Planta (1991) 184:105–112

Planta

Changes of cytoplasmic free Ca^{2+} in the green alga *Mougeotia scalaris* as monitored with indo-1, and their effect on the velocity of chloroplast movements*

U. Russ, F. Grolig, and G. Wagner

Membran- und Bewegungsphysiologie, Botanisches Institut 1, Justus-Liebig-Universität, Senckenbergstrasse 17–25, W-6300 Giessen, Federal Republic of Germany

Received 28 December 1989; accepted 24 November 1990

Abstract. The fluorescent calcium-sensitive dye 1-[2-amino-5-(6-carboxyindol-2-yl)-phenoxy]-2-(2'-amino-5'-methylphenoxy)-ethane-N,N,N',N'-tetraacetic acid (indo-1) was loaded by a transplasmalemma pH gradient into filamentous cells and protoplasts of *Mougeotia scalaris*, such that most of the indo-1 fluorescence originated from the cytoplasm. Incubation of *M. scalaris* filaments in ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)-buffered media ($-\log [Ca^{2+}]$ (=pCa) 8 versus pCa 3) caused a consistent and significant decrease in the cytoplasmic free $[Ca^{2+}]$. Pulses of the fluorescence excitation light (UV-A 365 nm, 0.7 s) caused an increase in cytoplasmic free $[Ca^{2+}]$ in *M. scalaris* that was nearly independent of the external $[Ca^{2+}]$ and of chloroplast dislocation by centrifugation. This calcium flux, highest in UV–A light, compared with blue or red light, probably resulted from a release of Ca^{2+} from intracellular stores. Increased cytoplasmic $[Ca^{2+}]$ may affect the velocity of chloroplast rotation since UV–A-light-mediated chloroplast movement was faster than in blue or red light. Consistently, the calcium ionophore A23187 and the calcium-channel agonist Bay-K8644 both increased the velocity of the red-light-mediated chloroplast rotation. Based on these and other observations, a Ca^{2+}-induced decrease in cytoplasmic viscosity in *Mougeotia* is presumed to occur.

Key words: Calcium (cytoplasmic free) – Chloroplast movement – Dye, calcium sensitive (Indo-1) – Light (blue, ultraviolet A) – *Mougeotia*

Introduction

The regulatory function of calcium has been demonstrated in many physiological processes, particularly in animal cells. In plant cells, our knowledge is less advanced, but the regulatory function of calcium has been proven here as well: besides metabolic pathways (Kauss 1987) and plant development (Hepler and Wayne 1985), calcium ions are involved in the regulation of cytoskeletal functions in intracellular motility (Williamson 1984).

Over the past 15 years, considerable indirect evidence has accumulated that in the green alga *Mougeotia* Ca^{2+} is part of the transduction process from the sensory pigments phytochrome and-or a blue-light pigment (Haupt 1987; Schönbohm 1987) to the effector of the chloroplast orientational movement, the actin-based force-generating system (Grolig and Wagner 1988). Direct measurements of free cytoplasmic Ca^{2+}, however, have not yet been performed in this organism.

The currently most favorable techniques for fast measurements of free cytoplasmic intracellular calcium in animal cells are those of calcium-sensitive microelectrodes and of calcium-sensitive dyes. Both methods have turned out to be difficult to use in plant cells (Felle 1989; Bush and Jones 1990), and the microelectrode technique was tried without success in *Mougeotia* (U. Russ and H. Felle, Botanisches Institut 1, Justus-Liebig-Universität, Giessen, FRG, personal communication; Russ et al. 1988).

In 1987, Bush and Jones introduced a plant-adequate method for loading the fluorescent dye 1-[2-amino-5-(6-carboxyindol-2-yl)-phenoxy]-2-(2'-amino-5'-methyl-

* Dedicated to Professor Wolfgang Haupt on the occasion of his 70th birthday

This paper is part of the Ph.D. thesis of U. Russ at the Justus-Liebig-Universität Giessen (FRG). Part of this work has been presented at a meeting on "Calcium and intracellular signalling in plants" in Plymouth, UK, Dec. 1990

Abbreviations: EGTA = ethylene glycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; indo-1 = 1-[2-amino-5-(6-carboxyindol-2-yl)-phenoxy]-2-(2'-amino-5'-methylphenoxy)-ethane-N,N,N',N'-tetraacetic acid; pCa = $-\log [Ca^{2+}]$; P_{fr} = far-red-absorbing form of phytochrome; P_r = red-absorbing form of phytochrome; $\bar{X}_G$ = geometric mean

phenoxy)-ethane-N,N,N',N',-tetraacetic acid (indo-1) into aleurone protoplasts using a pH-gradient between the external medium and the cytoplasm as the driving force. They suggested that this method would also be applicable to turgescent cells with intact cell walls. With little modification of their pH-method, we have succeeded in loading indo-1 into filamentous cells and protoplasts of *Mougeotia scalaris*.

Material and methods

Plant material. Mougeotia scalaris Hassel was grown as described by Russ et al. (1988). Protoplasts were prepared as described by Grolig and Wagner (1987).

Chemicals. Unless noted otherwise, all chemicals were from Merck, Darmstadt, FRG.

Light filtering. All interference and glass filters were from Schott, Mainz, FRG, unless noted otherwise.

Light measurements. Calibrations were done with an Optometer Model 161, equipped with the detectors 248 (450–950 nm) or 222AUV (200–400 nm; UDT, Hawthorne, Cal., USA).

Loading of indo-1. For dye loading, we applied the pH-gradient technique as suggested by Bush and Jones (1987). The loading medium was the acidified growth medium (Stabenau 1978) buffered by 5 mmol · l^{-1} 2-(N-morpholino)ethanesulfonic acid (Mes; Sigma, Deisenhofen, FRG); pH was adjusted to 4.0 with HCl. The fluorescent dye (indo-1) (Calbiochem, Frankfurt, FRG) was applied at 22 µmol · l^{-1}. The cell filaments or protoplasts were usually incubated for 2 h, and not longer than 4 h, in this solution prior to use.

