Plant protoplasts as a model system to study phytochrome-regulated changes in the plasma membrane

Planteprotoplasten als een model systeem om fytochroom-gereguleerde veranderingen in de plasmamembraan te bestuderen

ONTVANGEN

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Plantenfysiologisch Onderzoek

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STELLINGEN

1. Protoplasten zijn een waardevol model systeem voor onderzoek naar plasmamembraan gebonden processen in de plant.

   Dit proefschrift

2. De signaal transduktieketen geïnitieerd door fotomorfogenetisch licht heeft overeenkomsten met die van hormonen in dierlijke systemen.

   Morse et al. (1989) Light-stimulated inositol phospholipid turnover in *Samanea saman* pulvini. Plant Physiol. 90, 1108-1114

   Dit proefschrift

3. Bij het bestuderen van de effekten van extra-cellulair Ca^{2+} op fysiologische processen in planteweefsels is het gebruik van Ca^{2+}-EGTA buffers noodzakelijk .

   Viner, N., Whitelam, G., Smith, H. (1988) Calcium and phytochrome control of leaf unrolling in dark-grown barley seedlings. Planta 175, 209-213

4. De binding van fytochroom aan membranen, zoals beschreven in het model van Tokutomi & Mimuro, is niet in overeenstemming met de ongevoeligheid voor proteasen van fytochroom geassocieerd met mitochondrieën.

   Tokutomi, S. & Mimuro, M. (1989) Orientation of the chromophore transition moment in the 4-leaved shape model for pea phytochrome molecule in the red-light absorbing form and its rotation induced by the phototransformation to the far-red-light absorbing form. FEBS Lett. 255, 350-353

   Serlin, B.S. & Roux, S.J. (1986) Light-induced import of the chromoprotein, phytochrome, into mitochondria. Biochim. Biophys. Acta 848, 372-377

5. Voor het initieren van een respons is niet alleen de hormoon concentratie in een weefsel van belang, maar ook de concentratie van de receptoren.

   Medford, J.I., Horgan, R., El-Sawi, Z. & Klee, H.J. (1989) Alterations of endogenous cytokinins in transgenic plants using a chimeric isopentenyl transgerase gene. Plant Cell 1, 403-413

6. Zure regen verslechtert niet alleen de vitaliteit van de bossen op arme zandgronden, maar heeft ook gevolgen voor de broedresultaten van de standvogels.

   Drent, P.J. & Woldendorp, J.W. (1989) Acid rain and eggshells. Nature 339, 431

7. Het gebruik van tarwe als proefplant houdt niet zonder meer in dat het om landbouwkundig onderzoek gaat, of dat het onderzoek van belang is voor de landbouw.

8. Het woord "kadoshop" illustreert de veranderde houding van Nederlanders tav. het belang van de Franse en Engelse taal voor onze cultuur.

9. Studiefinanciering voor personen boven de 27 jaar is geen overbodige luxe.

10. Het eten van kaasfondue met een gezelschap van vier personen is gemakkelijker, indien er twee rechts- en twee linkshandigen aan tafel zitten.

Stellingen behorende bij het proefschrift van Margreet E. Bossen: " Plant protoplasts as a model system to study phytochrome-regulated changes in the plasma membrane".

Wageningen, 28 maart 1990.

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Margreet E. Bossen

Plant protoplasts as a model system to study phytochrome-regulated changes in the plasma membrane

Proefschrift
ter verkrijging van de graad van
doctor in de landbouw- en milieuwetenschappen,
op gezag van de rector magnificus,
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in het openbaar te verdedigen
op woensdag 28 maart 1990
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen.

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BIBLIOTHEEK
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WAGENINGEN

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en natuurlijk allen die voor kortere of langere tijd op de vakgroep PFO rondliepen, voor de gezellige sfeer.

CONTENTS

Abbreviations and symbols

ABA	abscisic acid
ACh	acetylcholine
Ad	adenine
BAP	benzylaminopurine
BCh	butyrylcholine
$[Ca^{2+}]_{cyt}$	cytoplasmic Ca^{2+}-concentration
cAMP	adenosine 3':5'-cyclic monophosphate
CCh	carbamylcholine
Ch	choline
CPZ	chlorpromazine
DB-cAMP	N^6,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate
DG	diacylglycerol
DPH	1,6-diphenyl-1,3,5-hexatriene
EDTA	ethylenediaminetetraacetic acid
FR	far-red light
GA_3	gibberellic acid
GDP-β-S	guanosine-5'-O-(2-thiodiphosphate)
G-protein	GTP-binding protein
GTP-γ-S	guanosine-5'-O-(3-thiotriphosphate)
H_7	1-(5-isoquinolinesulfonyl)-2-methylpiperazine
I_m	fluorescence intensity labeled protoplasts
IP_3	inositol 1,4,5-trisphosphate
I_s	fluorescence intensity non-labeled protoplasts
α-NAA	α-naphthaleneacetic acid
β-NAA	β-naphthaleneacetic acid
nov	non-osmotic-volume
PCh	propionylcholine
Pfr	FR-absorbing form of phytochrome
PIP_2	phosphatidyl-inositol 4,5-biphosphate
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
Pr	R-absorbing form of phytochrome
R	red light
r_f	anisotropy, corrected
r_m	anisotropy labeled protoplasts
r_s	anisotropy non-labeled protoplasts
TFP	trifluoperazine
W_5	N-(6-aminohexyl)-1-naphthalenesulfonamide
W_7	N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide

ABSTRACT

Protoplasts, isolated from the primary leaves of dark-grown wheat (*Triticum aestivum* L.), have been used as a model system to study phytochrome-regulated changes of the plasma membrane. Such protoplasts only swelled after red light (R)-irradiation, when Ca^{2+} was present in the medium. Far-red light (FR), after R, prevented swelling, indicating phytochrome involvement. Swelling was inhibited when La^{3+} or the Ca^{2+}-channelblocker Verapamil were added. Swelling was induced in darkness by the Ca^{2+}-ionophore A23187 and the calmodulin antagonist W_7. It is proposed that R-irradiation leads to opening of Ca^{2+}-channels, resulting in an increase of the cytoplasmic [Ca^{2+}] and protoplast swelling.

The effect of modulators of G-proteins and the phosphatidyl-inositol cycle, as known in animal cells, on the swelling response was examined. The R-induced swelling was inhibited by GDP-β-S and by neomycin, Li^+ and H_7. In darkness, swelling was found when GTP-γ-S or PMA were added to the protoplasts. All agonists and antagonists used, influenced the swelling response, as predicted by transposition of the animal model to plants. This suggests that R-irradiation, leads to activation of a G-protein, which results in the opening of Ca^{2+}-channels.

Plant hormones also induced protoplast swelling in the presence of Ca^{2+}, while swelling was inhibited by GDP-β-S. Acetylcholine induced, contrary to R-irradiation, swelling in the absence of Ca^{2+}, when K^+ or Na^+ were present in the medium. This swelling was not inhibited by GDP-β-S.

The Ca^{2+}-sensitive dye murexide, has been used to monitor phytochrome-regulated changes in the [Ca^{2+}] of the medium. Red light induced a Ca^{2+}-efflux, while FR reversed this effect. The R-induced efflux was inhibited by Verapamil and W_7 by approx. 75%. Therefore, the efflux, via a Ca^{2+}-ATPase, appears to be dependent on the activation of Ca^{2+}-channels and a Ca^{2+}-influx.

The fluidity of the protoplast plasma membrane was studied, using the fluorescent membrane probe DPH. After R the anisotropy of DPH (r_f) was higher, indicating a decrease in membrane fluidity. In darkness, r_f also increased upon osmotically induced protoplast swelling. It is not clear, whether R causes changes in membrane fluidity, independent of changes in volume.

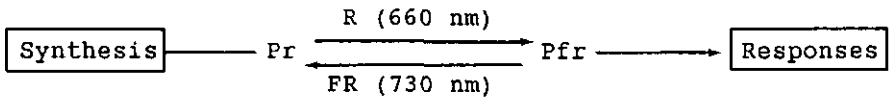
The observed changes in plasma membrane properties after R-irradiation, show that protoplasts are an useful tool for studying phytochrome action in higher plants.

Key words: Ca^{2+} (Ca^{2+}-fluxes), G-protein, membrane fluidity, phosphatidyl-inositol cycle, protoplast, phytochrome, *Triticum*

Chapter 1

General introduction

The photomorphogenetic pigment **phytochrome** is widespread throughout the plant kingdom. It has been shown to be functional in higher plants (Shropshire and Mohr 1983), ferns (Wada and Kadota 1989), mosses and liverworts (Hartmann and Jenkins 1984), green algae (Dring 1988) and a red alga (López-Figueroa and Niell 1989). Although fungi possess photoreceptors for UV- and blue light, there is no conclusive evidence that phytochrome is present in these organisms (Furuya 1986). The presence of phytochrome has been established in different ways. Based on the red light (R)-far-red light (FR) reversible induction of seed germination, Borthwick et al. (1952) postulated the existence of phytochrome. Phytochrome is synthesized in the red-absorbing form (Pr). Irradiation with R converts Pr to the FR-absorbing form (Pfr), which can be converted back to Pr by FR-irradiation. In general, Pfr is considered to be the physiologically active form (Hendricks and VanDerWoude 1983).



The presence and relative amount of phytochrome can be estimated by spectrophotometric measurements, since phytochrome displays repeatable R-FR reversible shifts in absorption. The absorbance difference between 660 and 730 nm (the absorption maximum of Pr and Pfr, respectively), after irradiation with actinic R or FR, can be used as an estimate for the relative amount of phytochrome present in the sample (Pratt 1983). As an alternative method, polyclonal and monoclonal antibodies raised against the phytochrome protein, have been used to detect the presence of a phytochrome-like pigment in several green algae and a red alga (López-Figueroa and Niell 1989).

Phytochrome is a biliprotein with an open-chain tetrapyrrole **chromophore**. The chromophore is covalently linked to the protein, via a cysteine residue, in the N-terminal domain. Absorption of R by the Pr chromophore is thought to result in a FR-reversible change of the stereometric configuration of the chromophore. The Z-E isomerization at the C-15, C-16 double bond results in a rotation of the chromophore relative to the protein moiety of the phytochrome molecule (Song 1988).

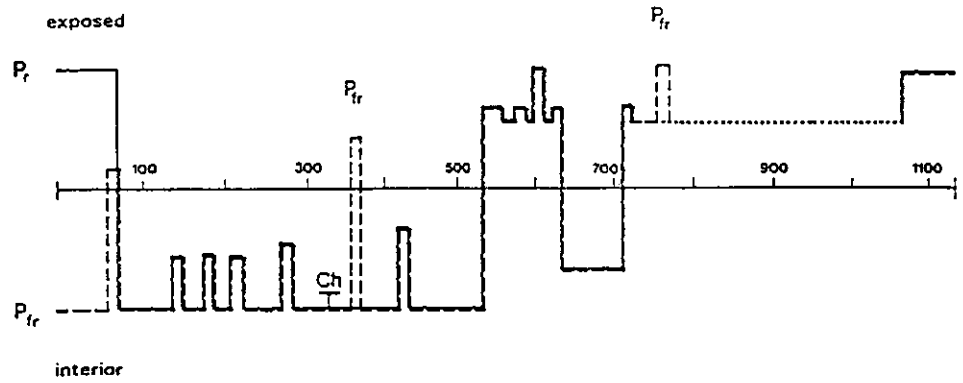


Fig. 1.1. Schematic drawing of exposed and interior parts of the peptide chain of phytochrome. On "exposed" parts of the molecule early cleavage sites for endoproteases are present, while in the "interior" parts the potential cleavage sites are not attacked. ——, Pr; ---, Pfr; Ch=chromophore. The scale shows the number of amino acid residues along the peptide. After Grimm et al. (1988)

Changes in the properties of the phytochrome protein, resulting from photoconversion, have been described. In the Pr and Pfr form, different cleavage sites are attacked when limited proteolysis is performed. The N-terminal domain is especially exposed in the Pr form, but much less accessible in the Pfr form, while part of the protein located near the chromophore is more exposed in Pfr, as is part of the C-terminal domain (Grimm et al. 1988; see Fig 1.1). Both forms also differ in the sites available for phosphorylation by protein kinases (Wong et al. 1986). A difference in surface charge between Pr and Pfr was described by Schendel and Rüdiger (1989). The isoelectric point for Pfr is in the range pH 5.80-5.85, whereas that of Pr is in the range 5.85-5.90. The surface of the phytochrome molecule appears to be more hydrophobic in the Pfr, than in the Pr form. A preferential binding of the Pfr form over the Pr form was found with neutral, but not with negatively charged liposomes (Kim and Song 1987; Singh et al. 1989). The changes in surface properties were ascribed to the exposure of the region near the chromophore attachment site in the Pfr form, which is in agreement with the model proposed by Grimm et al. (1988). Monoclonal antibodies which preferentially bind to Pr or Pfr have also been isolated (Cordonnier et al. 1989). The differences in antigenicity were mapped to at least four different domains, indicating a large change in phytochrome protein conformation upon photoconversion.

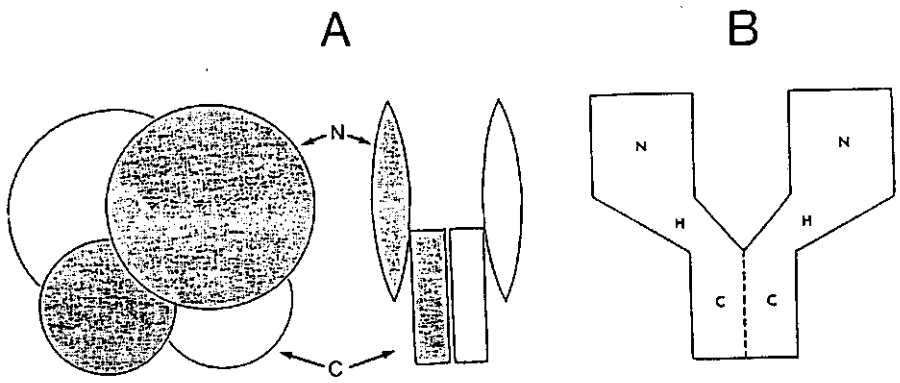


Fig. 1.2. Schematic diagram of the dimeric structure of phytochrome.
A. After Tokutomi et al. (1989), B. After Jones and Erickson (1989). N: amino-terminal domain, C: carboxyl-terminal domain, H: hinge region

Phytochrome is a water soluble protein, which exists as a dimer in solution. A molecular model has been proposed based on electronmicroscopic observations and on small-angle X-ray scattering, (Jones and Erickson 1989; Tokutomi et al. 1989). The N-terminal, chromophore bearing domain, and the C-terminal domain of the molecule are connected by a so called "hinge region". The contact site of the two monomers is located in the C-terminal domain (see Fig. 1.2).

Based on the different decay kinetics for the degradation of Pfr it has been proposed, that two types of phytochrome exist: so called "labile" phytochrome, which is readily degraded in the light and is abundant in dark-grown tissue, and "stable" phytochrome which is relatively more abundant in light-grown plants (Furuya 1989). However, it must be realized that both types of phytochrome are always present, even in dark-grown plants. The existence of at least two types of phytochrome has been confirmed by the isolation of monoclonal antibodies, which preferentially bind to labile or stable phytochrome (Abe et al. 1985; Konomi et al. 1987). Differences in the primary structure of the two types of phytochrome, isolated from pea, were detected with the aid of N-terminal micro-sequence analysis of peptide fragments obtained from highly purified phytochrome, by proteolytic digestion. It has been concluded that the proteins are transcribed from different genes (Abe et al. 1989).

The localization of phytochrome, especially physiologically active phytochrome, in the cell has been debated for a long time. About 95% of the phytochrome in etiolated tissue is readily extracted into a buffer solution. The remainder is associated with or trapped in membrane fractions. Addition of purified phytochrome to a plasma membrane fraction, isolated chloroplasts, mitochondria and nuclei has been reported to lead to "binding" of part of the phytochrome to the membranes. However, it was doubted if these binding events are part of the biological activity of phytochrome (for review see Pratt 1986). Immunostaining of etiolated coleoptile sections, revealed that phytochrome appears to be distributed uniformly throughout the cytosol and is not obviously associated with membrane sites. After conversion of Pr to Pfr, phytochrome is found sequestered at non-membrane sites (McCurdy and Pratt 1986). However, there is indirect evidence that physiologically active phytochrome is bound to or associated with the plasma membrane in the green alga *Mougeotia* (Haupt 1970) and in the protonemata of the fern *Adiantum* (Wada et al. 1983). These conclusions are based on the dichroic orientation of physiologically active phytochrome: Pr and Pfr are orientated differently with respect to the plane of the cell surface. Phytochrome in the Pr form is predicted to be orientated parallel, and in the Pfr form normal to the cell surface. Recently other evidence has emerged, based on the possibility to prepare highly purified plasma membrane vesicles, with the aid of aqueous two-phase partitioning. In such vesicles, isolated from etiolated pea, phytochrome was present in amounts independent of the phytochrome concentration of the soluble pool. Right-side out vesicles exhibited a R-FR reversible Ca^{2+}-efflux, as measured spectrophotometrically by murexide absorbance changes. In inside-out vesicles the Ca^{2+}-flux was in the opposite direction (Eisinger et al. 1989). These results suggest that the phytochrome associated with the plasma membrane of higher plants, is also physiologically active. Phytochrome was also found to be present in plasma membrane vesicles isolated from etiolated wheat, the plant used for the studies described in this thesis (Terry et al. 1989). The nature of the interaction between phytochrome and membranes appears to be both ionic and hydrophobic. This conclusion was reached for the binding studies of phytochrome to plasma membrane vesicles (Terry et al. 1989), to a crude membrane fraction from oat (Napier and Smith 1987) and to neutral liposomes (Singh et al. 1989).

A wide variety of physiological and developmental responses of both dark- and light-grown plants are thought to be regulated by phytochrome, with or without co-operation of other photomorphogenetic pigments. Some of these responses for organisms grown in darkness are: seed germination, fern spore germination, leaf unrolling of grasses, chloroplast biogenesis and hypocotyl hook opening. Examples for plants grown in the light are: stem elongation, leaf expansion, floral induction, nastic movements and chloroplast movement in algae (see Shropshire and Mohr 1983; Kendrick and Kronenberg 1986; Furuya 1987). At the molecular level, phytochrome appears to be involved in the regulation of transcription of many genes. The most thoroughly studied being: the phytochrome gene itself, genes coding for chlorophyll *a/b* binding protein (*cab*), the small subunit of ribulose-1,5-biphosphate-carboxylase (*rbc*S) and NADPH-protochlorophyllide oxidoreductase (for reviews see Tobin and Silverthorne 1985; Schäfer et al. 1986; Jenkins 1988; Nagy et al. 1988).

The signal transduction chain between phytochrome photoconversion and ultimate physiological responses is still largely unknown. At present, research on this subject is focused on two of the possible points of interaction. One is the regulation of gene expression by phytochrome (see above), the other the role of Ca^{2+} in phytochrome regulated responses. For an increasing number of phytochrome responses the involvement of Ca^{2+} has been shown. These are: leaflet closure in *Mimosa* (Toriyama and Jaffe 1972), membrane depolarization in *Nitella* (Weisenseel and Ruppert 1977), chloroplast rotation in *Mougeotia* (Dreyer and Weisenseel 1979), spore germination of the ferns *Onoclea* (Wayne and Hepler 1984) and *Dryopteris* (Scheuerlein et al. 1989), reduction of the surface charge of *Mesotaenium* cells (Stenz and Weisenseel 1986), leaf unrolling of dark-grown barley (Viner et al. 1988), regulation of gravitropism of maize "Merit" (Perdue et al. 1988), photoperiodic flower induction of *Pharbitis nil* (Friedman et al. 1989), regulation of light-off closure of the leaflets of *Cassia* (Roblin et al. 1989) and *Albizza* (Moysset and Simon 1989), regulation of NADH-glutamate dehydrogenase (Das et al. 1989) and the germination of turions of *Spirodela polyrhiza* (Augsten and Appenroth 1989). It is thought that changes in the permeability of the plasma membrane for Ca^{2+} play a role in these responses. This is mainly based on the observation that no response occurs in the absence of external Ca^{2+}, or when

specific blockers of Ca^{2+}-channels are added. Some of the plasma membrane related phytochrome responses are very rapid. Changes in the membrane potential after R have been measured with lag phases of 10 s or less (Racusen 1976; Weisenseel and Ruppert 1977; Newman 1981). Differences in Ca^{2+}-fluxes have been indicated as possible mediators of these changes. Transient enhancement of $^{45}Ca^{2+}$ uptake in protoplasts of dark-grown maize leaves, was measured 30 s after R (Das and Sopory 1985). At present it is not clear if the changes in plasma membrane properties (as described above), are in some way connected with the regulation of gene expression by phytochrome. The rapid changes of plasma membrane properties could be part of the transduction chain leading to changes in gene expression. As in animal cells a phosphorylation cascade, initiated by Ca^{2+}-regulated kinases located at the plasma membrane, could form the connection (Rasmussen 1989). In oat protoplasts the phosphorylation of two proteins after R-irradiation, was shown to be dependent on the presence of Ca^{2+} in the medium (Park and Chae 1989). However, it is also possible that phytochrome regulates different processes at different sites in the cell, independent from each other (Schäfer et al. 1986).

The aim of this thesis is to characterize phytochrome regulated changes of plasma membrane properties, which could be involved in the transduction chain between phytochrome photoconversion and physiological responses. These changes are of special interest, since some of them (e.g. membrane depolarization) show very short lag times, and may be close to the primary reaction of Pfr. Protoplasts isolated from the primary leaves of dark-grown wheat, were chosen as a model system. Blakeley et al. (1983) showed a phytochrome control of changes in osmotic behaviour of such protoplasts: they shrank less after R when transferred to a medium of higher osmotic potential. The plasma membrane of protoplasts is readily accessible for manipulation e.g. the controlled addition of chemical compounds, making them amenable for study by different techniques. Furthermore, during preparation of the protoplasts, the cell contents are relatively undisturbed. Therefore any phytochrome induced response observed, would be anticipated to be physiologically relevant.

Outline of this thesis

♦In Chapter 2 the protoplast system is introduced, and the involvement of Ca^{2+} and of Ca^{2+}-channels in the phytochrome regulated protoplast swelling is documented. A Boyle-van't Hoff analysis of the swelling response is presented.

♦In Chapter 3 the question how phytochrome regulates changes in Ca^{2+} permeability of the plasma membrane is addressed. The possible involvement of a GTP-binding protein and the phosphatidyl-inositol pathway of signal transduction in phytochrome-regulated protoplast swelling is described and discussed.

♦In Chapter 4 a comparison is made between phytochrome-regulated protoplast swelling and swelling induced by gibberellin, auxin, cytokinin, abscisic acid and acetylcholine. Research has been focused on the involvement of Ca^{2+} and of a GTP-binding protein.

♦Phytochrome-regulated changes in $[Ca^{2+}]$ of the protoplast medium are shown in Chapter 5. The dependence of these changes on Ca^{2+}-channel activity and on a plasma membrane located Ca^{2+}-ATPase was investigated.

♦Chapter 6 describes phytochrome-regulated changes of the fluidity of the plasma membrane. The anisotropic behaviour of the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) has been used as a measure of membrane fluidity.

♦Conclusions and summary of the results are given in Chapter 7.

Chapter 2

The role of calcium ions in phytochrome-controlled swelling of etiolated wheat (*Triticum aestivum* L.) protoplasts

Margreet E. Bossen, Hans H.A. Dassen, Richard E. Kendrick and Willem J. Vredenberg

Planta 174: 94-100 (1988)

Abstract

Protoplasts from dark-grown wheat (*Triticum aestivum* L.) maintained at a constant osmotic potential at 22°C, were found to swell upon red irradiation (R) and the effect was negated by subsequent far-red light (FR), indicating phytochrome involvement. Swelling only occurred when Ca^{2+}-ions were present in the surrounding medium, or were added within 10 min after R. Furthermore, Mg^{2+}, Ba^{2+} or K^{+} could not replace this requirement for Ca^{2+}. The presence of K^{+} did not enhance the Ca^{2+}-dependent swelling response. When the Ca^{2+}-ionophore A23187 was added to the medium, protoplasts swelled in the dark to the same extent as after R. Both the Ca^{2+}-channelblocker Verapamil and La^{3+} inhibited R-induced swelling. It is proposed that R causes the opening of Ca^{2+}-channels in the plasma membrane. Boyle-van't Hoff analyses of protoplast volume after R and FR are consistent with the conclusion that R irradiation causes changes in membrane properties.

