Plant Physiol. (1979) 64, 9–12
0032-0889/79/64/0009/04/$00.50/0

Rhizoid Differentiation in *Spirogyra*

III. INTRACELLULAR LOCALIZATION OF PHYTOCHROME

Received for publication January 30, 1978 and in revised form September 25, 1978

YOKO NAGATA[1]
Department of Biology, Faculty of Science, Osaka University, Toyonaka, Osaka 560 Japan[2]

ABSTRACT

Localization of phytochrome which mediates rhizoid differentiation in *Spirogyra* was investigated. The red-absorbing form of phytochrome (Pr) seems to be distributed all over the cell periphery which remained in the centripetal end part after the centrifugation, as rhizoids formed equally well with red spotlight irradiation of three different parts of an end cell, *i.e.* distal end, middle, and proximal end, and with irradiation of centrifugal and centripetal end parts of a centrifuged end cell. The Pr distribution was confirmed with an experiment using far red irradiation over the entire cell, centrifugation, and red spotlight irradiation. The Pr-phytochrome molecules appeared to be mobile because no dichroic orientation was shown with polarized red spotlight irradiation. On the contrary, it is suggested that far red-absorbing form of phytochrome molecules are evacuated from the centripetal end part by the centrifugation in an experiment involving red irradiation over the entire cell-centrifugation-far red spot irradiation. Rhizoid formation was repressed markedly by far red irradiation of the centrifugal end part but not of the centripetal end part.

The mode of function of the phytochrome in a physiological response, or a molecular description of the receptor/effector chain for a stimulus-response system remains unknown in spite of much work in this area. Reports on the distribution of phytochrome, especially within the plant cell, and on particle- or membrane-bound phytochrome are increasing, thus reflecting the importance of these subjects as clues toward understanding the mode of function.

Four methods are applicable to determine the localization of phytochrome: (*a*) a method using microspectrophotometer; (*b*) a physiological method based on the effect of local irradiation of R[3] or FR *in vivo* (3); (*c*) a biochemical method using cell fractionation by density (1, 4, 6, 9–13); (*d*) an immunocytochemical method (5). The first method cannot be used for a cell containing Chl. The third and the fourth methods involve the risk of intervention by phytochromes not involved in the physiological reaction or non-active ones. Thus, the second method seemed the best for determining the location of the phytochrome which mediated a physiological reaction.

Some species of the green alga, *Spirogyra*, grow in small streams attached to the beds by rhizoids. The rhizoids appear 3 to 8 hr after light irradiation mostly at the tips of end cells of the filaments (7, 8). Rhizoid cell differentiation is controlled by phytochrome as shown by the fact that the differentiation is induced by brief irradiation of R and reversed by subsequent FR (8).

Study of phytochrome in a system free of the complications of intercellular interactions should be valuable. The *Spirogyra* seems to be such a system since effect of light irradiation is restricted within the right cell irradiated as shown below, *i.e.* the effect can be detected in the cell. We applied the second physiological method of giving spotlight to determine the intracellular localization of phytochrome, together with a method utilizing centrifugal force. The latter method by Chen and Kamiya (2) introduced possible separate treatment of the cytoplasm of a large cylindrical cell of *Nitella* from the cortex, providing a chemical reagent or a physical factor.

In the present report, the term "cell periphery" is used to indicate cellular constituents which remained in centripetal end part after the centrifugation, *i.e.* cell membrane, cell wall, cytoplasm, etc.; and the term, "cell contents" for the mass which moved to the centrifugal end by the centrifugation, involving cytoplasm, bands of chloroplasts, nucleus, etc.

MATERIALS AND METHODS

The starting material, axenic filaments of *Spirogyra sp.*, was a gift from T. Ohiwa of our laboratory, and the filaments were cultured at 20 C under a 12 hr-12 hr light-dark cycle using fluorescent light (*cf.* 7).

