

# External $\text{Ca}^{2+}$ Is Essential for Chloroplast Movement Induced by Mechanical Stimulation But Not by Light Stimulation<sup>1[w]</sup>

Yoshikatsu Sato, Masamitsu Wada, and Akeo Kadota\*

Department of Biological Sciences, Graduate School of Science, Tokyo Metropolitan University, Minami-Osawa 1-1, Hachioji, Tokyo 192-0397, Japan (Y.S., M.W., A.K.); and National Institute for Basic Biology, Myodaiji, Okazaki 444-8585, Japan (M.W.)

In the fern *Adiantum capillus-veneris*, chloroplast movement is induced by mechanical stimulation as well as by light stimulation. Directional movement of both types depends on an actin-based motile system. To investigate the physiological relationship between mechanical and light signaling in the regulation of chloroplast movement, we examined the mechano-response of chloroplasts whose motility had been already restricted after photo-relocation. Chloroplast mechano-avoidance movement was induced under all of the photo-relocation conditions tested, indicating that mechano-specific signals generated by mechanical stimulation dominate over the light signals and reactivate the motility of chloroplasts. When the effects of external  $\text{Ca}^{2+}$  on the induction of mechano- and light responses were examined, strikingly different requirements of external  $\text{Ca}^{2+}$  were found for each. In medium without  $\text{Ca}^{2+}$ , the mechano-response was suppressed but no effects were observed on photo-response. Mechano-relocation movement of chloroplasts was inhibited by 100  $\mu\text{M}$  lanthanum ( $\text{La}^{3+}$ ), a plasma membrane calcium channel blocker, and by 10  $\mu\text{M}$  gadolinium ( $\text{Gd}^{3+}$ ), a stretch-activated channel blocker. However, the same concentrations of these drugs did not affect the photo-relocation movement at all. These results suggest that the influx of external  $\text{Ca}^{2+}$  is crucial for the early signaling step of chloroplast mechano-relocation but not for that of photo-relocation. This is the first report showing the separation of signaling pathways in mechano- and photo-relocation of chloroplasts.

Plants are non-motile and must thus suffer and cope with environmental changes to survive, whereas animals can move away from hazardous conditions. Among many environmental factors, light and mechanical stimulation are the most significant stresses to plants. Plants use these stimuli as signals for the regulation of development and morphology to adapt to the environment. Responses caused by these two environmental factors are known as photomorphogenesis and thigmomorphogenesis, respectively (Jaffe, 1973; Kendrick and Kronenberg, 1994). In addition to macroscopic responses, such as the morphology of individuals, plants also respond rapidly and reversibly at microscopic level

to these fluctuating signals. One of the most prominent responses at this level is an intracellular relocation of chloroplasts.

Chloroplast movement directed by light has been known over 100 years and was comprehensively described in the first decade of the 20th century (Senn, 1908). Chloroplasts move to a position where they can most effectively absorb light of moderate fluence rate for efficient photosynthesis, but they move away from regions of potentially hazardous light conditions where the fluence rate is too high. This photo-relocation movement of chloroplasts is well known in various plant cells and the effective wavelength is limited to the blue light (BL) region in many plants (Zurzycki, 1980). In some lower plants, however, phytochrome as well as a BL receptor also mediate chloroplast movement (Haupt and Scheuerlein, 1990; Wada et al., 1993; Kadota et al., 2000). In particular, in *Adiantum capillus-veneris*, similar effects of polarized light on chloroplast relocation are observed for both red light (RL) and BL, which are mediated by dichroic phytochrome and a dichroic BL receptor, respectively (Yatsuhashi et al., 1985).

Chloroplast movement in *A. capillus-veneris* is an excellent model system for studying the signal transduction pathways downstream of both light and mechano-perception. We recently discovered a mechano-relocation movement of chloroplasts in protonemal cells of the fern; that is, chloroplasts

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\* Corresponding author; e-mail kadota-akeo@c.metro-u.ac.jp; fax 81-426-77-2559.

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move away from the mechanically stimulated site (Sato et al., 1999). In this system, we can easily observe the response under the microscope and mechanical stimulation is readily applied with a capillary to portions of intact cells, which become the perception site of mechanical stimulation. Even brief stimulation can induce mechano-relocation of chloroplasts. The motile system of chloroplast mechano-relocation is found to be dependent on actin microfilaments, as is the case for photo-relocation movement (Kadota and Wada, 1992; Sato et al., 1999).

Until now, no studies have been performed on the relationship between light and mechano-signaling at the cellular level. In the present study, we show the utility of the fern protonemal cells in this regard. We first demonstrate the occurrence of mechano-relocation movement in photo-relocated chloroplasts, indicating that signaling after mechanical stimulus affects the actin-based motility of chloroplasts independent from light signaling. Examining the effects of external  $\text{Ca}^{2+}$  on mechano- and photo-relocation of chloroplasts, we report different requirement of external  $\text{Ca}^{2+}$  in signaling of mechano- and photo-relocation movement.

