PHOTOBIOLOGY AND PHOTOSYNTHESIS

Chloroplast relocation movement in the liverwort *Apopellia endiviifolia*

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Abstract  
Changes in the subcellular localisation of chloroplasts help optimise photosynthetic activity under different environmental conditions. In many plants, this movement is mediated by the blue-light photoreceptor phototropin. A model organism with simple phototropin signalling that allows clear observation of chloroplasts would facilitate the study of chloroplast relocation movement. Here, we examined this process in the simple thalloid liverwort *Apopellia endiviifolia*. Transverse sections of the thallus tissue showed uniformly developed chloroplasts and no air chambers; these characteristics enable clear observation of chloroplasts and analysis of their movements under a fluorescence stereomicroscope. At 22°C, the chloroplasts moved to the anticlinal walls of cells next to the neighbouring cells in the dark (dark-positioning response), whereas they moved towards weak light (accumulation response) and away from strong light (avoidance response). When the temperature was reduced to 5°C, the chloroplasts moved away from weak light (cold-avoidance response). Hence, both light- and temperature-dependent chloroplast relocation movements occur in *A. endiviifolia*. Notably, the accumulation, avoidance and cold-avoidance responses were induced under blue-light but not under red-light. These results suggest that phototropin is responsible for chloroplast relocation movement in *A. endiviifolia* and that the characteristics are similar to those in the model liverwort *Marchantia polymorpha*. RNA sequencing and Southern blot analysis identified a single copy of the PHOTOTROPIN gene in *A. endiviifolia*, indicating that a simple phototropin signalling pathway functions in *A. endiviifolia*. We conclude that *A. endiviifolia* has great potential as a model system for elucidating the mechanisms of chloroplast relocation movement.

1 | INTRODUCTION

Chloroplasts are organelles that perform photosynthesis to sustain plant growth and development. The intracellular locations of chloroplasts change in response to the ambient environments, a process that is important for the optimisation of light utilisation for photosynthesis. Various types of chloroplast relocation movement in response to environmental conditions (e.g., light and temperature) have been reported in model plants such as the thale cress *Arabidopsis thaliana*, the fern *Adiantum capillus-veneris*, the moss *Physcomitrella patens* and the...
liverwort *Marchantia polymorpha* (Kadota et al., 2000; Kagawa & Wada, 1994; Komatsu et al., 2014; Trojan & Gabrys, 1996). Under weak light, chloroplasts move to the periclinal wall of the cell to obtain sufficient light for photosynthesis, a phenomenon called the accumulation response (Gotoh et al., 2018; Wada, 2016). Conversely, chloroplasts move to the anticlinal wall under strong light to avoid photodamage, a phenomenon termed the avoidance response (Kasahara et al., 2002; Wada, 2016). Under low-temperature conditions, chloroplasts move away from weak light to the anticlinal wall of the cell to minimize low-temperature photoinhibition, which is referred to as the cold-avoidance response (Fujii et al., 2017; Kodama et al., 2008). Without light stimulation, chloroplasts move to the anticlinal walls of cells next to neighbouring cells in *A. capillus-veneris*, *P. patens* and *M. polymorpha* and to the bottoms of cells in *A. thaliana* (Komatsu et al., 2014; Sato et al., 2001; Suetsugu et al., 2005; Tsuboi & Wada, 2012). This phenomenon is called the dark-positioning response.

These model plants have been useful for studying chloroplast relocation movement. However, some of their photosynthetic organs, such as leaves and thalli, are morphologically complex. For instance, in *A. thaliana*, it is difficult to observe chloroplasts in palisade mesophyll cells in the second cell layer of the leaf because the first cell layer contains epidermal cells with undeveloped chloroplasts. In *M. polymorpha*, the thallus develops air chambers, and the gemma is composed of different types of meristematic cells such as thalli and rhizoids (Shimamura, 2016). The different types of cells and tissues present in these species make it difficult to observe internal chloroplast positioning clearly. By contrast, the protonemata and gametophyte prothallus of *P. patens* and *A. capillus-veneris* are morphologically simple, as both are composed of a single cell layer (Kadota et al., 2000; Kagawa & Wada, 1994; Kasahara et al., 2004). These photosynthetic organs allow clear and direct observation of chloroplasts, a useful characteristic for analysing the chloroplast relocation movement. Yet, the high genetic redundancy of *A. capillus-veneris* and *P. patens* is not beneficial for molecular genetic analysis (Kagawa et al., 2004; Li et al., 2015). Thus, there is a need for a model organism with morphologically simple photosynthetic organs and low genetic redundancy in which to analyse chloroplast relocation movement.

In many plants, chloroplast relocation movements are mediated by the blue-light (BL) photoreceptor phototropin (phot) (Jarillo et al., 2001; Kagawa et al., 2001; Sakai et al., 2001). Phot comprises the N-terminal light-sensing region containing two LOV (light, oxygen or voltage) domains that perceive BL and the C-terminal catalytic region containing a serine/threonine kinase domain (Christie, 2007). Multiple copies of *PHOT* are present in many plant species (Li et al., 2015); for instance, there are seven copies in *P. patens* (*PpPHOT1A-1, PpPHOT1A-2, PpPHOT1A-3, PpPHOT1B, PpPHOT2A, PpPHOT2C-1 and PpPHOT2C-2*), three copies in *A. capillus-veneris* (*AcPHOT1, AcPHOT2* and *AcNEOCHROME1*), a chimeric gene encoding phot with a phytochrome light-sensing domain) and two copies in *A. thaliana* (*AtPHOT1* and *AtPHOT2*) (Li et al., 2015; Sakai et al., 2001; Tsuboi et al., 2007). These *PHOT* genes react to different light conditions, resulting in a complex signalling pathway for chloroplast relocation movement. For example, in *A. thaliana*, the accumulation response induced by weak light is mediated by both *AtPHOT1* and *AtPHOT2*, whereas the avoidance response induced by strong light is mediated by only *AtPHOT2* (Jarillo et al., 2001; Kagawa et al., 2001; Sakai et al., 2001). This complexity hampers our elucidation of these signalling pathways, as it necessitates silencing other gene copies to study the gene of interest. Therefore, a model plant with a single copy of *PHOT* would be useful. Indeed, *M. polymorpha* is a model liverwort that has only a single copy of *PHOT* (M*PPHOT*). M*PPHOT* mediates chloroplast accumulation and the avoidance, cold-avoidance and dark-positioning responses in *M. polymorpha* (Fujii et al., 2017; Komatsu et al., 2014). However, as mentioned above, it is difficult to observe chloroplast positioning in the *M. polymorpha* thallus and gemma due to the presence of air chambers within the thallus and different types of cells in the gemma (Shimamura, 2016).

