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Correlation of Photosynthetic Products of Symbiotic *Chlorella* with the Mating Reactivity Rhythms in a Mutant Strain of *Paramecium bursaria*

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ABSTRACT—*Paramecium bursaria* shows many kinds of circadian rhythms, including a mating reactivity rhythm. *P. bursaria* cells normally contain several hundred *Chlorella* in the cytoplasm as endosymbionts. We found an interesting mutant strain (Ok2) from nature. *Chlorella*-contain green cells (Ok2) showed a normal mating reactivity rhythm in a constant light condition (LL) or constant darkness (DD). However, *Chlorella*-free white cells (Ok2w) derived from Ok2 did not display a mating reactivity rhythm in LL. In DD, they showed a normal mating reactivity rhythm. When Ok2w cells were infected with *Chlorella* isolated from green cells that show normal mating reactivity rhythms, they exhibited a circadian mating rhythm in LL. The green Ok2 cells reverted to the non-reactive state in LL by treatment with the herbicide paraquat. Sugar components in the cytosol of Ok2 and Ok2w were analyzed by HPLC, and four kinds of sugars were identified in Ok2 cells of day time. When maltose and maltotriose were added to Ok2w cell culture, the mating reactivity rhythms appeared in white cells in LL. These results suggest that the photosynthetic products of symbiotic *Chlorella* are closely related to the expression of circadian rhythms in a mutant strain of *P. bursaria*.

INTRODUCTION

Circadian rhythms are found in all levels of organisms, including both eukaryotes and prokaryotes (Kondo *et al*., 1993), as basic adaptive behavior to periodical changes in the global environment. Circadian rhythms are controlled by one or more endogenous oscillators, often called the "circadian clock." Circadian clock mechanisms have recently been investigated in molecular genetic studies (Gekakis *et al*., 1998; Rutila *et al*., 1998; Dunlap, 1998); however, the essential nature of the mechanisms has not yet been elucidated.

The unicellular ciliate *Paramecium bursaria* is an interesting model for the study of a coexisting system in intracellular symbiosis. The cells of *P. bursaria* collected from nature normally contain several hundred cells of the green alga *Chlorella* established in the cytoplasm as endosymbionts (Loefer, 1936). *Chlorella*-free white cells can be produced easily from natural green cells by rapid growth in constant darkness (DD). *Chlorella* can be isolated from host cells and re-infected easily to *Chlorella*-free white cells. Cloning culture of endsymbiotic algae isolated from *P. bursaria* was already accomplished (Nishihara *et al*., 1998). Since both green and white cells display many kinds of circadian rhythms, including mating reactivity and photoaccumulation rhythms (Miwa *et al*., 1987; Johnson *et al*., 1989), they are also suitable materials to analyze the cellular mechanisms of biological rhythms.

The sexual interaction of *Paramecium*, called mating reaction, occurs upon mixing of cells of complementary mating types when they are in the stationary phase and are sexually mature (Sonneborn, 1957; Hiwatashi, 1981). *P. bursaria* cells exhibit high mating reactivity in the light period and low reactivity in the dark period when they were exposed to a light/dark cycle (LD 12:12 hr). This rhythm is endogenously generated and continues for several days as a circadian rhythm in constant light (LL) (Ehret, 1953; Miwa *et al*., 1987). However, *Chlorella*-containing green cells of some strains show a normal mating reactivity rhythm in LL but a low mating reactivity in DD (Tanaka and Miwa, 1996).

We previously reported that symbiotic *Chlorella* forced the *Paramecium* cells to lengthen the period and to shift the phase of photoaccumulation rhythms in LL (Miwa *et al*., 1996). Furthermore, symbiotic *Chlorella* rescued the mating reactivity rhythms of arrhythmic mutant cells in LL but not in DD (Tanaka and Miwa, 1996). In this study, we found another interesting mutant strain (Ok2) from nature and their mating reactivity rhythms were characterized. *Chlorella*-free white cells (Ok2w) did not show a mating reactivity rhythm in LL. When Ok2w cells were infected with *Chlorella* isolated from green cells that showed normal mating reactivity rhythms, they exhibited a circadian mating rhythm in LL. We discuss the relationship between photosynthetic products of symbiotic *Chlo-*

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rella and the expression of circadian rhythms of Ok2w cells in LL.

