Photosynthesis-Dependent But Neochrome1-Independent Light Positioning of Chloroplasts and Nuclei in the Fern *Adiantum capillus-veneris*

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Chloroplasts change their positions in the cell depending on the light conditions. In the dark, chloroplasts in fern prothallia locate along the anticlinal wall (dark position). However, chloroplasts become relocated to the periclinal wall (light position) when the light shines perpendicularly to the prothallia. Red light is effective in inducing this relocation in *Adiantum capillus-veneris*, and neochrome1 (neo1) has been identified as the red light receptor regulating this movement. Nevertheless, we found here that chloroplasts in neo1 mutants still become relocated from the dark position to the light position under red light. We tested four neo1 mutant alleles (neo1-1, neo1-2, neo1-3, and neo1-4), and all of them showed the red-light-induced chloroplast relocation. Furthermore, chloroplast light positioning under red light occurred also in *Pteris vittata*, another fern species naturally lacking the neo1-dependent phenomenon. The light positioning of chloroplasts occurred independently of the direction of red light, a response different to that of the neo1-dependent movement. Photosynthesis inhibitors 3-(3,4 dichlorophenyl)-1,1-dimethyleurea or 2,5-dibromo-3-isopropyl-6-methyl-p-benzoquinone blocked this movement. Addition of sucrose (Suc) or glucose to the culture medium induced migration of the chloroplasts to the periclinal wall in darkness. Furthermore, Suc could override the effects of 3-(3,4 dichlorophenyl)-1,1-dimethyleurea. Interestingly, the same light positioning was evident for nuclei under red light in the neo1 mutant. The nuclear light positioning was also induced in darkness with the addition of Suc or glucose. These results indicate that photosynthesis-dependent nondirectional movement contributes to the light positioning of these organelles in addition to the neo1-dependent directional movement toward light.

In a variety of plant species, chloroplasts show relocation in the cell in response to the environmental light conditions (Haupt, 1982; Haupt and Scheuerlein, 1990; Wada et al., 2003; Gabrys, 2004; Wada and Sutetsugu, 2004). While in the dark chloroplasts locate along the anticlinal wall or the bottom of the cell depending on plant species (dark position), they move toward and accumulate along the periclinal cell wall that face incident illumination under weak or moderate light conditions (light position). This response is thought to optimize photosynthetic activity (Zurzycki, 1955). However, when the light intensity is too strong, chloroplasts avoid the light and locate along the anticlinal cell wall to reduce photodamage (Kasahara et al., 2002). Recently, photoprotective function of avoidance movement was analyzed measuring chlorophyll fluorescence parameters in several mutants (Sztatelman et al., 2010). Chloroplast photorelocation is sometimes accompanied by the movement of nuclei; light-regulated positioning of nuclei has been investigated in a few plants while physiological meaning of this phenomenon is still unclear (Kagawa and Wada, 1993, 1995; Iwabuchi et al., 2007).

Chloroplast photorelocation is generally induced by blue light, and phototropins have been identified as the photoreceptors (Jarillo et al., 2001; Kagawa et al., 2001, 2004; Sakai et al., 2001; Kasahara et al., 2004). Red light is usually ineffective in triggering chloroplast movement but some modulation through phytochrome phototropin-dependent movement was reported in Arabidopsis (Arabidopsis thaliana; DeBlasio et al., 2003; Luesse et al., 2010). In lower green plants (including ferns), however, red light, in addition to blue light, is also effective in chloroplast relocation (Sutetsugu and Wada, 2005, 2007). In the fern *Adiantum capillus-veneris*, the noncanonical phytochrome neochrome1 (neo1; formerly known as phytochrome3; Sutetsugu and Wada, 2007), which is a chimera of N-terminal chromophore-containing half of a canonical phytochrome plus a full-length phototropin downstream from it, has been identified as the receptor for the red-light-induced chloroplast and nuclei relocation (Kawai et al., 2003; Tsuboi et al., 2007).