Introduction

The signal transduction chain between phytochrome photoconversion and ultimate physiological responses such as seed and spore germination, flower induction, de-etiolation and inhibition of stem elongation, is still largely unknown. Some of the most rapid phytochrome responses appear to be membrane related. The conversion of phytochrome molecules from the red-light-absorbing form (Pr) into the far-red-light-absorbing form (Pfr) is completed within a few seconds of photon absorption (Spruit 1982; Scheuerlein et al. 1986). Changes in membrane potential after red light (R) have been measured with lag phases of less than 1, to 10 s (Racusen 1976; Weisenseel and Ruppert 1977; Newman 1981). Differences in Ca^{2+}-fluxes have been indicated as possible mediators of these changes. The reduction of the surface-charge of *Mesotaenium* cells after R, was also found to require the presence of Ca^{2+} in the surrounding medium (Stenz and Weisenseel 1986). The presence of Ca^{2+} in the medium, Ca^{2+} uptake or an increase in cytoplasmic [Ca^{2+}] have been shown to be essential for phytochrome mediated spore germination of *Onoclea* (Wayne and Hepler 1984), chloroplast rotation in *Mougeotia* (Dreyer and Weisenseel 1979) and leaflet closure in *Mimosa* (Toriyama and Jaffe 1972; for reviews see Hepler and Wayne 1985 and Roux et al. 1986). However, it is still unclear if Pfr is bound to or associated with the plasma membrane. After R, most of the

phytochrome appears to concentrate rapidly at non-membrane sites (McCurdy and Pratt 1986; Speth et al. 1986). There is indirect evidence that active phytochrome is bound to or associated with the plasma membrane in the green alga *Mougeotia* (Haupt 1970) and the protonemata of the fern *Adiantum* (Wada et al. 1983). Molecules of Pr are predicted to be orientated parallel and Pfr molecules normal to the cell surface. In *Adiantum* this change in orientation upon phototransformation has been shown to take place within 30 s of irradiation (Kadota et al. 1986).

To study changes in membrane properties induced by the photoconversion of Pr to Pfr, we have chosen protoplasts from the primary leaves of dark-grown wheat as a model system. Phytochrome control of changes in osmotic behaviour of such protoplasts were shown by Blakeley et al. (1983). After R, protoplasts shrank less than in darkness, when transferred to a medium of higher osmotic potential, while incubated at 4°C. In this paper we describe the results of studies on: (i) the volume changes of etiolated protoplasts after R or FR, incubated at constant osmotic potential at 22°C, (ii) the kinetics of protoplast swelling after R and (iii) the requirement of Ca^{2+} for this process.

Material and methods

Plant material. Wheat caryopses (*Triticum aestivum* L., cv. Arminda) were sown in a washed mixture of Vermiculite and Perlite (1:1), and placed in a dark growth room at 25°C and 60-65% humidity. After 8-10 d plants were harvested and the primary leaf was used for protoplast isolation.

Protoplast isolation. All manipulations were performed under dim green safe light (26 $nmol \cdot m^{-2} \cdot s^{-1}$). Protoplasts were isolated as described by Edwards et al. (1978). Primary leaves were cut in small pieces (1 mm) and incubated (2 g/10 ml) in 2% cellulase Onozuka R10, 0.2% macerozym (Kinky Yakult, Nishinomiya, Japan), 0.5 M sorbitol, 1 mM $CaCl_2$ and 1 mM KCl, pH 5.5 for 3-3.5 h at 22°C. After purification on a discontinuous sorbitol/sucrose gradient, protoplasts were resuspended in a medium consisting of 0.5 M sorbitol, 5 mM 2-(N-morpholino)ethanesulfonic acid (Mes) adjusted to pH 6.0 with 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) and 1 mM $CaCl_2$ (Medium A), and incubated in this medium unless otherwise indicated. Protoplasts were tested for viability with 0.2% phenosafranine (Widholm 1972). Experiments were carried out at 22°C except for those on the kinetics of protoplast swelling at 15°C. All experiments have been repeated at least three times with qualitatively similar results. Representative individual experiments are shown.

Light sources and irradiations. Monochromatic light was obtained from a 250 W quartz-iodine lamp in a custom-built projector using interference filters (Balzer B40, Liechtenstein) with a half bandwidth of approx. 10 nm. The fluence rates used were R(660 nm): 150 and FR (729 nm) 75 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for protoplast population studies. In the experiments with individual protoplasts, fluence rates were for both R(660 nm) and FR(732 nm): 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The fluence rates were measured with a photodiode meter, Optometer type 80 X (United Detectors Technology Inc., Santa Maria, Cal., USA). Irradiation times were 1 min for R and 5 min for FR, except in the experiments with individual protoplasts, where 3 min R was given.

Protoplast measurement. After irradiation or other treatment, protoplasts ($5 \cdot 10^5$ in 0.5 ml) were kept in darkness for 30 min, at 22°C. Samples were taken, placed on a haemocytometer (Fuchs-Rosenthal) and photographed. The diameters of 100 protoplasts were measured and the mean volume calculated, assuming protoplasts are spherical. In experiments without Ca^{2+} in the medium, protoplasts were washed twice with 0.5 M sorbitol, 5 mM Mes-Tris pH 6.0 and 50 μM ethylenediaminetetraacetic acid (EDTA), and resuspended in this medium without EDTA, as indicated. For kinetic studies of swelling, protoplasts were embedded in 0.6% agarose (low gelling, Sigma, St. Louis, MO., USA) in medium A, in a temperature-controlled flat cuvette. The medium was circulated through the cuvette and the temperature maintained at 15°C. Protoplasts were selected under the microscope, while irradiated simultaneously with green and FR by means of light guides. The microscopic image of the protoplasts was displayed on a TV screen and the diameter was measured at intervals of 1-2 min.

Boyle-van't Hoff analysis. The osmotic and non-osmotic volumes (nov) were estimated by using the Boyle-van't Hoff relation: $\pi(V-b)=nRT$ (Weyers and Fitzsimons 1982; Nobel 1983). This formula relates the osmotic potential of the medium (π) with protoplast volume (V) at a given temperature (T). R is the gas constant, n the apparent number of moles of osmotically active solutes, V and b are the cell volume and nov, respectively. A plot of V against π^{-1} is predicted to be linear with a y-intercept b. π was calculated as the solute potential of the medium, using the van 't Hoff relationship at T=295 K. After R or FR, protoplasts were transferred immediately to media with different sorbitol concentrations and maintained in darkness for 30 min before volume measurement. All media contained 5 mM Mes-Tris, pH 6.0 and 1 mM $CaCl_2$.

Phytochrome measurement. The phytochrome content of protoplasts was measured by using the spectrophotometer described by Spruit (1970), in the dual-wavelength mode. The measuring beam was set at 730 nm and the reference beam at 806 nm. Cuvettes with a light path of 1.6 mm were used. Calcium carbonate was added to the protoplast suspension as a scattering agent to enhance the signal (see Pratt, 1983). Phytochrome content is expressed as a change in absorbance difference ($\Delta\Delta A$; Spruit 1970).

Results

Protoplasts, were more than 90% viable, directly after isolation, as measured with phenosafranine (Fig. 2.1). Photoreversible changes, attributable to phytochrome, could be clearly demonstrated in these protoplasts. Protoplasts ($5 \cdot 10^6$ in 0.2 ml) gave a phytochrome signal of $2.6 \cdot 10^{-3}$ ($\Delta\Delta A$).

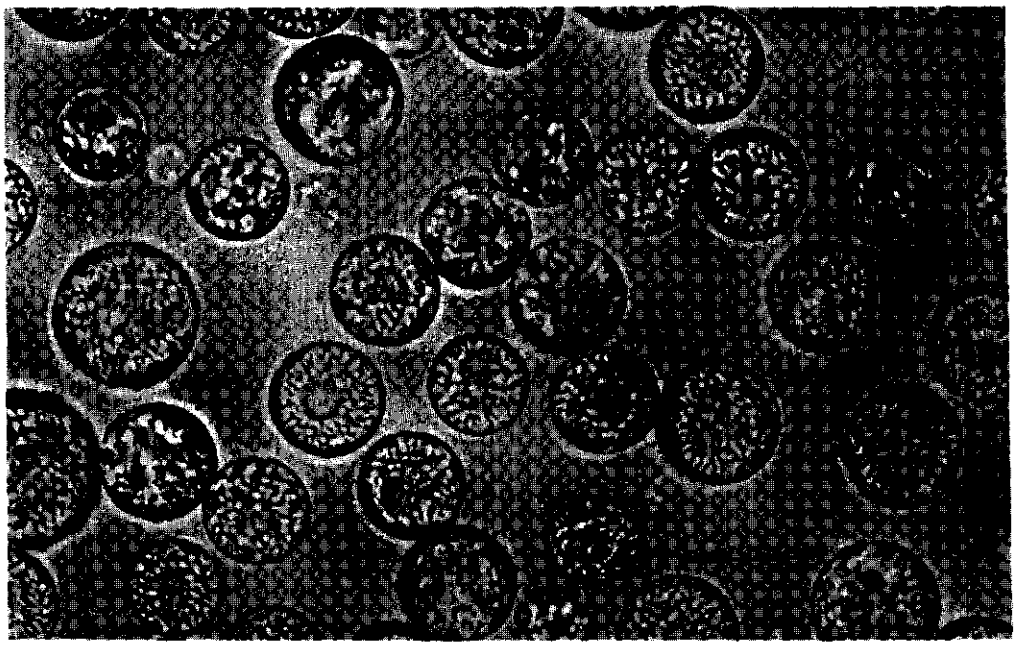


Fig. 2.1. Protoplasts of the primary leaves of dark-grown wheat, immediately after isolation. Bar=50 μm

Red-far-red reversibility. The volume of protoplasts irradiated with R and subsequently incubated in darkness for 30 min in medium A, were consistently 13-18% larger than those irradiated with FR, or of the dark-control (Fig. 2.2A). When FR was given immediately after R, swelling was not observed. Photoreversibility was observed for two R-FR cycles, indicating the involvement of phytochrome in the swelling response. That all protoplasts within the population swelled, is shown by the cumulative size-distribution curves (Fig. 2.2B). The percentage volume increase for small and medium-sized protoplasts was the same (14.5%), and for large protoplasts slightly higher (16.2%).

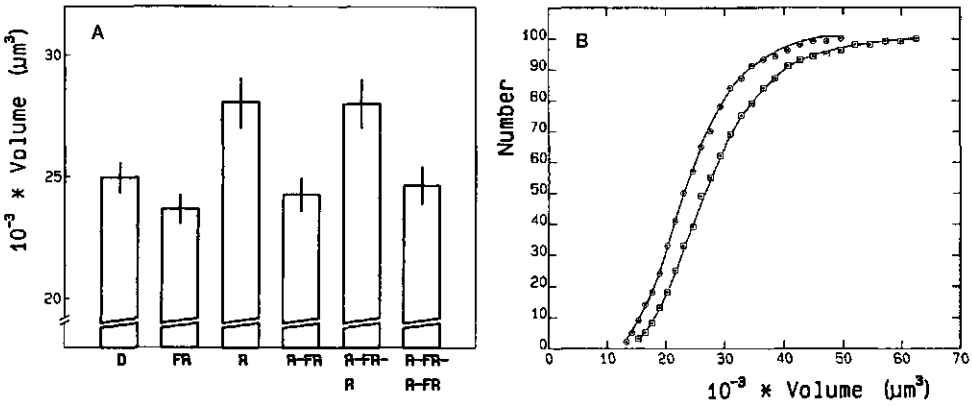


Fig. 2.2A, B. Red-far-red (R-FR) reversibility of protoplast swelling. **A** Protoplasts were maintained in darkness (D) or irradiated with schedules of R (1 min) and FR (5 min). After the last irradiation the protoplasts were incubated for 30 min in darkness in medium A at 22°C, before measurement. Mean volume ± SE is presented. **B** Cumulative size distribution of 100 protoplasts after R or FR irradiation. The mean numbers of eight independent experiments are presented. o, FR; □, R

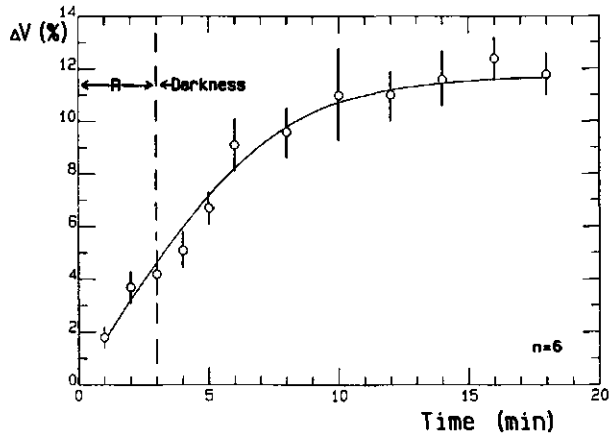


Fig. 2.3. Kinetics of protoplast swelling. The time course of protoplast swelling of individual protoplasts embedded in agarose in medium A at 15°C after 3 min red light (R), expressed as percent volume change (ΔV%) ± SE

Kinetics of swelling. To study the kinetics of protoplast swelling, the diameter of individual protoplasts was measured after R at 15°C (Fig. 2.3). Under these conditions protoplasts started to swell almost immediately after the start of irradiation (within 1 min) and swelling was virtually complete in 10 min. The half-time of swelling was 4.5 min.

Boyle-van't Hoff analysis of swelling. With the aid of Boyle-van't Hoff analysis, a distinction can be made between a volume change caused by a change in nov or induced by a change in solute content of the protoplasts. . Protoplasts were therefore transferred to media with different osmolarity, directly after R or FR irradiation. As shown in Fig. 2.4, FR-irradiated protoplasts had an apparent negative nov, whereas after R the nov was positive. Protoplasts maintained in darkness gave the same results as FR-irradiated protoplasts (results not shown). With these results it is not possible to say whether protoplast swelling after R at 0.5 M sorbitol ($1/\pi$=0.8) is caused by a change in nov or an increase in the solute content of the protoplasts, or a combination of both.

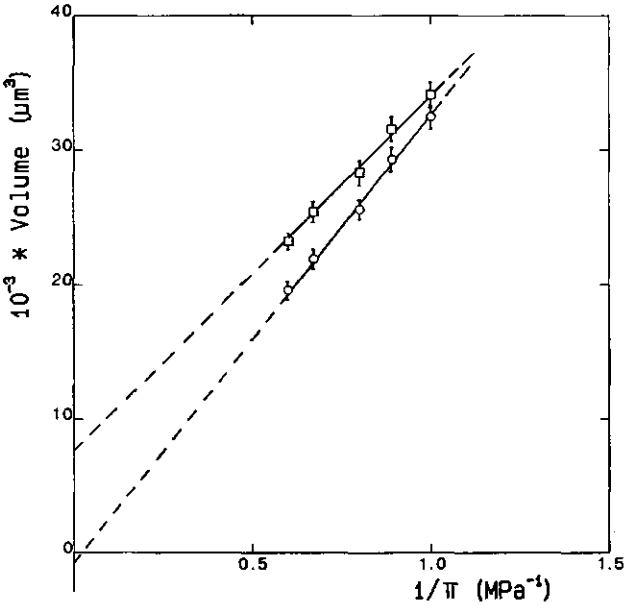


Fig. 2.4. Boyle-van't Hoff analysis of protoplast swelling. Immediately after 1 min red (R) or 5 min far-red (FR) irradiation, protoplasts were transferred to media with different sorbitol concentrations, having values of $1/\pi$ of 0.6-1.0 MPa^{-1}. After 30 min in darkness at 22°C the protoplasts were measured and the volume ± SE calculated. o, FR; □, R

Involvement of Ca^{2+}. To investigate a possible role of Ca^{2+} in the swelling response, Ca^{2+} was replaced by 1 mM of the chloride salts of K^+, Mg^{2+} or Ba^{2+}. In the absence of Ca^{2+}, no swelling after R occurred (Fig. 2.5). No significant enhancement of swelling was observed when the medium contained both Ca^{2+} and K^+. The requirement for Ca^{2+} in the medium indicates that influx of Ca^{2+} is a prerequisite for the response. This was confirmed by experiments with the Ca^{2+}-ionophore A23187 (Table 2.1).

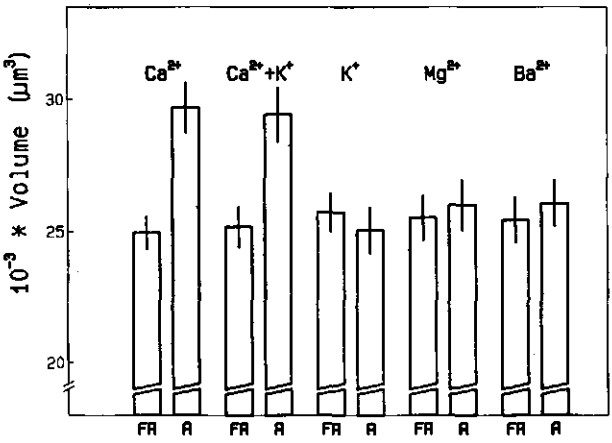


Fig. 2.5. The influence of the ionic content of the medium on protoplast swelling. Protoplasts were washed with medium A, without Ca^{2+} + 50 μM EDTA and resuspended in media with the indicated ions at a concentration of 1 mM. Protoplasts were irradiated with 1 min red (R) or 5 min far-red (FR) and measured after 30 min in darkness at 22°C. Mean volume ± SE is present

Protoplasts swelled to the same extent in darkness, when 10 μM A23187 was added to the medium containing 0.1 or 1 mM $CaCl_2$, as after R in the presence of Ca^{2+}. Addition of the Ca^{2+}-channelblocker Verapamil (10 μM) inhibited R-induced swelling. A similar effect was observed for 0.1 mM $LaCl_3$. In order to resolve whether the presence of Ca^{2+} in the medium is required during or after phytochrome conversion, protoplasts were washed with medium without Ca^{2+}, but with 50 μM EDTA, R-irradiated in Ca^{2+}-free medium A, and supplied with Ca^{2+} at different times after the irradiation. After the addition of Ca^{2+}, the protoplasts were incubated in darkness for 30 min before measurement (Fig. 2.6).

Table 2.1. The effect of ionophore A23187, La^{3+} and Verapamil on protoplast swelling, expressed as percent volume change (ΔV%). Protoplasts were suspended in media containing 1 mM $CaCl_2$, 1 mM $CaCl_2$ + 0.1 mM $LaCl_3$ or 1 mM $CaCl_2$ + 10 μM Verapamil and irradiated for 1 min with red (R) or 5 min with far-red (FR) light. The ΔV% after R is relative to the FR controls. A23187 (10 μM) was supplied in darkness (D) to protoplasts suspended in media with 0.1 or 1 mM $CaCl_2$. ΔV% is relative to the dark control without A23187

Medium	Light regime	ΔV%
$CaCl_2$ (1.0 mM)	R	13.6
$CaCl_2$ (1.0 mM), $LaCl_3$ (0.1 mM)	R	0.8
$CaCl_2$ (1.0 mM), Verapamil (10 μM)	R	-0.5
$CaCl_2$ (1.0 mM), A23187 (10 μM)	D	13.0
$CaCl_2$ (0.1 mM), A23187 (10 μM)	D	13.2

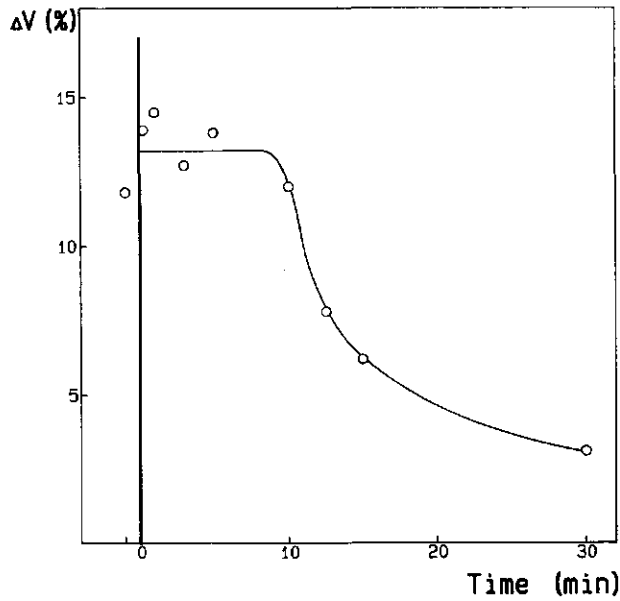


Fig. 2.6. The effect of Ca^{2+} addition after red (R) irradiation. Protoplasts were washed with medium without Ca^{2+}, but with 50 μM EDTA and irradiated with R for 1 min or far-red (FR) for 5 min. $CaCl_2$ (1 mM) was added at various times after irradiation, which terminated at t=0. Swelling is expressed as percent volume change (ΔV %) between FR and R

Calcium could be supplied up to 10 min after R to obtain a full swelling response. Longer delays resulted in a sharp decline of the swelling response and after 30 min no swelling occurred. Protoplasts incubated in medium without Ca^{2+} for a longer period retained the capacity for swelling, since if Ca^{2+} was added before R at the end of the experiment, protoplasts swelled normally (point -1 in Fig. 2.6). Protoplast volume after FR was the same for all times of Ca^{2+} application.

Discussion

The observed phytochrome-controlled swelling of etiolated wheat mesophyll protoplasts incubated at constant osmotic potential ($1/\pi$=0.8) at 22°C (Fig. 2.2) is in agreement with what would be predicted on the basis of the results of Blakeley et al. (1983). In their experimental procedure, protoplasts from dark-grown wheat were induced to shrink at 4°C on transfer to a medium of higher osmotic potential and R, but not FR, was found to prevent shrinkage. The R-induced swelling reported here, required the presence of Ca^{2+} in the medium. When both Ca^{2+} and K^+ were present, K^+ did not enhance the swelling response (Fig. 2.5). This makes it very unlikely that the swelling results from uptake of K^+ by the protoplasts, as is the case for the blue-light-induced swelling of guard-cell protoplasts (Zeiger and Hepler 1977). Blakeley et al. (1983) gave an indication, that the volume difference between FR- and R-irradiated protoplasts was larger when K^+, in addition to Ca^{2+} was present in the medium. They made the assumption that protoplast swelling or more precisely, prevention of shrinkage resulted from the uptake of solutes, especially K^+, from the medium.

When Ca^{2+} was displaced from the membrane by La^{3+} (Dos Remedios 1981), or when the Ca^{2+}-channelblocker Verapamil was added to the medium, no protoplast swelling occurred after R (Table 2.1). In darkness, swelling of protoplasts could be induced by the addition of the Ca^{2+}-ionophore A23187 to the Ca^{2+}-containing medium. These facts combined with the observation that Mg^{2+} and Ba^{2+} cannot replace the Ca^{2+} requirement of the response, leads us to conclude that Ca^{2+} uptake by the protoplasts is necessary for protoplast swelling. Calcium could be transported down the existing electrochemical gradient (Åkerman et al. 1983), through Ca^{2+}-channels, which become open as a result of R. Binding studies of [^{3}H]-Verapamil and [^{3}H]-nitrendipine have indicated the existence of Ca^{2+}-channels in the plasma membrane of plant cells

(Hetherington and Trewavas 1984; Andrejauskas et al. 1985). Other phytochrome-controlled processes have been reported to require external Ca^{2+}. Wayne and Hepler (1984) showed that external Ca^{2+} is required for the R-induced germination of Onoclea spores. A transient increase of total intracellular calcium was observed after R, using atomic absorption spectroscopy (Wayne and Hepler 1985). In *Mougeotia*, uptake of Ca^{2+} is associated with chloroplast rotation. Red light induces an uptake of $^{45}Ca^{2+}$, which is prevented by subsequent FR (Dreyer and Weisenseel 1979). A rise of cytoplasmic $[Ca^{2+}]$ after R could also result from the release of Ca^{2+} from Ca^{2+} containing vesicles (Wagner and Rossbacher 1980). An enhanced, transient uptake of $^{45}Ca^{2+}$ after R, by protoplasts from dark-grown maize leaves, was reported by Das and Sopory (1985), an effect prevented by FR given immediately after R. However, Hale and Roux (1980) have measured an increase of the $[Ca^{2+}]$ in the surrounding medium of oat coleoptile protoplasts after R, using the Ca^{2+}-sensitive dye murexide. Subsequent FR was found to reverse the effect. A Ca^{2+} efflux after R was only found when Ca^{2+} was present in the medium during irradiation or when the protoplasts were pre-incubated in Ca^{2+} before irradiation. Similar to Roux (1983), we reconcile these apparently conflicting results by assuming that R causes a small transient uptake of Ca^{2+}, which is followed by the release of a large amount of Ca^{2+} into the medium. The measurements of Newman (1981) on the electrical potential between the surface of oat coleoptiles and the bathing medium of the roots, are qualitatively in agreement with this model. He found that within seconds after R, a small surface depolarization was followed by a large hyperpolarization. These changes can be explained by a small Ca^{2+} influx, followed by a large Ca^{2+} efflux. A reduction of the negative zetapotential of *Mesotaenium* after R can also be explained by a release of Ca^{2+} into the medium (Stenz and Weisenseel 1986).

