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REGULAR PAPER

Temperature-dependent signal transmission in chloroplast accumulation response

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Abstract Chloroplast photorelocation movement, well-characterized light-induced response found in various plant species from alga to higher plants, is an important phenomenon for plants to increase photosynthesis efficiency and avoid photodamage. The signal for chloroplast accumulation movement connecting the blue light receptor, phototropin, and chloroplasts remains to be identified, although the photoreceptors and the mechanism of movement via chloroplast actin filaments have now been revealed in land plants. The characteristics of the signal have been found; the speed of signal transfer is about 1 μm min^{-1} and that the signal for the accumulation response has a longer life and is transferred a longer distance than that of the avoidance response. Here, to collect the clues of the unknown signal substances, we studied the effect of temperature on the speed of signal transmission using the fern *Adiantum capillus-veneris* and found the possibility that the mechanism of signal transfer was not dependent on the simple diffusion of a substance; thus, some chemical reaction must also be involved. We also found new insights of signaling substances, such that microtubules are not involved in the signal transmission, and that the signal could even be transmitted through the narrow space between chloroplasts and the plasma membrane.

Keywords Blue light · Chloroplast movement · Fern · Phototropin · Red light · Signaling

Introduction

Chloroplasts change their intracellular position in response to environmental light conditions. Under weak light, chloroplasts gather in the light irradiated area to perform efficient photosynthesis (the accumulation response) (Zurzycki 1955) but move away from strong light to avoid photodamage (the avoidance response) (Kasahara et al. 2002). This chloroplast photorelocation movement has been found in most land plants tested, including a liverwort, mosses, ferns and seed plants, and the mechanisms of this response have been well studied in recent years (Banaś et al. 2012; Gabryś 2012; Kong and Wada 2014; Suetsugu and Wada 2009).

In the terrestrial model plant *Arabidopsis thaliana*, chloroplast photorelocation movement is regulated by two blue/UV-A light-receptors, phototropin1 (phot1) and phot2. The accumulation response is redundantly mediated by both phototropins localized on the plasma membrane (Sakai et al. 2001), while phot2 probably localized on the chloroplast outer membrane is involved in the avoidance response (Kagawa et al. 2001; Jarillo et al. 2001; Kong et al. 2013a). In the fern *Adiantum capillus-veneris*, chloroplast avoidance movement is similarly regulated by phot2 (Kagawa et al. 2004) and, moreover, the accumulation response is induced by red light (Yatsuhashi et al. 1985; Kagawa and Wada 1996), which is captured by neochrome1 (neo1) (Kawai et al. 2003), a chimeric photoreceptor combining a full-length phototropin at the C-terminus and the

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chromophore binding domain of phytochrome in the N-terminus (Nozue et al. 1998; Suetsugu et al. 2005). phot1 is clearly involved in the accumulation response of *A. capillus-veneris* because a normal accumulation response was observed in the *phot2neo1* double mutant line (Tsuboi et al. 2007), but confirmation of a defective accumulation response in a *phot1phot2neo1* triple mutant line(s) and recovery from this deficiency with a phot1 sequence is necessary.

In recent years, not only the photoreceptors but also the motility systems of chloroplast photorelocation movement have been characterized (Kadota et al. 2009; Kong et al. 2013b). Chloroplast movement is dependent on short actin filaments specifically localized between the chloroplast outer envelope and the plasma membrane around the peripheral region of chloroplasts. The chloroplast actin (cp-actin) filaments also play an important role in chloroplast attachment to the plasma membrane and show dynamic changes in their distribution pattern and abundance on the leading edge of the chloroplast during movement (Kong et al. 2013b). Strong light induces the dissipation of cp-actin filaments and subsequent reappearance at the front edge of chloroplasts for the avoidance response, while during the accumulation response under weak light, accumulation of cp-actin filaments occurs at the front edge of chloroplasts without dissipation, and then the chloroplasts start their movement (Kadota et al. 2009; Kong et al. 2013b).

Despite of the abundance of knowledge about the photoreceptors and the motility system, the signal and its transmission pathway have not yet been clarified. Our studies using a microbeam irradiation system have revealed some characteristics of the signal(s), such as the speed of signal transmission (Tsuboi and Wada 2010), the signal lives of the accumulation and avoidance responses (Kagawa and Wada 1994, 1999; Higa and Wada 2015) and the distance travelled (Kagawa and Wada 1999). The speed of signal transfer for chloroplast photorelocation movement is approximately *c*. 1.0 μm min^{-1} irrespective of the light conditions (Tsuboi and Wada 2010). The lifetime of the signal in its active state is 5–7 times longer in the accumulation response than in the avoidance response in prothallial cells of *A. capillus-veneris* (Higa and Wada 2015), indicating that different signals are involved in these two responses. Chloroplasts monitor the signal continuously during accumulation movement (Tsuboi and Wada 2013), suggesting that the active forms of photoreceptors release the signal continuously until they are inactivated, depending on their photocycle or by far-red light irradiation in the case of neochrome1.

Other attempts to clarify the signaling of chloroplast movement have been performed pharmacologically. Calcium ions have long been thought to be a candidate secondary messenger of the signal for chloroplast movement (refer to the review Banaś et al. 2012). The involvement of Ca^{2+} ions has been shown in *Lemna trisulca* and *Nicotiana tabacum* cells by applying chelates, antagonists, and Ca^{2+} with ionophores (Tlałka and Gabryś 1993; Tlałka and Fricker 1999; Anielska-Mazur et al. 2009). Ca^{2+} ions play an important role as a secondary messenger in various cellular responses to several different types of stimulation including light (Hepler 2005; Trump et al. 1984; Banaś et al. 2012). A transient increase of cytosolic Ca^{2+} concentration was found to be mediated by phototropins upon blue light perception (Babourina et al. 2002; Baum et al. 1999; Harada et al. 2003; Stoelzle et al. 2003). In *A. thaliana*, phot1 induces transport of Ca^{2+} to the cytoplasm from the apoplast over 10 min, with a peak 3–5 min after blue light irradiation (Babourina et al. 2002). phot1 and phot2 increase the cytosolic Ca^{2+} concentration after low fluence rate light (0.1–50 μmol m^{-2} s^{-1}) and high fluence rate light (1–250 μmol m^{-2} s^{-1}) stimulation, respectively. Both phot1 and phot2 control influx of Ca^{2+} through the plasma membrane calcium channel, and phot2 alone induces an increase of cytosolic Ca^{2+} concentration within 10 s via induction of phospholipase C-mediated phosphoinositide signaling and subsequent release of internal Ca^{2+} stores, which continues for approximately *c*. 50–70 s (Harada et al. 2003). Although several experiments have shown a relationship between phototropins and Ca^{2+}, suggesting in a candidate for the chloroplast photorelocation signal, there is no conclusive evidence elucidating the signal and its transmission pathways. In *A. capillus-veneris* protonemal cells, the chloroplast avoidance response is induced by mechanical stress and is blocked by a plasma membrane calcium channel blocker, lanthanum (La^{3+}; 100 μM), and a stretch-activated channel blocker, gadolinium (Ga^{3+}; 10 μM) (Sato et al. 2001b). However, light-induced chloroplast movement including both the accumulation and avoidance responses is not affected by these channel blockers, indicating that calcium influx is not crucial in light-induced chloroplast movement, at least in fern gametophytes (Sato et al. 2001b).