In this study, to find a model species that would allow clear observation of chloroplasts that contain only a single copy of the *PHOT* gene, we focused on another liverwort species, *Apopellia endiviifolia* (homotypic synonym: *Pellia endiviifolia*). *A. endiviifolia* is a dioecious liverwort that can be found in moist and shady habitats (Alaba et al., 2015; Sierocka et al., 2011). This species, from a lineage of simple thalloid liverworts, belongs to the Jungermanniopsida class in the Marchantiophyta division (He-Nygrén et al., 2006; Parzych et al., 2018). The simple thallus of *A. endiviifolia* has uniformly developed chloroplasts and no air chambers (He-Nygrén et al., 2006; Shimamura, 2016). Furthermore, because the liverwort species *Pellia neesiana* and *M. polymorpha*, which are closely related to *A. endiviifolia*, each contain a single *PHOT* gene (Komatsu et al., 2014; Li et al., 2015), we expected that *A. endiviifolia* might also contain a single copy of this gene. Here, we describe our assessment of chloroplast relocation movement in this species and the number of *PHOT* copies in its genome. Our results highlight the potential of *A. endiviifolia* as a model organism for chloroplast relocation movement analysis.

## 2 | MATERIALS AND METHODS

### 2.1 | Plant material and growth conditions

*Apopellia endiviifolia* thalli were isolated from a field in Utsunomiya City, Tochigi, Japan and named the Utsunomiya male 1 (UTm1) strain. The thalli were sterilised with 3% H₂O₂ and axenically propagated on mineral growth medium (Sierocka et al., 2011). To prepare 500 mL of growth medium, 0.5 g (0.1%) of *Hyponex®* was combined with 250 μL (0.05%) of Gamborg's B5 medium, 5 g (1%) of sucrose (Wako Pure Chemical Industries) and 4 g (0.8%) of agar powder (BOP; SSK Sales) and adjusted to pH 7. Detached thallus (approximately 8 × 8 mm) was subcultured on the medium under 60 μmol photons m⁻² s⁻¹ of continuous white light (WL) (FL405W, NEC) in a culture room at 22°C. One- to two-month-old subcultured thallus tissue was used for the chloroplast relocation movement experiment. Gemmae of *M. polymorpha* (the Tak-1 strain) were cultured as described previously (Ogasawara et al., 2013). One-month-old
2.2 | Histological observation

One-month-old subcultured *A. endiviifolia* and *M. polymorpha* thalli were collected and subjected to paraffin fixation and tissue embedding. Specifically, the samples were fixed in FAA (5% formaldehyde, 5% acetic acid and 50% ethanol) and embedded in paraffin (Paraplast® Plus, Leica Biosystems) in moulds to form blocks. The blocks were sectioned transversely with an RM2245 rotary microtome (Leica Biosystems) to 10-μm thickness and mounted on glass slides. The sectioned tissues were dewaxed with Histoclear (National Diagnostics) and rehydrated through an ethanol series (100, 90, 70 and 50%). The tissues were stained with Safranin O stain (0.1% in 50% ethanol) and Alcian Blue counterstain (0.1% in water) (Buda et al., 2013). After the staining process, the tissues were dehydrated in an ethanol series (50, 70, 95 and 100%) and submerged in Histoclear (National Diagnostics) for 20 min. The dehydrated tissues were observed under a BX60 light microscope (Olympus), and images (1300 x 1024 pixels) were captured with a DP72 digital camera (Olympus).

2.3 | Extraction of genomic DNA and total RNA

Genomic DNA and total RNA were extracted from 1-month-old *A. endiviifolia* thalli. Genomic DNA was extracted from the samples using the cetyltrimethylammonium bromide (CTAB) method as described previously (Tsuboyama & Kodama, 2014). After measuring the DNA concentration with a Nanodrop One Spectrophotometer (Thermo Fisher Scientific), the DNA was stored at –80°C until use. Total RNA was extracted from the samples with Invitrogen™ Trizol™ Reagent (Thermo Fisher Scientific) according to the manufacturer’s protocol (Chomczynski, 1993). The RNA pellets were purified with a RNeasy Plant Mini kit (Qiagen®). The concentration of the RNA eluate was measured with a Nanodrop One Spectrophotometer. The RNA was also checked by agarose gel electrophoresis with DynaMarker RNA High for Easy Electrophoresis (BioDynamics Laboratory). The total RNA was stored at –80°C until use.

