Gametophytes of the fern *Asplenium trichomanes* exhibit guttation when illuminated. Membrane potential changes evoked by light were measured in the presence of ion channel and proton pump inhibitors to elucidate the nature of the response and a possible link to guttation. Light-induced depolarization was suppressed by the anion channel inhibitors: anthracene-9-carboxylic acid and niflumic acid. Potassium channel blockers: TEA and Ba\(^{2+}\) caused an increase of the amplitude of light-induced membrane potential changes. Calcium channel inhibitors, La\(^{3+}\), Gd\(^{3+}\), diltiazem, nifedipine and verapamil had no significant effect on the membrane potential changes. Similarly, proton pump inhibitors, diethylstilbestrol and vanadate, had only minor effects on the response. A possible role of Cl\(^{-}\) and K\(^{+}\) fluxes in light-induced guttation is discussed.

**Key words:** *Asplenium trichomanes* — Guttation — Ion channels — Light-induced membrane potential changes — Proton pump.

All green plant cells investigated so far react to sudden illumination/darkening by light-induced potential changes (LIPC) (reviewed by Bentrup 1974, Jeschke 1976). In most cases light causes a transient depolarization sometimes followed by a hyperpolarization (Felle and Bertl 1986, Trebacz et al. 1989, Elzenga et al. 1995). Darkening usually evokes a reversed response. There are plants, like Charophyta algae or *Egeria* where hyperpolarization in response to illumination predominates (Spanswick 1981, Mimura and Tazawa 1986). It seems that in many plant cells the final response observed is a superposition of at least two processes of different polarity and time constants. For example, in the liverwort *Conocephalum conicum* a slow hyperpolarization is recorded at low light intensities. It is gradually overlaid by a much faster depolarization whose amplitude increases depending on the intensity of a light stimulus (Trebacz et al. 1989). If depolarization exceeds a certain threshold action potentials can be triggered (Trebacz and Zawadzki 1985). This however, occurs only in a relatively narrow group of plant species (reviewed by Trebacz 1989). Light-induced action potentials were, among others, reported in gametophytes of the moss *Bryum pseudotriquetrum* and the fern *Asplenium trichomanes* (Sinyukhin 1973).

The mechanism of LIPC is not yet entirely understood, and probably there are somewhat different mechanisms in different groups of plants and even different tissues of the same species (Elzenga et al. 1995). This is well seen already at the first stage of the process, i.e. light reception. In most cases photosynthetic pigments, mainly chlorophylls, play a role as photoreceptors (Tazawa et al. 1986, Trebacz et al. 1989, Okazaki et al. 1994). However, there are well documented cases in which phytochrome (Racusen and Satter 1975, Ermolayeva et al. 1996) or a so-called blue light absorbing system (Spalding and Cosgrove 1989, Nishizaki 1996) is responsible for light reception. Different light absorbing systems are reported to coexist in the same plant species. In mesophyll cells of *Pisum sativum* LIPC's are governed by photosynthetic pigments, whereas in epidermal cells both the blue-light absorbing system and phytochrome participate in light perception (Elzenga et al. 1995).

The ionic mechanism causing LIPC was characterized in detail in the algal species: *Eremosphaera viridis* (reviewed by Schönknecht et al. 1998) and *Chara corallina* (Vanselow and Hansen 1989, Plieth et al. 1998).

*E. viridis* does not change its membrane potential after illumination but reacts with a drastic transient hyperpolarization (by up to 100 mV) in response to darkening (Geiswald et al. 1982, Köhler et al. 1983). The response resembles an action potential but with reversed polarity. The membrane potential reached at the peak of the hyperpolarization corresponds to the Nerst potential for potassium, E\(_K\).

In *Chara* (and some other Charophyta species) illumination evokes a long lasting hyperpolarization sometimes preceded by a small transient depolarization. It has been proposed for both *Chara* and *Eremosphaera* that Ca\(^{2+}\) uptake by chloroplasts upon illumination and release after darkening constitute a main link between chloroplasts and the plasma membrane. K\(^{+}\) channels in the plasma membrane are the targets for light-induced changes in [Ca\(^{2+}\)]\(_c\) (Köhler et al. 1983, Vanselow et al. 1989). In *E. viridis* Cl\(^{-}\) efflux near the peak of the dark-induced hyperpolarization supplements the ionic mechanism (Sauer...
et al. 1994).

The large cells of these algae are very specialized and their reactions to illumination differ in many aspects from those in terrestrial higher plants, thus the extrapolation of these mechanisms to higher plants is limited.