MATERIALS AND METHODS

Strains and Culture

Two strains of *Paramecium bursaria* (syngen 1) were used in the experiments. Strain Ok2 (mating type III, collected in Okazaki, Japan) and Sj2 (mating type I, collected in Shimane, Japan) contain *Chlorella* symbionts and are thus designated green. *Chlorella*-free white cells, Ok2w and Sj2w cells, were induced from natural green cells of Ok2 and Sj2, respectively, by rapid growth in DD.

All strains were cultured in a 1.25% fresh lettuce juice medium in K-DS solution (0.6 mM KH_2PO_4, 1.4 mM Na_2HPO_4, 2 mM $Na_3C_6H_5O_7$, 1.5 mM $CaCl_2$, pH 7.0), that had been inoculated with K*lebsiella pneumoniae* one day before use (Hiwatashi 1968). Cultures were kept at 25°C under a light/dark cycle (LD 12:12 hr, 1,500 lux of cool-white fluorescent light).

Test of mating reactivity

The mating reactivity of green Ok2 cells was tested by mixing them with white "tester" cells of a complementary mating type. When the white cells (Ok2w) were tested, green cells were used as "tester"

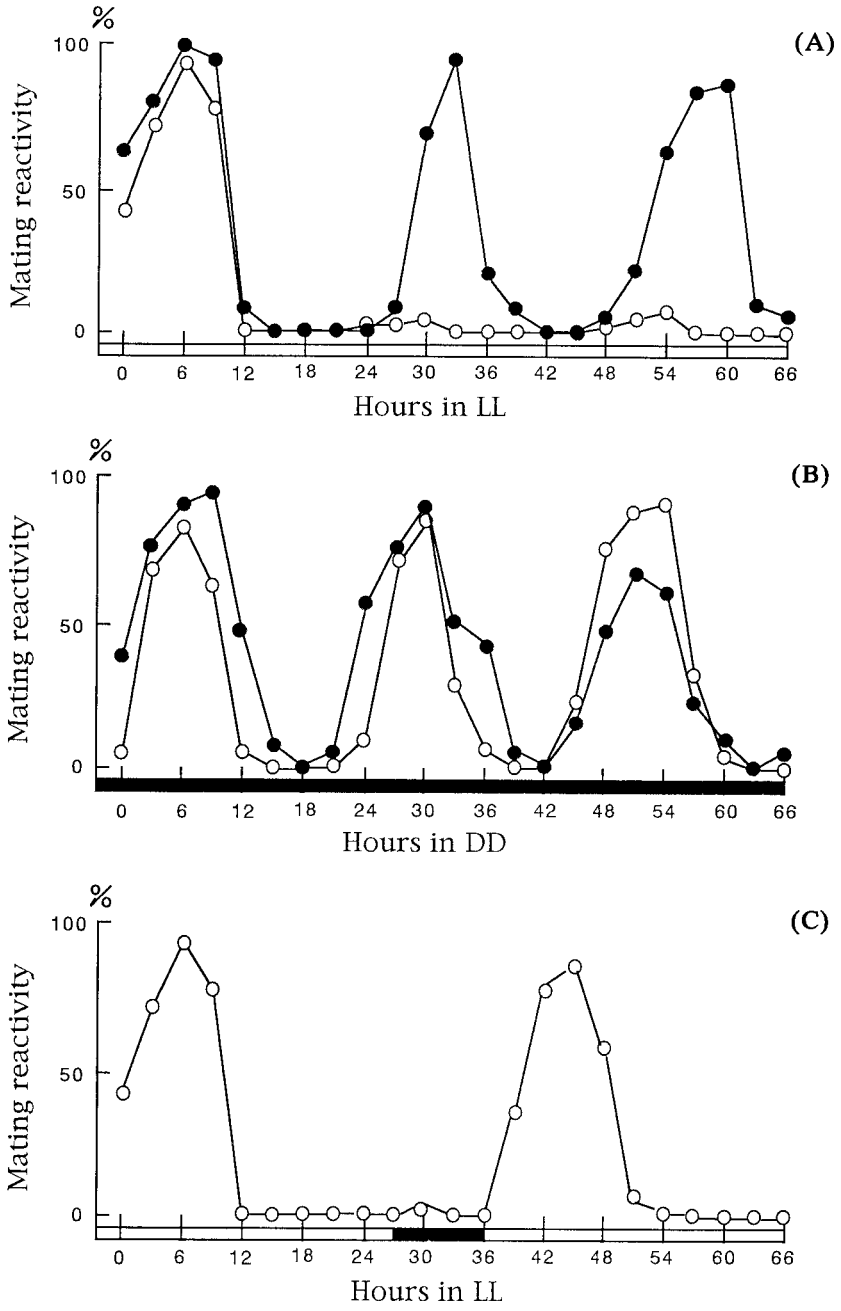

Fig. 1. Mating reactivity rhythms of strain Ok2. Mating reactivities of green Ok2 cells and *Chlorella*-free white cells (Ok2w) were tested in LL (A) and DD (B) every 3 hr by mixing them with "tester" cells of a complementary mating type. Ok2 cells (●) showed circadian mating reactivity rhythms in LL and DD, but Ok2w cells (○) showed almost no expression of mating reactivity in LL. (C): When Ok2w cells were given a dark pulse for 9 hr, they showed one cycle of mating reactivity rhythm.

cells. Mating reactivity in cell populations was measured every 3 hr as follows: 10 cells were placed in each of 6 different wells of a depression glass plate, and about 100 highly reactive tester cells were added to each well. After 5 min, the percentage of mating reactive cells clumping with tester cells was counted under a binocular microscope.

To prepare tester cells with high reactivity, four groups of green and white cells were entrained to four light/dark cycles (LD 12:12 hr, staggered by 6 hr). Each group of tester cells was used twice in the mating reactivity tests conducted at 3-hr consecutive intervals. Since each group of tester cells showed high reactivity for at least 6 hr in a day, tester cells were always highly reactive in every test.