In this study, we report that the neo1 mutant of *A. capillus-veneris* (Kadota and Wada, 1999; Kanegae and Wada, 2006) and the wild-type *Pteris vittata*, which...
lacks neo1-dependent responses (Kadota et al., 1989), still show light positioning of chloroplasts under red light regulated by photosynthesis and that relocation of nuclei accompanies the response.

RESULTS

Red-Light-Induced Chloroplast Relocation Movement in the neo1 Mutant of A. capillus-veneris and in P. vittata

When prothallia from the neo1-1 mutant and wild-type A. capillus-veneris kept in the dark for 3 d, with their chloroplasts in the dark position (i.e. positioned along the anticlinal walls), were continuously irradiated with red light (4.1 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) coming from above, chloroplasts of the wild-type prothallia showed relocation movement toward the periclinal wall facing the incident light via the neo1-dependent response, as reported previously (Kadota and Wada, 1999; Kawai et al., 2003). Surprisingly, many chloroplasts also became relocated from the anticlinal walls toward the periclinal wall in the neo1-1 mutant (Fig. 1, A and B). When the direction of the red light was changed and the prothallial cells were irradiated from the side with red light of 6.4 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), chloroplasts in the wild-type cells showed movement toward the light, and the chloroplasts became relocated to the anticlinal walls facing the red light (Fig. 1, A and B; Supplemental Movie S1). In the neo1-1 mutant, however, the chloroplasts did not become relocated to the anticlinal walls facing the red light but to the periclinal walls (Fig. 1, A and B; Supplemental Movie S1), demonstrating that chloroplast photorelocation in the neo1-1 mutant is independent of the direction of the light. Thus, the red light response in the neo1-1 mutant is apparently different from the neo1-dependent response and is a response not previously documented.

When a prothallial cell of the neo1-1 mutant was partially irradiated with a microbeam of red light of 20 \( \mu \text{m} \) in diameter (5.6 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)), the neo1-1 cell did not show chloroplast movement although chloroplast accumulation toward the microbeam region was apparent in the wild type (Fig. 2). However, chloroplast relocation in the neo1-1 mutant clearly occurred as it does in the wild type when whole cells were irradiated with the same light intensity. The speed of relocation movement was slower in the neo1-1 mutant; at 6 h after irradiation the wild-type cells showed chloroplast accumulation almost entirely on the periclinal wall, but the accumulation was not so obvious in the neo1-1

Figure 1. Red-light-induced chloroplast relocation movement in the neo1 mutant and the wild type of A. capillus-veneris. A, Prothallia of the neo1-1 mutant and wild type were irradiated with vertical red light (4.1 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) or horizontal red light (6.4 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)). Horizontal red light was irradiated from the left. Note that chloroplasts of the neo1-1 mutant show relocation to the periclinal walls irrespective of the direction of the light, while in the wild type, they became relocated depending on the direction of the light. The inset shows close-up of cells under horizontal red light irradiation and the arrows indicate the accumulation of chloroplasts on the anticlinal wall facing the red light. Prior to light treatment, the white-light-grown prothallia were kept in the dark for 3 d so that the chloroplasts in the cell initially showed the dark position, by accumulating on the anticlinal walls. Bar = 50 \( \mu \text{m} \). B, The bar graph shows the average number of chloroplasts per 1,000 \( \mu \text{m}^2 \) of the periclinal wall with the se. Cells of the second row from the edge of the prothallia were counted after red light irradiation for 11 h. At least 20 cells derived from three independent experiments were counted.
mutant (Fig. 2; Supplemental Movie S2). The red-light-induced chloroplast relocation was confirmed in the other neo1 mutant alleles, neo1-2, neo1-3, and neo1-4 (Fig. 3). Furthermore, the red light response was also observed in the prothallia of P. vittata (Fig. 3), another fern species naturally lacking the neo1-dependent chloroplast movement (Kadota et al., 1989).

