Infection of *Paramecium bursaria* by Symbiotic *Chlorella* Species

Yuuki Kodama and Masahiro Fujishima

Contents

Abstract *Paramecium bursaria* and endosymbiotic *Chlorella* species retain their ability to grow independently, but can reestablish endosymbiosis by mixing. Infection is induced through the host's digestive vacuoles (DVs). Acidosomal and lysosomal fusions to the DVs begin at 0.5 and 2–3 min after mixing, respectively.

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Pulse-labeling of algae-free paramecia with isolated symbiotic algae for 1.5 min and chasing for various times shows that some algae acquire temporal resistance to lysosomal enzymes in the DVs. They begin to escape from the DVs by budding of the vacuole membrane at 30 min after mixing. Then each small vacuole enclosing a green alga differentiates to a perialgal vacuole (PV), which gives protection from the host lysosomal fusion, and which translocates beneath the host cell surface. Algal cell division in the PV begins at about 24 h after mixing. Infection experiments with infection-capable and infection-incapable algae indicate that the infectivity is based on their ability to localize beneath the host surface after escaping from DVs. Algal proteins synthesized during photosynthesis serve some important functions to prevent expansion of the PV and to attach under the host surface, and to protect the PV from host lysosomal fusion. Although molecular mechanisms for these phenomena remain to be elucidated, accumulated evidence suggests that the symbiotic *Chlorella* sp. have a very long evolutionary history. This chapter mainly addresses studies of DV differentiation, the infection process of the algae, characteristics of the PV membrane, and related phenomena.

1 Introduction

Among ciliate *Paramecium* species, only *Paramecium bursaria* can maintain endosymbiotic algae in the cytoplasm. In fact, algae-free *P. bursaria* cells are rare in natural environments. Typically, *P. bursaria* cells harbor several hundred symbiotic algae in their cytoplasm (Fig. 1a); the association of *P. bursaria* with the symbiotic *Chlorella* sp. is a mutual symbiosis. For instance, the host can supply algal cells with nitrogen components and CO_2 (Albers and Wiessner 1985; Albers et al. 1982; Reisser 1976, 1980). Furthermore, when within the host, the host protects algae from infection of the *Chlorella* virus (Kawakami and Kawakami 1978; Reisser et al. 1988; Van Etten et al. 1983; Yamada et al. 2006). Also, algal carbon fixation is enhanced in the host (Kamako and Imamura 2006; see Kato and Imamura, this volume). The algae can supply the host with a photosynthetic product, maltose (Brown and Nielsen 1974; Reisser 1976, 1986). The algae in the host show a higher rate of photosynthetic oxygen production than in the isolated state, thereby guaranteeing an oxygen supply for the host (Reisser 1980). Algae-bearing *P. bursaria* can grow better than non-algae-bearing cells (Görtz 1982; Karakashian 1963, 1975); the alga has a photoprotective role for the host (Hörtnagl and Sommaruga 2007; see Sommaruga and Sonntag, this volume). Photosynthetic products of symbiotic *Chlorella* are related closely to the expression of circadian rhythms in host *P. bursaria* (Miwa et al. 1996; Tanaka and Miwa 1996, 2000; Miwa et al. 1996; see Miwa, this volume). Moreover, timing of cell divisions of both the algae and the host cells is well coordinated (Kadono et al. 2004; Takahashi et al. 2007).

Algae-free *P. bursaria* can be produced easily from algae-bearing cells using one of several methods: rapid fission (Jennings 1938); cultivation in darkness