When Ca^{2+} is absent from the medium during R-irradiation of the protoplasts, it can be added up to 10 min after irradiation to obtain a full swelling response (Fig. 2.6). If R leads to the opening of Ca^{2+}-channels in the plasma membrane as proposed here, it is necessary to conclude that these channels begin to close 10 min after opening, but how this closing is regulated is not clear. The observed R-FR reversibility indicates that FR, given directly after R, leads to closing of the Ca^{2+}-channels or prevents them opening. This is in agreement with the experiments of Das and Sopory (1985). Far-red light, given directly after R, causes an inhibition of the R-stimulated uptake of $^{45}Ca^{2+}$ by maize proto-

plasts. Whether phytochrome action is directly coupled with the opening/closing of the Ca^{2+}-channels or is coupled by other mediators cannot yet be answered. On the basis of escape studies, where FR was given at varying time intervals after R, Blakeley et al. (1983) and Wayne and Hepler (1984) conclude that, in their respective systems, other events occur between Pfr formation and ion uptake.

When a Boyle-van't Hoff analysis of our osmotic studies was carried out on FR-irradiated protoplasts, the nov appeared to be negative, whereas after R it was positive (Fig. 2.4). Since a negative nov is impossible, we assume that in FR-irradiated protoplasts the plasma membrane did not fulfil the prerequisites for the validity of the Boyle-van't Hoff relationship. Perhaps the plasma membrane of FR-irradiated protoplasts is not completely semi-permeable. In guard-cell protoplasts of *Commelina*, which swell upon irradiation, a rise in nov and a rise in solute content, especially K^+, has been observed (Fitzsimons and Weyers 1986). Whether the swelling of etiolated wheat protoplasts after R is also caused by a rise in both nov and solute content is not clear because no estimate of a nov change can be made. If it is assumed that the nov is the same for FR- and R-irradiated protoplasts (6900 μm^3), the osmotic volume would be 18400 and 21400 μm^3, respectively (data from Fig. 2.4). When possible changes in activity coefficients are neglected, the solute content of protoplasts after R is 14.4% higher. The total solute content of $5 \cdot 10^5$ FR-irradiated protoplasts has been calculated to be $4.6 \cdot 10^{-6}$ mol. An R-induced rise of 14.4% would then correspond to $0.65 \cdot 10^{-6}$ mol. The total amount of $CaCl_2$ in 0.5 ml medium in our experiment was $0.5 \cdot 10^{-6}$ mol. This means it is very unlikely that the rise in solute content of the protoplasts after R is directly caused by uptake of Ca^{2+} and accompanying co-ions. Other causes of an increase in internal solute concentration are uptake of sorbitol from the medium, hydrolysis of storage compounds or release of solutes from internal stores.

The results described in this paper clearly show the occurrence of relatively rapid changes in etiolated wheat protoplasts upon R irradiation at physiological temperatures. These changes are likely to be the consequence of alterations in plasma membrane properties. Protoplasts offer a convenient system for the study of phytochrome-mediated changes associated with the plasma membrane, which could be components of the transduction chain between phytochrome photoconversion and ultimate physiological responses.

Chapter 3

The involvement of a G-protein in phytochrome-regulated, Ca^{2+}-dependent swelling of etiolated wheat (*Triticum aestivum* L.) protoplasts

Margreet E. Bossen, Richard E. Kendrick and Willem J. Vredenberg

Abstract

The red light (R)-induced swelling of mesophyll protoplasts, isolated from dark-grown wheat (*Triticum aestivum* L.) leaves, was inhibited by guanosine-5'-O-(2-thiodiphosphate) (GDP-β-S). In darkness or after control irradiation with far-red light (FR), guanosine-5'-O-(3-thiotriphosphate) (GTP-γ-S) induced swelling to the same extent as after R. The possibility of R-induced activation of the phosphatidyl-inositol pathway of transmembrane signalling was investigated. Neomycin, Li^+ and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H_7) inhibited the R-induced swelling. Phorbol 12-myristate 13-acetate (PMA) induced swelling after control irradiation with FR. Neomycin and Li^+ also inhibited GTP-γ-S-induced swelling. These results suggest that a GTP-binding protein is involved in the phytochrome-regulated swelling response. Addition of N^6,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate (DB-cAMP) induced swelling to the same extent as after R-irradiation. The calmodulin antagonist N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W_7) induced swelling after FR, while R-induced swelling was not inhibited. The less active analogue N-(6-aminohexyl)-1-naphthalenesulfonamide (W_5) induced no swelling after FR. It is speculated that the protoplast volume is correlated with the cytoplasmic $[Ca^{2+}]$.

Introduction

Phytochrome is one of the plant pigments regulating photomorphogenesis. Much is known about the phytochrome molecule itself (Song 1988) and about the reversible photoconversion between its red light (R)-absorbing form (Pr) and the far-red light (FR)-absorbing form (Pfr) (Kendrick and Spruit 1977; Inoue 1987). However, little is known about the signal transduction chain between formation of active Pfr and ultimate physiological responses e.g. seed germination, germination of fern spores and de-etiolation. Some rapid phytochrome responses, occurring in the time range of seconds, appear to be associated with the plasma membrane, e.g. changes in membrane potential (Racusen 1976; Weisenseel and Ruppert 1977) and uptake of $^{45}Ca^{2+}$ by maize protoplasts (Das and Sopory 1985). These changes could be part of the signal transduction chain of phytochrome action. It has been shown that the presence of Ca^{2+} is essential for a number of phytochrome-regulated responses to occur, e.g. chloroplast rotation in *Mougeotia* (Dreyer and Weisenseel 1979), fern spore germination of *Onoclea* (Wayne and Hepler 1984) and *Dryopteris* (Scheuerlein et al. 1989), swelling of protoplasts isolated from

dark-grown wheat leaves (Bossen et al. 1988), leaf unrolling of dark-grown barley (Viner et al. 1988) and phytochrome regulated gravitropism of maize cv. 'Merit' (Perdue et al. 1988). An increase of the cytoplasmic Ca^{2+}-concentration ($[Ca^{2+}]_{cyt}$) and total cellular Ca^{2+}, have been measured in *Mougeotia* (Dreyer and Weisenseel 1979) and *Onoclea* (Wayne and Hepler 1985) respectively. While there is no evidence for a direct coupling of Ca^{2+} with the primary action of Pfr, it has been proposed that formation of Pfr leads to the opening of Ca^{2+}-channels in the plasma membrane, resulting in enhanced transport of Ca^{2+} into the cytoplasm and a rise in $[Ca^{2+}]_{cyt}$ (Roux 1983; Bossen et al. 1988). Scheuerlein et al. (1989) demonstrated a clear separation in time between phytochrome action and the requirement for Ca^{2+} in the case of fern spore germination.

Enhanced uptake of Ca^{2+} ions through Ca^{2+}-permeable channels and a rise in $[Ca^{2+}]_{cyt}$ are well known phenomena in animal cells, after interaction of the cells with several types of hormones, growth factors, light and other agonists (Gomperts 1983; Reuter 1983; see also Hepler and Wayne 1985 and references therein). It has been proposed that the primary event is the binding of the agonists to specific receptors on the plasma membrane, after which so called GTP-binding proteins (G-proteins) are activated, which in turn activate different enzymes, depending on the type of G-protein (see for reviews Stryer and Bourne 1986; Berridge 1987; Cockcroft 1987; Gilman 1987; see also Fig. 3.1). Activation of the G-proteins eventually leads, among other things, to opening or enhanced opening probability of Ca^{2+}-channels in the plasma membrane (Gomperts 1983; Dunlap et al. 1987; Meldolesi and Pozzan 1987).

We have investigated the possible involvement of a G-protein in the phytochrome-regulated, Ca^{2+}-dependent swelling of etiolated wheat protoplasts, by introducing the inhibitor GDP-β-S and the activator GTP-γ-S of G-proteins into the protoplasts. To obtain information about the type of G-protein which might be involved, the effect of neomycin, Li^+, H_7 and PMA, known as modulators of the phosphatidyl-inositol pathway in animal cells, have been tested. Chung et al. (1988) reported that phytochrome, dibutyryl-cAMP (DB-cAMP) and gibberellic acid induce swelling of etiolated oat protoplasts. We have made a comparison between the effect of neomycin and Li^+ on DB-cAMP-induced and phytochrome-regulated swelling. The influence of the anti-calmodulin compounds W_7 and W_5 was also investigated since Gilroy et al. (1987) showed, that W_7 causes an elevation of $[Ca^{2+}]_{cyt}$ in carrot cell protoplasts.

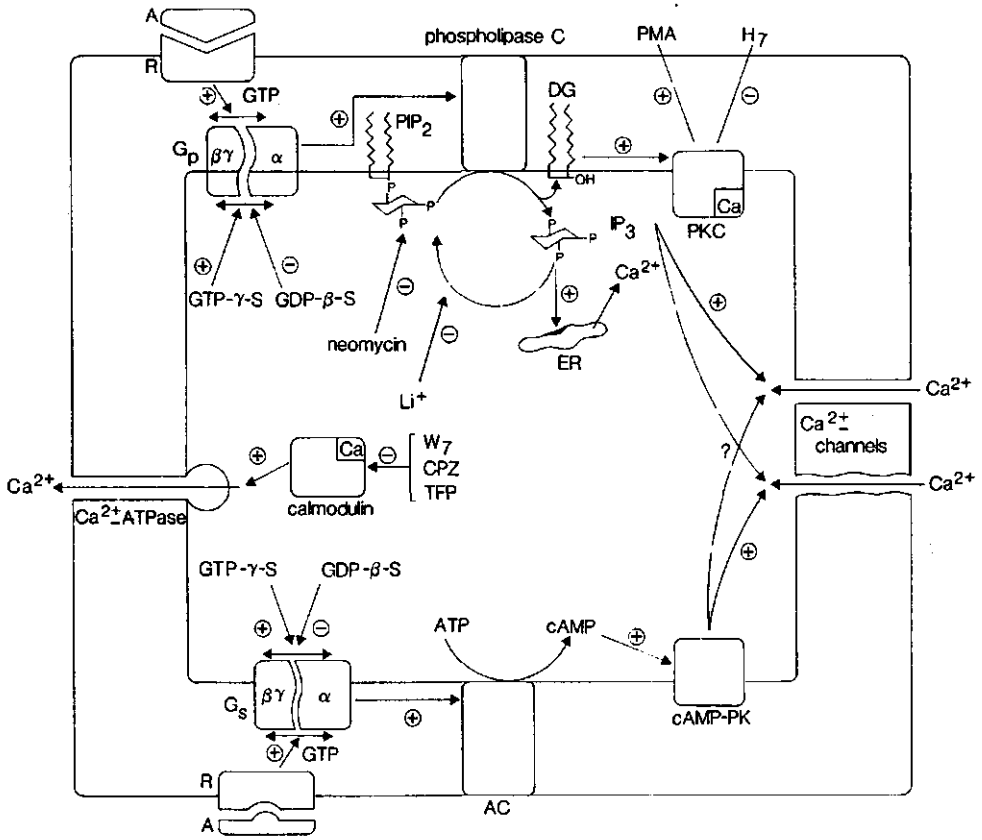


Fig. 3.1. Schematic representation of the phosphatidyl-inositol and adenylate cyclase pathway of signal transduction as known in animal cells. Phosphatidyl-inositol pathway: upon interaction of an agonist (A) with a receptor (R), a GTP-binding protein (G_p) is activated, by an exchange of bound GDP by GTP on the α-subunit. The subsequent dissociation of the α and β subunit can also be induced by GTP-γ-S, in the absence of an agonist. The dissociation can be inhibited by GDP-β-S. The activated G_p-α-subunit activates phospholipase C, which catalyses the hydrolysis of phosphatidyl-inositol 4,5-biphosphate (PIP_2), forming inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG). While IP_3 is released in the cytoplasm, where it interacts with receptors on the endoplasmic reticulum (ER) leading to release of Ca^{2+}, DG remains in the plasma membrane and activates protein kinase C (PKC). This kinase can also be stimulated by PMA. Ultimately the activation of G_p leads to opening of Ca^{2+}-channels in the plasma membrane. In the phosphatidyl-inositol cycle, PIP_2 is reformed from IP_3, which is inhibited by Li^+. Neomycin binds to PIP_2, inhibiting the action of phospholipase C. Protein kinase C is inhibited by H_7.

Adenylate cyclase pathway: the α-subunit of another G-protein, G_s, activates adenylate cyclase, which catalyses the formation of cAMP from ATP. The cAMP formed, stimulates a cAMP-dependent protein kinase (cAMP-PK). This kinase, which is also inhibited by H_7, activates a different type of Ca^{2+}-channel in the plasma membrane. A Ca^{2+}-calmodulin activated Ca^{2+}-ATPase is present in the plasma membrane. The calmodulin antagonists W_7, chlorpromazine (CPZ) and trifluoperazine (TFP) prevent the activation of this ATPase. This scheme is based partially on the model of Cockcroft (1987)

Material and methods

Plant material. Wheat (*Triticum aestivum* L., cv. Arminda) was sown in a mixture of Vermiculite and Perlite (1:1) in pots and placed, in darkness at 25°C, in a controlled air-flow cabinet. The air was purified and humidified by passing it first through a column with activated carbon Norit $RBAA_3$ (Norit, Amersfoort, the Netherlands) and then through a water column. After 8-11 d plants were harvested and the primary leaves were used for protoplast isolation.

Protoplast isolation. Protoplasts were isolated as described by Bossen et al. (1988). Primary leaves were cut in pieces (1 mm) and incubated in 3% cellulase Onozuka R10, 0.3% macerozym (Kinky Yakult, Nishinomiya, Japan), 0.5 M sorbitol, 1 mM $CaCl_2$ pH 5.6 for 3.5-4 h at 22°C. After purification on a discontinuous sorbitol/sucrose gradient, protoplasts were resuspended in the incubation medium consisting of 0.5 M sorbitol, 5 mM 2-(N-morpholino)ethanesulfonic acid (Mes) adjusted to pH 6.0 with 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) and 1 mM $CaCl_2$. Protoplasts were tested for viability with 0.2% phenosafranine (Widholm 1972). In the electroporation experiments 0.05% phenosafranine was used. All manipulations were performed under dim green safelight (26 $nmol \cdot m\text{-}2 \cdot s^{-1}$).

Light sources and irradiations. Monochromatic light was obtained from a 250 W quartz-iodine lamp in a custom-built projector using interference filters (Balzer B40, Liechtenstein) with a half bandwidth of approx. 10 nm. The fluence rates used were for R (660 nm): 150 and for FR (729 nm): 75 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The fluence rates were measured with a photodiode meter, Optometer type 80X (United Detectors Technology Inc., Santa Maria, CA., USA). Irradiation times were 1 min for R and 3 min for FR, sufficient to saturate phytochrome photoconversion.

Protoplast measurement. The estimation of protoplast volume was essentially as described by Bossen et al. (1988). After irradiation or other treatment, protoplasts ($0.5 \cdot 10^6$ in 0.5 ml) were kept in darkness for 30 min, at 22°C. Samples were taken, placed on a haemocytometer (Fuchs-Rosenthal) and photographed. The negatives were projected and the diameters of 100 protoplasts measured. The volume of each measured protoplast was calculated and results are expressed as mean volume ± SE. All experiments have been repeated at least three times with qualitatively similar results. Some results are expressed as percentage swelling in respect to a control (mean % ± SE), for the number of experiments indicated. Neomycin sulfate, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H_7), N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W_7), N-(6-aminohexyl)-1-naphthalenesulfonamide (W_5), phorbol 12-myristate 13-acetate (PMA) all from Sigma (St. Louis, USA) and LiCl, KCl, NaCl, K_2SO_4, $CaCl_2$ and *myo*-inositol (Merck, Darmstadt, BRD), were added from 10x concentrated stock solutions in incubation medium, 15 min before irradiation.

Electroporation. Before electroporation, protoplasts were washed twice with an ice-cold solution of 0.5 M sorbitol, adjusted to pH 6.0 with a few drops of 0.1 M KOH, without any further additions. Electroporation was performed in an electroporation chamber which consists of two gold-coated glass panel electrodes, placed 0.5 cm apart with a PVC spacer, and has a total volume of 1 ml. The power supply (Delta Electronics, Zierikzee, the Netherlands) was set at 225 V, yielding an electric field of 450 V/cm. Protoplasts, $(0.5 - 1.0) \cdot 10^6$ in 1 ml were pipetted into the precooled chamber and 0.47 μF capacitor was discharged three times at a rate of $1 \cdot s^{-1}$ (Rezelman et al. 1989). Protoplasts were left 10 min on ice, then incubated for 75-90 min at 26°C to allow recovery of their membrane integrity, and resuspended in incubation medium. Guanosine-5'-O-(2-thio-diphosphate) (GDP-β-S) and guanosine-5'-O-(3-thiotriphosphate) (GTP-γ-S) from Boehringer (Mannheim, BRD) were added from 100x concentrated stock solutions in 0.5 M sorbitol, directly after the electroporation pulses.

Results

Plant growth. Plants were grown in a controlled air-flow cabinet, through which purified air was passed continuously. Compared to plants grown in the same dark-room, but outside the cabinet, protoplast yield increased at least two fold, from $0.6 \cdot 10^6$ to more than $1.2 \cdot 10^6$ protoplasts/g leaves. Protoplast quality was also improved, especially in winter. The improved quality of the protoplasts was evident when they were purified on the discontinuous sorbitol/sucrose gradient. Protoplasts isolated from plants grown outside the cabinet, did not float on the sucrose layer, but sank, presumably due to sucrose uptake. Such protoplasts failed to swell when they were irradiated with R, in

the presence of Ca^{2+}, although they excluded the vital dye phenosafranine (results not shown). This could be due to damage to the plasma membrane, caused by air pollutants, such as ozone, present in the phytotron during the winter period. It has been reported that ozone causes loss of membrane integrity, solute leakage, breakdown of phospholipids, oxidation of polypeptides external to the plasma membrane and oxidative inactivation of protein kinase C (PKC) (Mudd et al. 1984; Gopalakrishna and Anderson 1987).

G-proteins. To investigate the possibility that a G-protein is involved in phytochrome regulated swelling of protoplasts, GDP-β-S was introduced into the cytoplasm. This compound competitively inhibits the binding of GTP to G-proteins, resulting in their inactivation. Activity of G-proteins was also tested with the aid of the non-hydrolysable GTP analogue, GTP-γ-S. Both GDP-β-S and GTP-γ-S are membrane impermeable and therefore these effectors were transported into the cytoplasm by means of electroporation. Poration was followed with the red exclusion dye phenosafranine in parallel samples. Before electroporation, approx. 95% of the protoplasts excluded phenosafranine. If the dye was added immediatedly after the electroporation pulses approx. 90% of the protoplasts were stained red, indicating that these protoplasts were porated. If the dye was added after the recovery period of 75-90 min, approx. 90% of the protoplasts excluded the dye, indicating that almost all the protoplasts had recovered their membrane integrity (Table 3.1).

Table 3.1. Effectiviness of protoplast electroporation. Protoplasts [$(0.5\text{-}1.0)\cdot10^6$/ml] were electroporated in an ice-cold medium of 0.5 M sorbitol pH 6.0. Phenosafranine (final concentration 0.05%) was added before electroporation, directly after the electroporation pulses or after a recovery period of 75-90 min at 26°C. The percent ± SE of stained cells in the population from six experiments is shown

Staining	Cells stained (% ± SE)
Before electroporation	4 ± 3
Directly after pulses	88 ± 2
After recovery	12 ± 4

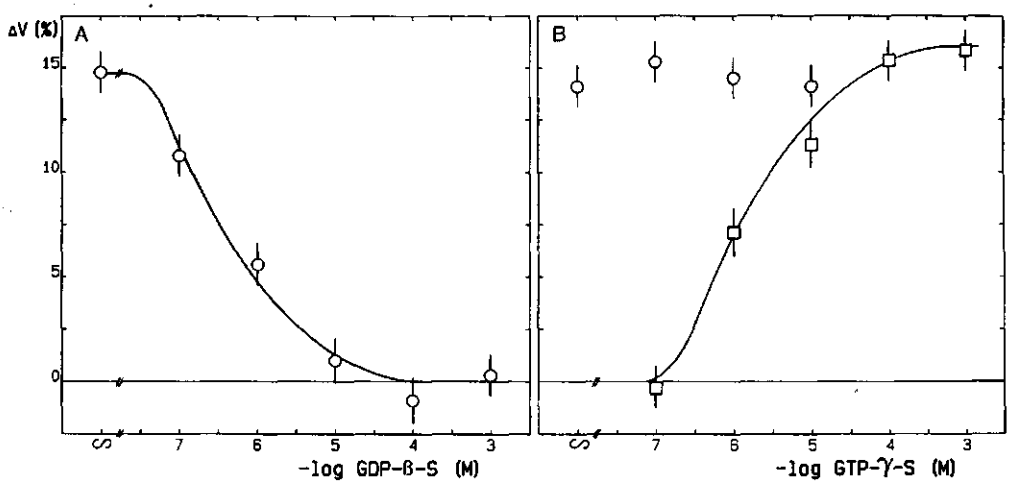


Fig. 3.2. Effect of G-protein inhibitor GDP-β-S and activator GTP-γ-S on protoplast swelling. These compounds were added directly after the electroporation pulses. After a recovery period of 75-90 min, protoplasts were irradiated with saturating far-red light (FR) or red light (R). After a further 30 min incubation in darkness, protoplast diam. were measured. **A** Effect of GDP-β-S on R-induced swelling. **B** Effect of G-protein activator GTP-γ-S on the volume of FR-(□——□) or R-(O——O) irradiated protoplasts. Swelling is expressed as percent volume change (ΔV%) compared to the FR control, and is the mean ± SE from three independent experiments

After recovery from the electroporation, the protoplasts were still able to swell after R irradiation, in the presence of Ca^{2+} (R control, Fig. 3.2A). Addition of GDP-β-S immediatedly after the pulses and before recovery, inhibited the R-induced swelling. Total inhibition was obtained with concentrations of 10 μM and higher. When GTP-γ-S was added to electroporated protoplasts, swelling occurred when they were incubated in darkness or after control irradiation with FR, only when Ca^{2+} was present in the medium (Table 3.2). This swelling was slightly greater than that induced by R. When sub-optimal concentrations of GTP-γ-S were used (< $3 \cdot 10^{-5}$ M) protoplasts swelled after R to the same extent as the control without addition of GTP-γ-S (Fig. 3.2B).

Table 3.2. The involvement of Ca^{2+} in GTP-γ-S-induced protoplast swelling. Directly after the electroporation pulses 0 (control) or 100 μM GTP-γ-S (final conc.) was added. After a recovery period of 75 min, protoplasts were washed with electroporation medium and resuspended in incubation medium with or without 1 mM $CaCl_2$. After a further 30 min incubation in darkness protoplast diam. were measured. Mean volume from a single, representative experiment is shown. The SE was between 600 and 700 μm^3

Compound	Volume (μm^3)	
	- Ca^{2+}	+ Ca^{2+}
Control	19 000	18 500
GTP-γ-S	18 800	21 400

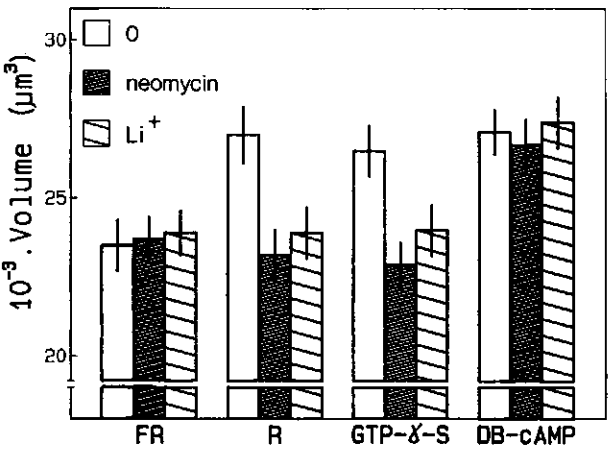


Fig. 3.3. Influence of neomycin and Li^+ on red light (R)-, DB-cAMP- and GTP-γ-S-induced swelling, as compared to the far-red-light (FR) control. Neomycin (10 μM) or LiCl (10 μM) were added 15 min prior to irradiation, addition of DB-cAMP or, together with GTP-γ-S immediatedly after the electroporation pulses. Protoplast diam. were measured after 30 min incubation in darkness. Mean volume ± SE from a single, representative experiment is presented

Phosphatidyl-inositol pathway. To investigate the possibility that the phosphatidyl-inositol pathway is either part of or associated with the events between phytochrome photoconversion and influx of Ca^{2+} through channels in the plasma membrane, the effect on protoplast swelling of inhibitors and activators of elements of this pathway, as known in animal cells, was studied (see Fig. 3.1). Neomycin inhibited R-induced swelling completely at a concentration of 10 μM (Fig 3.3), while no inhibition was observed at 1 μM. Concentrations higher than 30 μM were harmful to the protoplasts. In this case, protoplasts were no longer spherical and were unable to exclude the dye phenosafranine, indicating leakiness of the plasma membrane. Swelling after addition of GTP-γ-S was also inhibited by 10 μM neomycin, while the same concentration had no effect on the volume of FR-irradiated protoplasts. This observation is consistent with the specific inhibition of phospholipase C action by binding of neomycin to the substrate phosphatidyl-inositol 4,5-biphospate (PIP_2) (Schacht 1978; Cockcroft and Gomperts 1985). Lithium ions are known to inhibit inositol-1-phosphatase (Halcher and Sherman 1980) and could therefore diminish the availability of PIP_2. Addition of 10 μM LiCl inhibited both R- and GTP-γ-S-induced swelling in darkness, while there was no effect on the FR-irradiated control (Fig. 3.3). At this concentration (10 μM) the salts KCl, NaCl and K_2SO_4 had no inhibitory effect on the R-induced protoplast swelling. The last salt was tested because neomycin was added as the sulfate. The addition of 1 mM *myo*-inositol prevented the inhibitory effect of Li^+ on R-induced swelling, while alone it had no effect on protoplast volume after FR or R. (Table 3.3).