Effect of Photosynthesis Inhibitors on the Red-Light-Induced Chloroplast Photorelocation Movement in the neo1 Mutant and the Wild Type of A. capillus-veneris

Using inhibitors, we examined whether chloroplast photorelocation in the neo1 mutant was mediated by photosynthesis. Prothallia of the neo1-1 mutant and the wild type were treated with 100 \( \mu \text{M} \) 3-(3,4 dichlorophenyl)-1,1-dimethylurea (DCMU) and then irradiated with red light of 6.4 \( \mu \text{mol} \text{ m}^{-2} \text{s}^{-1} \). As shown in Figure 4, A and B, the neo1-1 mutant did not show chloroplast photorelocation regardless of the direction of the red light. In contrast, the wild-type chloroplasts showed directional movement toward the periclinal or anticlinal wall depending on the direction of the light; in fact, under vertical red light, the chloroplasts moved to the periclinal walls, and under horizontal irradiation from the side, they migrated to the anticlinal walls facing the irradiation (Fig. 4, A and B). Similar results were obtained with another photosynthesis inhibitor, 2,5-dibromo-3-isopropyl-6-methyl-p-benzoquinone (DBMIB; Supplemental Fig. S1). It should be noted that in the presence of the photosynthesis inhibitors, chloroplasts in wild-type cells rarely located on the periclinal wall under horizontal red light (Fig. 4A, inset; Supplemental Fig. S1). Without inhibitors, several chloroplasts were seen on the periclinal walls of wild-

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Partial and whole-cell irradiation with red light in the neo1 mutant and the wild type of A. capillus-veneris. Prothallial cells of the neo1-1 mutant and the wild type were partially irradiated with a microbeam of red light (white circle in the figure; 20 \( \mu \text{m} \) in diameter) or whole cells were irradiated with red light of the same fluence rate. Note that chloroplasts in the neo1-1 mutant showed relocation movement under whole-cell irradiation but not under microbeam irradiation. Under whole-cell irradiation, chloroplast relocation in the wild type was more rapid than that in the neo1-1 mutant. Pretreatment of the prothallia was performed as described in Figure 1. Bar = 50 \( \mu \text{m} \).

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Red-light-induced chloroplast relocation in other neo1 mutant alleles of A. capillus-veneris and in P. vittata. Prothallia of neo1-2, neo1-3, neo1-4, and P. vittata were irradiated horizontally with red light (6.4 \( \mu \text{mol} \text{ m}^{-2} \text{s}^{-1} \)) from the left. Note that in every prothallium, the chloroplasts became relocated to the periclinal walls. Pretreatment of the prothallium was performed as described in Figure 1. Bar = 50 \( \mu \text{m} \).
type cells, particularly in the inner cells of prothallia (see Fig. 1A, inset)—this is evidence that the photosynthesis-dependent nondirectional chloroplast movement also operates in the wild type.

Glc and Suc Induce Chloroplast Relocation in the Dark in *A. capillus-veneris*

As the red-light-induced, nondirectional chloroplast relocation in *neo1-1* depended on photosynthesis, we next examined whether the chloroplast relocation in the wild type could be induced in the dark with the addition of a sugar, the product of photosynthesis. We used 1% (w/v) Suc, Glc, and mannitol. After treatment with each sugar, the prothallia were kept in the dark for 2 d (Fig. 5A). In Suc- and Glc-treated prothallia, chloroplast relocation toward the periclinal wall occurred slowly (Fig. 5B). Although even after 2 d, the extent of chloroplast relocation was limited compared to the treatment by light irradiation, a significant number of chloroplasts migrated toward the periclinal wall from the anticlinal wall without any light irradiation (Fig. 5C; Supplemental Movie S3). It is noteworthy that the sugar effects could be detected even under the shorter period of treatment for 11 h (Fig. 5B). The chloroplasts did not show movement in the mannitol-treated prothallia, thus confirming that the above effects of Suc and Glc did not result from the indirect osmotic effect of the external medium (Fig. 5C).

