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Voltage-Regulated Short-Term Activation of Adenylyl Cyclase Effected by Phytochrome in the Lichen *Evernia prunastri* (L.) ACH.

By

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With 6 Figures

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Summary

VICENTE C., SEGOVIA M. & SOLAS M. T. 1999. Voltage-regulated short-term activation of adenylyl cyclase effected by phytochrome in the lichen *Evernia prunastri* (L.) ACH. – *Phyton* (Horn, Austria) 39 (2): 225–238, with 6 figures. – English with German summary.

Red irradiation of the tip of a thallus strip from the lichen *Evernia prunastri* increases adenylyl cyclase activity within 5 s of lighting as well as enhances K⁺ efflux that is not balanced by H⁺, Na⁺, or Ca²⁺ influxes. Both effects are reversed by far-red following red light and seem to be simultaneous, but not related consequences of P_r photoconversion to P_{fr} in the algal membrane. Signal induced by red light is translocated from the tip to the dark-maintained bottom of a lichen strip, although this translocation requires at least 15 s to be related to adenylyl cyclase activation. Transduction is related to the decrease of the electronegativity of the voltage difference between the tip and the bottom of lichen strips. A supply of exogenous GTP to thallus samples does not activate adenylyl cyclase for time periods lower than 1 min.

Zusammenfassung

VICENTE C., SEGOVIA M. & SOLAS M. T. 1999. Die spannungsregulierte Kurzzeitaktivierung der Adenylyl-Cyclase wird in der Flechte *Evernia prunastri* (L.) ACH.

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durch Phytochrom verursacht. – *Phyton* (Horn, Austria) 39 (2): 225–238, 6 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Die Bestrahlung der Spitze eines Thallusstreifens der Flechte *Evernia prunastri* mit Rotlicht läßt die Adenylyl-Cyclase-Aktivität innerhalb von 5-Sekunden-Beleuchtungsdauer ansteigen, gleichzeitig vergrößert sich auch der K^+ Efflux, welcher nicht durch H^+ , Na^+ oder Ca^{2+} Influx ausgeglichen wird. Beide Effekte können durch ein dem Rotlicht nachfolgendes Infrarot rückgängig gemacht werden und scheinen zwar gleichzeitig, jedoch nicht eine unmittelbare Folge der P_r - P_{fr} -Photokonversion in den Algenmembranen zu sein. Das Signal, welches vom Rotlicht induziert wurde, wird von der Spitze zum dunkelgehaltenen Grund des Flechtenstreifens weitergeleitet, wenn auch diese Translokation mindestens 15 Sekunden erfordert, um auf die Adenylyl-Cyclase-Aktivierung zurückgeführt werden zu können. Die Transduktion wird auf eine Abnahme in der Elektronegativität in den Spannungsdifferenzen zwischen der Spitze und dem Grund des Streifens zurückgeführt. Eine Zugabe von externen GTP zu den Thallusproben aktiviert nicht die Adenylyl-Cyclase über Zeiträume kürzer als eine Minute.

Introduction

Signal transduction in plants, effected by phytochrome, has sometimes been related to the activation of G-proteins, the occurrence of which has been reported for many plant species (BLUM & al. 1988, DOBRÁK & al. 1988, HASUNUMA & TAKIMOTO 1989, MILLNER 1987). In etiolated *Avena* seedlings, the binding of [^{35}S]GTP γ S to G-proteins was stimulated by 21 % in plants that were irradiated for 5 min with red light and negated by FR light (ROMERO & al. 1991a). Activation of G-proteins induced by P_{fr} seems to be related to protein phosphorylation in etiolated *Avena* seedlings (ROMERO & al. 1991b) or to the induction of stomatal opening in *C. communis* (LEE & al. 1993). Stomatal opening mediated by blue light was also induced by Sp-cAMPS in parallel to a decrease in cytosolic calcium concentration in guard cell protoplasts within 1–3 min (CURVETTO & al. 1994, MORSUCI & al. 1992). Moreover, activation of adenylyl cyclase seemed to be a consequence of G-protein activation. GTP γ S stimulated adenylyl cyclase in green flagellate *Chlorogonium elongatum* (GROMES & ZETSCHKE 1992).