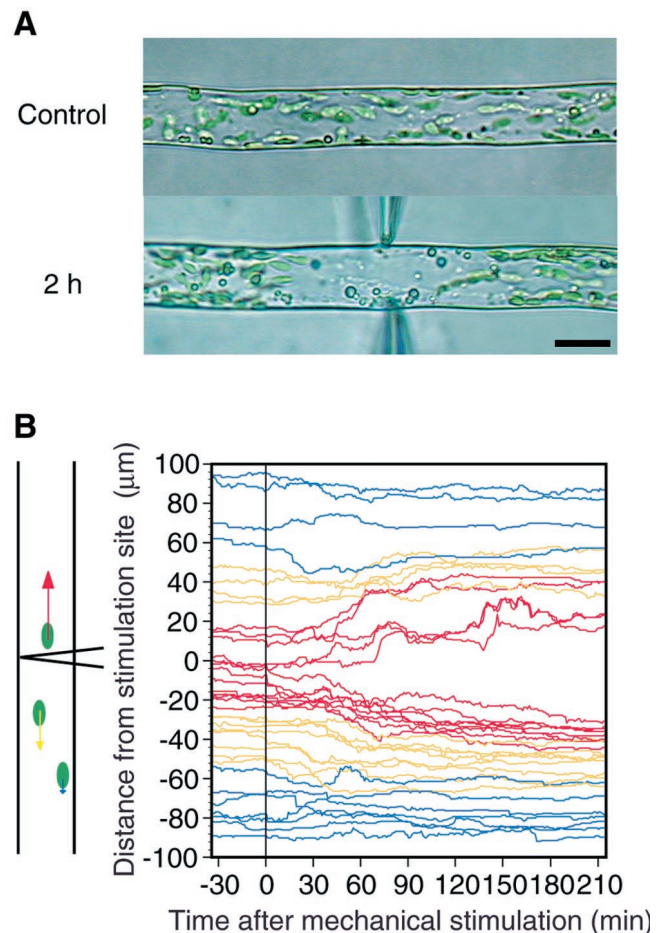
## RESULTS

### Tracking of Chloroplasts during Mechano-Relocation Movement

The avoidance response of chloroplasts induced by mechanical stimulation was analyzed using infrared video microscopy in the dark to exclude any light effects on chloroplast movement. When mechanical stimulation was continuously applied to part of a protonema from both sides with the flanks of bent capillaries, the chloroplasts moved away from the stimulated site, but oil drops kept moving constantly regardless of the stimulation site (Fig. 1A; see also video supplement). Brief stimulation periods shorter than 1 min were enough to induce the avoidance response to the maximum level (Sato et al., 1999). Therefore, the paths of individual chloroplasts along the long axis of the cell were analyzed by tracking each chloroplast at 1-min intervals before and after a 1-min mechanical stimulation (Fig. 1B). Directional movement of chloroplasts away from the mechanically stimulated site was apparent in an area within 25  $\mu\text{m}$  of the stimulation site, whereas the chloroplasts located further than 50  $\mu\text{m}$  from the site did not show any avoidance response. Thus, the avoidance signal generated by mechanical stimulation could be diffusible but may not reach beyond 50  $\mu\text{m}$  from the stimulation point.

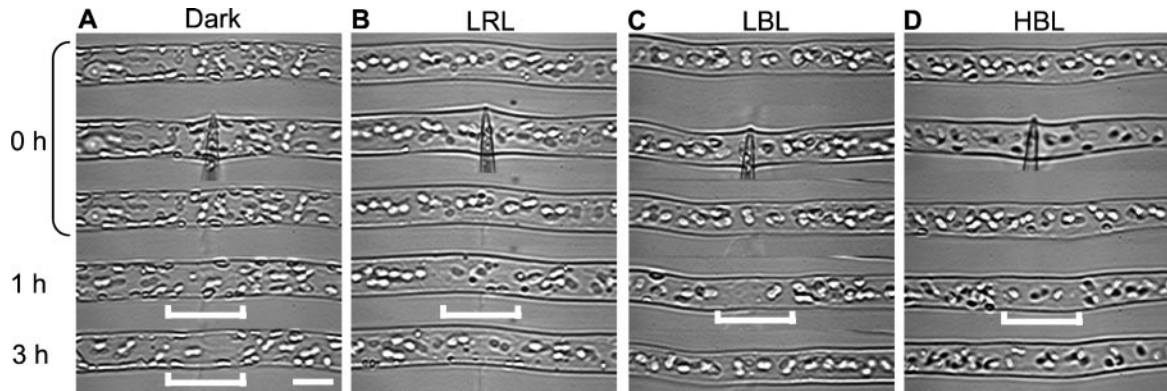
### Mechano-Relocation Movement of Photo-Relocated Chloroplasts

To investigate whether mechano-relocation movement was evoked in chloroplasts that have already



