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Cold-induced organelle relocation in the liverwort *Marchantia polymorpha* L.

Running title: Organelle cryorelocation in the liverwort

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ABSTRACT

Organelles change their sub-cellular positions in response to various environmental conditions. Recently, we reported that cold treatments alter the intracellular position of chloroplasts and nuclei (cold positioning) in the fern *Adiantum capillus-veneris*; chloroplasts and nuclei localized to the periclinal cell wall relocated to anticlinal cell wall after cold treatments. To further understand organelle positioning under cold conditions, we studied cold-induced organelle relocation in the liverwort *Marchantia polymorpha* L. When sporelings and gemmalings were treated under low temperature (5 °C), chloroplast cold positioning response was successfully induced both in the sporelings and the gemmalings of *M. polymorpha*. Using a genetic transformation, nuclei, mitochondria or peroxisomes were visualized with a fluorescent protein, and the transgenic gemmalings were incubated under the cold condition. Nuclei and peroxisomes, but not mitochondria, clearly relocated from the periclinal cell wall to the anticlinal cell wall after cold treatments. Our findings suggest that several organelles concurrently change their positions in the liverwort cell to cope with cold temperature.

Key-words: bryophytes, chloroplast, cold positioning, cryorelocation, environmental stress, *Marchantia polymorpha* L., mitochondria, nucleus, organelle relocation, peroxisome.

INTRODUCTION

Chloroplasts change their sub-cellular localization in response to environment alterations. For example, light, temperature, salinity and mechanical stimuli are known to induce chloroplast relocation (Senn 1908). Among those, light-induced chloroplast relocation has been well characterized (Suetsugu & Wada 2007). The light-induced chloroplast relocation occurs to optimize photosynthetic activity. Chloroplasts move away from high intensity (strong) light to avoid photodamage (avoidance response). On the other hand, chloroplasts accumulate in areas of low intensity (weak) light to efficiently capture light energy (accumulate response). The light-induced chloroplast relocation movements are mediated by blue light signaling via blue light receptors, phototropins (Suetsugu & Wada 2007), and the motility of the chloroplast is regulated by reorganization of short actin filaments (cp-actin filaments) localized at the periphery of the chloroplast (Kadota *et al.* 2009; Kodama *et al.* 2010; Suetsugu *et al.* 2010; Yamashita *et al.* 2011; Tsuboi *et al.* 2012). Similar light-dependent relocation movements of the nucleus and mitochondria were also reported. Together with chloroplasts, both nuclei and mitochondria change their locations in response to blue light (Kagawa & Wada 1993; Tsuboi *et al*. 2007; Iwabuchi *et al*. 2010; Islam *et al*. 2009). However, organelle relocation movements under various stimuli, other than light, are poorly understood.

Low temperature-induced chloroplast relocation was observed over one century ago (Haberlandt 1876; Senn 1908). In the report by Senn (1908), intracellular location of chloroplasts in the moss *Funaria hygrometrica* was changed in response to different temperature conditions under weak light. In response to cold treatments, chloroplasts localized along the periclinal cell wall, under weak light, relocated to the anticlinal cell wall (Senn 1908). Recently we rediscovered the low temperature-induced chloroplast relocation in the fern *Adiantum capillus-veneris*, and we specifically termed the positioning as chloroplast cold positioning (Kodama *et al.* 2008). Similar to the observation in *F. hygrometrica*, chloroplasts localized along the periclinal cell wall, under weak light, relocated to the anticlinal cell wall after cold treatments in *A. capillus-veneris* (Kodama *et al.* 2008). We observed that induction of the chloroplast cold positioning was dependent on light intensity and temperature (Kodama *et al.* 2008). As the light intensity increased, we observed a stronger induction of chloroplast cold positioning. Similarly, as the temperature decreased, there was a stronger induction of chloroplast cold positioning. It was also found that chloroplast cold positioning did not occur in *phototropin 2* (*phot2*) mutants of *A. capillus-veneris*, suggesting involvement of *phot2* signaling in the chloroplast cold positioning response (Kodama *et al.* 2008). Furthermore, using chemical staining, localization of nuclei and mitochondria were observed under low temperature, and a cold positioning of nucleus, but not mitochondria, was also observed in *A. capillus-veneris* (Kodama *et al.* 2008). However, because chemical staining of the organelles requires fixation of the cells, details of relocation movements of organelles other than chloroplasts remain to be determined.

In this study, we report low temperature-induced organelle relocation movements in the liverwort *Marchantia polymorpha* in which an *Agrobacterium*-mediated genetic transformation technique has been developed (Ishizaki *et al*. 2008; Kubota *et al*. 2013). The present study found the physiological response of chloroplast cold positioning in the liverwort *M. polymorpha*. Furthermore, with the use of yellow fluorescent protein, low temperature-induced relocation movements of nucleus and peroxisomes, but not mitochondria, were observed in the living liverwort cells. We also demonstrated coordinated relocation movements of several organelles in the liverwort cells under cold conditions. The significance and mechanism of such organelles' relocation movements will be discussed.

MATERIALS AND METHODS

Plant materials and growth conditions

Male, accession Takaragaike-1 (Tak-1), and female, accession Takaragaike-2 (Tak-2), of *M. polymorpha* were asexually maintained (Ishizaki *et al*. 2008). Tak-1 and Tak-2 thalli were cultured on M51C medium with 1% agar (M51C agar) (Ono *et al*. 1979) under 75 μmol photons m^{-2} s^{-1} continuous white light (FL40SW, NEC Corporation, Tokyo, Japan). The intensities of fluorescent lights were measured with a light meter (LI-250A, LI-COR Biosciences, Lincoln, USA). Formations of sexual organs were induced by the continuous white fluorescent light and another fluorescent light containing far-red spectrum (FL20S-FR-74, Toshiba Lighting & Technology Corporation, Kanagawa, Japan). F1 spores were obtained by crossing between Tak-1 and Tak-2 as described previously (Chiyoda *et al*. 2008). F1 spores were spread on M51C agar for germination, and 10-day-old sporelings (immature thalli grown from spores) were used for observation of chloroplasts. One-day-old gemmalings (immature thalli grown from gemmae) obtained from approximately 1-month-old thalli of Tak-1 or Tak-2, were also used for the observation.

Light and temperature treatments

Ten-day-old sporelings or 1-day-old gemmalings were incubated at 22°C or 5°C in the temperature-controlled incubators (IJ100, Yamato Scientific Co., Ltd., Tokyo, Japan). In the incubator, white-colored light-emitting diodes (LEDs) (OptoSupply Limited, Hong Kong, China) were set-up to irradiate the sporelings or the gemmalings with weak light (140 μmol photons m^{-2} s^{-1}). The light intensities of the LEDs were measured with the light meter LI-250A.

Plasmid constructions

Binary vectors for *Agrobacterium*-mediated transformation of *M. polymorpha* have been constructed (for vector information in details, contact to T.K. [tkohchi@lif.kyoto-u.ac.jp]), and these vectors are adapted to the gateway cloning system (Invitrogen, CA, USA). In this study, pMpGWB102 and pMpGWB106 were used (Supporting Information Fig. S1). For visualization of nucleus or peroxisomes, a DNA fragment for an archetypal nuclear localization signal (NLS) (Pro-Pro-Lys-Lys-Lys-Arg-Lys-Val) of the SV40 large tumour antigen (Kalderon *et al*. 1984) or a peroxisomal targeting signal 1 (PTS1) (Ser-Lys-Leu) (Gould *et al*. 1987; Gould *et al*. 1989) was respectively synthesized and fused to the C-terminal of Citrine by using polymerase chain reaction (PCR) with the following primers: 5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGTGAGCAAGGGCGAG-3’, and 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCACACCTTGCGCTTCTTCTTAGG AGGCTTGTACAGCTCG-3’ for *Citrine-NLS*; 5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGTGAGCAAGGGCGAG-3’, 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCACAACTTGGACTTGTACAGCT CGTCC-3’ for *Citrine-PTS1*. These PCR-amplified DNA fragments were cloned into a pDONR207 plasmid by BP reaction (Invitrogen, CA, USA). After checking the sequences, the resulting plasmids were mixed with pMpGWB102 vector, and LR reaction (Invitrogen, CA, USA) was performed to generate pMpGWB102-Citrine-NLS and pMpGWB102-Citrine-PTS1. For visualization of mitochondria, a gene fragment for N-terminal 50 amino acids of mitochondrial protein SD3 (Hamasaki *et al*. 2012), SD3(1-50), from *Arabidopsis thaliana* was amplified from genome of *A. thaliana* by PCR with primers: 5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGATGATGATGAATCT-3’ and 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTTTGAAAGAAATGAGTT-3’. The

N-terminal fragment of SD3 was predicted to have a mitochondrial targeting signal (MTS) (Supporting Information Fig. S2) (Hamasaki *et al*. 2012). The resulting *SD3(1-50)* gene fragment was cloned into the pDONR207 plasmid by the BP reaction, and the fusion sequence was confirmed. The *SD3(1-50)* gene was further transferred into pMpGWB106 vector harboring *Citrine* gene, generating a *SD3(1-50)-Citrine* fusion gene that termed as *MTS-Citrine* in this study.

