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The role of calcium ions in phytochrome-mediated germination of spores of *Onoclea sensibilis* L.

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Abstract. Phytochrome is confirmed to be the photoreceptor pigment in the germination response of *Onoclea sensibilis* L. by demonstrating red-far-red (R-FR) photoreversibility. External Ca^{2+} is required for this response with a threshold at a submicromolar concentration. Ethylene glycol-bis(β-amino-ethyl ether)-N,N,N′,N′-tetraacetic acid, La^{3+} and Co^{2+} reversibly inhibit germination. Lanthanum only inhibits germination when applied before or during irradiation, indicating that the external Ca^{2+} requirement is transient, although in the absence of Ca^{2+} the R-stimulated system remains maximally poised to accept the ion for over 4 h after irradiation. The ability to respond to Ca^{2+} 4.1 h after R-irradiation is not reversed by FR-irradiation, indicating that Ca^{2+} transport has been uncoupled from phytochrome. Barium and Sr^{2+}, but not Mg^{2+} can substitute for Ca^{2+}. Artificially increasing the concentration of intracellular free Ca^{2+} with the ionophore A 23187 stimulates germination in the dark. The Ca^{2+}-calmodulin antagonists, trifluoperizine and chlorpromazine, reversibly inhibit germination. Calcium is required in phytochrome-mediated fern spore germination; it may be acting as a second messenger.

Key words: Calcium – Calmodulin – Germination (spore) – *Onoclea* – Phytochrome and Ca^{2+} – Pteridophyta.

Introduction

Phytochrome controls a wide spectrum of developmental responses such as seed and spore germination, flowering, bud development, stomatal differentiation, formation of tracheary elements, anthocyanin synthesis, hair formation, leaf expansion and stem elongation (see e.g. Mohr 1972). How can phytochrome control such diverse responses? It is possible that the temporal and spatial specificity of the cell's response is preprogrammed according to its current state of differentiation and the far-red-light (FR) absorbing, physiologically active form of phytochrome (Pfr) triggers various responses by inducing an increase in the intracellular Ca^{2+} concentration (Anderson and Cormier 1978; Dreyer and Weisenseel 1979; Haupt and Weisenseel 1976; Dieter and Marmé 1981a; Roux et al. 1981; Weisenseel and Ruppert 1977).

In animals, Ca^{2+} acts as a second messenger in the coupling of many stimulus-response systems where the stimulus is a membrane effector (Rasmussen 1981; Kretsinger 1981). Upon activation by the primary stimulus, the intracellular concentration of free Ca^{2+} rises from 0.1 μM to 1–10 μM. At this concentration, Ca^{2+} binds to Ca^{2+}-binding proteins such as calmodulin which in turn may bind to protein kinases or other proteins and activate essential processes in the cell. Subsequently, the intracellular free Ca^{2+} concentration falls to that of the resting cell as a consequence of sequestration by the endoplasmic reticulum and mitochondria as well as the pumping out of Ca^{2+} by the plasmalemmal Ca^{2+}-ATPase (see Kretsinger 1981 for a review).

Photobiologically relevant phytochrome (Hughes 1965) is assumed to be localized on membranes (Etzold 1965; Haupt et al. 1969; Kadota et al. 1982) and may modulate ionic permeability (Georgevich and Roux 1982; Hale and Roux 1980; Roux et al. 1981; Weisenseel and Ruppert 1977), electrical potential and – or surface charge (Racusen 1976; Newman 1981; Dreyer and Weisenseel

Abbreviations: EGTA = ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; FR = far-red light; R = red light

1979), and hydraulic conductivity (Weisenseel and Smeibidl 1973). Calcium can modulate these same events (Rasmussen 1970). Moreover, Pfr and Ca^{2+} show other similarities. For example, they both modulate the enzymes NAD kinase and Ca^{2+}-ATPase (Anderson and Cormier 1978; Tezuka and Yamamoto 1969; Dieter and Marmé 1981a, b). Furthermore, Ca^{2+} is required for the phytochrome-mediated adhesion in mung-bean root tips (Tanada 1968) as well as the phytochrome-mediated membrane depolarization in *Nitella* (Weisenseel and Ruppert 1977). The Ca^{2+}-antagonist, La^{3+}, and the Ca^{2+}-chelator, ethylenediaminetetraacetic acid (EDTA), inhibit phytochrome-mediated leaf closure in *Mimosa* (Campbell and Thomson 1977). Moreover, Ca^{2+} fluxes in response to red light (R) have been demonstrated with $^{45}Ca^{2+}$ autoradiography in *Mougeotia* (Dreyer and Weisenseel 1979), and with the Ca^{2+}-sensitive metallochromic dye, murexide, in *Avena* coleoptile cells and isolated mitochondria (Hale and Roux 1980; Roux et al. 1981). It is thus reasonable to suggest that transient increases in intracellular Ca^{2+} may trigger phytochrome-mediated responses. In this paper we confirm that phytochrome is the photoreceptor pigment in *Onoclea* spore germination, and further suggest that its activity is mediated by Ca^{2+}.

Material and methods

Choice of experimental plant material. Spores have been selected for these studies because they are single cells at the same developmental stage and with the same developmental potential. It is our assumption that the photoreceptor exerts its effect at the cellular level and therefore the use of single, uniform cells eliminates averaging the responses of many different cells that occur in a multicellular organ. The spores of *Onoclea* have been chosen, in particular, because they germinate rapidly, because the germination response has been well studied (Huckaby et al. 1982; Miller et al. 1983; Raghavan 1980; Towill and Ikuma 1973. 1975) and because large amounts of material are readily available.

