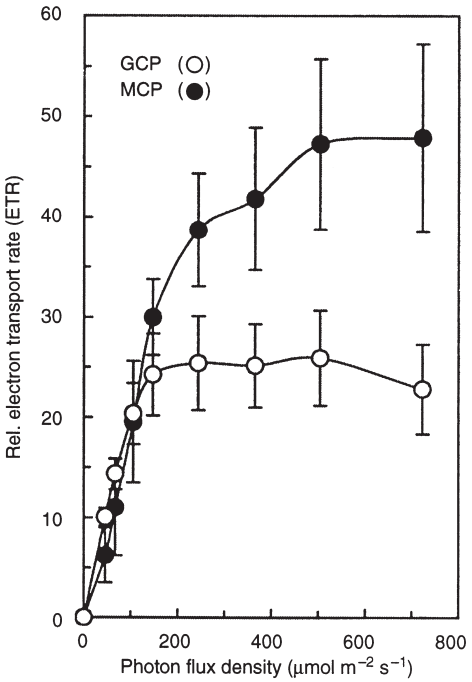

Figure 7. Rapid light response curves of the relative electron transport rate, ETR, of *Vicia* guard cell protoplasts (GCP) and mesophyll cell protoplasts (MCP). Actinic light was applied during consecutive 30 s periods with stepwise increasing intensity. At the end of each illumination period a saturation pulse was applied to assess the effective quantum yield on which the calculation of ETR is based. The data are displayed as means ± standard deviation (SD) for GCP (n = 11) and MCP (n = 11). Other conditions as for Fig. 2.

PGA/DHAP shuttle system (Shimazaki *et al*. 1989), which are oxidized by oxidative phosphorylation in the mitochondria. Oxidation of photosynthetically derived reducing equivalents by mitochondria has also been previously suggested for C_3 mesophyll cells (Krömer, Stitt & Heldt 1988). On the basis of the present data, we are unable to favour one of the two alternatives. A third alternative was suggested by Cardon & Berry (1992) who studied the steady-state fluorescence yield of single guard cells at 21% and 2% O_2 in dependence of CO_2 concentration. These authors interpreted a quenching of the steady-state fluorescence yield at a low CO_2 concentration upon transition from 21 to 2% O_2 as evidence for photorespiration in guard cells, analogous to related changes in mesophyll cells. Whether or not this suggestion is justified should be verified by corresponding experiments with the microscopy–PAM making use of fluorescence quenching analysis. In this context, it should be pointed out that the present study reports on the responses of isolated protoplasts and guard cells in epidermal peels only, which do not necessarily in all respects reflect the biochemical and physiological responses of guard cells in intact leaves. Therefore, it is intended to extend the present work to intact leaves, for example by studying guard cells in the albino parts of variegated leaves [see earlier work of Melis & Zeiger (1982) and Cardon & Berry (1992)].

While O_2 can function as an electron acceptor in the MAP cycle only during illumination (reduction at PS I acceptor side), the data presented in Figs 4 and 5 suggest that it also plays a role in the reoxidation of the intersystem electron transport chain in the dark. The observed increase of F_o argues for reduction of the plastoquinone pool by reduced stroma components which in air is compensated by O_2-dependent reoxidation. Such a 'chlororespiration' (Bennoun 1982) has been reported for higher plant chloroplasts (Garab *et al*. 1989; Gruszecki, Bader & Schmid 1994) and also for algae (Schreiber & Vidaver 1974; Ting & Owens 1993; Bennoun 1994). The present results suggest a rather strong flux of electrons from reduced substrates to O_2 via the plastoquinone pool, which displays the characteristics of chlororespiration.

Effect of FC on single guard cell fluorescence characteristics

The fungal toxin FC is well known to stimulate stomatal opening by hyperactivating the H^+-pumping ATPase in the plasma membrane of guard cells (Lohse & Hedrich 1992). The effect of FC on chlorophyll fluorescence characteristics of single guard cell pairs was studied in epidermal peels of *Vicia*. As shown in Fig. 6, there was a strong effect of 6·7 mmol m^{-3} FC on the dark–light fluorescence induction characteristics when applied for 3·5 h. The same amount of FC, when applied over several hours, induced pronounced stomatal opening in the dark, with saturation occurring at 3·5 h. The kinetics of the increase of stomatal aperture during dark incubation with 6·7 mmol m^{-3} FC are depicted in Fig. 8. Analogously, Fig. 9 shows the gradually developing suppression of effective PS II quantum yield, $\Delta F/F_m'$, measured during continuous illumination (as in the experiment of Fig. 6), after different incubation times with FC (compare corresponding data of stomatal aperture in Fig. 8). Control experiments with intact spinach chloroplasts as well as with *Vicia* MCP did not show any effect of FC on CO_2-dependent electron flow (data not shown). Phenomenologically, the FC effect on GCP fluorescence parameters resembles that of O_2 depletion, although not to the same extent as by the glucose/glucose oxidase trap (compare Fig. 4). This was not only true for the loss of photochemical and non-photochemical quenching, but also for the increase of F_o and of the steady-state fluorescence yield.