It is believed that the intracellular diffusivity of signaling substances such as Ca^{2+} ions and hydrogen peroxide (H_2O_2) is very fast (Vestergaard et al. 2012; Wen et al. 2008). Direct observation of anomalous diffusion in an artificial system using an inhomogeneous fluid, a solution of the aqueous polymer hyaluronan (molecular weight 300 kDa), revealed that the diffusion coefficient of the fluorescent dye Alexa 488 (which has a molecular diameter of 1.4 nm and an approximately spherical shape) in the polymer hyaluronan was in the order of 200–250 μm^2 s^{-1} (Masuda et al. 2005), which is very fast. However, the estimated speed of signal transmission for the accumulation response is very slow, about 1 μm min^{-1} (Tsuboi and Wada 2010), suggesting that the signaling pathway might not involve the diffusion of Ca^{2+} ions or other small substances. Hence,

we investigated the involvement of diffusion of signaling factor(s) in the accumulation response using gametophyte cells of the fern *A. capillus-veneris* and found that the signal transmission pathways in the chloroplast accumulation response did not involve the simple diffusion of substances but that a biochemical pathway must be involved.

Materials and methods

Plant materials

Spores of the fern *Adiantum capillus-veneris* L. were collected from a glasshouse of Tokyo Metropolitan University in September 2015. The spores were sterilized with 10% (v/v) sodium hypochlorite solution (Nacalai Tesque, Kyoto, Japan) for 30 s and were sown between two layers of agar-gelatin film, which was made from a solution of 0.5% (w/v) agar (Wako, Osaka, Japan) and 0.05% (w/v) gelatin (Wako) and spread on a cover glass (see details in Sato et al. 1999). The spores sandwiched between two gelatin-agar films were soaked in liquid White's medium (Tsuboi and Wada 2010) and cultivated under continuous red light (20 µmol m^{-2} s^{-1}) for 10 days to obtain single-celled protonemata. The protonemata were irradiated with white light from a fluorescent tube (FHF32EX-N-H; Toshiba, Tokyo, Japan) for 10 min at 10 µmol m^{-2} s^{-1} and then incubated in the dark for 2–3 days to induce cell division in the apical part of each protonema. All experiments were conducted using two-celled protonemata adapted to darkness by 2–3 days incubation in the dark. Two-dimensional prothallus was also used. Spores sown on the White's medium solidified with 0.5% agar (Wako) were cultivated under white light (100 µmol m^{-2} s^{-1}) at 25 °C in an incubator (Biotron LH300-RPSMP; Nippon Medical and Chemical Instruments, Osaka, Japan) for 2–3 weeks and then incubated in the dark for about 2 days. The dark-adapted prothallus was set in a custom-made cuvette (Wada et al. 1983) and used for microbeam irradiation experiments.

Partial cell irradiation

To calculate the speed of signal transmission in a linear protonemal cells, a *c*. 200 µm area from the septum between the apical and basal cells of two-celled protonemata was irradiated with either a blue microbeam (190 µmol m^{-2} s^{-1}) of 8 µm diameter for temperature-dependency experiments or a red microbeam (110 µmol m^{-2} s^{-1}) of 20 µm width for other experiments for 1 min using custom-made microbeam irradiators. The chloroplast movement on both sides of the microbeam was then recorded continually every 1 min with an IR-sensitive video camera (DFC340FX; Leica Microsystems, Wetzlar, Germany) under infrared light using an infrared filter (IR85, Hoya Corp., Tokyo, Japan) or 940 nm wavelength IR LED (OptoSupply Limited, Hong Kong, China), and the chloroplast behavior was analyzed on a monitor screen (see Tsuboi and Wada 2010 for details). To observe the signal transmission pattern in non-linear cells of two-dimensional prothallia, the center of the cells was irradiated with a red microbeam (110 µmol m^{-2} s^{-1}) of 5 µm in diameter for 1 min and then the chloroplast movement from the cell periphery to the microbeam-irradiated area was recorded and the speed of signal transfer to each chloroplast was calculated. To study whether the signal was transmitted over or under chloroplasts, or had to detour around them, an area close to a chloroplast was irradiated with a red microbeam (110 µmol m^{-2} s^{-1}) of 3 µm diameter for 1 min and then recorded. The recorded chloroplast behavior was analyzed by Image J (National institutes of Health, Bethesda, MD, USA).

Oryzalin treatment

The microtubule depolymerizing factor oryzalin was used to study the involvement of microtubules in the signal transmission pathway. Oryzalin dissolved in DMSO as a 50 mM stock solution was diluted in liquid White's medium to a final concentration of 5 µM. To facilitate drug import into the cells, a 0.2% (w/v) solution of the detergent Pluronic F-127 (Sigma–Aldrich, St. Louis, MO, USA) was also added to the medium. Dark-adapted protonemata growing between two agar-gelatin films were soaked in medium with or without oryzalin for 2 h in the dark before microbeam irradiation. To confirm the depolymerization of microtubules by oryzalin, the protonemata were fixed with 8% paraformaldehyde solution after treatment with oryzalin or DMSO as a control, and then the microtubules were immunostained using an anti-tubulin antibody (a mixture of N. 356 and N. 357; Amersham International plc, Buckinghamshire, England) and fluorescein-conjugated secondary antibody (N. 1031; Amersham International plc.). The method for immunostaining of microtubules followed Murata et al. (1997).

Microbeam irradiation under a temperature-controlled microscope

A custom-made microbeam-irradiator with a custom-made temperature-controlled stage was used to study the temperature-dependency of signal transmission. Because details of the microbeam-irradiator and temperature-controlled stage will be published in elsewhere (Tanaka and Kodama et al. submitted; Fujii, Hasegawa, Hayasaki and Kodama et al. submitted), they are described only briefly here. The observation system is composed of a blue LED at 450 nm, light guiding optics, a temperature-controlled stage and

a microscope (Leica DM IL LED). The irradiation area of blue light was controlled using an iris within the light guiding optics. The temperature-controlled stage was constructed with reference to a previous study (Buchner et al. 2007). Dark-adapted, two-celled protonemata cultured between two layers of gelatin-agar film were transferred to custom-made cuvettes (25 mm in diameter, 5 mm in height, see details in Wada et al. 1983) under dim light conditions, and then the cuvettes were placed on the temperature-controlled stage. An area *c*. 200 μm from the septum in the basal cell was irradiated with a blue microbeam (180 μmol m^{-2} s^{-1}) of 8 μm diameter for 1 min. Chloroplast movements at 15, 20, 25 and 30 °C were recorded under infrared light and analyzed.

