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INVITED ARTICLE

The fern as a model system to study photomorphogenesis

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Abstract The fern gametophyte is a good model system for studying cell biological, physiological, and photobiological aspects of the fundamental processes of plant development and physiological phenomena, because of its autotrophic characteristics and its simple structure. The cells, moreover, are not surrounded by tissue, so observation and manipulation of the cells are very easy. Here I summarize a part of my knowledge of fern systems, which I have studied for nearly 40 years.

Keywords *Adiantum capillus-veneris* · Fern · Photomorphogenesis

Introduction

Pteridophytes are, evolutionarily, in a pivotal position between bryophytes and seed plants (Pryer et al. 2001). Bryophytes have no vascular system for water transport, so growth in the vertical plane, resulting in a high and large plant, is severely limited. Seed plants, in contrast, have developed well-established vascular systems for transport of both water and nutrients, so some, for example plants in genus *Sequoia* or *Eucalyptus*, can grow to 100 m or more in height. With alternating generations fern gametophytes resemble mosses and sporophytes resemble seed plants developmentally and physiologically. Fern gametophytes have no vascular system, like bryophytes, and live on substrate surfaces as small individual plants, but their sporophytes have a vascular system enabling more vertical growth than gametophytes, resulting in a large herbaceous plant form. The origins of the plant vascular system must therefore have arisen during the evolution of primitive ferns (Kenrick 2000).

Similarly, bryophytes spend most of their lives as haploid (*n* phase) plants, even during the more leafy stage. Diploid (2*n*) tissue develops only on the top of stalks as spore capsules, which have a short life specifically for spore formation. The diplophase in moss is rather parasitic, being developed from within mother plants. Angiosperms, in contrast, spend most of their lives in diplophase, and the haplophase is found in flowers only. Ferns alternate these generations in a more balanced manner, because half their lives are spent in the gametophyte stage, or haplophase, and the other half in the sporophyte stage (Fig. 1). In conjunction with these differences, a variety of physiological phenomena, not to mention associated morphological and structural characteristics, must have been established and developed during evolution of the fern (Dyer 1979; Raghavan 1989; Wada and Kadota 1989; Banks 1999).

Ferns are, therefore, very interesting and critical plants to study for a variety of reasons (Dyer 1979; Raghavan 1989), especially to gain insight into the evolution of higher plants. Not many people currently study ferns, however. One reason for this may be the limited number of molecular biological techniques available to fern studies. Another may be that ferns are not so commercially valuable, with the exception of

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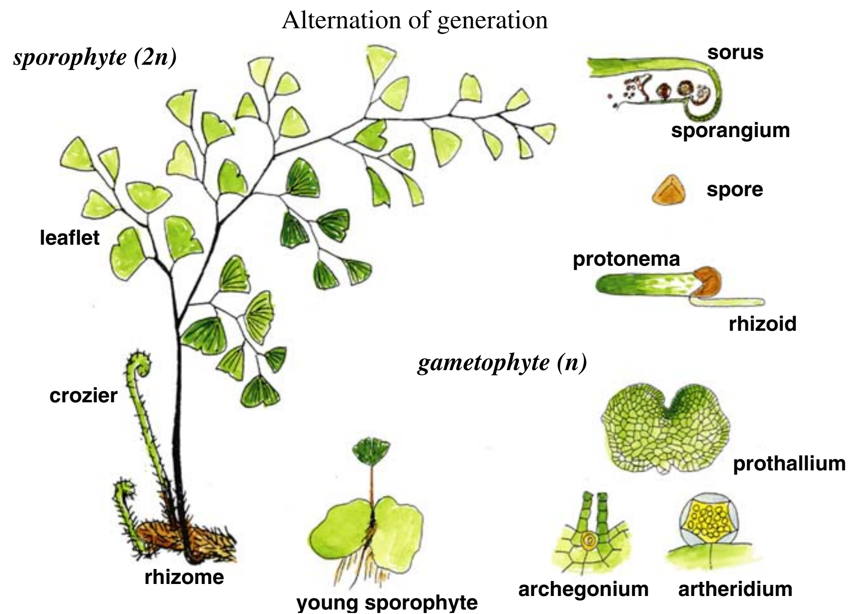

Fig. 1 Life cycle of *Adiantum capillus-veneris*. Spores are produced in sporangia under the leaves. The protonemal cell grows as a linear tube just after spore germination. If kept under continuous red light or very weak white light it grows as a long protonemal cell without cell division. When illuminated with white light it develops to a heart-shaped prothallium. Protonemata and prothalli are good materials for studying photomorphogenesis. Archegonia and antheridia are produced on the prothalli and sporophytes grow after fertilization of eggs by sperms

several species used as ornamental plants. In Asia *Pteridium aquilinum* (L.) Kuhn, *Osmunda japonica* Thunb., *Matheuccia struthiopteris* (L.) Todaro, and, recently, *Asplenium antiquum* Makino are well-known edible species, although *P. aqulinum* is reported to be carcinogenic (Evans and Mason 1965). Ferns do, however, contain a variety of secondary metabolic substances which might become valuable as medicine in the near future (Bresciani et al. 2003), and *Pteris vittata* L. has been reported to be useful for removing arsenic from contaminated soils (Ma et al. 2001; Gumaelius et al. 2004). Taken together, ferns may become useful plants for human beings in the near future if we continue to develop our understanding of them. I have been studying fern gametophytes for more than 35 years, mostly using *Adiantum capillus-veneris* L. as a model system. Many of the experiments performed in my laboratory have been designed to address questions that could be answered only by using fern gametophytes, not seed plants or bryophytes. Here, I summarize the knowledge that my colleagues and I have acquired from study of, predominantly, the fern *A. capillus-veneris*. More information is available in reviews by Wada (2003), Wada and Sugai (1994), and Kanegae and Wada (2006).

Light-induced physiological responses

Fern gametophytes are ideal for observation of intracellular structure, changes during a variety of developmental processes, and for manipulation of cells

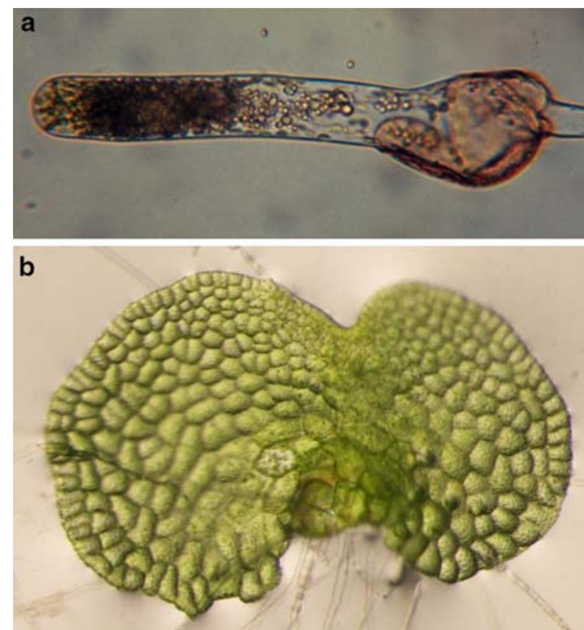

Fig. 2 Gametophytes of *A. capillus-veneris.* **a** A long protonemal cell cultured under continuous red light for 4 days. It grows toward the red light source. Cell thickness is approximately 15 μm. **b** A two-dimensional prothallus grown under white light. The width is approximately 1.5 mm

under a microscope for ligation (Wada 1988), micro-injection, or irradiation with light. This is partly because the cells are not surrounded by tissue and grow autonomously as filamentous cell lines or two-dimensional cell sheets made of a one-cell layer, at least in the early stages (Fig. 2). Fern gametophytes are quite sensitive to light and each developmental process and