Plant material and culture. Mature sporophylls of *Onoclea sensibilis* L. were collected in Amherst and Pelham, Mass., USA in January, 1981 and February, 1982 and stored in plastic bags in a freezer at −15° C for at least five months to eliminate dark germination. Prior to an experiment, one to four sporophylls were wetted with a 0.1% solution of Aerosol O.T. (Fisher Scientific Co., Pittsburg, Pa, USA) and sterilized with 1 l of a 20% (v/v) solution of commercial bleach (5.25% NaOCl) in a manner modified from Stockwell and Miller (1974) which yielded sterile spores. Unless otherwise stated, the sterile spores were prewashed in 2.5 mM EGTA (ethylene glycol-bis(β-amino-ethyl ether)-N,N,N′,N′-tetraacetic acid, pH 6.8) for 24 h at 23° C to remove any wall-bound Ca^{2+}. From this point on, all manipulations were carried out in sterile plasticware. The spores were collected by filtration through Millipore filters (5.0 μm pore size; Millipore Corporation, Bedford, Mass., USA) and approx. 2 mg were sown on 10 ml of experimental media in polystyrene Petri dishes, 60 mm diameter, 15 mm high (Falcon, Oxnard, Cal., USA) at 26.5° C. The spores floated on the surface of the liquid medium. The standard medium contained 1 mM $Ca(NO_3)_2$, 3.45 mM KNO_3, 810 μM $MgSO_4$, ±5 mM K-acetate buffer (pH 5.2). The $Ca(NO_3)_2$ was varied or replaced by $Ba(NO_3)_2$, $SrCl_2$, $MgSO_4$, $CaCl_2$, $CaSO_4$ or $Ca_{10}(OH)_2(PO_4)_6$. Antagonists were added as needed. Only deionized distilled (resistance = 2 MΩ) water was used. Organic compounds were sterilized by passage through a Millipore filter, pore size 0.22 μm. All inorganic solutions and EGTA were autoclaved at 1.04 bar for 35 min. Unless otherwise stated, the spores were left to equilibrate with the medium for 1 h prior to the light treatment.

Light treatments. All procedures were carried out under a dim green safelight which consisted of two "gold" fluorescent lamps (General Electric, Cleveland, O., USA; F40-T12-GO) and a green plexiglass filter (Rohm and Haas Co., Philadelphia, Pa., USA). Red light was obtained by passing light from two fluorescent lamps (General Electric; F20-T12-PL gro and sho) through one layer of a Roscolene medium red (No. 823) cellulose acetate filter (Capron Lighting Co., Needham, Mass., USA). Far-red light was obtained by passing light from a 300-W Reflector (Westinghouse, Bloomfield, N.J., USA) spot through 3 mm of a FRF 700 filter (Westlake Plastics, Lenni Mills, Pa., USA). Red light and FR were applied with an energy fluence rate of 2.4 J m^{-2} s^{-1} and 430 J m^{-2} s^{-1}, respectively, as measured with a Li-cor (Lincoln, Neb., USA) quantum-radiometer-photometer (Model LI 185 B) with a Li-cor pyranometer sensor (LI-200SB). The energy fluence rate of the safelight could not be detected with this sensor. The spores were irradiated for 5 min with red light at 24° C to ensure saturation, except where stated otherwise.

Reversal experiments with La^{3+} and Co^{2+}. Three sets of spores were run in parallel in various concentrations of $CoCl_2$ or $LaCl_3$ in the standard medium under continuous light from white fluorescent lamps (General Electric; F24T12-CW-HO; energy fluence rate = 100 J m^{-2} s^{-1}). After 48 h, one set was scored, and media were added to the second (reversed) set to bring the concentration of Ca^{2+} up to 3 mM, while the concentrations of all other ions remained unchanged. In the third (non-reversed) set, additional media were added, but the total concentration of all ions remained unchanged. Sets 2 and 3 were counted 48 h after the addition of the new media.

Determination of the timing of La^{3+} inhibition. Two sets of spores were run in parallel in the standard medium. Additional medium containing La^{3+} was added to one set at various times before, during or after irradiation with red light. The final concentration of La^{3+} was 1 mM. An equal volume of the standard medium without La^{3+} was added simultaneously to the control set.

Reversal experiments with chlorpromazine and trifluoperizine. Three sets of spores were run in parallel in various concentrations of chlorpromazine (2-chloro-N,N-dimethyl-10H-phenothiazine-10-propanamine) or trifluoperizine (10-[3-(4-methylpiperazin-l-yl)-propyl]-2-trifluomethylphenothiazine) in the standard medium under continuous light from white fluorescent lamps. After 48 h, one set was scored and spores in the second and third set were collected by Millipore filtration (5.0 μm pore size) and washed with deionized water. The second (reversed) set was placed into the standard medium without the antagonist; the third (non-reversed) set was placed in the standard medium with various concentrations of the antagonists.

Ionophore experiments. The spores were treated with 5–95.6 μM A 23187 (6S-[6α(2S*,3S*),8β(R*),9β,11α]-5-Methylamino)-2-[[3,9,11-trimethyl-8-[1-methyl-2-oxo-2-(1H-pyrrol-2-yl)ethyl]-1,7-dioxaspiro[5.5]-undec-2yl] methyl]-4-benzoxazolecarboxylic acid) in 0.03–2.5% DMSO (dimethyl sulfoxide) and 2 mM Pipes 1,4-piperazinediethanesulfonic acid, pH 7.0–7.4, 20 mM Hepes 4-(2-hydroxyethyl)-piperizine ethanesulfonic acid), pH 7.0–7.5 or 5 mM K-acetate buffer, pH 5.2, for 0.5–24 h. They were then collected by Millipore filtration (pore size = 5.0 μm), transferred into the standard medium, left to equilibrate for 1 h, and irradiated with continuous white light or 5 min R, or they were left in the dark; or, after 24 h, medium was added to the ionophore solution, the spores were left to equilibrate for 1 h, and were then irradiated with 5 min R or left in the dark. Since the variation in treatments did not produce any significant differences in results, the data from ten different experiments were grouped in Table 3.