2.4 | RNA sequencing

The RNA was used to construct mRNA-Seq libraries with a KAPA Stranded mRNA-Seq Kit (Illumina® Platforms). The size of the DNA fragments was verified using an Agilent 2100 bioanalyzer (Agilent Technologies) with an Agilent DNA 1000 Kit (Agilent Technologies). The concentration of the DNA library was quantified using a LightCycler 96 (Roche) with KAPA Library Quantification Kits (Illumina® Platforms). The concentration of the library was adjusted to 4 nM using elution buffer for subsequent sequencing. Next-generation sequencing (NGS) of the library was performed using MiSeq Reagent Kit v3 (150 cycles; Illumina® Platforms). The obtained raw reads were quality trimmed with Trimmomatic version 0.39 (Bolger et al., 2014). De novo transcript sequence reconstruction was performed using Trinity version 2.8.5 (Haas et al., 2013). The RNA-Seq reads of *A. endiviifolia* are available from the DDBJ Sequence Read Archive (DRA) under the accession number of DRA011765.

2.5 | Identification of male and female *A. endiviifolia* gametophytes

Primer sets used for PCR to amplify DNA fragments for genes expressed in male (PenB_Tua1 and PenB_Raba1/11) and female (PenB_MT3, PenB_CYSP and PenB_MT3) gametophytes were generated based on previous reports (Sierocka et al., 2011, 2014; Table S1). The *A. endiviifolia* Actin gene was used as a positive control (PenB_ACT1; accession number: DQ100290). Complementary DNA (cDNA) and genomic DNA of *A. endiviifolia* were subjected to PCR amplification with PrimeSTAR® Max DNA Polymerase (Takara Bio). Note that the cDNA was produced from RNA extracted from *A. endiviifolia* by reverse-transcription PCR (RT-PCR) using ReverTra Ace® (Toyobo); the genomic DNA was extracted from *A. endiviifolia* using the CTAB protocol (Tsuboyama & Kodama, 2014). The sizes of the amplified DNA fragments were verified by 2% TAE agarose gel electrophoresis with a 100-base pair DNA ladder (NEB3231; New England Biolabs).

2.6 | Light and temperature treatments

Five pieces of *A. endiviifolia* thalli were cut, placed flat onto the surface of a plate of culture medium, and incubated in the dark in a temperature-controlled incubator (IJ100 or IJ101, Yamato Scientific) at 22°C for 24 h. The dark-adapted thalli were grown under different light and temperature conditions for 24 h prior to microscopy. For light intensity and light quality tests, the thallus pieces were grown under white, blue and red light-emitting diodes (LEDs) (white LED-H2WWW, blue LED-BB45, red LED-RHB; CCS) at 22°C. The light spectrum of the LEDs was measured with a C-7000 spectrometer (Sekonic). The light intensities of each coloured LED were adjusted using an LI-250A light metre (LI-COR Bioscience). The low-temperature test, the thallus pieces were incubated at 5°C under weak WL (60 μmol photons m⁻² s⁻¹). For dark treatment, thalli were grown in an incubator with no supplemental light.

2.7 | Observation and evaluation of chloroplast positioning using thalli

The thallus pieces were observed under an MZ16F fluorescence stereomicroscope (Leica Microsystems). To observe chlorophyll autofluorescence of the chloroplasts, a 480/40-nm excitation filter and LP510-nm barrier filter were used (Ogasawara et al., 2013). RGB
digital images (800 × 600 pixels) were captured with a DP73 digital camera (Olympus). Each captured image was analysed with ImageJ software (http://rsb.info.nih.gov/ij/) (Rasband 1997–2018). The P/A ratio evaluation method was used to quantify the chloroplast positioning in the cells (Kodama et al., 2008). The brightness ratio of chlorophyll fluorescence from chloroplasts along the periclinal (P) and anticlinal (A) cell walls was calculated (Kodama et al., 2008). Fluorescent intensities from 30 points (42 μm each) and 30 areas (196 μm² each) along the periclinal and anticlinal cell walls were measured. The average P/A ratio with one standard deviation was calculated from three replicated experiments. All statistical analyses in this study were performed using Tukey’s HSD test. Furthermore, chlorophyll autofluorescence from chloroplasts from A. endiviifolia and M. polymorpha thalli was observed under an SP8X confocal laser scanning microscope (Leica Microsystems) with excitation at 488 nm and emission at 641 to 721 nm.

2.8 | Observation of chloroplast relocation movement under red-light microbeam irradiation

The temperature-regulated microscope with a BL microbeam irradiation system (Fuji et al., 2017; Tanaka et al., 2017) was modified to use a red-light (RL) LED microbeam (632 nm, FOLS-01-632 nm-600 μm, P. Photonics). Under the temperature-regulated microscope, thallus cells were maintained at 22°C and irradiated with a RL microbeam (approximately 10 μm in diameter) for 90 min. Two different intensities of RL microbeam (9 and 227 μmol photons m⁻² s⁻¹) were used to irradiate the A. endiviifolia cell. Images of chloroplast positioning in the cell before and after 90 min of RL microbeam irradiation were captured.

2.9 | Time-lapse observation of chloroplast relocation movement under BL microbeam irradiation

A piece of detached thallus was placed under a temperature-regulated microscope with a blue LED microbeam irradiation system and a charge-coupled device camera (Fujii et al., 2017, Tanaka et al., 2017). Three types of the chloroplast relocation movement were observed: the accumulation response (weak BL at 22°C), avoidance response (strong BL at 22°C), and cold-avoidance response (weak BL at 5°C). Under the temperature-regulated microscope, a thallus cell was irradiated by the BL microbeam (approximately 10 μm in diameter). For the accumulation response (weak BL: 30 μmol photons m⁻² s⁻¹) and avoidance response (strong BL: 430 μmol photons m⁻² s⁻¹), 90 images were taken every minute (for a total of 90 min) (Sakata et al., 2019). For the cold-avoidance response (weak BL: 60 μmol photons m⁻² s⁻¹), 138 images were taken every 5 min (for a total of 690 min). The images were compiled to form a time-lapse video with Fiji software (Schindelin et al., 2012). Five randomly selected chloroplasts were tracked in each time-lapse video and analysed with Fiji software to determine the distances they moved from the microbeam against time. The measurement data were analysed, and the correlation coefficient (r) between the average distance the chloroplasts moved from the microbeam and time was calculated with Microsoft Excel.

