

Calcium requirement of phytochrome-mediated fern-spore germination: No direct phytochrome-calcium interaction in the phytochrome-initiated transduction chain *

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Abstract. Phytochrome-mediated germination of fern spores of *Dryopteris paleacea* Sw. was initiated by a saturating red-light (R) irradiation after 20 h of imbibition. For its realization external Ca^{2+} was required, with a threshold at a submicromolar concentration, and an optimum was reached around 10^{-4} M. At concentrations $\geq 10^{-1}$ M only a reduced response was obtained, based probably on an unspecific osmotic or ionic effect. The germination response was inhibited by La^{3+} , an antagonist of Ca^{2+} . From these results it is concluded that Ca^{2+} influx from the medium into the spores may be an important event in phytochrome-mediated germination. In the absence of Ca^{2+} the R-stimulated system remained capable of responding to Ca^{2+} , added as late as 40 h after R. Moreover, Ca^{2+} was effective even if added after the active form of phytochrome, Pfr, had been abolished by far-red (FR) 24 h after R. Thus, the primary effect of Pfr, that initiates the transduction chain, does not require calcium. "Coupling" of Pfr to subsequent dark reactions has been investigated by R-FR irradiations with various dark intervals. The resulting "escape kinetics" were characterized by a lag phase (6 h) and half-maximal escape from FR reversibility (19 h). These kinetics were not significantly changed by the presence or absence of

calcium. Thus, direct interaction of Pfr and calcium is not a step in the transduction chain initiated by the active form of phytochrome.

Key words: Calcium and fern-spore germination – *Dryopteris* – Spore germination (fern) – Lanthanum – Phytochrome (fern-spore germination) – Pteridophyta

Introduction

The biliprotein phytochrome serves as an important regulator of many light-mediated growth and developmental responses in plants (Mohr and Shropshire 1983). This pigment exists in two interconvertible forms, the red-light-absorbing form (Pr) and the far-red-light-absorbing form (Pfr), the latter being generally considered as the physiologically active component. Phototransformation of Pr to Pfr with red light (R) occurs within seconds (Spruit 1982; Scheuerlein et al. 1986). For the mode of Pfr action three possibilities are being discussed: i) phytochrome acts as a regulator of gene expression (Lamb and Lawton 1983; Schäfer et al. 1986); ii) phytochrome acts as a protein kinase (Quail et al. 1978; Wong et al. 1986), and iii) phytochrome acts as a membrane effector (Hendricks and Borthwick 1967; Haupt and Weisenseel 1976). Whatever the actual mechanism, only little knowledge is available regarding the primary steps initiated by Pfr, however.

Some of the most rapid phytochrome responses appear to be related to membranes (Kendrick 1983), and in these cases the divalent ion, Ca^{2+} , seems to play an important role (Roux et al. 1986). Changes in membrane potential after R have been measured with lag phases as short as 1–10 s (Newman 1981; Racusen 1976; Weisenseel and Ruppert

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Abbreviations: EGTA = ethyleneglycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid; FR = far-red light, Pr = red-light-absorbing form of phytochrome; Pfr = far-red-light-absorbing form of phytochrome; Pipes = piperazine-1,4-bis(2-ethanesulfonic acid); R = red light

1977), and changes in Ca^{2+} fluxes have been reported as possible mediators of these changes (Roux et al. 1986). The reduction of the surface charge of *Mesotaenium* cells after R was also found to require the presence of Ca^{2+} in the external medium (Stenz and Weisenseel 1986). Furthermore, an involvement of Ca^{2+} in the transduction chain was demonstrated for some fast physiological responses including leaflet closure in *Mimosa* (Toriyama and Jaffe 1972), chloroplast movement in *Mougeotia* (Dreyer and Weisenseel 1979; Serlin and Roux 1984) and swelling of etiolated-wheat protoplasts (Bossen et al. 1988).