Determination of competence to respond to Ca^{2+} added after R irradiation. Two sets of spores were run in parallel in 2.5 mM EGTA (pH 5.2–6.8). Fifty μmol of Ca^{2+} were added to each replicate of the experimental set at various times before or after R irradiation. An equal volume (0.5 ml) of deionized water was added to each replicate of the control set. In order to determine whether the competence to respond to Ca^{2+} was based upon the stability of the phytochrome molecule or upon the stability of another structure, FR was applied at various times after R irradiation and Ca^{2+} was added either 1 h before or 4.1 h after irradiation. Equal volumes of deionized water were added to the control set.

Determination of percent germination. Percent germination was determined 48 h after irradiation, except where otherwise specified, by the acetocarmine-chloral hydrate method of Edwards and Miller (1972). Spores were considered germinated when two stained nuclei were observed. Two hundred spores per replicate were scored and there were two replicates per treatment. All treatments were run at least twice. Data are expressed as mean ± two standard errors of the mean.

Chemicals. A 23187 was purchased from Calbiochem-Behring (La Jolla, Cal., USA), EGTA, Pipes, Hepes and Chlorpromazine from Sigma Chemical Co. (St. Louis, Mo., USA). Trifluoperizine dihydrochloride was a gift from Smith Kline and French Laboratories (Philadelphia, Pa., USA).

Results

The nature of the photoreceptor pigment. Light triggers the germination of *Onoclea* spores. Low energy fluences of R stimulate germination, whereas high energy fluences of FR are ineffective (Fig. 1). The germination response for the spores shown in Fig. 1 is saturated at 288 J m^{-2} R; however, this is variable from batch to batch (the range is from 48 to 288 J m^{-2}).

Figure 2 shows that 60 s of FR given immediately after 60 s of R reverses the inductive effect of R. Under the above conditions, germination is only 50% reversible when there is a 5-min delay between the R and the FR treatments (data not shown). These data confirm that phytochrome is

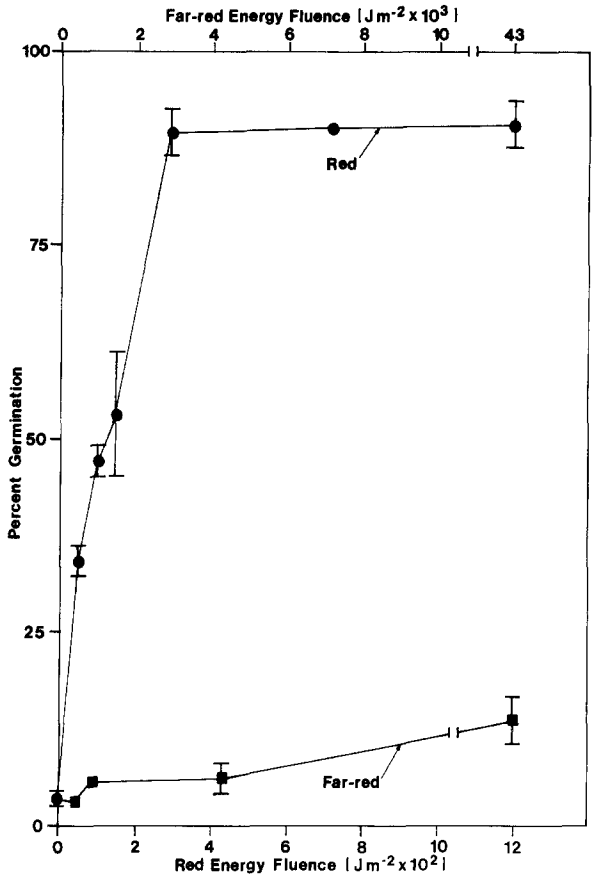


Fig. 1. The effect of energy fluence on spore germination in *Onoclea*. Energy fluence was varied by varying the time of irradiation. The media contained 3.45 mM KNO_3, 810 μM $MgSO_4$, and 1 mM $Ca(NO_3)_2$, pH 5.2. Note different abscissae for R and FR energy fluence. ●, R (2.4 J m^{-2} s^{-1}); ■, FR (430 J m^{-2} s^{-1})

the photoreceptor pigment in light-stimulated *Onoclea* spore germination (Huckaby et al. 1982; Wayne and Hepler 1982).

The role of Ca^{2+} in phytochrome-mediated spore germination. External Ca^{2+} is required for R-induced germination (Fig. 3a). The threshold concentration is submicromolar; 3 μM is the half-maximal concentration of external Ca^{2+}. This threshold is emphasized and the half-maximal concentration of Ca^{2+} is lowered to 0.6 μM when the spores are germinated in Ca^{2+}-EGTA buffered media (Fig. 3b). Red light-stimulated germination in inhibited by EGTA. The EGTA inhibition can be reversed by washing out the inhibitor and sowing the spores on the standard medium (Fig. 3a) or by increasing the concentration of free Ca^{2+} in the medium (Fig. 3b). Since the Ca^{2+}-EGTA-buffered media are not optimal for germination, the standard medium is used throughout these experiments. The Ca^{2+} requirement is not detected