Such studies on higher plants were recently intensified. The traditional attitude with application of intracellular recording and ion channel inhibitors was supplemented by other techniques such as patch-clamp, ion selective microelectrodes and optically active indicators (Spalding et al. 1992, Cho and Spalding 1996, Bauer et al. 1997, Blom-Zandstra et al. 1997, Bethmann et al. 1998, Plieth et al. 1998). There are two pictures which emerge from these investigations. In one group of cells activation of Cl\(^-\) and/or K\(^+\) channels predominates the electrogenesis of LIPC. It is often postulated that opening of those channels is Ca\(^{2+}\)-dependent (Elzenga and Van Volkenburgh 1997, Lewis and Spalding 1998). The other model covers cells where illumination affects mainly the proton pump via cytoplasmic pH and/or pCa changes (Felle and Bertl 1986, Nishizaki 1994, Bulychev and Vredenberg 1995).

What is the physiological significance of LIPC? For flagellated algal cells it is obvious that membrane processes are involved in optimal localization in a light environment, or, upon high stimuli, in the escape response (reviewed by Hagemann 1997). It is also well known that light is one of main factors regulating stomatal movement (reviewed by McAinsh et al. 1997, MacRobbie 1997). In the moss Physcomitrella patens red light evokes ion currents whose occurrence is necessary for formation of side branch initials in caulonemal filaments (Ermolayeva et al. 1996, 1997). Blue light-induced depolarization mediates growth inhibition in etiolated seedlings (Spalding and Cosgrove 1989). Red light induces depolarization of fern Onoclea sensibilis gametophytes in their early stage of development causing transitions from filamentous to two-dimensional forms (Racusen and Cooke 1982). Illumination evokes also guttation in gametophytes of mosses and ferns (Sinyukhin 1973). This is of special importance for fertilization, which is possible only in a water environment. Water enables movement of spermatozoids towards archegonia. Sinyukhin (1973) reported that gametophytes of the fern Asplenium trichomanes generate action potentials upon illumination. Several seconds after the passage of the action potential guttation becomes noticeable. Up to now the mechanism of gametophyte response to illumination remained unknown. Neither pigment systems nor ion channels involved in the response had been studied. The aim of our investigation was a reexamination of Sinyukhin's observations and an attempt to clarify the ion mechanism of the LIPC in Asplenium gametophytes. Possible link between light absorption and guttation is discussed.

Materials and Methods

Spores of Asplenium trichomanes L. were collected in the Botanical Garden, University of Bonn, Germany. The spores were sown into Petri dishes filled approx. half with a mixture of sand and soil. The mixture was sterilized at 150°C for 1.5 h before use. Gametophytes were grown in a vegetation chamber. Illumination 16:8 L:D provided by Power Star MQ1-T400 W/D lamps (Osrarn, Germany) was kept at 50 \(\mu\)mol m\(^{-2}\) s\(^{-1}\). The temperature was established at 24°C during days and 18°C at nights. Under such conditions fully grown gametophytes of 5-7 mm in diameter were obtained after 8-10 weeks.

Before every experiment individual gametophytes were detached from the ground, rinsed several times with distilled water and mounted in an experimental chamber. The chamber was put into a Faraday cage. The gametophytes were kept in darkness for 2 h before the registration started. During the dark adaptation and control measurements the gametophytes were equilibrated with a standard solution containing 1 mM KCl, 0.1 mM CaCl\(_2\), 50 mM sorbitol, 2 mM MES/tris pH 6.0 flowing through the chamber at a rate of approx. 50 ml min\(^{-1}\). Plants were illuminated with a xenon lamp (XBO 101 Wetron, Germany) equipped with a water filter 15 mm thick and an interference filter Calex C (Balzers, Liechtenstein), transmitting light between 350 and 750 nm. Broadband interference filters (\(T = 75 \pm 85\%\), \(A_{\lambda/2} = 43 \pm 54\) nm, Balzers, Liechtenstein) together with neutral, grey filters were applied to obtain quantum balanced monochromatic light beams. Photon fluence rate was measured by a quantummeter FF01 (Sonopan, Białystok, Poland). Light spots of approx. 10 mm in diameter were formed by a custom made system consisting of two focusing lenses and a concave mirror.