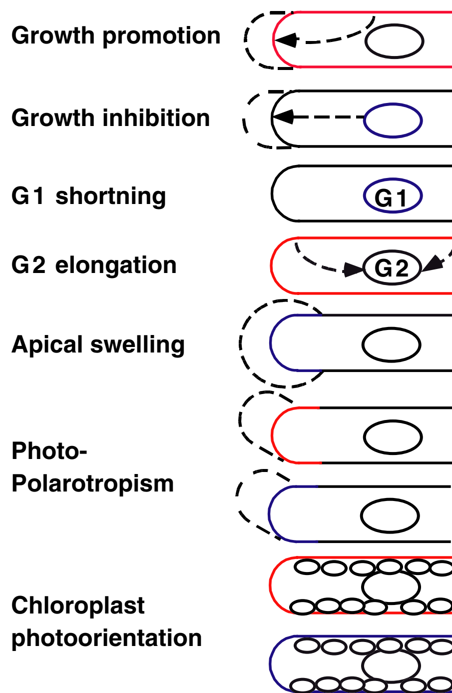

Fig. 3 Light-induced physiological phenomena in *A. capillus-veneris* protonemata cultured under red light. Photoreceptive sites of each phenomenon are indicated by *colored lines*. *Red* and *blue lines* indicate photoreceptive sites for red and blue light, respectively

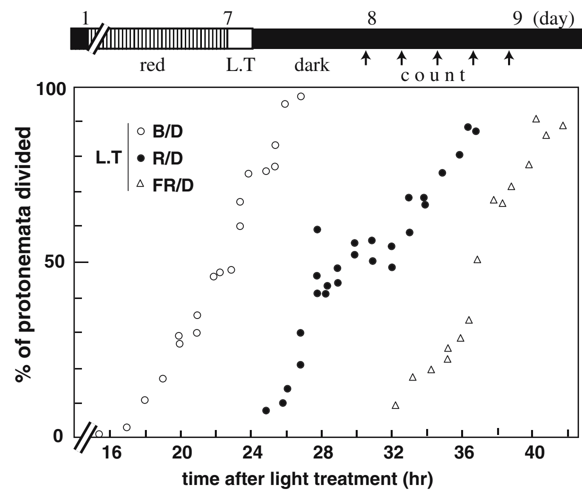

Fig. 4 Timings of cell division induced in red light-induced protonemata of *A. capillus-veneris* by irradiation with short pulses of blue, red, and far-red light before transfer to darkness. Blue light shortens the cell cycle (*white circles*) so that synchronous cell division occurs earlier than the division induced by transfer directly from red to darkness (*filled circles*). The timing of cell division was delayed in cells irradiated with far-red light (*triangles*), i.e. the cell cycle was extended. Modified from Wada and Furuya (1972)

physiological phenomenon is controlled by light as summarized in Fig. 3 (Kanegae and Wada 2006). In *A. capillus-veneris*, spore germination can be induced by irradiation with a short pulse of red light. This response can be inhibited by subsequent irradiation with far-red light, in a red/far-red reversible manner, indicating phytochrome-dependency (Furuya et al. 1997). Red light-induced spore germination is also inhibited by brief irradiation with a pulse of blue light, irrespective of the sequence of the two light pulses, i.e. either red followed by blue or blue followed by red (Sugai and Furuya 1985; Furuya et al. 1997). Although the specific photoreceptors involved in this phenomenon have not yet been identified, the red/far-red light response must be regulated by, at minimum, one of the three phytochromes (phy1, phy2, and/or phy4) already cloned (Okamoto et al. 1993; Kanegae and Wada 2006; Suetsugu and Wada 2005). Although the blue light receptor may be one of the five cryptochromes already reported (Kanegae and Wada 1998; Imaizumi et al. 2000), the possible redundancy of several cryptochromes has not yet been discounted. The other major plant blue light receptor class, phototropins, are not likely to be involved, because phototropins do not regulate gene expression, which must be essential for spore germination. When germination occurs, the gametophyte consists of a protonemal cell that develops into a two-dimensional prothallus, as a result of repeating cell division, and a rhizoid for which no cell division or any light-dependent characteristic responses are observed except negative phototropism (Tsuboi et al. 2006).

Almost all light-dependent responses in fern gametophytes are mediated by blue light and/or red/far-red light (Wada and Sugai 1994; Kanegae and Wada 2006). Although some red-light effects, for example spore germination (Furuya et al. 1997), are antagonistic to blue light (Wada and Furuya 1972; Wada and Sugai 1994), red and blue-light effects can also be synergistic (Kagawa and Wada 1996). Protonemal cells grow at their tip under red light without cell division but when the cells are irradiated with blue light cell growth is inhibited and cell division occurs at the apical region of the protonemata (Kadota et al. 1979). The timing of cell division is advanced, i.e. the cell cycle is shortened, by irradiation with blue light but is delayed (i.e. the cell cycle is extended) by red and far-red light (Fig. 4; Wada and Furuya 1972, 1974). It is not clear why these responses are antagonistic. My speculation is that blue light inhibits protonemal cell growth, which results in cell division, although the mechanism whereby this occurs is not clear. Blue light probably induces advancement of the cell cycle from G1 to S phase and, conversely, red light inhibits cell-cycle advancement, so that the cell cycle stays in early G1, promoting cell growth continuation (Miyata et al.

1979). Blue and red light must be a kind of switch controlling ‘‘on’’ and ‘‘off’’ of cell-cycle advancement, to overcome the barrier between the G1 to S or M phases, or to maintain the cell cycle at some point within the G1 phase. When red light-grown protonemata are transferred to darkness, cell division is induced through the S phase (Miyata et al. 1979), so cells under red light stay in the early G1 phase. If cells are still in G1 and have not yet entered the S phase, advancement of the cell cycle can be cancelled by irradiation with red light (Fig. 5). We named this position in the cycle the ‘‘point of no return’’. When advancement of the cell cycle is cancelled, the cycle not only stops at the advanced position, but returns to the start of the G1 phase to reset G1 from the beginning (Wada et al. 1984). Whether cell cycle stops or is reset to early G1 must depend on red light fluence given.

In contrast, in some responses, for example chloroplast photorelocation movement (Yatsuhashi et al. 1985; Kagawa and Wada 1994, 1996; Wada et al. 2003), as mentioned above, blue and red light works synergistically, so that additive effects are clearly observed when irradiation is conducted with red and blue light simultaneously (Kagawa and Wada 1996). For this to happen photoreceptors for red and blue light should occur at the same position.