**Figure 1.** Mechano-avoidance movement of chloroplasts in the dark. A, A protonemal cell was continuously stimulated from both sides of the cell with the flank of bent microcapillaries. The avoidance movement of chloroplasts can be seen in a video supplement at [www.plantphysiol.org](http://www.plantphysiol.org). B, Time course of avoidance movement of individual chloroplast induced by short-term (1 min) mechano-stimulation from one side (vertically) with the flank of a capillary. Bar, 20  $\mu\text{m}$ .

had certain intracellular positions after photo-relocation movement, we applied mechanical stimulation to cells under light irradiation. Photo-relocation of chloroplasts was induced by continuous irradiation from the tip of protonemata with polarized light. For low fluence rate responses, horizontally polarized light of low fluence rate RL (LRL, 0.44  $\text{W m}^{-2}$ ) and BL (LBL, 0.79  $\text{W m}^{-2}$ ) were used and vertically polarized light of high fluence rate BL (HBL, about 10  $\text{W m}^{-2}$ ) was employed for the high fluence rate response. As shown in Figure 2, chloroplasts accumulated in a line along the upper or lower surface of the cells, and movement at the relocation site ceased after 3 h of polarized light treatment. While maintaining irradiation with polarized light, we also applied mechanical stimulation to part of the cell using the flank of a capillary for 1 min. Mechano-avoidance responses of chloroplasts were observed 1 h after mechanical stimulation in all the light condition tested (Fig. 2, B–D). The relationship between



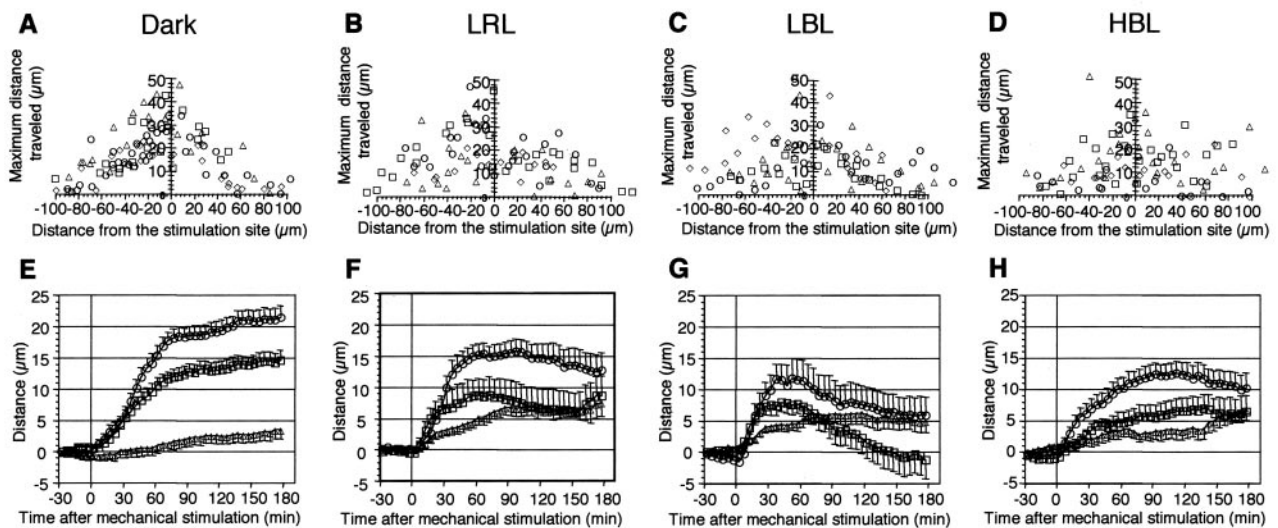
**Figure 2.** Serial images of mechano-avoidance response of photo-relocated chloroplasts. Mechanical stimulation was applied vertically with the flank of a capillary for 1 min. A, Dark control; B through D, cells were continuously pre-irradiated with polarized light for 3 h before mechanical stimulation and the light was kept on throughout the experiments. B, LRL ( $0.44 \text{ W m}^{-2}$ ); C, LBL ( $0.79 \text{ W m}^{-2}$ ); D, HBL (about  $10 \text{ W m}^{-2}$ ). Bar,  $20 \mu\text{m}$ .

chloroplast location from the stimulation site and the maximum distance traveled after mechanical stimulation was investigated (Fig. 3, A–D). There was a common feature. Regardless of the light conditions used, the nearer the chloroplasts were to the stimulation site, the further they moved away. Time courses of chloroplast mechano-relocation movement showed that under polarized light conditions, the chloroplasts moved away for about 1 h. However, they tended to relocate to the stimulated area in the next 1 to 2 h, particularly in LBL, whereas in cells stimulated in the dark, this recovery movement was not observed within 3 h of mechanical stimulation (Figs. 2 and 3, E–H). Thus, the avoidance response in