The *Spriogyra* filaments were cut into about 1-mm segments containing four to nine cells. The cut filaments were suspended in 0.65% agar (Difco Bacto-Agar) sol on a glass plate after 24-hr dark incubation at 20 C, then aliquots of them, more than five, were sucked together with the agar gel into a glass tube (about 0.08 cm in diameter, about 4.5 cm long) to be positioned parallel to the long axis of the tube, with the aid of green safelight (0.011 w/m^2, around 510 nm) obtained with fluorescent light and an interference filter (Hitachi EPS-2; half-bandwidth, 15 mm) in a dark room. The glass tube was sealed and the filaments were further incubated at 20 C in the dark for at least 24 hr before use (preincubation in the dark) to check whether they had already formed rhizoid(s) without R. Occasionally we found some filaments possessing rhizoids produced by unknown stimulus. The filaments preincubated in the dark in the glass tube are termed "specimen" below.

R (around 645 nm; half-bandwidth, 15 nm) and FR (around 720 nm; half-bandwidth, 75 nm) light for irradiation and green safelight (around 510 nm; half-bandwidth, 15 nm) were obtained by placing interference and glass filters (Toshiba) just before a tungsten light bulb of a microscope. See Figure 1 of reference 8 as to the transmission bands of the colored light. Spotlight irradiation was performed with the microscope by putting together a rectangular slit with the color filters, then using one of two identical objective lenses as an objective and the other as a condenser to obtain a clear rectangular light spot of Köhler's illumination.

Light energy was measured with a radiometer (United Detector Technology Inc., PIN-10RP).

[1] Present address: Department of Leprosy, Institute for Microbial Diseases, Osaka University Suita, Osaka 565 JAPAN
[2] Address for reprint requests.
[3] Abbreviations: R: red light, FR: far red light.

PROCEDURES OF SIX DIFFERENT EXPERIMENTS

Red Spotlight Irradiation. The glass tube containing the specimen was laid down in a vessel filled with water with a slide glass on the bottom and a coverslip on top. Each cell to be irradiated was identified by the location and cell number of the specimen using 0.0092 w/m^2 green safelight under an inverted microscope. The section of the cell, either of the three parts of the end cell or a part of the next cell of the specimen, was placed in the center of the microscopic field using dim green safelight (0.0053 w/m^2), then irradiated with 0.21 w/m^2 red spotlight for 1 min as shown in Figure 1, top.

The specimen irradiated was observed for rhizoid formation after postincubation in the dark for about 16 hr at 20 C. The rhizoid formation rate was calculated as the percentage of rhizoid cells to the total number of cells irradiated in the sealed glass tube. The same lengths of pre- and postincubation in the dark were used in the following experiments (*cf.* 8 for explanation of the need for pre- and postincubation).

Centrifugation—Universal White Light. Since identification of each segment was not needed in this case, the dark-preincubated segments were placed longitudinally between two pieces of pith using green safelight (0.011 w/m^2) and given 7,000*g* at 0 C for 15 min in the dark. The high centrifugal dose was to remove the cell contents from the centripetal end of the cell as completely as possible and was applicable with the aid of pith but not agar gel used throughout the present experiments except for this. The centrifuged segments were transferred into a vessel with the culture medium, and briefly given white fluorescent light at a time. In this, as well as in the following experiments, the end of a centrifuged cell which had formed a rhizoid could still be easily distinguished after postincubation in the dark because the chloroplast bands remained *in situ* in the centrifugal end.

Centrifugation—Red Spotlight Irradiation. The specimen in agar gel was centrifuged in the dark at 1,100*g* at 0 C for 10 min, after being chilled at 5 C for 10 min, to evacuate the cell contents from the centripetal end quickly. The location of the end cells of the centrifuged specimen was checked quickly with 0.95 w/m^2 FR under the inverted microscope. Check with FR was done also in other experiments except for two cases, red spotlight irradiation as mentioned above and R over the entire cell-centrifugation-far red spotlight irradiation. The reason that FR was used was to reverse all Pfr form of phytochrome molecules to Pr form before R irradiation. Immediately after the check either the distal end or proximal end part of the centrifugal and centripetal end cells of the specimen were irradiated with 0.30 w/m^2 R through a rectangular slit (70 × 40 μm) for 1 min (Fig. 1, bottom). The irradiated area of the cell was about one-third of the cell length in most cases, according to the size of cellular part where the cell contents were condensed by the centrifugation. The distal end part of the centrifugal end cell and the proximal end part of the centripetal end cell contained both the cell contents and the cell periphery, and the proximal part of the centrifugal end cell and the distal part of the centripetal end cell included only the cell periphery. Both the cell contents and the cell periphery were irradiated with the centrifugal end part-irradiation, and only cell periphery was illuminated with the centripetal end part-irradiation. The specimen was then centrifuged again in the reverse direction (recentrifugation) of the first centrifugation followed by postincubation in the dark as usual. The recentrifugation in the opposite direction was to eliminate effect of the cell contents particularly of cytoplasm which might act to some extent on rhizoid formation, being in contact with the site where the rhizoid formed, although I found later that the effect did not occur. This was shown in the centrifugation-white fluorescent light experiment below.