To analyze antagonistic red and blue light effects we constructed microbeam irradiators with which a small part of a cell could be irradiated with colored light (Wada and Furuya 1978; Yatsuhashi et al. 1985) (Fig. 6). Using these microbeam irradiators we found that for all antagonistic phenomena the photoreceptive sites for red and blue light are separated in the cell, such that blue light is perceived in the nuclear region but red light perception occurs in the cell periphery. This was found to be so for protonemal cell division (Wada and Furuya 1978), spore germination (Furuya et al. 1997), and apical growth of protonemata (Kadota and Furuya 1977; Kadota et al. 1979). The interpretation of these results is that the photoreceptions should occur separately, in different organelles or in separate sites of the cells. Direct photoreceptor–photoreceptor interaction, in all likelihood, does not occur in these circumstances. For synergistic effects, on the other hand, both light-perceptive sites were found to be in the same position. In one special case, to be described below, it was found that both red and blue light wavelengths are absorbed at the same time by a chimeric photoreceptor composed of phytochrome and phototropin (Nozue et al. 1998; Kawai et al. 2003).

Even if a photoreceptive site is identified in a very small area of the cell (for example the cell tip, cell periphery, or nuclear region) by using the microbeam irradiator, the light path passes through a part of the cell which includes many organelles. This makes determining exactly which organelle may be the photoreceptive site very difficult. If the photoreceptive site is at the very cell edge, polarized light irradiation has been used to distinguish whether the photoreceptors are located on the plasma membrane. Although this method is not conclusive, the principle has been commonly accepted for many years (Bünning and

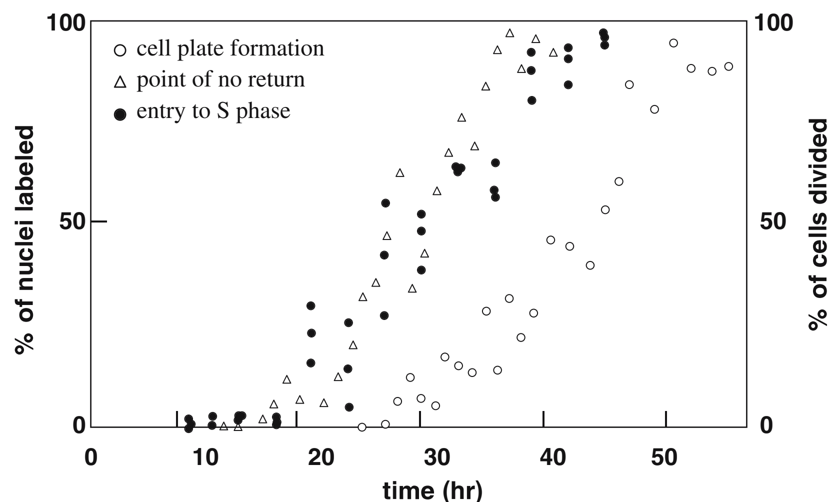

Fig. 5 The ‘‘point of no return’’ of cell division in red light-induced *A. capillus-veneris* protonemata. When red light-grown protonemata whose cell cycle is at the beginning of the G1 phase were transferred from red light to darkness, cell division was induced (*open circles*). But advancement of the cell cycle is cancelled, and returns to the beginning of G1, if the cells are irradiated with continuous red light before entering into S phase (*filled circles*). The timing is called ‘‘the point of no-return’’ (*triangles*). Modified from Wada et al. (1984)

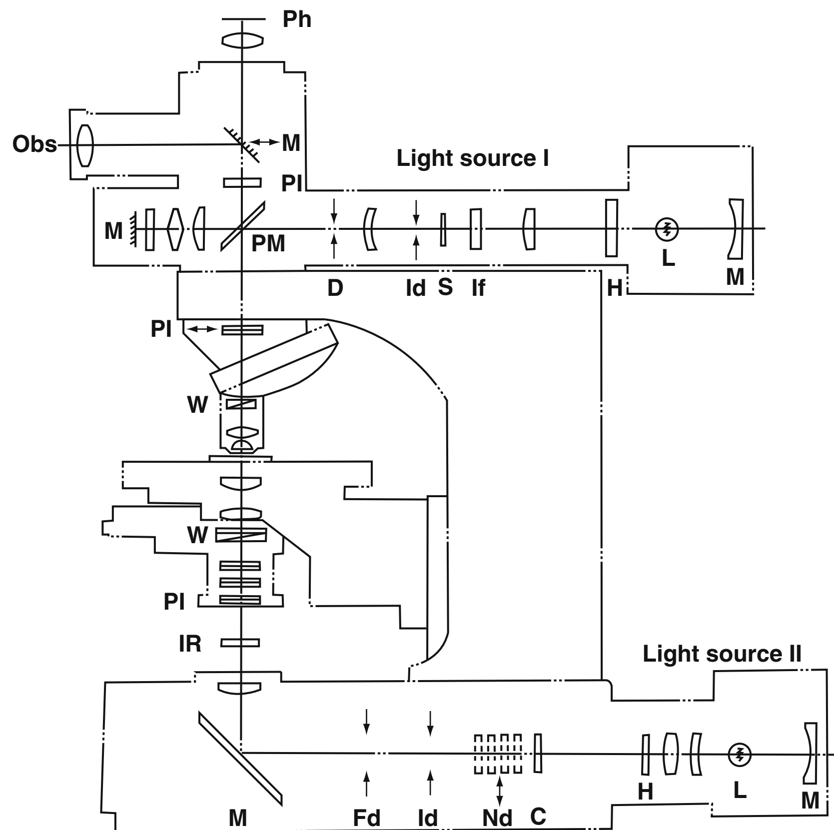

Fig. 6 Schematic diagram of a custom-made microbeam irradiator. It has two light sources: one for microbeam from above and the other for observation with infrared light from below. Samples for microbeam irradiation are placed on the stage. Images of the samples and a microbeam can be seen with the eye (*Obs*) and/or by taking a photograph or use of a monitor (*Ph*). The other abbreviations are not explained here. More information is available elsewhere (Yatsuhashi and Wada 1990)

Etzold 1958). When linearly polarized red or blue light (vibrating parallel or perpendicular to the cell axis) is irradiated along the edge of linear, tube-like cells, for example fern protonemal cells, the amount of light absorbed by photoreceptors located at the cell periphery depends on how the photoreceptors are arranged on or near the plasma membrane (i.e. parallel to the plasma membrane, perpendicular, or random; Kraml 1994). Light absorption and the resulting physiological effects depend on which direction the transition moment of the pigment is arranged. If the transition moment of the pigment is parallel to the plasma membrane, polarized light vibrating parallel to the cell axis is absorbed effectively but polarized light vibrating perpendicular is not. As a result, if photoreceptors are arranged in a particular manner on the plasma membrane, parallel vibrating polarized light can result in a greater effect than perpendicular polarized light, and vice versa. This is called a ‘‘dichroic effect’’ (Kraml 1994). When a dichroic effect has been observed it has been shown the photoreceptors are located on the plasma membrane (Kadota et al. 1982; Bünning and Etzold 1958).

