PART I DEVELOPMENT AND MORPHOGENESIS

1

Photoresponses in fern gametophytes

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1.1 Introduction

Fern gametophytes are ideal model systems for study of the mechanisms of photomorphogenesis from the standpoint of physiology, photobiology, and cell biology (Wada, 2003, 2007; Kanegae and Wada, 2006). Positive aspects of the fern system include the following. (1) Spores can be preserved at room temperature and they germinate under appropriate conditions within about a week in many species, becoming gametophytes that grow rapidly, at least in their critical early stages. (2) Gametophytes are nutritionally autonomous, facilitating ease of cultivation. (3) Gametophytes are not enclosed by other tissue, so that observation, light irradiation, and experimental manipulation are readily performed. (4) Each developmental step can be controlled synchronously because gametophytes are highly sensitive to light. Each step in development is completely dependent on light; indeed, without light, development does not proceed.

Since the nineteenth century, especially in Germany, fern gametophytes have been used (see Dyer, 1979a) to study photo-physiological phenomena, such as light dependent spore germination (Mohr, 1956a), differentiation from one-dimensional protonemata to two-dimensional prothalli (Mohr, 1956b), and intracellular dichroic orientation of phytochrome (Etzold, 1965). Even though fern gametophytes are very good materials for the study of both photobiology and cell biology, only a few laboratories use them presently, probably for the following reasons. (1) Although mutants can be obtained easily by phenomenological screening (gametophytes are haplophase), making crosses for genetic studies is difficult and time consuming. (2) The biochemistry is also challenging because

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collecting enough gametophyte tissue for biochemical analyses is difficult. (3) Molecular biological techniques are not yet established (e.g., stable transformation is not available, although transient gene expression is possible). (4) Most ferns are not commercially valuable plants, although some species, such as *Osmunda japonica*, *Pteridium aquilinum*, and *Matteuccia struthiopteris*, are edible and obtainable commercially in eastern Asia, or are used as ornamental plants, or for cleaning soil polluted by heavy metals including arsenic (Ma *et al.*, 2001).

Nevertheless, fern gametophytes have structural and physiological characteristics that seed plants do not have, making them more tractable systems for studying many phenomena that are common to ferns and seed plants. For example, we have analyzed factors controlling the pre-prophase band (PPB) formation and its disruption (Murata and Wada, 1989b, 1991a, 1991b, 1992) (Figure 1.1). The PPB is recognized as a factor controlling the attachment site of newly synthesized cell plates to mother cell walls (Mineyuki, 1999). It appears before prophase of the nuclear division cycle at the future site of cell plate fusion to the mother cell wall, but disappears before cell plate formation. The kind of information remaining at the PPB region has long been a mystery, as have the factors that determine the future cell plate attachment site and disrupt the PPB. To study this issue physiologically, Murata and Wada (1989b, 1991b, 1992) used a long protonemal cell cultured under red light in which cell division occurred at 40–60 μm from the tip where the division site is pre-determined by the PPB. During the period when the PPB was polymerizing, protonemal cells with a premature PPB were centrifuged to reposition the nucleus. A new PPB formed at the new nuclear site, distant from the original position, and then cell division occurred, suggesting that the nucleus must be close to the PPB polymerization site. In these cells the first PPB at the apical part did not de-polymerize even after cell division occurred, but if a dividing nucleus was returned to the former PPB site, the PPB de-polymerized. This result indicates that PPB de-polymerization requires a nucleus and/or surrounding cytoplasm. Experiments such as these could not be done using seed plant cells because long cells like protonemal cells are not found in seed plants, except in some special cases such as cambium cells, where cell division occurs periclinally, making them inappropriate for the experiment. Experiments using long protonemal cells were also performed to study the recovery of a nucleus elongated by cell centrifugation (Wunsch and Wada, 1989; Wunsch *et al.*, 1989).