Apart from these fast phytochrome-mediated responses, calcium is also important for Pfr-initiated germination in fern spores, both the green spores of *Onoclea* (Wayne and Hepler 1984, 1985) and the non-green spores of *Dryopteris* (Haupt et al. 1986). These single-celled systems can be of particular interest for the investigation of primary Pfr action since spores are quiescent at a well-defined stage. They are also easy to synchronize and to equilibrate to experimental conditions in the surrounding medium. Moreover, the late response expression (i.e. several days after the induction; Edwards and Miller 1972; Scheuerlein et al. 1988) may be advantageous to separate the different time steps of the transduction chain and thus to study the primary events of Pfr action.

In this paper a quantitative description for the dependence of germination in *D. paleacea* on the Ca^{2+} concentration in the external medium is given. Furthermore, the kinetics of this Ca^{2+} requirement are investigated by applying Ca^{2+} at various times in the Pfr-initiated transduction chain.

Material and methods

Plant material. Spores of *Dryopteris paleacea* Sw. (Dryopteridaceae) were collected from plants growing in the Botanical Garden of the University of Erlangen-Nürnberg, during the summer of 1985 and were stored in a desiccator at 5° C in darkness.

Culture conditions and light treatments. Spores were cultured on 3 ml of an aqueous medium. The standard medium contained 3.45 mM KNO_3 , 10 mM ethyleneglycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and 20 mM piperazine-1,4-bis(2-ethanesulfonic acid) (Pipes) at pH 6.0 ± 0.1 . Free- Ca^{2+} concentration was varied from $\leq 10^{-8}$ M to 1 M by adding $\text{Ca}(\text{NO}_3)_2$ from 0 (free $\text{Ca}^{2+} < 10^{-8}$ M) to 1.01 M (free $\text{Ca}^{2+} \approx 1$ M). Since in media with EGTA the free- Mg^{2+} concentration depends not only on the concentration of MgSO_4 but also on that of $\text{Ca}(\text{NO}_3)_2$, the MgSO_4 concentration was varied between 1.00 and 1.02 mM to have always a free- Mg^{2+} concentration of 1 mM (Wayne 1985).

Calcium-ion-free plastic ware (prewashed with 12.5 mM EGTA at pH 7.0) was used to prepare the solutions; spores were cultured in Ca^{2+} -free plastic Petri dishes (Falcon, Lincoln

Park, N.D., USA), prewashed as described above. All media were prepared with milli-Q water (Millipore Corporation, Bedford, Mass., USA) with a resistance ≥ 18 M Ω . All inorganic solutions and pure water were autoclaved (1 bar for 35 min).

In some experiments Ca^{2+} (10^{-4} M) was added to the culture medium various times after irradiation. In these cases the spores were sown on a medium without $\text{Ca}(\text{NO}_3)_2$ at pH 6.0 ± 0.1 , and the $\text{Ca}(\text{NO}_3)_2$ was added as 11.1 μl of a 2 M $\text{Ca}(\text{NO}_3)_2$ solution at pH 6.0 to give a final free- Ca^{2+} concentration of 10^{-4} M. For controls, 11.1 μl water was added to the medium. For the experiments with the Ca^{2+} antagonist, lanthanum, a culture medium without EGTA and with MgSO_4 reduced to 0.81 mM was used; LaCl_3 (in control experiments also AlCl_3) was added to give a final concentration between 10^{-8} M and 10^{-2} M to 0.1 mM $\text{Ca}(\text{NO}_3)_2$, 3.45 mM KNO_3 , 0.81 mM MgSO_4 and 20 mM Pipes at pH 6.0 ± 0.1 .