Another method, which avoids the ambiguity of organelle interference in determining the photoreceptive site, is cell centrifugation. In this technique cytoplasm is displaced by basipetal centrifugation such that almost all organelles are forced downward from the tip region of the cells. If partial cell irradiation still induces a phototropic response, at the apical region of protonema for example, the photoreceptive site is either on the plasma membrane or in the ectoplasm. Long protonemal cells have a big advantage in spinning down cytoplasm (which includes the nucleus and chloroplasts), because they can move a long distance, which is important for analytical resolution. This procedure can be made difficult, however, by oil droplets, which gather at the tip of protonemata by downward centrifugation, and ectoplasm, which stacks toward the plasma membrane even under centrifugation. To avoid accumulation of oil droplets at the top of the cells, L-shaped protonemata can be made by virtue of the phototropic response and changing the direction of red light by approximately 90° one day before using the materials for experiments (Wada et al. 1983) (Fig. 7). L-shaped cells can be centrifuged several times to remove almost all oil droplets from the protonemal tip or to accumulate them in the tip, furnishing non-oil-droplet cells or oil-stuffed cells (Fig. 8). When one side of the sub-apical part of these non-oil or oil-stuffed protonemal cells is irradiated with a red light microbeam, the phototropic response occurs toward the

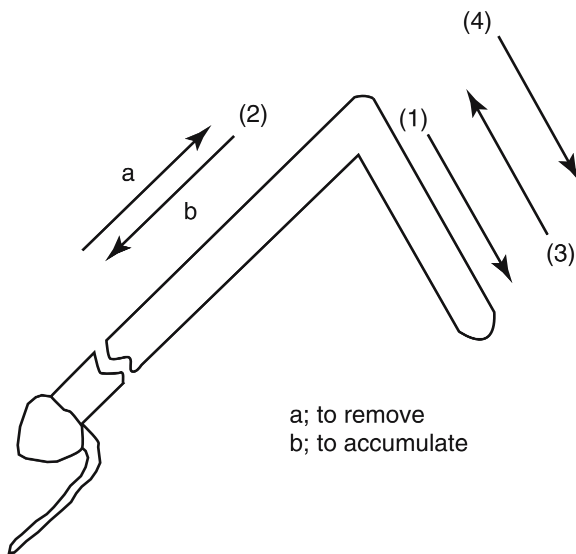

Fig. 7 An L-shaped protonema for cell centrifugation to remove oil droplets. The direction of red light irradiation was changed by 90° one day before using the protonema for experiments. *Arrows* and *numbers* mean the direction and sequence of centrifugation to remove oil droplets from (*a*) or accumulate oil droplets in (*b*) the tip of the protonema. Modified from Wada et al. (1983)

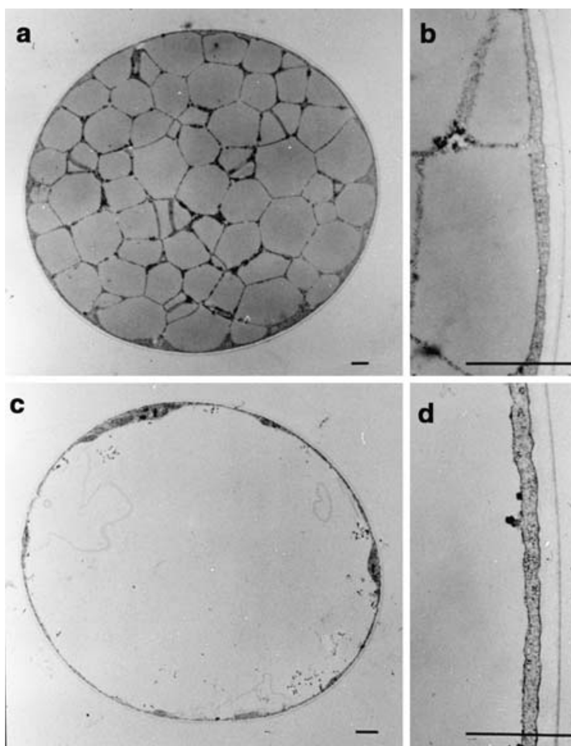

Fig. 8 Electron micrographs of cross-sections of sub-apical part of the protonema with **a** or without **c** oil droplets. **b** and **d** are high magnification of **a** and **c**, respectively. Each *bar* indicates 1 μm

irradiated side of the protonema if cytoplasm, including the nucleus, was moved back to the apical part of the cell by centrifugation. These experimental results indicate that the photoreceptor of this particular phenomenon should occur on the plasma membrane or in the ectoplasm but not on the tonoplast (Wada et al. 1983).

According to the results obtained by use of the microbeam, polarized light, and centrifugation experiments (Wada et al. 1983; Kadota et al. 1985; Yatsuhashi et al. 1987), phytochrome molecules mediate both red light-induced phototropic and polarotropic responses and chloroplast movement in *A. capillus-veneris* protonemal cells. We now know that one of these molecules, phytochrome 3 (renamed *Ac*neo1) is arranged on the plasma membrane, as shown in Fig. 9, (i.e. the red light-absorbing form is arranged parallel to the plasma membrane but the far-red light absorbing form is perpendicular to it).

Cytoskeletons

Cytoskeletal pattern changes in protonemal cells have been investigated by a variety of methods, including immuno-fluorescence microscopy and rhodamine-labeled phalloidin staining. Changes in microtubules and/or actin filaments have been investigated during cell growth (Murata et al. 1987), nuclear migration (Kadota and Wada 1995), tropistic response (Wada et al. 1990; Kadota and Wada 1992b), apical cell bulging (Murata and Wada 1989a; Kadota and Wada 1992a), pre-prophase band (PPB) formation (Murata and Wada 1989b, 1991a, b, 1992), and branch formation (Wada et al. 1998). Protonemal cells are fixed with formaldehyde and then treated successively with anti-tubulin antibody and a fluorescein-linked second antibody, or stained with rhodamine-labeled phalloidin. Recently, however, as for many organisms, observation of living cells transformed with constructs of GFP-tubulin for microtubules (Ueda et al. 1999) or GFP-talin (Kost et al. 1998) or GFP-fimbrin for actin filaments (Sheahan et al. 2004) has become a common technique in plants. Both microtubules and actin filaments can be observed simultaneously by use of different fluorescent proteins. In fern gametophytes stable transformation has not yet been achieved, as it has for several other plant species. We can, however, use a method of transient expression of these constructs, introduced by particle bombardment. The disadvantages of this technique are the rather short-lived expression and complications arising from damage during cell bombardment. For these reasons several

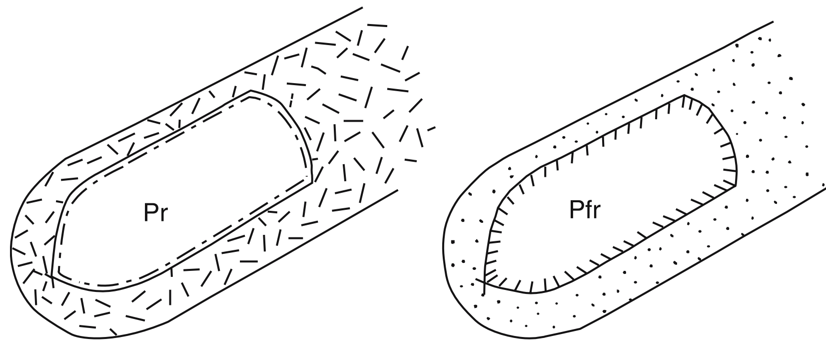

Fig. 9 Direction of transition moment of phytochrome3 chromophores located at the cell periphery in *A. capillus-veneris* protonemata. *Pr* (red light absorbing form of phytochrome) is parallel to the plasma membrane and *Pfr* (far-red light absorbing form of phytochrome) is perpendicular. From ''Phytochrome and Photoregulation in Plants'', Academic Press, 1987

