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Control of Mitosis by Phytochrome and a Blue-Light Receptor in Fern Spores[1]

Masaki Furuya*, Misao Kanno, Haruko Okamoto, Shunsuke Fukuda, and Masamitsu Wada

Hitachi Advanced Research Laboratory, Hatoyama, Saitama 350–03, Japan (M.F.); Department of Biology, Japan Women's University, 2–8–1 Mejirodai, Bunkyo-ku, Tokyo 112, Japan (M.K., H.O.); and Department of Biology, Faculty of Science, Tokyo Metropolitan University, 1–1 Minami Osawa, Hachioji-shi, Tokyo 192–03, Japan (S.F., M.W.)

The first mitosis in spores of the fern *A. capillus-veneris* was observed under a microscope equipped with Nomarski optics with irradiation from a safelight at 900 nm, and under a fluorescent microscope after staining with 4′,6-diamidino-2-phenylindole. During imbibition the nucleus remained near one corner of each tetrahedron-shaped dormant spore, and asymmetric cell division occurred upon brief irradiation with red light. This red light-induced mitosis was photoreversibly prevented by subsequent brief exposure to far-red light and was photo-irreversibly prevented by brief irradiation with blue light. However, neither far-red nor blue light affected the germination rate when spores were irradiated after the first mitosis. Therefore, the first mitosis in the spores appears to be the crucial step for photoinduction of spore germination. Furthermore, experiments using a microbeam of red or blue light demonstrated that blue light was effective only when exposed to the nucleus, and no specific intracellular photoreceptive site for red light was found in the spores. Therefore, phytochrome in the far-red absorbing form induces the first mitosis in germinating spores but prevents the subsequent mitosis in protonemata, whereas a blue-light receptor prevents the former but induces the latter.

One of the major obstacles in studies of plant photomorphogenesis has been our inability to monitor continuously any sequentially occurring process in a cell under a microscope for a long period of time without the influence of the visible light needed for such observation. The visible light has immediate effects on the photoresponses of the cell that is being monitored under the microscope (Furuya and Inoue, 1994). Therefore, we recently designed and custom-constructed a microscope that is equipped with Nomarski optics and uses light with a wavelength of 900 nm, a wavelength known to have no influence on photomorphogenesis in higher plants (Senger and Schmidt, 1994). The images observed under such illumination conditions can be stored in a computer and examined subsequently with a micro-image analyzer. Using this instrument, we have been able to monitor intracellular events continuously throughout the photoinduction of spore germination in the fern *A. capillus-veneris* L.

Spore germination in most Filicales ferns requires the presence of Pfr. The Pfr-dependent germination can be photoreversibly prevented by subsequent irradiation with far-red light, and can be photo-irreversibly prevented by brief irradiation with blue or near-UV light (for review, see Furuya, 1983). The first typical example of such phenomena was found in *Pteris vittata* by Sugai and Furuya (1967). The signal transduction pathway from Pfr to the critical process(es) that controls spore germination remains obscure, but a process involving Ca^{2+} ions has been reported as an intermediate step in *A. capillus-veneris* (Iino et al., 1989) and *Dryopteris paleacea* (Dürr and Scheuerlein, 1990). In addition, a nucleus sits in the center of each dormant spore of *Onoclea sensibilis*, but when the spores are hydrated and exposed to red light, the nucleus moves to a corner of each spore, with the consequent asymmetric division of the cells (Miller and Greany, 1974).

In the haploid generation of ferns, the division of cells is strongly influenced by environmental light (Furuya, 1983; Wada and Sugai, 1994). In the single-celled protonemata of *A. capillus-veneris*, local irradiation of only the nuclear region with a narrow beam of blue light was found to induce cell division (Wada and Furuya, 1978; Kadota et al., 1996). In these protonemata, phytochrome is located in the plasma membrane and/or the ectoplasm (Wada et al., 1983) and prevents cell division in protonemata upon exposure to red light (Miyata et al., 1979; Wada et al., 1984). Therefore, it is of interest to investigate the intracellular site of phytochrome and the blue-light receptor in *A. capillus-veneris* spores.