The standard microelectrode technique described earlier (Krol and Trebacz 1999) was used for measuring membrane potential changes in Asplenium gametophytes. A glass microelectrode and a reference electrode filled with 3 M KCl were connected to a high input resistance amplifier VF 4 (World Precision Instruments, Sarasota, FL, U.S.A.). The output signal was digitized by a custom made A/D converter and stored on a hard disk of a PC. The sampling frequency was 2 Hz.

The microelectrodes were inserted by an electrically driven micromanipulator (DC 3001/MS 314, World Precision Instruments, Sarasota, FL, U.S.A.) under the dissection microscope (MSt 130, PZO, Warsaw, Poland). Light spots of approx. 10 mm in diameter were obtained after 8-10 weeks. The values of the parameters are given as mean±SE. Differences between means were tested for statistical significance using a \(t\)-test.

Results

General characteristics of light-induced potential changes in gametophytes of Asplenium trichomanes—Illumination of Asplenium gametophytes caused transient depolarization of the membrane potential; darkening evoked transient hyperpolarization. The dependence between amplitudes of light-induced depolarization and stimulus strength showed saturation at approx. 300 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) (Fig. 1). No threshold was noticed, in contrast to the case of Conocephalum and other plants in which light triggers...
Light-induced membrane potential changes in *Asplenium*

action potentials. In *Asplenium* switching light off at the moment when illumination caused substantial depolarization caused always hyperpolarization (data not shown). In excitable plants turning light off after exceeding the threshold of excitation does not prevent development of the action potential (Trebacz and Zawadzki 1985, Trebacz and Sievers 1998).

In order to check the involvement of photosynthetic pigments in the reaction we blocked the photosynthetic electron transport chain with DCMU. DCMU applied at 25 μM concentration caused a complete blockage of responses to light stimuli after 30-50 min of treatment. Neither red nor blue light evoked detectable potential changes in DCMU treated plants (Fig. 2).

**Effects of ion channel and proton pump inhibitors on light-induced potential changes in Asplenium**—We checked the influence of both inorganic (La$^{3+}$, Gd$^{3+}$) and organic (diltiazem, nifedipine, verapamil) calcium channel inhibitors on LIPC in *Asplenium* gametophytes. We also applied EGTA, Ca$^{2+}$ chelator to reduce a residual free calcium concentration in the medium.

Of the inhibitors applied only La$^{3+}$ showed a substantial, statistically significant reduction of LIPC amplitudes (Fig. 3A). This, however, became obvious only after...
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Fig. 4 (A) Responses of gametophytes of Asplenium trichomanes to illumination (white bars) and darkening (black bars) treated with 2 mM A-9-C. Note change in polarity of membrane potential changes at 1 h 30' after application of the inhibitor. Other denotations as in Fig. 3A. (B) Relative changes of amplitudes of light-induced membrane potential changes in Asplenium trichomanes treated with anion channel inhibitors: A-9-C (2 mM) and niflumic acid (5 μM). The duration of treatment required to obtain constant amplitudes of LIPC was in the range 1.5–2 h. Average of 4 experiments. Denotations as in Fig. 3B.

A single flash of high intensity (450 μmol m\(^{-2}\) s\(^{-1}\)). Before that the amplitude remained fairly constant (115% of the control) during the first 2 h of treatment. Separate control experiments with the same sequence of light stimuli but without La\(^{3+}\) showed no significant changes in LIPC amplitudes (data not shown). Lowering the external Ca\(^{2+}\) concentration with EGTA caused a slight, insignificant reduction of the response to light stimuli. The average changes of LIPC amplitudes after the treatment with EGTA and calcium channel inhibitors are presented in Fig. 3B.

A-9-C, an anion channel inhibitor applied at 2 mM concentration caused gradual reduction of the responses to illumination/darkening. After approx. 90 min of treatment a reversed response was observed: light evoked hyperpolarization, and darkness transient depolarization (Fig. 4A). A-9-C caused a reduction of the membrane potential by 27±3.3 mV, \((n=3)\).

Niflumic acid (5 μM), another anion channel inhibitor showed a statistically significant but not so pronounced inhibition of LIPC amplitudes. In none of 4 experiments a reverse response, like that after A-9-C treatment, was recorded. The average responses of Asplenium cells treated with anion channel inhibitors are presented in Fig. 4B.

Ba\(^{2+}\) and TEA were applied to check the role of potassium channels in the electrogenesis of LIPC in Asplenium gametophytes. Both K\(^{+}\) channel inhibitors applied at 5 and 10 mM, respectively, caused a statistically significant increase of LIPC amplitudes in relation to control before the treatment. The sequence of original traces of LIPC obtained just before and after the application of Ba\(^{2+}\) is shown in Fig. 5A. Strontium ions are believed to release Ca\(^{2+}\) from internal stores, activating in this way, calcium-dependent potassium channels (Schönknecht et al.