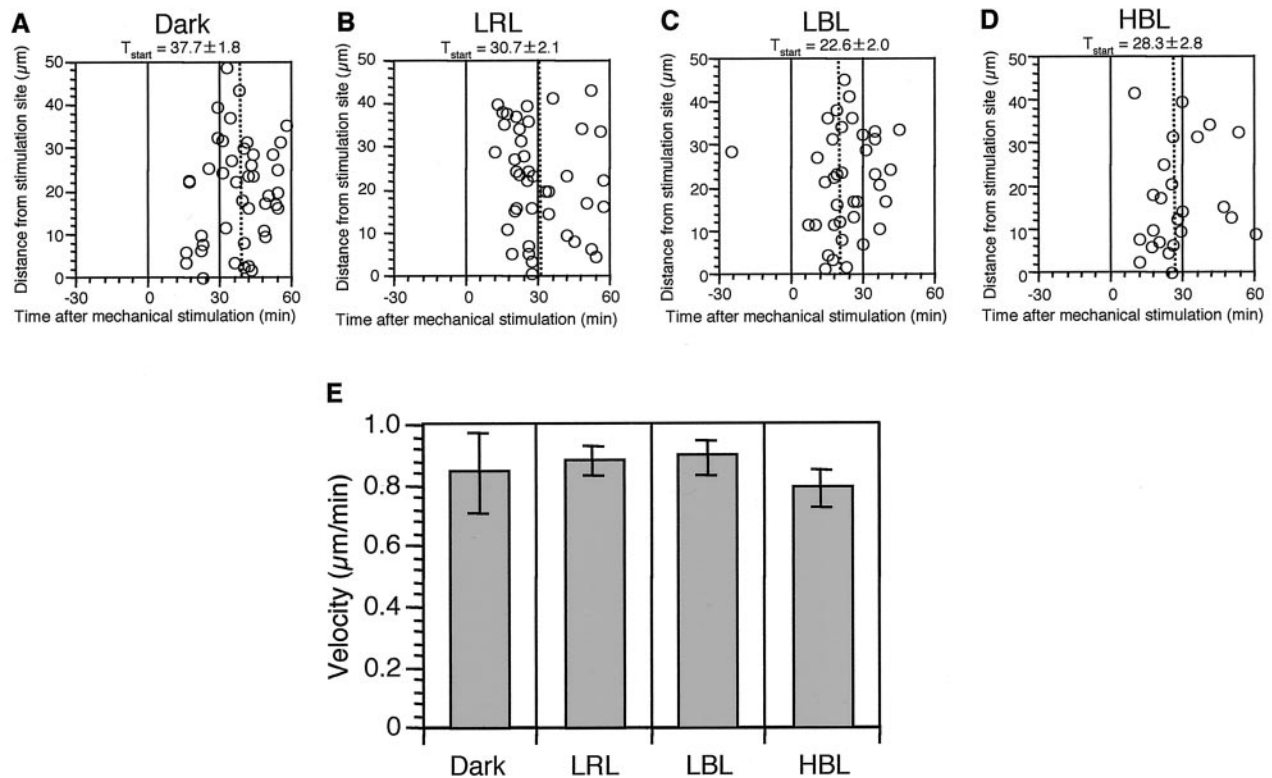
the dark-adapted cells was the largest among all light conditions tested (Fig. 3, E–H).

#### Lag Period in Mechano-Relocation Response and Velocity of Chloroplast Movement

Next, we measured how long it took for chloroplasts to start to move away after mechanical stimulation. We found that there was no relationship between the initial intracellular location of the chloroplasts and the lag period before chloroplasts started to move away under any of the light conditions tested (Fig. 4, A–D). When the lag periods are compared for different light treatments, they are longest in cells stimulated in the



**Figure 3.** Mechano-avoidance response of photo-relocated chloroplasts. A through D, Relationship between the initial location of chloroplasts and the maximum distance traveled. Different symbols indicate different cells analyzed. E through H, Time course of chloroplast avoidance response under various light conditions. Chloroplasts were divided into three groups with respect to their initial location before mechanical stimulation: those located with in  $25 \mu\text{m}$  ( $\circ$ ),  $25$  to  $50 \mu\text{m}$  ( $\square$ ), and over  $50 \mu\text{m}$  ( $\triangle$ ). Each point represents the mean  $\pm$  SE. A and E, Dark control; B and F, LRL; C and G, LBL; D and H, HBL.



**Figure 4.** Time lag and velocity of chloroplasts during avoidance response. A through D, Time lag between the mechanical stimulation and the start of chloroplast relocation movement. A, Dark control; B, LRL; C, LBL; D, HBL. Means and  $\text{SE}$  are also presented on the top of each figure and the mean is also indicated with dotted line in each graph. E, Velocity of chloroplasts during avoidance movement. Each bar represents the mean  $\pm$  SE derived from analyses of 24 to 42 chloroplasts from four protonemata. Data were obtained from the same cells used in Figure 3.