Red light-induced swelling was totally inhibited by $3 \cdot 10^{-6}$ M H_7, while half-maximal inhibition was at $3 \cdot 10^{-7}$ M (Fig. 3.4A). Protein kinase C is inhibited by H_7 (Hidaka et al. 1984), while phorbol 12-myristate 13-acetate (PMA) has been shown to activate PKC by binding to this enzyme (Castagna et al. 1982). Very low concentrations of PMA, in the range between $3 \cdot 10^{-11}$ and $3 \cdot 10^{-9}$ M caused swelling of protoplasts in darkness or after control irradiation with FR. This swelling was approx. 75% of R-induced swelling. The R-induced swelling was not influenced by PMA in the concentration range of 10^{-11} to $3 \cdot 10^{-8}$ M (Fig. 3.4B). As a control 4α-phorbol was used. This compound failed to induce protoplast swelling. When H_7 was added prior to PMA, protoplast swelling was inhibited (Table 3.4).

Table 3.3. The influence of Li^+, K^+, Na^+, SO_4^{2-} and *myo*-inositol on R-induced swelling. The salts KCl, NaCl and K_2SO_4 (all 10 μM) and *myo*-inositol (1 mM) were added 15 min prior to saturating far-red light (FR) or red light (R). *myo*-Inositol was added 5 min prior to LiCl when both compounds were used. After a further 30 min incubation in darkness protoplast diam. were measured. Mean volume from a single, representative experiment is presented. The SE was in all cases between 500 and 700 μm^3

Compound	Volume (μm^3)	
	FR	R
Control	18 100	20 700
LiCl	18 300	17 700
KCl	18 100	20 900
NaCl	18 300	20 500
K_2SO_4	17 900	20 700
myo-Inositol	18 000	20 600
myo-Inositol, LiCl	18 300	21 200

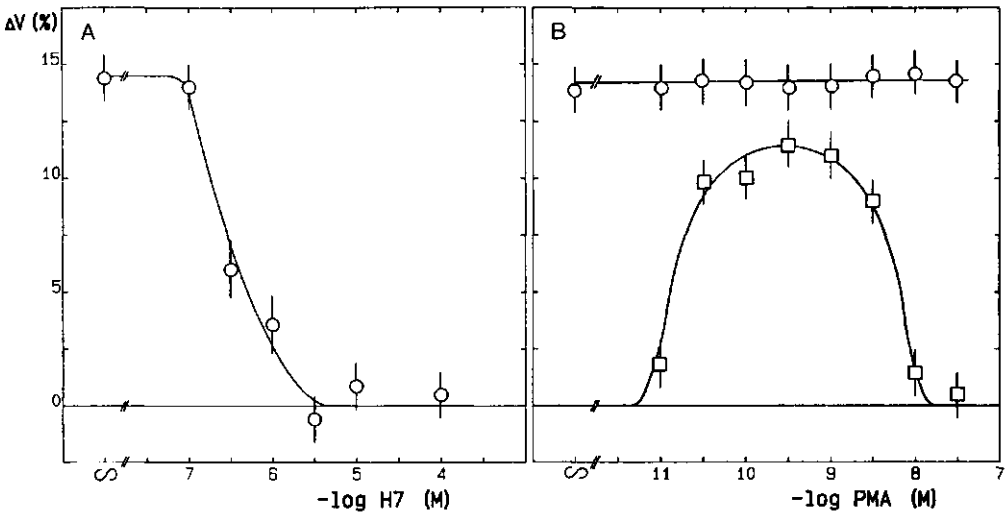


Fig. 3.4. The effect of a protein kinase C inhibitor (H_7) and of an activator (PMA) on protoplast swelling. The compounds were added 15 min prior to saturating far-red light (FR) or red light (R). After a further 30 min incubation in darkness protoplast diam. were measured. A Effect of H_7 on R-induced swelling. B Effect of PMA on protoplast swelling after FR (□——□) or R (O——O). Swelling is expressed as percent volume change (ΔV%) compared to the FR control. The mean ± SE of three independent experiments is shown

Table 3.4. Specificity of PMA-induced swelling. 4α-Phorbol and PMA were added prior to saturating control far-red light. The compound H_7 (10 μM) was added 5 min prior to PMA. After a further 30 min incubation in darkness, protoplast diam. were measured. Mean volume from a single, representative experiment is presented. The SE was in all cases between 600 and 700 μm^3

Compound	Volume (μm^3)
Control	22 900
PMA (10^{-10} M)	25 500
PMA (10^{-9} M)	25 100
4α-Phorbol (10^{-10} M)	22 800
4α-Phorbol (10^{-9} M)	23 100
H_7 (10^{-5} M), PMA (10^{-10} M)	22 800

Swelling induced by DB-cAMP. In animal cells activation of the G-protein G_s has been shown to result in activation of adenylate cyclase, which catalyses the formation of the second messenger cAMP. The latter is also involved in regulation of the influx of Ca^{2+} into the cytoplasm of these cells (Reuter 1983). A membrane permeable analogue of cAMP, DB-cAMP (30 μM) was added to the protoplasts. In the presence of 1 mM $CaCl_2$ they swelled to the same extent as after R (Fig. 3.3). This swelling was not inhibited by 10 μM neomycin or 10 μM LiCl when added 15 min prior to addition of DB-cAMP.

Calmodulin antagonists. It has been reported that calmodulin antagonists, such as chlorpromazine and trifluoperazine inhibit phytochrome-regulated responses, e.g. fern spore germination (Wayne and Hepler 1984). We therefore investigated the effect of calmodulin antagonists W_7 and W_5 on phytochrome-regulated protoplast swelling. These compounds are very similar, but W_7 binds to calmodulin with a much higher affinity (Hidaka et al. 1981). Surprisingly, the addition of 10 μM W_7 caused swelling of protoplasts incubated in darkness or after control irradiation with FR, and had no inhibitory effect on R-induced swelling (Fig. 3.5). No effect of W_5 (10 μM) on the swelling response was observed after FR or R irradiation.

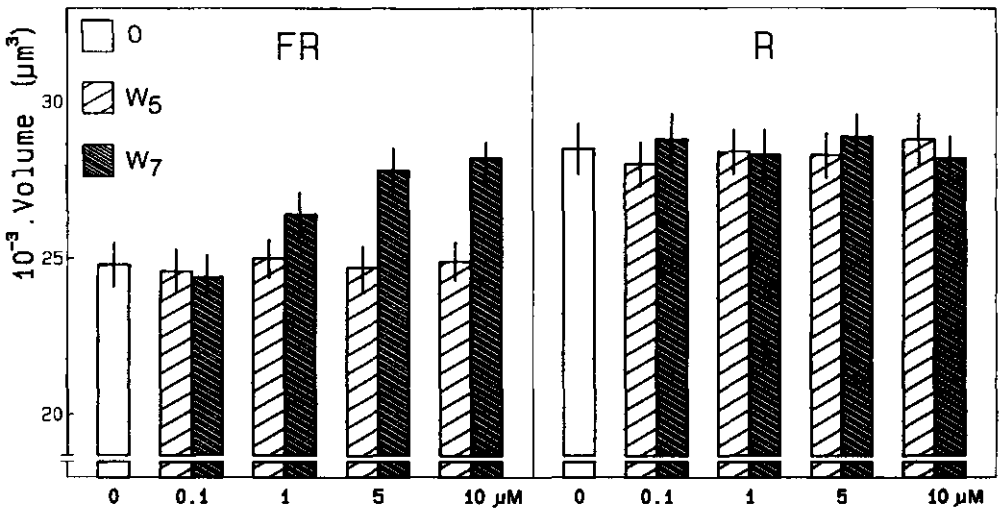


Fig. 3.5. The effect of the calmodulin antagonists W_7 and W_5 on protoplast swelling. The compounds were added 15 min prior to saturating far-red light (FR) or red light (R). After 30 min incubation in darkness, protoplast diam. were measured. Mean volume ± SE from a single, representative experiment is presented

Discussion

G-proteins play an important role as transducers in transmembrane signalling in animal cells (see for reviews Stryer and Bourne 1986; Gilman 1987). All types of G-proteins can be activated by the non-hydrolysable analogue of GTP, GTP-γ-S (Cockcroft 1987; Gilman 1987). The effect of GTP-γ-S is inhibited competitively by GDP-β-S (Holz et al. 1986; Taylor et al. 1988). The activation of G-proteins by hormones is also inhibited by GDP-β-S (Uhing et al. 1986). Recently, G-proteins have been identified in the plasma membrane of several higher plants (Blum et al. 1988). In isolated membranes from *Acer pseudoplatanus* the release of inositol-phosphate was found to be activated by GTP-γ-S and inhibited by GDP-β-S (Dillenschneider et al. 1986). In Fig. 3.2 it is shown that the R-induced swelling of etiolated wheat protoplasts is inhibited by GDP-β-S, while GTP-γ-S causes swelling after control irradiation with FR. The effective concentrations of these compounds were in the same range as those found to be effective

in animal cells. (Gomperts 1983; Mustelin et al. 1986). However, the actual cytoplasmic concentrations are unknown, because these compounds have to be transported into the cytoplasm by means of permeabilization of the plasma membrane. We conclude that it is likely that a G-protein is involved in phytochrome regulated protoplast swelling. This implies that the phytochrome molecule itself, or a product formed as a consequence of Pfr formation, is at least transiently bound to, or associated with the plasma membrane.

In plants there is also some evidence that the phosphatidyl-inositol transduction pathway is involved in hormone action. Components of this pathway, such as membrane-bound phospholipase C (Helsper et al. 1987; Melin et al. 1987; Pfaffman et al. 1987) and a PKC-like enzyme (Olah and Kiss 1986; Elliott and Kokke 1987) have been isolated. Rapid changes in phosphatidyl-inositol turnover have been reported after addition of auxins (Ettlinger and Lehle 1988; Zbell and Walter-Back 1988) and after irradiation of *Saman samanea* leaf pulvini with white light (Morse et al. 1989). As observed here, the swelling induced by R and by GTP-γ-S were inhibited specifically by 10 μM neomycin, as well as by 10 μM LiCl, both known as inhibitors of phospholipase C action in animal cells (Fig. 3.3, Table 3.3). These concentrations are much lower than those required in animal cells where concentrations of approx. 1-3 mM are necessary for inhibition (Cockcroft and Gomperts 1985; Mustelin et al. 1986). Such high concentrations of neomycin resulted in destruction of the plasma membrane of etiolated wheat protoplasts. Moreover, 1 mM LiCl failed to inhibit R-induced swelling. The reason for these differences in effective concentrations is as yet unknown, but may be attributed to differences in plasma membrane composition or to the sensitivity of the enzyme involved. In intact roots of maize 'Merit', phytochrome-regulated gravitropism was inhibited by Li^+, but only at concentrations >1 mM (Perdue et al. 1988). Addition of neomycin and Li^+ did not result in inhibition of swelling caused by DB-cAMP, indicating that these compounds do not inhibit the swelling response per se.

In darkness or after control FR irradiation swelling was stimulated specifically by PMA at very low concentrations (range $3 \cdot 10^{-11}$ to $3 \cdot 10^{-9}$ M) (Fig. 3.4 and Table 4.4). However, swelling induced by PMA was less than after R irradiation in the absence or presence of the same PMA concentration. This could mean that not all protoplasts are activated by PMA or that R-induced swelling is not or not only dependent on the activation of a

PKC-like enzyme. Furthermore, protoplasts also swelled after R irradiation in the presence of high PMA concentrations (>10^{-9} M), which alone induced no swelling. Reports in the literature on the effects of phorbol esters on activation of plant systems are very scarce. Addition of 10^{-11} to 10^{-9} M 12-O-tetradecanoyl-phorbol-13-acetate (TPA), another phorbol activator of PKC, to perfused *Nitella syncarpa* cells, resulted in changes of activity of Ca^{2+}-channels (Zherolova 1989). Phytoalexin formation was induced by addition of 30 nM PMA to carrot cells (Kurosaki et al. 1987b). Both R- and PMA-induced protoplast swelling were inhibited by the PKC-inhibitor H_7. However, this compound can also inhibit a cAMP-dependent protein kinase (Hidaka et al. 1984). Both kinases are involved in activation of different types of Ca^{2+}-channels in the plasma membrane of animal cells. The inhibition of R-induced swelling by H_7 suggests that one of these enzymes is involved in phytochrome-regulated swelling. Since swelling is also inhibited by neomycin and Li^+ (Fig. 3.3) it is more likely that the phosphatidyl-inositol pathway is activated after Pfr formation. However, the involvement of cAMP cannot be excluded, for addition of DB-cAMP also induced protoplast swelling in the presence of Ca^{2+} (Fig. 3.3). This is in agreement with the observation of Chung et al (1988), that DB-cAMP induces swelling of etiolated oat protoplasts, incubated for 2 d at 4°C.

All agonists and antagonists used influenced the swelling response, as predicted by transposition of the animal model to plants. The agonists GTP-γ-S and PMA induced swelling in darkness or after control FR irradiation and the antagonists GDP-β-S, neomycin, Li^+ and H_7 inhibited R-induced swelling. These results suggest that a signal transduction system is functional in plants, which is comparable to one occurring in animal cells. This does not necessarily imply, that the enzymes involved have exactly the same properties in plants and animals. The GTP-γ-S and PMA-induced swelling after control FR irradiation indicate that not only the presence, but the activation of a G-protein and a protein kinase are necessary for protoplast swelling to occur. The involvement of Ca^{2+}-channels in protoplast swelling has been shown previously: swelling induced by R is inhibited by Ca^{2+}-channel blockers (Bossen et al. 1988; Tretyn et al. in press) and swelling is induced in darkness or after control FR irradiation by the Ca^{2+}-channel agonist Bay K-8644, (Tretyn et al. in press). The results presented here are consistent with a model in which Pfr acts via a G-protein, phospholipase and protein kinase to activate Ca^{2+}-channels, which would imply that it is

unnecessary that Pfr and Ca^{2+} are present at the same time. This is in agreement with the results of Scheuerlein et al. (1989), who showed that, for Ca^{2+}-dependent fern spore germination, Ca^{2+} can be added some time after the active form of phytochrome, Pfr, has been removed by FR. However, our results give no indication whether activation of the G-protein is closely coupled to Pfr formation.

The calmodulin antagonist W_7 (10 μM) caused swelling of protoplasts after control irradiation with FR, while the analogue W_5, which has a much lower affinity for calmodulin, did not (Fig. 3.5). Gilroy et al. (1987) showed that within 15 min after addition of 10 μM W_7 to carrot cell protoplasts the $[Ca^{2+}]_{cyt}$ increased from approx. 0.35 to 3 μM, as measured with the Ca^{2+}-indicator quin-2. Since we added W_7 15 min prior to irradiation, this could mean that the $[Ca^{2+}]_{cyt}$ of etiolated wheat protoplasts was already elevated before FR or R irradiation. The rise of $[Ca^{2+}]_{cyt}$ could be a consequence of a plasma membrane located Ca^{2+}-ATPase being inactive, which is normally activated by calmodulin, as proposed by Gilroy et al. (1987). Rincon and Boss (1987) showed that in fusogenic carrot protoplasts the efflux of Ca^{2+}, evoked by IP_3, was also inhibited by the calmodulin antagonist trifluoperazine. Some calmodulin antagonists, such as chlorpromazine and trifluoperazine, have been shown to inhibit the PKC of animal cells (Schatzman et al. 1981) at relatively low concentrations (10-100 μM). It is therefore possible that the inhibition of the phytochrome-regulated fern spore germination (Wayne and Hepler 1984) by chlorpromazine and trifluoperazine is not a result of its anti-calmodulin activity, but of its inhibitory action on PKC. As shown previously (Bossen et al. 1988), protoplasts also swell after addition of the Ca^{2+}-ionophore A23187 in the presence of Ca^{2+} in the medium, or after activation of Ca^{2+}-channels by Bay K-8644 (Tretyn et al. in press). The swelling induced by W_7 is also thought to be a direct effect of an increase in the $[Ca^{2+}]_{cyt}$. We therefore propose that the protoplast volume is correlated with $[Ca^{2+}]_{cyt}$.

Chapter 4

Comparison between swelling of etiolated wheat (*Triticum aestivum* L.) protoplasts induced by phytochrome and α-naphthaleneacetic acid, benzylamino-purine, gibberellic acid, abscisic acid and acetylcholine

Margreet E. Bossen, Andrzej Tretyn, Richard E. Kendrick and Willem J. Vredenberg

Abstract

Swelling of mesophyll protoplasts, isolated from dark-grown wheat (*Triticum aestivum* L.) can be induced, if 0.5 mM Ca^{2+} is present in the medium, by (i) red light, (ii) the hormones α-naphthaleneacetic acid, benzylaminopurine, gibberellic acid, abscisic acid, (iii) acetylcholine (ACh). In the absence of Ca^{2+}, or when the Ca^{2+}-channelblocker Verapamil is present, no swelling was observed. However, when Ca^{2+} was replaced by K^+ or Na^+, ACh also induced swelling. The Ca^{2+}-dependent swelling was, in all cases, inhibited by guanosine-5'-O-(2-thiodiphosphate) (GDP-β-S), indicating the involvement of a G-protein. The K^+/Na^+-dependent ACh-induced swelling was not inhibited by GDP-β-S. Taken together these results suggest that all hormones tested, have receptors present on the plasma membrane of etiolated wheat protoplasts, which can activate a G-protein.

Introduction

The importance of Ca^{2+} in plant development and as "second messenger" in plant hormone action is now well recognized (Hepler and Wayne 1985; Trewavas 1986; Poovaiah and Reddy 1987; Marmé 1989). The involvement of Ca^{2+} has been shown for such diverse processes as mitosis (Hepler 1986), cytoplasmic streaming (Williamson 1984), callose synthesis (Kauss 1987) and phytoalexin formation (Kurosaki 1987a). For a review see Hepler and Wayne (1985). For different types of plant hormones it has been proposed that Ca^{2+} acts as second messenger. Some examples of Ca^{2+}-dependent, hormone-regulated responses are: gibberellin regulated α-amylase synthesis and secretion (Carbonell and Jones 1984), elongation of pea stem segments induced by auxins (Cunninghame and Hall 1986), abscisic acid (ABA) induced inhibition of stomatal opening (Owen 1988) and cytokinin regulated bud formation in the moss *Funaria* (Conrad and Hepler 1988). Furthermore, acetylcholine (ACh) influenced the uptake of $^{45}Ca^{2+}$ by oat coleoptile cells (Tretyn 1987). The involvement of Ca^{2+} has also been shown for photomorphogenetic responses. The presence of Ca^{2+} is a prerequisite for phytochrome regulated fern spore germination (Scheuerlein et al. 1989), leaf unrolling of dark-grown barley (Viner et al. 1988) and chloroplast rotation in *Mougeotia* (Dreyer and Weisenseel 1979), for the photo-inhibition of stem elongation of cucumber by the blue-light receptor cryptochrome (Shinkle and Jones 1988), as well as for the UV-B

photoreceptor regulated efflux of K^+ from rose cells (Murphy 1988).

Bossen et al. (1988) have presented evidence for the necessity of Ca^{2+} in phytochrome-regulated swelling of etiolated wheat protoplasts. The involvement of a GTP-binding protein (G-protein) in the signal transduction of phytochrome has been proposed (Chapter 3). A comparison has been made between phytochrome-regulated protoplast swelling and swelling induced by α-naphthaleneacetic acid (α-NAA), benzylaminopurine (BAP), gibberellic acid (GA_3), ABA and ACh. In particular the necessity of Ca^{2+} and the possible involvement of a G-protein in the hormone-induced swelling response have been studied.

Material and methods

Plant material. Wheat (*Triticum aestivum* L., cv. Arminda) was sown in a mixture of Vermiculite and Perlite (1:1) in pots and placed, in darkness at 25°C, in a controlled air-flow cabinet. The air was purified and humidified by passing it first through a column with activated carbon Norit $RBAA_3$ (Norit, Amersfoort, the Netherlands) and then through a water column. After 8-11 d plants were harvested and the primary leaves were used for protoplast isolation.

Protoplast isolation. Protoplasts were isolated as described by Edwards et al. (1978). Primary leaves were cut in pieces (1 mm) and incubated in 3% cellulase Onozuka R10, 0.3% macerozym (Kinky Yakult, Nishinomiya, Japan), 0.5 M sorbitol, 1 mM $CaCl_2$ pH 5.6 for 3.5-4 h at 22°C. After purification on a discontinuous sorbitol/sucrose gradient, protoplasts were resuspended in the incubation medium consisting of 0.5 M sorbitol, 5 mM 2-(N-morpholino)ethanesulfonic acid (Mes) adjusted to pH 6.0 with 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) and 0.5 mM $CaCl_2$. Protoplasts were tested for viability with 0.2% phenosafranine (Widholm 1972). All manipulations were performed under dim green safelight (26 $nmol \cdot m^{-2} \cdot s^{-1}$).

Light sources and irradiations. Monochromatic light was selected from a 250 W quartz-iodine lamp in a custom-built projector using interference filters (Balzer B40, Liechtenstein) with a half bandwidth of approx. 10 nm. The fluence rates used were for R (660 nm): 150 and for FR (729 nm): 75 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The fluence rates were measured with a photodiode meter, Optometer type 80X (United Detectors Technology Inc., Santa Maria, CA., USA). Irradiation times were 1 min for R and 3 min for FR, sufficient to saturate phytochrome photoconversion.

Protoplast measurement. The estimation of protoplast volume was essentially as described by Bossen et al. (1988). After irradiation or other treatment, protoplasts ($0.5 \cdot 10^6$ in 0.5 ml) were kept in darkness for 30 min, at 22°C. Samples were taken, placed on a haemocytometer (Fuchs-Rosenthal) and photographed. The negatives were projected and the diameters of 100 protoplasts measured. The volume of each protoplast was calculated and the results are expressed as mean volume ± SE. All hormones used were from Sigma (St. Louis, USA) and added from 10x concentrated stock solutions in incubation medium.

Electroporation. Before electroporation, protoplasts were washed twice with an ice-cold solution of 0.5 M sorbitol, adjusted to pH 6.0 with a few drops of 0.1 M KOH, without any further additions. Electroporation was performed in an electroporation chamber as described by Hibi et al. (1986). The chamber consists of two gold-coated glass panel electrodes, placed 0.5 cm apart with a PVC spacer, and has a total volume of 1 ml. The power supply (Delta Electronics, Zierikzee, the Netherlands) was set at 225 V, yielding an electric field of 450 V/cm. Protoplasts, $(0.5\text{-}1.0) \cdot 10^6$ in 1 ml were pipetted into the precooled chamber and 0.47 μF capacitor was discharged three times at a rate of $1 \cdot s^{-1}$ (Rezelman et al. 1989). Protoplasts were left 10 min on ice, then incubated for 75-90 min at 26°C to allow recovery of their membrane integrity, and resuspended in incubation medium with 0.5 mM $CaCl_2$ or washed with medium without Ca^{2+} + 50 μM ethylenediaminetetraacetic acid (EDTA) and resuspended in medium with 0.5 mM KCl or 0.5 mM NaCl. Guanosine-5'-O-(2-thiodiphosphate) (GDP-β-S) from Boehringer (Mannheim, BRD) was added from a 100x concentrated stock solution in 0.5 M sorbitol, directly after the electroporation pulses.

Results

Hormone induced swelling. The hormones α-NAA, BAP, GA_3, ABA and ACh induced protoplast swelling in darkness, comparable in magnitude to swelling after a R pulse, when protoplasts were incubated in a medium containing 0.5 mM $CaCl_2$ (Fig. 4.1). The hormone concentrations used were all 10 μM, except for ACh which was 1 μM. Swelling after addition of α-NAA was slightly greater than after R, while ABA-induced swelling was slightly less. The ACh analogue carbamylcholine, known to activate ACh-receptors in animal cells, also induced swelling at a concentration of 1 μM. Analogues of the hormones were used to test the specificity of the hormone-induced swelling response. No swelling was observed when β-NAA, adenine, choline, propionylcholine or butyrylcholine were added to the protoplasts.