Cell preparations. After dye accumulation, the cell filaments or protoplasts were transferred to identical medium without indo-1 (standard condition). In some experiments using filamentous cells, the chloroplast was centrifuged (500 · *g* for 2 min) into one half of the cylindrical cell (Fig. 1; compare Schönbohm 1987). To test the influence of external $-\log[Ca^{2+}]$ (=pCa) on cytoplasmic indo-1 fluorescence, the dye-loaded cells were transferred prior to centrifugation to the growth medium MXS (Neuscheler-Wirth 1970) without calcium and magnesium but with 1 mmol · l^{-1} 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes; Sigma) pH 7.8; pCa was adjusted with 1 mmol · l^{-1} ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA; Sigma) and

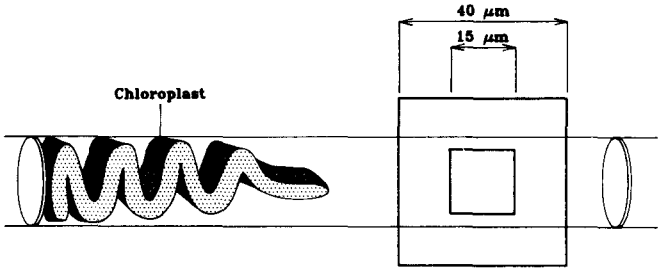

Fig. 1. Schematic drawing of part of a *Mougeotia* filament, showing a cell in which the chloroplast has become folded as a result of centrifugation. The *squares* indicate the size and position of the indo-1 exciting light beam (40 · 40 µm²) and of the photomultiplier window (15 · 15 µm²) used to detect the resulting fluorescence. The size and position of the photomultiplier window was chosen to minimize detection of fluorescence from outside the cell

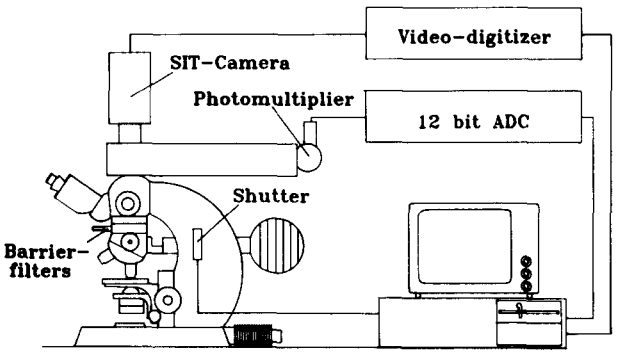

Fig. 2. Setup of the microspectrofluorometric equipment used. Pulses of excitation light were controlled by the computer via a mechanical shutter. The two barrier filters (Fig. 3a) for analyzing the Ca^{2+}-dependent spectral shift of indo-1 fluorescence were changed in a sliding holder by hand. The fluorescence output was either monitored by a silicon-intensified-target (SIT) camera with videodigitizer or detected by a photomultiplier with 12-bit analog-digital converter (ADC), and recorded and evaluated with a microcomputer

the appropriate amounts of $CaCl_2$ and $MgSO_4$ ($[Mg^{2+}]_{free}=0.2$ mmol · l^{-1}), as calculated according to constants in Martell and Smith (1974). The cell filaments then were transferred to microscope slides and covered by coverslips with spacers.

Calcium measurements. Fluorescence of indo-1 was monitored using an epifluorescence microscope (Zeiss, Oberkochen, FRG; Fig. 2). Though physically not optimal for indo-1 fluorescence measurements, the following parameters were chosen (see Fig. 3a) as a compromise with respect to the 365-nm Hg-line, the microscope setup, objective transmittance and the commercially available beamsplitter. Excitation light was generated by an HBO 100 lamp passing an interference bandpass filter (UV–II, $\lambda_{max}=365$ nm) as exciter; excitation intensity was 10 kW · m^{-2}. Irradiation, resulting in a fluence of 7 kJ · m^{-2}, was started and terminated by a computer-controlled shutter, with pulse duration of 0.7 s for each photomultiplier reading; a square of 40 · 40 µm² was irradiated (Fig. 1). The beamsplitter was an FT 395 (Zeiss), the objective was an NPL-Fluotar 40/1.3 Oil (Leitz, Wetzlar, FRG). The emitted light was measured from 390 to 450 nm and from 450 to 590 nm (½-power bandwidths), respectively (Fig. 3a), by changing the bandpass-filters KIF 450 versus KV 450+KIF 590 as optical barriers (Stern et al. 1989). Readings were performed either with a silicon-intensified-target (SIT) camera (2400–08–C; Hamamatsu, Herrsching, FRG) or a photomultiplier (type 1P28; Burle, Lancaster, Pa, USA) with a measuring area of 15 · 15 µm² (Fig. 1). Pictures or data were transmitted to a microcomputer, digitized, ratioed (390–450 nm/450–590 nm) and stored for further evaluation (Fig. 2). Preirradiation with blue light was given from an HBO 100 lamp and FT 510 beamsplitter (Zeiss) through the objective, filtered with BG 28 (2 mm)+GG 420 (2 mm; $\lambda_{max}=457$ nm). Orange light was given from the microscope lamp through the condenser, filtered with OG 590 (3 mm) and KG 1 (2 mm).

External calibration. Calibration of the signal ratio was done with the photomultiplier. Using methods similar to those of Grynkiewicz et al. (1985) and Popov et al. (1988), calcium buffers in the range of pCa_{free} 3 to 9, including 6 µmol · l^{-1} indo-1, 1.115 mmol · l^{-1} $MgSO_4$, 20 mmol · l^{-1} NaCl, 115 mmol · l^{-1} KCl, 1.115 mmol · l^{-1} EGTA, 10 mmol · l^{-1} 3-(N-morpholino)propanesulfonic acid (Mops; Sigma) pH 7.05 and the calculated amounts of $CaCl_2$ in aqueous solution with or without 25% (v/v) ethanol, were put on a microscopic slide (40 µl), and indo-1 fluorescence was determined in the same way as described for cell filaments (see above). The ratio (390–450)/(450–590) was plotted against pCa.