**Suc Promotes Red-Light-Induced Chloroplast Relocation Movement and Rescues from the Effect of DCMU Inhibition in the *neo1* Mutant of *A. capillus-veneris***

We further investigated whether inhibitory effect of DCMU on relocation movement could be rescued by simultaneous application of Suc in *neo1-1* mutant. Prothallia of *neo1-1* were treated with 1% (w/v) Suc in the presence or absence of 100 μM DCMU. As shown in Figure 6, Suc treatment greatly promoted chloroplast relocation movement in the absence of DCMU. In the presence of DCMU, chloroplasts resumed relocation movement from anticlinal wall to periclinal wall. These effects of Suc were clearly seen as early as 11 h after beginning of red light irradiation and provide further support for the involvement of photosynthesis in the red-light-induced chloroplast relocation in *neo1-1* mutant.
Red Light Induces Relocation Movement of Nuclei in the neo1 Mutant of *A. capillus-veneris*

In the dark, nuclei are positioned along the anticlinal cell wall, and the red-light-dependent relocation of nuclei as well as of chloroplasts has been reported in the wild type (Kagawa and Wada, 1993, 1995). Therefore, relocation of nuclei in the neo1-1 mutant located along the anticlinal wall in the dark, like the wild type. After vertical irradiation with red light (4.1 μmol m⁻² s⁻¹) for 20 h, the nuclei located along the periclinal cell wall or to a transitional region between the periclinal and anticlinal walls in the neo1-1 mutant, as observed in the wild type. When red light of 3.0 μmol m⁻² s⁻¹ was provided horizontally from the side, many nuclei in the wild type became positioned in the transitional region, between the periclinal wall and the anticlinal wall, which faces the incident red light (arrows in the figure). In the neo1-1 mutant, however, many nuclei became positioned in the transitional region (between the anticlinal wall and the periclinal wall), which was not necessarily facing red light; however, a small but significant number of nuclei became relocated on the periclinal wall. When strong red light (100 μmol m⁻² s⁻¹) was provided horizontally, most of the nuclei in the neo1-1 mutant became relocated along the periclinal walls (Fig. 7A). Seemingly, the light absorption gradient of neo1 is lost under red light of such a high intensity; therefore, the directional movements of nuclei and chloroplasts in the wild type became random.

Glc and Suc Induce Nuclear Relocation Movement in the Dark in the Wild Type and the neo1 Mutant of *A. capillus-veneris*

We next examined whether nuclear relocation can be induced by the addition of sugars, as it was in chloroplasts. After treatment with 1% (w/v) Suc or Glc for 2 d in the dark, many nuclei in the wild type were found to be located along the periclinal walls (Fig. 8, A and B). As for chloroplast movement (Fig. 5), mannitol treatment again produced no effect. The relocation movement of the nuclei, as well as the chloroplasts, under the Suc or Glc treatment was also observed in the neo1-1 mutant (Supplemental Fig. S2).

**DISCUSSION**

Intracellular chloroplast photorelocation is a ubiquitous phenomenon in multicellular plants. In most plants, blue light is effective in this photosresponse (Haupt, 1982; Haupt and Scheuerlein, 1990), and phototropins have been identified as the blue light receptors (Wada et al., 2003; Wada and Suetsugu, 2004). Red light is also effective in triggering intracellular chloroplast photorelocation in some plants, such
as the fern *A. capillus-veneris* (Yatsuhashi et al., 1985), the moss *Physcomitrella patens* (Kadota et al., 2000), and the green alga *Mougeotia scaralis* (Haupt, 1959, 1982). From analyzing mutants lacking the red light response, neo1, a chimeric photoreceptor of phototropin and phytochrome, was demonstrated to be the red light receptor in *A. capillus-veneris* (Kawai et al., 2003). NEO genes have also been identified in the genome of *M. scaralis* (Suetsugu et al., 2005). One feature of phototropin- and neochrome-dependent chloroplast photorelocation is that the movement is directional and occurs either toward or away from the light. Chloroplasts move in the cell according to the light absorption gradient of these receptors located on the plasma membrane, the most stable structure in the cell for receiving directional information (Haupt, 1982). Cell biological analyses of *phot1* - and *phot2-GFP* expression have consistently revealed their location on the plasma membrane (Sakamoto and Briggs, 2002; Kong et al., 2006).