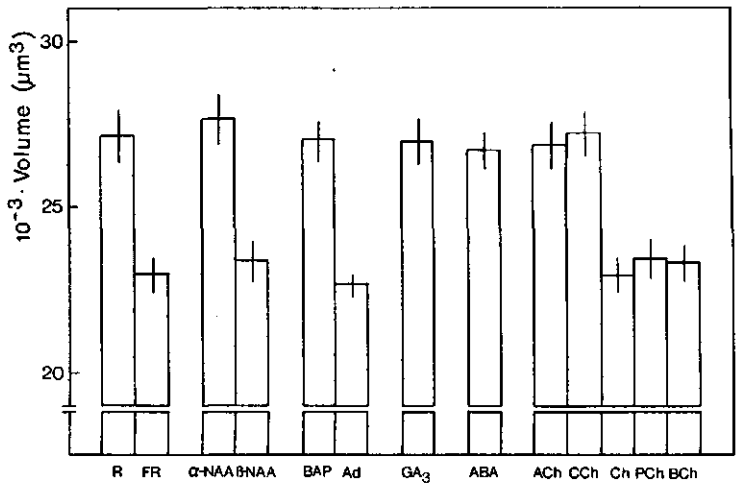


Fig. 4.1. Light and hormone-induced protoplast swelling. Protoplasts, suspended in a medium with 0.5 mM $CaCl_2$ and after irradiation with a red light (R) or far-red light (FR) pulse or after addition of hormones (final conc. 10 μM, except for the choline derivatives 1 μM), were incubated 30 min in darkness prior to measurement of protoplast diam. Mean volume ± SE is presented. (Ad=adenine; BCh=butyrylcholine; CCh=carbamylcholine; Ch=choline; PCh=propionylcholine)

Table 4.1. The involvement of Ca^{2+} in light- and hormone-induced swelling. Protoplasts were resuspended in a medium with 0.5 mM $CaCl_2$ + 10 μM Verapamil or washed in a medium without Ca^{2+} + 50 μM EDTA and resuspended in medium without Ca^{2+}. They were subsequently irradiated with a red light (R) pulse or hormones (final conc. 10 μM, except for 1 μM ACh) were added. After 30 min incubation in darkness, protoplasts diam. were measured. Mean volume is presented, SE was in all cases between 700 and 800 μm^3

Agonist	Volume (μm^3)		
	+ Ca^{2+}	- Ca^{2+}	+ Ca^{2+} + Verapamil
R	28 500	23 100	22 900
α-NAA	29 000	23 200	23 400
BAP	28 200	23 800	23 200
GA_3	28 400	23 600	23 600
ABA	27 200	23 800	23 500
ACh	28 400	23 200	22 900

Involvement of Ca^{2+}. The presence of Ca^{2+} in the medium has been shown to be a prerequisite for R-induced protoplast swelling (Bossen et al. 1988). No hormone-induced swelling was observed in the absence of Ca^{2+} or after addition of the Ca^{2+}-channelblocker Verapamil (10 μM) (Table 4.1), indicating the requirement of Ca^{2+} for hormone induced swelling.

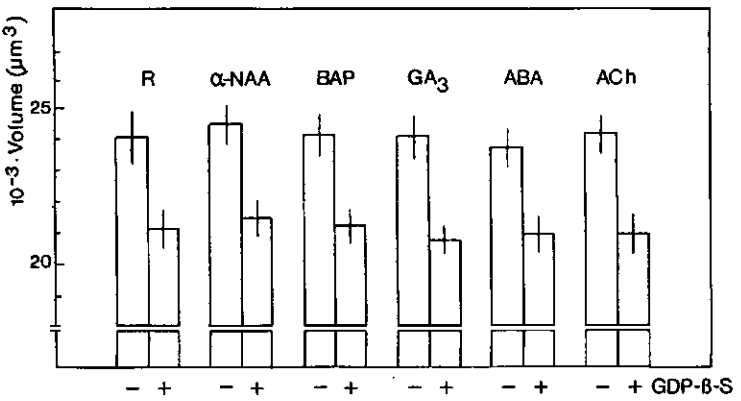


Fig. 4.2. Effect of G-protein inhibitor GDP-β-S on Ca^{2+}-dependent protoplast swelling. GDP-β-S was added immediatedly after the electroporation pulses. After a recovery period of 75-90 min protoplasts were resuspended in medium with 0.5 mM $CaCl_2$ and irradiated with red light (R) or hormones were added (final conc. 10 μM, except for 1 μM ACh). Protoplasts were incubated a further 30 min in darkness, prior to the measurement of protoplasts diam. Mean volume ± SE is presented

G-proteins. It has been proposed that a G-protein is involved in phytochrome-regulated protoplast swelling (Chapter 3). The R-induced swelling was inhibited by GDP-β-S. This compound competitively inhibits the binding of guanosine-5'-triphosphate to the G-protein, resulting in its inactivation. Since GDP-β-S is membrane impermeable, it was transported into the cytoplasm by means of electroporation. Directly after the electroporation pulses and before recovery, GDP-β-S (final concentration 100 μM) was added to the protoplasts. In the presence of Ca^{2+}, swelling induced by α-NAA, BAP, GA_3, ABA and ACh was in all cases inhibited by GDP-β-S (Fig. 4.2), indicating that activation of a G-protein is also involved in Ca^{2+}-dependent swelling, induced by hormones.

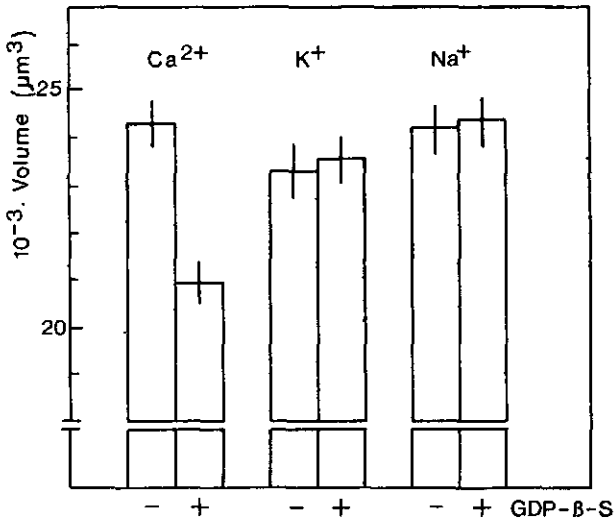


Fig. 4.3. Effect of G-protein inhibitor GDP-β-S on K^+- and Na^+-dependent swelling induced by ACh. The inhibitor GDP-β-S was added directly after the electroporation pulses. After a recovery period of 75-90 min protoplasts were washed with medium without Ca^{2+} + 50 µM EDTA and resuspended in medium with either 0.5 mM $CaCl_2$, 0.5 mM KCl or 0.5 mM NaCl. After addition of ACh (final conc. 1 µM) protoplasts were incubated 30 min in darkness prior to measurement of protoplasts diam. Mean volume ± SE is presented

K^+/Na^+-dependent swelling. The ACh-induced swelling was investigated in further detail. In animal cells two types of plasma membrane located ACh-receptors are known. One regulates Ca^{2+}-fluxes, the other activates K^+/Na^+-channels (Changeaux 1984). In the absence of Ca^{2+}, but in the presence of either 0.5 mM KCl or 0.5 mM NaCl, electroporated protoplasts also swelled after addition of 1 µM ACh, to an extent similar to the Ca^{2+}-dependent ACh-induced swelling (Fig. 4.3). The results were similar with non-electroporated protoplasts (Tretyn et al. 1989). When GDP-β-S was added to the protoplasts after the electroporation pulses, only the Ca^{2+}-dependent ACh-induced swelling was inhibited. On the contrary, the K^+/Na^+-dependent, ACh-induced swelling was not affected.

Discussion

All the hormones tested (α-NAA, BAP, GA_3, ABA, and ACh) induced swelling of etiolated wheat protoplasts similar to the phytochrome-regulated swelling. No swelling was observed in the absence of Ca^{2+} or when the Ca^{2+}-channelblocker Verapamil was added to the medium (Table 4.1). The swelling was specifically induced by active analogues of the hormones (Fig. 4.1). Protoplast swelling induced by GA_3 was previously shown by Blakeley et al. (1983) and Chung et al. (1988) with protoplasts isolated from the primary leaves of dark-grown wheat and oat, respectively. From the results presented by Iversen et al. (1983) it can be concluded that ABA also induces swelling of mesophyll protoplasts, isolated from light-grown *Vicia faba*. The Ca^{2+}-dependent protoplast swelling induced by different hormones is in agreement with the observed requirement of Ca^{2+} in several hormone-regulated physiological responses (Hepler and Wayne 1985; Elliott 1986).

Since the Ca^{2+}-dependent swelling could be inhibited by GDP-β-S (Fig. 4.2), we propose that activation of a G-protein is involved in hormone-induced protoplast swelling. This suggests that, for all hormones tested, receptors are present on the plasma membrane of etiolated wheat mesophyll protoplasts. Hornberg and Weiler (1984) have shown the presence of ABA-receptors on the outside of the plasma membrane in guard-cell protoplasts isolated from *Vicia faba*. Plasma membrane located binding sites/receptors for auxins have been described in protoplasts from tobacco leaves, (Vreugdenhil et al. 1980; Barbier-Brygoo et al. 1989). The G-protein involved in phytochrome-regulated protoplast swelling, has been proposed to be a G-protein connected with the phosphatidyl-inositol pathway of signal transduction (Chapter 3). After application of auxins (Ettlinger and Lehle 1988; Zbell and Walter-Back 1988) and light (Morse et al. 1987; Morse et al. 1989) hydrolysis of phosphatidyl-inositol has been reported to occur.

The similarity between swelling induced by phytochrome and the tested hormones, particularly in respect to the dependence on Ca^{2+} and the involvement of a G-protein, could explain why certain Ca^{2+}-dependent responses can be induced by either phytochrome or hormones. If such responses depend solely on changes of Ca^{2+}-fluxes and if their corresponding receptors are present, they could be induced by any "hormone". Examples of responses which can be induced by either phytochrome or hormone are: leaf

unrolling of barley (GA_3) (Viner et al. 1988), chloroplast development in etiolated cucumber cotyledons (cytokinin) (Cohen et al. 1988), gravitropism of maize "Merit" (ABA) (Leopold and LaFavre 1989), ethylene evolution from soybean leaf discs (ACh) (Jones and Stutte 1986; see also Hartmann and Gupta 1989). However, more detailed, comparative studies will have to be carried out with different plant systems to substantiate this proposal.

Unlike phytochrome after R-irradiation (Bossen et al. 1988), ACh also induced swelling in darkness, when instead of Ca^{2+}, either K^+ or Na^+ was present (Fig. 4.3). A clear difference was observed between the Ca^{2+}-dependent and K^+/Na^+-dependent, ACh-induced swelling, when GDP-β-S was added to the protoplasts. While the Ca^{2+}-dependent swelling was inhibited by GDP-β-S, the K^+/Na^+-dependent swelling was not. This would indicate that the Ca^{2+}-dependent swelling is dependent on the activation of a G-protein, while the K^+/Na^+-dependent swelling is regulated by a different mechanism. This is comparable to the situation in animal cells, where the so called "muscarinic" type of ACh-receptor is coupled to a G-protein and regulates Ca^{2+}-fluxes, while the "nicotinic" type is part of a K^+/Na^+ channel in the plasma membrane and regulates the channels activity (Changeaux 1984). However, it is difficult to envisage what mechanism underlies the K^+/Na^+-dependent swelling. It has been proposed that protoplast swelling is a consequence of a rise in $[Ca^{2+}]_{cyt}$, since both the Ca^{2+}-ionophore A23187 (Bossen et al. 1988) and the Ca^{2+}-channel agonist Bay K-8644 (Tretyn et al. in press) induce protoplast swelling in the presence of Ca^{2+} in darkness. Furthermore, swelling was observed when the calmodulin antagonist W_7 was added to the protoplasts in darkness. Inhibition of calmodulin could result in a rise of $[Ca^{2+}]_{cyt}$ because a plasma membrane located Ca^{2+}-ATPase, which is normally activated by calmodulin, stays inactive (Chapter 3). It therefore appears likely that activation of the K^+/Na^+ channels in the plasma membrane also leads, via an as yet unknown process, to a higher $[Ca^{2+}]_{cyt}$. Definite conclusions await experiments in which $[Ca^{2+}]_{cyt}$ in protoplasts is measured directly, after addition of ACh in the presence of either K^+ or Na^+.

Chapter 5

Phytochrome dependent changes in external Ca^{2+} -concentration in suspensions of etiolated wheat (*Triticum aestivum* L.) protoplasts

Margreet E. Bossen, Patricia A.P.M. Verhoeven-Jaspers, Richard E. Kendrick and Willem J. Vredenberg

Abstract. The Ca^{2+}-sensitive dye murexide, has been used to monitor phytochrome-regulated changes in the [Ca^{2+}] of the medium in which protoplasts, isolated from dark-grown wheat leaves were suspended. Red light (R) induced an efflux of Ca^{2+} from the protoplasts, far-red light (FR) reversed this effect, presumably by causing Ca^{2+}-uptake. The R-induced efflux was inhibited for approx. 75% by the Ca^{2+}-channelblocker Verapamil, as well as by the calmodulin antagonist N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W_7). The less active analogue N-(6-aminohexyl)-1-naphthalenesulfonamide (W_5) had no effect on the efflux. It is proposed that R causes the opening of Ca^{2+}-channels in the plasma membrane, and that the resulting enhanced influx of Ca^{2+} is followed by a large release of Ca^{2+} from internal stores and/or from Ca^{2+} bound to membranes. This Ca^{2+} is extruded from the protoplasts by means of a plasma membrane located Ca^{2+}-ATPase. The change in Ca^{2+}-fluxes is speculated to be associated with an activated state of the protoplasts. The formation of the R-absorbing inactive form of phytochrome (Pr) by FR, reverses the protoplasts to the inactivated state, leading to "re-uptake" of Ca^{2+}.

Introduction

Phytochrome is one of the pigments regulating photomorphogenesis in plants. There are two forms, the red light (R)-absorbing form (Pr) and the far-red light (FR)-absorbing form (Pfr), which can be interconverted by R and FR, respectively. In general, Pfr is considered the active form (Hendricks and VanDerWoude 1983). For an increasing number of phytochrome-regulated responses it has been shown that the presence of Ca^{2+} is a prerequisite. Examples are: leaflet closure in *Mimosa* (Toriyama and Jaffe 1972), chloroplast rotation in *Mougeotia* (Dreyer and Weisenseel 1979), spore germination of the ferns *Onoclea* (Wayne and Hepler 1984) and *Dryopteris* (Scheuerlein et al. 1989), reduction of the surface charge of *Mesotaenium* cells (Stenz and Weisenseel 1986), leaf unrolling of dark-grown barley (Viner et al. 1988) and phytochrome-regulated gravitropism of maize 'Merit' (Perdue et al. 1988). It has been proposed that formation of Pfr leads to the opening of Ca^{2+}-channels in the plasma membrane, resulting in enhanced transport of Ca^{2+} into the cytoplasm and a concomitant rise in cytoplasmic Ca^{2+}-concentration ($[Ca^{2+}]_{cyt}$) (Roux 1983; Bossen et al. 1988). A transient, enhanced uptake of $^{45}Ca^{2+}$ after R, has been shown to occur in protoplasts isolated from dark-grown maize leaves (Das and Sopory 1985) and in *Mougeotia* cells (Dreyer and Weisenseel 1979). In both cases the enhanced

uptake was prevented by FR, when given immediatedly after R. Using protoplasts, isolated from dark-grown oat coleoptiles (Hale and Roux 1980) or from *Vallisneria* leaves (Takagi and Nagai 1988), an increase in $[Ca^{2+}]$ of the medium after R was detected by means of the Ca^{2+}-selective dye murexide. This effect was also reversible by FR.

To clarify these apparently conflicting results, we have investigated the circumstances under which phytochrome regulated changes in $[Ca^{2+}]$ of the medium can be induced, using protoplasts isolated from dark-grown wheat leaves. Such protoplasts have been shown to swell only after R, when Ca^{2+} is present in the surrounding medium (Bossen et al. 1988).

Material and methods

Plant material. Wheat (*Triticum aestivum* L., cv Arminda) was sown in pots, in a washed mixture of Vermiculite and Perlite (1:1) and placed, in darkness at 25°C, in a controlled air-flow cabinet. The air was purified by passing it through a column with activated carbon Norit $RBAA_3$ (Norit, Amersfoort, the Netherlands) and then humidified by passing it through a water column. Plants were harvested after 10-11 d, the primary leaves were used for protoplast isolation.

Protoplast isolation. Protoplasts were isolated as described by Edwards et al. (1978). Primary leaves were cut in 1 mm pieces and incubated for 3.5-4 h at 22°C in 3% cellulase Onozuka R10, 0.3% macerozym (Kinky Yakult , Nishinomiya, Japan), 0.5 M sorbitol and 1 mM $CaCl_2$, pH 5.6. After purification on a discontinuous sorbitol/sucrose gradient, protoplasts were resuspended in incubation medium, consisting of 0.5 M sorbitol, 5 mM 2-(N-morpholino)ethane-sulfonic acid (Mes) adjusted to pH 6.0 with 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) and 1 mM $CaCl_2$. Protoplasts were tested for viability with 0.2% phenosafranine (Widholm 1972). All manipulations were performed under dim green light (26 $nmol \cdot m^{-2} \cdot s^{-1}$).

Light sources and irradiations. Monochromatic light was selected from a 250 W quartz-iodine lamp in a custom-built projector using interference filters (Balzer B40, Liechtenstein) with a half bandwidth of approx. 10 nm. The fluence rates used were 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for R (660 nm) and 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for FR (729 nm). The fluence rates were measured with a photodiode meter, Optometer type 80X (United Detectors Technology Inc., Santa Maria, USA). Irradiation times were 1 min for R and 3 min for FR, sufficient to saturate phytochrome photoconversion.

Spectrophotometric measurements. After isolation, protoplasts were resuspended in the incubation medium, with additional 50 μM murexide (Sigma, St. Louis, USA). Protoplasts [$(1.8 - 2.4) \cdot 10^6$ in

1.5 ml] were pipetted into a 3 ml cuvette and placed in a Aminco DW2a spectrophotometer. The protoplast suspension was stirred with a magnetic stirring device (Hellma, the Hague, the Netherlands). The measuring beam of the spectrophotometer, operated in the dual wavelength mode, was directed through the protoplast suspension. Temperature of the samples was kept constant at 22 ± 0.1°C. Protoplasts were pre-incubated 15-30 min prior to the start of the measurement. Irradiation was from above, while the high voltage of the photomultiplier was shut off. The compounds Verapamil, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W_7) and N-(6-aminohexyl)-1-naphthalenesulfonamide (W_5) all from Sigma (St. Louis, USA), were added from 100x concentrated stock solutions in the murexide incubation medium, approx. 15 min prior to irradiation.

Results

Choice of measuring wavelengths. The wavelength pair 544-473 nm was chosen for the measurements because different Ca^{2+}-concentrations show the largest changes in absorbance at these wavelengths (Fig. 5.1A, 1B).

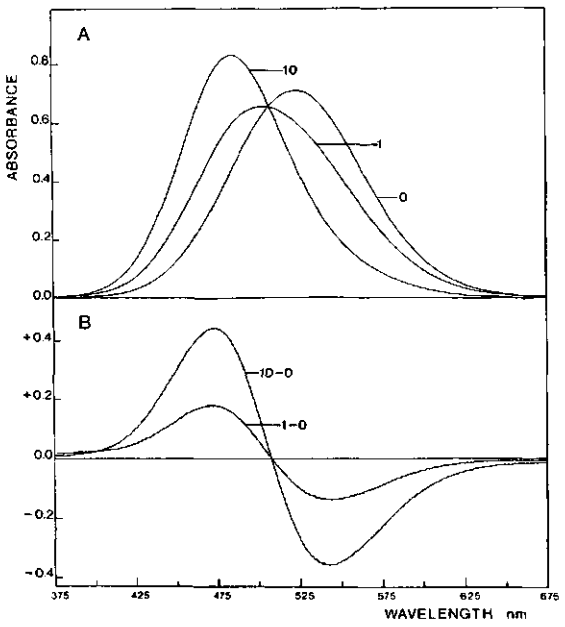


Fig. 5.1. Absorption and difference spectra of murexide. **A** Absorption spectrum of murexide (50 μM) at 0, 1 and 10 mM $CaCl_2$ in incubation medium. **B** Difference spectrum for (1 - 0 μM) and (10 - 0 μM) $CaCl_2$

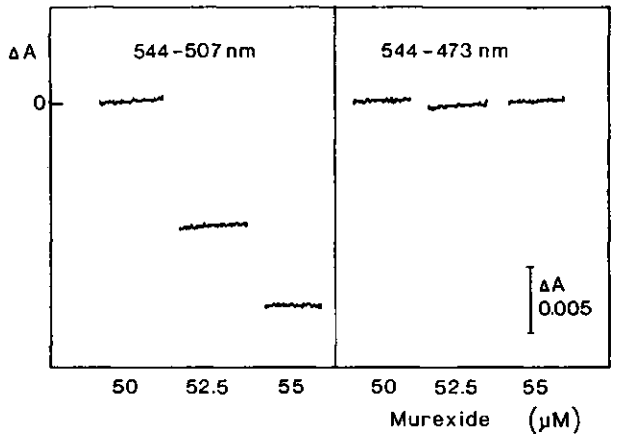


Fig. 5.2. The effect of changes of the murexide concentration on the absorbance difference at the wavelength pairs $A_{544-507}$ and $A_{544-473}$. Solutions of murexide (50, 52.5 and 55 μM) were made in incubation medium containing 1 mM $CaCl_2$

Small changes in the murexide concentration can mimic Ca^{2+}-induced changes in absorbance difference (Ohnishi 1978). Such changes in murexide concentration could occur during the measurement due to breakdown of the dye or to a change in protoplast volume after R (Bossen et al. 1988). To verify that such changes in murexide concentration do not interfere with the Ca^{2+}-dependent changes in absorbance difference at 544-473 nm, different murexide concentrations were applied and measured at a constant [Ca^{2+}] of 1 mM. A comparison was made with the wavelength pair 544-507 nm, which has been used by Hale and Roux (1980) and Takagi and Nagai (1988). As shown in Fig. 5.2 there was hardly any change in $\Delta A_{544-473}$, when the murexide concentration was changed from 50 to 52.5 or 55 μM. On the other hand quite a large change was observed at $\Delta A_{544-507}$. This is in agreement with the observation that the absorbance of murexide at 1 mM Ca^{2+} is the same at 544 and 473 nm (Fig. 5.1A).

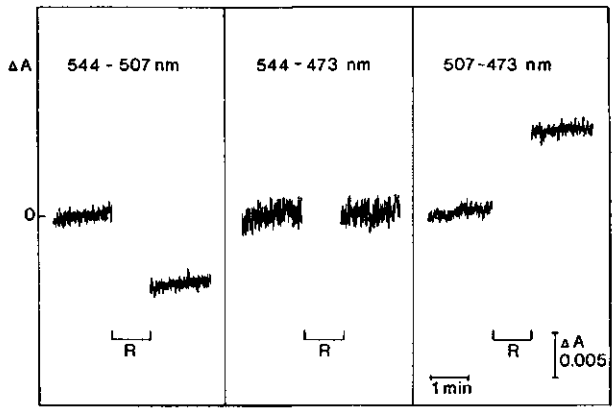


Fig. 5.3. The effect of red light (R) on the absorbance difference of protoplasts in the absence of murexide at the wavelength pairs $A_{544-507}$, $A_{544-473}$ and $A_{507-473}$. Protoplasts ($2.0 \cdot 10^6$) were suspended in 1.5 ml incubation medium and stirred with a magnetic bar. At the start of the measurement the absorbance difference at the indicated wavelength pair was set to zero. A saturating pulse of R was given at the indicated interval

Since measurements have been made with the measuring beam passing through the suspended protoplasts, changes in absorbance of the protoplasts themselves after R or FR irradiation, could interfere with the Ca^{2+}-dependent absorbance changes of the murexide. Therefore changes in the absorbance difference of the protoplasts were measured in the absence of murexide. Both at 544-507 nm and 507-473 nm there was an effect of R on the signal of equal, but opposite size. There was no effect when 544-473 nm was measured (Fig. 5.3). The protoplasts probably contain a compound which undergoes a change in absorbance at 507 nm after R. Therefore we have used the wavelength pair 544-473 nm for measurement of Ca^{2+}-dependent changes in murexide absorbance, in our protoplast suspension.