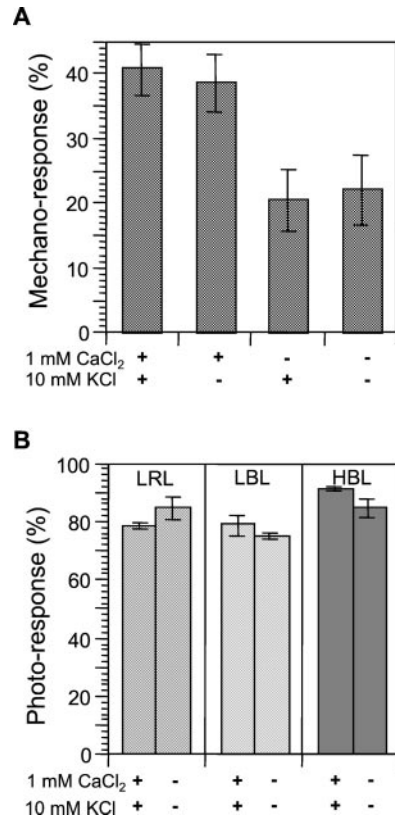
dark. We also calculated the velocity of chloroplast movement during the avoidance response (Fig. 4E). No apparent difference in chloroplast velocities was found between any of the light conditions, and the average value was about  $0.8$  to  $0.9 \mu\text{m min}^{-1}$ .

#### Effects of External $\text{Ca}^{2+}$ on the Mechano- and Photo-Relocation of Chloroplasts

The data for mechano-induced avoidance movement in photo-relocated chloroplasts indicate that mechanical stimulation dominates over the light signal in directing chloroplasts, and that further, mechano-relocation may be regulated differently from the actin-based chloroplast motility associated with photo-relocation. The actin cytoskeleton is known to be regulated by  $\text{Ca}^{2+}$  in many aspects of cell function. Thus, we tested the effects of external  $\text{Ca}^{2+}$  on mechano- and photo-relocation. Both mechano- and photo-relocation of chloroplasts was evident when cells were kept in 10 mM MES [2-(*N*-morpholino)-ethanesulfonic acid] buffer (pH 6.0) supplemented with 1 mM  $\text{CaCl}_2$  and 10 mM KCl. Chloroplast mechano-relocation caused by 1 min of stimulation was inhibited by omitting  $\text{CaCl}_2$  from the medium, whereas no effect was observed when KCl

was excluded (Fig. 5A). In contrast, chloroplast photo-relocation responses induced by continuous irradiation with polarized light were not affected at all by omission of both  $\text{CaCl}_2$  and KCl from the medium (Fig. 5B).

Next, we examined the effects of the calcium channel blocker, lanthanum ( $\text{La}^{3+}$ ), and the stretch channel inhibitor, gadolinium ( $\text{Gd}^{3+}$ ), in MES buffer containing 1 mM  $\text{CaCl}_2$  and 10 mM KCl (Fig. 6). Mechano-relocation of chloroplasts was inhibited by these ions in a dose-dependent manner.  $\text{La}^{3+}$  was inhibitory at a concentration of less than  $100 \mu\text{M}$  (Fig. 6A). The inhibitory effects of  $\text{Gd}^{3+}$  were stronger than  $\text{La}^{3+}$  and mechano-relocation was abolished at a concentration of less than  $10 \mu\text{M}$  (Fig. 6B). In contrast, these reagents did not inhibit chloroplast photo-relocation. These results suggest that external  $\text{Ca}^{2+}$  is crucial for induction of mechano-relocation of chloroplasts. To further confirm the differences in the effects of these drugs between mechano- and photo-relocation, mechanical stimulation was continuously applied while photo-relocation was induced by microbeam irradiation. With the latter, we could induce an avoidance response with RL because we could obtain a fluence rate of  $600 \text{ W m}^{-2}$ , which is necessary to induce the



**Figure 5.** External  $\text{Ca}^{2+}$  dependency of mechano- and photo-relocation movement. Cells were pre-incubated for 2 h in 10 mM MES with or without supplement of 1 mM  $\text{CaCl}_2$  and/or 10 mM KCl. A, Chloroplast mechano-relocation was inhibited in the medium without  $\text{Ca}^{2+}$ . Each bar represents the mean  $\pm$  SE obtained from 10 protonemata. B, Chloroplast photo-relocation was not affected by the external ions. Each bar represents the mean  $\pm$  SE derived from analysis of 150 or more protonemata from three dishes.

high fluence rate response with RL. Figure 7 shows representative images of mechano- and photo-relocation of chloroplasts. Clear mechano- and photo-relocation were observed in medium containing 1 mM  $\text{CaCl}_2$  (Fig. 7, A–E). When 100  $\mu\text{M}$   $\text{Gd}^{3+}$  was used in the medium containing 1 mM  $\text{CaCl}_2$ , mechano-relocation of chloroplasts was largely inhibited, but the inhibition was incomplete (data not shown). Complete abolition of mechano-relocation was observed when  $\text{Gd}^{3+}$  at 100  $\mu\text{M}$  added to the medium without supplementing with  $\text{CaCl}_2$  (Fig. 7F). We also examined photo-relocation movement under these conditions; namely, the least favorable for mechano-relocation (Fig. 7, G–J). There was no effect on photo-relocation movement in response to light quality, either via accumulation or avoidance. We obtained almost the same results when  $\text{La}^{3+}$  was used instead of  $\text{Gd}^{3+}$  (data not shown). Together, these data suggest that an influx of  $\text{Ca}^{2+}$  from extracellular sites is essential for induction of chloroplast mechano-relocation, but not for photo-relocation.

