RESEARCH ARTICLE

Symbiotic *Chlorella variabilis* incubated under constant dark conditions for 24 hours loses the ability to avoid digestion by host lysosomal enzymes in digestive vacuoles of host ciliate *Paramecium bursaria*

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Abstract

Endosymbiosis between symbiotic *Chlorella* and alga-free *Paramecium bursaria* cells can be induced by mixing them. To establish the endosymbiosis, algae must acquire temporary resistance to the host lysosomal enzymes in the digestive vacuoles (DVs). When symbiotic algae isolated from the alga-bearing paramecia are kept under a constant dark conditions for 24 h before mixing with the alga-free paramecia, almost all algae are digested in the host DVs. To examine the cause of algal acquisition to the host lysosomal enzymes, the isolated algae were kept under a constant light conditions with or without a photosynthesis inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea for 24 h, and were mixed with alga-free paramecia. Unexpectedly, most of the algae were not digested in the DVs irrespective of the presence of the inhibitor. Addition of 1 mM maltose, a main photosynthetic product of the symbiotic algae or of a supernatant of the isolated algae kept for 24 h under a constant light conditions, did not rescue the algal digestion in the DVs. These observations reveal that unknown factors induced by light are a prerequisite for algal resistance to the host lysosomal enzymes.

Introduction

Endosymbiosis is a major driving force behind eukaryotic cell evolution leading to acquisition of new intracellular components and cell diversity (Fujishima, 2009). Many aquatic multicellular organisms live in symbiosis with photosynthetic algae: corals, clams, *Hydra*, and spotted salamander (Muscatine *et al.*, 1975; Nowack & Melkonian, 2010; Kerney *et al.*, 2011). Algal symbioses are particularly beneficial for photic and oligotrophic environments (Nowack & Melkonian, 2010). However, for initiation of the establishment of endosymbioses, how the alga is recognized as a symbiont but not a prey by the host is not well known.

Among *Paramecium* species, *Paramecium bursaria* can maintain several hundred endosymbiotic *Chlorella* spp. in the cytoplasm. The association of *P. bursaria* with the symbiotic algae is a mutual symbiosis (Kodama & Fujishima, 2010). For instance, the host can supply algal cells with nitrogen components and CO_2 (Reisser, 1976a, b, 1980; Albers *et al.*, 1982; Albers & Wiessner, 1985). Furthermore, the host *Paramecium* protects the symbiotic algae from infection by the *Chlorella* virus (Kawakami & Kawakami, 1978; Reisser *et al.*, 1988; Van Etten *et al.*, 1991; Yamada *et al.*, 2006). Algal carbon fixation is enhanced in the host cell (Kamako & Imamura, 2006; Kato & Imamura 2009). The algae can supply the host with a photosynthetic product, mainly maltose (Brown & Nielsen, 1974; Reisser, 1976a, b, 1986). The algae in the host show a higher rate of photosynthetic oxygen production than in their isolated state, thereby guaranteeing an oxygen supply for the host (Reisser, 1980). Alga-bearing *P. bursaria* can grow better than alga-free cells (Karakashian, 1963, 1975; Görtz, 1982). The alga plays a photo-protective role for the host (Hörtnagl & Sommaruga, 2007; Summerer *et al.*, 2009). Irrespective of

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mutually beneficial relations between *P. bursaria* and symbiotic algae as described above, the alga-free cells and the symbiotic algae still can grow without a partner. Furthermore, endosymbiosis between the alga-free *P. bursaria* cells and the symbiotic algae isolated from the alga-bearing *P. bursaria* cells is easily re-established by mixing them together (Siegel & Karakashian, 1959; Karakashian 1975; Kodama & Fujishima, 2005). For these reasons, the symbiotic associations between the *Chlorella* and *P. bursaria* cells are excellent models for studying cell-to-cell interaction and the evolution of eukaryotic cells through secondary endosymbiosis.

Recently, details of the algal infection process were elucidated by pulse-labeling by addition of symbiotic *Chlorella* cells isolated from the alga-bearing *P. bursaria* to the alga-free *P. bursaria* for 1.5 min; then chasing for various periods of time (Kodama & Fujishima, 2005, 2007, 2009a, b, 2010, 2011, 2012a, b; Kodama *et al.*, 2007, 2011; Kodama, 2013). During the infection process, the first hurdle for the algae is acquisition of resistance to the host's lysosomal enzymes in the digestive vacuole (DV) (Kodama & Fujishima, 2005). In the DVs, some algae survive, even in the presence of others that are being digested. This differential fate of algae in the same DV is not an inherent property of the algae because this phenomenon occurs even with clonal symbiotic algae. Furthermore, this algal fate is independent of the cell cycle stage of the algae and location of the algae in the DV. Moreover, this resistance to digestion does not require algal protein synthesis (Kodama *et al.*, 2007). To date, the cause of different fates of algae in the same DV remains unclear. Gu *et al.* (2002) showed that degeneration of the symbiotic *Chlorella* under constant dark (DD) conditions is induced by the host lysosomal fusion to perialgal vacuole (PV) membranes enclosing the symbiotic algae (Gu *et al.*, 2002). This fact suggests that the photosynthetic activity and/or related cellular processes of the algae play important functions in protection from the lysosome fusion to the PV membrane. In the early infection process, the symbiotic algae are engulfed into the host DVs. The DV membrane allows fusion with host lysosomes (Kodama & Fujishima, 2005). However, some of the algae are not digested in DVs that had been fused with the host lysosomes. A difference in the experimental conditions used by Gu *et al.* (2002) and Kodama & Fujishima (2005) was the presence or absence of light: DD conditions (4 weeks) were used by Gu *et al.* (2002) and LL conditions by Kodama & Fujishima (2005). Therefore, in this study, we examined the effect of exposure to different illumination programs on algal persistence in alga-free paramecia.