Evaluation of the speeds of signal transmission and chloroplast movement

To analyze the speed of signal transmission from the light-irradiated area to chloroplasts and the speed of chloroplast movement, the accumulation movement of each chloroplast towards the light-irradiated area was recorded with an IR-sensitive video camera as mentioned above, and then the chloroplast paths were traced on a monitor screen and analyzed by Image J (National institutes of Health, Bethesda, MD, USA). The speed of signal transmission was calculated from the relationship between the distance of the chloroplast from the microbeam-irradiated area at the start point of experiment and the time when the chloroplast started moving after microbeam irradiation (refer to Tsuboi and Wada 2010). The slope of the regression line corresponds to the speed of signal transmission. It might seem confusing to calculate the signal transmission speed using the time taken to initiate the accumulation response because if there is a lag time between the timing of excitation of photoreceptors and the release of signal substance and/or signal acceptance by the chloroplasts and induction of the motility system required for chloroplast movement. However, these lag times might be constant irrespective of the distance of each chloroplast from the microbeam-irradiated area and could be cancelled in the same temperature. In this regard, the difference in the timings observed for the accumulation responses among chloroplasts will be equivalent to the speed of signal transmission. The speed of chloroplast movement during the accumulation response was calculated from the distance that chloroplasts moved in the first 5 min of the accumulation response.

Statistical analyses

Differences of the signal transmission distances were tested by analysis of covariance (ANCOVA), with temperature or with or without oryzalin as the main factor and the signal transmission time as a covariant.

Results

Speed of signal transmission under different temperatures

Red-light-grown, single-celled protonemata were irradiated with white light for 10 min and then kept in darkness for 2–3 days to induce cell division in the apical part of each protonema. The protonemata in a cuvette were put on the temperature-controlled stage of the microbeam irradiator for 5 min to adjust to the desired temperature (15, 20, 25 or 30 °C). Then, an area *c*. 200 μm from the cell plate of the long basal cell of each two-celled protonema was irradiated with a blue microbeam of 8 μm in diameter for 1 min at each temperature (Fig. S1). The accumulation response toward the microbeam-irradiated area was continually recorded under infrared light conditions. The times when individual chloroplasts in different parts of the cell started to show the accumulation response were detected using the image J software, and the data were plotted against the distance to where each chloroplast was located at the beginning of microbeam irradiation (Fig. 1). The data obtained from chloroplasts moving toward the microbeam-irradiated area from the septum (i.e., the signal transmitted from the microbeam-irradiated area with the direction of bottom-to-apex of the cell) (Fig. 1a, c, e, g) and from the cell bottom (i.e., the signal transmitted from the microbeam-irradiated area with the direction of apex-to-bottom of the cell) (Fig. 1b, d, f, h) were visualized in different graphs. The chloroplasts closer to the light-irradiated area clearly started moving earlier and vice versa at all temperatures tested. The slope of each regression line represents the speed of signal transmission. The distance of signal transmission per unit time period increased significantly with temperature elevation from 15 to 20 °C (ANCOVA; $P < 0.001$) and from 25 to 30 °C (ANCOVA; $P < 0.05$) but not from 20 to 25 °C (ANCOVA; $P > 0.1$), in both the apical and the basal directions (Fig. 1). There is no regression line across the ordinate axis at 0, which indicates that the signals from photoreceptors reach over several μm from the microbeam-irradiated region immediately after microbeam irradiation by stray light, or that it takes some time for chloroplasts to prepare moving machinery after signal acceptance, especially under low temperature conditions at 15 °C. However, the speed of signal transmission from apex to bottom at 20 °C is slower than 15 °C, although overall feature of the scatter plot data looks reasonable (ANCOVA; $P < 0.001$). Since

