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Effect of functional water treated with materials irradiating far-infrared light on the orientation of cortical microtubules in *Spirogyra* cells

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Effect of functional water on living organisms was investigated by using an alga, *Spirogyra*. The orientation of cortical microtubules (MTs) in *Spirogyra* cells repolymerized in functional water I containing 50mM NaCl treated by HIET plate, which irradiates far-infrared light [Nihon Reitou Yusou], after depolymerization with Amiprophos-methyl (APM), was different from that of cortical MTs repolymerized in 50 mM NaCl. Functional water I affected the orientation of cortical MTs in the same direction as the decrease of NaCl concentration. Functional water II in the presence 50mM NaCl prepared by diluting 1M NaCl with the DW treated by HIET plate also similarly affected the orientation of cortical microtubules (MTs) to functional water I. These results suggest that *Spirogyra* cells could be useful for investigating the effect of functional water on living organisms.

Key words: *Spirogyra*, cortical microtubules, functional water, far-infrared light, salt damage

Recently, some water given some function by treating with a weak energy, so-called functional water, is used in wide fields (1). Especially, in agriculture or livestock industry, functional water is used for increase of yield, improvement of quality and so on. However, there is not enough evidence that insures utility of functional water. Experimental system of estimating effects of functional water on living organisms in a short time is necessary.

In plant cells, cortical microtubules (MTs) exist just beneath the plasma membrane. The orientation of cortical MTs was found to be parallel to the orientation of cellulose microfibrils in some cases. Therefore, it is believed that cortical MTs play important role in cell

The abbreviations used are: APM, Amiprophos-methyl; APW, Artificial pond water; MT, microtubule; ORP oxidation-reduction potential.

morphogenesis (2). In *Spirogyra*, a species of algae, the orientation of cortical MTs is affected by constitution of ions (3,4), change of turgor pressure (5) and so on (4). In addition, detectable change of the orientation of cortical MTs appears within 90 min. These facts predict that subtle changes near the plasma membranes induced by water treatment may be detected in a short time. In the present study, the effect of functional water on the cortical MTs in *Spirogyra* cells is studied and then the possibility that *Spirogyra* cells could be used for evaluating functional water is examined.

The orientation of cortical MTs in *Spirogyra* cells is affected by Na^+ (3, 4). High Na^+ concentration can be harmful to some of fresh water algae and land plants and interferes with physiological processes (6,7,8,). Accumulation of salt in soil is one of the most serious problem (salt damage) inhibiting agriculture in the world. The most common salt composition of saline soils is NaCl (9). If functional water regulating the effect of Na^+ exist, it may be useful for lessening salt damage. In this study, we investigated how functional water influences the effect of Na^+ on the orientation of cortical MTs.

MATERIALS AND METHODS

Plant materials and treatments with solutions

Spirogyra fluviatilis Hilse (Fig. 1a) was isolated from a pond in Joyo, Kyoto. It was cultured in Ichimura medium (10) 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. Amiprophos-methyl (APM) was dissolved in DMSO to a concentration of 5mg/ml. Cells were treated with 3 μg/ml APM in order to depolymerize the cortical MTs for 24 h. Then the APM was removed by washing the cells quickly three times with distilled water. Cortical MTs were reorganized in various concentrations of NaCl or functional water for 1.5 h. When effects of control water and functional water were compared, the concentration of NaCl was adjusted to 50mM because MT orientation changes sensitively to NaCl concentration around 50mM (see Fig. 2). 50mM NaCl was prepared by diluting 1M NaCl with DW.

Preparation of functional water

Functional water I was prepared by placing 50mM NaCl solution on HIET plate (Nihon Reitou Yusou) irradiating far-infrared light (4 to over 20 μ

m[personal communication]) for 10min. Functional water II was prepared by placing DW on HIET plate for 10 min and then diluting 1M NaCl with the DW to 50mM NaCl.

Immunostaining

MTs were observed using the method of Hogetsu (11). Cells were fixed in 3.7% formaldehyde in 50 mM sodium phosphate buffer (pH7.0) at room temperature for 30 min. They were then cut in the same fixative with a razor blade to allow entry of antibodies. Cells were washed three times with 50mM sodium phosphate buffer and treated with a detergent solution (1% Triton X-100 and 0.4M mannitol in 50mM sodium phosphate buffer [pH7.0]) for 30 min at room temperature.

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) at room temperature, overnight. They were washed three times with 50mM sodium phosphate buffer and stained at room temperature for 3 h with FITC-conjugated antibodies raised in sheep against mouse IgG (Sigma, F2883) diluted 1:200 in PBS.

Cells were washed three times with 50mM sodium phosphate buffer and 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 are indicated as SO and ZO, respectively (see Fig.3).

Response of cells is slightly different in each experiment. MT orientation of cells treated with control water and functional water was observed at the same time in each experiment.