Materials and methods

Strains and cultures

An alga-free *P. bursaria* strain, Yad1w, was produced from *Chlorella* sp.-bearing *P. bursaria* strain Yad1g cells as described in our previous reports (Kodama & Fujishima, 2009a, b, 2011). An alga-bearing strain of Yad1g1N (syngen 3, mating type I) cells was used for isolation of their symbiotic algae. The strain Yad1g1N cell was produced by infection of cloned symbiotic *Chlorella variabilis* (formerly *Chlorella vulgaris*) strain 1N cells to the Yad1w cell. The culture medium used was red pea (*Pisum sativum*) extract culture medium (Tsukii *et al.*, 1995) in modified Dryl's solution (MDS; Dryl, 1959) (KH_2PO_4 was used instead of $NaH_2PO_4{\cdot}2H_2O$) inoculated with a non-pathogenic strain 6081 of *Klebsiella pneumoniae* 1 day before use (Fujishima *et al.*, 1990) for alga-bearing and alga-free *P. bursaria*. In ordinary cultures, several hundred cells were inoculated into 2 mL culture medium. Then 2 mL of fresh culture medium was added on each of the next 12 days. One day after the final feeding, the cultures were in the early stationary phase of growth. All cells used for this study were of this phase. Cultivation of alga-bearing *P. bursaria* strains was conducted at 25 ± 1 °C under fluorescent lighting (20–30 μmol photon m^{-2}) using an incandescent lamp. In some experiments, alga-bearing *P. bursaria* cells were cultivated under DD conditions for 24 h or 8 days at 25 ± 1 °C. *Paramecium* strains used for this study were provided by the Symbiosis Laboratory, Yamaguchi University, with support in part by the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Isolation of symbiotic *C. variabilis* cells from alga-bearing *P. bursaria* cells and the algal incubation under various conditions

Symbiotic *C. variabilis* strain 1N cells were isolated from alga-bearing *P. bursaria* strain Yad1g1N cultivated under LL or DD conditions (Kodama *et al.*, 2007). The cell numerical density was ascertained using a blood-counting chamber. Then, the isolated algae were treated as shown by Table 1 before mixing them with alga-free *P. bursaria* cells. Stock solutions of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (10^{-3} M in 100% ethanol) (Wako Pure Chemical Ind., Ltd, Japan) and maltose (1 mM in distilled water) were stored at −20 °C and room temperature, respectively, until use. The stock solutions of DCMU and maltose were diluted with MDS to give the final concentration.

Table 1. Incubation protocols for experiments

Corresponding result	Algal treatment conditions	Algal mixing light condition	Corresponding bar
Fig. 3a	LL for 24 h	LL	Left
	DD for 24 h	DD	Right
Fig. 3b	LL for 24 h	LL	Left
	DD for 24 h	DD	Middle
	DD for 24 h + supernatant of LL-incubated algae for 24 h	DD	Right
Fig. 3c	LL for 24 h	LL	Left
	DD for 24 h	DD	Middle
	DD for 24 h + 1 mM maltose	DD	Right
Fig. 3d	LL for 24 h	LL	First
	LL for 24 h + 10^{-5} M DCMU	LL	Second
	LL for 24 h + 1% EtOH	LL	Third
	DD for 24 h	DD	Fourth
Fig. 3e	LL for 48 h	LL	Left
	DD for 24 h, LL for 24 h	LL	Middle
	DD for 48 h	DD	Right

LL, denotes constant light at 25 ± 1 °C; DD, denotes constant dark at 25 ± 1 °C.

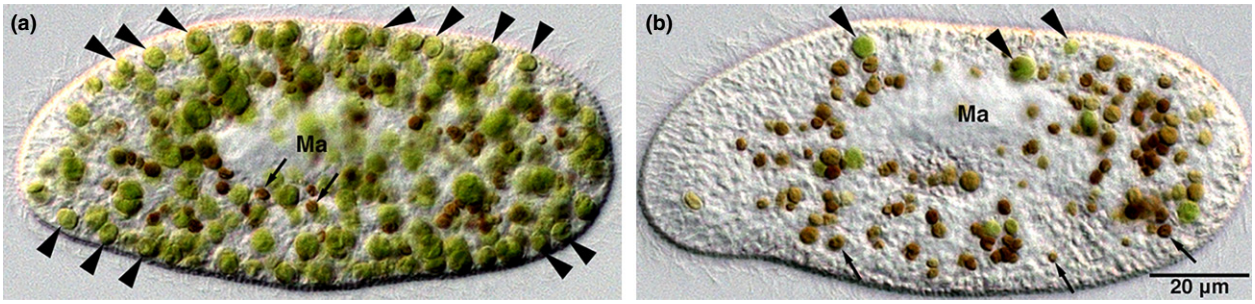

Fig. 1. Photomicrographs of alga-free *Paramecium bursaria* cells 6 h after mixing with LL- (a) or DD- (b) incubated algae for 24 h at 25 ± 1 °C. Both cells were mixed and kept under LL or DD conditions. As shown in (a), many LL-incubated algae showed resistance to the host lysosomal enzymes, and the undigested green algae localized beneath the host cell cortex (a, arrowheads). However, most of the DD-incubated algae were digested, and the algal color changed from green to brown (b, arrows). Few algae were able to avoid digestion and establish endosymbiosis (b, arrowheads). These results show that the algal incubation under LL conditions before ingestion by the alga-free *P. bursaria* cells is necessary to prevent algal digestion. *Arrowhead*, undigested single green alga localized beneath the cortex; *arrow*, digested brown alga; *Ma*, macronucleus. The reproducibility of this result was confirmed more than five times.

Fluorescence microscopy of LL- and DD-incubated algae stained with LysoSensor Yellow/Blue DND-160

Pellets of the LL-incubated and DD-incubated algae were stained respectively with LysoSensor Yellow/Blue DND-160 (Molecular Probes Inc.) as described in our previous study (Kodama & Fujishima, 2013). At 6 h after the staining, the cells were observed under a fluorescence microscope (BX51; Olympus Corp.). Their images were captured digitally using an Olympus DP73 camera system (Olympus Corp.).

Pulse label and chase of alga-free *P. bursaria* with symbiotic *C. variabilis*

In a centrifuge tube (volume, 10 mL), the treated algae (5×10^7 algae mL^{-1}) as shown in Table 1 were mixed with the alga-free *P. bursaria* cells (5000 paramecia mL^{-1}) for 1.5 min. After the mixing, the ciliate-algae mixture was transferred to a centrifuge tube equipped with a 15-µm-pore-size nylon mesh and filtered. By pouring 50 mL of fresh MDS per 1 mL ciliate-algae mixture into this tube, the paramecia were washed and algal cells outside the paramecia were simultaneously removed through the mesh. The paramecia retained in the mesh were transferred to a centrifuge tube (volume, 10 mL) and resuspended in MDS. The mixture was incubated under LL or DD conditions. Subsequently, using a differential-interference-contrast (DIC) and fluorescence microscopy, they were observed with or without fixation at various times after algal mixing. Fixation was conducted by mixing an equal volume of 8% (w/v) paraformaldehyde dissolved in phosphate-buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 8.1 mM $NaHPO_4 \cdot 12H_2O$, 1.47 mM KH_2PO_4, pH 7.2) with the cell suspension.