Fig. 5 (A) Light-induced membrane potential changes in gametophytes of Asplenium trichomanes after treatment with 5 mM BaCl\(_2\). Denotations as in Fig. 3A. (B) Relative changes of amplitudes of light-induced membrane potential changes evoked by illumination/darkening in gametophytes of Asplenium trichomanes treated with TEA (10 mM), BaCl\(_2\) (5 mM), SrCl\(_2\) (10 mM). The duration of treatment required to obtain constant amplitudes of LIPC was between 1 and 2 h. Average of 4–5 experiments.

Light-induced membrane potential changes in Asplenium trichomanes to illumination (white bars) and darkening (black bars) treated with 2 mM A-9-C. Note change in polarity of membrane potential changes at 1 h 30' after application of the inhibitor. Other denotations as in Fig. 3A. (B) Relative changes of amplitudes of light-induced membrane potential changes in Asplenium trichomanes treated with anion channel inhibitors: A-9-C (2 mM) and niflumic acid (5 μM). The duration of treatment required to obtain constant amplitudes of LIPC was in the range 1.5–2 h. Average of 4 experiments. Denotations as in Fig. 3B.

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If such channels play a role in *Asplenium* a reduction of LIPC amplitudes should be observed. Indeed, Sr$^{2+}$ caused a substantial decrease of LIPC amplitudes. The effects of Ba$^{2+}$, TEA and Sr$^{2+}$ on *Asplenium* responses to illumination/darkening are presented in Fig. 5B.

There are many reports pointing to the predominating role of the proton pump in the mechanism of LIPC (Spanswick 1981, Felle and Bertl 1986, Nishizaki 1996). We examined such possibilities in *Asplenium* gametophytes by applying two proton pump inhibitors: DES and orthovanadate. The results did not allow us to conclude unequivocally on the participation of the proton pump in LIPC in *Asplenium*. DES (25 μM) induced small, insignificant increase of LIPC amplitudes (Fig. 6A). Orthovanadate (3 mM) caused slight, statistically insignificant reduction of the response to illumination. The average amplitudes of LIPCs after applying the proton pump inhibitors are shown in Fig. 6B.

**Discussion**

In contrast to the report of Sinyukhin (1973) we never recorded action potentials in gametophytes of *Asplenium trichomanes*. The responses we observed have not the basic features of action potentials, such as consistence with the all-or-none law and ability of propagation. On the contrary, amplitudes of LIPCs recorded in *Asplenium* cells showed a distinct dependence on the stimulus strength. In excitable plants as *Conocephalum conicum* and *Dionaea muscipula* it is possible to switch off the light stimulus just after exceeding the threshold of excitation, and the action potential keeps on developing reaching a constant amplitude (Trebacz and Zawadzki 1985, Trebacz and Sievers 1998). This never occurred in *Asplenium* throughout our study. We also could not elicit action potentials using electrical stimulation, which is typical in excitable plants. The response to illumination in *Asplenium* was limited to the spot directly illuminated, as was the case in *Conocephalum*, but only after the blockage of action potentials with ion channel inhibitors (Krol and Trebacz 1999). Thus, it seems that the term "action potential" was misused by Sinyukhin (1973).

Clear indication of the involvement of photosynthesis in the light stimulus transduction was obtained after application of DCMU. Distortion of the photosynthetic electron transport chain blocked completely LIPC irrespective of the wavelength. In plant cells where phytochrome or a blue light absorbing system is responsible for photoreception DCMU has no influence on LIPC (Elzenga et al. 1995, Ermolayeva et al. 1996).

The link between light absorption by photosynthetic pigments and ion fluxes across the plasma membrane remains unknown. Hypotheses concerning such coupling were presented recently (Krol and Trebacz 1999). Among the factors which can participate in the transduction process changes in [Ca$^{2+}$], are often postulated. We began our investigation on *Asplenium* gametophytes with checking the involvement of Ca$^{2+}$ fluxes in the ionic mechanism of LIPC. Application of calcium channel inhibitors did not give an unequivocal indication that Ca$^{2+}$ fluxes across the plasma membrane play a role in transduction of the light signal. The participation of Ca$^{2+}$ from internal stores, such as chloroplasts, ER or vacuoles in the transduction process cannot be excluded. Such light-induced Ca$^{2+}$ fluxes were recently postulated (Schönknecht et al. 1998, Plieth et al. 1998). Basing on the experiments with isolated chloroplasts (Muto et al. 1982, Kreimer et al. 1985) it was concluded that upon illumination calcium ions are taken up by chlo-
Light-induced membrane potential changes in *Asplenium*

Arabidopsis thaliana +

by Sr

induced potassium channels are indirectly activated


cEremosphaera viridis

Application of such plants, in the absence of factor(s) enhancing [Ca

spikes (Thaler et al. 1989, Bauer et al. 1998). It is possible from internal stores (Schonknecht et al. 1998).