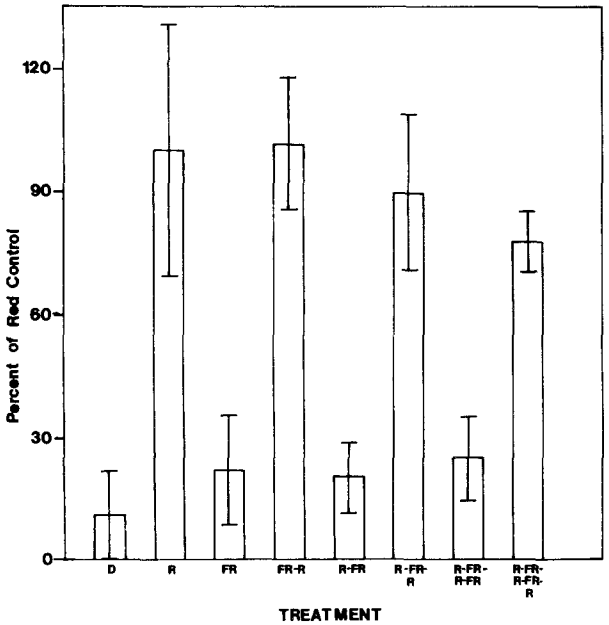
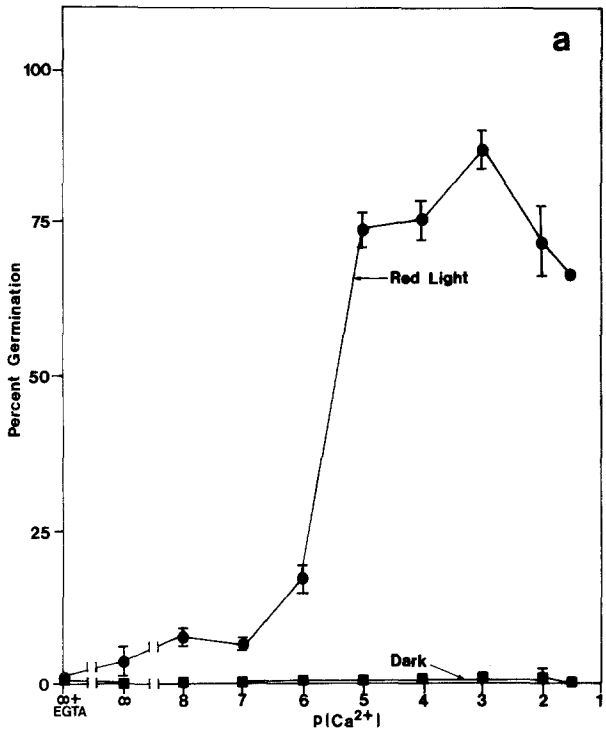


Fig. 2. The effect of sequential treatments of R and FR on *Onoclea* spore germination. Red light was applied with an energy fluence rate of 2.4 J m^{-2} s^{-1} for 1 min; FR was applied with an energy fluence rate of 430 J m^{-2} s^{-1} for 1 min. The spores were in the standard medium (see Fig. 1). Red control = 27% because of nonsaturating R conditions



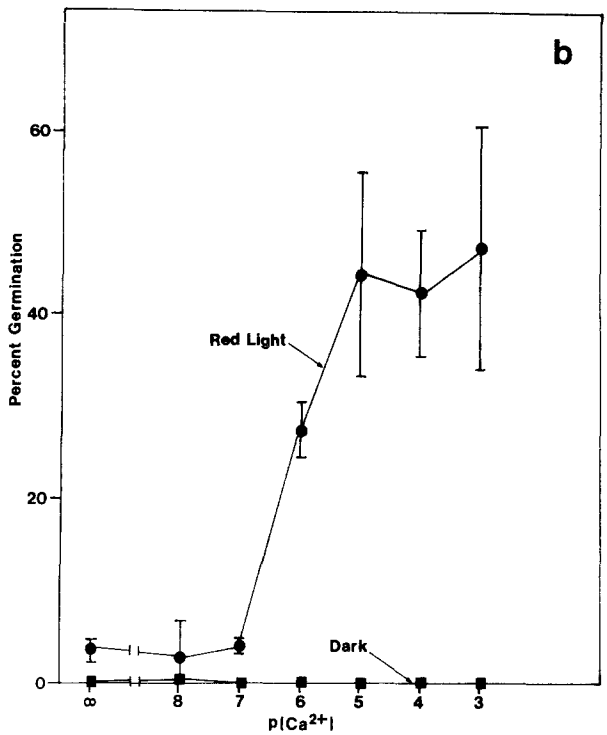


Fig. 3a, b. The effect of Ca^{2+} on *Onoclea* spore germination. **a** Using EGTA washed spores in the standard media (same as in Fig. 1). **b** Using spores put directly in Ca^{2+}-EGTA-buffered media which contained 100 mM KCl, 1 mM EGTA, 4 mM $MgSO_4$, 20 mM Pipes (pH 6.94) and $CaCl_2$. ●, R; ■, dark. ($p[Ca^{2+}] = -\log$ [free Ca^{2+}]). Note different ordinates in **a** and **b**

when the spores germinate in the standard medium unless they have been prewashed with EGTA (data not shown). Calcium is the effective ion, not NO_3^-, as $Ca(NO_3)_2$ can be replaced by $CaCl_2$, $CaSO_4$ and in part by the highly insoluble $Ca_{10}(OH)_2(PO_4)_6$ (Table 1a). Externally applied Ca^{2+} has no effect on dark germination.