Results and discussion

Effects of various treatments of isolated symbiotic *C. variabilis* before mixing with alga-free *P. bursaria*

Most of the isolated symbiotic *C. variabilis* incubated under constant light conditions for 24 h were able to resist digestion in the host DV. The undigested algae then started to translocate from the DVs as a result of budding of the membrane into the cytoplasm. The algae localized beneath the host cell cortex to establish endosymbiosis with alga-free *P. bursaria* cells (Fig. 1a, arrowheads) (Kodama & Fujishima, 2005, 2012a, b). However, by incubation of isolated symbiotic algae under constant dark conditions for 24 h before mixing with the host cells, most of the algae lost the capability of resistance to the host lysosomal enzymes in the DV. Only a few algae are able to avoid digestion and could be localized beneath the host cell cortex after escaping from the DVs (Fig. 1b, arrowheads). As demonstrated in our previous study, not only undigested algae but also digested algae can bud from the DVs (Fig. 1b, arrows).

Previously, some algae were reported as involving unknown, small, brown vesicles stained with Gomori's solution (Gomori, 1952) in the cytoplasm irrespective of presence or absence of the substrate for the acid phosphatase (AcPase) (Kodama & Fujishima, 2009a, b). The

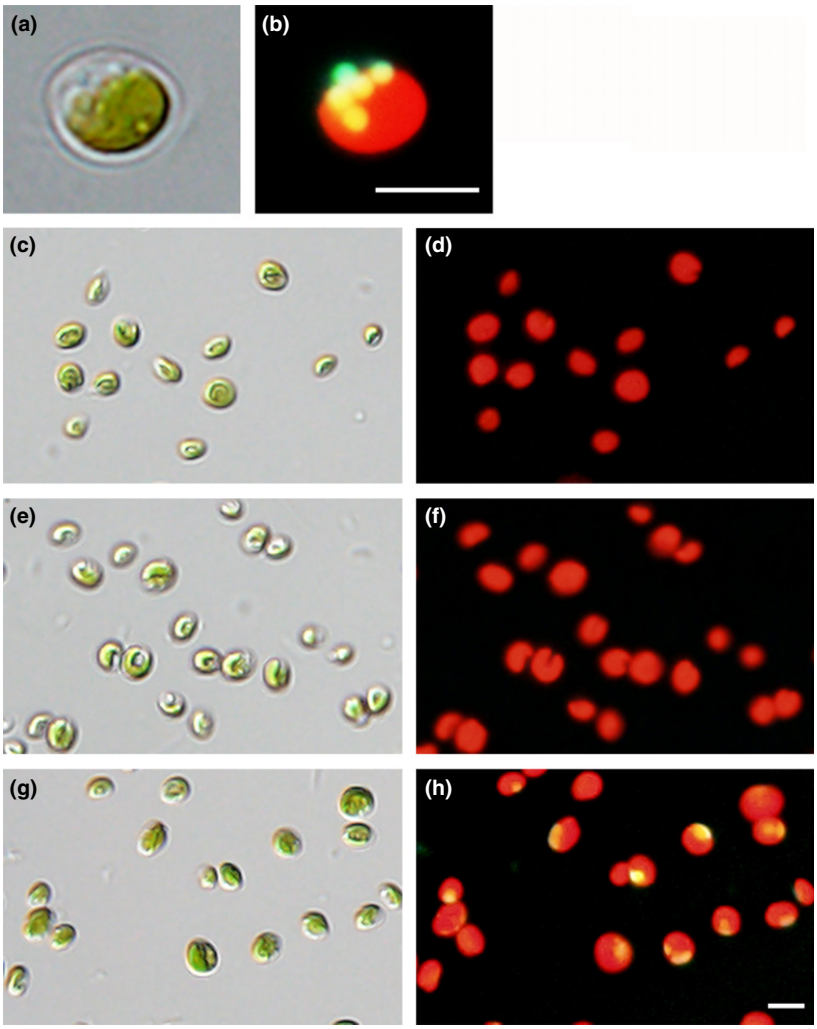

Fig. 2. DIC photomicrographs of LL-incubated (c), LL-incubated with 10^{-5} M DCMU (e), and DD-incubated (a and g) algae, and fluorescence photomicrographs of LysoSensor Yellow/Blue DND-160-treated LL-incubated (d), LL-incubated with 10^{-5} M DCMU (f), and DD-incubated (b) and (h) algae. LysoSensor accumulates in acidic vacuoles, and shows yellow fluorescence (b). Many vacuoles were observed in the DD-incubated algae (h), more than those incubated under LL conditions with (f) or without (d) DCMU. The red color shows chlorophyll autofluorescence in the chloroplast. Scale bars: 5 μm.