After sowing, spores remained in darkness for 20 h at 22° C and were then irradiated, using a 500-W projector lamp (DAY/DAK; General Electric, USA) combined with a heat-absorbing filter (KG 1, 2 mm, Schott & Gen., Mainz, FRG). Red light was obtained by passing the light through an AL 666 interference filter ($\lambda_m = 666$ nm, half-bandwidth = 23 nm, Schott & Gen.); the fluence rate was $5.5 \text{ W} \cdot \text{m}^{-2}$, as measured with a Li-Cor light meter (LI 185; Lincoln, Neb., USA). Far-red light (FR) was obtained by passing light through a cutoff filter RG 9 (3 mm thick, $\lambda_m = 740$ nm, bandwidth 80 nm, Schott & Gen.); the FR fluence rate was $3 \text{ W} \cdot \text{m}^{-2}$. Dark controls were run parallel to each irradiation experiment. Afterwards, spores were stored in darkness at 22° C until evaluation. All manipulations were carried out under dim-green light (area approx. 0.01 m^2 ; $\leq 10 \text{ mW} \cdot \text{m}^{-2}$).

Evaluation and statistics. Germination was examined with a Zeiss (Oberkochen, FRG) Universal microscope equipped with either brightfield or epifluorescence optics. With the epifluorescence mode, the presence or absence of a bright-red fluorescence, caused by chlorophyll, was used to determine germination in *D. paleacea* 3 d after irradiation (compare Scheuerlein et al. 1988). The exciting light was obtained from a 100-W mercury-vapor lamp and passed through a BP450-490 excitation filter combined with a dichroic mirror, giving excitation light of wavelengths less than 500 nm and the barrier filter LP520. Spores were counted in lots of 50 or 100 spores. At least 200 spores are represented in each datum point.

In the tables the mean $\pm$ one standard error of the mean (SE) is indicated. In the figures, standard error is not shown, as it is equal to or smaller than the symbols.

Results

Requirement for Ca^{2+} in phytochrome-mediated germination of *Dryopteris* spores. Induction of germination was studied using a R irradiation of 1 min which saturates the photoconversion of Pr to Pfr (see Scheuerlein and Koller 1988). The effect of R depended strongly on the concentration of free Ca^{2+} in the external medium (Fig. 1). With Ca^{2+} concentrations $\leq 10^{-7}$ M, only a weak germination response of approx. 5% was obtained, whereas with 10^{-6} M and higher Ca^{2+} concentrations a significantly enhanced germination was observed. As little as 3 μM free Ca^{2+} was required to support a half-maximal response, and the opti-

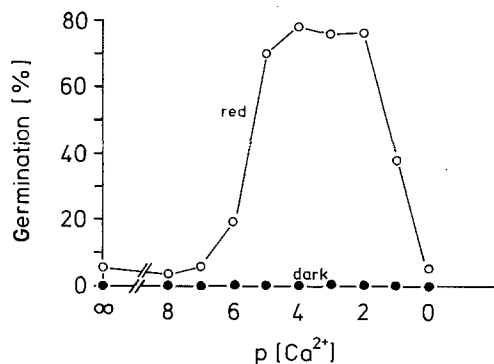


Fig. 1. The effect of Ca^{2+} on R-induced germination of *D. paleacea* spores. The spores were either irradiated 20 h after sowing on standard medium with saturating R (1 min; $5.5 \text{ W} \cdot \text{m}^{-2}$; $\circ-\circ$) or were kept in complete darkness ($\bullet-\bullet$). Standard medium: 3.45 mM KNO_3 ; 10 mM EGTA, 20 mM Pipes. MgSO_4 varied (free Mg^{2+} constant 1 mM), $\text{Ca}(\text{NO}_3)_2$ varied, its concentration is given on the abscissa as $p[\text{Ca}^{2+}] = -\log[\text{free Ca}^{2+}]$

