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Article

Effect of D_2O on the orientation of cortical microtubules in *Spirogyra* cells

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Effect of D_2O on the orientation of cortical microtubules (MTs) in *Spirogyra* cells was investigated. After MTs were depolymerized by 1 h treatment with an anti-MT reagent, MTs were repolymerized in various concentrations of D_2O diluted with H_2O. As the concentration of D_2O increased, cells having transverse MTs decreased and those having longitudinal MTs increased. When MTs were repolymerized in 25mM NaCl or 25mM KCl with various concentrations of D_2O, the effects of these salts apparently differed. When cells were treated with an anti-MT reagent for 24 h, most of the repolymerized MTs in 50mM NaCl with 50%(V/V) D_2O were Z-helix and were different from MTs repolymerized in the same concentration of NaCl with H_2O in their orientation. These results suggest that physicochemical properties of water molecules might be important for regulating MT orientation.

Key words: *Spirogyra*, cortical microtubules, D_2O

Water can be given a function by treating it with a weak energy. Changes in physicochemical properties are thought to be essential for these functions. Some of this water, so-called functional water, is used for living organisms (1). Therefore, it is necessary that effects of such water on living organisms should be investigated. We already reported that functional water, irradiated by far-infrared light, affected the orientation of cortical microtubules (MTs) in *Spirogyra* cells, a species of algae, in a short time (2). This result might be due to change of its physicochemical properties.

The abbreviations used are: APM, Amiprophos-methyl; APW, artificial pond water; MT, microtubule.

However, the deviation of data was large and the differences were not always clear. To determine whether the physicochemical properties of water affect the orientation of cortical MTs, water which has physicochemical properties quite different from control water (H_2O) should be investigated.

D_2O (heavy water) is different from H_2O in freezing point, boiling point, viscosity, density and so on (1). The hydrogen bond of D_2O is stronger and thermal motion is more limited. The effects of D_2O on living organisms are complicated. In the case of mammals, the level of spermatogenesis was reported to decrease (3, 4). Recently, the effect of D_2O on MTs was investigated. Tubulin, the main component of MTs, is an unstable protein. It is easily depolymerized to a monomer. However, it was reported that D_2O had an ability to stabilize MTs (5). D_2O also slows free-running rhythm (6).

In this paper, we show that D_2O clearly affected MT orientation and that the physicochemical properties of water might be important for regulating MT orientation.

Materials and methods

Plant materials and treatments with solutions

Spirogyra fluviatilis Hilse was the same species as used in the previous paper (2). Culture condition was also the same as described in the previous paper (2). It was cultured in Ichimura medium (7) under the light conditions of 16-h light and 8-h darkness at 25^0C. Cells were used for the experiments within 2 to 7 days (growing stage) after transferring them from the stock culture to the fresh Ichimura medium.

Artificial pond water (APW) (0.1mM each of KCl, NaCl, $CaCl_2$ and 5mM Hepes-Tris [pH7.5]) was used as the experimental medium when cortical MTs in *Spirogyra* cells were depolymerized. Cells were treated with 3 μg/ml Amiprophos-methyl (APM) in order to depolymerize the cortical MTs for 1 or 24 h. After APM was removed by washing the cells quickly three times with distilled water, cortical MTs were repolymerized in solutions of various constitutions for 1.5 h. D_2O (99.8%, Merck, Germany) was used for experiments. All the procedures were done at 25^0C.

Immunostaining

MTs were observed using the method of Hogetsu (8) as described in the previous paper (2). Cells were fixed in 3.7% formaldehyde in 50mM sodium phosphate buffer (pH7.0) for 30 min, cut in the same fixative with a razor blade to allow entry of antibodies and then washed three times with 50mM sodium phosphate buffer. They were treated with

a detergent solution (1% Triton X-100 and 0.4M mannitol in 50mM sodium phosphate buffer [pH7.0]) for 30 min.

After removal of the detergent, they were incubated with mouse monoclonal antibody against chick brain α-tubulin (Sigma, T9026) diluted 1:250 in phosphate-buffered saline (PBS, pH7.3) overnight. They were washed three times with 50mM sodium phosphate buffer and stained for 3 h with FITC-conjugated antibodies raised in sheep against mouse IgG (Sigma, F2883) diluted 1:200 in PBS. All the procedures were done at room temperature.