In the present study, we show that the first mitosis in the haploid generation of the fern *A. capillus-veneris* is induced by Pfr, and that such Pfr-dependent induction is blocked by brief irradiation with blue light. The second and subsequent mitoses in protonemata, however, are induced by blue/UV light rather than by phytochrome (Wada and Furuya, 1974). We will discuss the phenomena in terms of photoperception and light signaling.

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* Corresponding author; e-mail mfuruya@harl.hitachi.co.jp; fax 81–492–96–7511.

Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

MATERIALS AND METHODS

Plant Material, Aseptic Cultures, and Determination of Germination Rates

The spores of *A. capillus-veneris* L. used in this study were collected in a greenhouse in Tokyo Metropolitan University in June 1986, and stored in the dark at 4°C until use.

Spores were sterilized for a few minutes in a 10% solution of Haiter (Kao Co., Ltd., Tokyo, Japan), a commercially available solution of 4 to 6% (w/v) sodium hypochlorite. The spores were then rinsed three times with sterilized, distilled water and sown in plastic Petri dishes (30 mm in diameter × 10 mm in height) that contained 1.5 mL of liquid medium (10-fold diluted Murashige and Skoog mineral salts solution [Murashige and Skoog, 1962]) at pH 5.7. Unless otherwise stated, spores were incubated at 25°C for 7 d in darkness before use.

Germination rates were determined by monitoring the emergence and apical growth of protonemata and/or rhizoids in each population tested. At least three samples were analyzed for each experimental point. In the case of microbeam irradiation, the germination of each irradiated spore was determined from a printout of an image recorded at the time of microbeam irradiation.

Light Source and Light Treatments

Broad-range red light at 0.7 W m^{-2} was provided by fluorescent tubes (FL 20SW-G; Hitachi Ltd., Tokyo), the output from which was filtered through a 3-mm-thick red filter (Acrylite, no. 102; Mitsubishi Rayon, Tokyo) and a scattering plate (Acrylite no. 001; Mitsubishi Rayon). Broad-range blue light was obtained from the same type of fluorescent tube with a 3-mm-thick blue filter (Ryutelite no. 63; Ryuden-Sha, Tokyo). Alternatively, the tubes were wrapped with colored films: two layers of cerulean blue (Cutting Sheet no. 521C; Nakagawa Chemicals, Tokyo) and two layers of virijan (Cutting Sheet no. 431C; Nakagawa Chemicals). Far-red light was obtained from far-red fluorescent tubes (FL 20S/FR-74; Toshiba Ltd., Tokyo), the output of which was filtered through DeraglasA (Color no. 900; Asahi Kasei Ltd., Tokyo). The fluence rate was determined with a thermopile (MIR-1000; Mitsubishi Yuka Ltd., Tokyo). The safelight was provided by a 10-W green fluorescent tube (National FL 10G, Matsushita Electric Co., Ltd., Osaka, Japan), which was wrapped with four layers of lemon-yellow plastic film (no. 321C; Nakagawa Chemicals, Tokyo), two layers of the cerulean blue plastic film (no. 521C; Nakagawa Chemicals), and three layers of virijan plastic film (no. 431C; Nakagawa Chemicals).

Microscopy

Spores were observed with a microscope equipped with Nomarski optics under far-red light (Olympus FR-1; Olympus Optical Co., Ltd., Tokyo) using a micro-image analyzer (Argus 100; Hamamatsu Photonics, Hamamatsu, Japan) (Schmidt et al., 1990). The images were stored on a hard disk (C1990–06; Hamamatsu Photonics).

Nuclei in dormant and imbibed spores were stained for fluorescence microscopy using the method of Kuroiwa and Suzuki (1980). Spores were incubated in a solution of DAPI with 0.5% Triton-X 100 for 2 d at approximately 4°C in darkness. Fifteen milligrams of DAPI were dissolved in 1 L of a buffer that contained 0.25 M Suc, 1 mM EDTA, 0.6 mM spermine, 0.05% β-mercaptoethanol, and 10 mM Tris-HCl (pH 7.6).

Microbeam Irradiation

Spores were irradiated with a monochromatic microbeam light from a custom-made epi-microbeam irradiator, as described by Wada et al. (1983). Monochromatic blue light was provided by a tungsten-halogen lamp (JC12V100W; Iwasaki Electric Co., Tokyo) with a heat-cut filter and an interference filter (IF-BPF-453; Japan Vacuum Optics Co., Tokyo) that had a peak at 453 nm and a half-bandwidth of 31 nm. IR light was obtained by passing light through a glass filter (IR85; Hoya Corp., Akishima, Japan).