In this study, we revealed that chloroplasts of neo1 mutants in *A. capillus-veneris* and of *P. vittata*, an organism that lacks the neo1-dependent phenomenon (Kadota et al., 1989), retain the ability of light positioning under red light. The movement of chloroplasts is independent of the direction of red light. Under red light, either from above or from the side, chloroplasts became relocated toward the periclinal wall in the neo1 mutants. In the wild type of *A. capillus-veneris*, where neo1 is functional, chloroplasts accumulated on the periclinal or anticlinal cell walls, depending on the direction of the light. Thus, the relocation movement in the neo1 mutant is different from the neo1-dependent movement because it is not affected by the direction of the light. This can be easily understood considering that the photomovement was found to depend on photosynthesis, supplying the driving force for moving chloroplasts, and thus, directional information cannot be generated by the light direction.

In *A. capillus-veneris*, not only chloroplasts, but also nuclei are known to show photorelocation, depending on the direction of the light (Kagawa and Wada, 1993). The involvement of phototropins and neo1 in nuclear relocation was recently confirmed using neo1 and phototropin mutants (Tsuboi et al., 2007). The polarized light effect clearly reveals that the nuclei accumulate toward the region of higher light absorption of the photoreceptors neo1 and phototropin (Kagawa and Wada, 1995). The phototropin2-dependent photorelocation of nuclei in Arabidopsis mesophyll cells has also been reported (Iwabuchi et al., 2007). In the neo1 mutant, the nuclei also become relocated in the cells under red light. The positioning is again independent of the direction of the light and many of the nuclei located along the periclinal wall. The fact that the periclinal positioning of the nuclei is achieved in darkness by the addition of Glc or Suc strongly indicates the movement is also photosynthesis dependent, as in the case of chloroplasts.

The role of photosynthesis in chloroplast photorelocation was suggested in earlier studies, and several lines of evidence for this phenomenon have been presented (Haupt, 1982; Haupt and Scheuerlein, 1990; Takagi, 2003). However, the contribution of photosynthesis to the response is still not clear, because of the dominant regulation by phototropin and neo1 (phytochrome). Here, we clearly showed the involvement of photosynthesis in the photorelocation of chloroplasts and nuclei in the absence of neo1. Organelle movements in neo1 mutants are not dependent on the direction of the light. The nondirectional movements are likely to be the simple release of these organelles from the site of positioning in the dark. Chloroplasts and nuclei show a dark position in the dark; they position along the anticlinal walls or the bottom of the cell in the absence of light. How and why they accumulate in these specific parts of the cell in the dark remains to be determined. However, this study documented that the lack of photosynthesis and therefore, the lack of photosynthetic product is one of the factors regulating the positioning in the dark, because the chloroplasts and nuclei are liberated from their dark position even in the dark in the presence of Glc or Suc. The photosynthetic products seem to function as a signal for release from the site of dark positioning rather than as an energy source for movement. Wild-type cells showed neo1-dependent directional movement even under the conditions of photosynthesis inhibition with DCMU or DBMIB. Our findings indicate that the cells kept in the dark still have enough energy to drive chloroplasts.

In conclusion, using the neo1 mutants of *A. capillus-veneris* and wild type of *P. vittata*, this study clearly...
revealed the photosynthesis-dependent light positioning (or breakage of the dark position) of chloroplasts and nuclei, which is not obvious in the presence of neo1-dependent relocation movement.