R-FR reversibility. After a 1 min R pulse $\Delta A_{544-473}$ was lower, indicating a rise in $[Ca^{2+}]$ of the medium. When R was followed by a FR pulse, with a 15 s dark interval, no such change in $\Delta A_{544-473}$ was observed (Fig. 5.4). This photo-reversibility was apparent for at least two R-FR cycles, indicating the involvement of phytochrome.

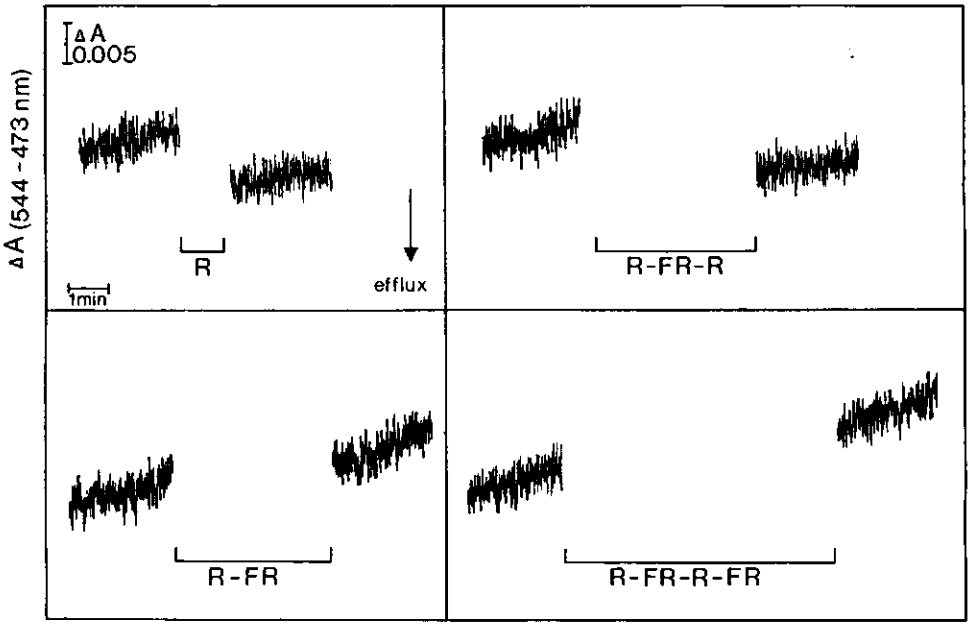


Fig. 5.4. Red-Far-red (R-FR) reversibility of Ca^{2+}-efflux. Protoplasts, $2.4 \cdot 10^6$ in 1.5 ml incubation medium, (containing 1 mM $CaCl_2$ and 50 μM murexide), were pre-incubated for 15-30 min while stirred in the cuvet before the measurement was started. After approx. 3 min recording, protoplasts were irradiated with saturating R and FR pulses as indicated. The results from a single, representative experiment are presented

Table 5.1. The effect of the Ca^{2+}-channelblocker Verapamil, and the calmodulin antagonists W_7 and W_5 on R-induced increase of $[Ca^{2+}]$ of the medium. The compounds (final concentration 10 μM) were added, approx. 15 min prior to a saturating red light (R) pulse, to the stirred protoplast suspension ($2.4 \cdot 10^6$ protoplasts in 1.5 ml incubation medium containing 50 μM murexide and 1 mM $CaCl_2$). The percent change of $\Delta[Ca^{2+}]$ of the medium ± SE relative to the control (=100%), from three independent experiments, is presented

Compound added	$\Delta[Ca^{2+}]$ (% ± SE)
Verapamil	25 ± 7
W_7	23 ± 9
W_5	103 ± 3

Ca^{2+}-channels and Ca^{2+}-ATPase. To investigate whether Ca^{2+}-channels and/or a plasma membrane located Ca^{2+}-ATPase are involved in the observed changes in $[Ca^{2+}]$ of the medium, the Ca^{2+}-channelblocker Verapamil and the calmodulin antagonists W_7 and W_5 were added to the protoplast suspension 15 min prior to R. The compound W_7 binds to calmodulin with a much higher affinity than the analogue W_5 (Hidaka et al. 1981). It has been proposed that the Ca^{2+}-ATPase in the plasma membrane remains inactive through the inhibition of calmodulin by W_7 (Gietzen and Bader 1985). After addition of 10 μM Verapamil or of 10 μM W_7, the R-induced change in $\Delta A_{544-473}$ was inhibited for approx. 75%, in both cases (Table 5.1). The less active analogue W_5 caused no inhibition. These observations suggest that an influx of Ca^{2+} into the protoplasts, as well as an efflux are both necessary steps in the observed changes in the $[Ca^{2+}]$ of the medium.

Discussion

The results show that R-irradiation induced an increase in $[Ca^{2+}]$ of the medium. This increase was reversed by FR, when given 15 s after R (Fig. 5.4). Since at the start of the FR pulse, the increase in $[Ca^{2+}]$ of the medium has already taken place, this means that FR really reverses the increase and Ca^{2+} is taken up by the protoplasts after FR. The measured change in $\Delta A_{544-473}$ is unlikely to be due to a change in murexide concentration, which could be induced by swelling of the protoplasts. Swelling has only just started at this time and requires almost 10 min for completion (Bossen et al. 1988). Moreover, changes in murexide concentration do not influence the absorbance difference at these wavelengths (Fig. 5.2). Similar results have been reported by Hale and Roux (1980) and Takagi and Nagai (1988) in protoplasts of oat coleoptiles and of *Vallisneria,* respectively. In both cases a R-induced efflux could be reversed by FR, even when FR was given 4-10 min after R. The increase in $[Ca^{2+}]$ of the medium, calculated from the changes in $\Delta A_{544-473}$, was approx. 10-20 μM, when $2.0 \cdot 10^6$ protoplasts were present in 1.5 ml. This amount of Ca^{2+} released by the protoplasts after R is quite large, especially if it is compared with the cytoplasmic Ca^{2+}-concentration ($[Ca^{2+}]_{cyt}$). A change of 10 μM Ca^{2+} in the medium corresponds with approx. 10^{-14} mol Ca^{2+}/protoplast. Assuming that: 75 % of the Ca^{2+} originates from within the protoplast (see below), the average volume of the protoplasts is 23000 μm^3 (see Bossen et al. 1988) and that the

cytoplasm accounts for 10 % of the protoplast volume, the amount of Ca^{2+} released corresponds to a concentration of 300 μM in the cytoplasm. In general it is thought that $[Ca^{2+}]_{cyt}$ is 50-300 nM (Williamson and Ashley 1982; Gilroy et al. 1987; Clarkson et al. 1988). The amount of Ca^{2+} released by oat coleoptile protoplasts was in the same order of magnitude: $5 \cdot 10^{-14}$ mol/protoplast (Hale and Roux 1980), while in *Vallisneria* protoplasts it was tenfold higher: $2 \cdot 10^{-13}$ mol/protoplast (Takagi and Nagai 1988). It is therefore very unlikely that the released Ca^{2+} originates from the cytoplasm. Possible sources are the endoplasmic reticulum, the vacuole and Ca^{2+} bound to, or associated with membranes. Release of Ca^{2+} from the endoplasmic reticulum and from the vacuole can be induced by inositol 1,4,5-trisphosphate (IP_3) (Drøbak and Ferguson 1985; Schumacher and Sze 1987). It has been proposed that IP_3 is formed after R in etiolated wheat protoplasts, by activation of a GTP-binding protein (Chapter 3). In carrot protoplasts IP_3 has been shown to induce a transient release of $^{45}Ca^{2+}$ (Rincon and Boss 1987). Release of Ca^{2+} bound to membranes could be caused by a change in the packing density of the lipids, as proposed by Buckhout et al. (1981). A release of Ca^{2+} from membranes was only observed by them, when physiological active auxins were added. The measured Ca^{2+} release after R could be associated with, what we call, an activated state of the membrane and of the protoplast. When membranes revert to the inactivated state after FR, Ca^{2+} could again bind to them. This would give a tentative explanation for the "re-uptake" of Ca^{2+} after FR. The observation of Takagi and Nagai (1988) that, if FR is given some minutes after R, the FR-induced decrease of the $[Ca^{2+}]$ of the medium is inhibited by the Ca^{2+}-channelblocker nifedipine, supports the notion that this decrease is the result of "re-uptake" of Ca^{2+}, through Ca^{2+}-channels, by the protoplasts.

The R-induced efflux was inhibited approx. 75% when the Ca^{2+}-channel blocker Verapamil was added to the medium. This indicates that an influx of Ca^{2+} is a prerequisite for the efflux to occur. The efflux was also inhibited by the calmodulin antagonist W_7. It has been proposed that calmodulin is involved in activation of a plasma membrane located Ca^{2+}-ATPase. Therefore a calmodulin antagonist could inhibit activation of this ATPase (Gietzen and Bader 1985; Gilroy et al. 1987). Apparently the larger part (approx. 75%) of the R-induced increase in $[Ca^{2+}]$ of the medium is dependent on Ca^{2+}-fluxes across the plasma membrane. The smaller part (approx. 25%) probably originates from the outside of the plasma membrane, due to changes in surface

properties as e.g. surface charge density. These results are in agreement with the reported inhibition of the Ca^{2+}-efflux by the ATPase inhibitor vanadate. The increase in $[Ca^{2+}]$ of the medium after R-irradiation, was only 17% as compared with the control without vanadate (Takagi and Nagai 1988). Hale and Roux (1980) have shown that the presence of Ca^{2+} in the medium is necessary for the R-induced efflux to occur. This is in agreement with the inhibition of the R-induced efflux by Verapamil.

The question remains as to why an influx of Ca^{2+} is required, for the release and efflux of a comparatively large amount of Ca^{2+}. The most logical answer appears to be, that the influx of Ca^{2+} is required for the release of Ca^{2+} from internal stores and/or for the release of Ca^{2+} bound to membranes. A consequence of the Ca^{2+}-influx could be a depolarization of the plasma membrane. It has been proposed that depolarization of the plasma membrane is part of the signal transduction chain induced by stress (Rincon and Hanson 1986), by ultraviolet light (Murphy 1988) and by fungal elicitors (Pelissier et al. 1986). A R-induced, Ca^{2+}-dependent depolarization of *Nitella* cells has been reported by Weisenseel and Ruppert (1977). If it is assumed that such a depolarization is caused only by a transient flux of Ca^{2+} across the plasma membrane, the amount of Ca^{2+} needed for a depolarization is given by: $Q = C_m \cdot E_m \cdot S/2F$, with Q = mol Ca^{2+}/cell; C_m = membrane capacitance (10^{-6} $F \cdot cm^{-2}$); E_m = membrane depolarization (assumed to be 0.03 Volt, the depolarization after R measured by Weisenseel and Ruppert 1977); S = protoplast surface ($40 \cdot 10^{-6}$ cm^2); F = Faraday constant. It can be calculated that, under our experimental conditions, such a depolarization, would cause a nanomolar change in the $[Ca^{2+}]$ of the medium, which is below the detection limit of the murexide dye. Such a small decrease, followed by a large increase, is at least qualitatively in agreement with the changes in membrane surface potential as measured by Newman (1981) in oat coleoptiles. After R a small depolarization was followed by a large hyperpolarization.

In conclusion, the phytochrome-regulated Ca^{2+} fluxes across the plasma membrane and its regulation, are in agreement with the necessity for the presence of Ca^{2+} in several phytochrome regulated processes such as fern spore germination, leaf unrolling and protoplast swelling. On the basis of the experiments reported here, it is predicted that phytochrome regulated changes in $[Ca^{2+}]_{cyt}$ occur. However, more definite conclusions await direct measurements of $[Ca^{2+}]_{cyt}$, as well as of their possible interaction with changes in membrane potential.

Chapter 6

The fluidity of the plasma membrane of etiolated wheat (*Triticum aestivum* L.) protoplasts before and after red light irradiation

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Abstract. The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was used to monitor the fluidity of the plasma membrane of etiolated wheat protoplasts. After correction for the non-specific contributions of non-labeled protoplasts, the corrected anisotropy of DPH (r_f) was independent of protoplast and DPH concentration. After red light irradiation r_f increased, indicating a decrease in membrane fluidity. In darkness, r_f also increased upon osmotically induced protoplast swelling. It is not clear, whether R causes changes in membrane fluidity independent of changes in volume.

Introduction

The photomorphogenetic pigment phytochrome exists in two forms, the red light (R)-absorbing form, Pr and the far-red light (FR)-absorbing form, Pfr, which can be interconverted by R and FR, respectively. Several plasma membrane properties have been reported to be regulated by phytochrome. For example, the membrane potential of oat coleoptile cells and of *Nitella* was found to depolarize within 10 s after R (Racusen 1976; Weisenseel and Ruppert 1977) and the surface charge of *Mesotaenium* cells was reduced (Stenz and Weisenseel 1986). The presence of Ca^{2+} in the medium is a prerequisite for these changes to occur. Red light-induced changes of Ca^{2+}-fluxes across the plasma membrane have also been observed (Hale and Roux 1980; Das and Sopory 1985; Takagi and Nagai 1988; Chapter 5). It is still unclear how, and by what mechanism, phytochrome regulates these membrane properties. It has been proposed that a GTP-binding protein and a protein kinase are involved in the R-induced changes of the Ca^{2+}-permeability of the plasma membrane (Chapter 3). In animal cells it has been shown that the activity of membrane enzymes (e.g. adenylate cyclase, Ca^{2+}-ATPase) and of carrier-mediated transport (e.g. glucose), as well as the accessibility of membrane receptors for their ligands, are modulated by artificially induced changes in the membrane lipid fluidity. Binding of ligands to their receptors on the plasma membrane has been shown to result in either an increase or a decrease in fluidity, dependent on ligand and cell type (Shinitzky 1984).

We have used the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH), to investigate whether phytochrome regulated changes in properties of the plasma membrane of plant

protoplasts, are connected with changes in membrane fluidity. The anisotropy of DPH, when incorporated in membranes, is considered to be a measure of membrane fluidity (Shinitzky and Barenholz 1978; Van Blitterswijk et al. 1981). The kinetics of the R-induced change of the membrane fluidity, and its dependency on the osmotic potential of the medium, have been the subject of the present study.

Material and methods

Plant material. Wheat (*Triticum aestivum* L., cv. Arminda) was sown in a mixture of Vermiculite and Perlite (1:1) in pots and placed, in darkness at 25°C, in a controlled air-flow cabinet. The air was purified and humidified by passing it first through a column with activated carbon Norit $RBAA_3$ (Norit, Amersfoort, the Netherlands) and then through a water column. After 8-11 d plants were harvested and the primary leaves were used for protoplast isolation.

Protoplast isolation. Protoplasts were isolated as described by Edwards et al. (1978). Primary leaves were cut in pieces (1 mm) and incubated in 3% cellulase Onozuka R10, 0.3% macerozym (Kinky Yakult, Nishinomiya, Japan), 0.5 M sorbitol, 1 mM $CaCl_2$ pH 5.6 for 3.5-4 h at 22°C. After purification on a discontinuous sorbitol/sucrose gradient, protoplasts were resuspended in the incubation medium consisting of 0.5 M sorbitol, 5 mM 2-(N-morpholino)ethane-sulfonic acid (Mes) adjusted to pH 6.0 with 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) and 0.5 mM $CaCl_2$. Protoplasts were tested for viability with 0.2% phenosafranine (Widholm 1972). All manipulations were performed under dim green safelight (26 $nmol \cdot m^{-2} \cdot s^{-1}$).

Light sources and irradiations. Monochromatic light was obtained from a 250 W quartz-iodine lamp in a custom-built projector using interference filters (Balzer B40, Liechtenstein) with a half bandwidth of approx. 10 nm. The fluence rates used were for R (660 nm): 60 and for FR (729 nm): 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The fluence rates were measured with a photodiode meter, Optometer type 80X (United Detectors Technology Inc., Santa Maria, CA., USA). Irradiation times were 1 min for R and 3 min for FR, sufficient to saturate phytochrome photoconversion.

Protoplast labeling. The probe DPH (Molecular Probes, Eugene, Or., USA) was stored as a $5 \cdot 10^{-3}$ M stock solution in acetonitrile (Merck, Darmstadt, BRD). For each experiment this stock was diluted to $5 \cdot 10^{-6}$ M in incubation medium under vigorous stirring. Protoplasts ($0.6 \cdot 10^6$ in 3 ml) were incubated with DPH for 45 min (final DPH concentration $2 \cdot 10^{-6}$ M; $=10^{-14}$ mol/protoplast), unless otherwise indicated. Protoplasts were then washed and resuspended in incubation medium. For each measurement the protoplast suspension was gently mixed and measured 3 min later.

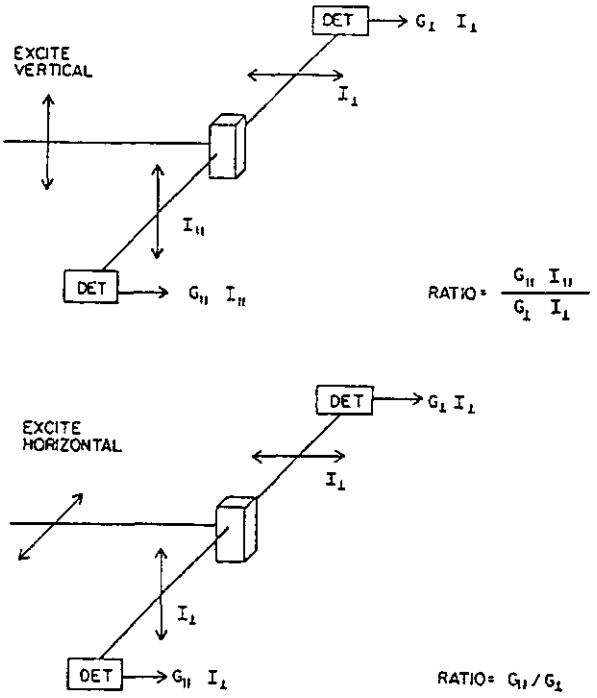


Fig. 6.1. Schematic diagram for T-format measurements of fluorescence anisotropy. DET, detectors; G, gains of the detectors. After Lakowicz (1983)

Fluorescence measurements. The measurements of steady-state fluorescence anisotropy were carried out with an SLM 4800s polarization/subnanosecond lifetime spectrofluorometer (SLM Instruments, Urbana, Il., USA) in the T-format. The ratio between the intensities of emission light oriented parallel ($I_\parallel$) and perpendicular ($I_\perp$) to the plane of the polarized excitation light was determined (Fig. 6.1). The anisotropy r is defined according to Lakowicz (1983), as

$$r = \frac{I_\parallel / I_\perp - 1}{I_\parallel / I_\perp + 2}$$

Excitation was at 360 nm (bandwidth 8 nm) and the emission light was selected by a combination of BG1 and GG420 cut off-glass filters (transmission maximum at 433 nm, bandwidth: 50 nm) (Schott, Mainz, BRD). The temperature of the sample was kept constant at 22 ± 0.2°C. Correction for autofluorescence and scattering was made by measuring non-labeled protoplasts under exactly the same conditions as the labeled protoplasts. Correction was according to Lakowicz (1983):

$$r_f = \frac{I_m r_m - I_s r_s}{I_m - I_s}$$

I indicates the fluorescence intensity ($I = I_{\parallel} + 2I_{\perp}$), r the anisotropy, while the subscripts m, s and f stand for measured values of labeled protoplasts, non-labeled protoplasts and of the fluorophore, respectively. The formula used by Kuhry et al. (1985): $r_m = f \cdot r_f + (1 - f) r_s$, with $f = (I_m - I_s)/I_m$, gives after rearrangement, the same expression as the one mentioned above. All experiments were repeated at least three times. Representative individual experiments are shown. The measuring points have been estimated with an average error of 2.5% for intensity (I) and 1% for anisotropy (r). The calculated error for r_f was 7.5%.

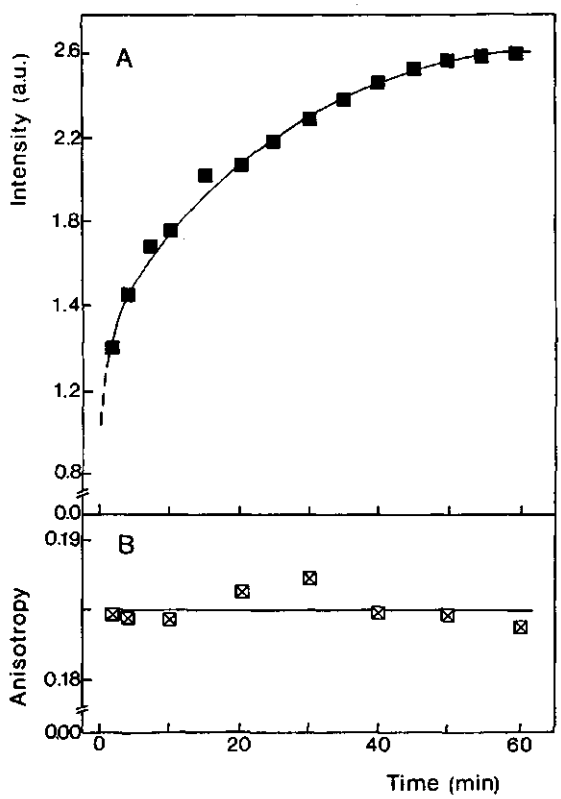


Fig. 6.2. Kinetics of DPH-incorporation into protoplasts. A Fluorescence intensity. B Anisotropy of DPH. Protoplasts ($0.2 \cdot 10^{-6} \cdot ml^{-1}$) were not washed before measurement of fluorescence intensity and anisotropy

Results

Measurement conditions. To establish optimal conditions for the fluorescence measurement, the kinetics of DPH-uptake and the dependency of the anisotropy of DPH (r_f) on protoplast and DPH concentration, were determined. As shown in Fig. 6.2A, the uptake of DPH by the protoplasts, measured as an increase in the fluorescence intensity, was nearly complete after 45 min incubation. During the uptake period r_f was constant (Fig. 6.2B). After washing and resuspension of the protoplasts in incubation medium r_f was lower, but then remained constant for at least 60 min (data not shown). The decrease of r_f after washing, can be accounted for by the contribution of the apparent fluorescence of DPH, dissolved in the sorbitol medium, to the fluorescence signal (Table 6.1). It has been shown that in some animal cell types (e.g. fibroblasts) DPH, after incorporation in the plasma membrane, is taken up in the cytoplasmic region (Kuhry et al. 1983), while in other cells (e.g. lymphocytes) DPH remains in the plasma membrane for a prolonged time (Shinitzky and Inbar 1976). We have no proof that DPH remains in the plasma membrane of etiolated wheat protoplasts. However, the result that r_f remains

Table 6.1. The contribution of DPH in sorbitol medium to the fluorescence signal. The fluorescence intensity (I) and anisotropy values (r) of the 0.5 M sorbitol containing incubation medium with 0 or $2 \cdot 10^{-6}$ M DPH, of non-labeled, of labeled and of labeled and subsequently washed protoplasts, were determined. Protoplasts ($0.6 \cdot 10^6$ in 3 ml) were incubated with DPH for 45 min (final conc. $2 \cdot 10^{-6}$ M DPH). To calculate the corrected anisotropy value r_f, the fluorescence intensity and anisostropy values of non-labeled protoplasts were used as correction for the measured values of labeled and labeled/washed protoplasts

Treatment	Fluorescence		
	I	r	r_f
sorbitol medium	0.217	0.1222	
sorbitol + $2 \cdot 10^{-6}$ M DPH	0.701	0.1940	
protoplasts, non-labeled	0.763	0.1190	
protoplasts, labeled	2.625	0.1660	0.1840
protoplasts, labeled/washed	2.185	0.1568	0.1770

constant during the whole incubation period suggests that, either DPH is not redistributed into the intracellular membranes of the protoplasts and remains in the plasma membrane, or if redistribution occurs, it not significantly affects the measured fluorescence signal. Therefore we assume that r_f is related to plasma membrane properties.