2.10 | Cloning and analysis of the PHOT gene from A. endiviifolia

Using the MpPHOT (accession no. AHZ63885) sequence as a reference, the PHOT gene sequence from A. endiviifolia (AePHOT) was identified from the RNA sequencing data (accession no. DRA011765). Multiple alignments between the nucleotide (AePHOT and MpPHOT) sequences and protein (AePHOT and MpPHOT) sequences were performed with ClustalW. Based on the multiple alignments, we designed a primer set (attB1_AePHOT_F and attB2_AePHOT_R) to amplify and clone the AePHOT sequence of A. endiviifolia (Table S1). The coding sequence of AePHOT was amplified from a cDNA library of A. endiviifolia that was transcribed from the extracted RNA by RT-PCR with ReverTra Ace® (Toyobo). AePHOT was amplified by PCR with the primers attB1_AePHOT_F and attB2_AePHOT_R (Table S1) and PrimeSTAR® Max DNA Polymerase (Takara Bio). The PCR product was extracted, purified and cloned into the pDONR™/ZEO vector using Gateway cloning technology (Invitrogen) to produce the pDONR™/ZEO-AePHOT plasmid; AePHOT in the plasmid was confirmed by sequencing. The verified sequence of AePHOT was subjected to an alignment test with the Basic Local Alignment Search Tool (BLAST). The results were used to construct a maximum likelihood phylogenetic tree using Mega7 and to re-test the multiple alignments with ClustalW (Kumar et al., 2016). The aligned sequences were shaded using ESPript 3.0 (Robert & Gouet, 2014).

2.11 | Southern blot analysis

One milligramme of genomic DNA from A. endiviifolia was digested with the restriction enzyme EcoRI-HF or EcoRV-HF in 10× CutSmart Buffer (NEB) at 37°C overnight (Tsukuyama & Kodama, 2014). The digested DNA was subjected to electrophoresis in 1% agarose gel with a 1-kb DNA ladder (NEB3232; New England Biolabs). Following electrophoresis, the DNA was transferred from the gel to a Hybond-N nylon membrane (GE Healthcare) using a vacuum transfer device (Bio Craft). Probe labelling and hybridisation were performed with AlkPhos Direct Labelling Reagents kits (GE Healthcare). A genomic DNA fragment (584 bp) of AePHOT between the LOV2 and kinase regions was amplified by PCR and used as a probe (Figure S1; Table S1) for labelling in the hybridisation of the membrane at 60°C. CDP-Star Detection Reagent (GE Healthcare) was used to detect the hybridised probes based on chemiluminescence.

2.12 | Quantitative RT-PCR

Apopellia endiviifolia thalli were incubated under four conditions: dark, weak WL (60 μmol photons m⁻² s⁻¹), strong WL (200 μmol photons m⁻² s⁻¹) and low temperature (5°C) with weak WL (60 μmol photons...
m⁻² s⁻¹). Four plates of 1-month-old pure A. endiviifolia cultures were pre-incubated in the dark at 22°C for 24 h, followed by incubation under the four conditions separately for another 24 h. After incubation, RNA was extracted from the cultures as described above. The RNA was reverse transcribed to cDNA using ReverTra Ace™ qPCR RT Master Mix with gRNA Remover (Toyobo). A primer set (AePHOT_F and AePHOT_R) was designed to amplify a 111-bp fragment of AePHOT (Table S1). The A. endiviifolia Actin gene (PenB_ACT1; accession number: DQ100290) was used as the reference gene. PCR was conducted in a final volume of 20 μL containing FastStart Universal SYBR Green Master (Roxy) (Roche), cDNA and gene-specific primers (10 μM) in the LightCycler®96 System (Roche) under the following cycling conditions: pre-incubation at 95°C for 10 min, following 45 cycles of 95°C for 15 s, and 60°C for 1 min. Following PCR, high resolution melting peak analysis was performed. The results were analysed with LightCycler®96 Software 1.1 (Roche) and Microsoft Excel. The Cq values of all transcripts were normalised to PenB_ACT1, and the relative AePHOT transcript levels (fold change values) were calculated using the comparative ΔΔCq method (Regier & Frey, 2010). The average of three technical replicates was obtained for each treatment, and three biological replicates were performed.

3 | RESULTS

3.1 | Identification of male and female A. endiviifolia gametophytes

Apopella endiviifolia thallus was isolated from the field, sterilised and axenically propagated. As A. endiviifolia is a dioecious liverwort, we identified the genders of the isolated A. endiviifolia gametophytes by PCR with specific primer sets to amplify genes that are specifically expressed in male or female gametophytes (Sierocka et al., 2011). PCR was conducted in a final volume of 20 μL containing FastStart Universal SYBR Green Master (Roxy) (Roche), cDNA and gene-specific primers (10 μM) in the LightCycler®96 System (Roche) under the following cycling conditions: pre-incubation at 95°C for 10 min, following 45 cycles of 95°C for 15 s, and 60°C for 1 min. Following PCR, high resolution melting peak analysis was performed. The results were analysed with LightCycler®96 Software 1.1 (Roche) and Microsoft Excel. The Cq values of all transcripts were normalised to PenB_ACT1, and the relative AePHOT transcript levels (fold change values) were calculated using the comparative ΔΔCq method (Regier & Frey, 2010). The average of three technical replicates was obtained for each treatment, and three biological replicates were performed.