Statistical Analysis

Statistical analysis was performed with Wilcoxon two-way analysis of variance followed by two-sample Wilcoxon test. Z_0>1.96 was considered as statistically significant.

Measurement of pH, conductivity and oxidation-reduction potential (ORP)

pH of solutions was measured by pH/ION meter F-23 (Horiba, Ltd.). Conductivity and ORP of solutions were measured by pH/Cond METER D-24 (Horiba, Ltd.).

RESULTS AND DISCUSSION

Cortical MTs in *Spirogyra* cells are

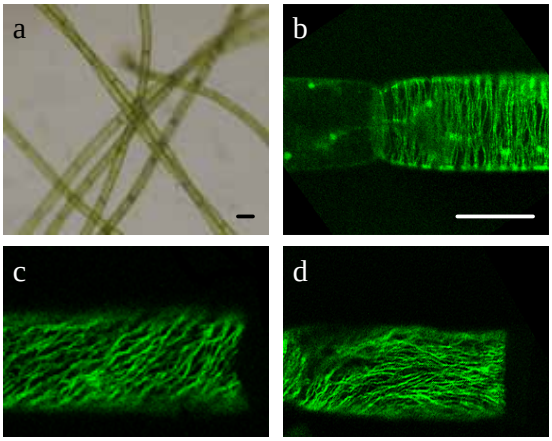

Fig. 1 Photographs of *Spirogyra* cells and immunofluorescence images of cortical MTs in *Spirogyra* cells. a, *Spirogyra* cells. b, Transverse (10^0-80^0 to the cell axis) MTs. c, oblique (10^0-80^0 to the cell axis) MTs. d, longitudinal (0^0-10^0 to the cell axis) MTs. Bars=30 μm.

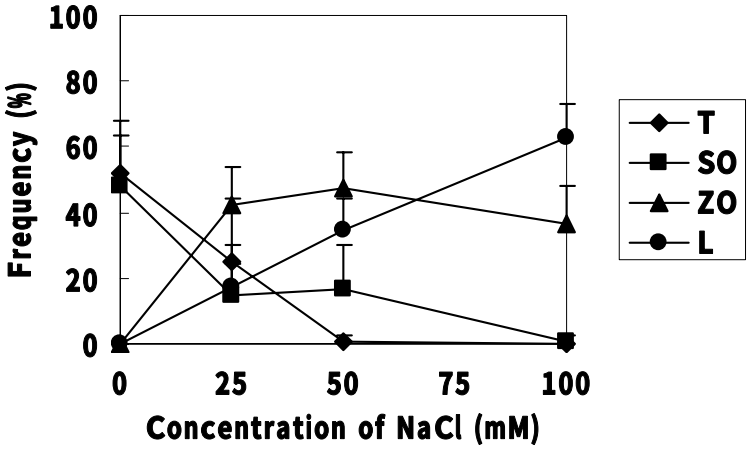

Fig. 2 The orientation of cortical MTs repolymerized in various concentrations of NaCl. T, SO, ZO and L indicate the cells having transverse, S-helix, Z-helix and longitudinal MTs, respectively. In one experiment, about 20 cells were examined and 5 experiments were repeated.

transverse in normal condition (Fig. 1b). However, the orientation of repolymerized MTs in NaCl after treatment of anti-MT agent, APM, for 24 h, changes from oblique (S-helix), oblique (Z-helix) (Fig. 1c) and then to longitudinal (Fig. 1d) as the concentration of NaCl increases (Fig.2, 3) (4).

Functional water prepared by placing 50mM NaCl solution on HIET plate for 10min (functional water I) or control water (50 mM NaCl in DW) were used as solutions for reorganization of the depolymerized MTs. In one experiment, 20 cells were examined and the

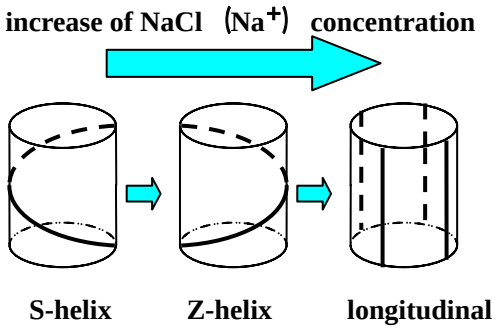

Fig. 3 Relationship between NaCl concentration and the orientation of cortical MTs