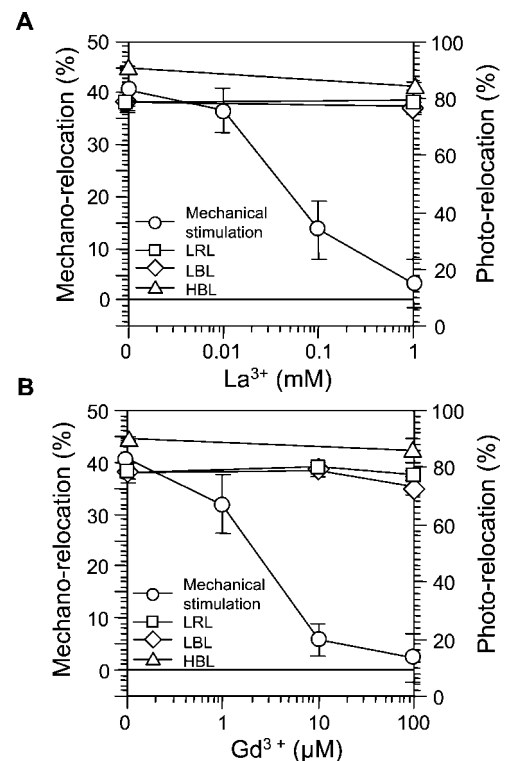
## DISCUSSION

### Chloroplast Behavior after Mechanical Stimulation

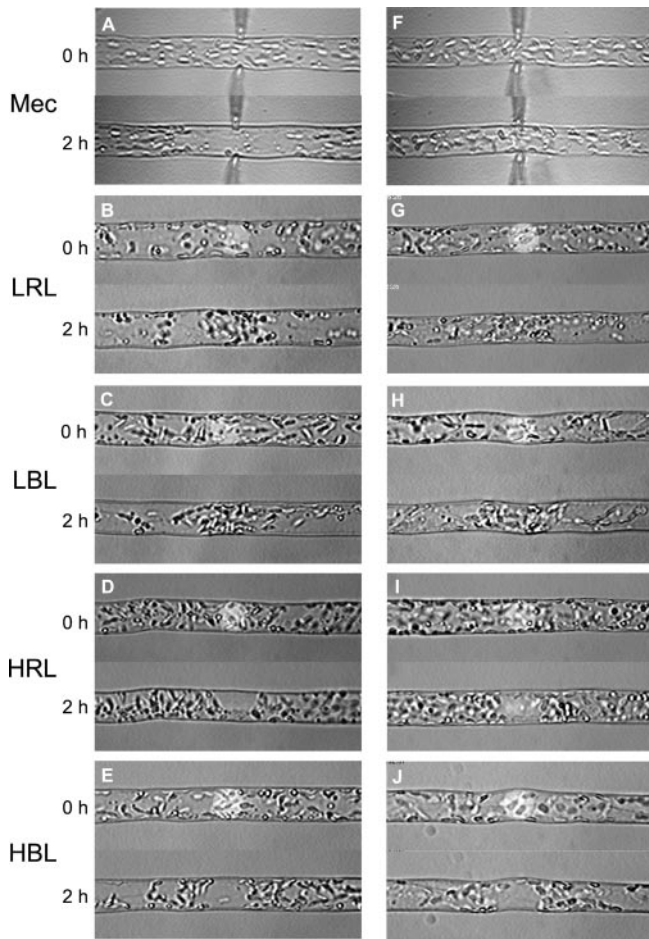
In this study, we first analyzed the behavior of individual chloroplasts during mechano-relocation using computer recording systems, which enable us to analyze at a higher time resolution than in our previous study (Sato et al., 1999). The kinetics of mechano-relocation movement showed similar features to those of photo-relocation. The directional movement of chloroplasts was clearly seen in mechano-relocation photo-relocation. The velocities of both movements were close to each other (Kagawa and Wada, 1996, 1999). Furthermore, both stimulation movements depend on the acto-myosin system (Kadota and Wada, 1992, Sato et al., 1999). Therefore, a common motile system may be shared by both responses, although the molecular motor responsible has yet to be identified.

### Mechano-Relocation Movement of Photo-Relocated Chloroplasts

In the previous study, when chloroplast photo-relocation response attained the stationary state, the photo-relocated chloroplasts were surrounded by actin filaments and their motility was reduced, indicat-



**Figure 6.** Effects of  $\text{Ca}^{2+}$  channel blockers on mechano- and photo-relocation of chloroplasts. Cells were pretreated with each drug in 10 mM MES containing 1 mM  $\text{CaCl}_2$  and 10 mM KCl for 2 h. A, Effects of  $\text{La}^{3+}$  on mechano- and photo-relocation. B, Effects of  $\text{Gd}^{3+}$  on mechano- and photo-relocation. Each point represents the mean  $\pm$  SE. Other details are the same as in Figure 5.