Red-light-induced germination still occurs when the external Ca^{2+} is replaced by the bivalent cations Sr^{2+} and Ba^{2+} but not by Mg^{2+} (Table 1b). The Sr^{2+}-treated spores grow into normal prothalli, but the Ba^{2+}-treated spores often develop only into prothalli consisting of only a few cells and which sometimes develop in an apolar fashion without rhizoid formation. Thus Ba^{2+} can only partially substitute for Ca^{2+}-mediated responses or it might fully substitute for the germination response but be detrimental for some other process(es).

In order to investigate further the role of Ca^{2+}, we treated the spores with the inorganic Ca^{2+}-antagonists, La^{3+} and Co^{2+}, in the presence of 1 mM Ca^{2+}. Lanthanum and Co^{2+} inhibit R-stimulated germination (Fig. 4). This inhibition can be reversed by increasing the concentration of external Ca^{2+} from 1 to 3 mM (Table 2). In order to be inhibitory, La^{3+} must be present prior to or during irradiation with R; La^{3+} added several sec-

Table 1. The effect of varying (a) anions and (b) cations on germination of *Onoclea* spores

Addition	Germination (%)	
	Light	Dark
a) The effect of varying anions		
No Ca^{2+}	0.33± 0.60	0.0±0.0
$Ca(NO_3)_2$	60.67± 1.99[a]	0.0±0.0
$CaSO_4$	61.00± 5.20	0.0±0.0
$CaCl_2$	61.00± 2.00	0.0±0.0
$Ca_{10}(OH)_2(PO_4)_6$	21.00±13.99	0.0±0.0
b) The effect of varying cations		
$Ca(NO_3)_2$	86.75± 0.50[a]	0.0±0.0
$MgSO_4$	10.50± 1.00	0.0±0.0
$Ba(NO_3)_2$	83.75± 4.50	0.0±0.0
$SrCl_2$	82.00±10.00	0.0±0.0

[a] Differences in control values are a consequence of differences between spore batches used in anion and cation experiments. Red light was applied for 5 min. The various compounds were present at 1 mM. All media contained 3.45 mM KNO_3 and 810 µM $MgSO_4$, pH 5.2. Values given are the means ± 2 SE

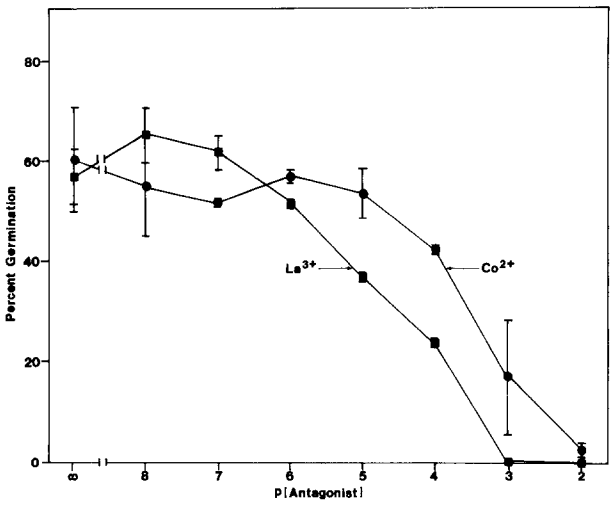


Fig. 4. The effect of La^{3+} or Co^{2+} on *Onoclea* spore germination. Red light was applied for 5 min. La^{3+} and Co^{2+} were added to the standard medium (same as in Fig. 1). ■, La^{3+}; ●, Co^{2+}. (p[ ] = −log [ ])

onds after irradiation is ineffective (Fig. 5). Rhizoidal and protonemal growth are inhibited in spores treated with La^{3+} after irradiation. Since Co^{2+} has been shown to mimic the effects of R (Kang and Ray 1969), we tested the effect of Co^{2+} and La^{3+} on dark germination. These cations have no effect on dark germination (data not shown). The lanthanides Ce^{3+} and Sm^{3+} also inhibit R-stimulated germination (data not shown).

Table 2. The effect of increasing the Ca^{2+} concentration on inhibition of *Onoclea* spore germination by La^{3+} and Co^{2+}

Antagonist (M)	Germination (%)		
	48 h	96 h	
	1 mM Ca^{2+}	1 mM Ca^{2+}	3 mM Ca^{2+}
$LaCl_3$			
0	62.0±6.0[a]	87.8± 1.5	89.0±2.0
10^{-6}	53.8±1.5	87.5± 4.0	91.3±4.5
10^{-5}	46.5±6.0	80.8±11.5	89.8±4.5
10^{-4}	20.5±7.0	21.8± 5.5	85.8±6.5
10^{-3}	0.8±1.5	8.0± 4.0	75.0±5.0
$CoCl_2$			
0	92.8± 1.0[a]	97.0±0.0	97.8±4.5
10^{-6}	83.5±17.0	99.0±2.0	98.3±3.5
10^{-5}	86.5± 9.0	92.3±2.5	95.8±3.5
10^{-4}	71.5± 5.0	75.3±7.5	89.0±0.0
10^{-3}	44.3±10.5	65.5±1.0	84.5±5.0

[a] Differences in control values between La^{3+} and Co^{2+} experiments are a consequence of differences between spore batches. Spores were germinated in continuous white light. Lanthanum and Co^{2+} were added to the standard medium (same as in Fig. 1). After 48 h, one set was scored and additional media were added to the other sets. The Ca^{2+} concentration was increased from 1 to 3 mM in the reversed set and remained at 1 mM in the non-reversed set