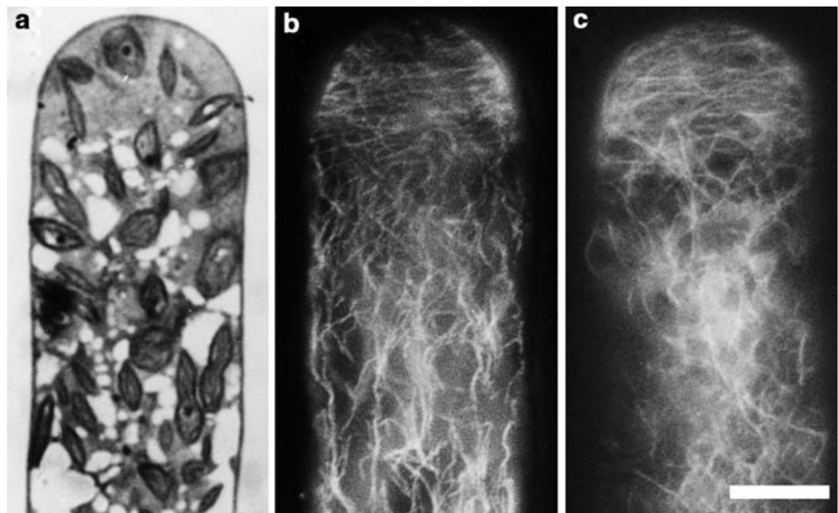

Fig. 10 Ring-like structures made of microtubules and actin filaments at the sub-apical part of red light-induced protonemal cells. **a** A light micrograph of a longitudinal section, **b** Fluorescence micrograph of microtubules. Note that microtubules perpendicular to the cell axis are seen in the sub-apical part of the cells. **c** Fluorescent micrograph of actin filaments. *Bar*: 10 μm. Modified from ''Cytoskeletal Basis of Plant Growth and Form'', Academic Press 1991

examples of results obtained by use of fixed fern cells are mentioned below.

The diameter of tip-growing cells, for example those of fern and moss protonema, rhizoids of a variety of plants, fungal hyphae, pollen tubes, and root hairs, are fairly stable, so it is probably under strict control (Wastneys and Galway 2003). The mechanism establishing and controlling cell diameter is, in most of these examples, not yet understood. At the sub-apical part of the growing fern protonemata we found a circular array of microtubules and microfilaments which may control the cell diameter (Murata et al. 1987; Murata and Wada 1989a; Kadota and Wada 1992b) (Fig. 10). If the microtubule bands of this array are destroyed by colchicine the apical portion of the protonemata swells as a balloon (Murata and Wada 1989c), indicating that cell diameter is controlled by this microtubule array. We therefore, observed the distribution pattern of microfibrils at the innermost layer of the cell wall, around the sub-apical part of the protonemata. Under red light without colchicine the microfibrils are arranged parallel to the microtubule array but when protonemata are treated with colchicine the microfibril pattern becomes random (Murata and Wada 1989a, c). Similarly, when red light-grown protonemata are irradiated with blue light, apical cell-bulging occurs. We observed whether the pattern of the microtubule array and that of microfibrils correlate, as it does for colchicine-treated cells, and confirmed the similarity between these patterns (Murata and Wada 1989a, c).

When protonemata are cultured under red light, they grow toward the light source. When the direction of red light irradiation is altered a phototropic response at the apical portion of the cell is observed—bending toward the new position of the red light. A similar tropistic response can be seen when protonemata are irradiated with polarized red light from above through a cover slip. Protonemata grow perpendicular to the vibration plane of such polarized light (Kadota et al. 1982, 1985) (Fig. 11), a phenomenon is called polarotropism. We studied the time course of pattern changes of microtubule and micro-

filament arrays in the subapical portion of protonemal cells and that of microfibrils during this tropistic response (Wada et al. 1990). Within 1 h oblique orientation of the microfilament arrays toward the light is observed; the microtubule array then becomes oblique and, finally, the microfibril pattern becomes random, even though no phototropic response has been observed for the protonemal tip (Fig. 12) (Wada et al. 1990).

In further studies directed at determining the role of microtubule and microfilament arrays in the regulation of cell diameter and the direction of growth, we studied their stability by treatment with colchicine and cytochalasin B (Murata and Wada 1989c; Kadota and Wada 1992a). When colchicine is used, only microtubule arrays are disrupted, but with cytochalasin B, both microfilament and microtubule arrays are disrupted (Kadota and Wada 1992a). This suggests that the stability of microtubules depends on the presence of microfilaments. Similarly, in reorganization of these cytoskeletal elements during phototropic responses, changes in microfilamentous structure precede that of microtubules, meaning that microtubule structure depends on microfilaments (Kadota and Wada 1992b).

A pre-prophase band (PPB) of microtubules appears before prophase along the cell periphery, at the site of future cell division, and disappears before nuclear division occurs. Although the function of the PPB is certainly critical to plant morphogenesis, nothing in detail is known about the mechanism of PPB site selection and appearance. Knowledge about the control of timing of the appearance and disappearance of the PPB has also been lacking (Mineyuki 1999). We therefore analyzed the behavior of the PPB with the advantage of long, single-celled fern protonemata (Murata and Wada 1989b, 1991a, b, 1992). In cells cultured under a continuous red light then transferred to blue or white light conditions cell division occurs synchronously at the apical region (Wada and Furuya 1972). During cell division a PPB appeared transiently in the future site of cell division (Murata and Wada 1989b). Because the mechanism and factors that control the PPB are unknown, we examined the importance of the cytoplasm, including the nucleus, to PPB behavior. At the moment the PPB appears in the nuclear region, approximately 40–60 μm from the protonemal tip, the cells were centrifuged basipetally to spin down the nucleus and cytoplasm and the PPB was then observed by immuno-fluorescence microscopy (Fig. 13) (Murata and Wada 1991b, 1992). It was found from these experiments that when a nucleus is relocated, a new PPB appears at the new nuclear position, and the former PPB remains for long time without disruption, even when cell division occurs in the lower

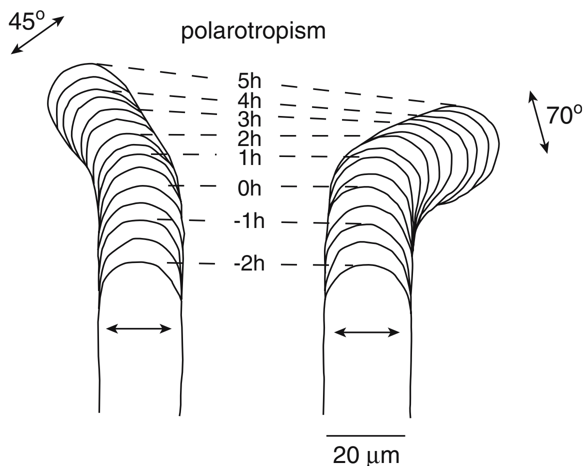

Fig. 11 Polarotropic response of the fern *A. capillus-veneris* protonemata. Protonemata were cultured under continuous red light then irradiated, through a cover glass, with polarized red light vibrating at 45° or 70° to the cell axis. One hour after irradiation with the polarized light the apical part of the cells started to bend in a direction perpendicular to the plane of vibration of the polarized light. From Wada et al. (1990)