Phytochrome in lichens regulates the opening of organic anion channels in the membrane of the algal partner as well as the activation of algal adenylyl cyclase (AVALOS & VICENTE 1987, SEGOVIA & VICENTE 1994). These actions are performed after photoconversion of P_r to P_{fr} . A small amount of phytochrome as P_{fr} form possibly binds to the plasma membrane (LAMPARTER & al. 1992, SPETH & al. 1986), although a substantial part of this P_{fr} remains in the cytoplasm where it binds to ubiquitin (MCCURDY & PRATT 1986, SINESHCHEKOV & al. 1994). Since both adenylyl cyclase regulation and the opening of ion channels might be related to G proteins, this possibility has been investigated in the present study by using the lichen *Evernia prunastri* as biological material.

Abbreviations: AMP-PNP, adenylyl imidodiphosphate; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; FR, far-red light; GTP γ S, guanosine 5'-O(3-thiotriphosphate); HPLC, high-performance liquid chromatography; P_r, the red light absorbing form of phytochrome; P_{fr}, the far-red light-absorbing form of phytochrome; PVP, polyvinylpyrrolidone; R, red light Sp-AMPS, Adenosine-3,5-monophosphorothioate, Sp isomer; Tris-HCl, tris (hydroxymethyl) aminomethane hydrochloride.

Material and Methods

Plant material and irradiation conditions

Strips of lichen thalli, of about 7.0 cm in length, were rehydrated over-night in a water vapour-saturated atmosphere at 24 °C in the dark and then immersed for 5 min in distilled water before use. Lichen strips were completely covered with aluminum sheet (Fig. 1C) or, alternatively, only in 2/3 of their length (Fig. 1D), leaving the rest open to the air. This open part was irradiated for 30 s with a red laser (3M, Saint Paul, MN), power output <5 mW, λ_{max} 680 nm, with a photon flux rate of 250 μmol

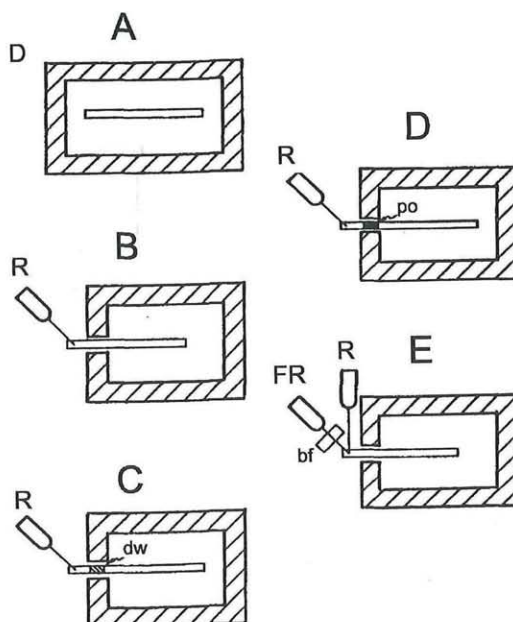


Fig. 1. For measuring adenylyl cyclase activity, lichen strips were maintained in the dark (A) or, alternatively, treated by irradiating the tip of a strip with red light (B) for 30 s, connecting the tip with the bottom with a drop of distilled water (dw, C) or paraffin oil (po, D). Finally, an intact strip was firstly irradiated with red light for 30 s, followed by 30 s with far-red light (E) obtained by filtering red light through a blue filter (bf).

$\text{m}^{-2} \text{s}^{-1}$ at the surface of the plant. Possible changes of temperature of the irradiated surface were recorded by using a Digitron thermo-couple. Alternatively, each strip was cut in two parts and connected with a drop of distilled water or a drop of paraffin oil before irradiation. Then, the irradiated zone of the thallus was immediately separated from that kept in the dark, washed with acetone for 1 min at room temperature to remove cortical phenols, and macerated with 1.0 ml 50 mM Tris-HCl buffer, pH 7.8, to prepare cell-free extracts. Moreover, thallus strips were irradiated for 30s with red laser and immediately with far-red light ($250 \mu\text{mol m}^{-2} \text{s}^{-1}$ at the level of plants) for 30s. The samples were processed as above and assayed for adenylyl cyclase activity.