As expected, the effect of FC on the apparent ETR in guard cell chloroplasts (determined by $\Delta F/F_m'$) was prevented by 100 mmol m^{-3} vanadate, a specific inhibitor of plasma membrane H^+-ATPase (Amodeo, Srivastava & Zeiger 1992) (Fig. 10). In parallel experiments, vanadate also prevented FC-induced stomatal opening in the dark (data not shown). These findings are in agreement with the suggestion that uncontrolled H^+-ATPase activity in response to FC and the resulting depletion of the ATP pool in the cytosol led to the suppression of photosynthetic electron flow in the guard cell chloroplasts. Indeed, when ATP

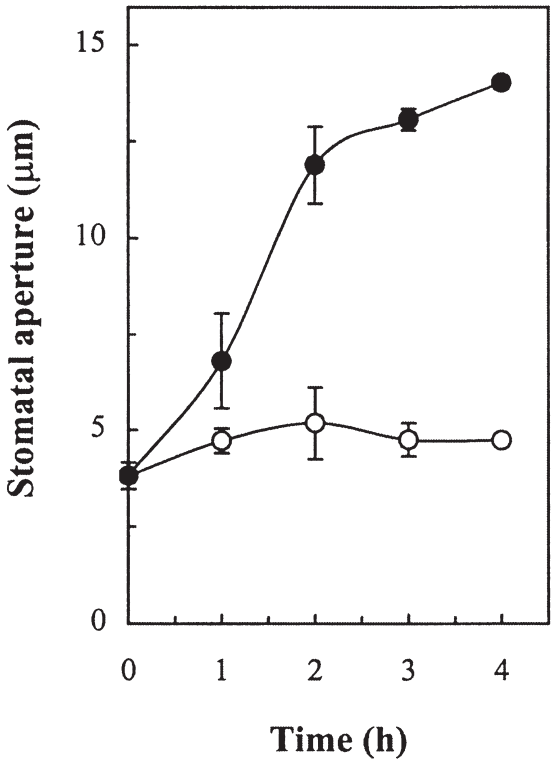

Figure 8. Kinetics of the fusicoccin (FC)-induced increase of stomatal aperture observed microscopically in *Vicia* epidermal peels. Closed symbols, epidermal peels submerged for the indicated time in buffer solution containing 6·7 mmol m^{-3} FC in the dark. Open symbols, control with epidermal peels submerged in buffer solution without FC in the dark (see Materials and methods). The displayed data are the means ± standard deviation (SD) of $n = 3$ (60 stomata).

depletion was counteracted by the addition of glucose (2 mol m^{-3}), which stimulates ATP generation by respiration, the FC-induced inhibition of guard cell photosynthetic electron flow was much less pronounced (Fig. 11). Furthermore, inhibition of oxidative phosphorylation by KCN restored the full effect of FC in the presence of glucose with a half-maximal effect at ≈ 25 mmol m^{-3} KCN (Fig. 12).

While all the data presented in Figs 6, 8–11 in principle seem to agree with the suggestion that the observed FC effect is related to ATP depletion of the cytosol, it is not clear why such ATP depletion should cause the inhibition of electron transport within the chloroplasts. As an alternative explanation, we have considered the possibility that, as a consequence of cytosolic ATP depletion, there is further stimulation of O_2 uptake due to the quasi-uncoupled state of oxidative phosphorylation, with the consequence of local O_2 depletion within the guard cells. However, this explanation, which would be in line with the observed phenomenology (compare Figs 5 and 6), appears to be ruled out by the effect of glucose, which restores photosynthetic electron transport in the presence of FC, while at the same time it is expected to stimulate O_2 uptake by respiration. Alternatively, the FC effect may be also seen in the context of the complex metabolic coupling between the photosynthetic activity of guard cell chloroplasts and oxidative phosphorylation in guard cell mitochondria, both of which are linked via the cytoplasma with sucrose–glucose metabolism on the one hand and stomatal opening via activation of the proton pumping ATPase, followed by ion uptake and malate biosynthesis on the other. Future studies on mutants defective in the FC response of guard cells, as well as on metabolic and photosynthesis mutants, will help to elucidate the molecular mechanisms underlying the FC effect on photosynthesis.