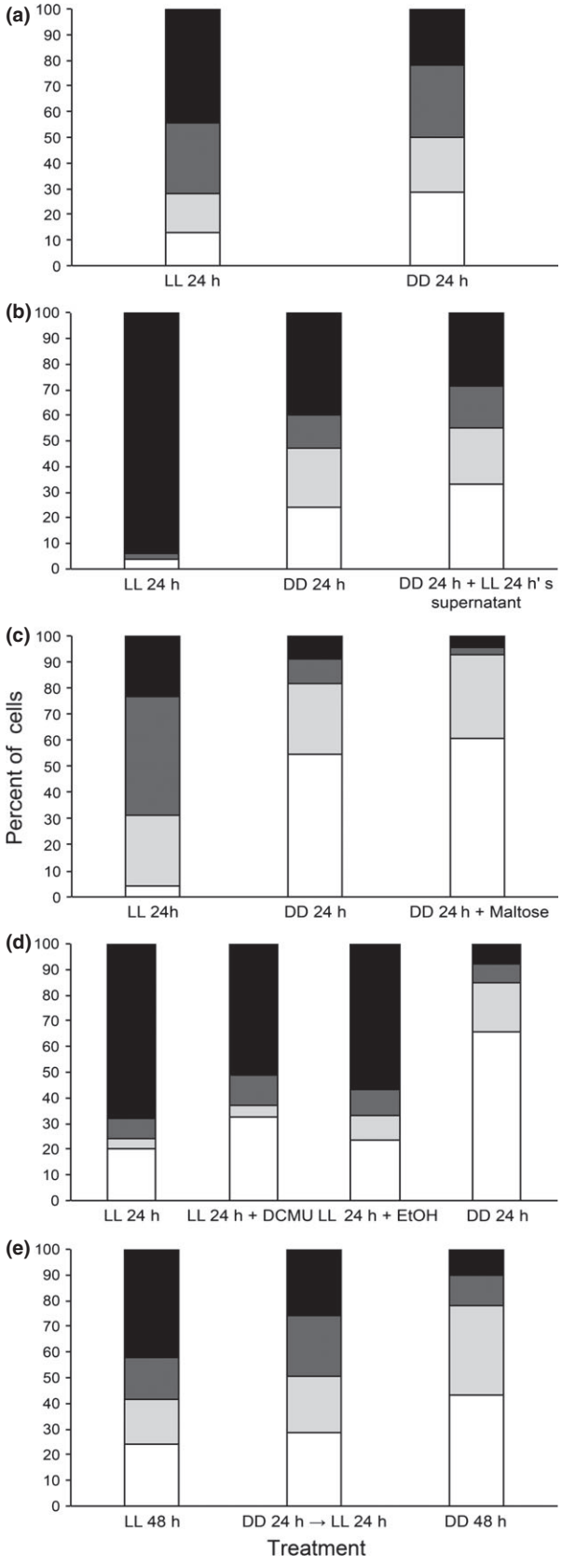

Fig. 3. Percentage of the cells with the green algae 6 h after mixing with alga-free *Paramecium bursaria* cells. The *Paramecium* cells were classified into four groups depending on the numbers of the undigested green algae per cell. The *x*-axis shows each treatment of isolated symbiotic algae isolated from alga-bearing *P. bursaria* cells before mixing with alga-free *P. bursaria* cells as shown in Table 1. (a) Effects of light conditions (LL or DD for 24 h). (b) Effects of culture supernatant from LL-incubated algae to DD-incubated algae. (c) Effects of maltose on DD-incubated algae. (d) Effects of photosynthesis inhibitor DCMU. (e) Effects of incubating light conditions: DD–LL. The *y*-axis shows the percentage of cells with each group. DD-incubated algae were digested more easily than the LL-incubated algae (a–c) even if the culture supernatant from LL-incubated algae (b) or maltose (c) was added to the DD-incubated algae. Resistance to the host lysosomal enzymes of LL-incubated algae was not inhibited by the addition of DCMU (d). It is noteworthy that the resistance to the host lysosomal enzymes of DD-incubated algae for 24 h is recovered after LL-incubation for 24 h (e). In each experiment, 150–200 cells were observed. The reproducibility of this result was confirmed more than twice.

results showed that algae with these vesicles were digested selectively in the host DVs in the early infection process (Kodama & Fujishima, 2009a, b). These vesicles exhibited similar shape and volume to those observed in *C. vulgaris* cells stained with neutral red or chloroquine, which are known as basic reagents specifically accumulated in acidic vacuoles by Kuchitsu *et al.* (1987). This observation raises the possibility that this small vesicle is an acidic vacuole and that an increased number are digested preferentially by the host lysosomal enzymes in early infection. To assess this possibility, we looked for morphological differences of the vacuole in LL-incubated, LL-incubated with photosynthesis inhibitor DCMU, and DD-incubated algae by staining with LysoSensor Yellow/Blue DND-160 (LysoSensor) (Fig. 2). In live cells, LysoSensor accumulates in acidic vacuoles of plant cells (Swanson *et al.*, 1998), and exhibits predominantly yellow fluorescence. As presented in Fig. 2b, several small spherical vacuoles with yellow fluorescence were observed in the algae. No differences in the algal color, shape or volume in LL-incubated (Fig. 2c), LL-incubated with 10^{-5} M DCMU (Fig. 2e), or DD-incubated (Fig. 2g) algae were observed using DIC microscopy. However, fluorescent microscopy clearly revealed that the number of vacuoles in the DD-incubated algae (Fig. 2h) increased more than those in algae incubated under LL conditions with (Fig. 2f) or without (Fig. 2d) DCMU. As a possible explanation for why the *Chlorella* with acidic vacuoles is readily digested in the host DVs, as presented in Fig. 2h, it is expected that the algal cell wall near the vacuoles might be thin and be easily digested by the host lysosome enzymes. However, to date, no relationship has been observed in the cell wall thickness of algae with or without the presence of the vacuoles (Karakashian *et al.*, 1968; Atkinson *et al.*, 1974).

Kuchitsu *et al.* (1987) reported that the number of the vacuoles increases in the algal cells at the stationary phase of growth compared with the cells in the log phase of growth. Furthermore, it has been shown that the vacuole volume becomes extremely large after a long period of sugar starvation in the plant cell (Yu, 1999). Taken together, algal starvation induced by the inhibition of photosynthesis under the DD conditions might be a cause of the vacuole development. Although the reason why the alga with the vacuole is digested preferentially in the host DV remains unknown, our results suggest that whether the algae are digested or not in the host DVs can de determined by staining the algae with LysoSensor.

Figure 3 presents a percentage of the cells with green algae 6 h after mixing with alga-free *P. bursaria* cells. The *Paramecium* cells were classified into four groups depending on the quantities of green algae per *Paramecium* cell, that is, 0 (white bar), 1–5 (light gray bar), 6–20 (dark gray bar), and more than 20 (black bar). At 6 h after mixing with alga-free *P. bursaria*, many algae incubated under LL conditions were able to establish endosymbiosis (Fig. 3a, left bar). However, most of the algae incubated under DD conditions were digested and were excreted from the *Paramecium* cytoproct. About 30% of the observed cells had no algae in their cytoplasm (Fig. 3a, white region of the right bar). The intracellular algae begin to undergo cell division about 24 h after mixing with alga-free *P. bursaria* (Kodama & Fujishima, 2005). Therefore, the algae remaining in the *Paramecium* 24 h after mixing can be regarded as undigested algae or already divided algae. These results suggest that the algal photosynthetic product might be correlated with resistance to the host lysosomal enzymes in the DVs fused with lysosomes. There was no difference in the shape or size of the LL-incubated and DD-incubated algae (Fig. 2).