Re-infection of *Chlorella*

Chlorella cells were prepared for re-infection into Ok2w cells as follows. Green Sj2 cells in a 100-ml culture were concentrated by low speed centrifugation. One ml of cell suspension was put into a micro test tube and then sonicated with an ultra-sonicator for 10 sec. *Chlorella* cells were collected through a 15-μm opening nylon mesh and washed with K-DS solution. About 4×10^7 *Chlorella* cells/ml were mixed with about 2×10^3 /ml of Ok2w cells.

Treatment with an inhibitor

Five μg/ml of the herbicide paraquat (1,1'-Dimethyl-4,4'-bipyridinium; methyl viologen) was added to the culture of green Ok2 cells to inhibit the photosynthetic activity of symbiotic *Chlorella*. The same amount of paraquat was added to the culture of white cells (Sj2w) as control. The cells treated with paraquat were tested for mating reactivity rhythms over a period of about 60 hr in LL.

Sugar analysis

Homogenates made from Ok2 and Ok2w cells by ultra-sonication were analyzed for their sugar components.

Each sample was centrifuged at 14,000 rpm, 4°C for 20 min to separate free-*Chlorella* and cell fragments. The supernatant was dried under vacuum at 40°C, and then a dried powder was dissolved in a little ultra-pure water. After centrifugation again at 1,4000 rpm, 4°C for 20 min, the extract was separated from nucleic acid, polysaccharides and free-peptides by an ultrafiltration. Finally, it was lyophilized. The lyophilized fractionation was analyzed for sugar components by HPLC using an amidosilica column.

Treatment with maltose

To investigate the effects of photosynthetic products of symbiotic *Chlorella*, cells were treated with maltose and maltose-type sugars. Ok2w cells in a 20-ml culture were added 0.5 ml of 10^{-2} M sugar every 3 hr during 12 hr of subjective day phase (0–12, 24–36 and 48–60 hr) in LL. Sugar amount is calculated at 3 mM final concentration after 60 hr. The same amounts of glucose were also tested as controls. Treated cells were assayed for mating reactivity rhythms in LL.