Transformation of the liverwort

For stable transformation of *M. polymorpha*, the binary vectors were transformed to *Agrobacterium tumefaciens* strain GV2260 (Deblaere *et al*. 1985). *Agrobacterium*-mediated transformation of *M. polymorpha* was performed as described previously (Kubota *et al*. 2013). For the transformation, Tak-1 was used. After selection with hygromycin, fluorescent transformants were selected under fluorescence microscopy MZ16F (Leica microsystems, Wetzlar, Germany), and used for following experiments. The resulting transformants of *M. polymorpha* (T1) were cultivated about for 1 month, and then gemmae (G1) were obtained (Supporting Information Fig. S3). Subsequently, the G1 gemmae were cultivated for 1 month, and then gemmae (G2) were obtained. For all experiments, transgenic G2 gemmae were used (Supporting Information Fig. S3).

Fluorescence observation using microscopes

Chloroplasts in sporelings and gemmalings of *M. polymorpha* were observed under the stereo fluorescence microscopy MZ16F (Leica microsystems, Wetzlar, Germany). Chloroplast positions were determined by emitting chlorophyll fluorescence by the excitation lights; excitation filter 480/40 nm and barrier filter LP 510 nm were used. For Citrine visualization of nuclei, mitochondria and peroxisomes, the inverted fluorescence microscope Axiovert 200M (Carl Zeiss, Oberkochen, Germany) was used. Filter set 41001 (EX 480/40 nm, EM535/50) and filter set 45 (EX 560/40 nm, EM630/75) were used in the Axiovert 200M for visualizations of Citrine and chloroplast, respectively. Fluorescence images were acquired as TIFF (Tagged Image File Format) files of RGB digital images by using the DP73 digital camera (Olympus, Tokyo, Japan) for the MZ16F and the AxioCam MRm CCD camera (Carl Zeiss, Oberkochen, Germany) for the Axiovert 200M. Image processing was carried out with the Java-based software ImageJ (http://rsb.info.nih.gov/ij/) (Rasband 1997-2012).

Evaluation of organelle positioning state

Chloroplast positions in *M. polymorpha* were evaluated by the P/A ratio method, which was previously reported using gametophytes of *A. capillus-veneris* (Kodama *et al*. 2008). Briefly, chloroplast position was quantified by the brightness ratio of chlorophyll fluorescence emitted from chloroplasts at the anticlinal and periclinal positions. In this study with the cells of *M. polymorpha*, fluorescent intensities from 30 points (0.625 μm each, equivalent to 1 pixel) and 30 areas (39.1 μm^2 each, equivalent to 10 x 10 pixels) were measured along the anticlinal and the periclinal walls in a sporeling, respectively. The P/A ratios with a standard deviation were obtained as an average of experiments repeated five times. Image processing and fluorescence measurement were performed with the ImageJ (http://rsb.info.nih.gov/ij/) (Rasband 1997-2012), and P/A ratios were calculated with Microsoft Excel (http://www.microsoft.com/).

Nuclear positions were evaluated by counting the number of nuclei along the anticlinal and the periclinal walls. Fluorescence images of the Citrine-visualized nuclei of the transgenic gemmalings were obtained under the Axiovert 200M with the AxioCam MRm CCD camera, and the numbers of nuclei were counted in 100 cells. The percentage with a standard

deviation was obtained as an average of experiments repeated three times.

Peroxisome positions were evaluated by counting number of peroxisomes along the periclinal wall. Fluorescence images of the Citrine-visualized peroxisomes of the transgenic gemmalings were obtained under the Axiovert 200M with the AxioCam MRm CCD camera, and the numbers of peroxisomes were counted in 30 cells. The peroxisome number with a standard deviation was obtained as an average of experiments repeated three times. The percentage of peroxisomes attached to chloroplasts was calculated by dividing the number of peroxisomes attached to chloroplasts by the number of all peroxisomes counted in 10 cells. The percentage with a standard deviation was obtained as an average of experiments repeated three times.

RESULTS

Chloroplast cold positioning in the liverwort

Although we previously found a chloroplast cold positioning response in *A. capillus-veneris*, molecular biology techniques have been limited in the *A. capillus-veneris*. In addition, a chloroplast cold positioning response could not be observed in several angiosperms including *Nicotiana tabacum*, *Taraxacum offinale*, *Oxalis acetosella*, *Viola odorata* and a model plant *Arabidopsis thaliana* (Haberlandt 1876; Senn 1908; Kodama *et al*. 2008). Therefore we searched for a suitable plant to study chloroplast cold positioning, and focused on the liverwort *M. polymorpha*. Molecular biology techniques, such as genetic transformation, have recently been developed in the *M. polymorpha* (Ishizaki *et al*. 2008, Kubota *et al.* 2013). To test whether chloroplast cold positioning responses occur in the *M. polymorpha*, the 10-day-old sporelings were incubated under cold conditions at 5°C after an accumulation response was induced at 22°C. The chloroplasts located along the periclinal cell wall at 22°C relocated to the anticlinal cell wall at 5°C for 24 h, indicating an induction of chloroplast cold positioning in the *M. polymorpha* (Fig. 1a). We evaluated the state of chloroplast positioning in *M. polymorpha* using the same method developed for *A. capillus-veneris* in the previous study (Kodama *et al.* 2008). The level was calculated as a ratio of brightness of chlorophyll fluorescence measured at areas along anticlinal (A) and periclinal (P) walls, and the unit was termed as P/A ratio (Kodama *et al.* 2008). When an accumulation response was induced in *M. polymorpha*, its P/A ratio was approximately 1.5 (Fig. 1b). In contrast, when chloroplast cold positioning occurred, its P/A ratio was approximately 0.3 (Fig. 1b). These results indicate that evaluation with P/A ratio for chloroplast positioning can be used in the *M. polymorpha*.

Because *M. polymorpha* is dioecious, the difference between male and female was verified using their gemmalings. Since the chloroplasts were likely dispersed to the entire cell in gemmae observed immediately after detachment from gemma cup of thalli, both male and female gemmae were cultured for 1 day under continuous weak white light at 22ºC to induce the accumulation response. After chloroplasts localized along periclinal walls during the accumulation response (Fig. 2a,c), male gemmalings were incubated at 5°C under the same weak white light for 24 h. Similar to the results in sporelings, the chloroplasts in gemmalings moved from periclinal to anticlinal positions, and the P/A ratio was approximately 0.2 in both male and female at 24 h (Fig. 2a-d). These results indicate that there is no difference in the degree of chloroplast cold positioning responses between male and female. When these gemmalings were incubated at 5°C for 48 h, the P/A ratio was comparable to that for 24 h (Fig. 2b,d), indicating that cold treatment for 24 h is enough for completion of chloroplast cold positioning in the *M. polymorpha*.

Organelle visualizations

To examine whether organelles other than chloroplasts experience cold-induced relocation, the nucleus, mitochondria and peroxisomes were observed under cold conditions in *M. polymorpha*. In this study, a yellow fluorescent protein variant, Citrine, was employed to visualize organelles in living cells, because Citrine is more resistant to acid quenching and is less sensitive to chloride (Griesbeck *et al*. 2001). To visualize nuclei, mitochondria and peroxisomes in the living *M. polymorpha*, genes for organelle-localized Citrine fluorescent proteins were constructed (Fig. 3). For nuclear visualization, a nuclear localization signal (NLS) was fused to the C-terminus of Citrine (Fig. 3). For visualizing mitochondria, the N-terminal region of a mitochondrial SD3 protein from *Arabidopsis thaliana* (Hamasaki *et al*. 2012) was fused to N-terminus of Citrine (Fig. 3). The iPSORT program [http://ipsort.hgc.jp/index.html] (Bannai *et al*. 2002), a subcellular localization site predictor for N-terminal sorting signals, predicted that the N-terminal region has a mitochondrial targeting signal (MTS) (Supporting Information Fig S2). For peroxisome visualization, the peroxisome targeting signal 1 (PTS1) was fused to the C-terminus of Citrine (Fig. 3). Expression of these genes was controlled by the cauliflower mosaic virus 35S promoter (P35S) and the *Agrobacterium tumefaciens* nopaline synthase terminator (TNOS) (Fig. 3). These reporter constructs were introduced into *M. polymorpha* using the *Agrobacterium*-mediated transformation technique. Transformants were selected using hygromycin resistance and Citrine fluorescence, and used for the following experiments.