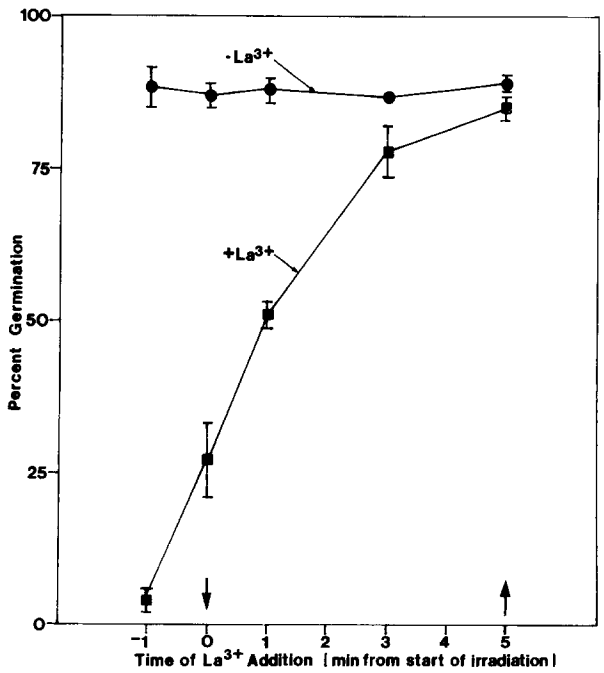


Fig. 5. The effect of time of addition of La^{3+} on *Onoclea* spore germination. Red light was applied for 5 min. The *down* and *up arrows* indicate the start and end of irradiation, respectively. ±La^{3+} solutions were added to the standard medium (same as Fig. 1). ■, +La^{3+} addition; ●, −La^{3+} addition

Table 3. The effect of the ionophore A 23187 on germination of *Onoclea* spores in the dark and in the light

Treatment	Germination (%)	
	Dark	Light
−A 23187	3.4±2.8	74.0±17.5
+A 23187	21.4±4.3	74.3±18.8

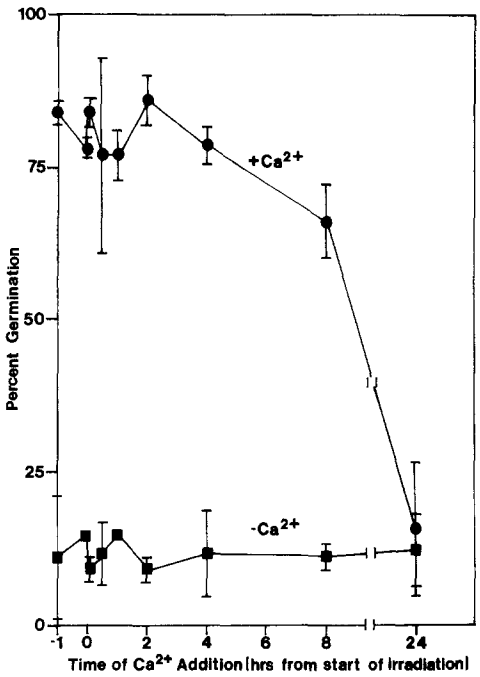


Fig. 6. The effect of varying time between the R treatment and Ca^{2+} addition on *Onoclea* spore germination. Red light was applied for 5 min. to spores incubating in 2.5 mM EGTA. Fifty μmol of $Ca(NO_3)_2$ or an equal volume of deionized water were added at various times after red irradiation. •, $+Ca^{2+}$ addition; ■, $-Ca^{2+}$ addition

The ionophore A 23187 stimulates an average of 21.4% of the spores to germinate in the dark (Table 3). Although maximal percent germination in the light is affected by pH, the pH has no effect on the percent germination stimulated by the ionophore in the dark (data not shown).

In order to characterize further the relationship between phytochrome and Ca^{2+} transport, we irradiated spores with saturating R in the presence of 2.5 mM EGTA, and added Ca^{2+} at various times before or after irradiation. Figure 6 shows that the spores are still fully competent to respond to Ca^{2+} 4 h after irradiation. Competence begins to fall off by 8 h and is completely lost by 24 h. In the treatments where Ca^{2+} is applied 4.1 h after the inductive irradiation, saturating FR given to the spores up through 5 min after the R treatment is partially capable of reversing the inductive effect

Table 4. The effect of time between R and FR irradiation on *Onoclea* spores differentially treated with Ca^{2+}. Red light (2.4 J m^{-2} s^{-1}) and FR (430 J m^{-2} s^{-1}) were applied for 5 min. Spores were incubated in 2.5 mM EGTA (pH 5.2); 50 μmol $Ca(NO_3)_2$ were added where indicated

Time between end of R and beginning of FR irradiation (min)	Ca^{2+} treatments		
	Ca^{2+} given 4.1 h after R	Ca^{2+} given 1 h prior to R	$-Ca^{2+}$
0	45.5±1.0	89.5±1.0	1.5±2.0
5	62.0±4.0	87.5±1.0	0.3±0.5
30	82.8±3.5	82.3±7.5	0.5±0.0
60	88.0±2.0	83.0±4.0	2.0±3.0
120	82.8±6.5	85.3±0.5	1.8±1.5
240	83.8±0.5	85.5±1.0	2.5±1.0
R only	87.0±0.0	85.0±8.0	2.0±0.0