MATERIALS AND METHODS

Plant Materials

Stock cultures of prothallia of Adiantum capillus-veneris of the wild type and neo1 mutant alleles (neo1-1, neo1-2, neo1-3, and neo1-4 according to the designation by Tsuoi et al., 2007; formerly rap2, rap27, rap7, and rap31, respectively) and of wild type of Pteris vittata were shattered to fragments by a razor blade with a few drops of sterilized water. neo1-1 mutant has a large deletion in 5’ region of the gene (Kanegae and Wada, 2006) and no mRNA expression is detected (T. Kanegae, personal communication). Each of neo1-2, neo1-3, and neo1-4 mutants has a point mutation that resulted in a premature stop codon or a missense mutation (Kanegae and Wada, 2006). The prothallial fragment suspension was spread evenly onto a cellophane sheet (55 mm in diameter) placed on the surface of White’s medium solidified with 0.5% agar (BA-30; Ina Food Industry Co., Ltd) in a petri dish (60 mm in diameter), and another cellophane sheet was overlaid on the prothallial fragments. These dishes were incubated under red light (4.1 μmol m⁻² s⁻¹) and horizontal red light (3.0 μmol m⁻² s⁻¹) for 20 h. The nuclei were stained with DAPI. Note that the nuclei in the neo1-1 mutant showed relocation to the periclinal walls both under vertical and horizontal irradiation. The arrows indicate the nuclei of the wild type positioned on the anticlinal wall, or the transitional region (between the periclinal wall and the anticlinal wall), which faces the incident horizontal red light. Pretreatment of the prothallia was performed as described in Figure 1. Bar = 50 μm. B, The bar graph shows the position of nuclei in the cells after irradiation with vertical red light (4.1 μmol m⁻² s⁻¹) and horizontal red light (3.0 μmol m⁻² s⁻¹). The intracellular location of nuclei was classified as periclinal (P), anticlinal (A), and intermediate (i.e. the position between the periclinal and the anticlinal positions [I]). The average and SE of each nuclear position was obtained from three independent experiments in which 20 cells were counted.

Light Sources

Red light was obtained by passing light from fluorescent tubes (FL40SD and FL10D; Toshiba Lighting and Technology Corp.) through a red plastic filter (Shinkolite A, #102; Mitsubishi Rayon Corp.) or by a LED light panel (ISL-150X150-RR and LDR2-74RD-LA, CCS Inc.). The wavelength range of the former light source was between 580 and 740 nm and that of the latter was between 610 and 710 nm.

Microbeam Irradiation of the Cell

Microbeam irradiation was performed on a custom-made microbeam irradiator as described previously (Kadota et al., 2000). Monochromatic red light was provided through an interference filter (Optical Coatings), with a peak at 633.2 nm and a half-band width of 32 nm. Neutral density filters (Inconel-coated quartz glasses, Fujitoku Corp.) were used to attenuate the fluence rate.
Nuclear Staining with DAPI

The prothallia were fixed with 3.7% (w/v) formaldehyde in 20 mM PIPES buffer (pH 7.0) for 30 min and then washed with the same buffer but containing 0.03% Triton X-100. The prothallia were first incubated for 1 h in the same buffer containing 100 ng mL\(^{-1}\) DAPI and 0.03% (v/v) Triton X-100, and then in the DAPI solution without Triton X-100 overnight. Prothallia stained with DAPI were observed under a fluorescence microscope (Axioplan with filter set, FS02, Zeiss) equipped with a digital color camera (FD-420 M, Flovel).

The position of the nucleus in the cell was classified as periclinal, anticlinal, and intermediate (i.e. the position between the periclinal and the anticlinal positions [I]). The average and s of each nuclear position was obtained from three independent experiments in each of which 20 cells were counted.

Photosynthesis Inhibitor Treatment

DCMU was dissolved in dimethyl sulfoxide at 100 mM as a stock solution and used at a concentration of 100 μM in White’s medium. The final concentration of dimethyl sulfoxide was 0.1% (v/v). DBMIB was dissolved in ethanol at 10 mM as a stock solution and used at a concentration of 0.4 mM in White’s medium. The final concentration of ethanol was 0.004% (v/v). The treatments were performed by transferring the prothallia onto the agar medium containing inhibitors. The prothallia were incubated for 1 to 2 h in the respective photosynthesis inhibitor medium before the experiments.

Treatment with Sugars

Suc, Glc, and mannitol were used at a concentration of 1% (w/v; 29, 56, and 56 mM, respectively) in White’s medium. The treatments were performed by transferring the prothallia onto the agar medium containing the sugar in the dark.

Supplemental Data

The following materials are available in the online version of this article.

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LITERATURE CITED