To analyze the physiological characteristics at each step of the developmental process or during transitions from one step to another of photobiological responses in fern gametophytes, various tools and special techniques have been developed. These include microbeam irradiators to stimulate only a small

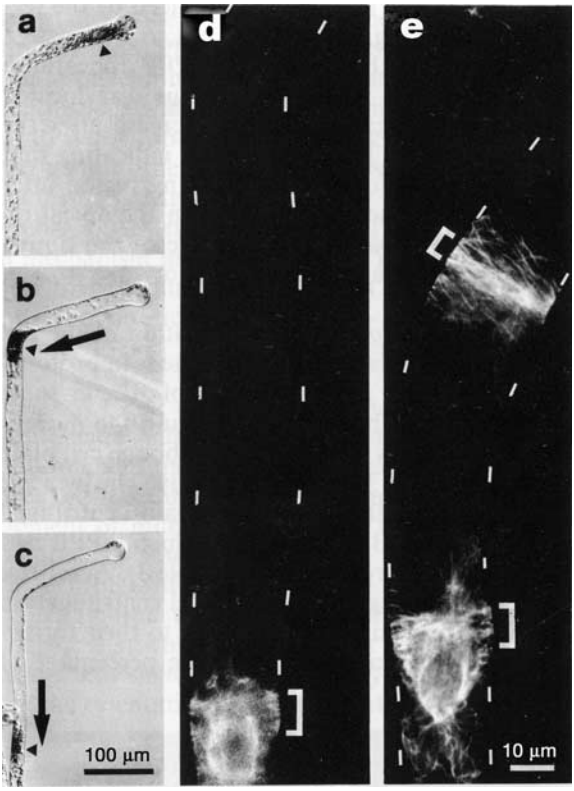

Figure 1.1 Photomicrographs of bent protonemata showing the effect of double centrifugation on pre-prophase band formation. (a)–(c) A bent protonema centrifuged parallel with the arrows to sediment cytoplasm, including a nucleus and chloroplasts. Note that a nucleus, indicated by small arrowheads, moved downward. (d), (e) Bent protonemata centrifuged 4 and 8.5 hours (d) and 4 and 12.5 hours (e) after the onset of blue light irradiation and fixed at 14.5 hours. The regions between the bend and the nucleus are shown. One pre-prophase band (marked with a bracket) was found in (d) and two bands were found in (e). The second centrifugation was applied before and during pre-prophase band formation, respectively. (After Murata and Wada, 1991b).

part of a cell and identify the photoreceptive site, i.e. the localization of photoreceptor molecules mediating a target phenomenon. The first machine was constructed in 1978 (Wada and Furuya, 1978) (Figure 1.2). Current microbeam projectors are now in their fourth or fifth generation, and are equipped with various accessories depending on their purpose (Iino *et al.*, 1990; Yatsuhashi and Wada, 1990). Photoreceptive sites revealed by experiments using microbeam irradiators are summarized in Figure 1.3, and will be explained in the following sections.