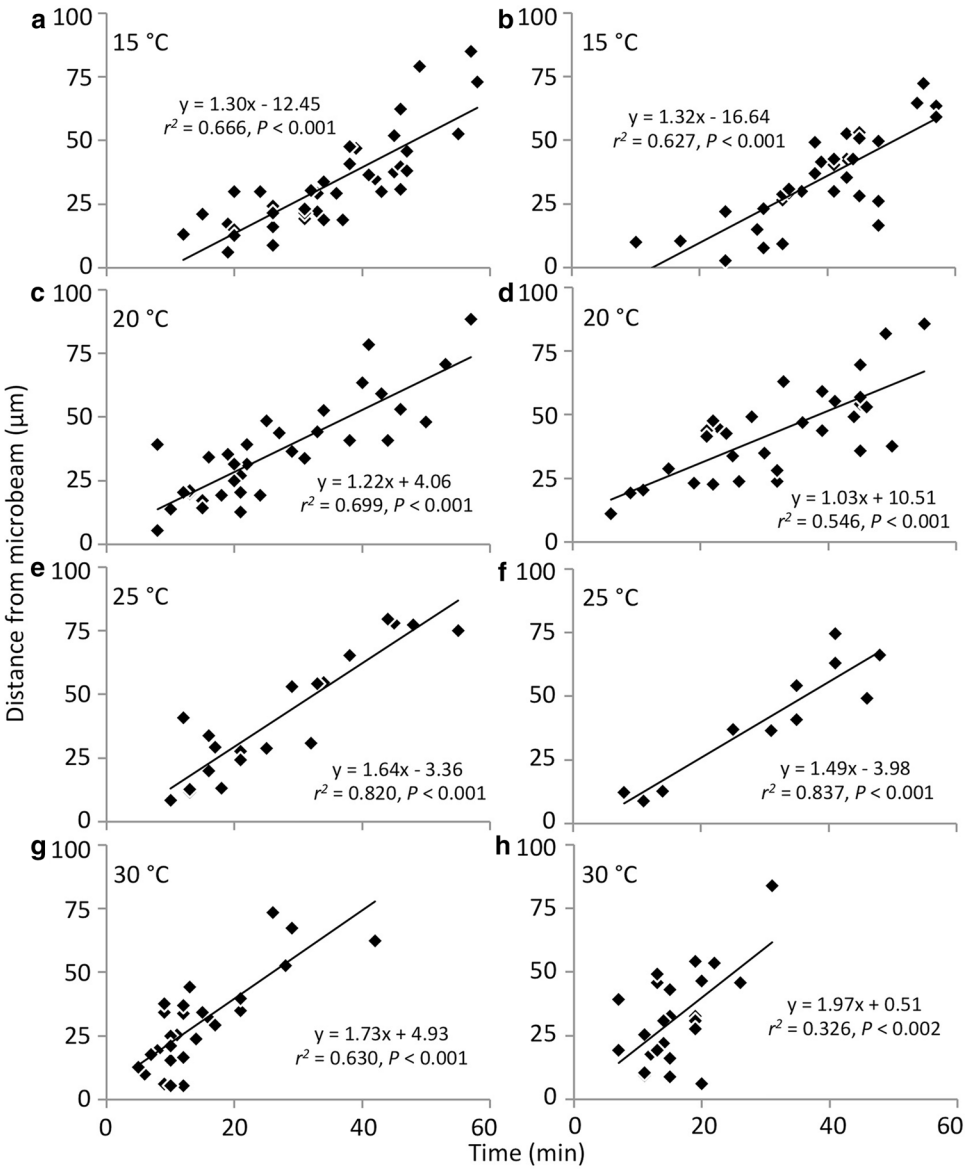

Fig. 1 The relationship between the chloroplast location (distance from the microbeam-irradiated area at 200 μm from the septum of the basal cell in two-celled protonemata of *Adiantum capillus-veneris*) and the start time of the accumulation response in each chloroplast under different temperatures. Ordinates indicate the distance of the chloroplast from the microbeam on the apical side (**a, c, e, g**) or basal side (**b, d, f, h**) of the protonema basal cell. Abscissae indicate the start time of the accumulation response in each chloroplast. The temperature was kept at 15 °C (**a, b**), 20 °C (**c, d**), 25 °C (**e, f**), and 30 °C (**g, h**)

the slope of regression line could easily be affected by unexpected data points caused by the mixture of the data from different protonemata, a different method for analysis of the speed of signal transmission under different temperature was applied and the results were shown in Fig. 2a, b.

The value in each temperature is the average of the data from 3 to 5 movies (i.e., 3–5 protonemata) in which the speed was calculated in each protonema with 5 to 10 chloroplasts. In both cases, from the cell bottom and from the cell apex, the speed of signal transmission was slow at 15 °C and more than twice as fast at 30 °C than at 15 °C, while the speeds at 20 and 25 °C were in between those at 15 and 30 °C, although the speeds at 20 and 25 °C were almost the same.

Speed of chloroplast accumulation movement at different temperatures

The speed of chloroplast accumulation movement was also dependent in temperature on both directions, from the cell bottom and from the cell apex. The movement speed at 30 °C was almost twice as fast than those at 15, 20 and 25 °C (Fig. 2c, d). It is curious that the speeds at 15, 20 and 25 °C were almost the same in both directions.

Effect of a microtubule inhibitor on the speed of signal transmission

Microtubules run parallel to the cell polarity, the growing axis of protonemal cells. Hence, the possible

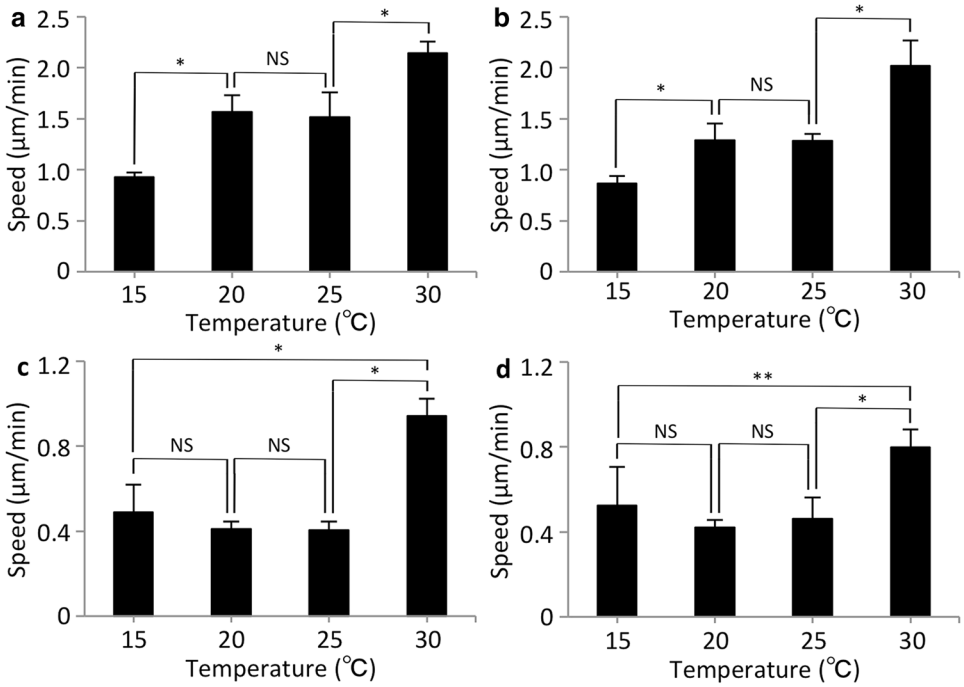

Fig. 2 The speed of signal transmission (**a, b**) and the speed of chloroplast accumulation movement (**c, d**) in the basal cell of two-celled protonemata at 15, 20, 25 and 30 °C. **a, b** The speeds of signal transmission from the cell bottom to the apex (**a**) and from the apex to the cell bottom (**b**). $*P<0.03$, *NS* no significant difference. **c, d** The speed of chloroplast accumulation movement from the apex to the cell bottom (**c**) and from the cell bottom to the cell apex (**d**). $*P<0.03$, $**P=0.13$, *NS* no significant difference. The speeds were calculated from the distance the chloroplasts migrated from the apical side (**c**) or basal side (**d**) toward the microbeam in the first 5 min of the accumulation response. All data were calculated from the time-lapse movies used for Fig. 1. *Error bars* indicate standard errors. Means and standard errors were obtained from 3 to 5 movies (i.e., 3–5 protonemata) in which the data of 5–10 chloroplasts in each movie were averaged

involvement of microtubules in the speed of signal transmission for the accumulation response was examined using oryzalin, a microtubule depolymerization factor. Before red microbeam irradiation for chloroplast accumulation induction, the protonemata were incubated in a low concentration DMSO solution with or without 5 μM oryzalin for 2 h in dark conditions, and then the speed of signal transmission was calculated. The results in Fig. 3 indicate that the speed of signal transmission from both sides, the cell bottom and the cell apex, was unaffected by the presence or absence of microtubules. Immunofluorescence microscopy was used to determine whether the concentration of oryzalin used in this study was high enough to depolymerize the microtubules (Fig. 3c, d). The microtubules were fragmented in protonemata treated with oryzalin, but long microtubules along the long axis of the protonemata were observed in the DMSO control (Fig. 3c, d), indicating that microtubules are not involved in the speed of signal transmission.