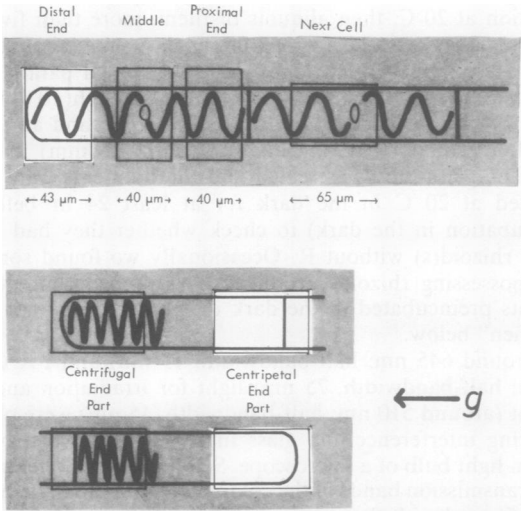

FIG. 1. Top: four parts of cells, irradiated with 0.21 w/m^2 R. The case of distal end irradiation is shown. Bottom two: four parts of cells, irradiated with 0.30 w/m^2 R or 14 w/m^2 FR for 1 min after centrifugation. The case of centripetal end irradiation is shown. Arrow indicates the direction of gravity. Only one chloroplast *band* is represented in the figure although the *Spirogyra sp.* used contains three chloroplast bands.

Centrifugation—Polarized Red Spotlight Irradiation. The entire procedure was the same as for the experiment, centrifugation-red spotlight irradiation except that polarized R was given instead of unpolarized R. Polarized light was obtained by putting a polaroid sheet together with the colored filters and the slit. The energy of light was adjusted to 0.30 w/m^2 changing the light intensity of the microscope. Only the centrifugal ends of end cells were used since it was unknown if the rear cell periphery of the cylindrical cells had the same dichroic orientation as the front cell periphery: *e.g.* it might be possible that the cell periphery had spiral dichroic orientation resulting in failure of detecting any polarized light favorable to rhizoid formation. The polarized R which may be absorbed completely by the front cell periphery and the centrifuged, packed chloroplasts, may not reach to the rear cell periphery.

FR Irradiation over the Entire Cell-Centrifugation-Red Spotlight Irradiation. Whole filaments of the specimen were irradiated with 1.7 w/m^2 FR for 1 min under the inverted microscope immediately before centrifugation ("quick experiment," 2,100*g* at 0 C for 5 min) or 40 to 140 min before centrifugation ("slow experiment," 1,100*g* at 0 C for 10 min). In the quick experiment greater centrifugal force was applied to perform the centrifugation more quickly. The centrifuged end cells were irradiated as soon as possible with 0.30 w/m^2 R for 1 min through a rectangular slit (65 × 35 μm) as shown in Figure 1, bottom, followed by recentrifugation in the reverse direction and postincubation in the dark. The irradiated area was about one-third of the cell as mentioned above.

R Irradiation over the Entire Cell-Centrifugation-Far Red Spotlight Irradiation. This was the counterpart of the above experiment. Whole filaments of the specimen were irradiated with 0.15 w/m^2 R for 1 min under the inverted microscope immediately before centrifugation (1,100*g* at 0 C for 10 min). The centrifuged end cells of which location had been checked using 0.0092 w/m^2 green light were irradiated with 14 w/m^2 FR for 1 min using a rectangular slit (65 × 35 μm) then recentrifuged and given postincubation.

Light dose and area were kept constant within a series of experiments so that it was possible to compare the data and form a conclusion. Where necessary the dose and area were changed: (*a*) Universal white fluorescent light was used to detect polarity of rhizoid formation on the direction of centrifugal force since spot light irradiation was not needed; (*b*) In all spotlight irradiations the width of the irradiated area of the cell was the cell width, *i.e.* 33 μm. A short length of spot area, 40 μm, was necessary in the

red spotlight irradiation of three different parts of the cell without overlapping (Fig. 1, top). (*c*) A longer length of spotlight, 65 or 70 μm, was used to cover the area of centrifugal or centripetal end part, about one-third of the cell length.