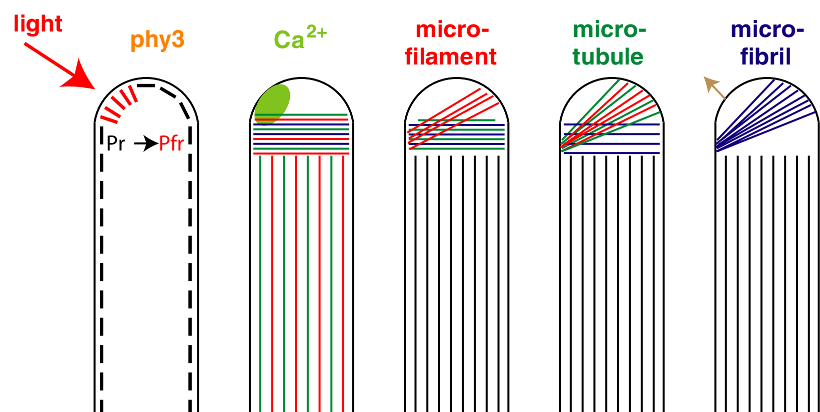

Fig. 12 Schematic model of phototropic response in *A. capillus-veneris* protonema. Red light is received by phy3 at the sub-apical part of the protonema, the concentration of calcium ions may increase in the light-irradiated area, and the orientation of microfilaments (*red lines*), microtubules (*green lines*), and, finally, microfibrils (*blue lines*) changes towards the bending direction

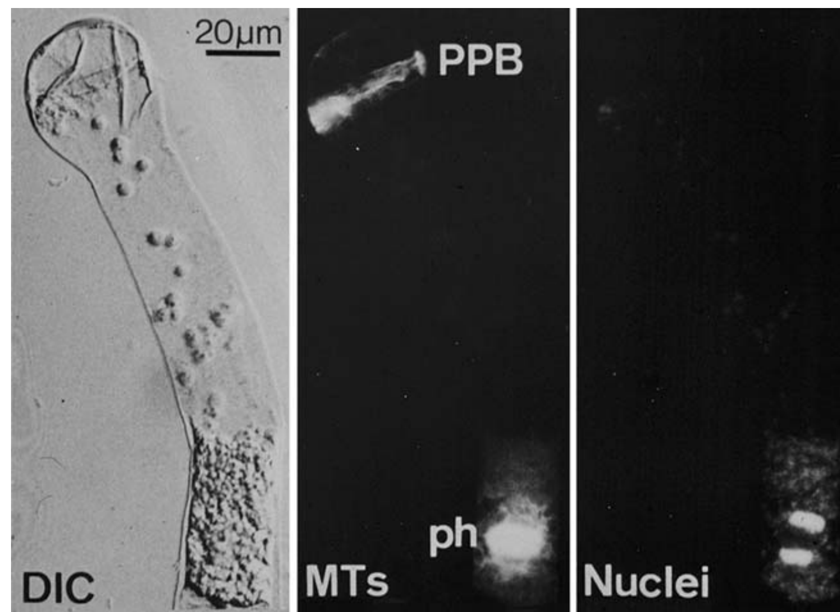

Fig. 13 Photomicrographs of an *A. capillus-veneris* protonema during cell division. A protonema was centrifuged basipetally during pre-prophase and then cultured until cell division occurred. *DIC*: Differential interference contrast image of protonema showing distribution of organelles. *Mts*: Protonema stained with anti-tubulin antibody. Pre-prophase band (*PPB*) and phragmoplast (*ph*) are observed. *Nuclei*: DAPI staining of daughter nuclei. Note that the PPB is still observed during telophase. Modified from Murata and Wada (1992)

part of the cell (Murata and Wada 1991b). If the nucleus is taken back from the basal position to the original position, however, the former PPB disappears (Murata and Wada 1992). Taken together, this suggests that for PPB formation and disruption the nucleus and/or surrounding cytoplasm is needed. These types of result emphasize the usefulness of fern protonemal cells, because such experiments would not be easy to perform using small and/or rectangular cells.

Photoreceptors

Photoreceptors known to regulate photomorphogenesis have been identified in many plant species. In *A. capillus-veneris*, three conventional phytochromes have been cloned (Okamoto et al. 1993; Nozue et al. 1997; Suetsugu and Wada 2005). Although their roles should be consistent with those of higher plant phytochromes, none of their functions has been ascertained. The forth phytochrome sequence, which is named phytochrome3 (phy3, and renamed *Ac*neo1, see below), was found to have an unusual gene structure—that of a chimeric photoreceptor. In phy3, the phytochrome chromophore-binding domain in the N terminus is fused to a full-length blue-light receptor, phototropin in the C-terminus (Nozue et al. 1998). This unusual photoreceptor has only been found in advanced ferns (Kawai et al. 2003), suggesting the *PHY3* gene arose during fern evolution. Similar chimeric photoreceptors named neochrome (neo1 and neo2) were found in the green alga *Mougeotia scalaris* Hassall, suggesting that a similar gene fusion has occurred twice in plant evolution, once either in *M. scalaris* or one of its ancestors, and once in a fern. The origins of these chimeric receptors are clearly independent, as judged from their gene structures (Suetsugu et al. 2005) (Fig. 14).

Two phototropins (as observed for *Arabidopsis thaliana* (L.) Heynh.; Kagawa et al. 2004) and five cryptochromes (Kanegae and Wada 1998; Imaizumi et al. 2000; where *A. thaliana* has only two) in total have been cloned in *A. capillus-veneris* as blue light receptors. Phototropin is a molecule that contains two LOV domains (named after sensitivity to light, oxygen, and voltage, and called LOV1 and LOV2) in the N terminus, which bind flavin mononucleotide (FMN) for light perception, and a serine/threonin protein kinase sequence in the C-terminus (Briggs et al. 2001). Cryptochrome has a sequence similar to photolyase, a DNA photo-repair enzyme, in the N terminus and an extension in the C terminus (Lin and Shalitin 2003). In *A. thaliana* it has been shown that cryptochrome has two chromophores in the N-terminus, one being flavin adenine dinucleotide (FAD) and the other possibly a pterin (Lin and Shalitin 2003), but chromophore identification in fern cryptochromes has not yet been studied.

It is now known that phy3 mediates red light-induced chloroplast movement and phototropism (Kadota and Wada 1999; Kawai et al. 2003; Tsuboi et al. 2006). Toward this understanding we screened mutants that were defective in phototropism under red light conditions but for which normal phototropism was maintained under blue light (Kadota and Wada 1999). For all the mutants studied the *PHY3* gene was

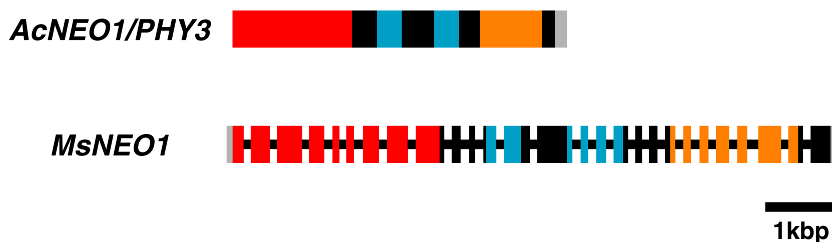

Fig. 14 Gene structures of *A. capillus-veneris* phytochrome3/*A. capillus-veneris* neochrome1 (phy3/*Ad*neo1) and *M. scalaris* neochrome1 (*Ms*neo1). Note that *Ms*neo1 has many introns but there is no intron in *A*cneo1

disrupted (Kawai et al. 2003). Transient expression of phy3 in these mutants rescued the red light responses, moreover, demonstrating that red light-induced phototropism and chloroplast movement are both mediated by this photoreceptor (Kawai et al. 2003). Interestingly, although only blue light is effective for most seed plant phototropisms, tropistic responses are observed for fern leaves under red or blue light. Fern *phy3* mutants are, however, defective in red light-induced tropistic responses (Kawai et al. 2003), indicating that both gametophytes and sporophytes use the same photoreceptor. The mechanisms of this response in tip-growing single cells and the intercalary growth in multi-cellular tissues are, however, assumed to be quite different.