The signal is transmitted evenly in all directions in prothallial cells

To determine whether the speed of signal transmission in prothallial cells (where cell polarity is not obvious), has direction-specificity, like the polarity-dependency in protonemal cells, or shows an equal speed in all directions, the accumulation response was induced with a red-light microbeam of 5 μm diameter (110 μmol m^{-2} s^{-1} for 1 min) in dark-adapted prothallial cells, and the time when each chloroplast started accumulation movement was measured. The speed of signal transmission to each chloroplast was calculated from the time period needed for the signal to reach the chloroplast (detected by the start of the accumulation response of the chloroplast) and the distance of the chloroplast from the microbeam. As shown in Fig. 4, chloroplasts moved towards the microbeam spot from any place, meaning that the signal was transmitted in every direction with almost the same speed; *c.* 1–1.5 μm min^{-1} on average.

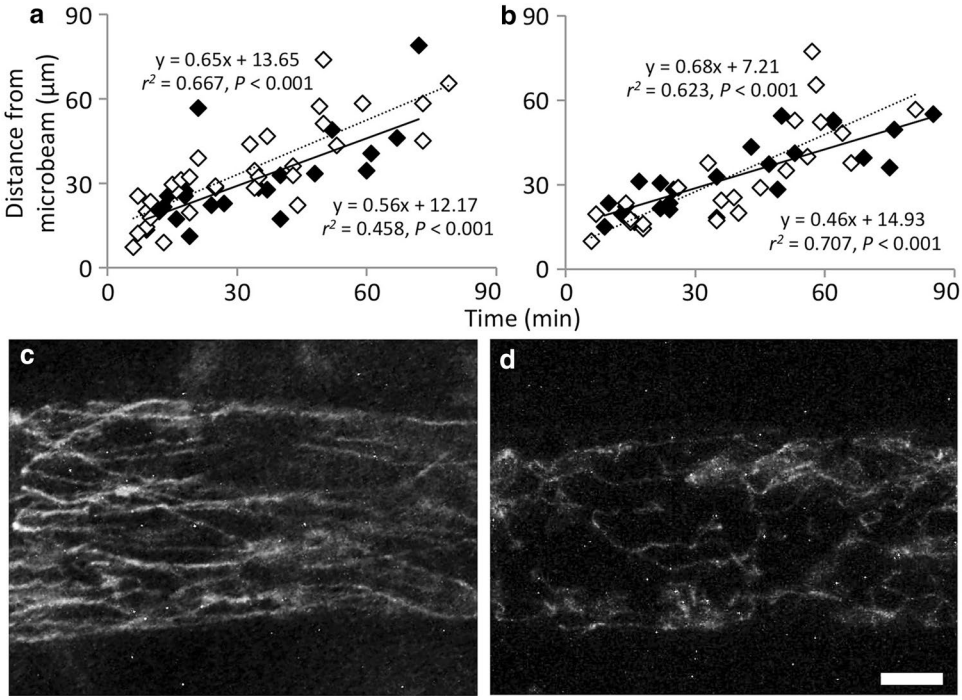

Fig. 3 The effect of oryzalin on the speed of signal transmission for the chloroplast accumulation response. The accumulation responses of chloroplasts were observed after 2 h incubation with oryzalin (*white squares*) or a low concentration of DMSO (closed *squares*) as a control. Ordinates indicate the distances between the microbeam and chloroplasts on the apical side (**a**) or basal side (**b**) of the basal cell in two-celled protonema. Abscissae indicate the start time of the accumulation response in each chloroplast. The regression lines were obtained from the data of oryzalin treatment (*dotted line*) or control (*solid line*). (**c, d**) Immunostaining of microtubules in protonemata treated with oryzalin (**d**) or a low concentration of DMSO (**c**). *Scale bar* 10 μm

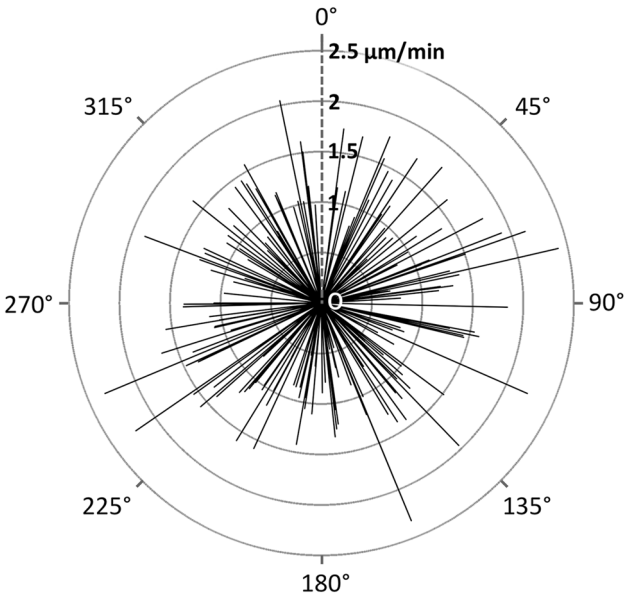

Fig. 4 The speed of signal transmission for the accumulation response on the surface of non-polar, two-dimensional cells of dark-adapted prothallia of *Adiantum capillus-veneris*. The accumulation response was induced by a short pulse of red microbeam (5 μm in diameter, 110 μmol m^{-2} s^{-1} for 1 min) irradiation. The speed of signal transmission to each chloroplast located at the cell periphery was calculated from 170 chloroplasts and plotted as a circle graph depending on the direction of signal transmission (i.e., the direction of the chloroplast at the cell periphery). The direction was measured as an angle between the prothallus polarity and a line connecting the center of the microbeam and each chloroplast. The speed is shown as the length of a radial bar. The direction 0° represents signal transmission from the apical side towards the basal side, parallel to the prothallus polarity

There were a few signals with rapid transmission, as indicated by chloroplasts that started accumulation movement early. This happened in random directions and did not have any directional specificity (Fig. S2).