All procedures except the counting of the rhizoid cells were carried out in a dark room equipped with dim green light (0.0010 w/m^2, Mitsubishi FL 10G) at 22 to 24 C. See references 7 and 8 for detailed information.

RESULTS

No difference appeared among the rhizoid formation rates when three different parts of an end cell were irradiated, *i.e.* the distal end, the middle, and the proximal end (Table I, *cf.* Fig. 1, top). The nucleus does not seem to be the immediate R acceptor which contains phytochrome. Almost all rhizoids appeared only at the tips of end cells out of relation to the irradiated sites. Irradiation of the next cell was not effective at all. A larger area of light was applied to show that irradiation of any part of the next cell could not induce a rhizoid even with the higher dose of R than that given in the end cell irradiation. Based on this result all of the results follow that can be considered not to be modified by interaction of cells which had not been irradiated.

In order to separate the cytoplasmic sol from the cortex, filaments were centrifuged (2). The cytoplasmic sol could not be precipitated completely, *i.e.* cytoplasm, chloroplasts, nucleus, etc. were moved to centrifugal end by the centrifugation whereas still some cytoplasm, cell membrane, cell wall, etc. were left at centripetal end part. It was shown by centrifugation-universal white light illumination that both end cells of a centrifuged segment could be used in the following experiments, since a rhizoid could form equally well at a centrifugal end of a cell as at a centripetal end under an identical light condition (Table II); that is, the rhizoid formation was not affected by the direction of centrifugal force, nor by the amount of cytoplasm in contact with the site where a rhizoid formed. Most of the chloroplast bands still remained in the centrifugal end when the number of rhizoid cell was counted after postincubation in the dark. No difference was found in the centrifugation-red spotlight experiment between the centrifugal and the centripetal end part-irradiation (Table II). The results suggest that the phytochrome, probably the Pr form because a long period of dark incubation was given, is localized in the cell periphery. Dichroic orientation of the phytochrome molecules could not be detected with a similar experiment using polarized R. All four polarized red lights of different vibration planes, *i.e.* perpendicular, parallel, and oblique (±45°) to the cell axis, were equally effective for rhizoid differentiation (Table II). This suggests that the Pr-phytochrome molecules are mobile.

We also studied the distribution of Pfr and Pr form of phytochrome (Table II). Cells were irradiated universally with FR then centrifuged and given red spot irradiation. In the slow experiment a 50- to 150-min dark period (20 C) intervened between the end of the FR irradiation and the end of the centrifugation, while there was only a 12-min dark interval in the quick experiment. No difference appeared between the centrifugal and the centripetal end part-irradiation in the slow experiment. This suggests that Pr is localized in the cell periphery and confirms the above results obtained in the experiments involving red spotlight and centrifugation-red spotlight irradiation. A similar result was shown in the quick experiment. The minimum dose of R to saturate the induction of rhizoid formation was found to be 0.14 w/m^2 and that of FR for complete reverse of R effect, 4.6 w/m^2 (8). Although the dose of FR given before centrifugation in the FR over the entire cell-centrifugation-red spotlight experiment was less than the min-

Table I. Red spotlight irradiation of three different parts of an end cell or a part of the next cell, at 0.21 W/m^2 for 1 min (See Fig. 1, top).

Rhizoid Formation Rate (%)[1]				
Distal End Irradiation	Middle Irradiation	Proximal End Irradiation	Next Cell Irradiation	Dark Control
61 ± 14	59 ± 7.4	57 ± 4.2		12 ± 4.2
	73 ± 7.1		2.1 ± 2.1	4.5 ± 3.0

[1] Each value represents mean ± standard error of 4 separate experiments, and for each experiments 4 to 6 glass tubes with specimen were served.

Table II. Effect of spotlight irradiation of centrifuged end cells. See "Materials and Methods" for details.