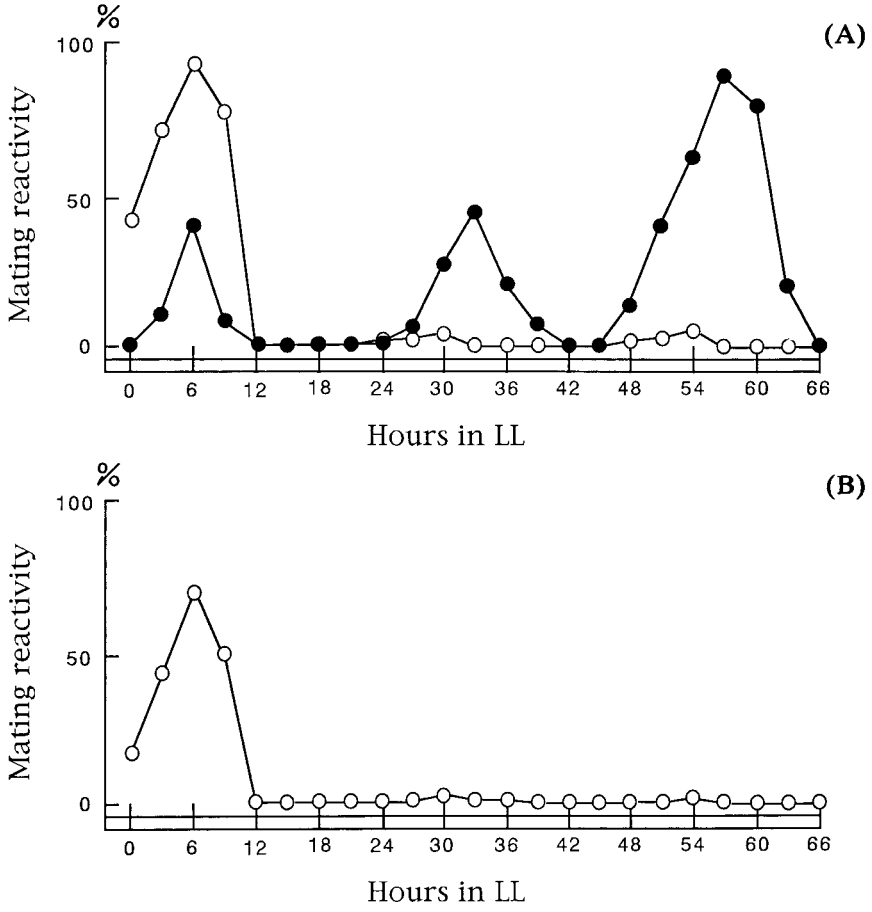

Fig. 2. Effects of infected *Chlorella* on the expression of mating reactivity of Ok2w cells in LL. (A) Mating reactivity rhythms of Ok2w cells (○) and OkwS cells (●) infected with *Chlorella* isolated from Sj2. Ok2w cells showed almost no expression of mating reactivity in LL. OkwS cells infected with *Chlorella* showed mating reactivity rhythm in LL. (B) Mating reactivity of Ok2w cells mixed with sonicated Sj2w cells. They showed no mating reactivity in LL.

RESULTS

Characteristics of a mutant strain

It is generally thought that *Chlorella*-free white cells derived from natural green cells show a normal mating reactivity rhythm in LL or DD and that *Chlorella*-containing green cells of many strains also show a normal mating reactivity rhythm in LL but a low mating reactivity in DD. In the strain Ok2 corrected from nature, unusual mating reactivity rhythms were observed. White Ok2 cells (Ok2w) showed no circadian rhythm of mating reactivity in LL except for the first cycle (Fig. 1A) and green Ok2 cells showed a normal mating reactivity rhythm in DD (Fig. 1B). After the Ok2w cells had been exposed to a dark pulse for 9 hours, they expressed one cycle of mating reactivity rhythm (Fig. 1C).

Mating reactivity rhythms appeared by infection with *Chlorella*

Ok2w cells were infected with *Chlorella* isolated from natural green cells (Sj2) that exhibited a normal rhythm in LL. The cells that had become green (OkwS cells) were tested for mating reactivity every 3 hr. It has been reported that a period of about five days is needed for infected *Chlorella* to be established as endosymbionts (Nishihara *et al.*, 1998). However, we previously showed that infected *Chlorella* affected