To examine the function of the photosynthetic product, culture supernatant from LL-incubated algae was added to the DD-incubated algae. Effects on the algal digestion were examined by mixing with alga-free paramecia (Fig. 3b). After 24 h under an LL or DD conditions, the pH of the culture supernatant from LL-incubated algae became higher (pH 7.0–7.4, $n = 2$) than that from DD-incubated algae (pH 6.2, $n = 2$). Therefore, the isolated symbiotic algae cultivated under LL conditions photosynthesized actively because photosynthesis by aquatic plants under LL conditions removes carbon dioxide (CO_2) from the medium. The pH can be expected to increase (Araoye, 2009). Most of the LL-incubated algae were able to resist the host lysosomal enzymes, as explained above (Fig. 3b, left bar). As shown by the right bar in Fig. 3b, DD-incubated algae were digested more than LL-incubated algae, even if the culture supernatant from LL-incubated algae was added. No difference was observed in the number of undigested green algae between DD-incubated algae (Fig. 3b, middle bar) and DD-incubated algae with culture supernatant from LL-incubated algae (Fig. 3b, right bar).

Symbiotic *Chlorella* of *P. bursaria* releases photosynthetic products, mainly maltose and oxygen, contributing to host energy (see Introduction). Therefore, we examined the effects as shown of the addition of 10 mM maltose on DD-incubated algae in accordance with previous reports (Tanaka & Miwa, 1996, 2000). As shown in the right bar of Fig. 3c, the addition of maltose to the DD-incubated algae had no effect on the algal digestion. Most of the ingested algae were digested. No significant difference was found in the number of undigested green algae between DD-incubated algae (Fig. 3c, middle bar) and DD-incubated algae with maltose (Fig. 3c, right bar). It is reported that addition of maltose to the alga-free *P. bursaria* cells can induce some physiological changes such as a rescue of a circadian rhythm of mating reactivity of arrhythmic mutant alga-free *P. bursaria* cells or an acquisition of various stress resistances (Miwa, 2009). In contrast, our result shows that addition of maltose does not affect the inhibition of algal digestion.

DCMU is a blocker of electron flow in photosystem II (Kleinig & Sitte, 1986). We added 10^{-5} M DCMU to the isolated symbiotic algae and incubated under LL conditions for 24 h. As a control experiment, 1% (v/v) ethanol,

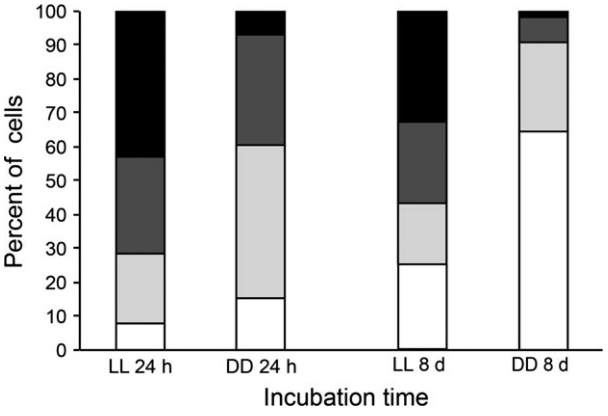

Fig. 4. Percentage of the cells with the green algae 6 h after mixing with alga-free *Paramecium bursaria* cells. The *Paramecium* cells were classified into four groups depending on the quantities of green algae per cell as in Fig. 3. The *x*-axis shows incubation time of alga-bearing *P. bursaria* cells under LL or DD conditions. The *y*-axis shows percentage of the cells with each group. Isolated symbiotic algae from DD-incubated alga-bearing *P. bursaria* cells for 24 h were digested more easily (second bar) than that from LL-incubated *Paramecium* (first bar). A longer incubation period of alga-bearing *P. bursaria* cells under DD conditions produced easier digestion of the algae (fourth bar). In each experiment, 150–200 cells were observed. The reproducibility of this result was confirmed twice.

used as a solvent of DCMU, was added to the algae. Although this concentration of DCMU was described as inhibiting photosynthesis of the symbiotic *Chlorella* spp. in previous reports (Tanaka & Miwa, 1996; Kodama & Fujishima, 2008), it did not affect the acquisition of resistance to the host's lysosomal enzymes of LL-incubated algae (Fig. 3d, first to third bars). At that time, most of the DD-incubated algae were digested (Fig. 3d, right bar). These results show that some unknown physiological processes under LL conditions that are not inhibited by DCMU might be necessary for algal resistance to the host lysosomal enzymes.

How long an LL-period is necessary for the acquisition of the resistance to the host a time of the lysosomal enzymes? To examine this question, the algae isolated under LL conditions were incubated under DD condi-