Experimental Procedure[2]	Rhizoid Formation Rate (%)[1]		
	Centrifugal End Irradiation	Centripetal End Irradiation	Control
CEN-white light	42 ± 6.9	45 ± 6.6	61 ±12 (Without CEN)
CEN-R SL	43 ± 9.3	39 ± 4.9	2.8 ± 1.5 (Dark)
CEN-polarized R SL	33 ± 8.3 (↕)		
	37 ± 9.7 (↔)		4.1 ± 2.3 (Dark)
	40 ± 7.8 (↗)		
	42 ± 4.8 (↖)		
F-CEN-R SL, Slow	56 ± 7.7	60 ± 10	8.6 ± 4.2 (Dark)
F-CEN-R SL, Quick	51 ± 10	38 ± 12	12 ± 4.4 (Dark)
R-CEN-F SL	15 ± 8.0	60 ± 12	74 ± 7.5 (Without F SL)

[1] Each value is mean ± standard error of 3 to 12 separate experiments, and for each of the experiment 7 to 14 glass tubes with specimen were served.

[2] R, red light; F, far red light; SL, spotlight; CEN, centrifugation.

imum dose, it might be enough to reverse all Pfr to Pr because a long preincubation in the dark had been provided to the specimen.

On the other hand, a marked difference appeared in the counterpart experiment using R irradiation over the entire cell, centrifugation, and far red spotlight irradiation. In R-irradiated cells, rhizoid formation was much more effectively reversed by the subsequent FR irradiation of centrifugal end part than the centripetal end part. Dark periods, 20 to 24 min long, intervened between the end of R irradiation and the end of centrifugation. The same result was obtained in a quicker experiment with dark intervals as short as 10 min (data not shown). These results suggest that Pfr is localized not in the cell periphery but in the cell contents. The phytochrome which had been localized in the cell periphery as Pr at least 12 to 150 min after FR irradiation, seems to have moved into the cell contents quite rapidly, within about 10 min after R irradiation, to exist there as Pfr.

DISCUSSION

Bound phytochrome to some cellular particulate or membranous vesicles in higher plants were reported by Rubinstein (12), Boisard *et al.* (1), Pratt and Marmé (9), Lehmann and Shäfer (4), and Quail (10, 11). In most cases the R-induced binding of phytochrome could not be fully reversed by FR and the remaining, bound Pr was slowly released from the membrane. According to Quail (11) FR immediately terminated further development of the R-induced "potential to pellet" but no immediate reduction of amount of pellet was observed: and two operationally defined sequential stages, the *in vivo* development of the potential to pellet and the expression of this potential *in vitro*, are suggested. The latter stage was enhanced by the presence of Mg^{2+} or a divalent cation during homogenization, and thus the divalent cation-mediated induction of an artifactual, *in vitro* association should be considered (10). Only Lehmann and Shäfer (4) showed a quick, but partial reverse of amount of pelletable phytochrome by FR pulse. In comparison with the extraction method applied to the above investigations the method used in the present experiment has the advantages of (*a*) being free of such a stage, in which the artifactual association of phytochrome with membrane fraction might be considered: and (*b*) making possible the investigation of the very phytochrome which is active and controlling the physiological reaction, although the method has the disadvantage that it is difficult to perform a clear fractionation of subcellular components in the cell. Mackenzie *et al.* (5) showed immunocytochemically that Pr was associated with discrete regions of the cell. The regions did not seem to be nuclei, plastids, or mitochondria. Also, in this case, associated phytochrome was released slowly by FR irradiation. Manabe and Furuya (6) and Williamson *et al.* (13) reported the association of Pfr with mitochondria-rich fraction and with rough-surfaced ER fraction of higher plants, respectively.

The present investigation indicated that Pr remains in the cell periphery at least up to 150 min after FR irradiation and is quickly released into the cell contents by R as Pfr, and that Pfr in the cell contents associates with the cell periphery as Pr quite rapidly, in 10 min, following FR. Thus, in our experimental system change in the intracellular localization of phytochrome seems to fit the physiological interconversion of the pigment, Pr $\rightleftarrows$ Pfr, as to the length of period required for the change. Pfr may not be bound to the cell membrane, because the rhizoid formation was repressed by FR given on centrifugal but not centripetal end part of the cell.