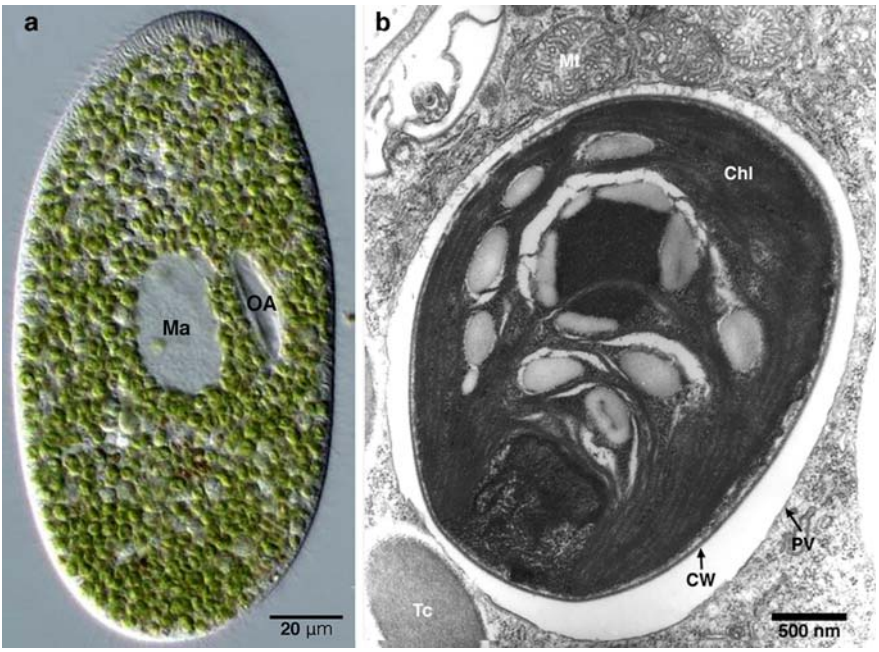

Fig. 1 Light and transmission electron micrographs of *Paramecium bursaria*: **a** Differential interference contrast (DIC); **b** Transmission electron microscopy (TEM); *Ma,* macronucleus; *OA*, oral apparatus; *Chl, Chlorella*; *PV,* perialgal vacuole membrane; *CW,* cell wall; *Mt,* mitochondrion; *Tc*, trichocyst. (**b** provided by Y. Kodama and I. Inouye, University of Tsukuba)

(Karakashian 1963; Pado 1965; Weis 1969); X-ray irradiation (Wichterman 1948); treatment with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a blocker of electron flow in photosystem II (Reisser 1976); treatment with the herbicide paraquat (Hosoya et al. 1995; Tanaka et al. 2002); or treatment with cycloheximide (Kodama and Fujishima 2008; Kodama et al. 2007; Weis 1984). Irrespective of mutual relationships between *P. bursaria* and symbiotic algae, the algae-free cells and the symbiotic algae retain the ability to grow without a partner. Furthermore, endosymbiosis between the algae-free *P. bursaria* cells and the symbiotic algae isolated from the algae-bearing *P. bursaria* cells is easily reestablished by mixing them (Karakashian 1975; Siegel and Karakashian 1959). Therefore, the symbiotic associations between these eukaryotic cells are excellent models for studying cell-to-cell interaction and the evolution of eukaryotic cells through secondary endosymbiosis between different protists. However, the mechanisms and timings used by the algae to escape from the host DV and to protect themselves from host lysosomal fusion were not revealed for a long time. In *P. bursaria*, each symbiotic alga is enclosed in a PV derived from the host DV, which is assumed to have the ability to protect the alga from lysosomal fusion (Gu et al. 2002; Karakashian and

Rudzinska 1981) (Fig. 1b). Recently, important cytological events needed for establishing endosymbiosis and their timings in the infection process were clarified by pulse-labeling with symbiotic *Chlorella* cells isolated from the algae-bearing *P. bursaria* for 1.5 min; then chasing for various times (Kodama and Fujishima 2005, 2007, 2008, 2009a, 2009b; Kodama et al. 2007). This review specifically examines these four checkpoints for establishing stable endosymbiosis between *P. bursaria* and the symbiotic *Chlorella* species.

2 Differentiation of DVs of *P. bursaria*

Infection of the symbiotic algae to the host *P. bursaria* cells is performed through the host's phagocytosis. To investigate the infection process, we must first know the differentiation process of the host's DVs in phagocytosis.