Nuclear positioning under cold condition

A nucleus was successfully visualized by Citrine in each cell of transgenic gemmalings, in which induction of chloroplast cold positioning was confirmed (Fig. 4a). Under weak light at 22°C, each nucleus was localized at the center of the periclinal cell wall (Fig. 4a). When the gemmalings were then incubated under weak light at 5°C for 24 - 48 h, cold-induced relocation of nuclei was observed; nuclei moved from the periclinal wall to the anticlinal wall (Fig. 4a). The result observed in living cells of *M. polymorpha* is consistent with the previous results in the fixed cells of *A. capillus-veneris* (Kodama *et al*. 2008). When nuclear positioning was evaluated, approximately 70 % and 90 % of nuclei were located along the anticlinal wall after incubation at cold temperature for 24 h and 48 h, respectively (Fig. 4b). This observation demonstrates that *M. polymorpha* has a nuclear cold positioning response.

Mitochondria positioning under cold condition

Numerous mitochondria visualized with Citrine were observed along the periclinal wall in the transgenic gemmalings at 22°C (Fig. 5), and chloroplast cold positioning occurred in the transgenic gemmalings under weak light at 5°C for 24 - 48 h (Fig. 5). Although our time course observations suggest that the number of mitochondria at the periclinal wall was slightly reduced under cold treatment, many mitochondria remained on the periclinal wall (Fig. 5). Thus, we concluded that there is no cold positioning response of mitochondria in *M. polymorpha*. This is consistent with the previous results in the fixed cells of *A. capillus-veneris* (Kodama *et al*. 2008).

Peroxisome positioning under cold condition

Citrine with PTS1 was localized to peroxisomes in *M. polymorpha*; observed in a punctate fluorescence pattern (Fig. 6a). As was the case with chloroplasts, the fluorescent peroxisomes were observed at the periclinal wall under 22°C (Fig. 6a). When chloroplast cold positioning occurred, peroxisomes relocated to the anticlinal wall from the periclinal wall, indicating peroxisome relocation in response to cold (Fig. 6a). The number of peroxisomes on the

periclinal wall was gradually reduced after cold treatments (Fig. 6b). When peroxisome positioning was evaluated, approximately 60 % and 80 % of peroxisomes were relocated at 24h and 48 h after cold treatments, respectively (Fig. 6b). During the relocation, over 95 % of peroxisomes were in close proximity to the chloroplasts (Fig. 6a,c), suggesting a physical interaction between chloroplasts and peroxisomes during their relocation under cold conditions.

DISCUSSION

The present study demonstrated cold-induced relocation movements of chloroplasts, nucleus and peroxisomes, but not mitochondria, in the liverwort *M. polymorpha*. Although the physiological significance is not understood to date, our findings revealed evidence that cold-induced relocation of various organelles occurs in plant cells.

In 1908, Gustav Senn (1875-1945; Thompson 1945) described a phenomenon of low temperature-induced chloroplast relocation in *Funaria hygrometrica* (Senn 1908). It was reported that chloroplasts were relocated from the periclinal wall to the anticlinal wall in *F. hygrometrica* under low temperature (Senn 1908). After just 100 years from that report, we rediscovered a similar relocation in the fern *A. capillus-veneris* in 2008 (Kodama *et al*. 2008). In the previous study we developed an evaluation method, a calculation of P/A ratio, for chloroplast positioning in the cells, and a quantitative analysis of chloroplast cold positioning was completed (Kodama *et al*. 2008). Note that P/A ratio is an arbitrary value, and represents the state of chloroplast positioning in the cells. In the present study, we found low temperature-induced chloroplast relocation movements in the liverwort *M. polymorpha*. Similar to *F. hygrometrica* and *A. capillus-veneris,* the chloroplasts relocated from the periclinal wall to the anticlinal wall in *M. polymorpha* under low temperature. Because the obtained P/A ratios of *M. polymorpha* in this study were similar to that of *A. capillus-veneris* in the previous study, the P/A ratio method for an evaluation of chloroplast positioning might be useful in various photosynthetic organisms and cells.

In *A. thaliana*, *Physcomitrella patens* and *A. capillus-veneris*, light–induced chloroplast relocation movements are regulated by cp-actin filaments, which are relocalized to the leading edge of chloroplasts before and during the relocation (Kadota *et al.* 2009; Kodama *et al.* 2010; Suetsugu *et al.* 2010; Yamashita *et al.* 2011; Tsuboi *et al.* 2012). It is unknown whether motility of cold-induced chloroplast relocation movement is regulated by the cp-actin filaments. Recently, filamentous actins were successfully visualized in *M. polymorpha* by using the Lifeact peptide fused to a fluorescent protein (Era *et al*. 2009). The Lifeact is a peptide that consists of the first 17 amino acids of the yeast protein Abp140p, and can localize to filamentous actin (Riedl *et al.* 2008). The use of the fluorescent protein-tagged Lifeact peptide will clarify whether cp-actin filaments are involved in motility of chloroplasts in *M. polymorpha* under cold conditions.

The light-induced chloroplast relocation movements occur to optimize photosynthetic activity. Chloroplasts move away from strong light to avoid photodamage, and accumulate toward weak light to capture light energy efficiently (Suetsugu & Wada 2007). While the physiological significance of chloroplast relocation movements under cold conditions remains to be determined, our previous study showed that the chloroplast cold positioning response is induced in evergreen ferns, but not summer green ferns (Kodama *et al*. 2008). Based on these observations, we propose that the chloroplast cold positioning response could be involved in plant wintering and/or resistance to cold. To clarify this hypothesis, a physiological comparison between a wild-type plant and a mutant plant having an impaired chloroplast cold positioning response will be necessary.

As in the case of the chloroplast relocation, nuclear relocation under environmental alterations has been well characterized. Several studies on light-induced nuclear positioning were reported in *A. capillus-veneris* and *A. thaliana* (Kagawa & Wada 1993; Tsuboi *et al*. 2007; Iwabuchi *et al*. 2010). Similar to chloroplasts, nuclei relocate depending on light intensity; nuclei accumulate to the periclinal wall under weak intensity and to the anticlinal wall under strong light. Also, we previously reported cold-induced nuclear relocation, called nuclear cold positioning response, in *A. capillus-veneris* (Kodama *et al.* 2008). In the previous study, *A. capillus-veneris* cells were fixed, and the nuclei were stained by 4',6-diamidino-2-phenylindole (DAPI) to visualize cold-induced nuclear relocation (Kodama *et al*. 2008). The present study successfully visualized nuclei using a NLS-tagged Citrine fluorescent protein in the living *M. polymorpha*. Nuclei of *M. polymorpha* were relocated from the periclinal wall to the anticlinal wall under cold conditions, which is consistent with the previous results in the fixed *A. capillus-veneris*. Although the physiological significance of nuclear relocation remains to be determined, Tsuboi *et al*. discussed that strong light-induced nuclear relocation might be involved in avoidance from ultraviolet-induced DNA damage (Tsuboi *et al*. 2007). As low temperature is assumed to diminish some protein activities that repair damaged DNA, the nuclear cold positioning response could contribute to the reduction of the ultraviolet-induced DNA damage response under cold conditions. Further studies examining the DNA damage response under cold conditions would provide evidence for the significance of cold-induced nuclear relocation.