After three washes with 50mM sodium phosphate buffer, cells were mounted with a solution (50% glycerol in PBS containing 0.1% *p*-phenylenediamine dihydrochloride). The orientation of MTs was determined using an epifluorescence microscope. Orientation of MTs was classified into three categories based on MT angles to the cell axis, longitudinal (L) for 0^0-10^0, oblique (O) for 10^0-80^0, transverse (T) for 80^0-90^0. Oblique (O) was further classified into S-helix and Z-helix, which were called as SO and ZO, respectively.

Results and Discussion

Spirogyra cells have transverse MTs in their normal condition (8,9,10). After cells were incubated in APM solution for 1 h,

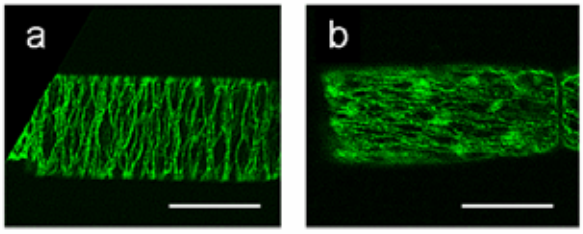

Fig. 1 Immunofluorescence images of cortical MTs in *Spirogyra* cells. a, Transverse (10^0-80^0 to the cell axis) MTs. b, longitudinal (0^0-10^0 to the cell axis) MTs. Bars=30 μm.

MTs were repolymerized in various concentrations of D_2O diluted with H_2O for 1.5 h. When they were repolymerized in H_2O, all the cells had transverse MTs (Fig. 1a, 2). As the concentration of D_2O increased, the ratio of transverse MTs decreased and that of longitudinal MTs increased (Fig. 1b, 2). It was clearly shown that D_2O affected MT orientation. Especially, MT orientation changed sensitively to D_2O concentrations

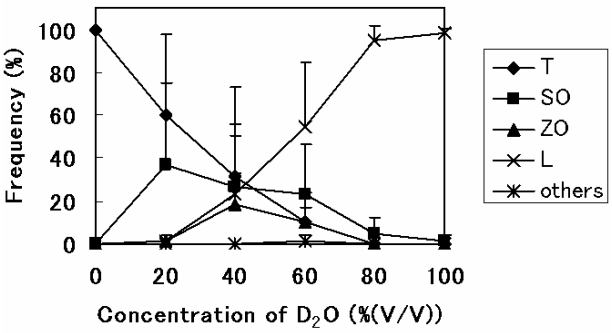

Fig. 2 The orientation of cortical MTs repolymerized in various concentrations of D_2O. T, SO, ZO and L indicate the cells having transverse, S-helix, Z-helix and longitudinal MTs, respectively. In one experiment, 20 cells were examined and 3 experiments were repeated.

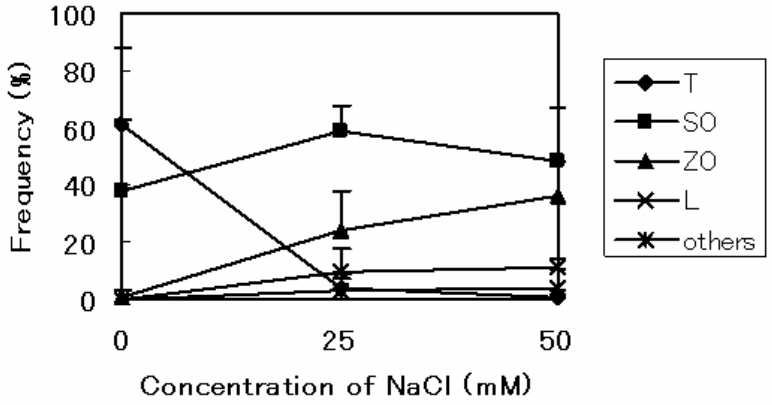

Fig. 3 The orientation of cortical MTs repolymerized in various concentrations of NaCl with 30%(V/V) D_2O. T, SO, ZO and L indicate the cells having transverse, S-helix, Z-helix and longitudinal MTs, respectively. In one experiment, 20 cells were examined and 5 experiments were repeated.

between 20 and 40%(V/V). The concentration of 30%(V/V) D_2O was adopted in the next experiment (Fig. 3).