To measure spectral changes in vivo conditions, small pieces of thallus (about 2.0 cm^2) were placed in the corresponding chambers of a diffuse reflectance accessory in a dualbeam Varian DMS 90 spectrophotometer in such a manner that the beam was firstly received on the upper cortex. The chlorophyll density of the samples used in these analyses was $6.3 \mu\text{g cm}^{-2}$.

Assay of adenylyl cyclase activity

Adenylyl cyclase activity was measured in reaction mixtures containing 2.0 mM ATP, 5.0 mM Mg^{2+} , 50 mM Tris-HCl, pH 7.8, 1.0 mM EDTA and 0.1 mg protein in a final volume of 2.0 ml. Reaction was carried out at 30°C for 30 min and stopped by adding 2.0 ml cold acetonitrile (VICENTE & MATEOS 1992). Protein was measured by the Folin phenol reaction (LOWRY & al. 1951). Protein precipitate was removed by centrifuging the mixture at $38,000 \times g$ for 30 min at 2°C and the supernatant was dried in air flow. Residue was redissolved in 2.0 ml distilled water, filtered through a column ($8 \text{ cm} \times 1 \text{ cm}$) of active neutral alumina and eluted with distilled water (SPITERI & al. 1989). Fractions showing absorbance at 260 nm were collected and lyophilized. Residue was redissolved in 2.0 ml methanol (HPLC grade), filtered and used to quantify cyclic AMP. Guanylyl cyclase activity was measured in the same way but 2.0 mM ATP was substituted by 2.0 mM GTP in the reaction mixtures. GTPase assay was carried out in 1.0 ml reaction mixtures containing 20 mM TrisHCl buffer, pH 7.5, 1.0 mM EDTA, 1.0 mM DTT, 5.0 mM MgCl_2 , 0.8 M NaCl, 3.0 mM L- α -dimyristoylphosphatidyl choline, and 0.2 mM GTP for 10 min at 30°C (SONG & al. 1996).

Samples were chromatographed by using a Varian 5000 liquid chromatograph equipped with a Vista CDS 401 computer. Chromatographic conditions were as follows: reverse phase column ($300 \text{ mm} \times 4 \text{ mm i.d.}$) packed with MicroPak MCH-10; loading, 10 μl ; mobile phase, methanol:acetic acid:water (80:0.5:19.5 v/v) isocratically; pressure, 88 bars; temperature, 20°C ; flow rate, 1.0 ml min^{-1} ; detector, UV set at 254 nm; internal standard, 0.1 mg ml^{-1} AMP. Retention time for cyclic AMP was 2.38 min (VICENTE & MATEOS 1992).

Cytochemical localization of adenylyl cyclase

Cytochemical localization of adenylyl cyclase activity was performed according to AL-AZZAWI & HALL 1976. Segments of lichen thalli, of about 25 mm^2 in area, were fixed in ice-cold 1 % glutaraldehyde in 50 mM sodium cacodylate-nitrate buffer, pH 7.4, for 2h. After fixation, the segments were washed thoroughly for 6h before staining for adenylyl cyclase activity at 30°C for 60 min. The staining medium consisted of 80 mM Tris-maleate buffer, pH 7.4, containing 8 % glucose (w/v), 4 mM $\text{Pb}(\text{NO}_3)_2$,

2 mM MgSO_4 and 0.5 mM adenylyl-imidodiphosphate (AMP-PNP). At the end of the incubation period, the segments were washed briefly before post-fixation in 1 % OsO_4 buffered at pH 7.4. The material was dehydrated in ethanol and embedded in Epon/Araldite. Thin sections were cut with a LKB Ultratome I and examined in a electron microscope Carl Zeiss EM 902 (Germany) at 80 kV.