For the microbeam experiment, spores were sown on the surface of a solidified medium that consisted of 10-fold-diluted Murashige and Skoog mineral salt solution and 0.5% agar, cultured in a Petri dish, covered with a coverslip (3 × 18 mm^2), kept on a specimen stage of the microbeam irradiator, and then observed under IR light using a monitoring system with an IR-light-sensitive video camera (C2400–07ER; Hamamatsu Photonics). The position of the microbeam spot on a sample cell was chosen using an IR beam, and the spot was focused on a nucleus or other appropriate site. Then, the safety IR light was changed to blue light by turning a filter cassette. The size, shape, and fluence rate of the microbeam were 10 × 10 μm^2, square, and 40 W m^{-2}, respectively.

To prepare centrifuged samples for microbeam experiments, sterilized spores were kept in a microcentrifuge tube (1 mL) in darkness for 1 week and then centrifuged for 20 min at 2800*g*. The centrifuged spores were sown on the surface of solidified medium and covered with a piece of coverslip. Centrifugation for 20 min under these conditions did not affect the germination rate that was induced by red light or prevented by blue light, nor did such centrifugation affect the germination rate in darkness (data not shown). For handling in the dark room, a dim green safelight was used.

RESULTS

Positioning of Nuclei in Dormant and Imbibed Spores

Spores were inoculated aseptically onto the surface of the agar-solidified medium and incubated in darkness at 25°C. The position of the nucleus and the first mitosis during the imbibition of spores were observed under a microscope equipped with Nomarski optics at 900 nm, which allowed us to observe a single sample sequentially with the least influence of observing light (Fig. 1).

Some of the spores were collected at various times during incubation on the solidified medium in darkness. They were fixed immediately and stained with DAPI, and their position was observed under a fluorescence microscope

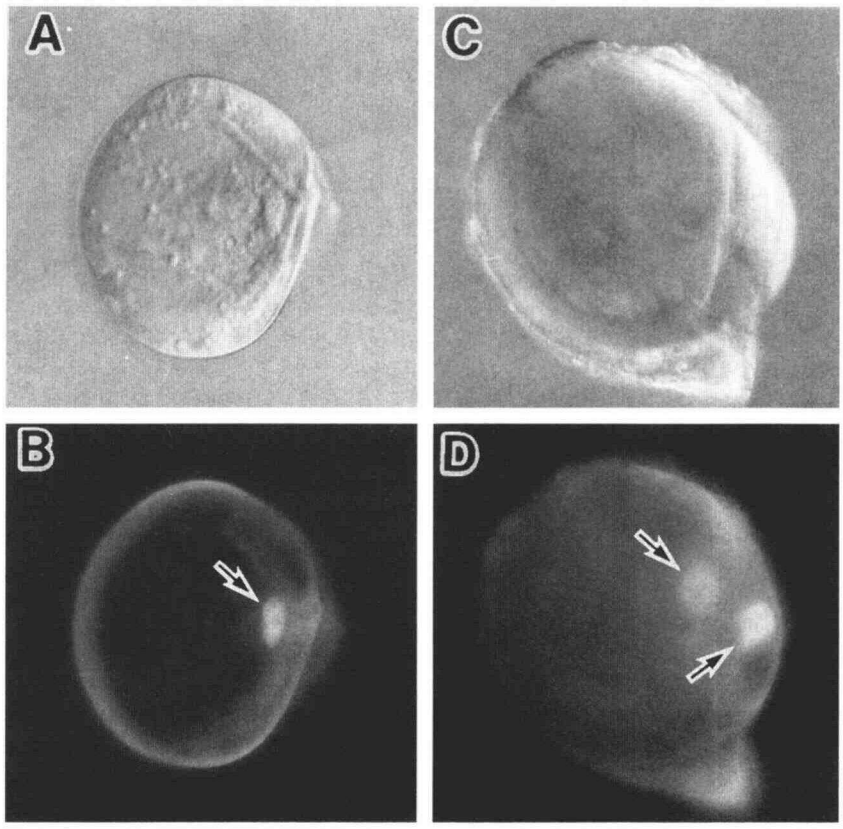


Figure 1. Microphotographs of dormant spores (A and B) and of germinating spores (C and D) in *A. capillus-veneris*. The arrow shows the nucleus. A, Nomarski image (×600) of the nucleus and a tetrahedral structure showing three furrows. B, Fluorescence microphotograph (×600) of a nucleus stained with DAPI. C, The spore was imbibed for 4 d in darkness at 25°C, exposed to red light for 5 min, and finally incubated in darkness for 72 h. D, Fluorescence microphotograph (×600) of nuclei stained with DAPI.