In *A. thaliana*, chloroplast movement is mediated by phototropin. The phot2 photoreceptor mediates the chloroplast avoidance response (Kagawa et al. 2001; Jarillo et al. 2001), and phot1 and phot2 redundantly mediate the accumulation response (Sakai et al. 2001). Two *A. capillus-veneris* mutants defective in the avoidance response were identified by screening and used to show that phot2 was also the photoreceptor regulating the avoidance response in fern (Kagawa et al. 2004). In these two plants the mutant phenotype was rescued when the *PHOT2* gene was transiently expressed. This transient expression system has additional value in determining which domain or amino acid is critical for function (Kagawa et al. 2004). In this regard, mutated constructs of *PHOT2* cDNA were transiently expressed in *phot2* mutants by particle bombardment. A construct containing a deletion between the N-terminus and the LOV1 domain was still functional in the avoidance response, but the same construct in which LOV2 was exchanged for LOV1 was not functional (Kagawa et al. 2004). These results indicate that LOV2 is essential for photoreception in phot2 in *A. capillus-veneris*, and similar results have been obtained in phot1 in *A. thaliana* (Christie et al. 2002). When a cysteine in LOV1, to which FMN binds, is changed to alanine, the cDNA is still functional, but when this cysteine of LOV2 is changed, the construct is not functional (Christie et al. 2002). Similar experiments with a C-terminal deletion reveal that only 20 amino acids in the C-terminus are critical for phot2 function in the chloroplast avoidance response (Kagawa et al. 2004).

Molecular studies

Molecular studies in fern are still very difficult because:

1. stable transformation is not available (although transient expression of introduced genes is possible by particle bombardment, as already mentioned);
2. fern gene databases are very poor, except for some EST collections in *A. capillus-veneris* (Yamauchi et al. 2005) and *Ceratopteris richardii* Brongniart (Salmi et al. 2005); and
3. a genome project in ferns has not yet been undertaken, perhaps because of their extraordinary large genome sizes.

A genome project in *Selaginella moellendorffii* Hieron, is now in progress (Wang et al. 2005), but Selaginellaceae belong to the Lycophytes, a group with microphylls, phylogenetically different from ferns which have macrophylls. There is, on the other hand, an advantage reported only for ferns, a phenomenon called ‘‘DNA interference (DNAi)’’, described below (Kawai-Toyooka et al. 2004).

Fern gametophytes are in haplophase, thus contain only one genome set per cell, making them useful in screening for mutant plants. When fern spores are treated with ethylmethane sulfonate (EMS) or exposed to a heavy ion beam, such as nitrogen, the resulting mutated gametophytes have a mutant phenotype that can be observed even if the gene is recessive. We have screened many different kinds of mutant, especially those defective in light-induced phenomenon (Kadota and Wada 1999; Kagawa et al. 2004). Identification of the mutant genes is almost impossible, however, except when we have *A. thaliana* mutants whose phenotype is quite similar to that in ferns (Kagawa et al. 2001). The fern *phot2* mutants are a good illustration of this. We had identified the photoreceptor for the chloroplast avoidance response in *A. thaliana*, i.e. phot2, before we

screened fern mutants that were defective in the same response (Kagawa et al. 2004). We sequenced the *PHOT2* gene in the available fern mutants and found that their *PHOT2* genes were disrupted (Kagawa et al. 2004). Complementation tests with the wild type *PHOT2* gene in *A. capillus-veneris*, then was used to demonstrate that phot2 was, indeed, also the photoreceptor for chloroplast avoidance.

Particle bombardment is the only method for fern transformation at the moment. We believe *Agrobacterium* should work in fern, but no one has yet had success. Although we have tried stable transformation in *A. capillus-veneris* several times by particle bombardment, it also has not yet been successful. We usually use transient expression of genes, driven by the cauliflower mosaic virus 35S promoter, and this has been very successful, within its limits (Kawai et al. 2003; Kagawa et al. 2004; Suetsugu et al. 2005).

For an effective molecular approach, methods like stable transformation and targeted gene knockouts are indispensable. To knockout genes in fern gametophytes we have attempted homologous recombination, because this technique is reportedly less troublesome in haplophase plants than in diplophase plants (Schaefer 2002). We created a construct for homologous recombination, such that a full-length *PHY3* gene was interrupted with a hygromycin resistant gene (*HYG*), driven by the 35S promoter, and this construct was bombarded into *A. capillus-veneris* gametophytes. Drug-resistant prothalli were screened after a few weeks, and the red light-induced phototropic response was not observed for protonemata grown from these prothalli, indicating that knockout of the *PHY3* gene was successful (Fig. 15). The results were very curious, however, because the phenotype was reproducible and it was defective in both red light-induced chloroplast movement and the tropistic response. Sequencing of the *PHY3* gene in these lines indicated it had not been disrupted, i.e. was as in the wild type. We then measured the expression level of *PHY3* mRNA in these lines and found it was very much reduced, indicating that he phenomenon is more a type of gene silencing. Much like the now well-known RNA interference (RNAi), which is induced by double stranded RNA, we believe this type of silencing to have been induced by double-stranded DNA (similarly dubbed DNAi; Kawai-Toyooka et al. 2004). Although it has been shown that RNAi can be used for gene silencing in the water fern *Marsilea vestita* (Klink and Wolniak 2000) and in *C. richardii* (Stout et al. 2003), DNAi is relatively easy, because fragments of double-stranded DNA can be obtained by polymerase chain reaction (PCR). We have tested the effect of PCR-amplified *PHY3* fragments, and whether silencing occurs using DNA fragments co-bombarded with the *HYG* gene. Amazingly, PCR products are also very effective when the fragment length is longer than, on average, 500–1,000 bp (Kawai-Toyooka et al. 2004). We have introduced five different target genes at once into a cell, with effective results for each gene fragment (Kawai-Toyooka et al. 2004). Although this method seems very simple, the mechanism of action of DNAi is not known yet. Note that DNAi-like gene silencing has also been demonstrated in *C. richardii* (Rutherford et al. 2004).