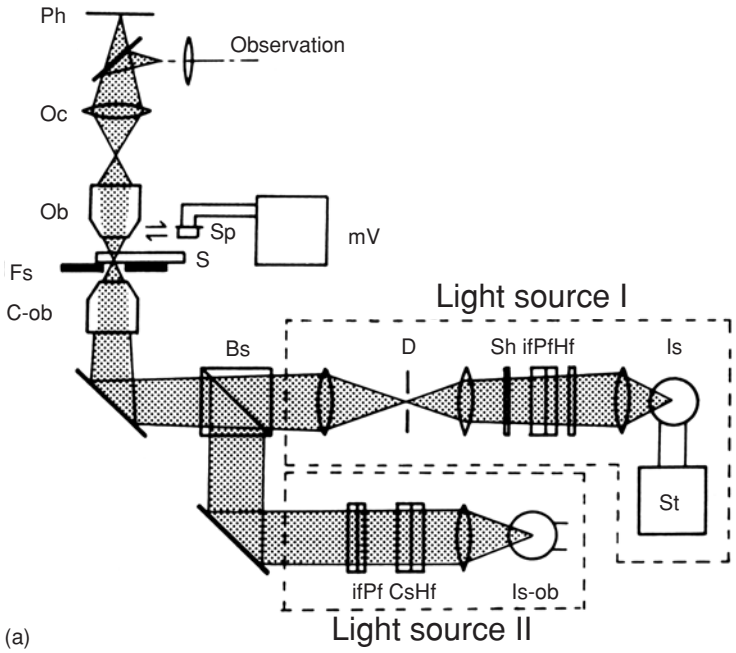

Figure 1.2 Diagrams showing microbeam irradiators. (a) The first generation irradiator. An ordinal light microscope was remodeled for microbeam irradiation by inserting a diaphragm, and another light source was added for observation. Ph, photographic camera; Oc, ocular lens; Ob, objective lens; S, specimen; Fs, focusing stage; C-ob, condenser objective lens; Sp, silicon photocell; mV, millivolt meter; Bs, beam splitter; D, diaphragm; Sh, shutter; if, interference filter; Pf, plastic filter; Hf, heat filter; Is, irradiation source; St, stabilizer; Cs, $CuSO_4$ solution; Is-ob, irradiation source for observation. (b) A third generation irradiator. Four different wavelength lights can be irradiated simultaneously at one point or two mixed lights can be irradiated side by side. CF, cut-off filter; Dp, depolarizer; Fs, field stop; HM, half-silvered mirror; IF, interference filter; IRV, infrared viewer; LS, light source; M, mirror; Obs, observation point; P, polarizer; PC, photographic camera; Sh, shutter; Sl, slit; St, sample stage; WF, water cell; WP, pump for circulation of cooled water to water cell. See Fig. 6 of Wada (2007) for a diagram of the second generation irraditor. ((a) After Wada and Furuya, 1978. (b) After Iino *et al.*, 1990.)

This chapter will focus on recent analyses performed mostly by my laboratory group using *Adiantum capillus-veneris*. I also include some results that have not been published but are based on a synthesis of nearly 40 years of my experience with fern gametophytes. Our knowledge, mostly obtained from *A. capillus-veneris*, assumes that this species follows a pattern of development that is typical of most ferns. However, because of the large diversity in species and gametophytes, numerical data such as the growth rate of protonemata mentioned here may