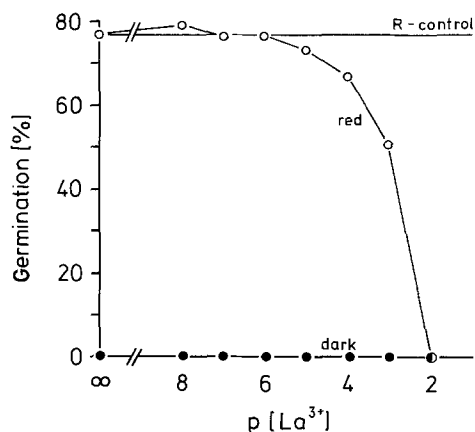


Fig. 2. The effect of La^{3+} on R-induced spore germination in *D. paleacea*. Lanthanum as different concentrations of LaCl_3 was added to the medium. The saturating R treatment was given after 20 h imbibition ($\circ-\circ$); dark controls were run at the same time ($\bullet-\bullet$). Culture medium: 0.1 mM $\text{Ca}(\text{NO}_3)_2$, 3.45 mM KNO_3 , 0.81 mM MgSO_4 , 20 mM Pipes. The concentration of La^{3+} is given on the abscissa as $p[\text{La}^{3+}] = -\log[\text{La}^{3+}]$

mum was reached around 10^{-4} M . The reduction in response at $\geq 10^{-1} \text{ M}$ is probably based upon an unspecific osmotic or ionic effect, as corresponding concentrations of other ions are inhibiting as well (C.J. Haas, University of Erlangen-Nürnberg, FRG, personal communication). There was no germination of unirradiated samples over the whole range of Ca^{2+} concentrations used.

The importance of Ca^{2+} for the expression of the germination response, initiated by a R treatment, is emphasized by the lanthanum experiment in Fig. 2. The effect of a saturating irradiation, applied at an optimal Ca^{2+} concentration (10^{-4} M), could be cancelled by addition of lan-

Table 1. The effect of Cl^- on R-induced spore germination in *D. paleacea*. Chloride at different concentrations of AlCl_3 or LaCl_3 was added to the culture medium (same as described for Fig. 2). The saturating R treatment (1 min, $5.5 \text{ W} \cdot \text{m}^{-2}$) was given after 20 h imbibition

Salt concentration (M)	Germination (%)	
	AlCl_3	LaCl_3
10^{-2}	61.0 ± 2.4	2.0 ± 0.7
10^{-4}	62.0 ± 2.4	46.4 ± 2.2
10^{-6}	62.5 ± 2.4	58.0 ± 2.6
10^{-8}	60.8 ± 2.2	60.0 ± 2.4
0 (Red)	60.2 ± 1.0	
0 (Dark)	0.0 ± 0.0	

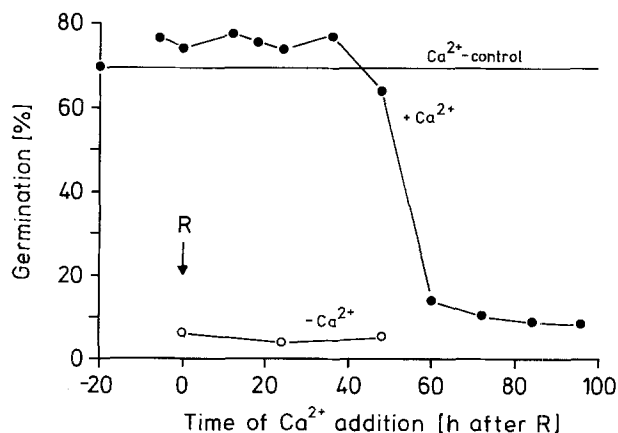


Fig. 3. The effect of varying the time between R treatment and the addition of Ca^{2+} on spore germination in *D. paleacea*. Germination was induced by a saturating R treatment (1 min, $5.5 \text{ W} \cdot \text{m}^{-2}$) 20 h after sowing on standard medium (arrow). Calcium (free $\text{Ca}^{2+} = 10^{-4} \text{ M}$) was present from the beginning (Ca^{2+} control) or given at various time intervals from R treatment ($\bullet-\bullet$); alternatively, pure water was added ($\circ-\circ$). Dark control with Ca^{2+} from the beginning = 0%

thanum to the medium. At concentrations $> 10^{-5} \text{ M}$ a significant inhibition was obtained, and R-initiated germination was completely blocked at 10^{-2} M . When, instead of LaCl_3 , AlCl_3 was added (Tab. 1), no inhibition was observed for the concentrations tested. Thus, the lanthanum effect is specific, indicating that a Ca^{2+} influx from the external medium into the cells may be an important event in phytochrome-mediated germination of *Dryopteris* spores.