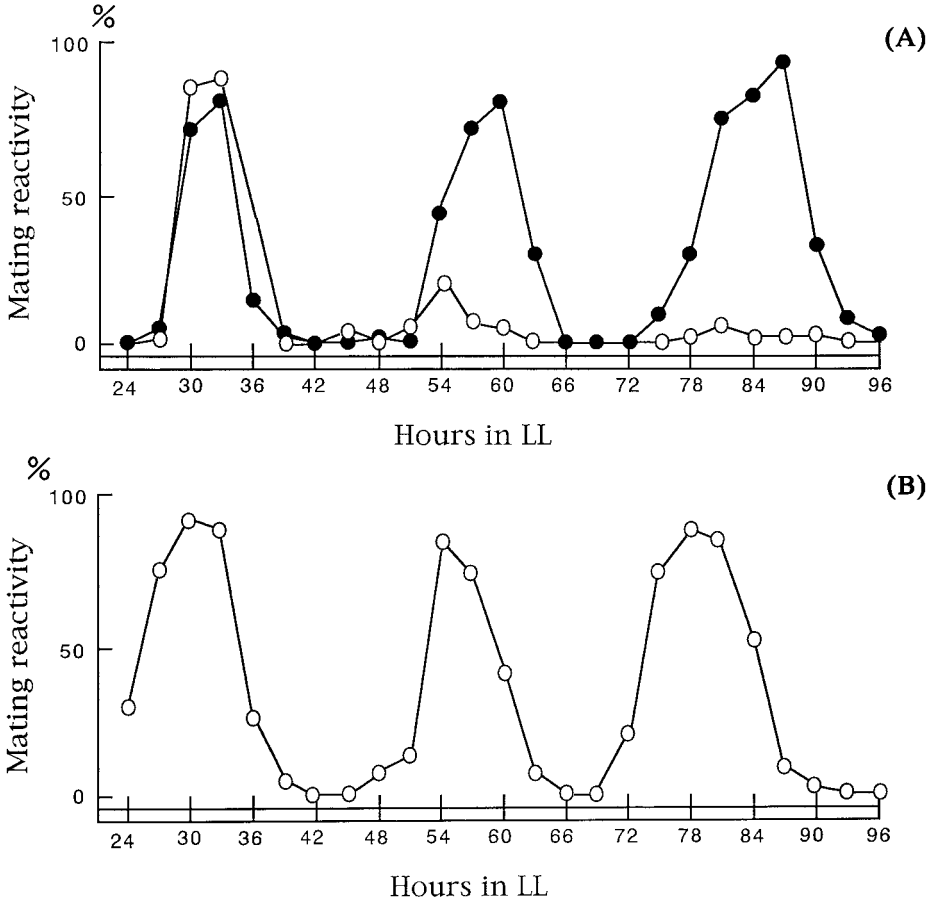

Fig. 3. Effects of a photosynthesis inhibitor on the expression of mating reactivity in Ok2. (A) Mating reactivity of Ok2 cells (●) and cells treated with 5 μg/ml of paraquat (○). Mating reactivity of the treated cells declined gradually and finally disappeared, as was seen in Ok2w cells. (B) Mating reactivity of Sj2w cells that show a normal mating reactivity rhythm in LL treated with paraquat. These cells showed a normal mating reactivity rhythm in LL. Thus, paraquat itself has no effect on the expression of mating reactivity.

Table 1. Sugar components in the cytosols of Ok2 and Ok2w cells analyzed by HPLC.

Samples	Saccharide			
	Glucose	Maltose	Tri-saccharide	Tetra-saccharide
Green cells (Ok2 day cells)	+	+	+	+
Green cells (Ok2 night cells)	+	−	−	−
White cells (Ok2w day cells)	+	−	−	−