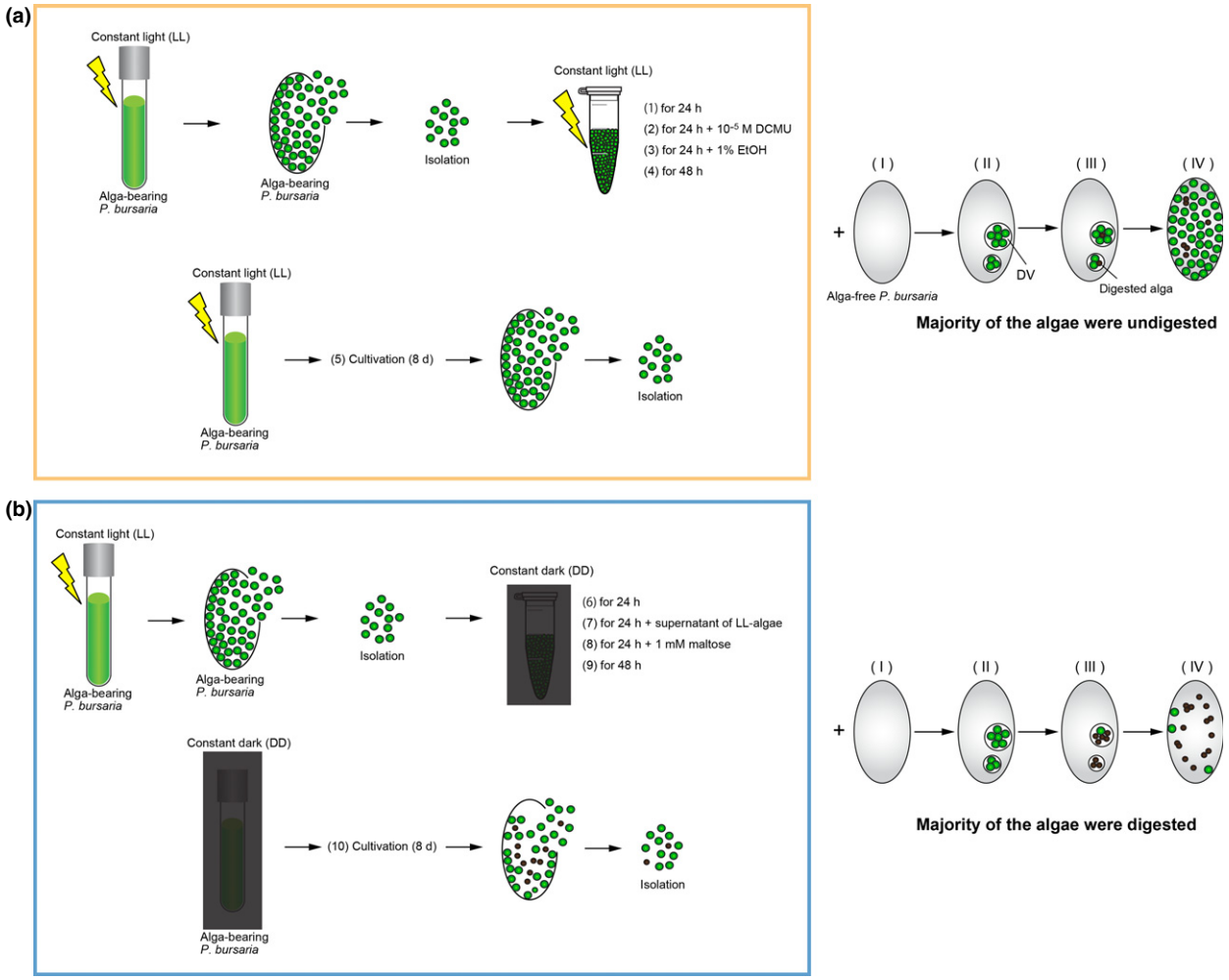

Fig. 5. Schematic representation of the algal digestion patterns in the DVs. Under LL conditions (a), the isolated symbiotic algae from LL-incubated alga-bearing *Paramecium bursaria* cells were incubated for 24 h (1), for 24 h with 10^{-5} M DCMU (2), for 24 h with 1% EtOH (3), for 48 h (4), and for 8 days (5) after (1–4) and before (5) isolation from alga-bearing *P. bursaria* cells. After mixing with alga-free *P. bursaria* cells (a: I), some algae were passed through the host cytopharynx and enclosed in the DVs (a: II). After the lysosomal fusion with the DVs, few algae were digested (brown alga in a: III), but most of the algae showed resistance to the host lysosomal enzymes and were undigested (green alga in a: III). Finally, most of the algae ingested in the DVs were able to establish endosymbiosis with alga-free *P. bursaria* cells (a: IV). Under DD conditions (b), the isolated symbiotic algae from LL-incubated alga-bearing *P. bursaria* cells were incubated for 24 h (6), for 24 h with supernatant of LL-incubated algae (7), for 24 h with 1 mM maltose (8), for 48 h (9) and for 8 days (10) after (6–9) and before (10) isolation from alga-bearing *P. bursaria* cells. After mixing with alga-free *P. bursaria* cells (b: I), some algae were enclosed in the DVs as with the LL-incubated algae (b: II). After the lysosomal fusion, most of the algae were digested (brown alga in b: III) and a few algae showed resistance to the host lysosomal enzymes and undigested (green alga in b: III). Finally, most of the algae ingested in the DVs were digested. A few of the algae were able to establish endosymbiosis with alga-free *P. bursaria* cells (b: IV).

tions for 24 h and then incubated under LL conditions for an additional 24 h before mixing with the alga-free *P. bursaria* cells. At 24 h after mixing, results showed that the algae acquired a lysosomal enzyme-resistant nature (Fig. 3e, middle bar), but algae incubated under DD conditions for 48 h were digested (Fig. 3e, right bar). This result demonstrates that 24 h exposure to LL is sufficient to renew algal resistance to the host lysosomal enzymes.

The reproducibility of these results was confirmed three times.

Effects of cultivation of alga-bearing *P. bursaria* under LL and DD conditions

Incubation of isolated symbiotic algae under DD conditions for 24 h preceding mixing with the alga-free *P. bursaria* cells induces algal digestion in the host DVs, as shown in Figs 1 and 3. To assess the effects of the cultivation of the alga-bearing *P. bursaria* under LL or DD conditions on the algal digestion, symbiotic algae were isolated from the LL- or DD-cultivated paramecia, and were mixed with alga-free *P. bursaria* cells. As portrayed in Fig. 4, the isolated symbiotic algae from alga-bearing *P. bursaria* cells cultivated under DD conditions for 24 h (Fig. 4, second bar) tended to be digested more easily than those from LL-cultivated alga-bearing *P. bursaria* cells for 24 h (Fig. 4, first bar). When the alga-bearing *P. bursaria* cells were kept under DD conditions for 8 days, most of the isolated symbiotic algae from the host cells were digested by mixing with alga-free *P. bursaria* cells and were unable to establish endosymbiosis (Fig. 4, fourth bar). After long-term DD cultivation of the alga-bearing cells, most of the symbiotic algae became pale green. Some became brown because of digestion by the host lysosomal fusion with the PV membrane (data not shown). These results demonstrate that the symbiotic algal resistance to the host lysosmal enzymes is highly influenced by light conditions, even in the host cells.

It is reported that the endosymbiotic *Chlorella* sp. of *P. bursaria* can excrete maltose both in the light and in the dark (Ziesenisz *et al.*, 1981). Experiments on photosynthetic $^{14}CO_2$ fixation and $^{14}CO_2$ pulse-chase experiments showed that maltose is synthesized in the light directly from compounds of the Calvin cycle, whereas in the dark it is synthesized from starch degradation. Their results suggest that maltose continues to synthesize until the starch depletion even under DD conditions. The results presented in this paper demonstrate that external addition of maltose to the DD-incubated algae was unable to inhibit algal digestion. No effects were observed for acquisition of resistance to the host lysosomal enzymes (Fig. 3c). Consequently, addition of maltose might not directly prevent algal digestion in the host DVs. Figure 5 shows the results in this study.