3.2 | Morphological characteristics of A. endiviifolia thallus

To assess the differences between the simple and complex thallus tissues of A. endiviifolia (Jungernanniopsida) versus M. polymorpha (Marchantiopsida), we fixed and transversely sectioned detached thalli of these liverworts and compared the characteristics of the cell layers (Figure 1A,B). The cell walls were stained red with Safranin O, and the intracellular contents were stained blue with Alcian blue counterstain. In transverse sections of A. endiviifolia thallus, the chloroplasts developed uniformly in the parenchyma cell layer, and no air chambers were present above the parenchyma cell layer (Figure 1C,E). By contrast, in transverse sections of M. polymorpha thallus, the chloroplasts developed unevenly in the parenchyma cell layer, where more chloroplasts were developed in the upper parenchyma layer than the lower parenchyma layer. Also, air chambers were developed above the parenchyma cell layer (Figure 1D,F). These characteristics of A. endiviifolia and M. polymorpha are consistent with previous reports (Alaba et al., 2015; He-Nygrén et al., 2006; Shimamura, 2016; Sierocka et al., 2011, 2014). Due to the absence of air chambers in the thalloid tissue of A. endiviifolia, uniformly developed chloroplasts could be clearly observed based on chlorophyll fluorescence, unlike the chloroplasts in the thalloid tissue of M. polymorpha (Figure 1G,H). These observations distinguish the characteristics of the simple thalloid of A. endiviifolia from the complex thalloid of M. polymorpha and highlight the capacity for clear observation of chloroplasts in A. endiviifolia.

3.3 | Chloroplast relocation movement in A. endiviifolia

The ease of observation of A. endiviifolia chloroplasts allowed us to fully assess chloroplast relocation movement. We analysed light- and temperature-dependent chloroplast relocation movements: the dark-positioning response, the accumulation response, the avoidance response and the cold-avoidance response. We quantified chloroplast positioning using the P/A ratio method, which is based on the ratio of chlorophyll fluorescent intensities from the chloroplasts near the periclinal wall (P) to the anticlinal wall (A) (Kodama et al., 2008). Briefly, more chloroplasts accumulated near the periclinal wall, resulting in a high chlorophyll fluorescence intensity from the periclinal wall to the anticlinal wall and, thus, a high P/A ratio.

We observed the dark-positioning response of A. endiviifolia following incubation in the dark for 24, 48 and 72 h at 22°C (Figure 2A). Initially, chloroplasts accumulated at the periclinal wall under weak WL (60 μmol photons m⁻² s⁻¹) with a P/A ratio of 1.3 prior to incubation in the dark (Figure 2A,B). When we incubated the thalli in the dark, the chloroplasts moved to the edges of the cells bordering neighbouring cells in the first 24 h of dark treatment, significantly decreasing the P/A ratio to 0.8 (Figure 2A,B). During 48 and 72 h of incubation in the dark, the chloroplasts continually shifted around the anticlinal area, resulting in a P/A ratio of 0.8 and 0.9, respectively (Figure 2A,B). During this response, chloroplasts were not observed near the edge of the thallus, as the outermost anticlinal cell wall is not bordered by neighbouring cells (Figure 2A and S3). This chloroplast relocation pattern is consistent with the previously reported dark-positioning responses in A. capillus-veneris and M. polymorpha (Komatsu et al., 2014; Tsuboi & Wada, 2012).
To determine the light intensities that induce the accumulation and avoidance responses in *A. endiviifolia*, we irradiated dark-adapted thalli with 10–210 μmol photons m⁻² s⁻¹ of white LEDs (Figure S4). The transition phase of the accumulation-to-avoidance responses was observed when the light intensity was switched from 110–120 μmol photons m⁻² s⁻¹ of WL (Figure 2C,D and S4). The chloroplasts localised along the periclinal cell wall under light intensities of up to 110 μmol photons m⁻² s⁻¹, indicating the accumulation response (accumulation towards weak light) (Figure 2C). The average P/A ratios during this response were high, ranging from 1.2 to 1.8 (Figure 2D and S4). Under light intensities of 120 μmol photons m⁻² s⁻¹ and above, the chloroplasts moved to the anticlinal position, indicating the avoidance response (avoidance from strong light) (Figure 2C). This chloroplast response resulted in low average P/A ratios ranging from 0.3 to 0.7, which were significantly different from the P/A ratios detected during the accumulation response (Figure 2D and S4). Based on the observed accumulation and avoidance
responses of *A. endiviifolia*, we defined weak WL as that from 10 to 110 μmol photons m⁻² s⁻¹ and strong WL as that greater than 120 μmol photons m⁻² s⁻¹ under our experimental conditions.

The cold-avoidance response in *A. endiviifolia* was observed after 24 h of incubation under weak light at low temperature (5°C). The chloroplasts moved to the anticlinal position even under weak light.
FIGURE 3  Blue-light-dependent chloroplast relocation movement in A. endiviifolia. Detached thalli were incubated under 10–70 μmol photons m⁻² s⁻¹ of blue- and red-light irradiation at 22 °C for 24 h. (A) Quantitative analysis of chloroplast positioning resulting from blue-light irradiation based on the average P/A ratio from three replicates. (B) Quantitative analysis of chloroplast positioning resulting from red-light irradiation based on the average P/A ratio from three replicates. Asterisks above the error bars indicate significant difference (Tukey’s HSD test; p < 0.05). Average P/A ratio of the dark-adapted thallus before treatment can be referred to the average P/A ratio of dark-positioning response of 24 h in Figure 2B.