Measurement of ion fluxes

Ion fluxes were measured from lichen strips to a total volume of 60 ml distilled water, with continuous stirring, by using a Metrohm Ionometer with selective liquid membrane electrodes for K^+ and Ca^{2+} , selective glass membrane electrode for Na^+ and a combined pH glass electrode for H^+ . An Ag/AgCl electrode was used as reference and the temperature was recorded with a probe resistance thermometer during experimentation. Changes of voltage difference between two different zones of an individual strip were measured with surface-contact electrodes (FROMM & SPANSWICK 1993) by using a Cole Palmer 26833-00 Digit Multimeter.

Results

As shown in Fig. 2A, FR-irradiated thalli had a sharp peak of absorbance at 667 nm which became broad at the same wavelength after R exposure. Simultaneously, a net increase in the absorbance at 731 nm was observed. The difference spectrum, FR-R, showed a net peak at 675 nm and a small but clear minimum at 727 nm (Fig. 2B).

Adenylyl cyclase activity was not detected (data are not shown) in thalli rehydrated in the dark. However, low activity values were obtained

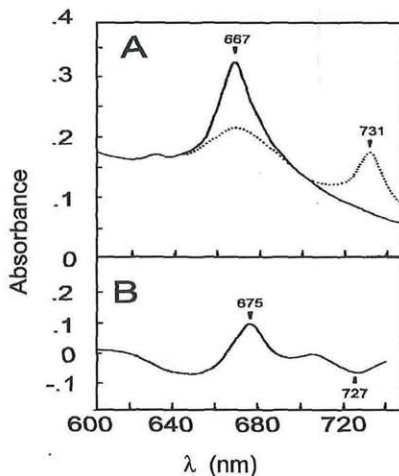


Fig. 2. Absorption and difference spectra of intact thalli of *Evernia prunastri*. Absorption spectra (A) were measured after far-red light (continuous line) and red light (dashed line). The difference spectrum (B) was automatically recorded by subtracting the spectrum after R from that after FR.

after 5s red irradiation (Fig. 3), though this activity was only restricted to the thallus zone which directly received red light. After 30s red irradiation, enzyme activity was detected throughout the complete strip, though the value obtained for irradiated zone is twice that found for the dark one (Fig. 3). When a strip of lichen thallus was cut in two pieces and both connected by a drop of distilled water before irradiation, adenylyl cyclase activity in the dark zone was 1.25 times higher than that found for the irradiated part of the thallus whereas a drop of paraffin oil connecting both pieces impeded the appearance of enzyme activity in the zone kept in the dark (Fig. 3). Activation of adenylyl cyclase after 30s red irradiation was reversed by 30s far-red light following red treatment (inset in Fig. 3). Surface temperature of the irradiated zone remained almost constant at 22.6°C during all the treatments. Electric potential established between the irradiated and dark zones of one thallus strip was estimated by applying on those zones the external electrodes of a voltmeter. Voltage difference between two points in a lichen strip, one of them irradiated and another remaining in the dark, became less electronegative after red irradiation but it reached values near the initial one after far-red irradiation (Fig. 4). Similar changes were recorded when light treatments were applied

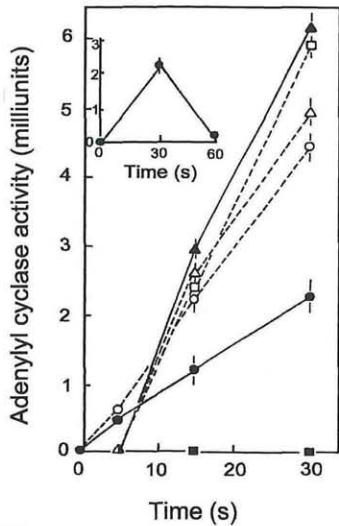


Fig. 3 Time-course of adenylyl cyclase activity obtained from the tips (filled symbols) and their distal zones (empty symbols) of thallus strips irradiated with red light at the tip, using intact strips (circles), cuts strips connected with a drop of distilled water (triangles) or paraffin oil (squares). The inset shows adenylyl cyclase activity of an intact strip irradiated with far-red light for 30s following 30 s of red light. Values are the mean of three replicates. Vertical bars give standard error where larger than the symbols.