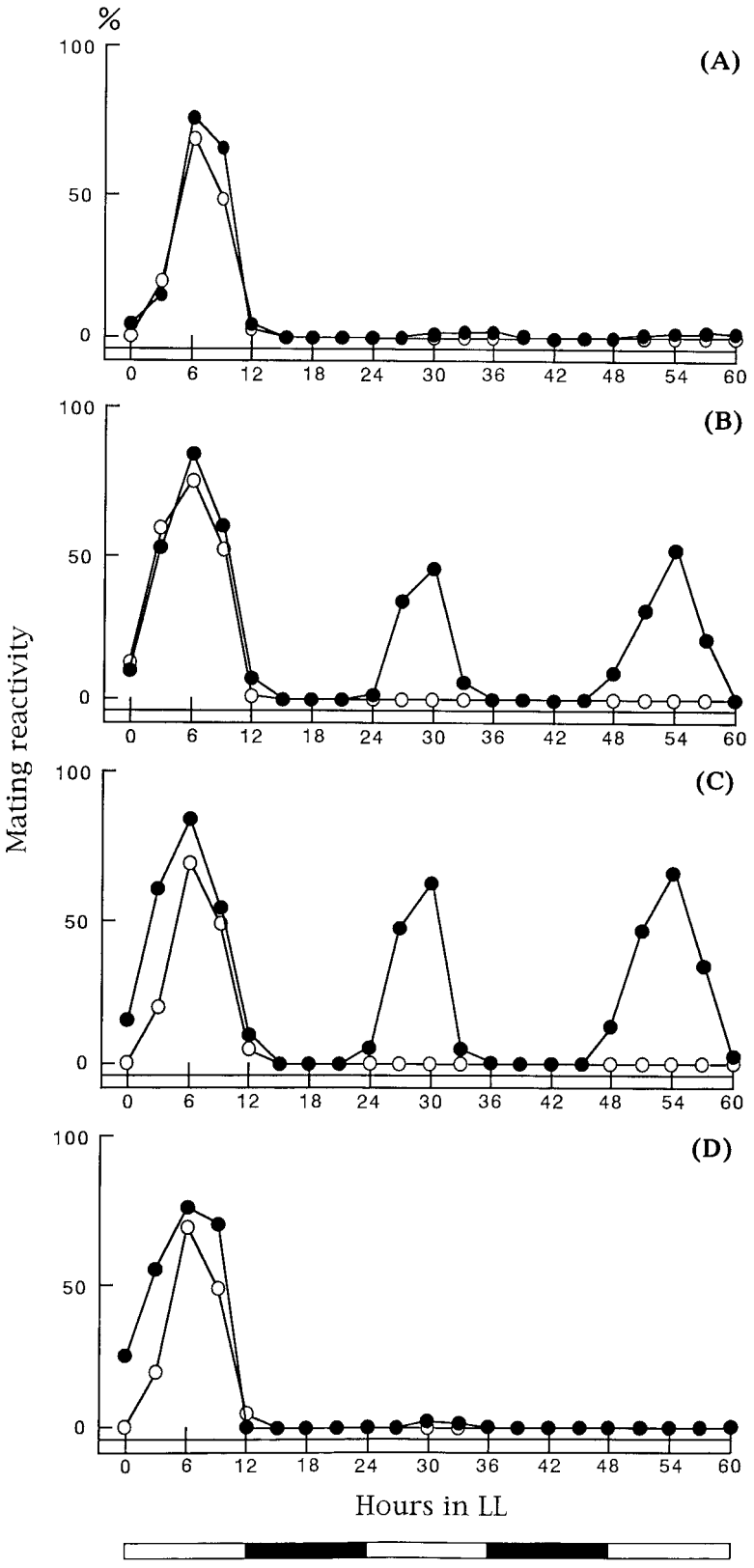

Fig. 4. Mating reactivity rhythms of Ok2w cells treated with sugars. To 20 ml cell suspensions were added 0.5 ml of 10^{-2} M sugars every 3 hr during subjective day phases (0-12, 24-36 and 48-60 hr) in LL. The lower bar indicates subjective day and night phases that are the light/dark cycle given in previous to LL. Closed circles (●) show mating reactivity rhythms of Ok2w cells treated with glucose (A), maltose (B), maltotriose (C) and maltotetraose (D). Open circles (○) show mating reactivity rhythms of untreated Ok2w cells as controls. Maltose and maltotriose induced a mating reactivity rhythm in Ok2w cells. Glucose and maltotetraose had no effects on the expression of mating reactivity.

the expression of circadian photoaccumulation rhythms in *P. bursaria* at three days after infection (Miwa *et al*., 1996). Moreover, the effects of infected *Chlorella* on expression of mating reactivity rhythms appeared 24 hr after infection (Tanaka and Miwa, 1996). Therefore, Ok2w cells were infected with *Chlorella* one day before the beginning of measurements in this experiment. On the following day, the cells contained many *Chlorella* cells and were clearly designated green, and they were assayed for mating reactivity rhythms. It was observed that cells of *Chlorella* began to propagate in the host cells two days after infection. The OkwS cells expressed a circadian rhythm of mating reactivity in LL (Fig. 2A). To exclude the possibility that the expression of mating reaction was caused by sonicated cytoplasm and nuclei of donor cells (Sj2), Ok2w cells were infected with sonicated white cells (Sj2w). As seen in Fig. 2B, they did not show a mating reactivity rhythm in LL. These results show that the expression of a mating reactivity rhythm in the recipient cells is caused by infected *Chlorella*.