Light-induced depletion of Ca

was detected after turning light on and off (Trebcz et al. 1994, Bauer et al. 1997, Lewis et al. 1997). In *Eremosphaera viridis* [Ca

increased rapidly upon darkening but only in those cases when the transient hyperpolarization was generated. Otherwise, there was no detectable change in a cytosolic free calcium concentration, especially it never occurred after illumination (Bauer et al. 1997, Schönknecht et al. 1998).

The role of chloride fluxes in LIPC seems less disputable. Niflumic acid at micromolar concentration showed a significant blockage of LIPC, similarly as in *Physcomitrella patens* (Ermolayeva et al. 1996). A-9-C not only reduced the amplitudes of LIPC but also reversed their polarity: hyperpolarization was recorded after illumination and depolarization after darkening. In many instances LIPCs are multiphasic, as if they were a superposition of at least two processes of different kinetics and probably different nature. It seems thus possible that application of A-9-C completely blocks one of them exposing the other one, which was previously masked by a high conductance of anion channels susceptible to A-9-C. Chloride channels taking part in light signal transduction were recently identified in the plasma membrane of *Pisum sativum* and *Arabidopsis thaliana* by a patch-clamp technique (Cho and Spalding 1996, Elzenga and Van Volkenburgh 1997). Application of the same technique allowed Pottosin and Schöenknecht (1995) characterizing chloride channels in thylakoid membranes.

Application of either potassium channel inhibitors TEA and Ba

or Sr

gave a quite clear picture. Both inhibitors caused an increase of LIPC amplitudes. This is consistent with the possibility that, after depolarization caused by Cl

channel activation, potassium channels are opened and repolarization occurs. Suppression of K

conductance damps the repolarization process allowing more pronounced depolarization. A similar effect was recently observed in *Conocephalum conicum*, where application of TEA significantly increased the amplitudes of light-induced voltage transients (Trebcz et al. 1997, Krol and Trebcz 1999). Strontium is believed to cause a release of Ca

from internal stores (Schönknecht et al. 1998). Application of Sr

evokes repetitive transient hyperpolarizations in *Eremosphaera viridis* coinciding with [Ca

] spikes (Thaler et al. 1989, Bauer et al. 1998). It is possible that in gametophytes of *Asplenium*, Ca

induced potassium channels are indirectly activated by Sr

 treatment which suppresses LIPC. In untreated plants, in the absence of factor(s) enhancing [Ca

] such K

channels would remain closed allowing LIPC to develop.

Except ion channels, the proton pump is often mentioned as responsible for LIPC (Nishizaki 1994, Bulychev and Vredenberg 1995). In *Asplenium* proton pump inhibitors, vanadate and DES did not alter significantly LIPC. Chlorophyll fluorescence measurements showed a very low rate of photosynthesis in *Asplenium* gametophytes as compared to sporophytes (data not shown). Reduced metabolism of gametophyte cells probably does not supply enough ATP for efficient functioning of H

ATPase. This may be the reason that in gametophytes of *Asplenium* rather ion channels than the proton pump are responsible for LIPC. This may explain the guttation being the consequence of illumination. Equilibrium potential for Cl

in *Asplenium* is positive in relation to the resting potential as in all plants investigated so far (Thaler et al. 1992, Trebcz et al. 1994, Bethmann et al. 1995). Thus, opening Cl

channels leads to Cl

efflux and depolarization. Activation of K

channels by depolarization and/or increase of [Ca

] results also in an efflux, this time the efflux of K

. Combined efflux of both ion species together with water surrounding them is probably the basis of guttation. Such a scheme of responses to different stimuli is widespread in plants. It includes movements being the consequence of turgor reduction, such as stomata closure, shrinking of leaf pulvini, closing leaf-traps in carnivorous plants, and many other processes.

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References


, K

, Ca

and Cl


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