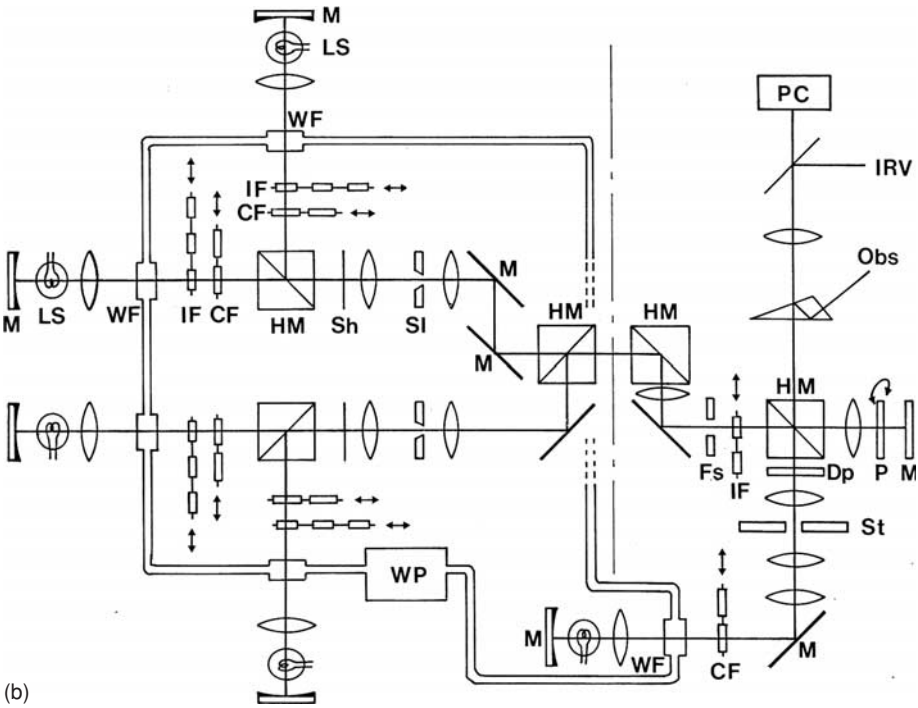

Figure 1.2 (*cont.*)

or may not be applicable to other fern species. For more information refer to books by Dyer (1979b) and Raghavan (1989) and the following reviews: Wada and Kadota (1989), Wada and Sugai (1994), Kanegae and Wada (2006), and Wada (2007).

1.2 Spore germination

There are two kinds of fern spores based on their color: one is green (chlorophyllous) and the other is brown (non-chlorophyllous). Green spores have chloroplasts even before water imbibition and, unless refrigerated, their germination ability (spore viability) does not persist long after harvest. See Raghavan (1989) for more information.

Most fern spore germination is light dependent. In a tetrahedral, non-chlorophyllous, dormant spore, the nucleus sits in one corner surrounded by three furrows. When spores are irradiated with red light after imbibition in the dark, they become round, and the nucleus, still in its corner position, divides, followed by cell division to produce large protonemal and small rhizoidal mother cells (Furuya *et al.*, 1997). In *A. capillus-veneris, Pteris vittata*, and probably other

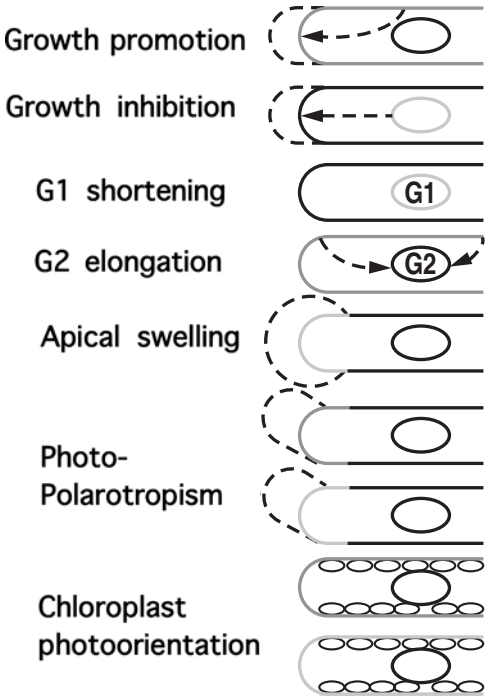

Figure 1.3 Photoreceptive sites for light-induced phenomena in an *Adiantum capillus-veneris* protonema. Light grey (blue) and dark grey (red) indicate photoreceptive sites of blue and red light photoreceptors, respectively. (After Wada, 2007.)

species, red-light induced germination is inhibited by far-red light in a red/far-red reversible manner, indicating the involvement of phytochrome (Sugai and Furuya, 1967; Furuya *et al.*, 1997). The red light effect is inhibited by blue light, on exposure before or after the red light treatment (Sugai and Furuya, 1967; Furuya *et al.*, 1997). The blue light inhibition effect, however, cannot be reversed instantaneously by subsequent exposure to a pulse of red light, suggesting the involvement of a blue light receptor, but not a phytochrome system. Inhibition can be prevented when the spores are kept in the dark for about a week (Sugai and Furuya, 1968; Furuya *et al.*, 1997). The time period required for prevention of blue light inhibition is very much reduced if the spores are irradiated with red light. The red light effect can be reversed by far-red light, indicating phytochrome dependence (Sugai and Furuya, 1968; Furuya *et al.*, 1997). The inhibitory effects of far-red and blue light could not be observed after the first mitosis in spores, suggesting that cell division is a crucial step for spore germination (Furuya *et al.*, 1997). Partial spore irradiation with red or blue microbeam lights showed that the blue light receptor is located in the nucleus, but the