Inhibition of photosynthetic activity in symbiotic *Chlorella*

To determine whether the photosynthetic activity of *Chlorella* is related to the expression of a mating reactivity rhythm, Ok2 cells were treated with paraquat. Their mating reactivity rhythms disappeared in LL, as was the case with Ok2w cells (Fig. 3A). In a previous study (Miwa *et al*., 1996), DCMU was used to inhibit the effects of *Chlorella* on the period length of photoaccumulation rhythm. However, it was found that DCMU was toxic for expression a mating reactivity in *P. bursaria*. Therefore paraquat was used in this experiment. Paraquat forms free radical in plant cells and destroys chloroplast. Thus it was used as photosynthetic inhibitor in this experiment. To confirm that paraquat has no effects on the ability of mating reaction in *P. bursaria*, *Chlorella*-free white cells (Sj2w) that showed normal mating reactivity rhythm in LL were treated with paraquat. As shown in Fig. 3B, they showed a normal mating reactivity rhythm in LL. Paraquat, therefore, affects only the photosynthetic activity of symbiotic *Chlorella*. These results suggest that the photosynthetic activity of symbiotic *Chlorella* in Ok2 cells has relation to the expression of mating reactivity rhythms of Ok2w cells in LL.

Sugar analysis in the cytosol

It is known that symbiotic *Chlorella* affects the circadian rhythms in many natural strains and mutant strain (MC1w) in LL but not in DD (Miwa *et al*., 1996; Tanaka and Miwa, 1996). This fact suggests that the photosynthesis of symbiotic *Chlorella* is related to the expression of circadian rhythms. To clarify that symbiotic *Chlorella* cells release photosynthetic products in the cytoplasm of Ok2 cells, sugar components in the cytosol were analyzed by HPLC. For the analysis, the following three kinds of samples were prepared: 1) Ok2 cells in light phase (day cells), 2) Ok2 cells in dark phase (night cells), 3) Ok2w cells in light phase (day cells). The results are shown in Table 1. Glucose, maltose, and unidentified tri-saccharide and tetra-saccharide were detected in the cytosol of Ok2 day cells, while only glucose was detected in Ok2 night cells and Ok2w day cells. These results suggest that symbiotic *Chlorella* cells release maltose in Ok2 cells according to the LD cycle. Unidentified tri-saccharide and tetra-saccharide were thought to be maltose-type sugars, maltotriose and maltotetraose, respectively. These sugars were also released in Ok2 cells in the light phase.

Effects of maltose on the expression of mating reactivity rhythm

The effects of sugar components in Ok2 cells on the expression of mating reactivity rhythms were investigated. Glucose, maltose, maltotriose and maltotetraose were added to a culture medium of Ok2w cells every 3 hr during the subjective day phases (0–12, 24–36, and 48–60 hr), and their mating reactivity rhythms were assayed in LL. The results are shown in Fig. 4. Mating reactivity rhythms of Ok2w cells were induced by the supply of both maltose and maltotriose in LL. However, the other sugars, glucose and maltotetraose, had no effects on the induction of mating reactivity in Ok2w. Thus, the secretion of maltose and maltotriose from symbiotic *Chlorella* has close connection with the expression of circadian rhythms in Ok2w cells.

DISCUSSION

P. bursaria shows many kinds of circadian rhythms, including mating reactivity and photoaccumulation (Miwa *et al*., 1987; Johnson *et al*., 1989). In this study, we showed the correlation of photosynthetic products of symbiotic *Chlorella*, maltose and maltose-type sugar, with the expression of mating reactivity rhythms in a mutant strain (Ok2) of *P. bursaria*.