FIGURE 4  Time-lapse observation of chloroplast relocation movement in A. endiviifolia under blue-light microbeam irradiation. (A) The accumulation response of chloroplasts towards the weak BL microbeam-irradiated spot in the cell at 22 °C. (B,C) Quantitative analysis of the chloroplast movement in (A) calculated from the five numbered chloroplasts; the average result is shown in the graph. (D) The avoidance response of the chloroplasts away from the strong BL microbeam-irradiated spot in the cell at 22 °C. (E,F) Quantitative analysis of the chloroplast movement in (D) calculated from the five numbered chloroplasts; the average result is shown in the graph. (G) The cold-avoidance response of the chloroplasts away from the weak BL microbeam-irradiated spot in the cell at 5 °C. (H,I) Quantitative analysis of the chloroplast movement in (G) calculated from the five numbered chloroplasts; the average result is shown in the graph. Scale bars, 1 μm. Error bars represent standard deviation; r represents correlation between the average distances the chloroplasts moved and the time of irradiation. Black circle indicates the irradiated BL microbeam-irradiated spot (A, D, E, G and H).
(60 μmol photons m⁻² s⁻¹) at 5°C (Figure 2E). The average P/A ratio was 0.3, which is significantly different from the P/A ratio observed during the accumulation response at 22°C (Figure 2F). In conclusion, all the expected types of chloroplast relocation movements were successfully observed in A. endiviifolia.

3.4 | Blue-light-dependent chloroplast relocation movement in A. endiviifolia

We tested the accumulation response, avoidance response and cold-avoidance response of A. endiviifolia using WL composed of a combination of different light spectra (Figure S5). To determine the type of photoreceptor that likely induces the chloroplast relocation movements in A. endiviifolia, we incubated dark-adapted thalli under different intensities of BL or RL. Like WL, BL also induced chloroplast accumulation and the avoidance response (Figures 2C and 3A). The accumulation response was induced at BL intensities of 10–30 μmol photons m⁻² s⁻¹, resulting in a high average P/A ratio of 1.6 at 10 and 20 μmol photons m⁻² s⁻¹ of BL (Figure 3A). By contrast, the avoidance response was induced at BL intensities of 40–70 μmol photons m⁻² s⁻¹ (Figure 3A). Under RL, inconsistent chloroplast relocation movements were observed, resulting in fluctuating average P/A ratios with increasing light intensities (Figure 3B). To further explore this response, we treated the thalli with RL microbeam irradiation, but no chloroplast relocation movement was observed (Figure S6). These results indicate that chloroplast relocation movement in A. endiviifolia is dependent on BL, but not RL.

To validate the BL-dependent chloroplast relocation movement in A. endiviifolia, we took time-lapse videos of the chloroplast movement induced by a BL microbeam. The accumulation response was induced

![Diagram](image-url)

**FIGURE 5** The single-copy PHOTOTROPIN gene (AePHOT) in A. endiviifolia. (A) Schematic illustration of the AePHOT protein. aa, amino acids. (B) Maximum likelihood phylogenetic tree. Phylogenetic relationships of A. endiviifolia (red border) with 47 PHOT genes from non-vascular and vascular plants were assembled from an alignment of AePHOT sequences in BLAST. The bootstrap values are indicated at the nodes. Bar represents 0.10 substitutions in each site. (C) Southern blot analysis of AePHOT. A single band was observed in each blot of EcoRI- or EcoRV-digested DNA products. The blot was hybridised to a probe of the AePHOT genomic fragment between the LOV2 and kinase regions (Figure S1)
using weak BL microbeam irradiation (30 μmol photons m$^{-2}$ s$^{-1}$), as the chloroplasts moved towards the microbeam spot (Figure 4A). To quantify chloroplast movement, we calculated the average distance of five randomly selected chloroplasts from the centre of the microbeam spot. A strong negative correlation was observed between the average distance and the irradiation period ($r = -0.97$) (Figure 4B,C). On the other hand, strong BL microbeam irradiation (430 μmol photons m$^{-2}$ s$^{-1}$) stimulated the chloroplasts to move away from the microbeam spot (Figure 4D), indicating the avoidance response. A strong positive correlation was observed between the average distance and the irradiation period ($r = 0.95$) (Figure 4E,F). Based on quantitative analysis of the accumulation and avoidance responses in *A. endiviifolia*, the chloroplasts reacted strongly during the first 60 min of light treatment and moved steadily during the subsequent 30 min of treatment (Figure 4C,F). To analyse the BL dependency of the cold-avoidance response, we treated thalli with weak BL microbeam irradiation (30 μmol photons m$^{-2}$ s$^{-1}$) at 5°C. The chloroplasts moved away from the weak BL microbeam (Figure 4G). A positive correlation was detected between the average distance and the irradiation period ($r = 0.95$) (Figure 4H,I). As previously reported for *A. capillus-veneris* and *M. polymorpha*, chloroplast movement at this temperature was slower than the dark-positioning, accumulation and avoidance responses of chloroplasts at standard temperatures (Figure 4C,F,I), and the chloroplasts aggregated during the cold-avoidance response (Figure S7) (Fujii et al., 2017; Kodama et al., 2008; Tanaka et al., 2017).

These results indicate that the accumulation, avoidance and cold-avoidance responses of *A. endiviifolia* chloroplasts are induced in a BL-dependent manner, suggesting that chloroplast relocation movement is mediated by a BL photoreceptor (i.e., phot).