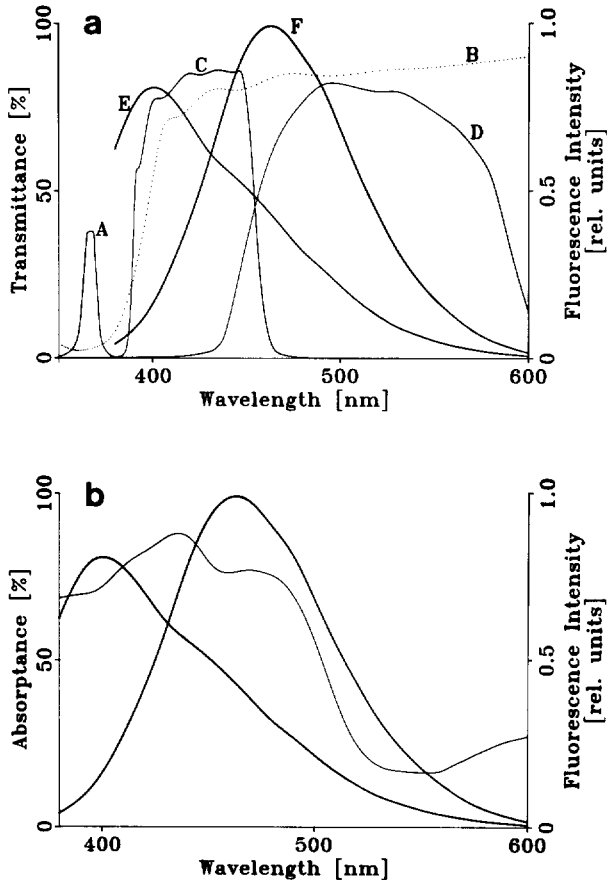

Fig. 3a, b. Spectral characteristics of the microspectrofluorometric equipment used, compared with the absorptance of the *Mougeotia scalaris* cell. **a** Optical properties of the microscope setup, as used under the limitations imposed by the line spectrum of the HBO Hg-lamp, exciting light-source, the transmittance of the NPL-Fluotar objective, and transmittance/reflectance characteristics of the beamsplitter: *A*, excitation filter matching the 365-nm Hg-line; *B*, beamsplitter with half transmittance/reflectance at 395 nm; *C*, barrier-filter I with ½-power bandwidth of 390–450 nm; *D*, barrier-filter II with ½-power bandwidth of 450–590 nm. Transmittance of the latter filter was 0.7% and 0.1% at 620 nm and 630 nm, respectively, with no detectable transmittance at longer wavelengths. Indo-1 fluorescence emission spectra in 25% ethanol are shown for the calcium-saturated *(E)* and the calcium-free *(F)* forms of indo-1 after excitation at 365 nm. **b** Absorptance of a *Mougeotia* cell with chloroplast in face-on position, as compared to the indo-1 emission spectra, shown also in **a** as *E* and *F*

Using a computer program for nonlinear regression, a curve according to:

$$[Ca^{2+}] = K_d \cdot \frac{R - R_{min}}{R_{max} - R} \cdot \frac{S_{f2}}{S_{b2}}$$ (Grynkiewicz et al. 1985)

was fitted to the data (Fig. 5), and the following values were obtained: $R_{min} = 0.237$, $R_{max} = 1.007$, $K_d \cdot (S_{f2}/S_{b2}) = 2.64 \cdot 10^{-6}$ (in the absence of ethanol); $R_{min} = 0.417$, $R_{max} = 1.406$, $K_d \cdot (S_{f2}/S_{b2}) = 2.44 \cdot 10^{-6}$ (in the presence of 25% ethanol).

R_{min} and R_{max} are the fluorescence ratios of indo-1 at Ca^{2+} depletion and at Ca^{2+} saturation, respectively. K_d is the dissociation constant of indo-1. S_{f2} and S_{b2} are the absolute fluorescence values from 450–590 nm under conditions of Ca^{2+} depletion and Ca^{2+} saturation, respectively. Solving the equation for K_d under both conditions results in values of $2.77 \cdot 10^{-7}$ mol $\cdot$ l^{-1} and $2.43 \cdot 10^{-7}$ mol $\cdot$ l^{-1}. Though this is not the method of choice to evaluate the K_d, the obtained values are in good agreement with values given by Grynkiewicz et al. (1985) as $2.5 \cdot 10^{-7}$ mol $\cdot$ l^{-1} and by Popov et al. (1988) as $2.4 \cdot 10^{-7}$ mol $\cdot$ l^{-1}.

Examination of chloroplast movement. Preparation of microscope slides and preorientation of the chloroplasts were basically done as described by Grolig and Wagner (1989). For the experiments of Fig. 7, the chloroplasts were oriented to face-on position by white light from above (10 W $\cdot$ m^{-2} for 30 min), generated by an electric bulb and filtered by KG 1 heat-absorbing glass (2 mm). For the experiments of Figs. 8 and 9, stock solutions of Bay-K8644 (10 mmol $\cdot$ l^{-1}; Bayer, Wuppertal, FRG) and A23187 (1 mmol $\cdot$ l^{-1}; Calbiochem) in ethanol were prepared. Test solutions were prepared freshly every day using growth medium (Stabenau 1978) without EDTA (Sigma), Fe^{2+}, trace elements, vitamins, and buffered with 1 mmol $\cdot$ l^{-1} Hepes, pH 8. In the experiments shown in Fig. 9, EGTA (1 mmol $\cdot$ l^{-1}) was added to the Stabenau growth medium, resulting in a pCa of 9.3; in one experiment of Fig. 9, $[Ca^{2+}]$ was increased from 0.1 mmol $\cdot$ l^{-1} to 1.1 mmol $\cdot$ l^{-1} Ca^{2+} (pCa $\approx$ 4). After the process of chloroplast preorientation in the test solutions, the microscope slides were transferred to an epifluorescence microscope (Ortholux II; Leitz). For the experiment with orange light alone, shown in Fig. 7, irradiation was generated by a halogen bulb, filtered with OG 590 (3 mm) and KG 1 (2 mm) glass filters (Haupt 1970) and applied horizontally to the microscope slides using a light guide (40 W $\cdot$ m^{-2}); observations were made in the light produced by passing the light of the microscope lamp through an infrared glass filter (093; B&W, Wiesbaden, FRG).

For the experiments with UV-A or blue light, shown in Fig. 7, red background light was given simultaneously from the microscope lamp through the condenser and an RG 630 glass filter (3 mm, 20 W $\cdot$ m^{-2}) to establish a tetrapolar P_r/P_{fr} gradient (Haupt 1970; P_r and P_{fr} are the red- and far-red-absorbing forms of phytochrome, respectively) and to saturate photosynthesis (Zurzycki 1955). Ultraviolet-A or blue light was generated by a mercury lamp and the following filter combinations: BG 12 (2 mm) + UG 1 (1 mm; $\lambda_{max} = 373$ nm) for UV-A light and BG 28 (2 mm) + GG 420 (2 mm; $\lambda_{max} = 457$ nm) for blue light. Irradiation was adjusted to 20 W $\cdot$ m^{-2} with neutral-density filters (Balzers, Lichtenstein; fluence of 36 kJ $\cdot$ m^{-2} for 30 min), and applied through the objective. Low-irradiance chloroplast movement, shown in Figs. 8 and 9, was mediated by continuous orange light from the microscope lamp through the condenser together with OG 590 (3 mm) and KG 1 (2 mm) glass filters (1 W $\cdot$ m^{-2}). Chloroplast movement was recorded via a video camera (Grundig, Fürth, FRG) by a video recorder (U-Matic; Sony, Tokyo, Japan) equipped with a time-lapse unit (UPG 10/60; Omtec, Giessen, FRG) and a video-timer (FOR.A, Tokyo, Japan). The videotapes were evaluated by an independent person to determine the number of cells with chloroplasts in their final position. Because of the logarithmical Gaussian distribution of the data in Figs. 7–9, $\bar{X}_G$ indicates the geometric mean of the time span needed to perform full chloroplast movement.