(Fig. 1, B and D). The position in three dimensions of the nucleus in each spore was carefully determined by changing the focus of the microscope. A single nucleus approximately 8 μm in diameter was always observed to be surrounded by three furrows in a corner of the tetrahedral dormant spore (approximately 50 μm in diameter; Fig. 1A). The nucleus remained near the corner of tetrahedron-shaped dormant spores and dark-incubated spores. In contrast to a previous observation of spores of *O. sensibilis* (Miller and Greany, 1974), there was no evidence of any nuclear movement during imbibition for 167 h.

Pfr-Dependent Induction of the First Mitosis

When spores were incubated on the agar-solidified medium for 4 d in darkness and then irradiated with red light for 5 min, the first mitosis occurred in the above-described corner of the cell, with the resultant asymmetric division of the cell (Fig. 1, C and D). The induction of this mitosis by brief irradiation with red light was reversed by a subsequent brief exposure to far-red light. The effects of red and far-red light were repeatedly reversible (Table I), an indication that the induction of the first mitosis in imbibed

Table I. *Effects of brief irradiation with red, far-red, and blue light on the first mitosis and germination in spores of A. capillus-veneris*

Irradiation[a]	Mitosis Rate[b]			Germination Rate
	69 h	94 h	Duncan's test[c]	168 h
	%			%
None (dark control)	0	0	a	0
R	42.1 ± 2.2	50.0 ± 1.9	b	58.3
FR	0	0	a	1.2
R/FR	0	0	a	0.4
FR/R	46.2 ± 2.0	59.5 ± 2.4	b	58.3
R/FR/R	36.8 ± 1.8	58.2 ± 2.3	b	54.9
R/R/FR	0	0	a	0
B	0	0	a	0
R/B	0	0	a	2.6
B/R	0	0	a	2.4
R/B/R	0	2.1 ± 0.4	a	5.4

[a] R, Red light (0.7 W m^{-2}) irradiation for 5 min; FR, far-red light (0.7 W m^{-2}) irradiation for 5 min; B, blue light (0.7 W m^{-2}) irradiation for 5 min. [b] Spores were incubated on agar-solidified medium in darkness for 7 d, irradiated with light at different wavelengths as indicated, and incubated in darkness until observations were made. [c] a and b are different groups identified by Duncan's multiple range test (1%).

spores is photoreversibly regulated by phytochrome. The spores eventually germinated in darkness and the larger cell developed into a protonema, whereas the smaller one developed into a rhizoid.

In the next experiment, spores were imbibed for 4 d in darkness and exposed to red light at 0.7 W m^{-2} either continuously or for 5 min prior to incubation in darkness. The time course of the first mitosis under continuous irradiation with red light showed that 50% of the cells divided 33 h after the beginning of the irradiation, and the rate of cell division reached a maximum by 38 h (Fig. 2). The synchrony of cell division was less marked when spores were exposed to red light for shorter periods of time. No mitosis was observed in the dark-incubated control spores during this experiment (data not shown).

Inhibition by Blue Light of Pfr-Dependent Mitosis

The Pfr-dependent spore germination of *Pteris vittata* is blocked by brief irradiation with blue/UV-A light (Sugai and Furuya, 1967). Considering this, we examined the Pfr-induced first mitosis to determine whether a brief exposure to blue light could influence the effect of the phytochrome. Spores were incubated in darkness for 4 d and then irradiated with red light for 5 min and/or with blue light at 0.7 W m^{-2} for 5 min. The proportion of spores that underwent the first mitosis in each sample population was determined 69 and 94 h after the light treatment (Table I). It was evident that a brief irradiation with blue light totally prevented the Pfr-induced mitosis, irrespective of whether irradiation was applied prior to or after red-light treatment.