### 3.6 Relative quantification of *AePHOT* expression level under different light and temperature conditions

To explore the expression pattern of *AePHOT* during the dark-positioning, accumulation, avoidance and cold-avoidance responses, we performed quantitative RT-PCR analysis of *A. endiviifolia* samples incubated under four conditions: the dark at 22°C, weak WL (60 μmol photons m$^{-2}$ s$^{-1}$) at 22°C, strong WL (200 μmol photons m$^{-2}$ s$^{-1}$) at 22°C and weak WL (60 μmol photons m$^{-2}$ s$^{-1}$) at 5°C (Figure 6). The lowest expression level of *AePHOT* was detected in the dark (Figure 6). *AePHOT* was expressed at higher levels in the light at 22°C and 5°C, and its expression levels were highest under weak WL at

![Figure 6](image-url)  
**Figure 6** Quantitative RT-PCR analysis of *AePHOT* from *A. endiviifolia*. Relative expression levels (fold change values) of *AePHOT* in the dark, weak white light (WL), strong WL, and low temperature (5°C) with weak WL. All transcript levels were normalised against PenB_ACT1. Error bars represent standard deviation.

In the maximum likelihood phylogenetic tree, *A. endiviifolia* was clearly separated from the vascular plants and grouped with the liverwort (Marchantiophyta) family, which is characterised by a single-copy *PHOT* gene (Figure 5B) (Komatsu et al., 2014; Li et al., 2015). The phylogenetic analysis also showed that *A. endiviifolia* shares the same clade with the closely related species *Pellia neesiana*, which also contains a single-copy *PHOT* gene (Figure 5B) (Li et al., 2015). These results suggest that *A. endiviifolia* contains only one *PHOT* gene. To confirm the number of *AePHOT* genes, we performed Southern blot analysis using genomic DNA from *A. endiviifolia* digested by EcoRI or EcoRV. Only a single band was detected in blots generated using both types of digested genomic DNA, indicating that a single copy of the *AePHOT* gene is present in *A. endiviifolia* (Figure 5C). Given that it contains a single copy of *AePHOT*, we conclude that *A. endiviifolia* has a simple phototropin signalling pathway that regulates chloroplast relocation movement.

### 3.5 *A. endiviifolia* contains a single copy of PHOTOTROPIN

To identify *PHOT* genes in *A. endiviifolia* (*AePHOT*), we performed next-generation sequencing of mRNA from thalli. Only a single copy of the *AePHOT* gene was identified in the RNA sequencing data from *A. endiviifolia* when we compared the data with sequence information for the *M. polymorpha* gene of *M. polymorpha* (Figure 5A). Based on the RNA sequencing data, we cloned the coding sequence of *AePHOT* and performed multiple sequence alignment between *AePHOT* and *MpPHOT* (nucleotide sequences) and *AePHOT* and *MpPHOT* (protein sequences) (Figures S8 and S9) (Komatsu et al., 2014). The *AePHOT* and *MpPHOT* sequences share 73% similarity, and the *AePHOT* and *MpPHOT* sequences share 71% similarity. Based on sequence alignment with *MpPHOT*, the LOV1, LOV2 and kinase domains were also identified in *AePHOT* (Figures S8 and S9). Notably, the cysteine residues for flavin mononucleotide (FMN) binding in the LOV domains of *MpPHOT* were found in the LOV domains of *AePHOT* (Figure S9) (Fujii et al., 2017). In the kinase domain of *ApPHOT*, the asparagine residue for functionality of serine/threonine kinase domain of *MpPHOT* was also found (Figure S9) (Komatsu et al., 2014). Upon the similarity of the functional amino acids between *AePHOT* and *MpPHOT*, the function of the LOV and kinase domains were believed to be conserved in *AePHOT*.
22°C (Figure 6). The expression levels of AePHOT were similar under strong WL at 22°C and weak WL at 5°C (Figure 6). These results suggest that AePHOT expression increases in response to light irradiation and is not dependent on temperature. Overall, these results are consistent with the finding that the expression of A. thaliana PHOT1 and PHOT2 increases in response to light irradiation and that the expression of M. polymorpha PHOT is unchanged in response to cold treatment (Fujii et al., 2020; Jarillo et al., 2001; Kagawa et al., 2001; Labuz et al., 2015).

DISCUSSION

In this study, we focused on the liverwort species A. endiviifolia, which possesses a simple thallus with no developed air chambers and a single-copy PHOT gene. Previous studies have focused on phylogenetic analysis and gender identification in this liverwort, but not on physio-morphological analysis (Alaba et al., 2015; Sierocka et al., 2011, 2014). Therefore, this is the first report describing the physiological functions in A. endiviifolia, including its simple thallus morphology and chloroplast relocation movement related to photosynthetic optimisation.

Plant species with chloroplasts that can be clearly observed and have a single-copy PHOT signalling pathway are preferable for studying chloroplast relocation movement, but not all plant species possess both of these characteristics. The hornwort Anthoceros punctatus was reported to contain a single-copy PHOT gene, but it also contains a PHOT derivative gene (NEOCHROME gene) (Li et al., 2014, 2015). The NEOCHROME gene of hornwort comprises a fusion of the PHOT and PHYCHROME sequences, pointing to its involvement in chloroplast relocation movement (Li et al., 2014). Therefore, the analysis of PHOT in hornwort is complicated by the presence of NEOCHROME, as also been observed in a study of A. capillus-veneris (Tsuboi et al., 2007). Moreover, A. punctatus cells contain only one large chloroplast with a pyrenoid, so its light-induced chloroplast relocation movement can hardly be observed (Li et al., 2014; Vaughn et al., 1990). Furthermore, although the liverwort M. polymorpha contains only a single-copy PHOT gene without a derivative gene, air bubbles are produced from the air chambers in its complex thallus that disrupt the observation of chloroplasts (Komatsu et al., 2014; Li et al., 2015; Shimamura, 2016). These types of disruptions can be avoided by using photosynthetic organs that allow a clear view of chloroplasts, such as the single-cell-layer tissue of the moss P. patens and the simple thallus of the liverwort A. endiviifolia (Figure 1E) (Buda et al., 2013). However, P. patens has high genetic redundancy, with seven PHOT genes (PpPHOT1A-1, PpPHOT1A-2, PpPHOT1A-3, PpPHOT1B, PpPHOT2A, PpPHOT2C-1 and PpPHOT2C-2) that function in the chloroplast relocation movement signalling pathway (Kasahara et al., 2004; Li et al., 2015). Notably, we identified only one copy of the PHOT gene in A. endiviifolia, indicating that a simple phototropin signalling pathway is involved in chloroplast relocation movement in this species (Figure 5A,C). Hence, A. endiviifolia exhibits both beneficial characteristics for chloroplast relocation movement analysis.