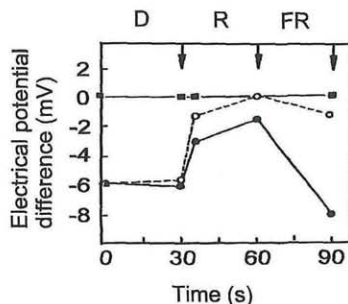
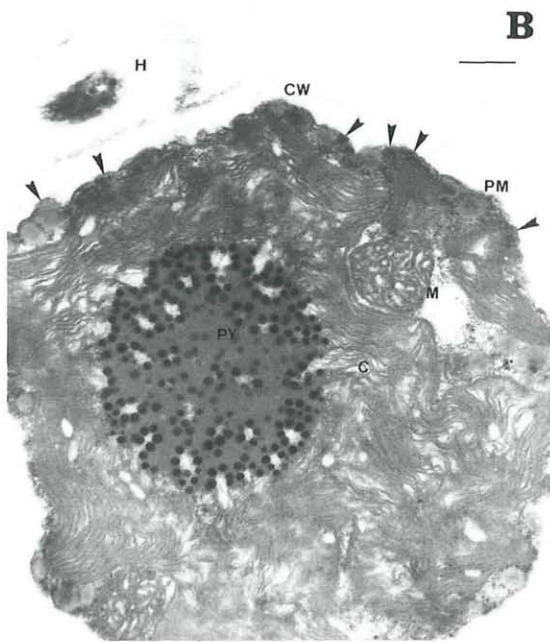
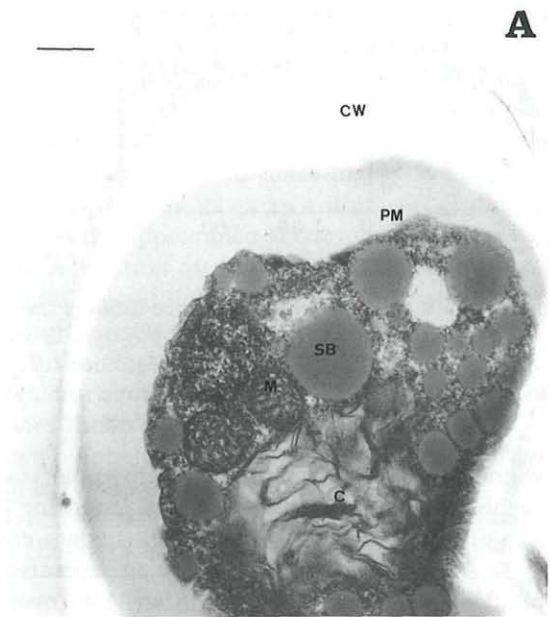


Fig. 4. Measurement of the electric potential difference between the tip and the bottom by using surface contact electrodes as effected by R light or R+FR light treatments. Changes of electric potential difference were recorded for an intact lichen strip (●) a broken strip connecting the tip with the bottom with a drop of distilled water (○) or a drop of paraffin oil (■).

on a cut strip connected with water, although hyperpolarization after FR treatment was achieved in less extent than that found for intact thallus strips. No voltage difference was found for thallus zones connected with paraffin oil (Fig. 4). A supply of $0.4 \mu\text{mol}$ GTP to a rehydrated thallus strip did not activate adenylyl cyclase in the dark, at 24°C , over 1 min treatment, even when strips were previously permeabilized with 2 % (v/v) isopropanol. Moreover, neither GDP nor cyclic GMP were detected in thallus extracts obtained after dark or light treatments for 30 s (data are not shown), in spite of the very sensitive method of analysis used here (PEDROSA & al. 1992). Thus, GTPase and guanylyl cyclase activities can be discarded as a part of reactions occurring during a 30s period of irradiation.