Evolutionary aspects

As already mentioned, ferns are in a special position in plant evolution, connecting bryophytes and seed plants

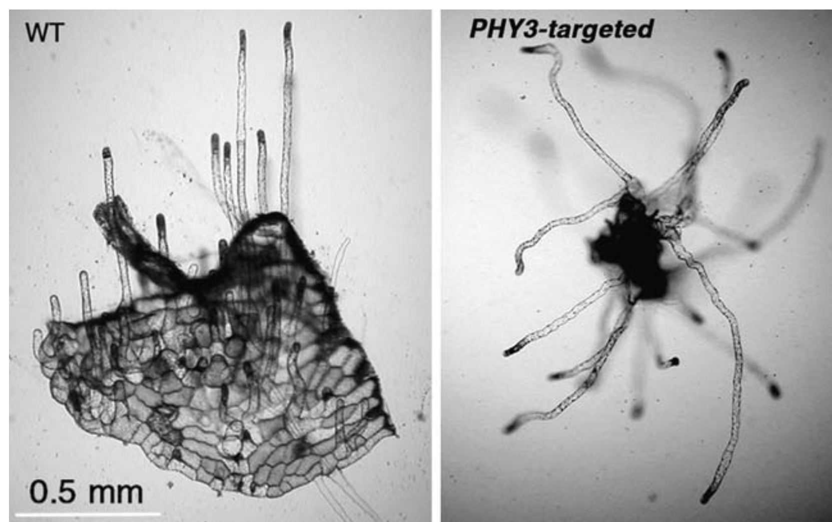

Fig. 15 Silencing of *A. capillus-veneris* phytochrome3 by DNAi. PCR fragments of *PHY3* cDNA and hyg^r gene (*HYG*) or *HYG* only for control were introduced by particle bombardment into fern prothalli and these were cultured under continuous red light. Protonemal cells were regenerated from prothalli, and whereas control cells grew toward the red light source *phy3* silenced cells did not

(Pryer et al. 2001). Thus, fern study is essential for the understanding of seed plant origins, because many characteristic features of seed plants are based on the genes developed in an ancestor common to both groups. The entire fern lineage has diverged from seed plants, however, so some characteristics, and the genes that support them, should be specific to ferns. One example of this is phy3-dependent phenomena. The chimeric photoreceptor phy3, as discussed above, is found in advanced ferns only (i.e. not below the relatively primitive tree fern), suggesting that the gene fusion between the phytochrome chromophore binding domain and phototropin may have occurred during fern evolution, probably just after the divergence of tree ferns (Kawai et al. 2003). Wild type plants with *PHY3* are quite sensitive to white light in response to phototropism, compared with phy3 knockout lines in *A. capillus-veneris*. With the *PHY3* gene, the sensitivity of the phototropic response to white light is increased by at least one order of magnitude, nearly two (Kawai et al. 2003) (Fig. 16). It is unknown why and how phy3 renders ferns so sensitive, but the *PHY3* gene must have played a big role in fern evolution. The benefit of increased sensitivity to white light, afforded by phy3 in conjunction with phot1 and phot2, under forest canopy conditions, probably made it possible for ferns to perform in a niche previously under-utilized, resulting in their proliferation (Kawai et al. 2003; Schneider et al. 2004). Interestingly, phototropism and chloroplast photorelocation movement by phy3 may have played a big role in the success of ferns, but stomatal opening likely did not contribute, because stomatal opening is believed to be controlled by photosynthesis, and not by phototropin or phy3, although the three phenomena are controlled by photoropins in higher plants (Doi et al. 2006). Ferns may have met with evolutionary success under canopy conditions because of the acquisition of the *PHY3* gene and the integration of red light photoperception with blue light signal transduction.

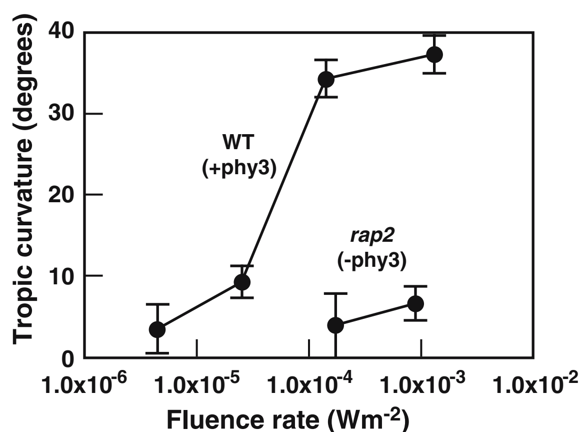

Fig. 16 Fluence response curve of phototropic response under white light in *A. capillus-veneris* leaves with (wild-type) or without (*phy3* mutant) *PHY3* genes. Note that the sensitivity to white light is nearly 100 times greater in wild-type plants than in *phy3*-deficient mutant plants. Modified from Kawai et al. (2003)

This situation becomes even more interesting now we have found a similar chimeric photoreceptor, called neochrome, in *M. scalaris* (Suetsugu et al. 2005), the algae famous for red light-induced chloroplast movement studied for many years by the late Professor Wolfgang Haupt. *M. scalaris* has two neochromes (*Ms*neo1 and *Ms*neo2) that are similar to fern phy3 in their structures, so we renamed phy3 in *A. capillus-veneris Ac*neochrome1 (*Ac*neo1). If we analyze the genome structures of the *AcNEO* and *MsNEO* genes, however, we find they are quite different. *AcNEO1* has no intron in its genome sequence (Nozue et al. 1998) whereas *MsNEO* genes have many introns, both in the phytochrome chromophore binding domain and within the full-length phototropin (Suetsugu et al. 2005). *AcNEO1* could be detected in advanced ferns but no *PHY3*-like gene could be found in an EST collection of the moss *Physcomitella patens* (Hedw.) Bruch & Schimp. subsp. *patens* Tan, indicating that fern neochrome must have arisen during fern evolution as mentioned above (Suetsugu et al. 2005). Thus, *Ms*neo genes must have evolved independently from fern neochrome. *Ms*neo has not yet been shown to function as the photoreceptor for red light-induced chloroplast movement in *M. scalaris*, because mutant screening and/or RNAi are very difficult. We have, however, tested whether *Ms*neo can rescue chloroplast movement in *A. capillus-veneris* phy3 defective mutants, using transient expression, and found that *Ms*neo can replace *Ac*neo1 in *A. capillus-veneris*. This illustrates that they are functionally equivalent, although their origins are different (Suetsugu et al. 2005).

Future perspectives in fern studies

Fern gametophytes, both linear protonemal cells and the two-dimensional prothalli, make a very useful experimental system for cell biological and photobiological analysis. Not only is this because of characteristic light regulation in developmental and physiological processes, and its unique alteration of generations, but simply because fern spores are stable for many years once harvested. Even if they are put on a shelf at room temperature (preferably kept in a refrigerator, of course, the green spores being exceptional), they germinate under proper conditions. This makes the storage and handling of individual lines

exceptionally trouble-free. With techniques such as gene silencing by DNAi, ferns are an ideal system for complementing our knowledge of higher plants, especially plant vascular systems and their origins. Several issues must be addressed, however, before ferns can become one of the best model systems among plant species. Stable transformation is a top priority. As mentioned, transformation by *Agrobacterium tumefaciens* has not yet been successful, but it should be possible to generate stable transformants in some way, if proper attention is directed to clearing this hurdle. The second issue is lack of a full-length genome sequence. An EST collection is now being generated in our laboratory using *A. capillus-veneris* gametophytes. This will undoubtedly yield new and potentially critical additions to our knowledge of plant and fern gene families, but the information will be very limited in comparison with that from sequencing of the entire genome. Given that genome projects have, in recent years, been completed with increasing speed, it may not be long before the entire genome of the first fern species will become available. With it will come an explosion in our understanding of ferns, fern gene families, and their unique contributions to our world.

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