CONCLUSIONS

In conclusion, the recently developed microscopy–PAM chlorophyll fluorometer has allowed the investigation of guard cell photosynthesis and physiology at the level of a single stoma and GCP. The signal/noise ratio was sufficiently high to apply the methodology of chlorophyll fluorescence quenching analysis, which originally was developed for whole-leaf measurements. There was a surprising similarity between the fluorescence induction characteristics of *Vicia* GCP and MCP, indicating a similar functional organization of the thylakoid membranes in

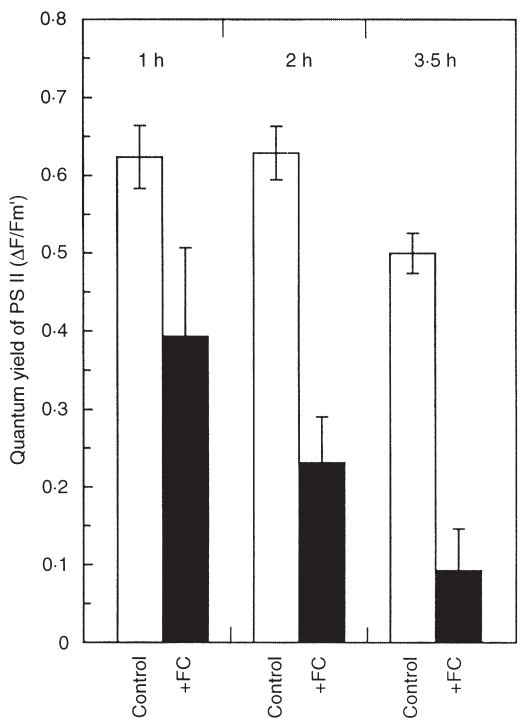

Figure 9. Effective quantum yield during illumination, $\Delta F/F_m'$, in single *Vicia* guard cell pairs as a function of the preceding dark incubation time in the absence and presence of 6·7 mmol m^{-3} fusicoccin (FC). The quantum yield was determined at the end of a 4·7 min illumination period. The displayed data are the means ± standard deviation (SD) of $n = 6$ (1 h incubation), $n = 8$ (2 h) and $n = 10$ (3·5 h). Other experimental conditions as for Fig. 6.

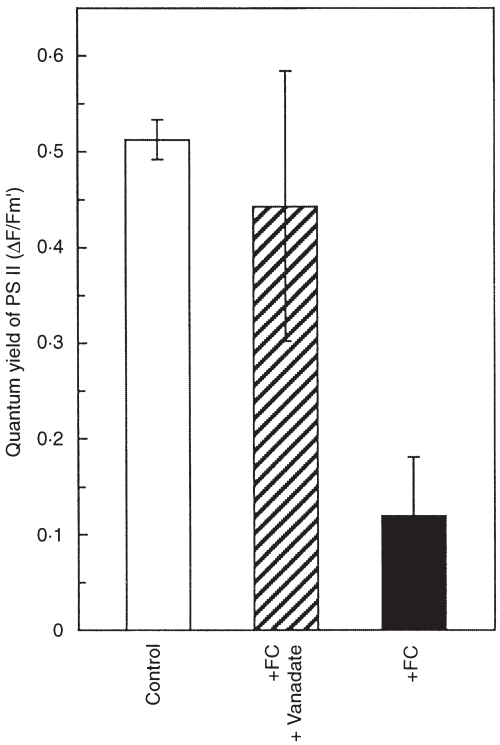

Figure 10. Reversal of the fusicoccin (FC) effect on effective quantum yield, $\Delta F/F_m'$, in single *Vicia* guard cell pairs by vanadate. Incubation was for 3·5 h. Vanadate dissolved in water was added to a final concentration of 100 mmol m^{-3}. Other experimental conditions as for Fig. 6. The displayed data are the means ± standard deviation (SD) of $n = 5$.