Location of adenylyl cyclase is shown in Fig. 5. The PNP liberated as a result of enzyme activity on the substrate AMP-PNP was precipitated by lead ions as electron-dense deposits at its production sites. No deposits were found in cells without AMP-PNP during the staining period (Fig. 5A). Deposits were found associated with the plasma membrane of the algal partner (Fig. 5B and 5C). A slow efflux of K^+ from the lichen strip was found in the dark (Fig. 6A). This efflux was significantly enhanced by red light and delayed by far-red light (Fig. 6A). The enhanced efflux of K^+ was not found in the dark zone of a strip connected by living tissue to that receiving the red light (Fig. 6B). A parallel efflux of H^+ was detected, although its was not significantly enhanced by light (Fig. 6C). Since several lichen acids were superficially adhered to the upper cortex, they were previously removed by washing thallus samples with 2 % (w/v) polyvinylpyrrolidone for 1h in the dark (Fig. 6C). After this, proton efflux was maintained. No proton flux was found from the dark zone of the thallus strip (Fig. 6D). Ca^{2+} and Na^+ uptake or efflux were not detected for 1 min of experimentation.



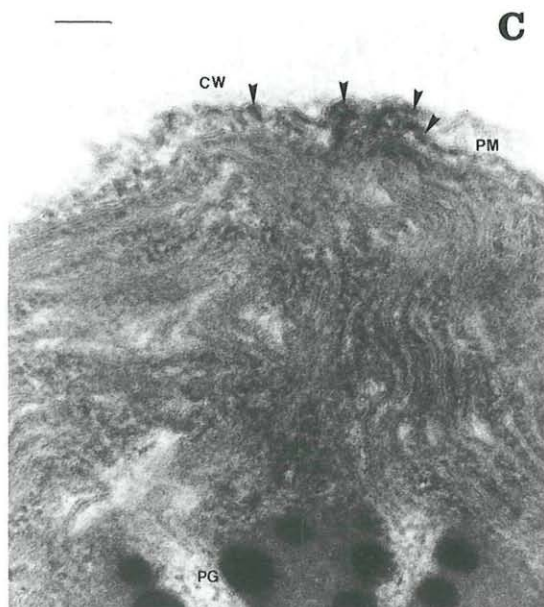


Fig. 5. Cytochemical localization of adenyl cyclase activity. In A) an algal cell from a lichen thallus that was used as a control (without staining reaction). Bar = 0.6 μm . In B), staining reaction was performed (bar = 0.6 μm). After this, some electron-dense bodies associated to the inner surface of the plasma membrane can be observed (arrows). In C), a magnification of the same preparation, in which bar = 0.09 μm . C = chloroplast; CW = cell wall; H = fungal hypha; M = mitochondria; PM = plasma membrane; PG = pyrenoglobuli; PY = pyrenoid; SB = storage bodies.

Since activation of adenyl cyclase occurred in parallel to the increased K^+ efflux, at least from the irradiated zone, P_{fr} should induce a loss of K^+ to the medium and the enzyme activation should be produced by the change in the membrane potential effected by charge redistribution. Alternatively, activation of adenyl cyclase induced by the photoconversion of P_r to P_{fr} could promote the K^+ efflux. Thus, a substitution of red light by an exogenous supply of 0.5 mM cyclic AMP in order to promote K^+ might be expected. However, this supply did not promote cation losses from the lichen strip to the medium during 30s. On the contrary, no activation of adenyl cyclase was found after loading lichen thallus with 2.5 mM, 10 mM and 25 mM KCl in the dark (data are not shown).