MT orientation in *Spirogyra* cells was affected by ions (Na^+, K^+ etc.) (2, 9, 10). Therefore, the effect of D_2O on MT orientation in solutions containing NaCl or KCl was investigated. The concentration of NaCl and KCl was adjusted to 25mM because MT orientation changed sensitively to NaCl concentration around 25mM in 30%(V/V) D_2O (Fig. 3). This species of *Spirogyra* is Na^+-sensitive (2, 9, 10). In 100mM NaCl, many cells died (data not shown).

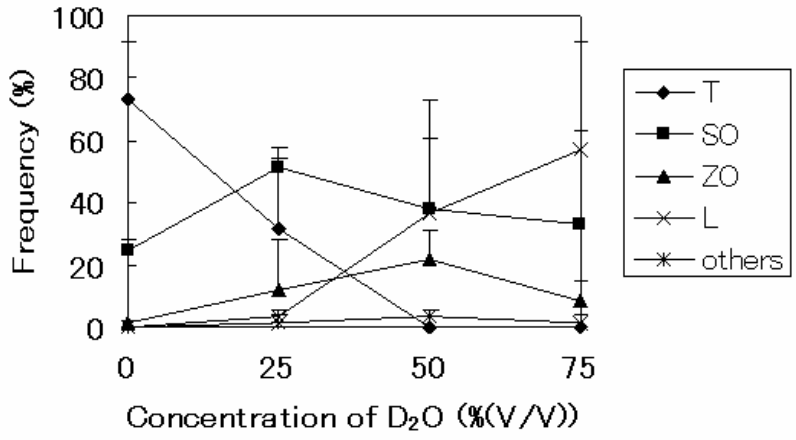

Fig. 4 The orientation of cortical MTs repolymerized in various concentrations of D_2O with 25mM NaCl. T, SO, ZO and L indicate the cells having transverse, S-helix, Z-helix and longitudinal MTs, respectively. In one experiment, 20 cells were examined and 3 experiments were repeated.

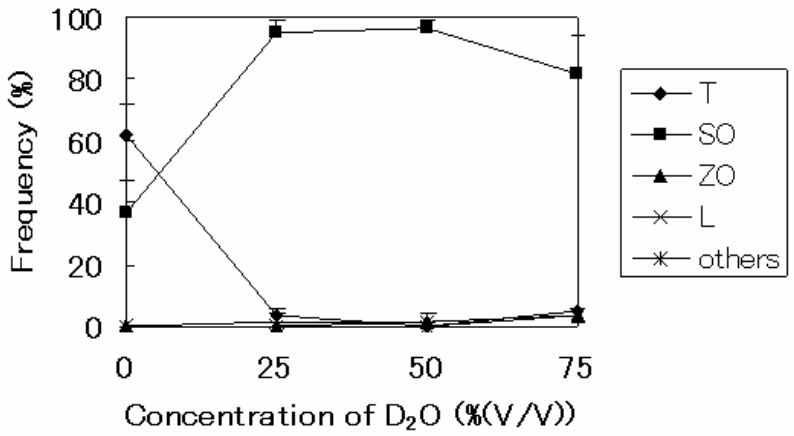

Fig. 5 The orientation of cortical MTs repolymerized in various concentrations of D_2O with 25mM KCl. T, SO, ZO and L indicate the cells having transverse, S·helix, Z·helix and longitudinal MTs, respectively. In one experiment, 20 cells were examined and 3 experiments were repeated.

After cells were incubated in APM solution for 1 h, MTs were repolymerized in 25mM NaCl with various concentrations of D_2O for 1.5 h. As the concentration of D_2O increased, cells having transverse MTs decreased and those having longitudinal MTs increased (Fig. 4). However, in the case of 25mM KCl, quite a different phenomenon was observed. When the concentration of D_2O was above 25%(V/V), MTs in about 90% of cells were S-helix (Fig.5). In H_2O, there was no apparent difference in MT orientation between NaCl and KCl (Fig.4, 5). These results suggest that Na^+ and K^+ affect MT orientation in D_2O in a different way.