chloroplasts of both cell types. On the other hand, the capacity of photosynthetic electron transport was distinctly higher in MCP than in GCP. The presence of molecular oxygen was found to be essential for photosynthetic electron flow in GCP, confirming previous work of Mawson (1993). It appears likely that O_2-dependent electron flow in guard cell chloroplasts involves the MAP cycle. However, in agreement with the findings of Mawson (1993) close metabolic coupling between photosynthetic electron transport, export of reducing equivalents via a PGA/DHAP shuttle and oxidative phosphorylation in the mitochondria may also be considered. O_2 removal induced a strong reduction of intersystem electron carriers in the dark, suggesting that chlororespiration occurs in guard cells. The observed strong O_2 sensitivity calls for strict control of the O_2 concentration in all studies of guard cell photosynthesis and physiology, which may be a problem in work with submersed epidermal peels when O_2 removal by respiration may exceed O_2 uptake from the air by diffusion. Another important finding is the strong inhibition of guard cell photosynthesis by FC, which appears to correlate with the depletion of the ATP pool in the cytosol. However, it remains to be clarified why cytosolic ATP is required for photosynthetic electron flow in guard cell chloroplasts, and other explanations, for example involving metabolic coupling between chloroplasts and mitochondria, have to be tested by future work. The presented results clearly show that chlorophyll fluorescence provides a highly sensitive indicator to monitor key functions of stomatal movement which are located outside the chloroplasts, such as H^+-ATPase activity, glucose uptake and oxidative phosphorylation. Combining microfluorimetry with the patch-clamp technique, photosynthetic activity can be monitored simultaneously with the H^+ pump and channel currents. Applying metabolites which shuttle between chloroplasts and cytosol through the patch pipette should enable us to gain new insights into metabolic interactions between these compartments. Therefore, it may be predicted that this very sensitive and selective method will be of considerable practical value in future studies on stomatal physiology. While the present study was restricted to the responses of isolated protoplasts and guard cells in epidermal peels, future work will also deal with guard cells in intact leaves, the biochemical and physiological responses of which should be most relevant for the *in vivo* functioning of stomates.

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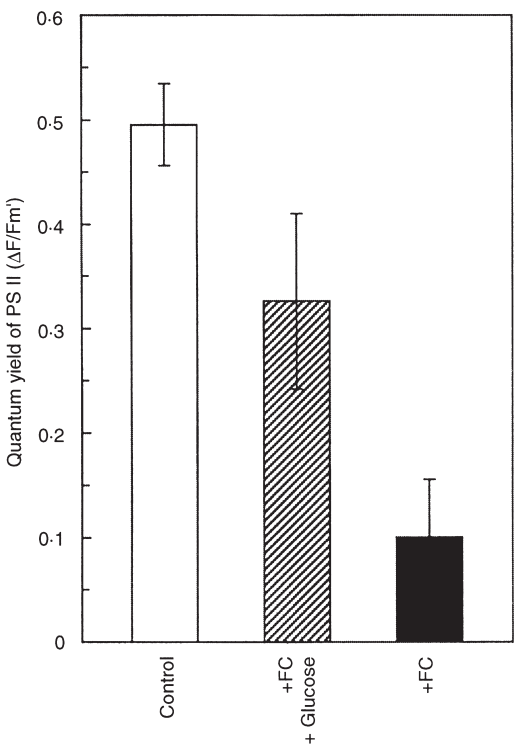

Figure 11. Effect of glucose on the suppression of $\Delta F/F_m'$ by fusicoccin (FC) in single *Vicia* guard cell pairs. Incubation was for 3·5 h. Glucose was added to a final concentration of 2 mol m^{-3}. Other experimental conditions as for Fig. 6. The displayed data are the means ± standard deviation (SD) of $n = 8$.

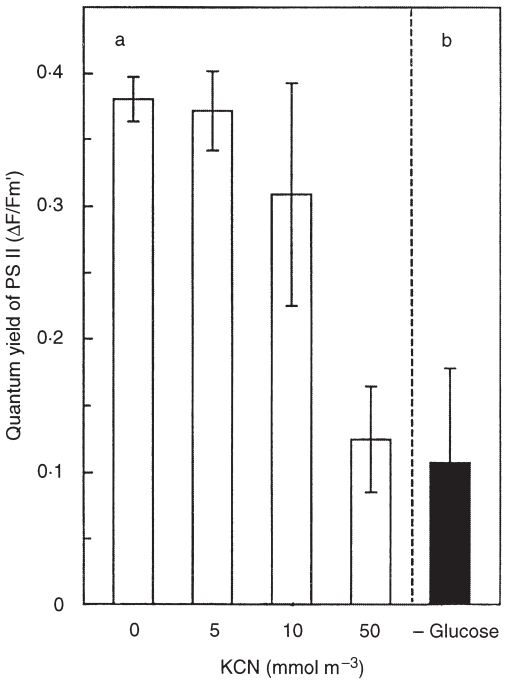

Figure 12. Effect of increasing KCN concentration on $\Delta F/F_m'$ in single *Vicia* guard cell pairs in the presence of fusicoccin (FC) and glucose (a). The control value of $\Delta F/F_m'$ in the absence of glucose is shown in (b). Incubation was for 3·5 h in the dark. Other experimental conditions as for Fig. 10. The displayed data are the means ± standard deviation (SD) of $n = 5$.

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