2.1 Morphological Classification of DVs and Timing of Appearance of Each DV

To classify stages of DVs that appear during infection by *Chlorella* sp. and to determine the timing of the appearance of each stage, symbiotic algae isolated from algae-bearing *P. bursaria* cells were mixed with algae-free paramecia at densities of 5×10^7 algae/ml and 5×10^3 paramecia/ml under fluorescent light (1,500 lx) at 25 ± 1°C and fixed with 4% (w/v) paraformaldehyde as shown in Fig. 2 (Kodama and Fujishima 2005). The DVs during the infection process are classified into eight different stages on the basis of their morphological characteristics and algal color changes in the DVs (Fig. 3). The DV-I vacuole has a rounded vacuole membrane containing green algae. Its membrane is clearly visible under a DIC microscope. In contrast, DV-II has a contracted vacuole, which renders the vacuole membrane barely visible; the algae are green. In DV-III, the vacuole has increased size, making the vacuole membrane visible; the algae are discolored faint yellow or green, or both. DV-III, is further classified into three substages: DV-IIIa contains green algae only; DV-IIIb contains both discolored faint yellow and green algae; and DV-IIIc contains discolored faint yellow algae only. In the final stage, DV-IV, the membrane is again contracted, as in DV-II, rendering the vacuole membrane barely visible under a microscope; the algae are green or brown, or both. Unlike DV-II, this vacuole was not observed in cells before 2 min after mixing at 25 ± 1°C but after 20–30 min. DV-IV was also further classified into three substages: DV-IVa contains green algae only; DV-IVb contains both green and brown algae; and DV-IVc contains brown algae only. DVs containing single green *Chlorella* (SGC) were observed in cells fixed 30 min after mixing, but all SGCs present in cells before 30 min after mixing were digested for 30 min. The algae-free cells were mixed with the isolated symbiotic