Discussion

Perhaps, a cascade pathway involving P_{fr} GTP, G-protein, adenyl cyclase, cAMP and protein kinase was in the basis of the activation of some

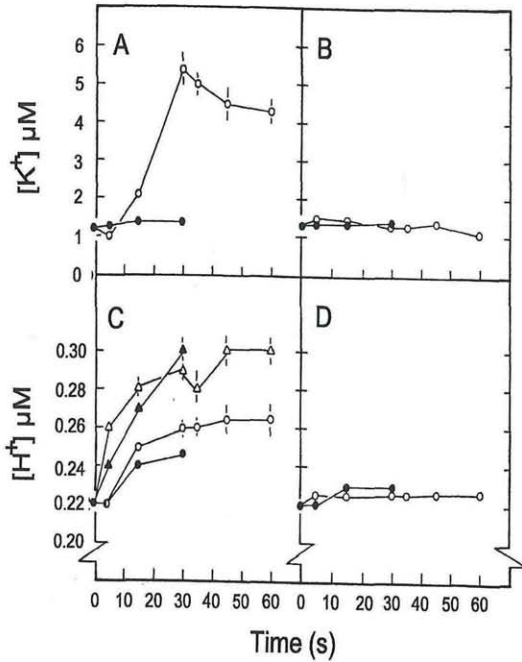


Fig. 6. K^+ and H^+ concentration measured in 60 ml distilled water in which thallus strips were immersed during experimentation. Lichen tissue was maintained in the dark (filled circles) or irradiated at the tip with red light (empty circles). K^+ efflux was measured from the irradiated tip (A) or from the bottom maintained in the dark (B). Proton efflux was measured, by using untreated strips (circles) or lichen tissue washed with 2 % (w/v) PVP for 1h in the dark (triangles) to remove cortical lichen phenols, from the irradiated tip (C) or from the bottom maintained in the dark (D). Values are the mean of three replicates. Vertical bars give the standard error where larger than the symbols.

ion channels in plants (VICENTE 1993). For example, red light regulated Ca^{2+} -activated K^+ channels in the plasma membrane of *Mougeotia*, activation that was reversed by far-red light (LEW & al. 1990, 1992). Cyclic GMP mediated phytochrome phototransduction for stimulating full chloroplast development and anthocyanin biosynthesis in tomato plants (BOWLER & al. 1984), and the increase of GTPase activity has been described as a response to red irradiation (SONG & al. 1996). But the results obtained by using *E. prunastri* thalli do not correspond to this scheme, although the involvement of adenylyl cyclase in the cascade seems to be supported by the localization of this enzyme as adhered to the inner surface of algal membrane (Fig. 5). In 5s, photoactivation of adenylyl cyclase has been found in the red-irradiated zone of lichen thallus but the signal did not appear to be transported to the non-photostimulated tissues (Fig. 3). After

30s red irradiation, the primary (physical or chemical) product of light perception seemed to be transported to the thallus zone kept in the dark. This transport was sustained by water connecting two separated zones of the thallus but barred by paraffin oil. This fact implied that the immediate response to P_{fr} formation could be an electric impulse which was stopped by a bad conductor (Fig. 4), or a very polar chemical, a second messenger that did not move through an apolar, connecting substance (Fig. 1). However, no activation of adenylyl cyclase by GTP has been proved for short time periods, although this activation was performed after 1h treatment. In addition, neither GTPase activity nor cyclic GMP production have been found for 30s of red irradiation. This would exclude G-proteins from the first rapid activation of adenylyl cyclase after P_{fr} formation. Activation of adenylyl cyclase, located on the inner surface of plasma membrane, could be the result of the hyperpolarization caused by the binding of P_{fr} to plasmalemma. The binding could be explained by supposing that a part of phytochrome might be an internal ligand to plasma membrane (SINESHCHEKOV & al. 1994). The receptor seems to be a hydrophobic protein. This binding should be accompanied by the opening of a voltage-regulated ion channel (a channel for K^+ since a net efflux of K^+ to the medium has been found from illuminated lichen thalli, as shown in Fig. 6A). Voltage operated selective channels have been described for Ca^{2+} in higher plants (PINEROS & TESTER 1995, THULEAU & al. 1994) but, in this case, no exchange of Ca^{2+} with the medium has been found. The loss of K^+ seems to be exclusively accompanied by an efflux of protons. This is not an unusual process, since a co-transport of both K^+ and H^+ has previously been found in higher plants (SCHACHTMAN & SCHROEDER 1994) using a complementary DNA encoding a membrane protein (HKT1) from wheat roots. A slow efflux of protons from isolated *Evernia* phycobionts was produced by red light whereas internal concentration of both protons and K^+ remained unchanged in the dark (BROWN & al. 1987, VICENTE & MOLINA 1993). By using guard-cell protoplasts of *Vicia faba*, an inward current of K^+ has been shown after membrane hyperpolarization, whereas cation efflux was effected by depolarization (SCHROEDER & al. 1987). These fluxes imply voltage-dependent K^+ channels, the opening of which seems to be similar to that found for the Shaker B protein of *Drosophila* or *Xenopus* oocytes (ISACOFF & al. 1990, JAN & JAN 1989, MILLER 1991, YOOL & SCHWARTZ 1991).