As one of the authors has reported, the

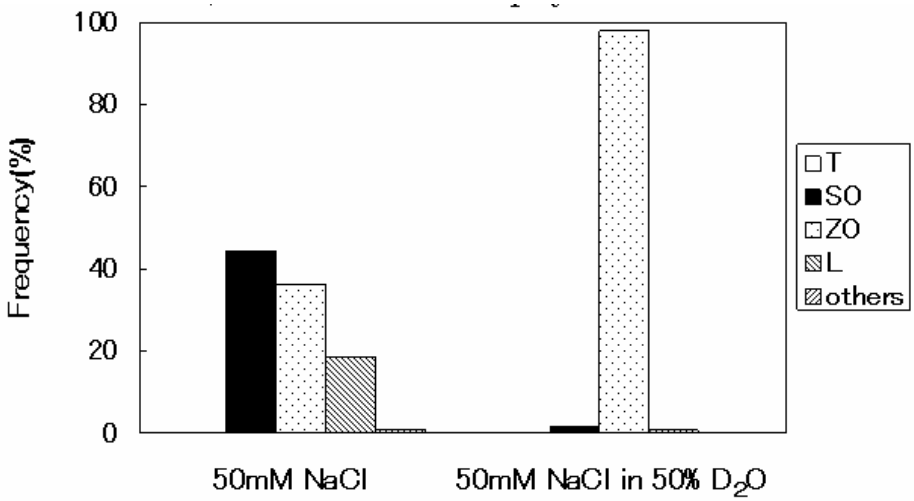

Fig. 6 The orientation of cortical MTs repolymerized in 50mM NaCl or 50mM NaCl in 50%(V/V) D_2O. T, SO, ZO and L indicate the cells having transverse, S·helix, Z·helix and longitudinal MTs, respectively. In one experiment, 20 cells were examined and 7 experiments were repeated.

effect of Na^+ and K^+ on the initial crystallization process of lysozyme was similar to this case (11). In the case of H_2O, the initial aggregation rate was about the same in both Na^+ and K^+. In the case of D_2O, the initial aggregation rate was affected by the ion species and the value was lower in K^+ than in Na^+. It is probable that the repolymerization process of MTs might be affected by these ions in a similar way as crystallization process of lysozyme, resulting in the phenomenon of the MT orientation described above. It is interesting to investigate the effect of these ions on the repolymerization process of MTs in H_2O and D_2O.

As already reported, functional water, irradiated by far-infrared light, affected the orientation of cortical microtubules (MTs) in *Spirogyra* cells (2). In this case, after cells were incubated in APM solution for 24 h, MTs were repolymerized in 50mM NaCl of control water or of functional water. The effect of D_2O was also investigated in the same conditions.

After cells were incubated in APM solution for 1 h, MTs were repolymerized in 50mM NaCl for 1.5 h. MTs of S-helix, Z-helix and longitudinal orientations were observed intermingled. However, when MTs were repolymerized in 50mM NaCl with 50%(V/V) D_2O, the orientation of MTs in above 90% of cells was Z-helix (Fig. 6). It is known that the orientation of MTs in *Spirogyra* cells changes from S-helix, Z-helix to longitudinal, as the concentration of NaCl increases (2, 10). These results suggest that the effect of D_2O on MT orientation is not through inhibiting or promoting the effect of NaCl.

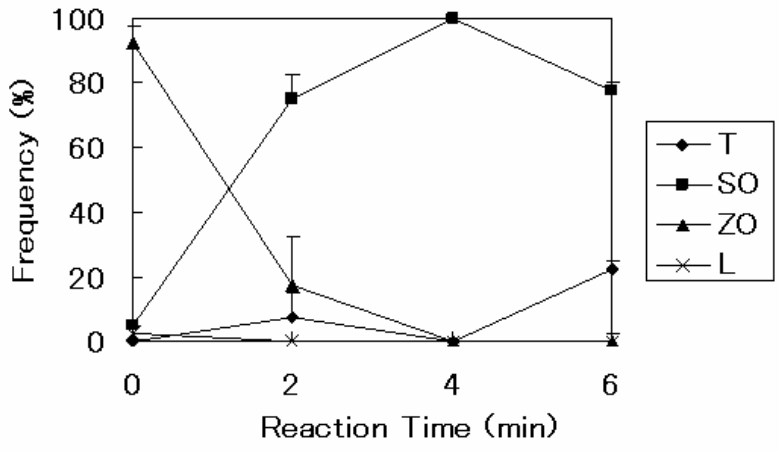

Fig. 7 Time course change of the orientation of cortical MTs repolymerized and incubated in 50mM NaCl in 50%(V/V) D_2O. T, SO, ZO and L indicate the cells having transverse, S-helix, Z-helix and longitudinal MTs, respectively. In one experiment, 20 cells were examined and 2 experiments were repeated.