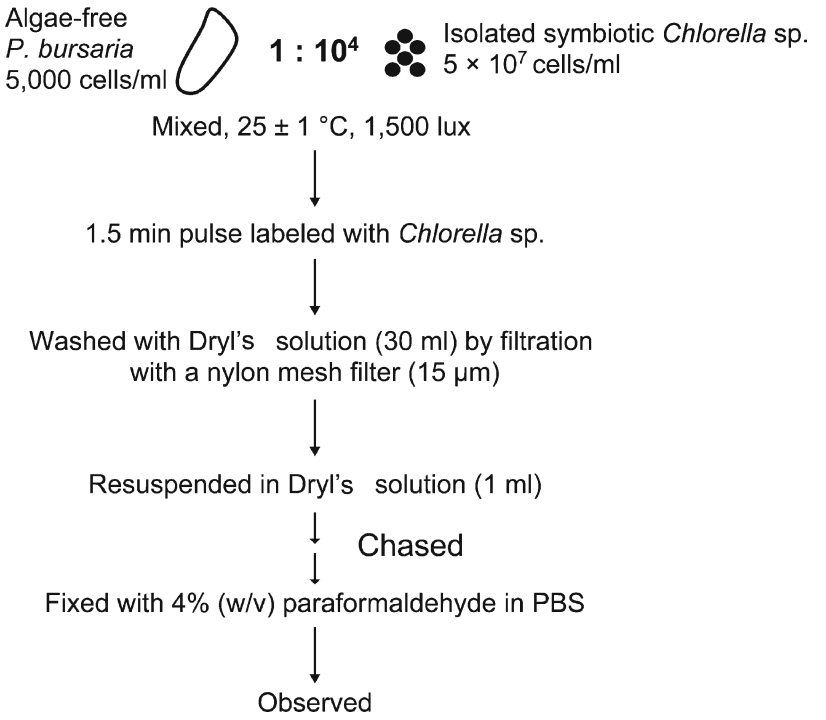

Fig. 2 Pulse-labeling and chasing with isolated *Chlorella* sp. Algae-free *P. bursaria* cells were mixed at a density of 5×10^3 per milliliter with isolated *Chlorella* sp. at 5×10^7 algae/ml under a fluorescent light (1,500 lx) for 1.5 min at 25 ± 1°C. The ciliate–algae mixture was transferred to a centrifuge tube equipped with a 15-μm pore size nylon mesh and filtered. By pouring fresh modified Dryl's solution (MDS) (Dryl 1959) (KH_2PO_4 was used instead of $NaH_2PO_4 \cdot 2H_2O$) 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 and resuspended in MDS, and then chased for various times under a fluorescent light (1,500 lx) at 25 ± 1°C. The cell suspension was fixed by mixing it with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) (137 mM NaCl, 2.68 mM KCl, 8.1 mM $Na_2HPO_4 \cdot 12H_2O$, 1.47 mM KH_2PO_4, pH 7.2) at various time points, and the cells were observed under a DIC microscope

algae and fixed at 10-s intervals for 60 s to determine the timing of the DV-II appearance. Actually, DV-II appeared in cells fixed at 30 s after mixing. To determine the timings of DV-III and DV-IV appearance, the algae-free cells were pulsed with isolated algae for 1.5 min, washed, chased, and fixed at every 1-min interval after mixing. Both DV-III and DV-IV appeared in cells at 2–3 and at 20–30 min after mixing, respectively. Because of changes in the number of each stage of DV, the majority of stage DV-I vacuoles become DV-II, DV-IIIa, DV-IIIb, and then DV-IVb, in that order. Other changes, for example, from DV-IIIa to DV-IVa, from DV-IVa to DV-IVb, and from DV-IIIb to DV-IIIc, were few if they existed at all (Kodama and Fujishima 2005, 2007, 2009a).