The signal is transmitted without detouring chloroplasts

As the signal was transmitted in all directions but with variability in speed as shown in Fig. 4, the question arose whether the signal was transmitted freely irrespective of the presence of organelles or had to detour around organelles as obstacles. Because chloroplasts are the largest organelles in cells excepting nuclei and firmly attach to the plasma membrane at their peripheral region with CHUP1 (Chloroplast Unusual Positioning 1) proteins, we tested whether the signal dispersed to avoid and detour chloroplast-containing areas or was transmitted straight to the microbeam-irradiated area, passing through the narrow space between the chloroplasts and the plasma membrane. The chloroplast accumulation response was induced with a red microbeam located near to a chloroplast as an obstacle in prothallial cells. The speeds of signal transmission to the chloroplasts from the microbeam with or without the chloroplast as an obstacle were calculated (Fig. 5a). Surprisingly, chloroplasts whose paths to the microbeam were blocked by the obstructing chloroplast moved straight towards the microbeam (Fig. 5a). Moreover, the speeds of signal transmission were the same irrespective of the presence of the obstructing chloroplast (Fig. 5b), meaning that the signal was

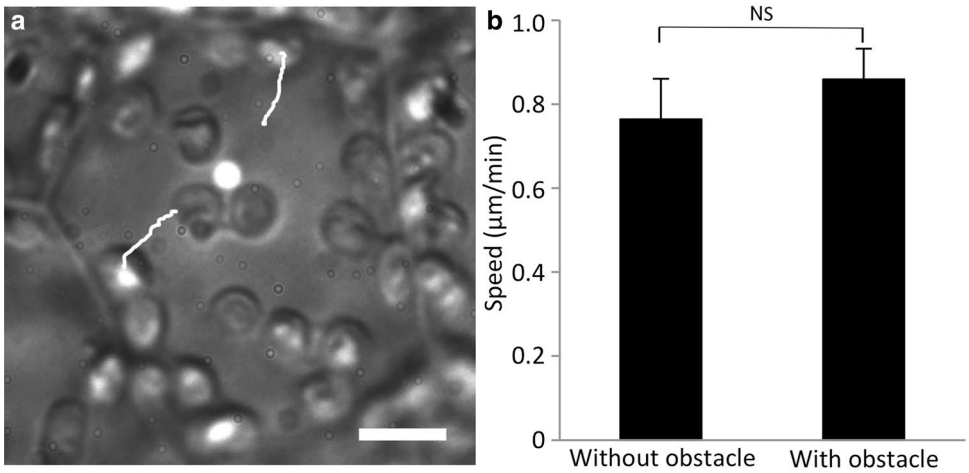

Fig. 5 The speed of signal transmission through chloroplasts. The accumulation response was induced in dark-adapted prothallial cells by a short irradiation with a red microbeam (3 µm in diameter, 110 µmol m^{-2} s^{-1} for 1 min) illuminated close to a chloroplast as an obstacle. **a** The paths of the two chloroplasts were traced and shown with *white lines*. *Scale bar* 10 µm. **b** The speeds of signal transmission from the microbeam to chloroplasts at the cell periphery with or without the obstacle chloroplast in between them were calculated. *Error bars* indicate standard errors obtained from four movies. Each movie includes 3–5 chloroplasts "with obstacle" or "without obstacle". *NS* no significant difference

spread evenly without detouring the obstructing chloroplast and reached chloroplasts beyond the obstructing chloroplast, probably through the space between the chloroplast and the plasma membrane. Similar chloroplast behavior was repeatedly observed in all experiments performed.

Discussion

Ca^{2+} ions, phosphoinositides, and reactive oxygen species have all been thought to be part of the signaling pathway of chloroplast movement (Banaś et al. 2012), but no concrete evidence has been presented about the signal(s). More information on the signal characteristics would be valuable to infer the real second messenger for chloroplast movement; thus, that the temperature dependency of signal transmission was examined in this work.

In our results, the speed of signal transmission for the accumulation response increased roughly as much as two times with a 10 °C increase. This fits the Arrhenius equation in chemical reactions, suggesting the involvement of a chemical reaction during the signal transmission. Conversely, the diffusion coefficient is proportional to the absolute temperature, known as the Stokes–Einstein equation, meaning that a temperature rise of 10 °C affects on the diffusion speed by only 3–4%. On the other hand, the viscosity is depending on Andrade equation and shows that the higher temperature causes the lowering of liquid viscosity. Previous study reported that the viscosity of cytosol of the animal muscles was increased by 1.35-fold, and the diffusion coefficient for $^{45}Ca^{2+}$ was increased by 2.04-fold with a 10 °C increase (Sidell and Hazel 1987). Therefore, it is still possible that the diffusion of unknown substances is involved in chloroplast accumulation response. However, the diffusion coefficient of 247 µm^2 s^{-1} for $^{45}Ca^{2+}$ at 25 °C is very fast compared to the signal transmission speed of 1.02–1.97 µm s^{-1} at 15–30 °C in the accumulation response calculated in this study. Thus, there is a possibility that an enzymatic reaction, not only the simple diffusion of a signaling substance such as Ca^{2+} ions, phosphoinositides, or reactive oxygen species, is involved in the signal transmission of accumulation response. Furthermore, we showed that signals could be transmitted along the plasma membrane being attached to or embedded in the membrane or through a narrow space of ectoplasm between the plasma membrane and a chloroplast whose peripheral region was attached tightly to the plasma membrane when observed under electron microscopy (Wada and O'Brien 1975; Wada and Kong 2011) probably with an N-terminal half of CHUP1 (Oikawa et al. 2003). Based on these characteristics, the signal might be transmitted by relaying from one factor to another factor localized on or close to the plasma membrane. Specifically, some proteins localized on the plasma membrane such as KINESIN-LIKE PROTEIN FOR ACTIN-BASED CHLOROPLAST MOVEMENT (KAC) (Suetsugu et al. 2010) and PLASTID MOVEMENT IMPAIRED 1 (PMI1) (DeBlasio et al. 2005) are possible candidates, because their mutants are deficient in or have strongly altered chloroplast movement, although the precise roles of these proteins are still to be identified.

The speed of signal transmission is dependent on cell polarity in *A. capillus-veneris* protonemal cells.

Transmission from the cell bottom to the cell apex is faster than in the opposite direction, from the cell apex to the cell bottom (Tsuboi and Wada 2010), however the speed of signal transmission from the cell bottom and from the cell apex were similar in this work (Fig. 2). This is probably because, in the previous study, the speeds of signal transmission both from the bottom and from the apex were measured in the same region—from the septum down to 200 µm—with microbeam irradiation at 200 µm from the septum and near the septum, respectively (Tsuboi and Wada 2010). In this study, however, the 200 µm region from the septum was irradiated with a spot of 8 µm diameter and the speed of signal transmission as well as chloroplast movement were measured on both sides, from the septum to the 200 µm region and in the area from 200 to *c*. 400 µm. It is plausible that different parts of protonemal cells have different sensitivities for signal transmission, because the speed of chloroplast accumulation movement in protonemata is faster farther down from the septum (Tsuboi and Wada 2010), although the reason for this is not known.