Mitochondrial behavior under cold temperature has only been reported in *A. capillus-veneris* (Kodama *et al.* 2008). Unlike chloroplasts and nuclei, the mitochondria visualized with 3,3'-dihexyloxacarbocyanine iodide remained along the periclinal wall after cold treatment (Kodama *et al*. 2008). In this study, we also found that mitochondria could not change their positions in *M. polymorpha* after cold treatment. Thus, the previous and present studies suggest there is no cold positioning response of mitochondria in either pteridophytes or bryophytes. Our time course observations, in *M. polymorpha*, also suggested a slight reduction in the number of mitochondria at the periclinal wall under cold conditions. Some mitochondria might be dragged by chloroplasts during the chloroplast cold positioning. Future studies are necessary to clarify whether the reduction of mitochondria at the periclinal wall correlates with cold temperature treatment. Although mitochondrial behavior under cold temperatures has not been reported in seed plants, light-induced mitochondria relocations were studied in *A. thaliana* (Islam *et al*. 2009). In the palisade cells of Arabidopsis, mitochondria visualized with green fluorescent protein changed their distribution patterns in response to blue light (Islam *et al*. 2009). Similar to chloroplasts, almost all mitochondria accumulate to the periclinal under weak light and to the anticlinal walls under strong light (Islam *et al*. 2009). However, when we analyzed light-induced mitochondrial relocation in *M. polymorpha*, to examine whether the mitochondria have light-dependent responses as reported in *A. thaliana*, dissimilar light-induced relocations were observed (Supporting Information Fig. S4). Although mitochondria were observed at the periclinal wall under weak light, which is consistent with the result in *A. thaliana* (Islam *et al*. 2009), many mitochondria remained at the periclinal wall even after strong light irradiations (Supporting Information Fig. S4). In this context, the underlying mechanism(s) of mitochondrial relocation movements, affected by environmental alterations, might be different between *M. polymorpha* and *A. thaliana*.

Peroxisome movement and positioning in plant cells has been reported previously. It was observed that peroxisomes frequently move in the cells of *Arabidopsis thaliana*, *Allium cepa* and *Allium ampeloprasum* (Collings *et al*. 2001; Jedd & Chua 2002; Mano *et al*. 2002; Mathur *et al*. 2002). The peroxisomes likely have various movements such as directional, non-directional or stop-and-go movements (Wada & Suetsugu 2004). However,

environmental alteration–induced peroxisome relocation has not been reported so far. The present study revealed that peroxisome positioning could be changed by cold treatment. Most Citrine-visualized peroxisomes were relocated from the periclinal wall to the anticlinal wall by cold treatment under weak light. Our observation also suggests that the peroxisomes interact with chloroplasts during the relocation. Studies on the physical interaction between chloroplasts and peroxisomes have been reported (Wada & Suetsugu 2004; Schumann *et al*. 2007). The strength of the interaction between chloroplasts and peroxisomes is likely strong (Wada & Suetsugu 2004), and they are thought to exchange some molecules through the interaction (Raghavendra & Padmasree 2003). Given that interaction between chloroplasts and peroxisomes was observed to be strong in *M. polymorpha*, cold-induced peroxisome relocation might involve passive movements due to dragging by chloroplasts, or vice versa. To clarify this idea, further investigation with mutant plants that impair interaction between chloroplasts and peroxisomes will be necessary. Nevertheless, the tight interaction between chloroplasts and peroxisomes might be crucial for plant life even during the relocation process.

Our previous study revealed that phot2, a blue light receptor, is essential for the chloroplast cold positioning response in *A. capillus-veneris* (Kodama *et al*. 2008). Although the present study found cold-induced relocation movements in chloroplasts, nuclei and peroxisomes in *M. polymorpha*, it remains to be determined whether the cold-induced organelle relocations are mediated by phototropin. To address this issue, a phototropin-deficient mutant of *M. polymorpha* should be generated in the future. When we carried out a BLAST search against ESTs database of *M. polymorpha* to find sequence(s) similar to *PHOT2* of *A. capillus-veneris*, a candidate *PHOT* sequence was found (Accession: BJ867210). Random or target mutagenesis technologies will enable the isolation of phototropin-deficient mutant in *M. polymorpha*.

As for experimental techniques, it is noteworthy that some organelle localization signal peptides (NLS, MTS and PTS1), which function in angiosperms and/or other organisms, were successfully utilized to visualize nuclei, peroxisomes and mitochondria in *M. polymorpha*. This was the first time the organelle localization signal peptides were employed for use in the liverwort species. Our observations suggest that typical methods with fluorescent proteins to visualize organelles are available for the liverwort plants.

In summary, we found cold-induced chloroplast relocation in the liverwort *M. polymorpha*, and similar relocations of nuclei and peroxisomes, but not mitochondria, were also observed. Based on our finding in *M. polymorpha,* several organelles likely synchronize to change their subcellular locations in response to cold temperature. Given that these cold-induced organelle relocations are induced in winter, the synchronized organelle relocations might facilitate cold tolerance in plants. Further studies using *M. polymorpha* will shed light on the molecular mechanism and the physiological significance of cold-induced organelle relocation common to many land plants.

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Figure legends

Figure 1. Chloroplast cold positioning in the liverwort *M. polymorpha.* (a) Ten-day-old sporelings under two different temperatures (22°C and 5°C) with weak white light (140 μmol photons m^{-2} s^{-1}). Chloroplast positions were visualized using chlorophyll fluorescence, and the images were obtained under the stereo fluorescence microscopy MZ16F with the DP73 digital microscope camera. Chloroplasts localize along the periclinal wall at 22°C and along the anticlinal wall at 5°C. Bar represents 100 μm. (b) Quantitative analysis of chloroplast positioning using P/A ratio method. Chloroplast positioning at 22°C and 5°C were quantified. Bars represent standard deviation.

Figure 2. Comparison between male and female of *M. polymorpha*. (a) Chloroplast cold positioning in the male of *M. polymorpha.* Gemmalings of male, accession Tak-1, were treated with two different temperatures (22°C and 5°C) under weak white light (140 μmol photons m^{-2} s^{-1}), and chlorophyll images were obtained. (b) Quantitative analysis of chloroplast positioning in the Tak-1 using P/A ratio method. Chloroplast positioning at 22°C and 5°C (24 h and 48 h) were quantified. Bars represent standard deviation. (c) Chloroplast cold positioning in the female of *M. polymorpha.* Gemmalings of female, accession Tak-2, were treated with two different temperatures (22°C and 5°C) under weak white light (140 μmol photons m^{-2} s^{-1}), and chlorophyll images were obtained. (d) Quantitative analysis of chloroplast positioning in the Tak-2 using P/A ratio method. Chloroplast positioning at 22°C and 5°C (24 h and 48 h) were quantified. Bars represent standard deviation.

Figure 3. Schematic illustration of T-DNA region of binary vectors. Two expression elements, each of which consists of cauliflower mosaic virus 35S promoter (P35S) and *Agrobacterium tumefaciens* nopaline synthase terminator (TNOS), were used in the T-DNA region between right boarder (RB) and left boarder (LB). For selection of transformants, a gene for hygromycin phosphotransferase (HPT) was used, and controlled by the expression element near the LB. Genes for Citrine-NLS, MTS-Citrine and Citrine-PTS1 were controlled by the expression element near the RB. Bar represents 200 bp.

Figure 4. Nuclear positioning under cold condition. (a) Nucleus-visualized gemmalings under two different temperatures (22°C and 5°C) with weak white light (140 μmol photons m^{-2} s^{-1}). Chloroplast and nuclear positions were visualized using chlorophyll fluorescence and Citrine fluorescence, respectively. Nuclei localize along the periclinal wall at 22°C and along the anticlinal wall at 5°C. Bar represents 100 μm. (b) Quantitative analysis of nuclear positioning. The percentage of cells whose nuclei localized at the periclinal (gray bars) and the anticlinal (white bars) walls were calculated. Bars represent standard deviation.

Figure 5. Mitochondrial positioning under cold conditions. Mitochondria-visualized gemmalings under two different temperatures (22°C and 5°C) with weak white light (140 μmol photons m^{-2} s^{-1}). Chloroplast and mitochondrial positions were visualized using chlorophyll fluorescence and Citrine fluorescence, respectively. Mitochondria stay along the periclinal wall both at 22°C and 5°C. Bar represents 100 μm.

Figure 6. Peroxisome positioning under cold conditions. (a) Peroxisome-visualized gemmalings under two different temperatures (22°C and 5°C) with weak white light (140 μmol photons m^{-2} s^{-1}). Chloroplast and peroxisome positions were visualized using chlorophyll fluorescence and Citrine fluorescence, respectively. Peroxisomes localize along

the periclinal wall at 22°C and along the anticlinal wall at 5°C. Bar represents 100 μm□ (b) Quantitative analysis of peroxisome positioning. The number of peroxisomes localized to the periclinal wall were quantified at 22°C and 5°C. Bars represent standard deviation. (c) The percentage of peroxisomes attached to chloroplasts. The percentage was calculated by dividing the number of peroxisomes attached to chloroplasts by the number of all peroxisomes counted in 10 cells. The experiments were repeated three times (the counts: 399/403, 324/327 and 300/305 at 22°C; 358/366, 407/410 and 348/352 at 5°C, 24 h; 338/343, 285/296 and 276/285 at 5°C, 48 h). Bars represent standard deviation.