Results and discussion

Methodical aspects of the use of indo-1. As observed for many other plant cells (Bush and Jones 1987), the walled filamentous cells and the protoplasts of *Mougeotia scalaris* also tolerate low pH; incubations for at least 16 h at pH 4 to 5 gave no detectable loss of viability (Grolig and Wagner 1987). Hence, a pH difference between the external medium and cytoplasm in the order of 3.5 units could be used successfully to accumulate indo-1 in the cytoplasm of *Mougeotia*. Fura-2 or fluo-3 (Minta et al. 1989) could not be loaded into *Mougeotia* by this tech-

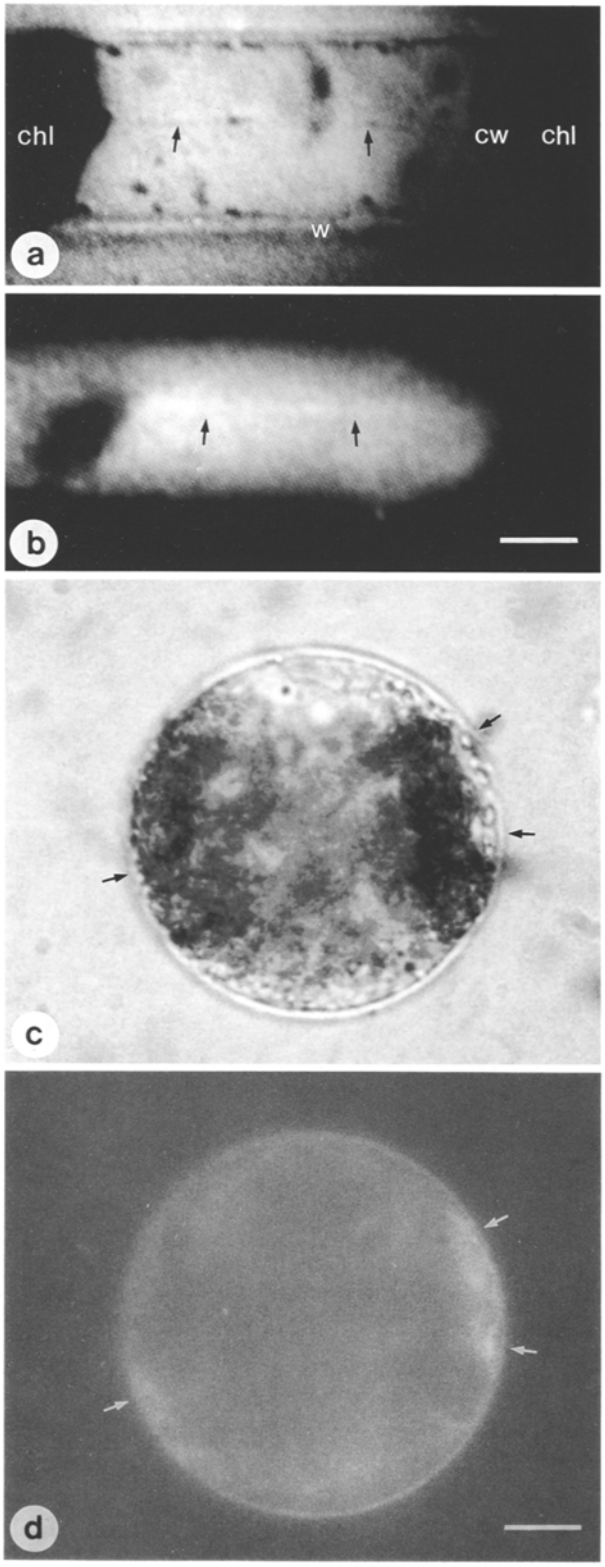

Fig. 4a–d. Micrographs of a *Mougeotia scalaris* filamentous cell **(a, b)**, and of a protoplast **(c, d)**, after loading of indo-1 **(b, d)**. Indo-1-specific fluorescence originates from the cytoplasm, and is most readily seen is strands **(b,** *arrows***)** occasionally running longitudinally through the vacuole in filamentous cells after centrifugation, or in protoplasts, from the large cytoplasmic flaps **(d,** *arrows***)** between plasmalemma and chloroplast. *chl*, chloroplast after centrifugation; *w*, longitudinal cell wall; *cw*, cross wall. Bars indicate 10 μm; × 1000

nique, as also reported by Bush and Jones (1987) for aleurone protoplasts with fura-2.

Figure 4 shows micrographs of a *Mougeotia* filamentous cell (Fig. 4a) and of a *Mougeotia* protoplast (Fig. 4c) after loading with indo-1. Indo-1-specific fluorescence is readily assigned to the cytoplasm when originating from cytoplasmic strands, occasionally stretching through the vacuole in filamentous cells after centrifugation (Fig. 4b), or from the large cytoplasmic flaps between plasmalemma and chloroplast in the spherical protoplasts (Fig. 4d).

For calibration of indo-1-specific fluorescence (see *Material and methods*), direct measurement of calcium in the cytoplasm of a permeabilized cell (internal calibration) is the most adequate method, but has proved difficult in many systems (Cork et al. 1989), and impossible so far for *Mougeotia*. Therefore, as a first approximation, we measured indo-1 fluorescence, in EGTA-buffered Ca^{2+} solutions, according to Grynkiewicz et al. (1985). To minimize the apparent mismatch of this external calibration with respect to the hydrophobicity of the solutions (Popov et al. 1988; Owen and Shuler 1989), the indo-1 signal was measured in the presence of the slightly lipophilic solution of 25% (v/v) ethanol: for the known calcium concentrations, function (4) of Grynkiewicz et al. (1985) was fitted to the measured ratios (Fig. 5), yielding values for further calculation (see *Material and methods*). The calibration in the absence of ethanol is shown for comparison.

Using the calibration curve obtained under mimicked lipophilic conditions of 25% ethanol (Fig. 5), cytoplasmic free $[Ca^{2+}]$ in *Mougeotia* cells was determined to be 0.92 ± 0.29 μmol · l^{-1} (SD; standard condition), during the first monitoring period of 3 s, without centrifugation (see below). Extended measurements resulted in higher values, as the pulses needed to excite indo-1 fluorescence turned out to be a calcium-related photostimulus to *Mougeotia* as well (see below). This measured $[Ca^{2+}]$ is