**Figure 7.** Representative images of chloroplast mechano- and photo-relocation. Mechanical and microbeam light stimulation was continuously applied for 2 h. A through E, Mechano- and photo-relocation of chloroplasts in 10 mM MES containing 1 mM  $\text{CaCl}_2$  and 10 mM KCl. F through J, Mechano- and photo-relocation of chloroplasts in 10 mM MES containing 10 mM KCl and 100  $\mu\text{M}$   $\text{Gd}^{3+}$ . A and F, Mechanical stimulation (Mec); B and G, LRL (1  $\text{W m}^{-2}$ ); C and H, LBL (1  $\text{W m}^{-2}$ ); D and I, HRL (600  $\text{W m}^{-2}$ ); E and J, HBL (10  $\text{W m}^{-2}$ ). A through J, Each panel shows the response in the same cell.

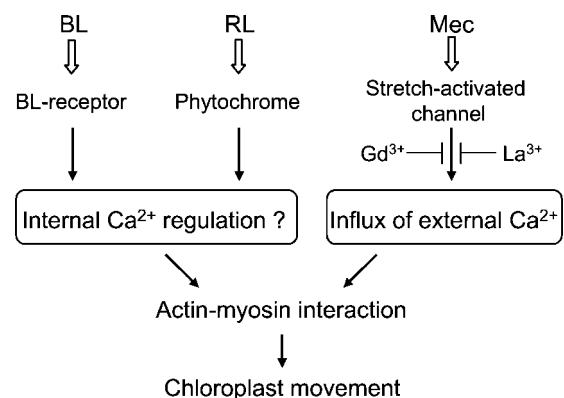
ing that they were mechanically anchored to the location by actin cytoskeleton (Kadota and Wada, 1989, 1992). This notion is also supported by studies showing that photo-relocated chloroplasts become resistant to centrifugal force in the epidermal cells of *Vallisneria gigantea* (Takagi et al., 1991; Dong et al., 1998). In this study, we clearly showed that the mechano-avoidance response occurred in these anchored chloroplasts under all the photo-relocation conditions tested (Fig. 2), suggesting that different signal transduction pathways are activated and regulate chloroplasts in mechano- and photo-relocation. It is surprising that the lag time before avoidance movement occurs was longer in the dark than that under any of the photo-relocation conditions tested, whereas no significant difference in the velocity of chloroplast avoidance movement was observed among the conditions (Fig. 4). These findings may

indicate that reactivation of a motor apparatus by the mechano-signal leads to avoidance of chloroplasts using the actin tracks already arranged around them by photo-relocation, rather than the availability of energy for movement under photosynthetic conditions.

### Roles of $\text{Ca}^{2+}$ in Mechano- and Photo-Relocation of Chloroplasts

Many pharmacological studies have been performed to investigate the role of  $\text{Ca}^{2+}$  on chloroplast movement induced exclusively by light (Haupt and Scheuerlein, 1990; Nagai, 1993; Wada et al., 1993, and references therein). The presence of  $\text{Ca}^{2+}$  is essential for chloroplast photo-movement, but whether it functions in the light-signaling processes remains unclear because of the problem on the specificity of drugs used to disturb  $\text{Ca}^{2+}$  level. Our fern protoneural cell experimental system has the advantage that actin-based directional system has the advantage that actin-based directional movement of chloroplasts can be induced with different stimuli, which allowed us to demonstrate the distinct contribution of external  $\text{Ca}^{2+}$  in the mechano- and photo-relocation of chloroplasts. A model for  $\text{Ca}^{2+}$  contribution in the signaling events of photo- and mechano-relocation of chloroplast is shown in Figure 8. Extracellular  $\text{Ca}^{2+}$  is necessary for mechano-relocation but not for photo-relocation. We propose that the entry of external  $\text{Ca}^{2+}$ , possibly through the stretch-activated channels (Morris, 1990), in response to localized deformation of a cell is essential for directional chloroplast movement in an early step of mechano-signaling. Because  $\text{Ca}^{2+}$  mobility in the cytoplasm is much lower than that in free solution, the result that only chloroplasts near the stimulation site respond may indicate that a localized stimulus establishes a localized  $[\text{Ca}^{2+}]_i$  gradient in the cell.