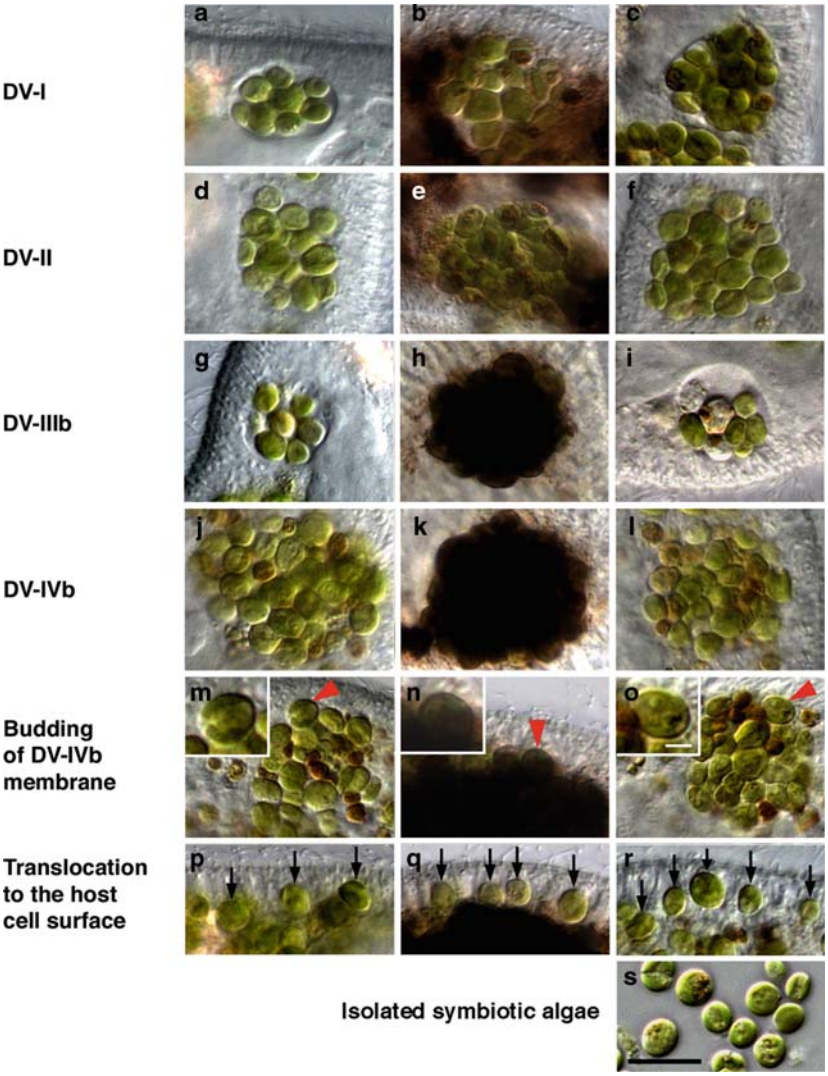

Fig. 3 DIC micrographs of the infection process of symbiotic *C. vulgaris* cells to the algae-free *P. bursaria* cells. *Chlorella*-free paramecia were mixed with isolated symbiotic algae and fixed at 0.5 min (**a**–**c**), 1 min (**d**–**f**), 10 min (**g**–**i**), 30 min (**j**–**l**), and 3 h (**m**–**r**) after mixing. **a**, **d**, **g**, **j**, **m**, **p** Cells non-treated with Gomori's solution. **b**, **e**, **h**, **k**, **n**, **q** Cells treated with Gomori's solution. **c**, **f**, **i**, **l**, **o**, **r** Cells treated with Gomori's solution lacking sodium β-glycerophosphate, a substrate for the acid phosphatase (AcPase) (control experiment). **s** Isolated symbiotic algae treated with Gomori's solution lacking the substrate. Experiments were repeated more than ten times; the results were reproducible. **a**–**c** DV-I; **d**–**f** DV-II; **g**–**i** DV-IIIb; **j**–**l** DV-IVb; **m**–**o** An alga is just escaping by budding of the DV-IVb membrane (*red arrowhead*). *Insets* in **m**–**o** show enlarged photomicrographs of the escaping alga. **p**–**r** show algae attached just beneath the host cell surface (*black arrows*). DV-I (**b**) and DV-II (**e**) are AcPase activity-negative; DV-IIIb (**h**) and DV-IVb (**k**, **n**) are AcPase activity-positive. The single green *Chlorella* (SGC) that escaped from the host digestive vacuoles (DVs) and translocated just beneath the host cell surface are AcPase activity-negative (**q**, *black arrows*). *Bars* 10 μm (**s**) and 2 μm (*inset* in **o**). (Updated from Kodama and Fujishima 2009a)

2.2 Timing of Acidosomal Fusion to DVs

To determine the time taken for the intravacuolar pH to change, yeast cells labeled with the pH indicator dyes bromcresol green (BCG), bromophenol blue, or Congo red were given to the algae-free *P. bursaria*. The color changes of the ingested yeasts were observed (Fig. 4). The Intravacuolar pH rapidly decreases from 6.4–7.0 to 2.4–3.0 at 0.5–1.0 min by acidosomal fusion to DVs; this occurs simultaneously with morphological differentiation into a DV-II vacuole. However, the intravacuolar pH begins to increase again before differentiation into a DV-III vacuole (Kodama and Fujishima 2005).