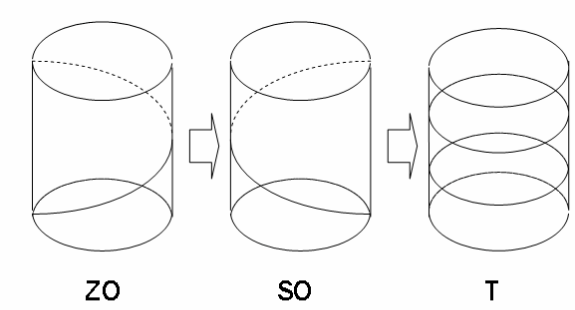

Fig. 8 Schematic drawing of change of the orientation of cortical MTs repolymerized and incubated in 50mM NaCl with 50%(V/V) D_2O.

When cells were incubated in 50mM NaCl with 50%(V/V) D_2O further, MT orientation changed peculiarly. After 4 h further incubation, most cells had MTs of S-helix. Then, they seemed to slowly change from S-helix to transverse (Fig. 7, 8). This change of MT orientation is hard to understand. However, this implies that *Spirogyra* cells might have mechanisms to adjust to Na^+ and D_2O because MT orientation in the normal condition is transverse (8, 9, 10). Further investigation of such an adjustment may be informative to understand how MT orientation might be regulated.

We showed that the physicochemical properties of water molecules surely have effects on MT orientation. We have no information about the main property of D_2O affecting MT orientation. However, if the dynamic structure of the plasma membrane could regulate MT orientation as one author described (10), the strong hydrogen bond of D_2O compared with that of H_2O might limit the motion of molecules in the plasma membrane and affect MT orientation. It is interesting to investigate the relationship between physicochemical properties of water molecules and MT orientation to know the effects of water molecules on living organisms.

References

1. Mizu Handobukku (in Japanese) pp. 573-677 (2003) Edited by Mizu Handobukku Hensyuu Iinkai. Maruzen, Tokyo.
2. Iwata and Sano (2005) J. Biol. Macromol. in press.
3. Amarose, A.P., Czajka, D.M. (1962) Cytopathic effects of deuterium oxide on the male gonads of the mouse and dog. Exp. Cell Res. 26: 43-61.
4. Oakberg, E.F., Hughes, A.M. (1968) Deuterium oxide effect on spermatogenesis in the mouse. Exp. Cell Res. 50: 306-314.
5. Chakrabarti, G., Kim, S., Gupta, Jr. M.L., Barton, J.S. and Himes, R.H. (1999) Stabilization of tubulin by deuterium oxide. Biochemistry 38: 3067-3072.
6. Enright, J.T. (1971) Heavy water slows biological timing processes. Z. Vergl. Physiol. 72: 1-16.
7. Ichimura, T. (1971) Sexual cell division and conjugation-papilla

formation in sexual reproduction of *Closterium strigosum In* K. Nishizawa, ed., Proc. of the 7th Int. Seaweed Symp. University of Tokyo Press, Tokyo, pp.208-214.

8. Hogetsu, T. (1987) Re-formation and ordering of wall microtubules in *Spirogyra* Cells. Plant Cell Physiol. 28: 875-883.
9. Iwata, K. and Itoh, T. (1998) Effect of ion on the orientation of cortical microtubules in *Spirogyra* cells. Plant Cell Physiol. 39:1099-1103.
10. Iwata, K. (2001) Studies on factors affecting the orientation of cortical microtubules in selected plant cells. Doctoral thesis for Kyoto University
11. Liu,X.Q. and Sano, Y. (1998) Effect of Na^+ and K^+ on the initial crystallization process of lysozyme in the presence of D_2O and H_2O. J. Protein Chem. 17:479-484.

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