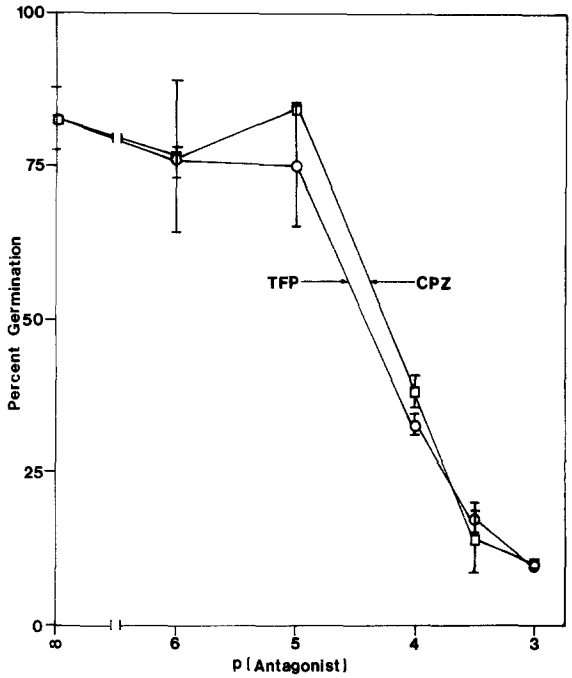


Fig. 7. The effect of trifluoperizine (TFP) or chlorpromazine (CPZ) on *Onoclea* spore germination. Red light was applied for 5 min; TFP or CPZ were added to the standard medium (same as in Fig. 1). o, TFP; •, CPZ. (p[ ]= −log [ ])

of R; but, FR given 4 h after R irradiation is incapable of reversing the inductive effect of red light (Table 4, column 2), a result which indicates that Ca^{2+} transport has been uncoupled from phytochrome. The ability of FR to reverse the inductive effect of R is not observed when a saturating concentration of Ca^{2+} is present at the time of irradiation with saturating R (Table 4, column 3; compare Fig. 2). These data indicate that escape from photoreversibility is a function of time of irradiance and external Ca^{2+} concentration. Since

Table 5. The effect of washing on reversal of inhibition of germination of *Onoclea* spores by trifluoperizine (TFP) and chlorpromazine (CPZ). Spores were germinated under continuous white light; TFP and CPZ were added to the standard medium (same as in Fig. 1). One set of spores was scored after 48 h, while the reversed set was washed after 48 h with deionized water and put into the standard medium without the antagonist. The non-reversed set was washed with deionized water after 48 h and put into fresh standard medium that contained the antagonist. The reversed and non-reversed sets were scored 96 h after the start of irradiation

Antagonist (M)	Germination (%)		
	48 h	96 h	
		Non-reversed	Reversed
TFP			
0	99.0±0.0	100.0±0.0	100.0± 0.0
10^{-6}	98.5±3.0	99.3±1.5	100.0± 0.0
10^{-5}	98.5±1.0	99.5±0.5	98.5± 3.0
10^{-4}	44.0±7.0	47.8±4.5	100.0± 0.0
$10^{-3.5}$	4.3±0.5	3.8±0.5	80.3±10.5
10^{-3}	0.0±0.0	0.0±0.0	17.3± 0.5
CPZ			
0	93.8±0.5	98.5± 2.0	99.0± 2.0
10^{-6}	94.5±1.0	100.0± 0.0	100.0± 0.0
10^{-5}	93.8±2.5	99.5± 1.0	98.8± 0.5
10^{-4}	36.0±2.0	50.0±15.0	93.8± 3.0
$10^{-3.5}$	15.3±3.5	15.5± 1.0	67.0±11.0
10^{-3}	4.0±1.0	7.0± 4.0	20.0± 2.0

Ca^{2+} need not be present until 4 h after the R-treatment, Ca^{2+} is not required for phytochrome phototransformation.

Trifluoperizine and chlorpromazine, antagonists of the Ca^{2+}-calmodulin complex (Weiss et al. 1982), inhibit R-induced germination (Fig. 7). Specifically, they inhibit the nuclear migration and the subsequent division, but not the initial swelling. Inhibition by $1 \cdot 10^{-4}$ and $1 \cdot 10^{-3.5}$ M trifluoperizine or chlorpromazine is reversed by transferring the spores to inhibitor-free medium (Table 5). Inhibition by $1 \cdot 10^{-3}$ M trifluoperizine and chlorpromazine is not reversible. Trifluoperizine and chlorpromazine have no effect on dark germination (data not shown).

Discussion

Red light triggers the germination of quiescent spores of *Onoclea sensibilis*. Our results confirm that phytochrome is the photoreceptor pigment (Huckaby et al. 1982; Wayne and Hepler 1982) and show that Ca^{2+} is required for this phytochrome-mediated response.

The role of Ca^{2+} in phytochrome-mediated germination of fern spores. It is usually assumed that external ions are not required for fern spore germination because spores germinate in distilled water (Miller et al. 1983; Raghavan 1980; Towill and Ikuma 1973, 1975). However, the magnitude of the ion-storage capacity of the wall (Miller et al. 1983), the Ca^{2+} bound to glassware (Shimomura and Johnson 1976) and the traces of Ca^{2+} in distilled water have not been appreciated. By using deionized water (resistance = 2 MΩ), EGTA-washed plasticware, and spores that are prewashed with EGTA to remove wall-bound Ca^{2+}, we show a requirement for external Ca^{2+}. The approximately micromolar threshold concentration of Ca^{2+} occurs within the range of the k_d(Ca)s of intracellular Ca^{2+}-binding proteins and is within the range of physiological concentrations where Ca^{2+} functions as a second messenger in the coupling of many stimulus-response systems (Kretsinger 1981; Rasmussen 1981; Schmidt and Eckert 1976; Tominga and Tazawa 1981). If Ca^{2+} is only a necessary cofactor for a component in the normal signal chain, and is chelated by the routine EGTA prewash, then the half-maximal concentration of Ca^{2+} required would be 10- to 100-fold less than is shown to be required for germination (Fig. 3; see Kretsinger 1981 for a discussion on Ca^{2+} binding affinities).