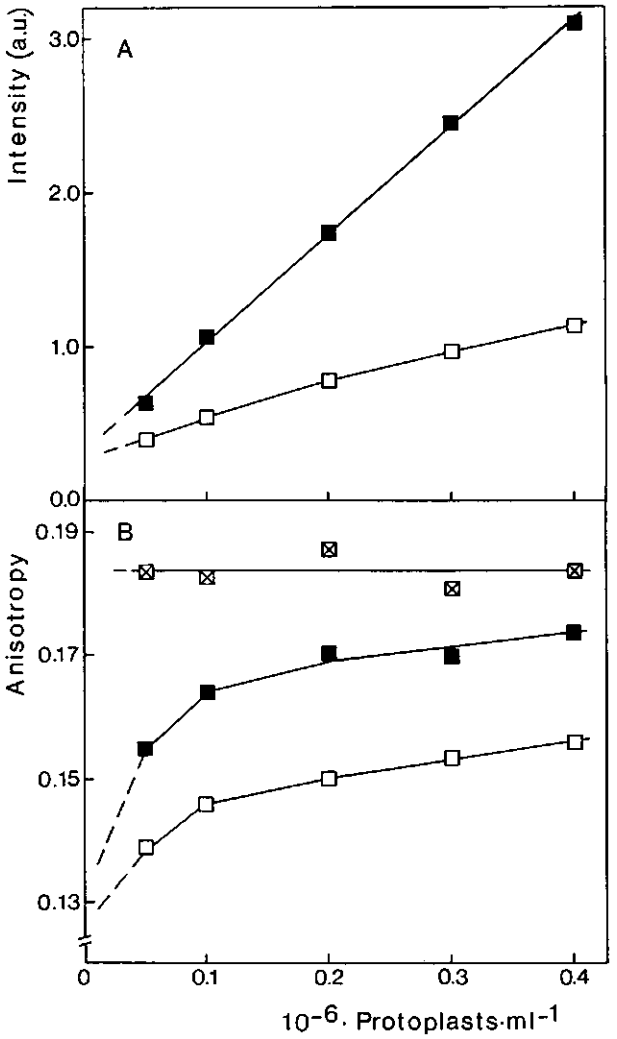


Fig. 6.3. The influence of protoplast concentration on fluorescence intensity and anisotropy. A Fluorescence intensity. B Fluorescence anisotropy. By varying the concentration DPH, the amount of DPH/protoplast was the same for all protoplast concentrations: 10^{-14} mol. □, non-labeled; ■, labeled; ⊠, corrected

The fluorescence intensity of both non-labeled (I_s) and labeled protoplasts (I_m) was strongly dependent on protoplast concentration (Fig. 6.3A), as were the anisotropy values of non-labeled (r_s) and labeled (r_m) protoplasts. However, the anisotropy of DPH (r_f) was constant over the range of $(0.05 - 0.4) \cdot 10^6$ protoplasts$\cdot$ml^{-1} (Fig. 6.3B). With increasing DPH concentration, both I_m and r_m increased, while r_f was constant (Fig 6.4A, 4B).

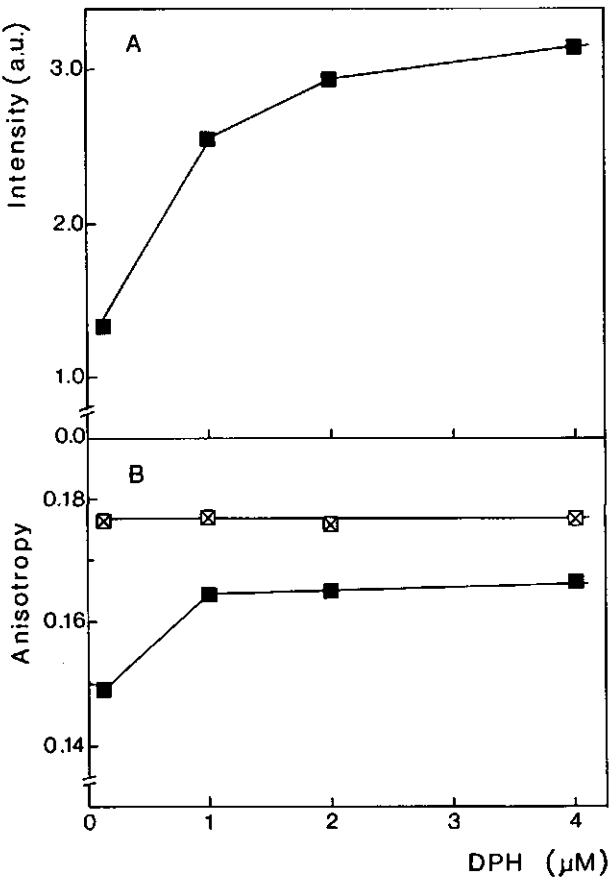


Fig. 6.4. The influence of DPH concentration on fluorescence intensity and anisotropy. **A** Fluorescence intensity. **B** Fluorescence anisotropy. The protoplast concentration was $0.2 \cdot 10^{-6} \cdot ml^{-1}$. ■, labeled; ⊠, corrected. Non-labeled protoplasts were treated with corresponding amounts of the DPH solvent acetonitrile. For all concentrations the anisotropy r_s was: 0.1316 and the intensity I_s: 0.742

When treated with corresponding amounts of the DPH solvent acetonitrile, I_s and r_s were found to be constant (data not shown). These results are in agreement with the results of Kuhry et al. (1985), who investigated the incorporation of trimethylammonium-diphenylhexatriene (TMA-DPH) in mouse fibroblasts. As a compromise between fluorescence intensity and the available amounts of protoplasts in our experiments, $0.2 \cdot 10^6$ protoplasts$\cdot$ml^{-1} and a DPH concentration of $2 \cdot 10^{-6}$ M were chosen as standard conditions.

Anisotropy changes after red light. The kinetics of anisotropy changes after control-FR- or R-irradiation were estimated (Fig. 6.5). After FR hardly any change in the anisotropy of DPH (r_f) was observed, while the change of r_f after R was much larger and nearly complete within 4 min.

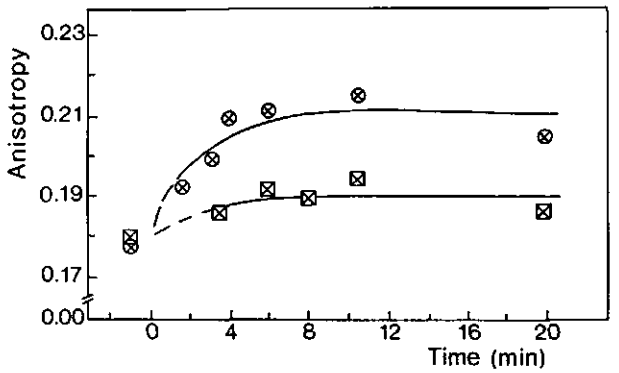


Fig. 6.5. The effect of red light (R) and far-red light (FR) on the anisotropy of DPH-labeled protoplasts. Prior to irradiation (t = -1) and after a 1 min R (⊗) or a 3 min FR (⊠) pulse, the fluorescence intensity and anisotropy of non-labeled and labeled protoplasts were measured. Irradiation was started at t = 0. The corrected anisotropy values are presented

It has been shown that R-induced protoplast swelling is influenced by the osmotic potential of the medium. At higher osmolarity of the medium the R-induced swelling was larger (Chapter 2). Therefore the influence of the osmotic potential on r_s, r_f and R-induced changes therein, was studied.

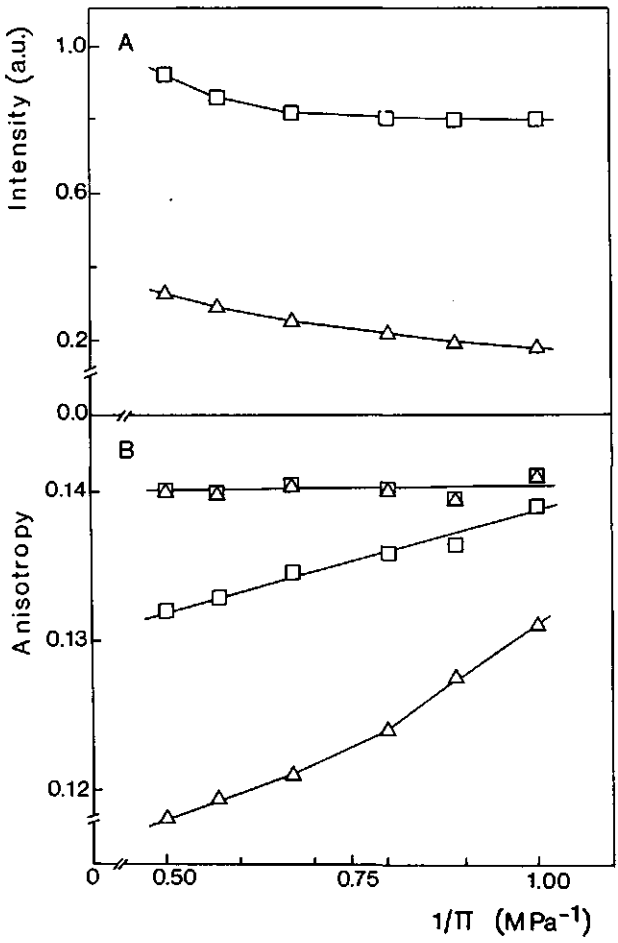


Fig. 6.6. The effect of the osmotic potential on the fluorescence anisotropy of non-labeled protoplasts. Protoplasts were resuspended in media with different sorbitol concentrations. After 30 min in darkness, fluorescence intensity and anisotropy were measured. The anisotropy values of non-labeled protoplasts were corrected for the measured values of the sorbitol media alone. A Fluorescence intensity. B Anisotropy values. Δ, sorbitol medium; □, non-labeled protoplasts; ▨, corrected anisotropy

At the wavelengths used, solutions of D-sorbitol showed a concentration dependent, apparent fluorescence intensity and anisotropy (Fig. 6.6A, 6B). Non-labeled protoplasts, suspended in media with these sorbitol concentrations, and measured after 30 min incubation, showed higher values of both fluorescence intensity (I_s) and anisotropy (r_s), when compared to those of the sorbitol solutions. When the anisotropy of non-labeled protoplasts was corrected for the apparent fluorescence intensity and anisotropy of the corresponding sorbitol solution, using the correction formula described in the Material and methods, the anisotropy appeared to be independent of the sorbitol concentration in the range 0.4 - 0.8 M sorbitol ($1/\pi$ = 1.0 - 0.5 MPa^{-1}).

To investigate the effect of the osmotic potential on the anisotropy of labeled protoplasts, protoplasts were resuspended in incubation media with sorbitol concentrations varying between 0.4 and 0.8 M sorbitol. After 30 - 60 min incubation in darkness, protoplasts were labeled with DPH, washed and measured. The protoplasts showed an osmotic potential dependent anisotropy: at lower sorbitol concentration the anisotropy value (r_m) was higher (Fig. 6.7). After measurement, protoplasts were R-irradiated and measured again 10 min later. At sorbitol concentrations higher than 0.4 M ($1/\pi$ < 1.0), r_m was higher after R. The increase appeared to be larger at higher osmotic potential. The corrected values (r_f) showed the same tendency. At all osmotic potentials used, the fluorescence intensity (I_m) increased by approx. 10% after R (data not shown). At 0.8 M sorbitol ($1/\pi$ = 0.5 MPa^{-1}) the anisotropy values, both before and after R-irradiation, were higher than expected. The membranes are probably wrinkled at this sorbitol concentration, due to extensive shrinkage of the protoplasts.

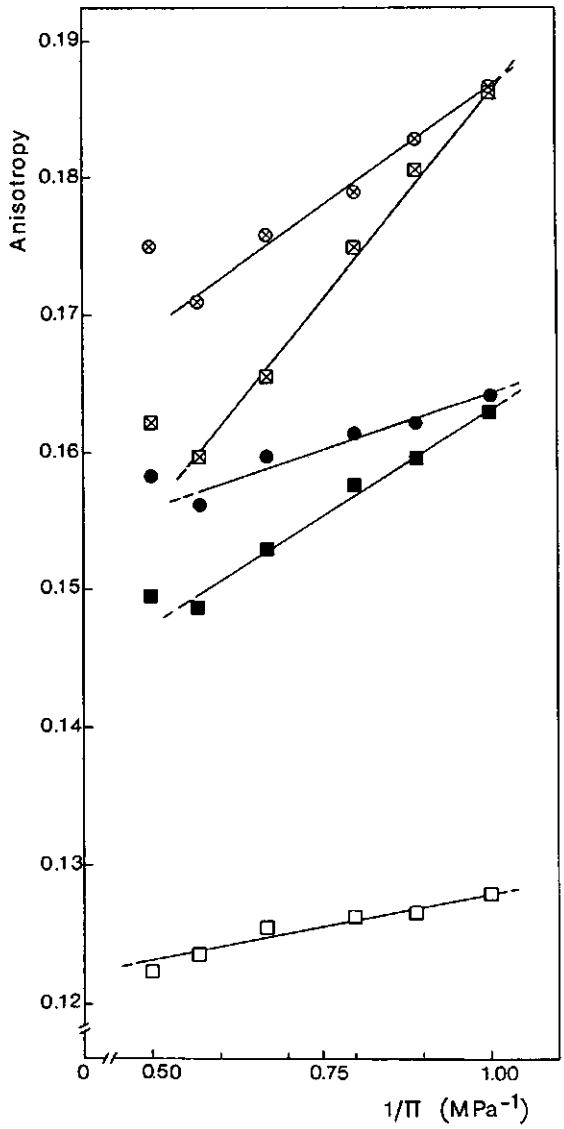


Fig. 6.7. An example of the effect of the osmotic potential on the red light (R)-induced anisotropy increase. Protoplasts were resuspended in media with different sorbitol concentrations and incubated in darkness for 30 - 60 min. After DPH-labeling protoplasts were measured, and the same protoplasts were then R-irradiated and measured again after 10 min. The same procedure was executed with non-labeled protoplasts. □, Dark, non-labeled; ■, Dark, labeled; ⊠, Dark, corrected; ●, R, labeled; ⊗, R, corrected. After R, the anisotropy values of non-labeled protoplasts were the same as in darkness

Discussion

The steady-state fluorescence anisotropy of DPH (r_f), incorporated into lipid membranes, is thought to be mainly determined by the molecular packing, i.e. the degree of order of the lipids, restricting the rotation of the fluorophore (Van Blitterswijk et al. 1981). An increase of r_f can either result from an increase of the degree of order of the lipids or from a decreased lifetime of the excited DPH molecules. However, this last possibility is unlikely to have occurred in the R-irradiated protoplasts, because an increase in fluorescence intensity was measured. The term "membrane fluidity" will be used here as a general description, in which an increase in anisotropy represents a decrease in fluidity. The R-induced increase in anisotropy, which was almost complete after 4 min (Fig. 6.4), corresponds to a decrease in fluidity of the membrane. A similar result was described for plasma membrane vesicles from soybean hypocotyls, in which indole acetic acid induced an increase in anisotropy of the lipophilic probe N-phenyl-1-naphthylamine. This was correlated with a change in membrane conformation: thinning of the membrane (Helgerson et al. 1976). In some animal cell types such an increase in anisotropy has also been observed, e.g. after addition of insulin to liver plasma membrane (Hyslop et al. 1984), after stimulation of lymphocytes with succinyl-concanavalin A (Cherenkevich et al. 1982) and of neutrophils stimulated with phorbol myristate acetate (Stocker et al. 1982). However, no change in DPH anisotropy was observed, after peptide stimulation of human polymorphonuclear leukocytes (Valentino et al. 1986), and after addition of cholinergic agonists to adrenal chromaffin cells, a decrease in anisotropy was found (Schneeweiss et al. 1979).

The anisotropy of non-labeled protoplasts (r_s) seems to be independent of the osmotic potential of the medium, when corrected for the apparent anisotropy of the medium itself (Fig. 6.6B). The anisotropy of labeled protoplasts (r_m) and the corrected anisotropy (r_f) appear to be strongly dependent on the osmotic potential (Fig. 6.7). The anisotropy is highest at low potentials, indicating that swelling of protoplasts is accompanied by a decrease in the fluidity of the membrane. This is in contrast to the results of Borochov and Borochov (1979), who found in protoplasts of rose petals an increased fluidity upon osmotically-induced swelling. These protoplasts were incubated with DPH at 37°C, while in our case the wheat

protoplasts were maintained throughout at 22°C. It is doubtful if this is the explanation for the observed discrepancy. It is also necessary to keep in mind that, although r_f has been corrected for autofluorescence (including the apparent fluorescence of the medium) and scattering, values cannot be corrected for the effect of sorbitol dependent polarization of the emitted fluorescence. Therefore, the values presented are only relative and cannot be regarded as absolute.

After R, the corrected anisotropy (r_f) was higher at sorbitol concentrations higher than 0.4 M: the increase was larger at higher osmotic potential. If these results are compared with the Boyle-van't Hoff analysis of swelling (see Chapter 2), there is a remarkable resemblance. In swelling experiments, R-induced swelling was also larger at higher osmolarity. The figures 6.7 and 2.4 (Chapter 2) suggest a simple relationship between volume change and change in r_f. However, when volume and r_f of several experiments were compared, different relationships between these parameters were observed. The observed influence of osmotic potential on r_f may interfere with a R-induced change in r_f, independent of changes in osmotic potential or volume. To investigate the effect of R, independent of volume change, it could be better to resuspend the protoplasts in a medium with 0.4 M sorbitol, since at this concentration swelling of protoplasts, as well as the change in r_f, is small or even zero. The change in membrane properties can than be studied varying external conditions, such as temperature, osmotic potential or membrane potential. Because of the observed interference with volume changes, temperature might be a better choice to study R-induced changes in r_f. To investigate further the role of changes in membrane fluidity in phytochrome action, it has to be established under what circumstances these changes occur. For R-induced swelling it was shown that activation of Ca^{2+}-channels is a prerequisite for swelling. A study of the effect of depletion of Ca^{2+} from the medium, or addition of the Ca^{2+}-channelblocker Verapamil, or the protein kinase inhibitor 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine (H_7) on R-induced anisotropy changes in anisotropy, could give an indication if changes in anisotropy are also dependent on Ca^{2+}-channel activation, or that the formation of Pfr itself influences membrane fluidity. The effect of the calmodulin antagonist N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W_7) on changes in r_f, is also of interest, since this compound induced protoplast swelling, probably without activation of Ca^{2+}-channels in the plasma membrane.

Chapter 7

Conclusions and summary

Isolated plant protoplasts proved to be useful in different fields of scientific research. In plant biotechnology, they are an important tool for studying plant regeneration and for introducing foreign genes (Friedt and Brune 1987; Puite et al. 1988), and in plant virus research they are used for minute studies of the virus infection process (Sander and Mertens 1984). In plant physiology, protoplasts have been mainly used for the study of plasma membrane related topics, e.g. cell wall regeneration, transport of ions and of organic compounds such as amino acids and sugars, and for the study of the chemical structure and physical properties of the plasma membrane itself (Galun 1981; Pilet 1985). The introduction of the patch-clamp technique and its application to plant protoplasts, has accelerated our knowledge about ion transport across the plasma membrane and the tonoplast (Hedrich and Schroeder 1989; Schroeder and Hedrich 1989).

In this thesis mesophyll protoplasts, isolated from the primary leaves of dark-grown wheat, have been used to investigate phytochrome-induced changes of plasma membrane properties. These changes could be involved in the transduction chain between phytochrome photoconversion and ultimate physiological responses. In Chapter 2, it is shown that protoplasts swell after red light (R) irradiation. When far-red light (FR) is given immediately after R, swelling does not occur, indicating the involvement of phytochrome in this response. No R-induced swelling occurs in the absence of Ca^{2+} or when Ca^{2+}-channelblockers are added to the medium. Furthermore, swelling was induced in darkness or after control FR irradiation, by the Ca^{2+}-ionophore A23187 (Chapter 2) and by the Ca^{2+}-channel agonist Bay K-8644 (Tretyn et al. in press). Protoplast swelling after control FR was also observed when the calmodulin antagonist N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W_7) was added to the protoplast suspension (Chapter 3). Gilroy et al. (1987) have proposed that such a treatment results in a rise of the cytoplasmic Ca^{2+}-concentration ($[Ca^{2+}]_{cyt}$), due to the inactivity of a plasma membrane located Ca^{2+}-ATPase, which is normally activated by calmodulin. Based on these results it was proposed, that the R-induced formation of the FR-absorbing form of phytochrome (Pfr) results in the activation of Ca^{2+}-channels, which in turn leads to enhanced uptake of Ca^{2+} and a rise in $[Ca^{2+}]_{cyt}$, and ultimately to protoplast swelling.

To obtain further information about phytochrome-regulated Ca^{2+}-fluxes, changes in the $[Ca^{2+}]$ of the protoplast incubation medium were monitored spectrophotometrically with the Ca^{2+}-sensitive dye murexide (Chapter 5). After R-irradiation the $[Ca^{2+}]$ of the medium increased. A subsequent FR irradiation reversed this effect. The R-induced efflux was inhibited by the Ca^{2+}-channelblocker Verapamil and by the calmodulin antagonist W_7. Although the observed R-induced efflux seems not to be in agreement with the hypothesis that R-irradiation results in activation of Ca^{2+}-channels and a Ca^{2+}-influx, the observed inhibition of the efflux by the Ca^{2+}-channel blocker Verapamil can explain this apparent contradiction. A Pfr-induced influx seems to be a necessary step which precedes the observed efflux. It is speculated that the influx causes a depolarization of the plasma membrane. The observed complete R-FR reversibility of the change in the $[Ca^{2+}]$ of the medium (Chapter 5; Hale and Roux 1980; Takagi and Nagai 1988) is difficult to explain. A tentative explanation, given in Chapter 5, is the postulation of an inactivated state of the membrane in darkness or after FR-irradiation (phytochrome is present as Pr) and an activated state after R-irradiation (phytochrome is present as Pfr). These different states of activation are speculated to determine the binding of Ca^{2+} to membranes and as a consequence the $[Ca^{2+}]$ of the medium, by an as yet unknown mechanism. The possibility that such activation states do exist, is supported by the following observations. When wheat protoplasts are irradiated with a 1 min R pulse in the absence of Ca^{2+}, Ca^{2+} can be added up to 10 min after the irradiation, to obtain a full swelling response (Chapter 2). This could be interpreted as that 10 min after R, the protoplast membrane begins to revert from the activated to the inactivated state, possibly due to destruction of phytochrome (as Pfr). In *Vallisneria* protoplasts, the reversion of the R-induced increase of the $[Ca^{2+}]$ of the medium, is found to begin approx. 5 min after the end of the R-irradiation. If FR is given directly after R (and Pfr is reverted to Pr), the reversion seems to start immediately (Takagi and Nagai 1988). Although phytochrome-regulated protoplast swelling and the phytochrome-regulated change in $[Ca^{2+}]$ of the medium have features in common (e.g. involvement of Ca^{2+}-channels) it is doubtful if these responses are directly correlated with each other. Protoplast swelling was also observed in darkness when activation of a plasma membrane located Ca^{2+}-ATPase, was inhibited by addition of the calmodulin antagonist W_7. This compound did not inhibit the R-induced

swelling. However, W_7 inhibited approx. 75% of the R-induced increase in $[Ca^{2+}]$ of the medium. It remains to be established which resemblances and which differences there are between protoplast swelling and the changes in $[Ca^{2+}]$ of the medium, particularly with respect to the time between R- and FR-irradiation, which can be passed in order to revert the response. This is especially of interest because swelling of wheat protoplasts was completed within 10 min (Chapter 2) and the reversion of the R-induced increase in the $[Ca^{2+}]$ of the medium was still possible after 4-10 min in the case of oat and *Vallisneria* protoplasts (Hale and Roux 1980; Takagi and Nagai 1988). As yet, it is unknown if the completed protoplast swelling can be reversed by FR-irradiation. The involvement and function of different Ca^{2+}-pools, intracellular (membrane-bound, cytoplasmic, in organelles and vacuolar) and extracellular (apoplastic), in phytochrome-regulated responses and especially in protoplast swelling await detailed studies of Ca^{2+}-influx, Ca^{2+}-efflux and changes of the $[Ca^{2+}]$ of the different pools.

The primary event in many hormone-regulated processes in animal cells, has been proposed to be the binding of hormone molecules (or agonists) to specific receptors on the plasma membrane, after which **GTP-binding proteins** (G-proteins) are activated, which in turn activate different enzymes, depending on the type of G-protein (Stryer and Bourne 1986; Berridge 1987; Cockcroft 1987; Gilman 1987). This activation eventually leads, among other things, to the opening of Ca^{2+}-channels in the plasma membrane. The R-induced protoplast swelling was found to be inhibited by the G-protein inhibitor GDP-β-S (Chapter 3). The G-protein activator GTP-γ-S induced, in the absence of hormones, protoplast swelling in darkness or after control FR-irradiation. This makes it likely that a G-protein is involved in phytochrome regulated protoplast swelling. Prelimanary observations made by L.C. Romero and P.-S. Song (pers. commun.) show an enhanced GTP-binding activity after R-irradiation in etiolated oat seedlings. This effect was R/FR reversible. Both the physiological and biochemical evidence suggest that G-proteins are involved in phytochrome mediated signal transduction in plants.