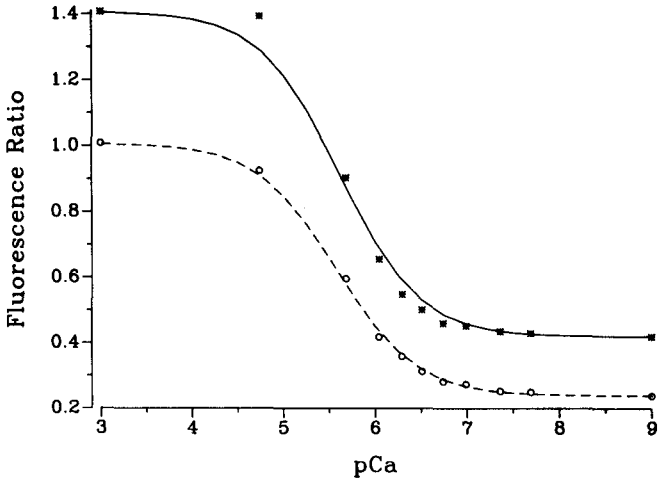

Fig. 5. Fluorescence ratio (390–450 nm/450–590 nm) of indo-1 as a function of pCa in a lipophilic calibration medium of 25% (v/v) ethanol (—). The curve was fitted to the recorded data using nonlinear regression according to function (4) of Grynkiewicz et al. (1985). For comparison, the calibration curve of indo-1 in ethanol-free medium is shown, too (---)

almost an order of magnitude higher than results reported from higher-plant cells, e.g. 112–195nmol · l^{-1} (Felle 1988), 120–361 nmol · l^{-1} (Gilroy et al. 1989) and 250 nmol · l^{-1} (Bush and Jones 1987). In lower-plant cells, the reported values are 360 nmol · l^{-1} in *Physarum* plasmodia (Kuroda et al. 1988), 280 to 2470 nmol · l^{-1} in *Fucus* rhizoids (Brownlee and Wood 1986), 220 nmol · l^{-1} (6.7 μmol · l^{-1} during an action potential) in *Chara* and 1.1 μmol · l^{-1} (43 μmol · l^{-1} during an action potential) in *Nitella* (Williamson and Ashley 1982).

A relatively high cytoplasmic free [Ca^{2+}] seems reasonable for *Mougeotia* because Roberts (1989) has detected a calcium-activated protein kinase here with a K_m of 1.5 μmol · l^{-1}. In the closely related alga *Mesotaenium*, Berkelman and Lagarias (1990) demonstrated that the calcium-transport efficiency of tonoplast-derived tight vesicles was unable to produce free [Ca^{2+}] below 0.4 μmol · l^{-1}.

Although the cytoplasmic free [Ca^{2+}] calculated here seems plausible for *Mougeotia*, the following sources of possible error have to be taken into account: (i) The used technique of signal calibration by means of mimicked lipophilic solutions, to match in vivo conditions, may not reflect adequately the cytoplasmic environment. But even in vivo, calcium-unrelated spectral shifts of indo-1 have been reported (Owen and Shuler 1989). In the same nerve cell, Cork et al. (1989) determined the resting level of cytoplasmic [Ca^{2+}] as 24 nmol · l^{-1} and 416 nmol · l^{-1}, based on the simultaneous use of fura-2 and indo-1. (ii) The measured-volume element within the photomultiplier window consists of more than 90% vacuole and less than 10% cytoplasm. Trace amounts of calcium-saturated indo-1 in the vacuole ($pCa_{vacuole} \approx 3$) cannot be excluded (Zhang et al. 1990) and may have falsified the recordings, especially as the microscope focus was set to the center of the cell cylinder. (iii) Preferential chloroplast transmittance for fluorescence from Ca^{2+}-free versus Ca^{2+}-loaded indo-1 from the half-cell underneath the chloroplast has to be considered (Fig. 3b) as well as chloroplast autofluorescence. Centrifugation of the chloroplast to one half of the cell (Fig. 1), which was used in some of the experiments (see below), did not allow these difficulties to be avoided: upon chloroplast dislocation these cells showed a decrease in fluorescence from 450–590 nm by about 20%, normalized to the fluorescence from 390–450 nm (Table 1). This decrease in fluorescence is neither explainable by the minor change in autofluorescence (Table 1), nor by the small "inner filter" effect attenuating the fluorescence output from the half-cell underneath the chloroplast only (Fig. 3b); also both effects together do not add up to the recorded fluorescence change of 20%. Hence, dislocation of the chloroplast appears to be a calcium-related stimulus, which adds to the calcium-related 365-nm photostimulus (see below). It is of interest to note that the here-evaluated increase of cytoplasmic free calcium upon dislocation of the chloroplast up to about 2 μmol · l^{-1} leaves the cell capable of chloroplast orientational movement (Schönbohm 1987). Independent of the state of chloroplast dislocation, the ratio of Ca^{2+}-loaded versus Ca^{2+}-unloaded indo-1 increased by about 0.015 per measurement (compare Fig. 6). (iv) The time resolution may have been too poor to detect the initial, resting value

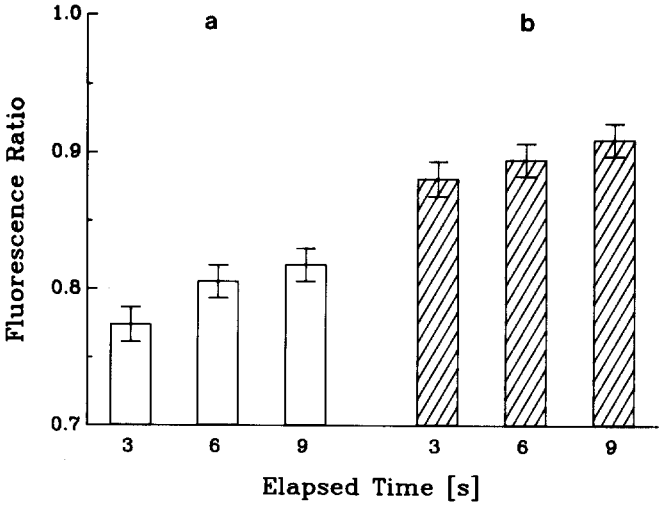

Fig. 6. Ultraviolet-A-light-mediated change in the ratio of indo-1 fluorescence (390–450 nm/450–590 nm) in *Mougeotia* as a function of time and of external [Ca^{2+}]. Shown are the data obtained by three consecutive, paired measurements within 9 s; the elapsed time shows the end of each paired measurement under the conditions of $[Ca^{2+}]_{external}$ = 10 nmol · l^{-1} (**a**) and $[Ca^{2+}]_{external}$ = 1 mmol · l^{-1} (**b**), respectively. During the period of 9 s, the blue-light-mediated *increase* in cytoplasmic [Ca^{2+}] was found to be independent of the applied external pCa (**a** versus **b**), although the cytoplasmic calcium *level* significantly depended on $[Ca^{2+}]_{external}$ ($P < 0.0001$, $n = 40$; bars show ± SE). Autofluorescence contributed close to 20% of the fluorescence signal (Table 1), and no significant shift was detected within the time span of measurements

Table 1. Total fluorescence in indo-1-loaded and autofluorescence in indo-1-unloaded *Mougeotia* cells with and without dislocated chloroplast. An average of 30 photomultiplier readings each in the wavelength ranges 390–450 nm and 450–590 nm was used for ratio determination (R). Indo-1-specific fluorescence was calculated as the difference of total fluorescence minus autofluorescence (= Δ fluorescence). The chlorophyll fluorescence, avoided by dislocation of the chloroplast, contributes only about 10% to the dislocation-mediated change in fluorescence ratio ΔR

	Cells with dislocated chloroplast			Cells with chloroplast in face-on position			ΔR
	390–450 nm	450–590 nm	R	390–450 nm	450–590 nm	R	
Total fluorescence	854	1084	0.788	882	1319	0.669	0.119
Autofluorescence	214	211	1.014	220	261	0.843	–
Δ fluorescence	640	873	0.733	662	1058	0.626	0.107

of cytoplasmic free calcium in *Mougeotia* cells, since the Ca^{2+} level rose rapidly upon irradiation with the 365-nm light used for fluorescence excitation of indo-1 (Fig. 6).