SUPPORTING INFORMATION

Figure S1. Schematic illustration for T-DNA of binary vectors. (a) T-DNA structure of pMpGWB102. RB: right border, *P35S*: cauliflower mosaic virus 35S promoter, *attR1* (in blue): a recombination site for LR reaction of Gateway system, *Cm*^{r} (in red): chloramphenicol resistance gene, *ccdB*: gene for a lethal protein that poisons topoisomerase II (DNA gyrase), *attR2* (in blue): a recombination site for LR reaction of Gateway system, *TNOS*: *Agrobacterium tumefaciens* nopaline synthase terminator, HPT: hygromycin phosphotransferase, LB: left border. (b) T-DNA structure of pMpGWB106. A yellow box indicates *Citrine* gene.

Figure S2. Prediction of a mitochondrial targeting sequence within N-terminal of SD3 from *Arabidopsis*. The amino acid sequence shows full-length sequence of SD3 protein (AT4G00026). An amino acid region predicted for a mitochondrial targeting sequence by the iPSORT program [http://ipsort.hgc.jp/index.html] (Bannai *et al*. 2002) is shown in red. Bar indicates the first 50 amino acids, which was used as a mitochondrial targeting sequence in this study.

Figure S3. Procedure to obtain transgenic G2 gemmae. The transformed thallus of *M. polymorpha* (T1) were cultivated, and then gemma (G1) was obtained from gemma cup (T1). The gemma (G1) was cultivated for approximately 1 month, and then gemma (G2) was obtained from gemma cup (G1). The gemmae (G2) was used for all analyses after growth for 24 h under white weak light at 22°C.

Figure S4. Mitochondria positioning in *M. polymorpha* under strong light irradiation. Mitochondria-visualized gemmalings were irradiated by strong white light (300 μmol photons m^{-2} s^{-1}) for 4 h at 22°C. Chloroplast and mitochondria positions were visualized using chlorophyll fluorescence (red) and Citrine fluorescence (green), respectively. While chloroplasts relocated to the anticlinal wall, mitochondria stayed along the periclinal wall after the irradiation. Bar represents 20 μm.

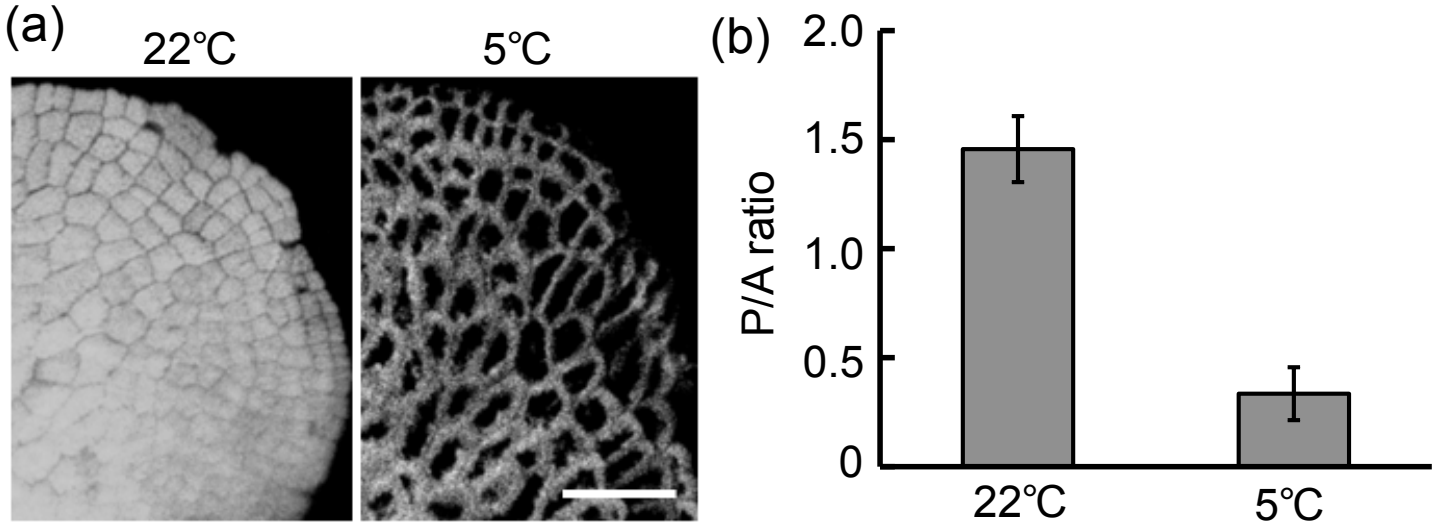


Fig.1

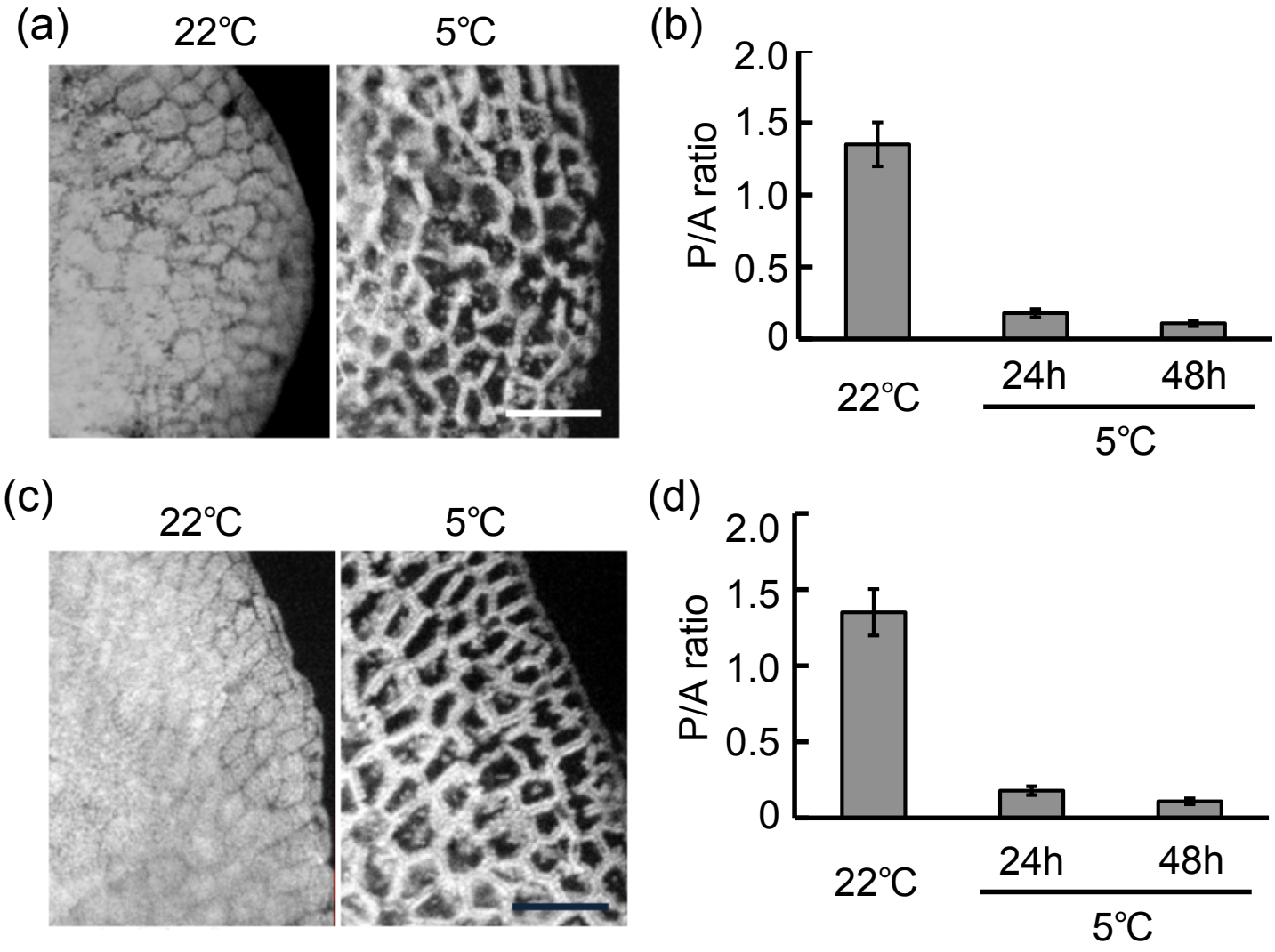

(a)
22℃
5℃
(b)
P/A ratio
2.0
1.5
1.0
0.5
0
22℃
24h
48h
5℃
(c)
22℃
5℃
(d)
P/A ratio
2.0
1.5
1.0
0.5
0
22℃
24h
48h
5℃