location of the red light photoreceptor could not be identified (Furuya *et al.*, 1997). The photoreceptors mediating spore germination (both phytochrome and blue light receptors) have not yet been identified, although several candidate genes have been cloned and sequenced. The details of fern photoreceptors will be described in Section 1.10.

1.3 Cell growth

In most homosporous ferns, after spore germination under red light, a filamentous protonemal cell grows at the apical dome towards a red light source without (or at least with a low frequency of) cell division. The cell is about 15–20 μm in diameter, although this varies with species and culture conditions. The nucleus is always located about 60 μm from the tip during cell growth in *A. capillus-veneris*, indicating that the nucleus migrates in the cell toward the tip, maintaining a constant distance (Figure 1.4) (Wada and O'Brien, 1975; Wada *et al.*, 1980). In a growing protonema, microtubule and microfilament strands connect the nucleus to the cortex of the apical and basal parts of the cell (Kadota and Wada, 1995), although how these cytoskeletal strands control nuclear migration is not yet known. The growth rate varies with species and also with environmental conditions in the same species. In the case of *A. capillus-veneris* under continuous red light (0.5 W m^{-2} s^{-1}) at 25 °C the protonemata grew at an average rate of about 200 μm/day (Wada, 1988a).

In some species (including those of *Anemia, Osmunda*, and *Lygodium*), even under red light conditions, gametophytes germinate as two-dimensional prothallia, and no protonemal stage is observed (Raghavan, 1989). In *Ceratopteris*, when spore germination was induced by white light irradiation for 1 day after imbibition and then the spores were kept in the dark, gametophytes germinated as a two-dimensional, strap-shaped prothallium in four cell-columns. A cell mass proliferated at the apical part of the gametophyte and each cell at the basal part of the cell mass grew in the dark parallel to the cell polarity (Murata *et al.*, 1997). In this species, cells can grow in the dark, similar to protonemal cells of *A. capillus-veneris* grown under red light, but the cells are not protonemata.

The cell diameter under red light is reasonably constant. How do cells know the diameter and how do they maintain it? At the basal part of the apical dome of protonemata, a circular array of microtubules and microfilaments is observed (Murata *et al.*, 1987; Kadota and Wada, 1992b). Because this will be discussed in detail in Section 1.6, it is sufficient to note that these cytoskeletal structures play a key role in maintaining a constant diameter, as has been well established in higher plant cells (Shibaoka, 1994).

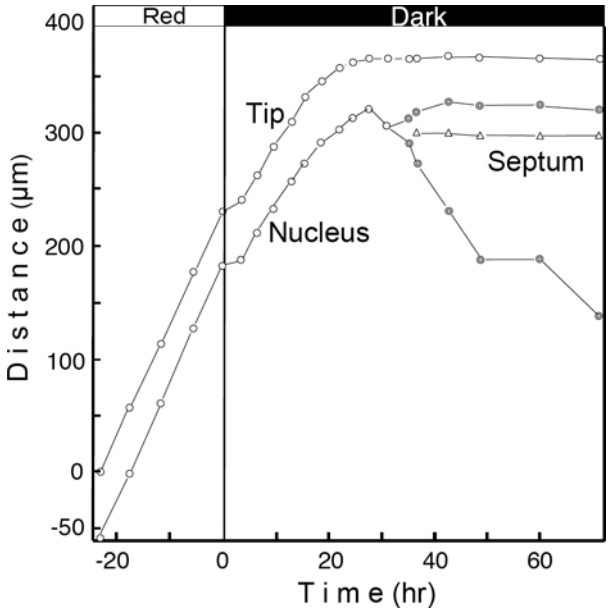

Figure 1.4 Time courses of interacellular nuclear movement and apical growth during the cell cycle in *Adiantum capillus-veneris* protonema. A red-light grown protonema was transferred into the dark to induce cell division and the positions of a nucleus and the tip of the protonema were traced under infrared light microscopy. (After Wada *et al.*, 1980.)