Lower levels were reported not only of chlorophyll *a*/*b* content but also of cell quantity, RNA content, and protein content in algal cells grown in complete darkness compared with those grown under light (Piotrowska & Czerpak, 2009). Furthermore, it was demonstrated that low-light intensities and darkness reduced expression in light-dependent genes, and that darkness stimulated RNase activity and RNA degeneration (Thomas *et al.*, 1997; Jayabaskaran, 1998). Regarding the algal resistance to the host lysosomal enzymes, a previous study reported that live algae can delay the digestion of dead algae when both algae have been ingested in the same DV (Karakashian & Karakashian, 1973). They assumed that this interference of live algae might be affected by their secretion of an inhibitor of the digestive enzymes, or that the algae might induce a change in the DV membrane so that it can no longer fuse with host lysosomes. Our previous studies showed that host lysosomal fusion to the DVs is not inhibited during early algal reinfection process, and that some algae can resist the host lysosomal enzymes in lysosome-fused DVs (Kodama & Fujishima, 2005, 2009a, b). Therefore, the latter hypothesis of Karakashian & Karakashian (1973) is not supported. Regarding the former hypothetical inhibitor of the digestive enzymes, comparison of gene expressions by transcriptome analysis and proteome analysis between isolated symbiotic algae under LL and DD conditions might be useful for identifying the hypothetical inhibitor. Our results provide some keys to ascertaining a mechanism for recognition as a symbiont by aquatic host organisms with photosynthetic algae.

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References

Albers D & Wiessner W (1985) Nitrogen nutrition of endosymbiotic *Chlorella* spec. *Endocytobiosis Cell Res* **2**: 55–64.

Albers D, Reisser W & Wiessner W (1982) Studies of the nitrogen supply of endosymbiotic chlorellae in green *Paramecium bursaria*. *Plant Sci Lett* **25**: 85–90.

Araoye PA (2009) The seasonal variation of pH and dissolved oxygen (DO_2) concentration in Asa lake Ilorin, Nigeria. *Int J Phys Sci* **4**: 271–274.

Atkinson AW, John PCL & Gunning BES (1974) The growth and division of single mitochondrion and other organelles during the cell cycle of *Chlorella*, studied by quantitative stereology and three dimensional reconstitution. *Protoplasma* **81**: 77–109.

Brown JA & Nielsen PJ (1974) Transfer of photosynthetically produced carbohydrate from endosymbiotic *Chlorellae* to *Paramecium bursaria*. *J Protozool* **21**: 569–570.

Dryl S (1959) Antigenic transformation in *Paramecium aurelia* after homologous antiserum treatment during autogamy and conjugation. *J Protozool* **6**: 25.

Fujishima M (2009) Infection and maintenance of *Holospora* species in *Paramecium caudatum*. *Endosymbionts in Paramecium*. *Vol. 12* (Fujishima M, Ed), pp. 201–225. Springer, Berlin.

Fujishima M, Nagahara K & Kojima Y (1990) Changes in morphology, buoyant density and protein composition in differentiation from the reproductive short form to the infectious long form of *Holospora obtusa*, a macronucleus-specific symbiont of the ciliate *Paramecium caudatum*. *Zool Sci* **7**: 849–860.

Gomori G (1952) *Microscopic Histochemistry. Principles and Practice*. University of Chicago Press, Chicago, IL.

Görtz HD (1982) Infections of *Paramecium bursaria* with bacteria and yeasts. *J Cell Sci* **58**: 445–453.

Gu F, Chen L, Ni B & Zhang X (2002) A comparative study on the electron microscopic enzymo-cytochemistry of *Paramecium bursaria* from light and dark cultures. *Eur J Protistol* **38**: 267–278.

Hörtnagl PH & Sommaruga R (2007) Photo-oxidative stress in symbiotic and aposymbiotic strains of the ciliate *Paramecium bursaria*. *Photochem Photobiol Sci* **6**: 842–847.

Jayabaskaran C (1998) Light- and phytohormone-induced variations in the level and modified nucleotide content of plastid tRNAs. *Plant Sci* **131**: 115–122.

Kamako S & Imamura N (2006) Effect of Japanese *Paramecium bursaria* extract on photosynthetic carbon fixation of symbiotic algae. *J Eukaryot Microbiol* **53**: 136–141.

Karakashian SJ (1963) Growth of *Paramecium bursaria* as influenced by the presence of algal symbionts. *Physiol Zool* **36**: 52–68.

Karakashian MW (1975) Symbiosis in *Paramecium bursaria*. *Symp Soc Exp Biol* **29**: 145–173.

Karakashian MW & Karakashian SJ (1973) Intracellular digestion and symbiosis in *Paramecium bursaria*. *Exp Cell Res* **81**: 111–119.

Karakashian SJ, Karakashian MW & Rudzinska MA (1968) Electron microscopic observations on the symbiosis of *Paramecium bursaria* and its intracellular algae. *J Protozool* **15**: 113–128.

Kato Y & Imamura N (2009) Metabolic control between the symbiotic *Chlorella* and the host *Paramecium*. *Endosymbionts in Paramecium*. *Vol. 12* (Fujishima M, Ed), pp. 57–82. Springer, Berlin.

Kawakami H & Kawakami N (1978) Behavior of a virus in a symbiotic system, *Paramecium bursaria*–zoochlorella. *J Protozool* **25**: 217–225.

Kerney R, Kim E, Hangarter RP, Heiss AA, Bishop CD & Hall BK (2011) Intracellular invasion of green algae in a salamander host. *P Natl Acad Sci USA* **108**: 6497–6502.

Kleinig H & Sitte P (1986) *Zellbiologie*. Gustav Fischer Verlag, Stuttgart.

Kodama Y (2013) Localization of attachment area of the symbiotic *Chlorella variabilis* of the ciliate *Paramecium bursaria* during the algal removal and reinfection. *Symbiosis* **60**: 25–36.

Kodama Y & Fujishima M (2005) Symbiotic *Chlorella* sp. of the ciliate *Paramecium bursaria* do not prevent acidification and lysosomal fusion of host digestive vacuoles during infection. *Protoplasma* **225**: 191–203.

Kodama Y & Fujishima M (2007) Infectivity of *Chlorella* species for the ciliate *Paramecium bursaria* is not based on sugar residues of their cell wall components, but on their ability to localize beneath the host cell membrane after escaping from the host digestive vacuole in the early infection process. *Protoplasma* **231**: 55–63.

Kodama Y & Fujishima M (2008) Cycloheximide induces synchronous swelling of perialgal vacuoles enclosing symbiotic *Chlorella vulgaris* and digestion of the algae in the ciliate *Paramecium bursaria*. *Protist* **159**: 483–494.

Kodama Y & Fujishima M (2009a) Localization of perialgal vacuoles beneath the host cell surface is not a prerequisite phenomenon for protection from the host's lysosomal fusion in the ciliate *Paramecium bursaria*. *Protist* **160**: 319–329.