Fig.2

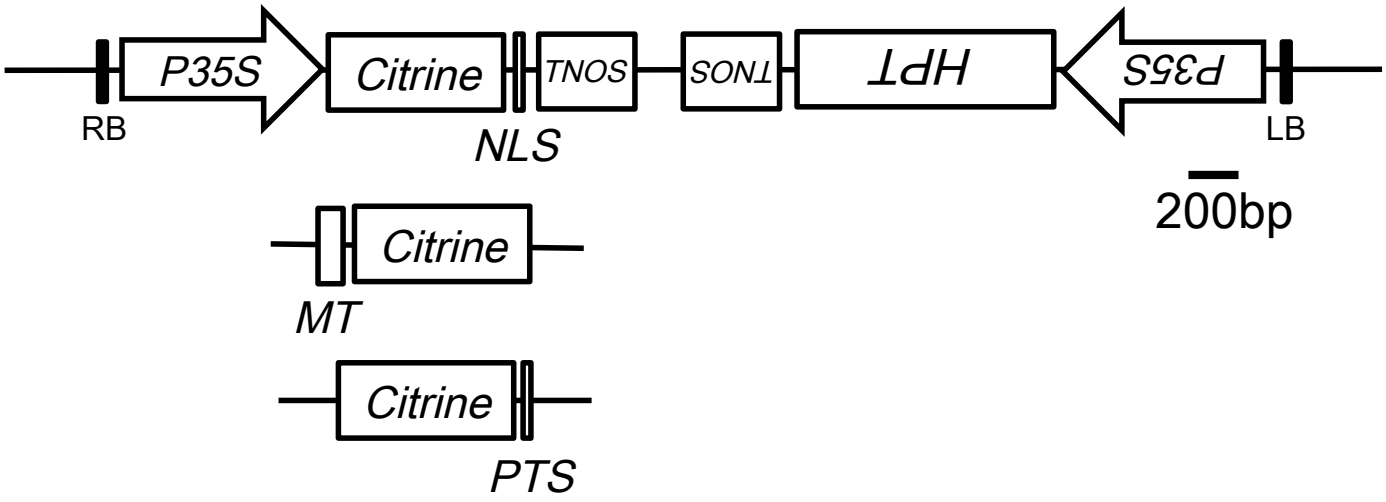
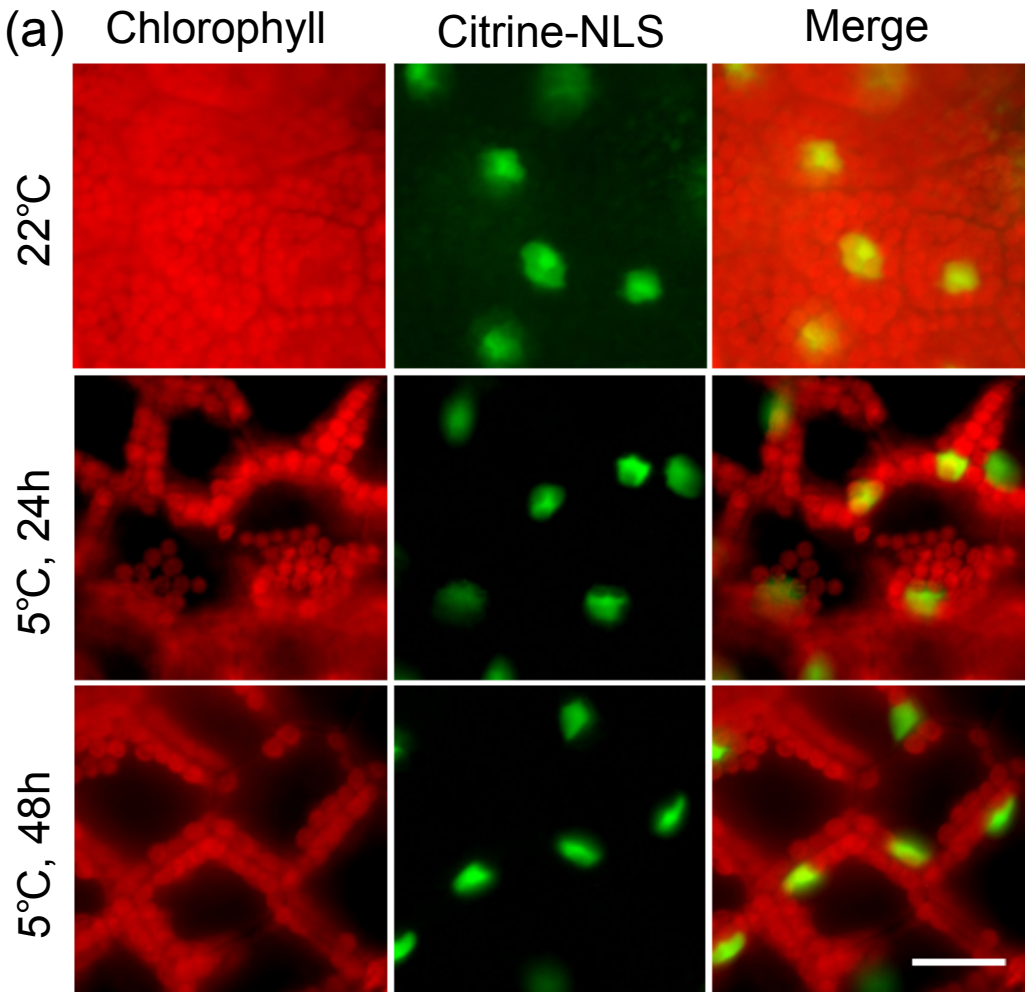