Microtubules in the long protonemal cells of *A. capillus-veneris* run parallel to the cell axis (Murata et al. 1987; Murata and Wada 1989), suggesting they are involved in the signal transmission because they play an important role in intracellular transportation using kinesin and/or dynein (Romagnoli et al. 2003; Shimmen and Yokota 2004) as motor proteins. Furthermore, microtubules and actin filaments are used for chloroplast movement in the moss *Physcomitrella patens* (Sato et al. 2001a). However, our results shown in Fig. 3 contradict this possibility because the depolymerization of microtubules by oryzalin had no effect on the speed of signal transmission. Interestingly, the slope of the regression lines corresponding to the speed of signal transmission decreased compared to the results of Fig. 1. It is assumed that the solvent DMSO and/or detergent Pluronic F-127 affect the signal transmission for chloroplast accumulation response.

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Author contributions Authors have made the following contributions to the manuscript: TH, YK, MW planned and designed the research. TH performed the experiments. SH, YH, YK designed and constructed a system for microbeam irradiation in a temperature-controlled microbeam irradiator.

References

Anielska-Mazur A, Bernaś T, Gabryś H (2009) In vivo reorganization of the actin cytoskeleton in leaves of *Nicotiana tabacum* L. transformed with plastin–GFP: correlation with light-activated chloroplast responses. BMC Plant Biol 9:1–14. doi:10.1186/1471-2229-9-64

Babourina O, Newman I, Shabala S (2002) Blue light-induced kinetics of H^+ and Ca^{2+} fluxes in etiolated wild-type and phototropin-mutant *Arabidopsis* seedlings. Proc Natl Acad Sci USA 99:2433–2438. doi:10.1073/pnas.042294599

Banaś AK, Aggarwal C, Łabuz J, Sztatelman O, Gabryś H (2012) Blue light signalling in chloroplast movements. J Exp Bot 63:1559–1574. doi:10.1093/jxb/err429

Baum G, Long JC, Jenkins GI, Trewavas AJ (1999) Stimulation of the blue light phototropic receptor NPH1 causes a transient increase in cytosolic Ca^{2+}. Proc Natl Acad Sci USA 96:13554–13559. doi:10.1073/pnas.96.23.13554

Buchner O, Lütz C, Holzinger A (2007) Design and construction of a new temperature-controlled chamber for light and confocal microscopy under monitored conditions: biological application for plant samples. J Microsc 225:183–191. doi:10.1111/j.1365-2818.2007.01730.x

DeBlasio SL, Luesse DL, Hangarter RP (2005) A plant-specific protein essential for blue-light-induced chloroplast movements. Plant Physiol 139:101–114. doi:10.1104/pp.105.061887

Gabryś H (2012) Blue-light-activated chloroplast movements: progress in the last decade. In: Lüttge U, Beyschlag W, Büdel B, Francis D (eds) Progress in Botany 73. Springer-Verlager, Berlin, pp 189–205

Harada A, Sakai T, Okada K (2003) Phot1 and phot2 mediate blue light-induced transient increases in cytosolic Ca^{2+} differently in *Arabidopsis* leaves. Proc Natl Acad Sci USA 100:8583–8588. doi:10.1073/pnas.1336802100

Hepler PK (2005) Calcium: a central regulator of plant growth and development. Plant Cell 17:2142–2155. doi:10.1105/tpc.105.032508

Higa T, Wada M (2015) Clues to the signals for chloroplast photorelocation from the lifetimes of accumulation and avoidance responses. J Integr Plant Biol 57:120–126. doi:10.1111/jipb.12310

Jarillo JA, Gabryś H, Capel J, Alonso JM, Ecker JR, Cashmore AR (2001) Phototropin-related NPL1 controls chloroplast relocation induced by blue light. Nature 410:952–954. doi:10.1038/35073622

Kadota A, Yamada N, Suetsugu N, Hirose M, Saito C, Shoji K, Ichikawa S, Kagawa T, Nakano A, Wada M (2009) Short actin-based mechanism for light-directed chloroplast movement in *Arabidopsis*. Proc Natl Acad Sci USA 106:13106–13111. doi:10.1073/pnas.0906250106

Kagawa T, Wada M (1994) Brief irradiation with red or blue light induces orientational movement of chloroplasts in dark-adapted prothallial cells of the fern *Adiantum*. J Plant Res 107:389–398. doi:10.1007/BF02344062

Kagawa T, Wada M (1996) Phytochrome- and blue light-absorbing pigment-mediated directional movement of chloroplasts in dark-adapted prothallial cells of fern *Adiantum* as analyzed by microbeam irradiation. Planta 198:488–493. doi:10.1007/BF00620067

Kagawa T, Wada M (1999) Chloroplast-avoidance response induced by high-fluence blue light in prothallial cells of the fern *Adiantum capillus-veneris* as analyzed by microbeam irradiation. Plant Physiol 119:917–923. doi:10.1104/pp.119.3.917

Kagawa T, Sakai T, Suetsugu N, Oikawa K, Ishiguro S, Kato T, Tabata S, Okada K, Wada M (2001) *Arabidopsis* NPL1: a phototropin homolog controlling the chloroplast high-light