Ok2 is an interesting natural strain. *Chlorella*-containing Ok2 cells showed a normal mating reactivity rhythm in LL or in DD. *Chlorella*-free Ok2w cells displayed normal mating reactivity rhythms in DD but they did not show mating reactivity in LL. This fact indicates that Ok2w cells have a normal circadian oscillator and that it shows ordinary functions irrespective of the presence of symbiotic *Chlorella*. A continuous light condition may inhibit the output route from a circadian oscillator to the expression of mating reactivity rhythms. On the other hand, Ok2w cells displayed a normal mating reactivity rhythm in LL after infection of *Chlorella*. Thus, it was suggested that infected *Chlorella* could affect the output process in the circadian systems of Ok2w cells. Ok2w cells could be suitable materials for investigating the output systems from a circadian oscillator. Nishihara *et al*. (1998) reported a method for establishing cloned culture of algae from heterogeneous symbiotic algae in *P. bursaria* cells, and they did their infection experiments using cloned symbiotic algae. In the present study, we used uncloned *Chlorella* for the re-infection experiments. It is important to investigate whether cloned symbiotic *Chlorella* induce a mating reactivity rhythm in Ok2w cells in LL.

In our previous paper, we reported that green cells of two

strains (T316 and Sj2) showed low or no mating reactivity in DD (Tanaka and Miwa, 1996). It was thought that these results were due to the digestion of endosymbiotic *Chlorella* in DD. In the present study, however, Ok2 cells displayed normal mating reactivity rhythms in DD. It is possible that the interaction between host cells and symbiotic *Chlorella* varies according to the strain. When the mating reactivity rhythm of Ok2 cells was observed after 60 hr in DD, their mating reactivity became to low gradually. It would be interesting to clarify the transition of density of symbiotic *Chlorella* of Ok2 in DD.

When Ok2 cells were treated with paraquat, their mating reactivity disappeared in LL. It has been reported that symbiotic *Chlorella* was destroyed and that green cells changed into *Chlorella*-free white cells when green cells were treated with paraquat for a period of 4–10 days (Hosoya *et al*., 1995). However, in the present study, Ok2 cells treated with paraquat remained green during the period of measurement of mating reactivity (about 60 hr). Furthermore, paraquat itself had no effect on the expression of mating reactivity in *Chlorella*-free white cells (Sj2w). It is thought that the disappearance of mating reactivity in Ok2 cells is caused by interruption of the supply of photosynthetic products from symbiotic *Chlorella*.

It has been reported that symbiotic *Chlorella* cells provide their photosynthetic products, maltose, to the host cells as living energy (Weis, 1979, 1980). We analyzed the sugar components in the cytosol of Ok2 and Ok2w cells by HPLC, and we found maltose and maltose-type sugars in Ok2 day cells. These sugars were not detected in the cytosol of Ok2 night cells and Ok2w day cells. When the Ok2w cells were exposed to maltose or maltotriose, they showed mating reactivity rhythms in LL. However, glucose and maltotetraose had no effect on the expression of mating reactivity rhythms. These results are possibly due to the recognition range of sugar receptors in *P. bursaria*. It is generally thought that the sugar receptor (lectin) on cell-surface recognizes disaccharide and trisaccharide but that it is difficult for the receptor to recognize monosaccharide, tetrasaccharide. In the present study, since Ok2w cells induced mating reactivity after infection of *Chlorella* in LL, it is considered that the cells also have a sugar receptor like a lectin in cytoplasm. It is interesting to make sure the receptor of sugars in *P. bursaria* cells.

The circadian system in higher organisms may involve several oscillators, as a hierarchical combination of a master and slave oscillators, or as coupled circadian oscillators, each of which drives different rhythms (Pittendrigh, 1981). Furthermore, it has been reported that the unicellular alga *Gonyaulax polyedra* has two oscillators (Roenneberg and Morse, 1993). Analysis of the effects of intracellular interaction between *P. bursaria* and their symbiotic *Chlorella* on the expression of circadian rhythms is valuable as a model for the coupling system of plural oscillators in higher organisms. In a previous paper (Tanaka and Miwa, 1996), we proposed that symbiotic *Chlorella* might regulate the mating reactivity rhythms via mitochondria in *P. bursaria*. On the basis of the results of the present study, we propose another hypothesis that maltose and maltotriose released from symbiotic *Chlorella* might be a trigger to express a mating reactivity rhythm in Ok2w cells. However, we have no evidence how the sugar signal is recognized in Ok2w cells. Further experiments are needed to clear the process of intracellular signal transduction of sugars in *P.bursaria* cells.

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