In the phosphatidyl-inositol pathway, as known in animal cells, the G-protein G_p activates phospholipase C, which catalyzes the hydrolysis of phosphatidyl-inositol 4,5-biphosphate (PIP_2), yielding inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG). In turn, DG activates a protein kinase C (PKC). This ultimately leads to opening of Ca^{2+}-channels in the plasma membrane. Specific modulators of this pathway were tested for their effect on the protoplast-swelling response. The antagonists of phospholipase C action, neomycin and Li^+, were found to inhibit the R-induced, Ca^{2+}-dependent protoplast swelling (Chapter 3). Other salts such as KCl, NaCl and K_2SO_4 had no effect on the R-induced swelling, while *myo*-inositol (1 mM) prevented the inhibition by Li^+. The agonist 12-phorbol myristate 13-acetate (PMA) of protein kinase C, induced swelling of protoplasts after control FR irradiation, while the antagonist 1-(5-isoquinolinesulfonyl)-2-methyl-piperazine (H_7) inhibited both the R-induced and PMA-induced swelling. These results suggest that a signal-transduction system is functional in plants, which is comparable to one occurring in animal cells. Moreover, there is some biochemical evidence that the phosphatidyl-inositol pathway is utilized in the signal transduction of light and especially of R via phytochrome. A transient, light-induced increase of IP_3 and DG has been reported by Morse et al. (1989). A R-FR reversible, transient increase of IP_3 and DG was also found in the moss *Ceratodon purpureus* (Hartmann and Pfaffmann 1989), while Sopory et al. (1989) have isolated a protein kinase from maize, the activity of which was increased by the analogue of DG, oleyl acetyl glycerol and also by PMA.

As discussed in Chapter 3, the activation by phytochrome, of a G-protein and the phosphatidyl-inositol cycle "would imply that the phytochrome molecule itself, or a product formed as a consequence of Pfr formation, is at least transiently bound to, or associated with, the plasma membrane". The proposed association of phytochrome (as Pr and Pfr) with the plasma membrane (see Chapter 1) supports the idea that the phytochrome molecule itself interacts with the membrane. If this is the case, Pfr could directly activate a G-protein or bind to a receptor and the activated receptor in turn would activate the G-protein. Both possibilities are consistent with the conformational changes of the protein moiety of the phytochrome molecule after photo-conversion (see Chapter 1). In general, plasma membrane located hormone receptors of animal cells are present on the outside of

the membrane. However, if phytochrome binds to a receptor on this membrane it is more likely that the receptor is on the cytoplasmic face, or within the membrane. Tokutomi and Mimuro (1989) have proposed a model in which dimeric phytochrome is attached on the membrane with the C-terminal domain. If phytochrome is attached on the membrane, it is conceivable that it would be attacked by proteases. Serlin and Roux (1986) found that phytochrome associated with mitochondria is protected against proteases, and therefore propose that in this case, phytochrome is localized within the membrane. This phytochrome has been proposed to modulate several mitochondrial activities: NADH dehydrogenase, release of Ca^{2+} and ATPase activity. When studying phytochrome-membrane interactions it should also be kept in mind that, phytochrome associated with membranes could differ in conformation with phytochrome in solution (Jordan et al. 1984). The interaction of phytochrome with synthetic membranes (Roux and Yguerabide 1973; Singh et al. 1989) shows that the interaction per sé is independent of membrane proteins.

In some plant systems a resemblance between responses induced by **phytochrome and by cAMP** has been noted. In the lichen *Evernia prunastri*, both Pfr and cAMP induced the activity of the enzyme d-usnic acid dehydrogenase (Avalos and Vicente 1987). Addition of the membrane permeable cAMP analogue dibutyryl-cAMP to protoplasts isolated from dark-grown wheat (Chapter 3) or dark-grown oat (Chung et al. 1988), resulted in protoplast swelling to an extent comparable to that after R. In roots of etiolated *Cicer arietium* seedlings the endogenous concentration of cAMP increased after R-irradiation, while FR or the presence of EDTA prevented this effect. The addition of cAMP in darkness or R-irradiation resulted in both cases in the enhanced uptake of ammonia by the roots of these plants (Vaello and Vicente 1989). In animal cells the activation by hormones of either phospholipase C (IP_3 and DG) or adenylate cyclase (cAMP), induces activation of different types of Ca^{2+}-channels. It is therefore possible that in plant cells phytochrome (Pfr) and cAMP activate Ca^{2+}-channels independently, resulting in the same response, e.g. protoplast swelling. This is consistent with the involvement of the phosphatidyl-inositol pathway in phytochrome action, as proposed in this thesis. However, I don't exclude the possibility that there are interactions between the phytochrome initiated transduction chain and cAMP, as seems to be the case in the roots of *Cicer*.

It has been shown (Chapter 4) that the hormones gibberellic acid, benzylaminopurine, α-naphthaleneacetic acid, abscisic acid and acetylcholine (ACh) all induce swelling of protoplasts in darkness, to an extent comparable to that after R, when incubated in a medium containing Ca^{2+}. No hormone-induced swelling was observed in the absence of Ca^{2+} or when the Ca^{2+}-channelblocker Verapamil was added to the medium. For all hormones tested, the Ca^{2+}-dependent swelling was inhibited by GDP-β-S. Unlike phytochrome, ACh also induced swelling when, in the absence of Ca^{2+}, K^+ or Na^+ were added to the medium. This K^+/Na^+-dependent swelling was not inhibited by GDP-β-S. These results are in agreement with the existence of two types of ACh-receptors, as known in animal cells. The "muscarinic" type of ACh-receptor is coupled to a G-protein and influences Ca^{2+}-fluxes, while the "nicotinic" type is part of a K^+/Na^+-channel (Changeaux 1984). Tretyn et al. (1989) have presented evidence for the existence of both types of receptors on protoplasts, isolated from the primary leaves of dark-grown wheat. The observed Ca^{2+}-dependency of hormone induced protoplast swelling is in agreement with the reported requirement of Ca^{2+} for other hormone induced responses (Hepler and Wayne 1985; Elliott 1986).

The similarity between phytochrome and hormone induced protoplast swelling, leaf unrolling and some other responses (see Chapter 4), poses the question which signal, alone or in concert, is operating in the whole plant growing under natural conditions. In the absence of light or hormones Ca^{2+}-channel agonists have been reported to induce protoplast swelling (Tretyn et al. in press), leaf unrolling (Tretyn and Kendrick 1989), initiation of gametophore buds in the moss *Funaria* (Conrad and Hepler 1988) and to influence leaflet closure of *Cassia* (Roblin et al. 1989). Based on these observations it is speculated that any treatment which leads to activation of Ca^{2+}-channels, induces these particular responses.

The induction of leaf unrolling in the primary leaves of wheat and of swelling of protoplasts, isolated from these leaves, show many resemblances. The presence of Ca^{2+} is a prerequisite for both these phytochrome induced responses (Chapter 2; Viner et al. 1988). Furthermore, protoplast swelling (Tretyn et al. in press) and leaf unrolling (Tretyn and Kendrick 1989) are both induced by the Ca^{2+}-channel agonist Bay K-8644. The observed protoplast swelling after addition of hormones (Chapter 5) has its

equivalent in leaf unrolling. Leaf unrolling has been found to be induced by gibberellin, cytokinin and acetylcholine (Beevers et al. 1970; Viner et al. 1988; Tretyn and Kendrick 1989). However, abscisic acid (ABA) inhibited phytochrome, gibberellin and cytokinin induced leaf unrolling, as well as the slight unrolling of segments maintained in darkness in the absence of hormones (Beevers et al. 1970; Poulson and Beevers 1970). The inhibition by ABA could be aspecific since parameters such as pH and [Ca^{2+}] of the medium were not controlled and the unrolling in the absence of hormones in darkness, was also inhibited. In the absence of Ca^{2+}, both protoplast swelling and leaf unrolling are inducible by ACh, if K^+ or Na^+ is present in the medium (Tretyn et al. 1989; Tretyn et al. in press). These resemblances make it likely that the swelling of protoplasts and the process of leaf-unrolling have a common basis.

It has been proposed that the irreversible **enlargement of plant cells**, as is the case for phytochrome-regulated leaf unrolling and leaf expansion, results from two interdependent processes: i.e. cell wall yielding and water absorption (Sangeetha and Sharma 1988). In such a model, cell expansion starts with a reduction in wall stress, resulting in water absorption and expansion. Changes in osmotic potential will induce only a transient, reversible water flow and is not thought to be the cause of cell expansion (Cosgrove 1986). The protoplast swelling, described in this thesis, after R-irradiation or after addition of hormones, is therefore probably not directly involved in leaf unrolling, especially since protoplast swelling is less, or even zero at the osmotic potential of the cells in plants (Chapter 2). The mechanism of cell wall yielding is largely unknown, a change of cell wall composition by deposition of new material has been proposed (Dale 1988). The proposed increase in cytoplasmic Ca^{2+} and of phosphorylation of proteins may play a role in the deposition of new cell wall material. Such a model has been described for auxin-induced growth of dicotyledons (Brummel and Hall 1987). Whether the observed efflux of Ca^{2+} after R in the protoplast suspension (Chapter 5), which would enhance the apoplastic [Ca^{2+}] in the plant tissue, is important for the regulation of cell wall yielding and thus cell expansion, is not clear.

The involvement of Ca^{2+}-channels in phytochrome regulated protoplast swelling and leaf-unrolling, indicates that the plasma membrane is an important cellular component for phytochrome regulated responses. Other plasma membrane properties regulated by phytochrome are the transmembrane potential (Weisenseel and Ruppert 1977) and the surface potential (Stenz and Weisenseel 1986). However, the connection between these changes and the responses discussed previously, is not clear and awaits further research. Other plasma membrane properties may also play a role in phytochrome action. In this thesis (Chapter 6) it is shown that R-irradiation leads to a decrease in the "fluidity" of the plasma membrane. The decrease in "fluidity" was larger at higher sorbitol concentrations. The R-induced protoplast swelling was also dependent on the osmotic potential of the medium. Whether there is a simple relationship between R-induced changes in volume and in "fluidity" is not clear as yet. A comparison of the circumstances under which changes in volume and "fluidity" occur, could help to establish whether or not R-irradiation influences membrane "fluidity", independently of volume changes. A decrease in membrane "fluidity" was also observed when auxins were added to membrane vesicles of soybean hypocotyls (Helgerson et al. 1976).

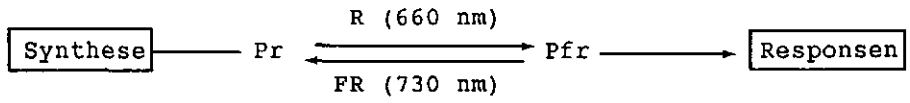
The results presented in this thesis show that protoplasts are an interesting object and useful tool for studying signal transduction in the case of phytochrome and hormones. It is necessary to obtain protoplasts of constant good quality throughout the year, to make such studies possible. Controlled environmental conditions for plant growth are important prerequisites for achieving this. In our laboratory it was necessary to grow plants in a controlled air-flow cabinet, through which purified air was passed continuously, to obtain physiological active protoplasts throughout the year (Chapter 3). Air pollutants were implicated as the possible source of damage to the plants grown in non-purified air. The exclusion dyes phenosafranine and Evans blue, generally used to test the viability of protoplasts, have only a limited use for this purpose. Some protoplast preparations that did exclude phenosafranine were inactive in our swelling experiments. Enzymes used for the protoplast preparation can have the same effect on protoplasts as the air pollutants. Some enzyme batches or enzyme types yield protoplasts which do exclude vital dyes, but are physiologically inactive (Fitzsimons and Weyers 1985).

Protoplasts provide the possibility to use different techniques to study, in higher plants, phytochrome-regulated changes in molecular and cellular properties, within a single system. Hitherto, phytochrome-induced changes in membrane properties have been studied mainly in lower plants, e.g. the transmembrane potential of the alga *Nitella* (Weisenseel and Ruppert 1977) and the surface potential of the alga *Mesotaenium* (Stenz and Weisenseel 1986). For higher plants these types of studies have been hampered by the inaccessibility of the plasma membrane. With protoplasts and perhaps also with single-cell suspensions, studies on membrane potential can be performed and the results compared with changes in e.g. ion fluxes and membrane fluidity. However, insights obtained in this way should be tested and further explored in whole plants, growing under natural conditions, to verify the validity of the model obtained with protoplasts.

Samenvatting

De ontwikkeling van een plant wordt op velerlei manieren gestuurd door uitwendige faktoren, zoals licht, zwaartekracht en het aanbod van voedingsstoffen. Licht wordt door planten op twee manieren gebruikt, nl. als energiebron in de fotosynthese en als informatiebron in de fotomorfogenese. Planten beschikken over pigmenten om het licht te kunnen waarnemen. Voor de fotosynthese is dit oa chlorofyl a. Voor de fotomorfogenese zijn er tenminste drie pigmenten, die gevoelig zijn voor respektievelijk ultraviolet, blauw of rood licht. Het pigment dat gevoelig is voor rood licht wordt fytochroom genoemd en is het onderwerp van dit proefschrift. Het wordt gevonden in zowel hogere, als lagere planten (mossen, varens, algen), en het reguleert ondermeer kieming, lengtegroei en deëtiolatie. Dit laatste proces wordt gekenmerkt door chlorofyl synthese (groen worden), remming van de lengtegroei en bij grasachtigen, het ontrollen van het blad, en treedt op bij planten, die vanuit het donker (ondergronds) in het licht (bovengronds) komen.

Fytochroom komt voor in twee vormen, de rood licht absorberende vorm (Pr) en de ver-rood licht absorberende vorm (Pfr). Het wordt, in het donker, gesynthetiseerd in de Pr-vorm en na R-belichting omgezet in de Pfr-vorm. Door FR-belichting wordt Pfr weer omgezet in Pr.



Er is nog weinig bekend over hoe fytochroom de fysiologische processen reguleert. De vorming van Pfr gaat gepaard met veranderingen in het fytochroom molekuul en dit leidt, via een keten van reakties, uiteindelijk tot de genoemde fysiologische responsen. Het onderzoek, hoe dit precies gebeurt, richt zich vooral op twee van de mogelijke aangrijpingspunten, de regulatie van gen expressie en de rol van Ca^{2+}. Van beide is aangetoond dat ze betrokken zijn bij fytochroom gereguleerde processen.

In dit proefschrift wordt onderzoek beschreven naar fytochroom gereguleerde veranderingen van plasmamembraan eigenschappen, zoals veranderingen in Ca^{2+}-fluxen en membraan vloeibaarheid. Er is gebruik gemaakt van protoplasten, geïsoleerd uit in donker gegroeide tarwe. Protoplasten zijn plantecellen waarvan de celwand met enzymen wordt verwijderd, waardoor de plasmamembraan direkt toegankelijk is voor onderzoek.

Deze protoplasten bleken na R-belichting een groter volume te hebben, ze waren gezwollen tov protoplasten, die in donker waren gebleven, of met controle FR waren belicht (Hoofdstuk 2). Als direkt na R, FR werd gegeven trad deze zwelling niet op. Dit is een aanwijzing dat fytochroom de zwelreaktie reguleert. De reaktie trad alleen op wanneer Ca^{2+} in het medium aanwezig was of werd toegevoegd binnen 10 min na de R-belichting. Bovendien, konden Mg^{2+}, Ba^{2+} en K^{+} de behoefte aan Ca^{2+} niet vervangen. Indien geen Ca^{2+} aanwezig was, of wanneer Verapamil (een blokkeerder van Ca^{2+}-kanalen in de plasmamembraan) werd toegevoegd, werd geen zwelling waargenomen. Zwelling kon ook in donker geïnduceerd worden door toevoeging van de Ca^{2+}-ionofoor A23187 en door de calmoduline antagonist N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W_7). Van beide behandelingen wordt verondersteld dat ze leiden tot een verhoging van de Ca^{2+}-concentratie [Ca^{2+}] van het cytoplasma. De R-geïnduceerde zwelling was afhankelijk van de osmotische waarde van het medium. Bij hogere sorbitol concentraties was de zwelling respons groter, terwijl bij lagere concentraties de zwelling kleiner was dan bij de als standaard gebruikte 0,5 M sorbitol.

Als hypothese werd voorgesteld, dat de vorming van Pfr door R-belichting leidt tot de aktivering van Ca^{2+}-kanalen in de plasmamembraan, met als gevolg een verhoogde opname van Ca^{2+}, een stijging van de [Ca^{2+}] van het cytoplasma, en uiteindelijk zwelling van de protoplasten.

Aktivering van Ca^{2+}-kanalen en een verhoogde opname van Ca^{2+}-ionen zijn verschijnselen, die in dierlijke cellen optreden na bij voorbeeld binding van hormonen aan de receptoren in de plasmamembraan. Het hormoon-receptor complex aktiveert zogenaamde "GTP-binding proteins" (G-proteins), die op hun beurt verschillende enzymen aktiveren, afhankelijk van het type G-protein. Het effekt op de zwelrespons van tarwe protoplasten van de G-protein remmer guanosine-5'-O-(2-thiodiphosphate) (GDP-β-S) en de G-protein aktivator guanosine-5'-O-(3-thiotriphosphate) (GTP-γ-S), werd onderzocht. De R-geïnduceerde zwelling werd geremd door GDP-β-S en bovendien induceerde GTP-γ-S zwelling van protoplasten in donker of na een controle FR-belichting. Aktivering van een G-protein lijkt noodzakelijk te zijn voor de fytochroom gereguleerde zwelling van protoplasten (Hoofdstuk 3).

Eén van de G-proteinen uit dierlijke cellen (G_p) aktiveert de phosphatidyl-inositol cyclus. Bekende modulatoren van deze cyclus werden getoetst op hun effekt op de R-geïnduceerde zwelling van protoplasten. Remmers zoals Li^+, neomycine en 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine (H_7) remden de R-geïnduceerde zwelling specifiek. Een aktivator 12-myristate 13-acetate (PMA) induceerde zwelling in donker. De zwelling geïnduceerd door PMA, werd, op zijn beurt, ook geremd door H_7. Dit zou kunnen betekenen dat de phosphatidyl-inositol cyclus betrokken is bij de keten van reakties, tussen de vorming van Pfr en de fysiologische responsen.

Voor verschillende typen plantehormonen wordt verondersteld dat Ca^{2+} als "second messenger" fungeert. Daarom werd getoetst of gibberelline, auxine, cytokinine, abscicinezuur en acetylcholine, net als R-belichting, zwelling van protoplasten kan induceren (Hoofdstuk 4). Als Ca^{2+} in het medium aanwezig was, induceerden aktieve vormen van alle genoemde hormonen zwelling van de protoplasten. Zonder Ca^{2+} of na toevoeging van Verapamil (een blokkeerder van Ca^{2+}-kanalen) werd geen zwelling gevonden. Deze Ca^{2+}-afhankelijke zwelling werd in alle gevallen geremd door GDP-β-S, wat wijst op een mogelijke betrokkenheid van een G-protein bij de induktie van zwelling door de hormonen. Bovendien zou dit kunnen betekenen dat, op/in de plasmamembraan van de geëtioleerde tarwe protoplasten receptoren voor de hormonen aanwezig zijn, die een G-protein aktiveren.

Acetylcholine induceerde ook zwelling wanneer Ca^{2+} werd vervangen door K^+ of Na^+, wat niet het geval was na R-belichting. Deze K^+/Na^+-afhankelijke zwelling werd niet geremd door GDP-β-S, wat wijst op een andere reaktieketen.

Om de Ca^{2+} fluxen, die optreden na R-belichting, verder te karakteriseren en te kwantificeren bepaalden we de $[Ca^{2+}]$ van het medium, met behulp van de Ca^{2+}-gevoelige kleurstof murexide (Hoofdstuk 5). Na R-belichting werd de $[Ca^{2+}]$ van het medium hoger. Indien na R, direkt FR werd gegeven, trad deze verandering niet op. Zowel de blokkeerder van Ca^{2+}-kanalen Verapamil, als de calmoduline antagonist W_7, remden de stijging van de $[Ca^{2+}]$ van het medium na R-belichting, voor ca. 75%. Dit wijst erop dat zowel een Ca^{2+}-influx door Ca^{2+}-kanalen, als een Ca^{2+}-efflux via een Ca^{2+}-ATPase, een rol spelen in de R-geinduceerde toename van de $[Ca^{2+}]$ van het medium. Bovendien is het waarschijnlijk dat eerst een kleine influx moet plaats vinden, die gevolgd wordt

door een veel grotere efflux. De vrijkomende Ca^{2+} zou afkomstig kunnen zijn uit opslagplaatsen in de cel, bv de vacuole, of bestaan uit Ca^{2+} dat vóór de R-belichting aan membranen was gebonden. Na FR-belichting zou de cel terug kunnen keren naar een "geïnaktiveerde" toestand, wat leidt tot het opnemen van de eerder vrijgekomen Ca^{2+}.

Omdat de plasmamembraan een belangrijke rol lijkt te spelen in de keten van reakties tussen de vorming van Pfr en de zwelrespons, werd het effekt van R-belichting op de "membraan vloeibaarheid" onderzocht (Hoofdstuk 6). De anisotropie van de fluorescerende membraan probe 1,6-diphenyl-1,3,5-hexatriene (DPH) wordt beschouwd als een maat voor de vloeibaarheid van de membraan. Na R-belichting nam de anisotropie van DPH toe. Dit zou betekenen dat de membraan minder vloeibaar, dus meer star is, als gevolg van de R-belichting. Deze toename in anisotropie was afhankelijk van de osmotische waarde van het medium. Bij hogere sorbitol concentraties was het verschil groter, bij lagere kleiner, dan bij de standaard gebruikte 0,5 M sorbitol. In het donker, werd een verandering in de anisotopie gevonden, na osmotisch geïnduceerde zwelling van de protoplasten. Het is nog niet duidelijk of R-belichting de membraan vloeibaarheid verandert, onafhankelijk van de volume verandering. De omstandigheden en faktoren die de toename in anisotropie van DPH na R-belichting bepalen en beïnvloeden, moeten nog nader in detail onderzocht worden.

In hoofdstuk 7 wordt nader ingegaan op een aantal punten. Het is lang onduidelijk geweest of fytochroom aan membranen bindt en of dit fysiologisch van betekenis is. Fytochroom is een water oplosbaar eiwit en wordt in donker-gegroeide planten, vooral in het cytoplasma van de cel gevonden. In geisoleerde vesikels van de plasmamembraan is ook fytochroom aangetoond. De in dit proefschrift beschreven aktivering van een G-protein, gevolgd door de aktivering van Ca^{2+}-kanalen tgv de vorming van Pfr door R-belichting, ondersteunt het idee dat binding van fytochroom aan de plasmamembraan belangrijk is voor fytochroom aktie. Het fytochroom eiwit ondergaat konformatie veranderingen na R-belichting. Hierdoor is het mogelijk dat fytochroom in de Pfr vorm aan een receptor in de plasmamembraan bindt, of direkt een G-protein aktiveert.

Zowel de zwelling van protoplasten als de ontrolling van het primaire blad van donker-gegroeide tarwe, kunnen geinduceerd worden door R-belichting, of door toediening van gibberelline, cytokinine en acetylcholine, indien Ca^{2+} aanwezig is. Beide processen worden geremd door toevoeging van blokkeerders van Ca^{2+}-kanalen, en kunnen in de afwezigheid van hormonen/R worden geinduceerd door een agonist van Ca^{2+}-kanalen. Deze overeenkomsten maken het waarschijnlijk dat beide processen een gemeenschappelijke basis hebben. Of er echter een direkt verband is tussen de zwelrespons van de protoplasten en de blad-ontrolling, is twijfelachtig. Vergroting van plantecellen zou vooral veroorzaakt worden, door veranderingen in de celwand, en niet door toename van het volume van de protoplast. Een toename van de $[Ca^{2+}]$ in het cytoplasma en van de fosforylatie van eiwitten, zouden wel een rol kunnen spelen bij dit proces.

In dit proefschrift zijn protoplasten van donker-gegroeide tarwe gebruikt, om veranderingen in eigenschappen van de plasmamembraan, als gevolg van R-belichting, te bestuderen. De toegankelijkheid van de plasmamembraan bij protoplasten maakt het mogelijk, onderzoek te doen naar bv ionfluxen en ion-concentraties, membraanpotentiaal en membraanvloeibaarheid in hogere planten, in één systeem. De zo verkregen inzichten moeten echter wel getoetst worden aan de hele plant, zoals die onder natuurlijke omstandigheden groeit.

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Curriculum vitae

Margaretha Elisabeth Bossen werd op 15 mei 1947 te Alkmaar geboren en groeide op in Winkel (NH). Na het behalen van het diploma HBS-B aan de RHBS te Alkmaar, werd de Middelbare Tuinbouwschool voor Meisjes "Huis te Lande" te Rijswijk bezocht. Vanaf 1969 werkte zij al analiste achtereenvolgens op de vakgroep Virologie van de Landbouw Universiteit Wageningen en het Centrum voor Plantenfysiologisch Onderzoek (nu CABO) in Wageningen. In de avonduren werd een studie gevolgd voor botanisch analist en biochemisch laboratorium assistent. In 1977 werd begonnen aan een studie biologie-cel aan de Landbouw Universiteit Wageningen, die werd afgerond in 1985. Het vakkenpakket bestond uit moleculaire biologie, celbiologie en plantenfysiologie. Van september 1985 tot september 1988 werkte zij met een beurs van BION-NWO op de vakgroep Plantenfysiologisch Onderzoek van de Landbouw Universiteit Wageningen. Thans is zij als toegevoegd onderzoeker op een projekt van het Nationaal Programma Verzuringsonderzoek verbonden aan dezelfde vakgroep.