Our finding that external  $\text{Ca}^{2+}$  plays important roles in the mechano-response in *A. capillus-veneris* does not support the hypothesis based on work with transgenic plants expressing apoaquorin that  $\text{Ca}^{2+}$  influx from apoplastic sites is not required for the mechano-response (Knight et al., 1992; Haley et al.,



**Figure 8.** A model for the signaling events of photo- and mechano-relocation of chloroplast in *A. capillus-veneris*.

1995). This difference between the two studies may occur because a plant is constructed of many types of differentiated cells, and the response in specialized cells may be occluded by responses in other cells. We currently cannot detect the response of the minor cells in the analysis of a whole plant or of cell masses. Another reason may be the different ways in which mechanical stimulation was applied in the two studies: Direct touch on a cell that caused localized deformation of the cell was applied in this study, whereas wind or application of isotonic medium was used as the mechanical stimulation in the transgenic plant studies. Light-induced elevation of  $[Ca^{2+}]_i$ , on the other hand, was reported in transformed plants with an aequorin gene (Russell et al., 1998; Baum et al., 1999). The elevation in these studies was completely abolished by  $La^{3+}$  at concentrations close to those having no effects on chloroplast photo-relocation in the present study. This suggests that  $[Ca^{2+}]_i$  elevation is not involved in early signaling of photo-relocation, or the increase of  $[Ca^{2+}]_i$  in signaling for photo-relocation of chloroplasts was too small to detect in the tissue level analysis. An experimental system enabling us to detect the spatial information of  $[Ca^{2+}]_i$  at the subcellular level would be required at this point.

Finally, chloroplast movement induced by an artificial increase of  $[Ca^{2+}]_i$  via the  $Ca^{2+}$  ionophore A23187 has been reported in *Mougeotia* sp. and *Lemna trisulca* (Serin and Roux, 1984; Tlalka and Fricker, 1999). In these plants, however, the external  $Ca^{2+}$  does not play a major role in chloroplast photo-movement (Schönbohm et al., 1990a, 1990b; Tlalka and Gabryś, 1993; Tlalka and Fricker, 1999). Therefore, we can suggest the possibility that  $Ca^{2+}$  ionophore-induced chloroplast movement may result from mimicry of the signaling pathway for mechano-relocation rather than that for photo-relocation.

## MATERIALS AND METHODS

### Plant Material and Aseptic Culture

Two-celled protonemata of *Adiantum capillus-veneris* were cultured using the same procedure as described previously (Sato et al., 1999). In brief, spores of *A. capillus-veneris*, sterilized with a sodium hypochlorite, were sown in a line between two layers of agar-gelatin film on a coverslip. They were submerged in modified Murashige and Skoog liquid medium. Spores were cultured under continuous RL ( $0.5 W m^{-2}$ ) for 9 d. The protonemata were irradiated with white light ( $4.5 W m^{-2}$ ) for 6 h for induction of cell division and then kept in the dark for 2 d. The resultant two-celled protonemata were composed of a short apical cell and long basal cell. The basal cells of the protonemata were used for the present study.

### Light Sources

Fluorescent lamps (FL40SD or FL10D, Toshiba Lighting and Technology Corp., Tokyo) were used as the source of

white light. A halogen lamp (15 V, 150 W; Philips Japan, Tokyo) was also used as a light source for the experiments on the high-fluence rate response of chloroplasts. Colored light was obtained from the lamps through a red plastic filter (Shinkolite A, no. 102; Mitsubishi Rayon Co., Ltd., Tokyo) or a blue plastic film (Ryutate no. 63; Ryudensha, Tokyo). The colored lights were polarized through a linear polarizer (Polaroid HN 32 or HN38; Polaroid Corporation of Japan, Tokyo).

### Chloroplast Photo-Relocation Induced by Polarized Light

Protonemata were irradiated horizontally from the tip with either vertically or horizontally vibrating polarized light. The response of chloroplast photo-relocation was observed 3 h after the onset of polarized light irradiation in the apical 100- $\mu m$  region of the basal cell. The photo-relocation response was quantified as the percentage of protonemata in which all chloroplasts within the 100- $\mu m$  region gathered at the central zone when seen from above.

### Chloroplast Photo-Relocation Induced by Microbeam Irradiation

Microbeam irradiation was performed on a custom-made microbeam irradiator as previously described (Kadota et al., 2000). Monochromatic RL and BL were obtained through interference filters, and neutral density filters were used to attenuate the fluence rate.

### Mechanical Stimulation

Mechanical stimulation was applied to individual cells as described by Sato et al. (1999). In brief, cells were pressed with the flank of a capillary until deformation of the cell was observed under the microscope. For continuous stimulation, individual cells were stimulated with bent capillaries from both sides. The avoidance response was determined as the percentage of chloroplasts that had moved away from the stimulus region, as previously described (Sato et al., 1999). For detailed analysis of the movement of individual chloroplasts, we used the computer recording system described by Sato et al. (2001). Lag periods in chloroplast avoidance were determined as the time when they moved more than 10  $\mu m$  following the mechanical stimulation.

### Inhibitor Treatments

Lanthanum chloride and gadolinium chloride (both from Sigma, St. Louis) were dissolved in a deionized, distilled water as stock solutions of 10 mM and 100 mM, respectively. After diluting to an appropriate concentration, cells were incubated with the solution for 2 h before light and mechanical stimulation.

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