P35S
Citrine
TNOS
TNOS
HPT
P35S
RB
NLS
LB
200bp
Citrine
MT
Citrine
PTS

Fig.3


(a)
Chlorophyll
Citrine-NLS
Merge
22°C
5°C, 24h
5°C, 48h

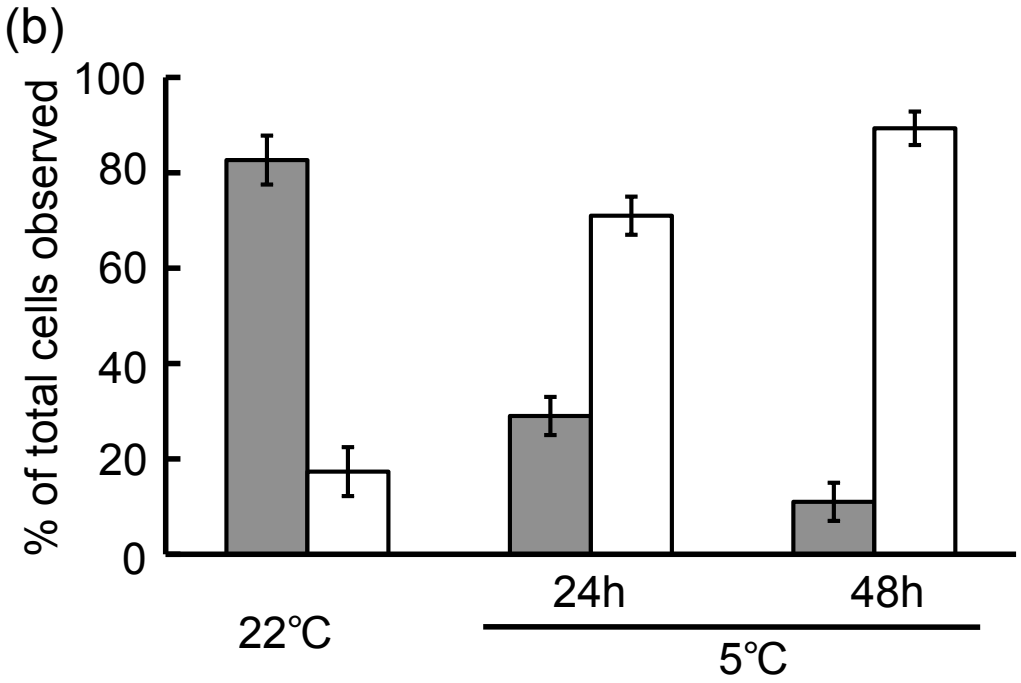

(b)
% of total cells observed
100
80
60
40
20
0
22°C
24h
48h
5°C

Fig.4

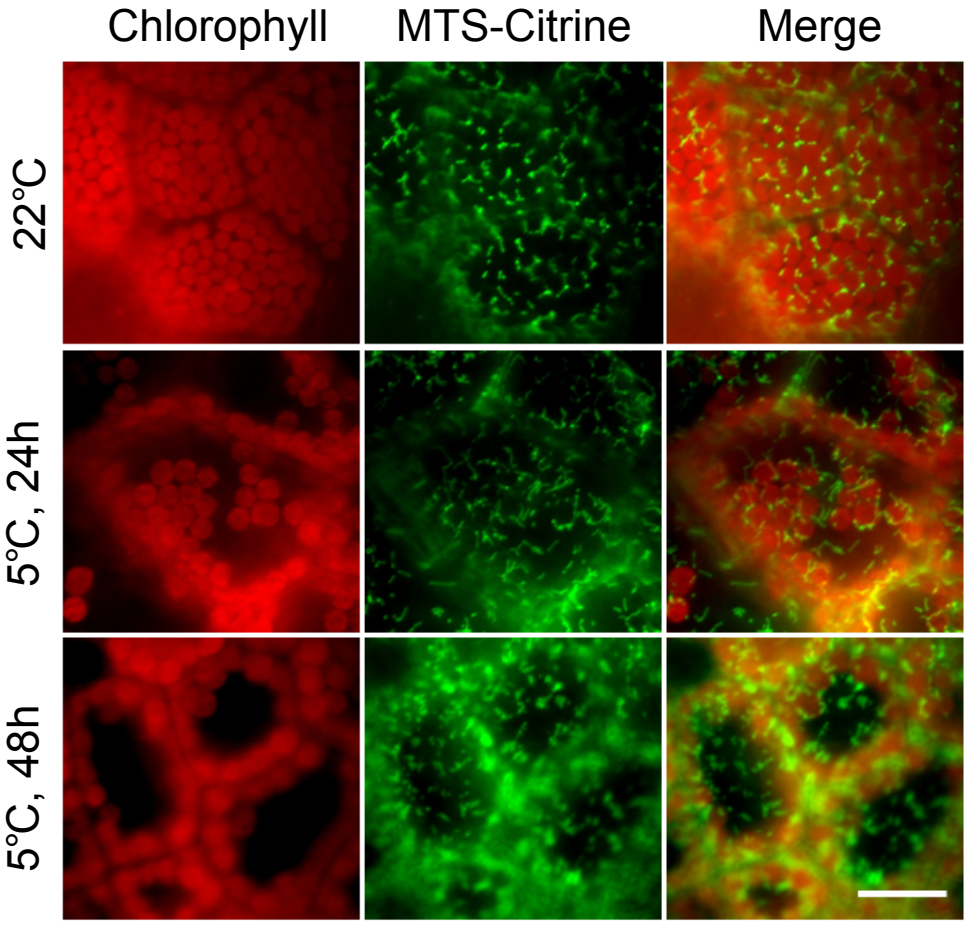


Fig.5

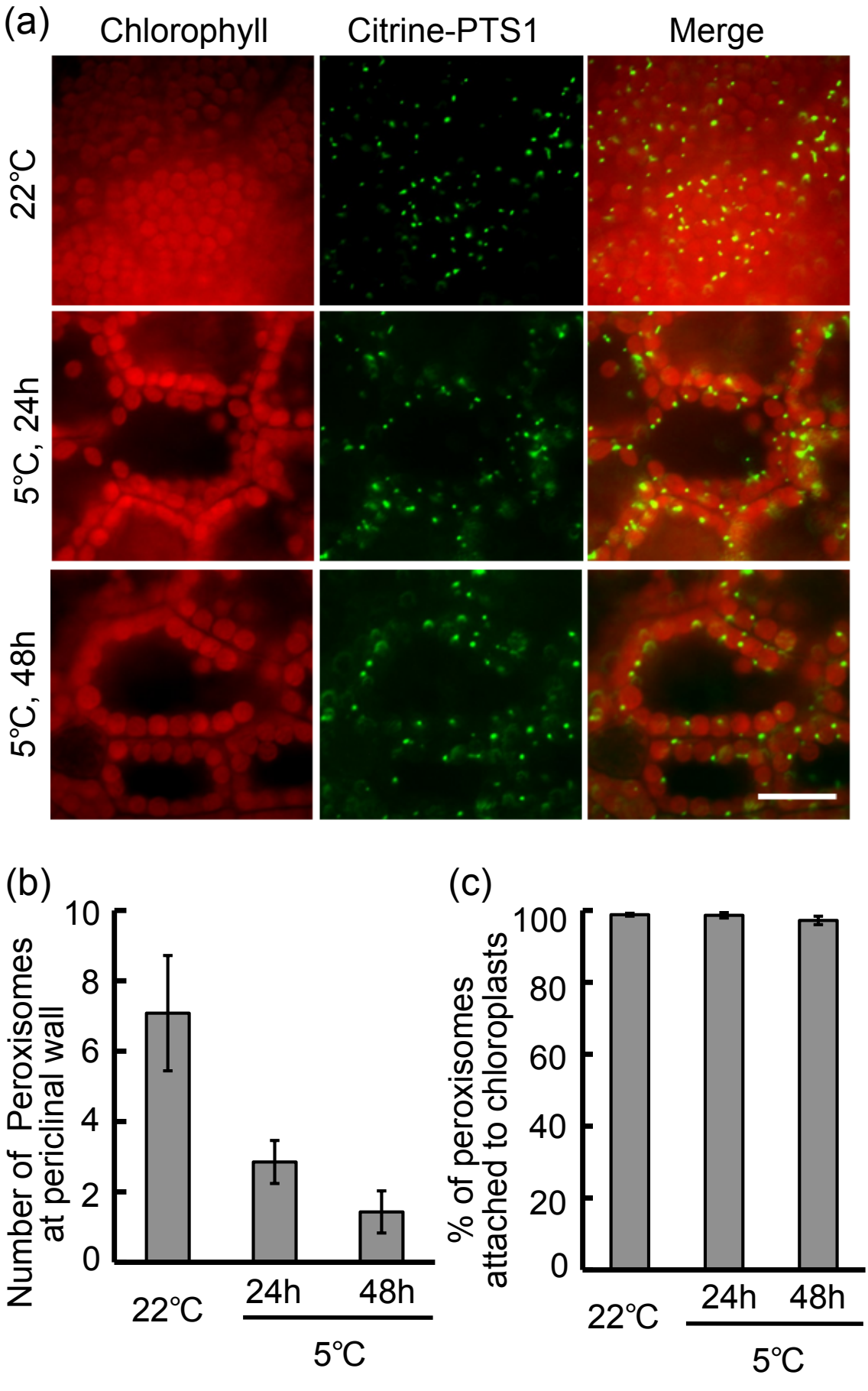
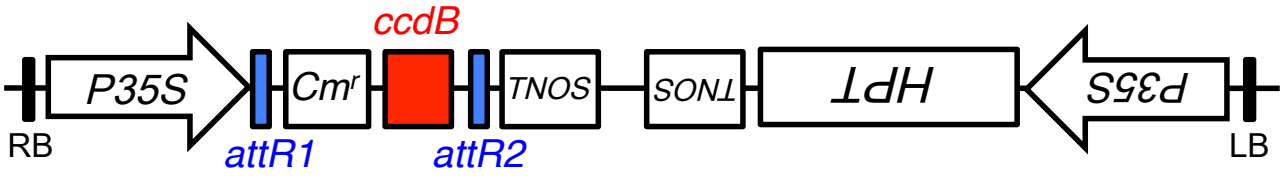
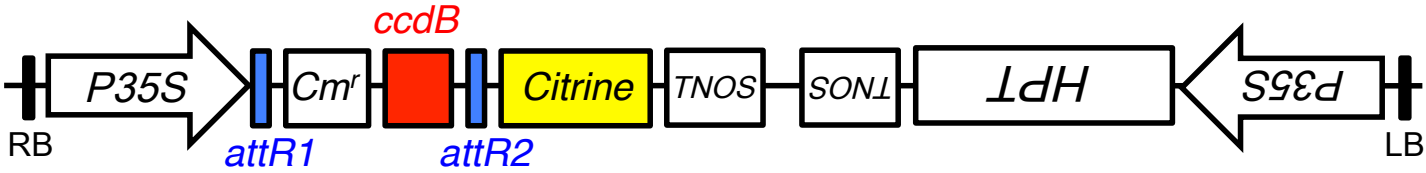

(a)
Chlorophyll
Citrine-PTS1
Merge
22°C
5°C, 24h
5°C, 48h
(b)
Number of Peroxisomes at periclinal wall
10
8
6
4
2
0
22°C
24h
48h
5°C
(c)
% of peroxisomes attached to chloroplasts
100
80
60
40
20
0
22°C
24h
48h
5°C