The Absence of Any Effect on Germination Rates of Red, Far-Red, and Blue Light when Given after the First Mitosis

The next experiments were performed to determine whether any further photoregulatory process(es) might be involved in the photoinduction of spore germination after the first mitosis has taken place.

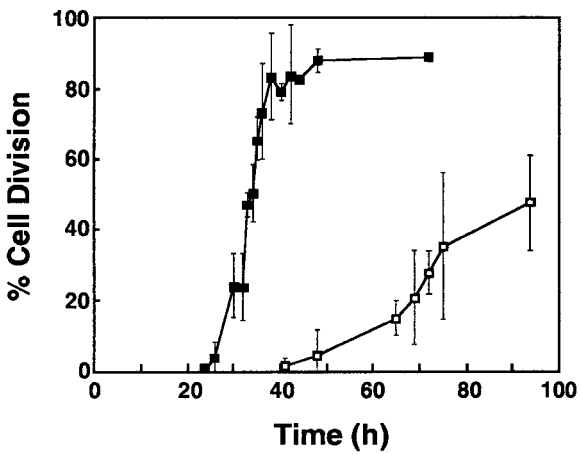


Figure 2. Time course of mitosis in *A. capillus-veneris* spores that had been imbibed in darkness for 4 d at 25°C and then exposed to red light at 0.7 W m^{-2} for 5 min (□) or continuously (■). Time 0 means the onset of the red-light treatment.

Table II. *Effects of brief irradiation with red, far-red, and blue light after the first mitosis induced by continuous irradiation with red light in spores of A. capillus-veneris*

Irradiation[a]	Germination Rate ± SE[b]	Duncan's Test[c]
	%	
Red (38 h)		
+ Dark	78.3 ± 2.6	a
+ FR	83.0 ± 2.2	a
+ FR/R	72.0 ± 2.6	a
+ FR/R/FR	82.6 ± 2.2	a
+ B	87.3 ± 1.9	a
+ B/R	73.9 ± 3.1	a
+ B/R/B	67.9 ± 3.3	a
Dark		
+ Dark	0	b

[a] R, Red light (0.8 W m^{-2}) irradiation for 5 min; FR, far-red light (0.8 W m^{-2}) irradiation for 10 min; B, blue light (0.8 W m^{-2}) irradiation for 5 min. [b] Observed 38 h after the light treatment. [c] a and b are different groups identified by Duncan's multiple range test (1%).

Assuming that the first mitosis was complete within 40 h of continuous red irradiation (Fig. 2), we irradiated spores with red light for 38 h and then briefly with red, far-red, or blue light for 5, 10, and 5 min, respectively. The rate of germination was determined 38 h after the second light treatment. The results in Table II show that such exposure to far-red or blue light had no effect on the rate of germination. This seemed to imply that once the first mitosis has occurred, no other photoregulatory process is functional in the photoinduction of spore germination. Essentially the same result was obtained 72 h after the light treatment (data not shown).

Microbeam Irradiation with Blue Light

When a nucleus in the corner of a tetrahedral spore is irradiated with a microbeam of light, the light must pass not only through the target nucleus, but also through other surrounding organelles in the spore. Therefore, to avoid possible misleading results of such irradiation, one-half of a sample of spores was centrifuged prior to microbeam irradiation so that the nuclei were displaced from their original sites. Nuclei in the corners of noncentrifuged spores and those located at other sites in centrifuged spores were similarly irradiated with a microbeam of light. The results for noncentrifuged spores (Table III) were always similar to those for centrifuged spores. Therefore, we felt confident that we would be able to identify the true effect of a beam of blue light on the nucleus.