avoidance response. Science 291:2138–2141. doi:10.1126/science.291.5511.2138
Kagawa T, Kasahara M, Abe T, Yoshida S, Wada M (2004) Function analysis of phototropin2 using fern mutants deficient in blue light-induced chloroplast avoidance movement. Plant Cell Physiol 45:416–426. doi:10.1093/pcp/pch045
Kasahara M, Kagawa T, Oikawa K, Suetsugu N, Miyao M, Wada M (2002) Chloroplast avoidance movement reduces photodamage in plants. Nature 420:829–832. doi:10.1038/nature01213
Kawai H, Kanegae T, Christensen S, Kiyosue T, Sato Y, Imaizumi T, Kadota A, Wada M (2003) Responses of ferns to red light are mediated by an unconventional photoreceptor. Nature 421:287–290. doi:10.1038/nature01310
Kong SG, Wada M (2014) Recent advances in understanding the molecular mechanism of chloroplast photorelocation movement. Biochim Biophys Acta 1837:522–530. doi:10.1016/j.bbabio.2013.12.004
Kong SG, Suetsugu N, Kikuchi S, Nakai M, Nagatani A, Wada M (2013a) Both phototropin 1 and 2 localize on the chloroplast outer membrane with distinct localization activity. Plant Cell Physiol 54:80–92. doi:10.1093/pcp/pcs151
Kong SG, Arai Y, Suetsugu N, Yanagida T, Wada M (2013b) Rapid severing and motility of cp-actin filaments are required for the chloroplast avoidance response. Plant Cell 25:572–590. doi:10.1105/tpc.113.109694
Masuda A, Ushida K, Okamoto T. (2005) Direct observation of spatiotemporal dependence of anomalous diffusion in inhomogeneous fluid by sampling-volume-controlled fluorescence correlation spectroscopy. Physi Rev E 72, 060101(R). doi:10.1103/PhysRevE.72.060101
Murata T, Wada M (1989) Organization of cortical microtubules and microfibril deposition in response to blue-light-induced apical swelling in a tip-growing *Adiantum* protonemal cell. Planta 178:334–341. doi:10.1007/BF00391861
Murata T, Kadota A, Hogetsu T, Wada M (1987) Circular arrangement of cortical microtubules around the subapical part of a tip-growing fern protonema. Protoplasma 141:135–138. doi:10.1007/BF01272895
Murata T, Kadota A, Wada M (1997) Effects of blue light on cell elongation and microtubule orientation in dark-grown gametophytes of *Ceratopteris richardii*. Plant Cell Physiol 38:201–209. doi:10.1093/oxfordjournals.pcp.a029153
Nozue K, Kanegae T, Imaizumi T, Fukuda S, Okamoto H, Yeh KC, Lagarias JC, Wada M (1998) A phytochrome from the fern *Adiantum* with features of the putative photoreceptor NPH1. Proc Natl Acad Sci USA 95:15826–15830
Oikawa K, Kasahara M, Kiyosue T, Kagawa T, Suetsugu N, Takahashi F, Kanegae T, Niwa Y, Kadota A, Wada M (2003) CHLOROPLAST UNUSUAL POSITIONING1 is essential for proper chloroplast positioning. Plant Cell 15:2805–2815. doi:10.1105/tpc.016428
Romagnoli S, Cai G, Cresti M (2003) In vitro assays demonstrate that pollen tube organelles use kinesin-related motor proteins to move along microtubules. Plant Cell 15:251–269. doi:10.1105/tpc.005645
Sakai T, Kagawa T, Kasahara M, Swartz TE, Christie JM, Briggs WR, Wada M, Okada K (2001) Arabidopsis nph1 and npl1: blue light receptors that mediate both phototropism and chloroplast relocation. Proc Natl Acad Sci USA 98:6969–6974. doi:10.1073/pnas.101137598
Sato Y, Kadota A, Wada M (1999) Mechanically induced avoidance response of chloroplasts in fern protonemal cells. Plant Physiol 121:37–44. doi:10.1104/pp.121.1.37
Sato Y, Wada M, Kadota A (2001a) Choice of tracks, microtubules and/or actin filaments for chloroplast photo-movement is differentially controlled by phytochrome and a blue light receptor. J Cell Sci 114:269–279
Sato Y, Wada M, Kadota A (2001b) External Ca^{2+} is essential for chloroplast movement induced by mechanical stimulation but not by light stimulation. Plant Physiol 127:497–504. doi:10.1104/pp.010405
Shimmen T, Yokota E (2004) Cytoplasmic streaming in plants. Curr Opin Cell Biol 16:68–72. doi:10.1016/j.ceb.2003.11.009
Sidell BD, Hazel JR (1987) Temperature affects the diffusion of small molecules through cytosol of fish muscle. J Exp Biol 129:191–203
Stoelzle S, Kagawa T, Wada M, Hedrich R, Dietrich P (2003) Blue light activates calcium-permeable channels in *Arabidopsis* mesophyll cells via the phototropin signaling pathway. Proc Natl Acad Sci USA 100:1456–1461. doi:10.1073/pnas.0333408100
Suetsugu N, Wada M (2009) Chloroplast photorelocation movement. In: Sandelius AS, Aronsson H (eds) The Chloroplast-Interaction with Environment. Plant Cell Monographs. Sprinter, Berlin, pp 235–266
Suetsugu N, Mittmann F, Wagner G, Hughes J, Wada M (2005) A chimeric photoreceptor gene, NEOCHROME, has arisen twice during plant evolution. Proc Natl Acad Sci USA 102:13705–13709. doi:10.1073/pnas.0504734102
Suetsugu N, Yamada N, Kagawa T, Yonekura H, Uyeda TQP, Kadota A, Wada M (2010) Two kinesin-like proteins mediates actin-based chloroplast movement in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 107:8860–8865. doi:10.1073/pnas.0912773107
Tlałka M, Fricker M (1999) The role of calcium in blue-light-dependent chloroplast movement in *Lemna trisulca* L. Plant J 20:461–473. doi:10.1046/j.1365-313x.1999.00621.x
Tlałka M, Gabryś H (1993) Influence of calcium on blue-light-induced chloroplast movement in *Lemna trisulca* L. Planta 189:491–498. doi:10.1007/BF00198211
Trump BF, Berezesky IK, Sato T, Laiho KU, Phelps PC, DeClaris N (1984) Cell calcium, cell injury and cell death. Environ Health Perspect 57:281–287
Tsuboi H, Wada M (2010) Speed of signal transfer in the chloroplast accumulation response. J Plant Res 123:381–390. doi:10.1007/s10265-009-0284-y
Tsuboi H, Wada M (2013) Chloroplasts continuously monitor photoreceptor signals during accumulation movement. J Plant Res 126:557–566. doi:10.1007/s10265-012-0542-2
Tsuboi H, Suetsugu N, Kawai-Toyooka H, Wada M (2007) Phototropins and neochrome1 mediate nuclear movement in the fern *Adiantum capillus-veneris*. Plant Cell Physiol 48:892–896. doi:10.1093/pcp/pcm057
Vestergaard CL, Flyvbjerg H, Møller IM (2012) Intracellular signaling by diffusion: can waves of hydrogen peroxide transmit intracellular information in plant cells? Front Plant Sci. doi:10.3389/fpls.2012.00295
Wada M, Kong SG (2011) Analysis of chloroplast movement and relocation in Arabidopsis. Methods in Molecular Biology 774, "Chloroplast Research in Arabidopsis", Methods and Protocols, vol 1. Jarvis RP. Humana Press, Totowa NJ, pp 87–102
Wada M, O'Brien TP (1975) Observations on the structure of the protonema of *Adiantum capillus-veneris* L. undergoing cell division following white-light irradiation. Planta 126:213–227. doi:10.1007/BF00388964
Wada M, Kadota A, Furuya M (1983) Intracellular localization and dichroic orientation of phytochrome in plasma membrane and/or ectoplasm of a centrifuged protonema of fern *Adiantum capillus-veneris* L. Plant Cell Physiol 24:1441–1447
Wen F, Xing D, Zhang LR (2008) Hydrogen peroxide is involved in high blue light-induced chloroplast avoidance movements in *Arabidopsis*. J Exp Bot 59:2891–2901. doi:10.1093/jxb/ern147

Yatsuhashi H, Kadota A, Wada M (1985) Blue- and red-light action in photoorientation of chloroplasts in *Adiantum* protonemata. Planta 165:43–50. doi:10.1007/BF00392210

Zurzycki J (1955) Chloroplast arrangements as a factor of photosynthesis. Acta Soc Bot Pol 24:27–63. doi:10.5586/asbp.1974.052