However, activation of adenylyl cyclase in the non-photostimulated zone of the thallus might be the consequence of the transfer of an electric current generated by phytochrome light perception (Fig. 4). However, this transfer is not enough to open the ion channel which promotes K^+ efflux. Thus, activation of adenylyl cyclase and K^+ efflux for very short time periods are two independent consequences of P_{fr} formation (compare Figs. 3 and 6B).

Acknowledgements

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Phyton (Horn, Austria) 39 (2): 238 (1999)

Recensio

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Fortsetzung von p. 216.

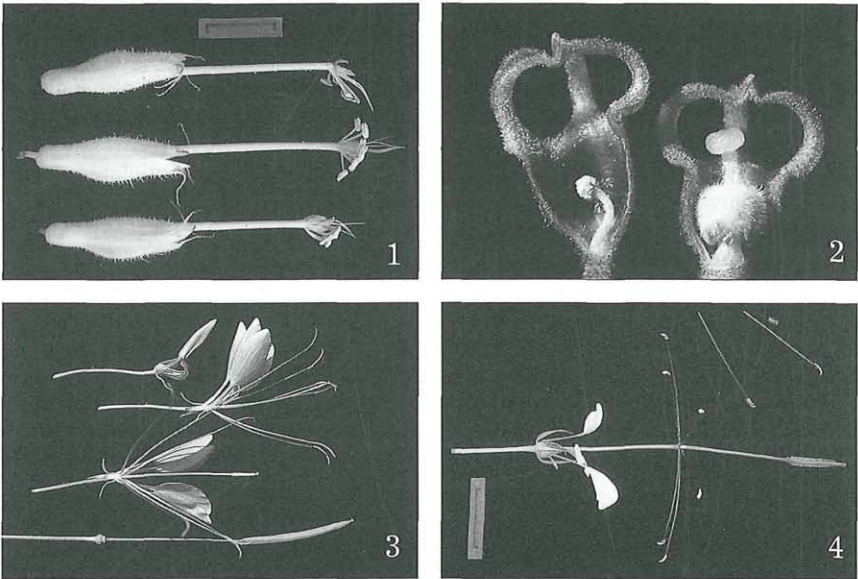


Abb. 1–4. Androphor, Gynophor und Androgynophor. – Abb. 1. *Helicteres pentandra* L. mit Androphor. Die sechs (!) Antheren sind durch die am Scheitel verschmolzenen Theken scheinbar monothezisch. – Abb. 2. *Sterculia tragacantha* LINDL., langer Androphor in der funktionell männlichen, kurzer in der funktionell weiblichen Blüte. – Abb. 3. *Cleome spinosa* JACQ. mit Gynophor. – Abb. 4. Zwitterblüte von *Gynandropsis gynandra* (L.) BRIQ. mit Androgynophor.

Cirrhus: an zweiter Stelle einzufügen: als Kletterhilfe dienender Rhachisfortsatz am Blatt von *Calamus*-Arten (*Arecaceae-Calamoideae*).

Columna: zu ergänzen um: → *Columniferae*.

Columniferae: zu verbessern, z.B. in folgender Weise: leitet sich her von den auffällig säulenartig getragenen Stamina (Filamentröhre bei *Malvaceae*, Androphor bei *Sterculiaceae*).

Fortsetzung p. 249.

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