Irradiation with a microbeam (10 × 10 μm^2) of blue light (40 W m^{-2}) for 10 s (Fig. 3) was applied to nuclear and nonnuclear regions of both centrifuged and noncentrifuged spores, and then the entire surface of the spores was irradiated with red light for 5 min. The results shown in Table III reveal that exposure of the nuclear region to blue light in both centrifuged and noncentrifuged spores was significantly more effective than exposure of areas in which no nucleus was detected. When the site of the intersection of the three furrows in the corner of a tetrahedral spore in

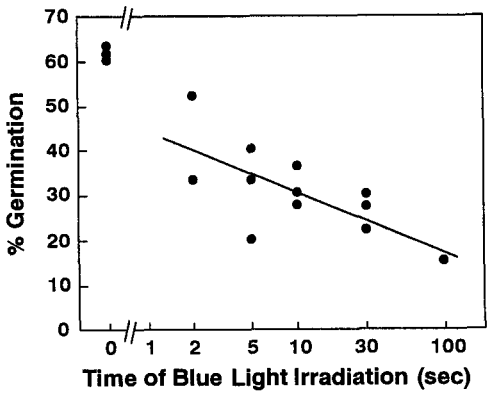


Figure 3. Fluence-response curve for the inhibitory effect of blue light on red light-induced germination of an *A. capillus-veneris* spore. The nuclear region of a spore that had been imbibed for a week was irradiated with a microbeam (10 × 10 mm^2) of blue light (40 W m^{-2}) for various periods of time, and then the whole spore was irradiated with red light (0.6 W m^{-2}) for 5 min. The germination rate was determined after incubation for 5 d in darkness.

which a nucleus had been located before centrifugation was irradiated with a microbeam of blue light, the blue light was not effective in preventing germination. This result indicates that neither the corner where the three furrows intersected nor that part of the cellular microstructure that was not dislocated by centrifugation was the photoreceptive site, but that the site must be the nucleus or another organelle that had been moved by the centrifugation. Similar experiments were performed with a beam of red light, but they gave no definitive results about the localization of phytochrome (data not shown). It is possible, however, that stray light from the microbeam might have caused minor effects as a result of refraction during passage through the many small granules, mostly oil droplets, in the spores.

DISCUSSION

Premitotic Positioning of the Nucleus in Fern Spores

The pattern of cell division is critical in the morphogenesis of multicellular plants (for review, see Furuya, 1984). Sinnott and Bloch (1941) discovered that premitotic positioning of the nucleus prior to mitosis is very important to the position and orientation of the cell plate that is newly formed during cytokinesis.

The nuclei in germinating spores of the fern *O. sensibilis* were reported to move from the center to the corner of spores prior to the first mitosis (Miller and Greany, 1974). If this movement was prevented, no differentiation of a rhizoid occurred during the subsequent mitosis (Miller and Greany, 1976). By contrast, the present study clearly showed that the nucleus remained in the corner of the tetrahedron-shaped dormant spore of *A. capillus-veneris* (Fig. 1), and no intracellular movement of the nucleus took place prior to mitosis. The discrepancy between *O. sensibilis* and *A. capillus-veneris* might be associated with the different shapes of the spores. Fern spores can be classified into two groups in terms of shape: the tetrahedral structure (three-furrow type) and the two-faced structure (single-furrow type). The former type may have evolved into the latter (Mitsui, 1982).

The First Mitosis Is Crucial for Photoinduction of Spore Germination

The timing of germination of dormant spores is critical to the survival of fern species, because their life cycles must match seasonal changes in the environment. It is well known that the induction of spore germination in Filicales ferns requires light, that the effect of light is mediated by phytochrome, and that phytochrome-induced germination can be prevented by brief irradiation with blue and UV-A light (Furuya, 1983). The present study demonstrated clearly, and for the first time, to our knowledge, that the crucial step in this photoregulation by phytochrome and a blue-light photoreceptor is the induction of the first mitosis in spores (Table I), and, in fact, that light no longer has any effect after the first mitosis (Table II). Thus, environmental light controls the first mitosis in fern spores and, as a consequence, indirectly regulates the germination of spores. Therefore, previous reports on the photoinduction of spore germination actually provided indirect observations on the photoregulation of the first mitosis. In fact, the time course (Fig. 2), the fluence/response relationship, and the effects of wavelength (Table I) as related to photoinduction of the first mitosis obtained in the current study agree quite well with the results of previous studies of the photoregulation of spore germination (Sugai and Furuya, 1967).