Kodama Y & Fujishima M (2009b) Timing of perialgal vacuole membrane differentiation from digestive vacuole membrane in infection of symbiotic algae *Chlorella vulgaris* of the ciliate *Paramecium bursaria*. *Protist* **160**: 65–74.

Kodama Y & Fujishima M (2010) Secondary symbiosis between *Paramecium* and *Chlorella* cells. *Int Rev Cell Mol Biol* **279**: 33–77.

Kodama Y & Fujishima M (2011) Endosymbiosis of *Chlorella* species to the ciliate *Paramecium bursaria* alters the distribution of the host's trichocysts beneath the host cell cortex. *Protoplasma* **248**: 325–337.

Kodama Y & Fujishima M (2012a) Cell division and density of symbiotic *Chlorella variabilis* of the ciliate *Paramecium bursaria* is controlled by the host's nutritional conditions during early infection process. *Environ Microbiol* **14**: 2800–2811.

Kodama Y & Fujishima M (2012b) Characteristics of the digestive vacuole membrane of the alga-bearing ciliate *Paramecium bursaria*. *Protist* **163**: 658–670.

Kodama Y & Fujishima M (2013) Synchronous induction of detachment and reattachment of symbiotic *Chlorella* spp. from the cell cortex of the host *Paramecium bursaria*. *Protist* **164**: 660–672.

Kodama Y, Nakahara M & Fujishima M (2007) Symbiotic alga *Chlorella vulgaris* of the ciliate *Paramecium bursaria* shows temporary resistance to host lysosomal enzymes during the early infection process. *Protoplasma* **230**: 61–67.

Kodama Y, Inouye I & Fujishima M (2011) Symbiotic *Chlorella vulgaris* of the ciliate *Paramecium bursaria* plays an important role in maintaining perialgal vacuole membrane functions. *Protist* **162**: 288–303.

Kuchitsu K, Oh-hama T, Tsuzuki M & Miyachi S (1987) Detection and characterization of acidic compartments (vacuoles) in *Chlorella vulgaris* 11 h cells by ^{31}P-*in vivo* NMR spectroscopy and cytochemical techniques. *Arch Microbiol* **148**: 83–87.

Miwa I (2009) Regulation of circadian rhythms of *Paramecium bursaria* by symbiotic *Chlorella* species. *Endosymbionts in Paramecium*. *Vol. 12* (Fujishima M, Ed), pp. 83–110. Springer, Berlin.

Muscatine L, Cook CB, Pardy RL & Pool RR (1975) Uptake, recognition and maintenance of symbiotic *Chlorella* by *Hydra viridis*. *Symp Soc Exp Biol* **29**: 175–203.

Nowack ECM & Melkonian M (2010) Endosymbiotic associations within protists. *Philos Trans R Soc B* **365**: 699–712.

Piotrowska A & Czerpak R (2009) Cellular response of light/dark-grown green alga *Chlorella vulgaris* Beijerinck (*Chlorophyceae*) to exogenous adenine- and phenylurea-type cytokinins. *Acta Physiol Plant* **31**: 573–585.

Reisser W (1976a) The metabolic interactions between *Paramecium bursaria* Ehrbg. and *Chlorella* spec. in the *Paramecium bursaria*–symbiosis. I. The nitrogen and the carbon metabolism. *Arch Microbiol* **107**: 357–360.

Reisser W (1976b) The metabolic interactions between *Paramecium bursaria* Ehrbg. and *Chlorella* spec. in the *Paramecium bursaria*–symbiosis. II. Symbiosis-specific properties of the physiology and the cytology of the symbiotic unit and their regulation. *Arch Microbiol* **111**: 161–170.

Reisser W (1980) The metabolic interactions between *Paramecium bursaria* Ehrbg. and *Chlorella* spec. in the *Paramecium bursaria*-symbiosis. III. The influence of different CO_2-concentrations and of glucose on the photosynthetic and respiratory capacity of the symbiotic unit. *Arch Microbiol* **125**: 291–293.

Reisser W (1986) *Endosymbiotic Associations of Freshwater Protozoa and Algae*. Biopress Ltd, Bristol.

Reisser W, Klein T & Becker B (1988) Studies of phycoviruses I. On the ecology of viruses attacking *Chlorellae* exsymbiotic from a European strain of *Paramecium bursaria*. *Arch Hydrobiol* **111**: 575–583.

Siegel R & Karakashian SJ (1959) Dissociation and restoration of endocellular symbiosis in *Paramecium bursaria*. *Anat Rec* **134**: 639.

Summerer M, Sonntag B, Hortnagl P & Sommaruga R (2009) Symbiotic ciliates receive protection against UV damage from their algae: a test with *Paramecium bursaria* and *Chlorella*. *Protist* **160**: 233–243.

Swanson SJ, Bethke PC & Jones RL (1998) Barley aleurone cells contain two types of vacuoles: characterization of lytic organelles by use of fluorescent probes. *Plant Cell* **10**: 685–698.

Tanaka M & Miwa I (1996) Significance of photosynthetic products of symbiotic *Chlorella* to establish the endosymbiosis and to express the mating reactivity rhythm in *Paramecium bursaria*. *Zool Sci* **13**: 685–692.

Tanaka M & Miwa I (2000) Correlation of photosynthetic products of symbiotic *Chlorella* with the mating reactivity rhythms in a mutant strain of *Paramecium bursaria*. *Zool Sci* **17**: 735–742.

Thomas TH, Hare PD & van Staden J (1997) Phytochrome and cytokinin responses. *J Plant Growth Regul* **23**: 105–122.

Tsukii Y, Harumoto T & Yazaki K (1995) Evidence for a viral macronuclear endosymbiont in *Paramecium caudatum*. *J Eukaryot Microbiol* **42**: 109–115.

Van Etten JL, Lane LC & Meints RH (1991) Viruses and viruslike particles of eukaryotic algae. *Microbiol Rev* **55**: 586–620.

Yamada T, Onimatsu H & Van Etten JL (2006) *Chlorella* Viruses. *Adv Virus Res* **66**: 293–336.

Yu SM (1999) Cellular and genetic responses of plants to sugar starvation. *Plant Physiol* **121**: 687–693.

Ziesenisz E, Reisser W & Wiessner W (1981) Evidence of *de novo* synthesis of maltose excreted by the endosymbiotic *Chlorella* from *Paramecium bursaria*. *Planta* **153**: 481–485.