Three views on the distribution of phytochrome may be suggested based on the present results. First, Pr is bound to the cell membrane and is quickly released into cytoplasm by FR as Pfr. We do not know whether the Pfr of the present study is bound to some subcellular organelles in the cytoplasm. If almost all cytoplasm was precipitated by the centrifugation and the remaining cytoplasm at the centripetal end did not include enough Pfr to reverse rhizoid formation by FR irradiation, then the first view may be most feasible. Second, Pr is diffusible and uniformly distributed throughout the cytoplasm whereas Pfr becomes somehow sedimentable in the centrifugal end of the cell by virtue of aggregation or binding to some particulate cell structure. This view may be acceptable if much cytoplasm was left at the centripetal end and enough Pr to induce rhizoid formation by R existed there. Third, both Pr and Pfr are soluble and uniformly distributed throughout the cytoplasm, and the centrifugation-caused, a clearly unequal population of Pfr in the cytoplasm, *i.e.* many more phytochrome molecules in centrifugal end than centripetal end, brought about the failure of FR irradiation of centripetal end part to reverse the response induced by universal R irradiation. This hypothesis seems to be unfeasible because the unequal population of phytochrome should also cause failure of R irradiation of centripetal end part to induce rhizoid formation. This hypothesis may explain the data only when Pr has much greater sensitivity to R than Pfr's sensitivity to FR. Note that near minimum saturation dose of R, 0.30 w/m^2, was used to induce rhizoid formation in the present experiment (the minimum dose, 0.14 w/m^2 [8]), and the irradiated area was only a part of the whole cell whereas the dose of FR, 14 w/m^2, far exceeded the minimum dose (4.6 w/m^2 [8]) to reverse the R action completely. To know the distribution of phytochrome more precisely, correct information about the volume of cytoplasm in the centrifugal and the centripetal ends is necessary. Also comparative R dose-response curves for the both ends is required to distinguish if Pr is stuck in the cell periphery. It may be better to irradiate the centrifugal and the centripetal ends being centrifuged, by means of a microscope attached to a centrifuge.

Acknowledgments—I am grateful to N. Kamiya for his valuable discussions and encouragement to perform this work, and to O. Matsumoto, Faculty of Engineering Science for loan of the equipment to measure light energy.

LITERATURE CITED

1. Boisard J, D Marmé, WR Briggs 1974 *In vivo* properties of membrane-bound phytochrome. Plant Physiol 54: 272–276
2. Chen JCW, N Kamiya 1975 Localization of myosin in the internodal cell of *Nitella* as suggested by differential treatment with N-ethylmaleimide. Cell Struct Function 1: 1–9
3. Haupt W 1972 Perception of light direction in oriented displacements of cell organelles. Acta Protozool 11: 179–188
4. Lehmann U, E Schäfer 1978 Kinetics of phytochrome pelletability. Photochem Photobiol 27: 767–773
5. Mackenzie JM Jr, RA Coleman, WR Briggs, LH Pratt 1975 Reversible redistribution of phytochrome within the cell upon conversion to its physiologically active form. Proc Nat Acad Sci USA 72: 799–803
6. Manabe K, M Furuya 1974 Phytochrome-dependent reduction of nicotinamide nucleotides in the mitochondrial fraction isolated from etiolated pea epicotyls. Plant Physiol 53: 343–347
7. Nagata Y 1973 Rhizoid differentiation in *Spirogyra*. I. Basic features of rhizoid formation. Plant Cell Physiol 14: 531–541
8. Nagata Y 1973 Rhizoid differentiation in *Spirogyra*. II. Photoreversibility of rhizoid induction by red and far-red light. Plant Cell Physiol 14: 543–554
9. Pratt LH, D Marmé 1976 Red light-enhanced phytochrome pelletability. Re-examination and further characterization. Plant Physiol 58: 686–692
10. Quail PH 1975 Interaction of phytochrome with other cellular components. Photochem Photobiol 22: 299–301
11. Quail PH 1978 Irradiation-enhanced phytochrome pelletability in *Avena*: *in vivo* development of a potential to pellet and the role of Mg^{2+} in its expression *in vitro*. Photochem Photobiol 27: 147–153
12. Rubinstein B, KS Drury, RB Park 1968 Evidence for bound phytochrome in oat seedlings. Plant Physiol 44: 105–109
13. Williamson FA, DJ Morré, MJ Jaffe 1975 Association of phytochrome with rough-surfaced endoplasmic reticulum fractions from soybean hypocotyls. Plant Physiol 56: 738–743