Evidence that Ca^{2+} transport from the extracellular space is needed is provided by the experiments showing that the inorganic Ca^{2+}-antagonists, La^{3+} and Co^{2+}, reversibly inhibit germination. Lanthanum does not enter plant cells (Thomson et al. 1973) and may be inhibiting the action of Ca^{2+} on the external side of the plasmalemma, or more specifically it may be inhibiting Ca^{2+} influx (Lettvin et al. 1964). Lanthanum also inhibits the phytochrome-mediated leaflet closure in *Mimosa* (Campbell and Thomson 1977) and the phytochrome-mediated depolarization in *Nitella* (Weisenseel and Ruppert 1977).

Lanthanum must be present prior to or during irradiation by R in order to inhibit germination. Lanthanum reaches its target and acts quickly; however, when presented to the spores within seconds after the 5 min irradiation it is without effect. These data indicate that external Ca^{2+} is required for a very short time, and support the idea that ion plays a signalling role and that the signal is a transient one. The Ca^{2+} signal is also transient in the volume-regulating response of *Poterioochromonas* since addition of EDTA (ethylenediaminotetraacetic acid) less than 1 min after the stimulus is without effect (Kauss 1981). In *Onoclea*, Ca^{2+} is needed further along in development for rhizoid elongation and protonemal growth (Miller et al.

1983). Although the concentrations of Co^{2+} and La^{3+} that are required to inhibit spore germination are relatively high, the inhibition is reversible, indicating that the spores are not irreversibly damaged. The spore coat may be acting as a high-capacity binding site for these cations that must be saturated before the Co^{2+} and La^{3+} can get to the plasmalemma. Our results on the reversible inhibition of germination by EGTA support the presence of a high-capacity binding site, and emphasize the importance of the wall space as a reservoir of Ca^{2+}.

Our studies on restricting Ca^{2+} entry or activity during germination have been complemented by experiments aimed at increasing the intracellular free-Ca^{2+} concentration artificially through the use of the ionophore A 23187 (Pressman 1976). In *Onoclea,* A 23187 plus 1 mM Ca^{2+} stimulates germination in the dark, indicating that the mode of action of phytochrome, in part, is to induce an increase in the concentration of intracellular free Ca^{2+}. That A 23187 and Ca^{2+} induce only a portion of the full germination response is probably a consequence of the inability of the large ionophore molecule to penetrate the spore coat (see below).

One of the novel observations to emerge from our studies is the finding that the phytochrome transformation can be uncoupled from the Ca^{2+} transport process. The red-light-absorbing form of phytochrome (Pr) is rapidly converted to Pfr and that in turn poises the transport system in a form that is insensitive to FR light. Interestingly, the transport component can remain maximally poised to except Ca^{2+} for 4 h or longer after R-irradiation. We interpret these data to mean that phytochrome (Pfr) sets in motion one or more rapid events which enable the membrane to transport Ca^{2+}, and Ca^{2+}, in turn, acts as a "second messenger" to activate essential processes leading to germination.

The ability of trifluoperizine and chlorpromazine to inhibit germination indicates that calmodulin might be involved. However, any conclusion from our data must also keep in mind the nonspecific detergent action of these agents, especially since high concentrations are required. Calmodulin is a pleiotropic mediator of Ca^{2+}-dependent processes and can provide a mechanism for the multiplicity of phytochrome-mediated responses. The calmodulin antagonists inhibit the nuclear migration that occurs approx 24 h after irradiation. Calmodulin may also be involved in the early events of germination but the molecular size of the calmodulin antagonists may limit their ability to reach the protoplast at this time (see below). It is tempting to speculate that Ca^{2+}-calmodulin activates NAD kinase in an early stage of the phytochrome-mediated germination of fern spores. Its activation may play a pivotal in the activation of dormant seeds (Taylorson and Hendricks 1977). An increase in NAD-kinase activity is a very early step in the activation of sea-urchin eggs (Epel 1964).

Throughout this study, the impermeability of the spore coat has been a major barrier to entry of large macromolecules, and has thus limited the kinds of experiments that can be performed. The *Onoclea* spore coat has a pore size of 0.8 nm (Miller 1980) that is sufficient to allow the passage of elemental ions (ionic radii = 1–4 Bohr radius) and water, but impedes the passage of larger molecules. The spore coat splits approx. 24 h after irradiation, which coincides with nuclear migration. Until this time the protoplast is essentially inaccesable to externally applied compounds larger than 0.8 nm. If the increase in intracellular free Ca^{2+} that activates the spores is transient and occurs during or immediately after irradiation (Fig. 5), drugs that will presumably interfere with an increase in intracellular free Ca^{2+} will not be not accessable to the protoplast, although Ca^{2+} and water will freely pass through the spore coat and the spores will germinate. Thus, while we see pronounced effects with La^{3+}, Co^{2+}, or Ca^{2+}, we do not see any effect with the Ca^{2+}-antagonists, TMB-8, D 600, nitroprusside or procaine (data not shown). The impermeability of the spore coat probably also accounts for the relatively small stimulation of germination by A 23187. The timing of spore-coat splitting explains the inhibition by the calmodulin antagonists of nuclear migration but not of the initial swelling. In support of these ideas, the dyes neutral red and acetocarmine do not penetrate the spore until the spore coat splits.

In conclusion, our results indicate that phytochrome activates fern spores by inducing an increase in the concentration of intracellular free Ca^{2+}. Here is an example where a phytochrome-mediated change in membrane properties leads to a developmental response, indicating that short-term effects of phytochrome may have primary importance in regulating development while changes in gene expression may be a secondary effect. This conclusion is substantiated by the ineffectiveness of RNA synthesis inhibitors on germination (Raghavan 1980; Towill and Ikuma 1975).

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