1.3.1 Growth cessation

When protonemal cells cultured under continuous red light are transferred to darkness, the growth rate is reduced and ultimately cell growth stops (Kadota and Furuya, 1977). Simultaneously, the nucleus moves a short distance towards the cell base and after a period of time cell division occurs (Figure 1.5) (Wada *et al.*, 1980). The timing of cell division is controlled by phytochrome (Wada and Furuya, 1972), as will be discussed in Section 1.5. The timing of growth retardation and the length of cell growth during the dark period before cell division occurs are also controlled reversibly by red/far-red light irradiation just before transferring to the dark (Kadota and Furuya, 1977). Protonemal cell growth may be controlled in conjunction with the timing of cell division by the same phytochrome system. It is curious, however, that the timing of cell division is delayed by far-red light whereas growth retardation is advanced and, consequently, growth is reduced. This result indicates that cell cycle advancement and cell growth retardation (i.e., cell growth) are not really parallel. Protonemal cell growth is also controlled by blue light; this will be discussed in Section 1.5.

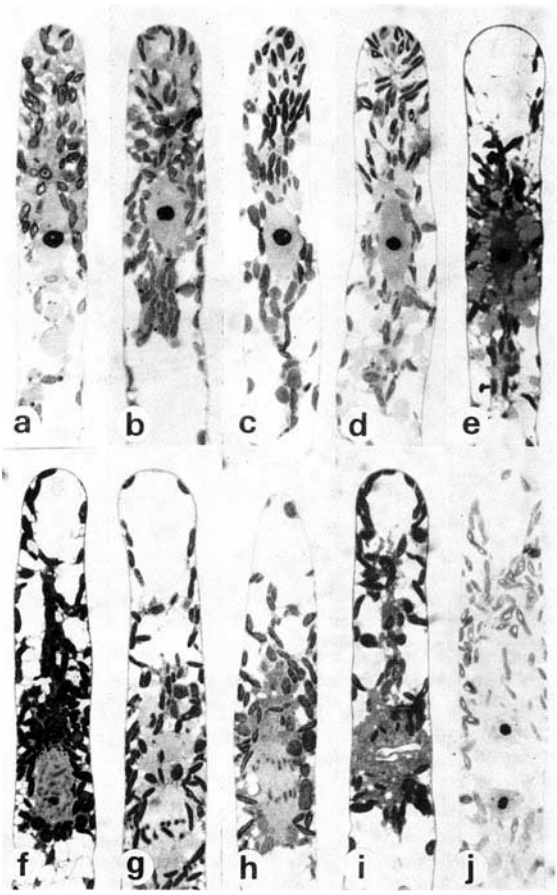

Figure 1.5 Light micrographs of a longitudinal section of *Adiantum capillus-veneris* protonemata during cell cycle in the dark. Nuclear migration towards the base of the cell and structural changes of organelle patterns are shown. (After Wada *et al.*, 1980.)

1.3.2 Resumption of cell growth

When red-light grown protonemata are transferred into the dark, cell division occurs in the apical region of the linear protonemata (Wada and Furuya, 1972). If protonemal cells are kept in the dark after cell division for several days, almost all cytoplasm in the apical cells, including chloroplasts and a nucleus, moves toward the cell plate at the basal end, so that the cells are occupied by a large vacuole and become transparent. These cells appear dormant and neither grow nor divide until light is provided. It is not known how long the cells can survive without light. When protonemata are irradiated with red light continuously, the nucleus moves toward the cell tip, the cytoplasm disperses