Fig.6

(a) pMpGWB102



(b) pMpGWB106



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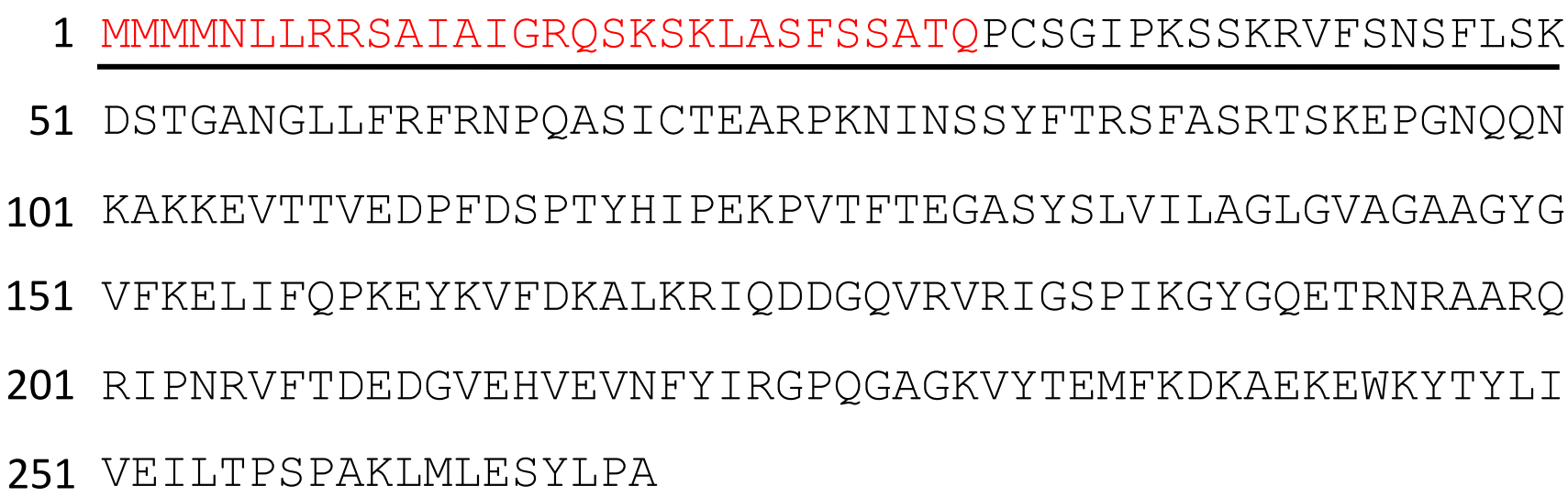

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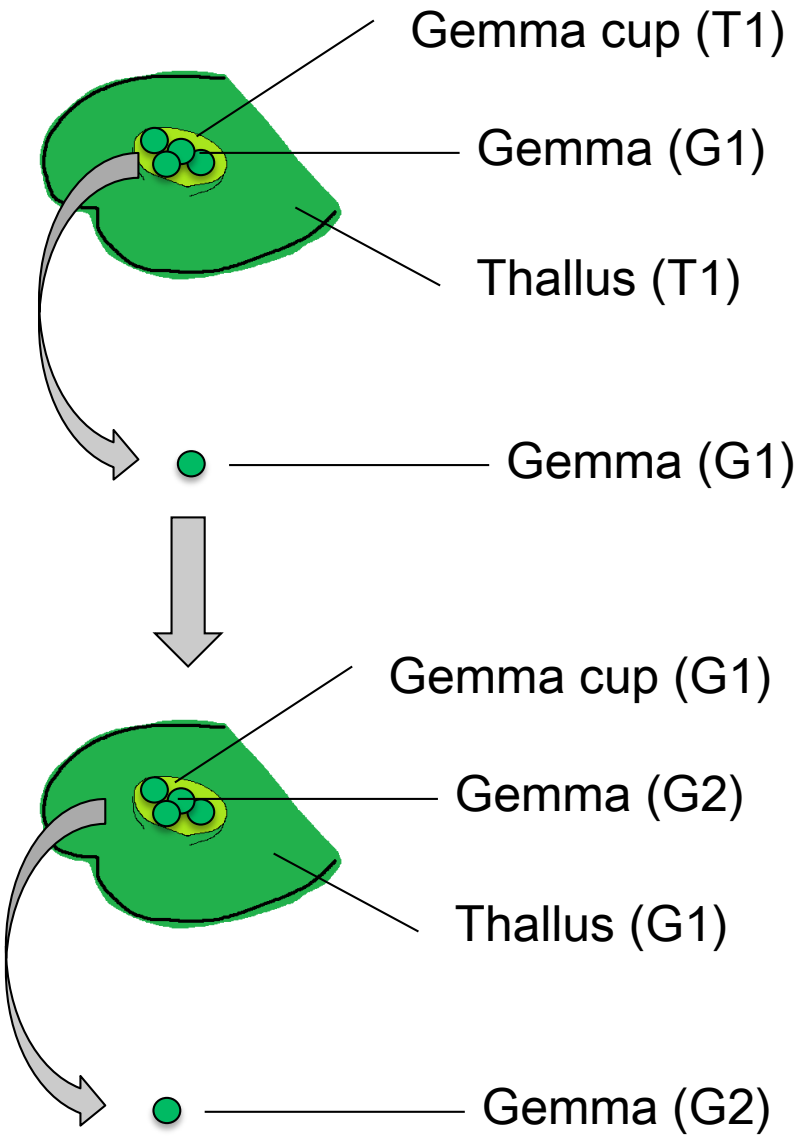

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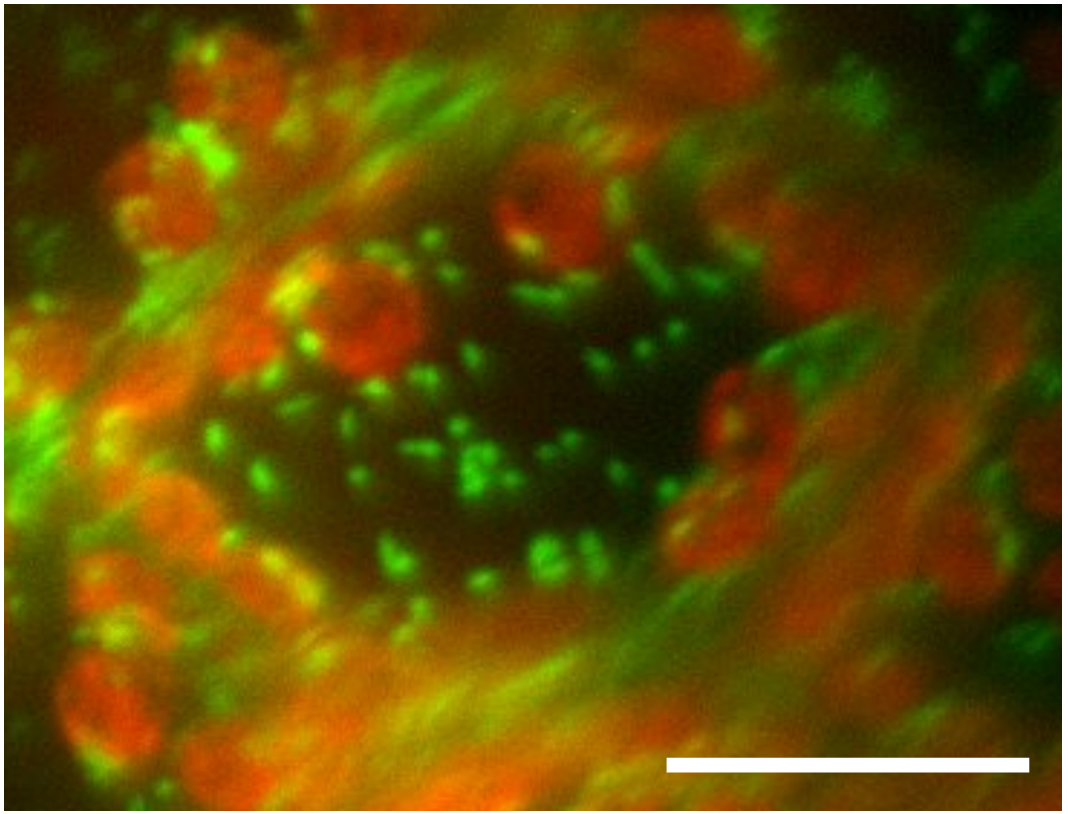

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