On the whole, although the signal of indo-1 could not be calibrated exactly (compare Zhang et al. 1990), the signal ratio was useful for monitoring relative changes of pCa upon various treatments of the cell. The highly significant ($P < 0.0001$) difference in cytoplasmic pCa, as seen 10 min after incubation of *Mougeotia* filaments in media of pCa 3 versus pCa 8 (Fig. 6), certainly was not due to changes of vacuolar pCa because indo-1 is insensitive in detecting differences in $[Ca^{2+}]$ above pCa 4.5 (Fig. 5). Therefore, the difference most probably results from changes in cytoplasmic $[Ca^{2+}]$ in *Mougeotia*.

Physiological aspects. Pulsed irradiation of *Mougeotia* cells with light of 365 nm (=UV-A), as used for excitation of indo-1 fluorescence, caused a fast increase of cytoplasmic free $[Ca^{2+}]$ in *M. scalaris* within less than 1 s (Fig. 6), and was independent of the state of chloroplast centrifugation (not shown) or the external $[Ca^{2+}]$ used (Fig. 6). These observations indicate that a photosynthesis-independent release of calcium from intracellular stores may be occurring, perhaps involving the calcium-binding vesicles of *Mougeotia* (Grolig and Wagner 1987, 1989; Russ et al. 1988). Light of 365 nm turned out to be at least twice as effective as 450-nm light in raising the cytoplasmic free Ca^{2+} of cells with centrifuged chloroplasts under comparable conditions. The efficiency of red-light here was beyond the limit of detection, although red/far-red reversible fluorescence changes of chlorotetracycline-stained calcium-binding vesicles are reported (Wagner et al. 1987).

The question arises, whether and how, the observed light-induced rise in cytoplasmic free calcium may be related to chloroplast movement. Not only has the vectorial feature of chloroplast movement, *orientation* with respect to light, been reported to depend on light conditions but so has the scalar feature, *velocity* of movement (Haupt 1982). In accordance with Schönbohm (1987), we observed that blue-light-mediated movement is faster than that mediated by red light, and that UV–A-mediated movement is fastest (Fig. 7). The slope of the phytochrome gradient (Haupt and Trump 1975) and-or the cellular state of energy (Schönbohm 1987), which are of measurable influence on the velocity of chloroplast movement, appear not to be a major factor here, as an identical irradiance of red-light was used as background light for the 365-nm, the 450-nm and the 660-nm response in order to establish a tetrapolar P_r/P_{fr} gradient

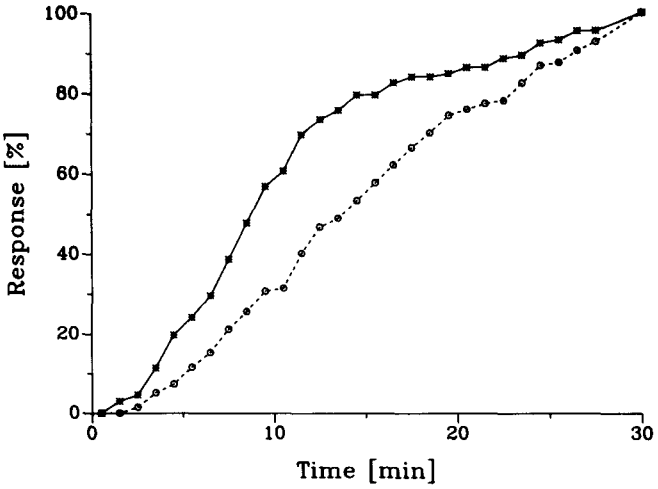

Fig. 8. Kinetics of low-irradiance chloroplast movement in *Mougeotia* in the presence (x—x, $\bar{X}_G = 8.1$ min, $n = 127$) and absence (○---○, $\bar{X}_G = 12.2$ min, $n = 126$) of 1 μmol · l^{-1} Bay-K8644. Difference in $\bar{X}_G$ is highly significant ($P < 0.0001$)

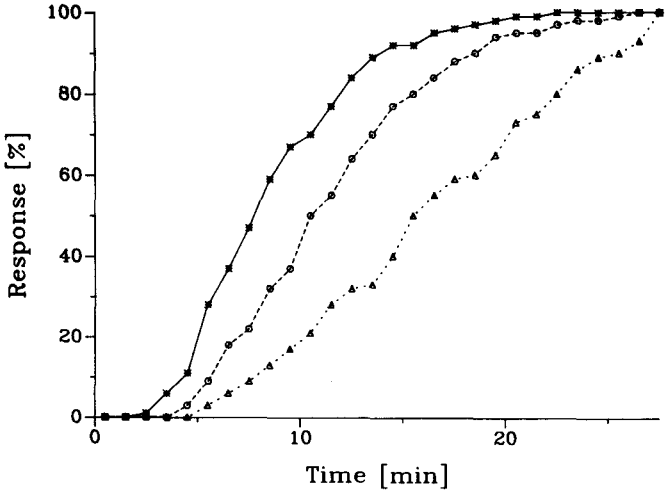

Fig. 7. Kinetics of UV–A-light-mediated (x—x, $\bar{X}_G = 8.2$ min, $n = 107$), blue-light-mediated (○---○, $\bar{X}_G = 11.0$ min, $n = 114$) and red-light-mediated (△....△, $\bar{X}_G = 15.4$ min, $n = 97$) chloroplast movement in *Mougeotia*. In UV–A, blue and red light, red background light was given additionally, in order to saturate the P_r/P_{fr} gradient and photosynthesis (see *Material and methods*). Differences in $\bar{X}_G$ are highly significant ($P < 0.0001$)