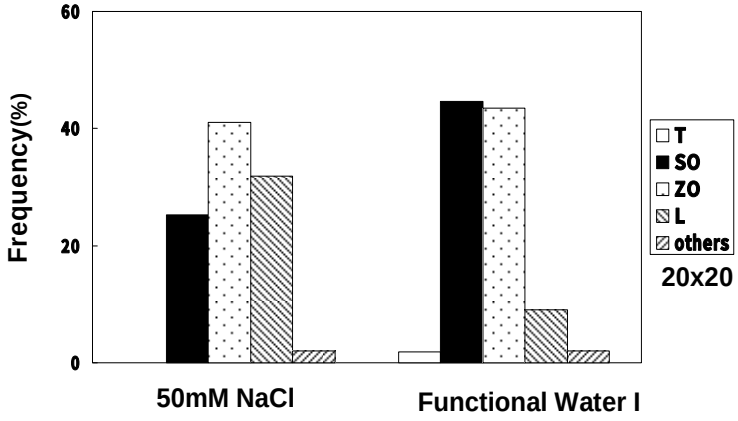

Fig. 4 The orientation of cortical MTs repolymerized in 50mM NaCl and functional water I. T, SO, ZO and L indicate the cells having transverse, S-helix, Z-helix and longitudinal MTs, respectively. In one experiment, 20 cells were examined and 20 experiments were repeated.

orientation of cortical MTs in 400 cells in total was decided in 20 experiments. In cells treated with functional water I, the ratio of cells having longitudinal MTs decreased and that of cells having oblique (S-helix) MTs increased, compared with cells treated in control water (Fig. 4). Statistic analysis showed appreciable differences. In each experiment, significant differences were observed in 10 experiments but not in the other 10 experiments. However, the changes of the orientation of cortical MTs in the direction shown by arrows in Fig. 3 were not observed after treatment with functional water. Conductivity of solution did not change by HIET treatment (Table 1). These results imply that functional water I could lessen the effect of Na^+. pH and ORP did not change, either (Table 1). Na^+ is known as an element inhibiting growth of some of fresh algae and land plants (salt damage) (6,7,8). Salt damage is one of the most serious problem limiting the amount of agricultural products. This species of *Spirogyra* is also

Table 1 pH, Conductivity and ORP of each solution. They were measured three times.

	pH	conductivity (mS/cm)	ORP(mV)
50mM NaCl	5.48±0.14	5.68±0.07	348±3
Functional Water I	5.47±0.12	5.65±0.08	347±3
Functional Water II	5.43±0.07	5.71±0.11	348±3

Na^+-sensitive. Many cells died after incubation for 24 h in 100mM NaCl solution (4). The present results may indicate a possibility that HIET-treated water may lessen salt damage without change of ionic strength.

Next, functional water II, which was prepared by placing DW on HIET plate for 10 min and diluting 1M NaCl by HIET-treated DW to 50mM, was also examined. Depolymerized MTs were reorganized in functional water II or control water (50mM NaCl in DW). In one experiment, 20 cells were examined and the orientation of cortical MTs in 200 cells in total was decided in 10 experiments. In the case of cells treated in functional water II, the ratio of cells having longitudinal MTs decreased and that of cells having oblique (S-helix) MTs increased, compared to the case of cells treated in control water (Fig. 5). Statistic analysis showed that appreciable differences existed. In each experiment, significant differences existed in 4 experiments but not in the other 6

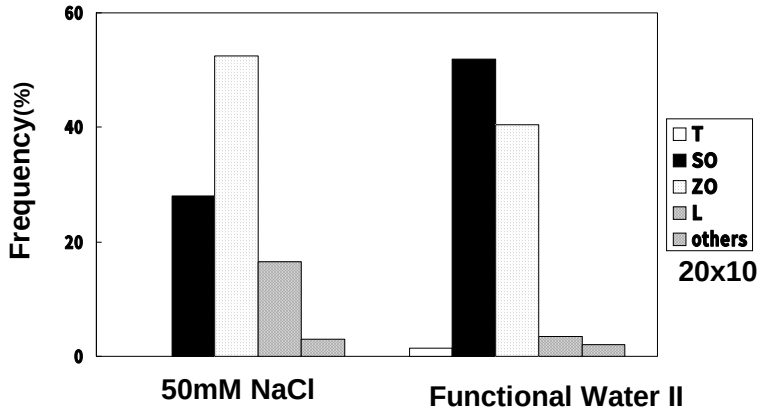

Fig. 5 The orientation of cortical MTs repolymerized in 50mM NaCl and functional water II. T, SO, ZO and L indicate the cells having transverse, S-helix, Z-helix and longitudinal MTs, respectively. In one experiment, 20 cells were examined and 10 experiments were repeated.

experiments. However, there was no experimental result that the orientation of cortical MTs after treatment with functional water II change significantly in the direction shown by arrows in Fig. 3, which was similar to the case of functional water I. This result may imply that HIET-treated DW also keeps the similar function to HIET-treated 50mM NaCl. As mentioned in materials and methods, response of cells is slightly different in each experiment. Frequency of cells in 50mM NaCl in Fig. 5 was different from that in Fig. 4.

The present study suggests that *Spirogyra* cells are useful for judging whether functional water differs from control water and inferring effects of functional water on living organisms.

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