Differences in Photoperception and Signaling for the Photoinduction of Mitosis in Spores and Protonemata

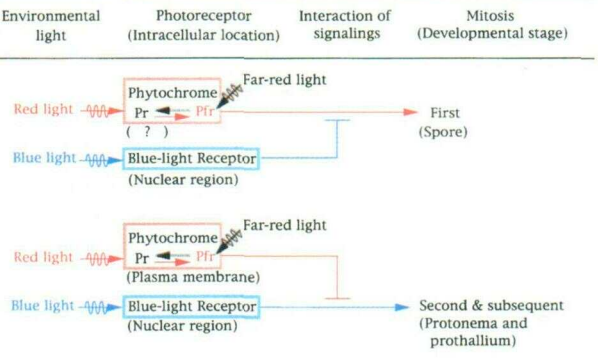
Mitosis in ferns is antagonistically regulated by phytochrome and a blue-light receptor throughout the haploid

Table III. *Inhibitory effect of irradiation with a microbeam of blue light on red-light-induced spore germination in A. capillus-veneris*

Irradiation		Noncentrifuged Spores			Centrifuged Spores		
Microbeam[a] (target)	Inductive light[b]	Germination rate ± SE[c]	N[d]	Duncan's test[e]	Germination rate ± SE	N[d]	Duncan's test[e]
		%					
None	R	54.7 ± 2.4	664	a	56.4 ± 5.4	999	a
B (nucleus)	R	21.4 ± 2.3	166	b	31.3 ± 4.7	136	b
B (not nucleus)	R	40.5 ± 1.3	169	a	45.7 ± 5.4	102	a

[a] B, Spores were incubated for a week in the dark and then irradiated with a blue microbeam (10 × 10 μm^2, 40 W m^{-2}) for 10 s. [b] R, Spores were irradiated with red light (0.6 W m^{-2}) for 5 min. [c] Germination rate was counted after 5 d of incubation in the dark. [d] N, Sample size. [e] a and b were different groups identified by Duncan's range test (1%).

Table IV. *Interaction of phytochrome and a blue-light receptor on the induction of mitosis in haploid generation of A. capillus-veneris*



development of these plants. It is noteworthy that the physiological role of these two photoreceptors in the first mitosis in germinating spores is totally different from their role in the second and subsequent mitoses in protonemata and prothallia. Namely, phytochrome in the Pfr form induces the former (Table I) but prevents the latter (Wada et al., 1984), whereas the blue-light receptor prevents the former (Table I) but induces the latter (Wada and Furuya, 1974). With respect to the intracellular sites of these photoreceptors, however, we showed that in spores as well as in protonemata, the blue-light photoreceptor is located in the nuclear region (Table III) and phytochrome is located in the plasma membrane and/or the ectoplasm (Wada et al., 1983). Table IV summarizes the available information on this subject, comparing the results for the first mitosis in the present study with those for the second and subsequent mitoses in previous studies.

Why do photoperception and signaling alternate between the first mitosis and the subsequent mitoses in the haploid development of this fern? We have no clear-cut answer to this question yet, but we can speculate as to why different photoperception and signaling might occur during fern development in view of current knowledge about phytochromes and blue-light receptors. First of all, phytochromes in higher plants are now known to be encoded by small gene families, for example, *PHYA-PHYE* in *Arabidopsis thaliana* (Sharrock and Quail, 1989; Clack et al., 1994), and different phytochromes play different physiological roles within a single plant species (Furuya, 1993). Four phytochrome genes in *A. capillus-veneris* have been cloned and partially sequenced (K. Nozue, unpublished data), and one of them is *PHYB*-like (Okamoto et al., 1993). A blue/UV-A light receptor in Arabidopsis was also characterized as a protein that binds flavin and deazaflavin derivatives as chromophores (Ahmad and Cashmore, 1993); similar photoreceptors have been cloned from *A. capillus-veneris* (T. Kanegae, unpublished data). Because multiple photoreceptors exist in *A. capillus-veneris*, as they do in higher plants, they might share roles during development as in phytochrome A- and B-specific induction of seed germination in Arabidopsis (Shinomura et al., 1996). However, if the photoperception of red, far-red, and blue light involves the same photoreceptors throughout their haploid generation, light signaling from phytochrome and the blue/UV-A light receptor must cross somewhere between the photoreceptors and the mitosis, as reported previously in the photoorientation of chloroplasts in the gametophytes of *A. capillus-veneris* (Kagawa and Wada, 1996). The scheme presented in Table IV provides a basis for future attempts to define the molecular species of photoreceptors and to characterize the signaling pathways from the photoreceptors to switches in the regulation of the cell cycle.

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