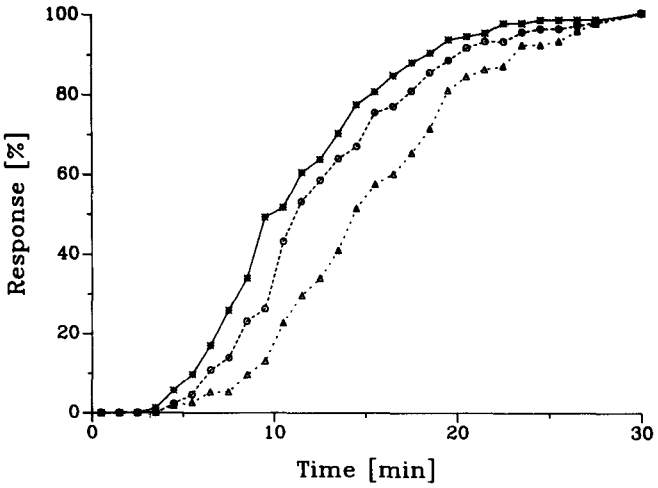

Fig. 9. Kinetics of low-irradiance chloroplast movement in *Mougeotia* in the presence of 1 μmol · l^{-1} A23187 at pCa 4 (x—x, $\bar{X}_G = 10.6$ min, $n = 115$) or pCa 9.3 (○---○, $\bar{X}_G = 12.2$ min, $n = 130$), and in the absence of A23187 at pCa 9.3 (△...△, $\bar{X}_G = 14.7$ min, $n = 124$). Difference in $\bar{X}_G$ is significant for A23187 at pCa 4 versus pCa 9.3 ($P < 0.02$). Difference in $\bar{X}_G$ is highly significant for the presence and absence of A23187 at pCa 9.3 ($P < 0.001$)

(Haupt 1970, 1987) and to saturate photosynthesis (Zurzycki 1955). Thus, the UV–A- and the blue-light-mediated increases in the velocity of chloroplast movement may reflect a rise of cytoplasmic free calcium.

Consistent with this conclusion, use of the calcium-channel drug Bay-K8644, at a concentration suitable for it to act as a channel-agonist (1 $\mu mol \cdot l^{-1}$; Tretyn et al. 1990), increased the velocity of low-irradiance chloroplast movement (Fig. 8). The calcium ionophore A23187 had the same effect as Bay-K8644 (Fig. 9). Interestingly, chloroplast velocity increased in the presence of A23187 even under conditions of low extracellular calcium (pCa 9.3). Hence the drug is presumed to release more Ca^{2+} from intracellular stores, probably free calcium mainly from the vacuole, into the cytoplasm than from the cytoplasm to the outer medium. A similar intracellular effect of A23187 has been shown for *Tradescantia* (Dorée and Picard 1980) and onion epidermial cells (Quader 1990).

The rise of $[Ca^{2+}]_{free}$ in the cytoplasm after treatment with 365-nm light or 450-nm light at fluences comparable to those needed for high-irradiance movement in continuous UV–A light or blue light (see *Material and methods*) is of particular interest with respect to reported changes in the mechanical properties of the cytoplasm under the same light conditions: Schönbohm (1987) has shown an immediate decrease of chloroplast anchorage in high-irradiance blue light, in contrast to a slow increase of chloroplast anchorage after red-light irradiation. Weisenseel (1968) has shown, that *Mougeotia* cells at low temperature are still able to carry out reorientational movement in blue light, but not in red light. The difference is thought by Schönbohm and Hellwig (1979) and Kraml et al. (1988) to reflect a difference in light-mediated cytoplasmic viscosity. Kraml et al. (1988) reported a considerable increase in the velocity of phytochrome-mediated chloroplast movement in the closely related alga *Mesotaenium* when blue light was provided in addition to red-light. The light-mediated difference in cytoplasmic viscosity may reflect cytoskeletal changes.

Microtubules in various systems are known to depolymerize after a rise in the cytoplasmic $[Ca^{2+}]$ (Zhang et al. 1990). In *Mougeotia* interphase cells, the microtubules are located just beneath and in close contact with the plasmalemma (Foos 1970; Galway and Hardham 1986). Microtubules adhering to plasma-membrane ghosts of *Mougeotia* protoplasts have been shown to be very stable and to depolymerize upon raising the $[Ca^{2+}]$ only after pretreatment with Triton X–100 (Kakimoto and Shibaoka 1986). On the other hand, in *Mougeotia* depolymerization of microtubules upon application of specific drugs speeds up red-light-mediated chloroplast movement from a response time of more than 20 min to less than 10 min (Serlin and Ferrell 1989).

With respect to the details mentioned above, we suggest that a physically or chemically induced rise in the free cytoplasmic $[Ca^{2+}]$, e.g. upon light-activation of the calcium-binding vesicles, may cause a possibly calmodulin-mediated (Wagner et al. 1984) microtubule depolymerization and thus a decrease of cytoplasmic viscosity in *Mougeotia*.

We are indebted to Dr. G. Seibold and Dipl. Phys. H. Weintraut for their advice on the technique of microspectrofluorometry and for allowing access to the microspectrophotometric facilities in the Strahlenzentrum der Justus-Liebig-Universität, Giessen, FRG. We thank Mrs. A. Quanz for reliable culture of the algae and evaluation of the videotapes. Bay-K8644 was a generous gift of Bayer AG, Wuppertal, FRG. U. Russ was supported by a scholarship according to the Hessisches Graduierten Förderungsgesetz. This work was supported by the Deutsche Forschungsgemeinschaft.

References

Berkelman, T., Lagarias, J.C. (1990) Calcium transport in the green alga *Mesotaenium caldariorum* – Preliminary characterization and subcellular distribution. Plant Physiol. **93,** 748–757

Brownlee, C., Wood, J.W. (1986) A gradient of cytoplasmic free calcium in growing rhizoid cells of *Fucus serratus*. Nature **320,** 624–626

Bush, D.S., Jones, R.L. (1987) Measurement of cytoplasmic calcium in aleurone protoplasts using Indo-1 and Fura-2. Cell Calcium **8,** 455–472

Bush, D.S., Jones, R.L. (1990) Measuring intracellular Ca^{2+} levels in plant cells using the fluorescent probes, Indo-1 and Fura-2 – progress and prospects. Plant Physiol. **93,** 841–845

Cork, R.J., Strautman, A.F., Robinson, K.R. (1989) Measuring cytoplasmic calcium: a review of three methods with emphasis on the practical aspects of their use. Biol. Bull. **176,** Suppl., 25–30

Dorée, M., Picard, A. (1980) Release of Ca^{2+} from intracellular pools stops cytoplasmic streaming in *Tradescantia* staminal hairs. Experientia **36,** 1291–1292

Felle, H. (1988) Cytoplasmic free calcium in *Riccia fluitans* L. and *Zea mays* L.: Interaction of Ca^{2+} and pH? Planta **176,** 248–255

Felle, H. (1989) Ca^{2+}-selective microelectrodes and their application to plant cells and tissues. Plant Physiol. **91,** 1239–1242

Foos, K. (1970) Mikrotubuli bei *Mougeotia* spec. Z. Pflanzenphysiol. **62,** 201–203

Galway, M.E., Hardham, A.R. (1986) Microtubule reorganization, cell wall synthesis and establishment of the axis of elongation in regenerating protoplasts of the alga *Mougeotia*. Protoplasma **135,** 130–143