Previous studies observed different chloroplast migration patterns during the avoidance and cold-avoidance responses of M. polymorpha (Tanaka et al., 2017). During the avoidance response, the chloroplasts spread out and move to the nearest anticlinal edge of the cell (Tanaka et al., 2017). By contrast, during the cold-avoidance response, chloroplasts aggregate into groups and then move to all sides of the anticlinal wall (Tanaka et al., 2017). Here, we observed similar chloroplast movement patterns during the avoidance and cold-avoidance responses in A. endiviifolia through time-lapse observation (Figure S7). Notably, chloroplast aggregation during the cold-avoidance response was also observed in the fern A. capillus-veneris (Kodama et al., 2008). Furthermore, in A. endiviifolia, chloroplasts moved to the anticlinal side of the cell during the dark-positioning response and accumulated at the side of the anticlinal wall next to the neighbouring cells (Figure 2A). This chloroplast relocation pattern is also consistent with the previously reported dark-positioning response in the fern A. capillus-veneris (Tsuboi & Wada, 2012). Overall, the patterns of all chloroplast relocation movements observed in A. endiviifolia match those reported in the model plants A. thaliana, A. capillus-veneris and M. polymorpha, indicating that these processes were highly conserved throughout the evolution from non-vascular to vascular plants.

Based on our observation, one of the photoreceptors responsible for inducing chloroplast relocation movements in A. endiviifolia is the BL photoreceptor phot. A previous study reported that the presence of a single PHOT sequence is ancestral to the duplication of PHOT in vascular plants (seed plants, lycophytes and ferns) and non-vascular plants (mosses and hornworts) (Li et al., 2015). This evolutionary pattern highlights the significant inheritance of the function of a single-copy PHOT in both liverworts and land plants during plant evolution (Alaba et al., 2015; Li et al., 2015). Furthermore, PHOT1 and PHOT2 expressions were enhanced in A. thaliana exposed to light following growth in the dark, also can be upregulated by the weak BL irradiation (Jarillo et al., 2001; Kagawa et al., 2001; Labuz et al., 2015). Positive light-dependent phot protein expression was also observed in M. polymorpha (Komatsu et al., 2014). A similar phenomenon was also revealed by our quantitative RT-PCR analysis of AePHOT, as this gene was expressed at higher levels under all light conditions (weak WL at 22 and 5°C, and strong WL at 22°C) compared to the dark at 22°C (Figure 6). These data suggest that the light-dependent upregulation of PHOT expression is conserved in A. endiviifolia. However, AePHOT was expressed at lower levels under strong WL at 22°C (i.e., increased light intensity) than under weak WL at 22°C, indicating that strong light induces the avoidance response and weakens AePHOT expression (Figure 6). Similar to the weak AePHOT expression under strong WL at 22°C, weak WL at 5°C, which induces the cold-avoidance response, also resulted in low levels of AePHOT expression (Figure 6). These results indicate that cells showing the accumulation response had higher AePHOT expression levels than cells showing the avoidance and cold-avoidance responses. On the other hand, based on the general property of the photocycle of the LOV domain (Christie, 2007; Salomon et al., 2000), the level of the photoactivated form of AePHOT under strong WL at 22°C (for the avoidance
response) or weak WL at 5°C (for the cold-avoidance response) appears to be higher than that under weak WL at 22°C (for the accumulation response). Taken together, when the photoactivated form of AePHOT is accumulated, its expression will be downregulated. Perhaps a feedback control mechanism exists in which the level of phot expression is adjusted according to the level of the photoactivated form of phot.

In this study, we fully elucidated the ranges of light intensities and temperatures needed to induce different chloroplast relocation movements (the dark-positioning response, accumulation response, avoidance response and cold-avoidance response) in A. endiviifolia. A single copy of AePHOT was identified in A. endiviifolia, as in M. polymorpha. Given the ability for the clear observation of chloroplasts and the single gene copy of AePHOT, A. endiviifolia represent a useful plant species for studying chloroplast relocation movements. To analyse the function of AePHOT in detail, an efficient genetic transformation technique for A. endiviifolia is needed. Genetic transformation techniques have been developed for M. polymorpha using particle bombardment and Agrobacterium (Chiyoda et al., 2008; Ishizaki et al., 2008, 2015; Kubota et al., 2013; Tsuboyama-Tanaka et al., 2015). Perhaps these techniques could be used as a reference to develop genetic transformation technology for A. endiviifolia in the future, which would enhance its utility for the analysis of chloroplast relocation movement.

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DATA AVAILABILITY STATEMENT
The data that support the findings of this study are openly available in the DDBJ Sequence Read Archive (DRA) under the accession number of [DRA011765]. The data that supports the findings of this study are available in the supplementary material of this article.

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Supporting Information
Additional supporting information may be found online in the Supporting Information section at the end of this article.