Kinetics of the Ca^{2+} dependence. Next, it was tested at which stage Ca^{2+} acts in the transduction chain. Spores were sown on a Ca^{2+} -free medium, and Ca^{2+} was added at various times. The results are compared to controls with Ca^{2+} present from the beginning (Fig. 3). Full competence to respond to

Table 2. The effect of FR given immediately or 24 h after R on spores of *D. paleacea* differentially treated with Ca^{2+} . Red (1 min, $5.5 \text{ W} \cdot \text{m}^{-2}$) and FR (5 min, $3 \text{ W} \cdot \text{m}^{-2}$) were given as saturating treatment. Ca^{2+} (10^{-4} M) was in the standard medium from the beginning or was added 24 h after R (immediately after FR)

Light treatment	Germination (%)	
	Ca^{2+} given from the beginning	Ca^{2+} given 24 h after R
Dark-control	0	0 ^a
FR-control	0	0 ^a
R-control	77.7 ± 2.4	76.9 ± 1.9
R/FR, $\Delta t = 0$	0	0
R/FR, $\Delta t = 24 \text{ h}$	68.5 ± 3.3	66.7 ± 2.7

^a Ca^{2+} added in darkness, 44 h after start of experiment

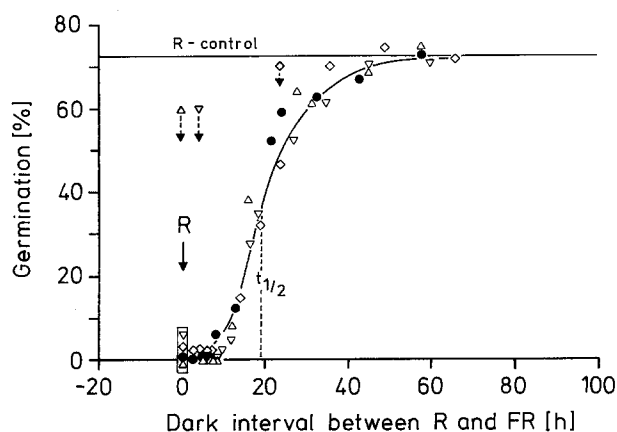


Fig. 4. R/FR kinetics for the induction of fern-spore germination. Germination was studied on a standard medium with free $\text{Ca}^{2+} = 10^{-4} \text{ M}$. Red treatment was given 20 h after sowing as saturating irradiation (1 min, $5.5 \text{ W} \cdot \text{m}^{-2}$) (R-control). Saturating FR (5 min, $3 \text{ W} \cdot \text{m}^{-2}$) was given at variable intervals after R. Ca^{2+} was present from the beginning (●, standard experiment) or given immediately after R (▽), 5 h after R (△) or 24 h after R (◇), as indicated by arrows. No significant differences were obtained for the R-controls by varying the time of adding Ca^{2+} and the R-control shown represents the mean value of the four Ca^{2+} treatments

to Ca^{2+} was found up to 40 h after R; thereafter, the competence disappeared, and 60 h after R no significant enhancement was obtained as compared to controls without addition of Ca^{2+} .

Since in *Dryopteris* spores Pfr is remarkably stable (Haupt et al. 1988), the results in Fig. 3 do not exclude a possible late interaction between Pfr and Ca^{2+} . In the next experiment, therefore, both factors were separated in time.

Spores were sown on a Ca^{2+} -free medium and Ca^{2+} was added 24 h after R; the response was compared to controls with Ca^{2+} added from the beginning. Saturating R was followed by saturating FR immediately ($\Delta t = 0$) or 24 h after R ($\Delta t =$

24 h), thus phototransforming Pfr almost completely back to Pr (see Scheuerlein and Koller 1988). Table 2 shows that the Pfr effect was nearly saturated after 24 h (compare R/FR, $\Delta t = 24 \text{ h}$ with the R control) and that Ca^{2+} was fully effective even when given after FR had reversed Pfr to Pr. Thus, Pfr and Ca^{2+} act in a sequential way, and Ca^{2+} has to interact with a product of Pfr rather than with Pfr itself.