Gilroy, S., Hughes, W.A., Trewavas, A.J. (1989) A comparison between Quin-2 and Aequorin as indicators of cytoplasmic calcium levels in higher plant cell protoplasts. Plant Physiol. **90,** 482–491

Grolig, F., Wagner, G. (1987) Vital staining permits isolation of calcium vesicles from the green alga *Mougeotia*. Planta **171,** 433–437

Grolig, F., Wagner, G. (1988) Light-dependent chloroplast reorientation in *Mougeotia* and *Mesotaenium:* Biased by pigment-regulated plasmalemma anchorage sites to actin filaments? Bot. Acta **101,** 2–6

Grolig, F., Wagner, G. (1989) Characterization of the isolated calcium-binding vesicles from the green alga *Mougeotia scalaris,* and their relevance to chloroplast movement. Planta **177,** 169–177

Grynkiewicz, G., Poenie, M., Tsien, R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. **260,** 3440–3450

Haupt, W. (1970) Hellrot- und Dunkelrot-Wechselwirkungen bei der Chloroplastendrehung von *Mougeotia*. Wiss. Z. Ernst-Moritz-Arndt-Univ. Greifswald, Math. Naturwiss. Reihe **19,** 47–54

Haupt, W. (1982) Light-mediated movement of chloroplasts. Annu. Rev. Plant Physiol. **33,** 205–233

Haupt, W. (1987) Phytochrome control of intracellular movement. In: Phytochrome and photoregulation in plants, pp. 225–237, Furuya, M., ed. Academic Press, London

Haupt, W., Trump, K. (1975) Licht-orientierte Chloroplasten-

bewegung bei *Mougeotia*: Die Größe des Phytochromgradienten steuert die Bewegungsgeschwindigkeit. Biochem. Physiol. Pflanz. **168,** 131–140

Hepler, P.K., Wayne, R.O. (1985) Calcium and plant development. Annu. Rev. Plant Physiol. **36,** 397–439

Kakimoto, T., Shibaoka, H. (1986) Calcium-sensitivity of cortical microtubules in the green alga *Mougeotia*. Plant Cell Physiol. **27,** 91–101

Kauss, H. (1987) Some aspects of calcium-dependent regulation in plant metabolism. Annu. Rev. Plant Physiol. **38,** 47–72

Kraml, M., Büttner, G., Haupt, W., Herrmann, H. (1988) Chloroplast orientation in *Mesotaenium:* the phytochrome effect is strongly potentiated by interaction with blue light. Protoplasma Suppl. 1, 172–179

Kuroda, R., Hatano, S., Hiramoto, Y., Kuroda, H. (1988) Change of cytosolic Ca-ion concentration in the contraction and relaxation cycle of *Physarum* microplasmodia. Protoplasma Suppl. 1, 72–80

Martell, A.E., Smith, R.M. (1974) Critical stability constants. Plenum Press, New York

Minta, A., Kao, J.P.Y., Tsien, R.Y. (1989) Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromatophores. J. Biol. Chem. **264,** 8171–8178

Neuscheler-Wirth, H. (1970) Wachstumsgeschwindigkeit und Wachstumsrhythmik bei *Mougeotia*. Z. Pflanzenphysiol. **63,** 352–369

Owen, C.S., Shuler, R.L. (1989) Spectral evidence for non-calcium interactions of intracellular Indo-1. Biochem. Biophys. Res. Commun. **163,** 328–333

Popov, E.G., Gavrilov, I.Y., Pozin, E.Y., Gabbasov, Z.A. (1988) Multiwavelength method for measuring concentration of free cytosolic calcium using the fluorescent probe indo-1. Arch. Biochem. Biophys. **261,** 91–96

Quader, H. (1990) Formation and disintegration of cisternae of the endoplasmic reticulum visualised in live cells by conventional fluorescence and confocal laser scanning microscopy: evidence for the involvement of calcium and the cytoskeleton. Protoplasma **155,** 166–175

Roberts, D.M. (1989) Detection of a calcium-activated protein kinase in *Mougeotia* by using synthetic peptide substrates. Plant Physiol. **91,** 1613–1619

Russ, U., Grolig, F., Wagner, G. (1988) Differentially absorbed vital dyes inhibit chloroplast movement in *Mougeotia scalaris*. Protoplasma Suppl. 1, 180–184

Schönbohm, E. (1987) Movement of *Mougeotia* chloroplasts under continuous weak and strong light. Acta Physiol. Plant **9,** 109–135

Schönbohm, E., Hellwig, H. (1979) Zum Photorezeptorproblem der Schwachlichtbewegung des *Mougeotia*-Chloroplasten im Blau bzw. Hellrot bei niedrigen Temperaturen. Ber. Dtsch. Bot. Ges. **92,** 749–762

Serlin, B.S., Ferrell, S. (1989) The involvement of microtubules in chloroplast rotation in the alga *Mougeotia*. Plant Sci. **60,** 1–8

Stabenau, H. (1978) Wachstum von *Mougeotia* in der Durchlüftungskultur. Ber. Dtsch. Bot. Ges. **91,** 251–255

Stern, M.D., Spurgeon, H.A., Hansford, R., Lakatta, E.G., Capogrossi, M.C. (1989) Optimum spectral windows to minimize quantum noise of ratiometric intracellular fluorescent probes. Cell Calcium **10,** 527–534

Tretyn, A., Kendrick, R.E., Bossen, M.E. (1990) The effect of a calcium-channel antagonist, nifedipin and agonist, Bay K-8644, on the phytochrome-controlled swelling of etiolated wheat protoplasts. Physiol. Plant. **78,** 230–235

Wagner, G., Grolig, F., Altmüller, D. (1987) Transduction chain of low irradiance response of chloroplast reorientation in *Mougeotia* in blue or red light. Photobiochem. Photobiophys. Suppl., 183–189

Wagner, G., Valentin, P., Dieter, P., Marmé, D. (1984) Identification of calmodulin in the green alga *Mougeotia* and its possible function in chloroplast reorientational movement. Planta **162,** 62–67

Weisenseel, M. (1968) Vergleichende Untersuchungen zum Einfluß der Temperatur auf lichtinduzierte Chloroplastenverlagerungen. Z. Pflanzenpysiol. **59,** 56–69 and 153–171

Williamson, R.E. (1984) Calcium and the plant cytoskeleton. Plant Cell Environ. **7,** 431–440

Williamson, R.E., Ashley, C.C. (1982) Free Ca^{2+} and cytoplasmic streaming in the alga *Chara*. Nature **296,** 647–651

Zhang, D.H., Callaham, D.A., Hepler, P.K. (1990) Regulation of anaphase chromosome motion in *Tradescantia* stamen hair cells by calcium and related signaling agents. J. Cell Biol. **111,** 171–182

Zurzycki, J. (1955) Chloroplast arrangement as a factor in photosynthesis. Acta Soc. Bot. Pol. **24,** 27–63