It remains to be tested, however, whether the coupling of Pfr to the transduction chain might be influenced by Ca^{2+} . This coupling has been investigated by R/FR-escape kinetics (Fig. 4); i.e. Pfr was reversed to Pr by FR at various times after R (abscissa). During a lag phase of 6 h, Pfr appeared to be without an effect; half-maximal escape from FR reversibility was reached approx. 19 h after R. This curve, with Ca^{2+} present from the time of sowing (closed circles) was not significantly changed when Ca^{2+} was added at later times, i.e. immediately, 5 h or 24 h after R (open symbols). This result indicates that the kinetics of this coupling are not influenced by the presence or the absence of external Ca^{2+} .

Discussion

We have shown a strong dependence of the Pfr-induced spore germination in *Dryopteris* on calcium. A threshold response occurs at $0.1 \mu\text{M}$ Ca^{2+} and $3 \mu\text{M}$ support a half-maximal effect.

Since Ca^{2+} was added as $\text{Ca}(\text{NO}_3)_2$ and since NO_3^- strongly enhances germination in *Dryopteris* (Haupt et al. 1986), this Ca^{2+} effect might be interpreted as a result of the increase in NO_3^- . However, NO_3^- was always present in the millimolar range, thus saturating the NO_3^- effect (e.g. Haupt et al. 1986) whereas the half-maximal saturating Ca^{2+} effect is obtained already in the micromolar range. This Ca^{2+} requirement is in agreement with the results of Wayne and Hepler (1984) in their investigation of R-mediated germination in *Onoclea*, and the obtained threshold value correlates well with the concentration of free Ca^{2+} measured in the cytoplasm of plant cells (reviewed by Kauss 1987). On the other hand, the inhibiting effect found at concentrations $\geq 10^{-1} \text{ M}$ can be explained as a general osmotic or ionic effect.

The Pfr-mediated germination is inhibited by lanthanum. Lanthanum has been found to block other phytochrome responses in which calcium may be involved, namely, phytochrome-mediated leaflet closure in *Mimosa* (Campbell and Thomson 1977), phytochrome-mediated depolarization in *Nitella* (Weisenseel and Ruppert 1977) and phyto-

chrome-mediated swelling of etiolated-wheat protoplasts (Bossen et al. 1988). Thus, our results with lanthanum could be taken as evidence that calcium uptake is an important step in Pfr-initiated fern spore germination. It must be admitted, however, that this is not completely conclusive as long as the mechanism of lanthanum action is not fully understood. Usually, it is assumed that lanthanum does not enter the cell, rather binding to membrane proteins (Thomson et al. 1973) where it can block rather specifically the influx of calcium and thus its action in the cell (Lettvin et al. 1964; Wayne and Hepler 1985). However, it cannot be excluded that Ca^{2+} itself is acting at the external side of the plasmalemma; in this case an inhibition by lanthanum would also be expected. Thus, final proof of calcium uptake into the spores as a result of Pfr action still needs to be provided.

Whatever the site and mechanism of Ca^{2+} action, it should be noted that there is no interaction between Pfr and Ca^{2+} . The full effect of Ca^{2+} is found even if Ca^{2+} is added after Pfr has been fully coupled to the transduction chain and the latter has escaped from FR reversibility. Thus, Ca^{2+} appears to interact with a product of Pfr rather than with Pfr itself; in other words, there is a dark reaction that is started by Pfr and is required before Ca^{2+} can act. It may be speculated that Pfr induces formation or activation of membrane proteins that are necessary to allow or to facilitate Ca^{2+} action or, more specifically, Ca^{2+} influx. Experiments are in progress to investigate kinetics concerning the proposed Pfr-initiated Ca^{2+} influx and the formation of Pfr-initiated early proteins during germination.

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