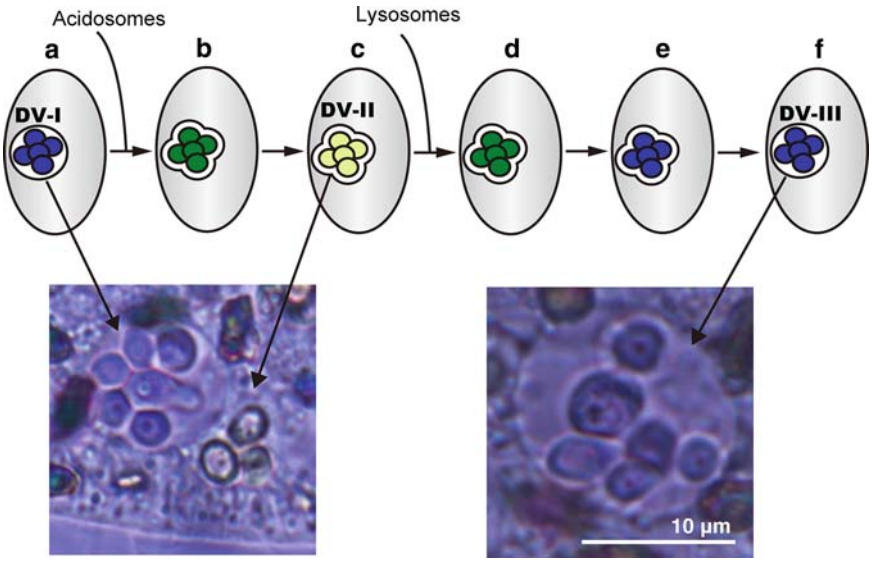

Fig. 4 Color changes of bromcresol green (BCG)-labeled yeast cells in DVs of *P. bursaria*. Algae-free paramecia and BCG-labeled yeast cells were mixed and the color of the yeast in the host DVs was observed in living cells. **a** DV-I – soon after mixing, the yeast cells are blue, indicating that the intravacuolar pH is 6.4–7.0. **b**–**e** DV-II: **b** Blue-green yeast cells at 0.5–1 min after mixing, indicating that the intravacuolar pH is 4.4–5.0; **c** Yellow-green yeast cells at 1–2 min after mixing, indicating that the intravacuolar pH is 2.4–3.0; **d** Blue-green yeast cells at 2–3 min after mixing, indicating that the intravacuolar pH has risen to 4.4–5.0; **e** DV-II bearing blue yeast cells at 2–3 min after mixing, indicating that the intravacuolar pH rises to 6.4–7.0 before morphological differentiation to DV-III. **f** DV-III bearing blue yeast cells at 3 min after mixing, indicating that the intravacuolar pH had risen to 6.4–7.0. The changes in yeast color indicate that acidosomes fuse between the stages depicted in *panels* **a** and **b**, and lysosomes fuse between the stages depicted in *panels* **c** and **d**. Photomicrographs are of DVs ingesting BCG-labeled yeasts. *Bar* 10 µm. (Updated from Kodama and Fujishima 2005)

2.3 Timing of Lysosomal Fusion to DVs

Partially digested algae appear first in DV-IIIb at 2–3 min after mixing; the increase in pH in acidified DV-II begins in this late DV-II. These results suggest that lysosomal fusion might start before 2–3 min after mixing. Gomori's staining shows that DV-I and DV-II vacuoles are AcPase-activity-negative, but all substages of DV-III and DV-IV are positive (Fig. 3) (Kodama and Fujishima 2008, 2009a). These results suggest that the timing of lysosomal fusion occurs at or immediately before DV-III at 2–3 min after mixing.

3 Fates of *Chlorella* Cells in Infection

Each alga that is maintained in the host cell is surrounded by a PV membrane in the host cytoplasm (Karakashian et al. 1968; Meier and Wiessner 1989; Meier et al. 1984; Reisser 1981). This indicates that DVs containing SGC appear from DVs containing many green algae in the early infection process. To determine the timing of the appearance of the SGCs, algae-free paramecia were pulsed with symbiotic algae for 1.5 min, chased, then fixed at 0.05, 0.5, 1, 1.5, 2, 3, 6, 9, 24, 48, and 72 h after mixing. The percentages of cells with SGC, single digested *Chlorella* (SDC), DV-IIIa, DV-IIIb, DV-IVa, and DV-IVb are portrayed in Fig. 5a (Kodama and Fujishima 2005). All SGCs that were present in the host cytoplasm before 30 min after mixing are digested. One hour after mixing, however, SGCs reappear in the host cytoplasm. Figure 5a depicts that the SGCs appearing after 0.5 h are derived from DV-IVa or DV-IVb because no green algae are present in other DVs. At 24 h, the SGCs begin to multiply by cell division, indicating that these algae had established endosymbiosis. In contrast to results of an earlier study (Meier and Wiessner 1989), Fig. 5a shows that the algal escape from the DV-IVa or the DV-IVb vacuole occurs after acidosomal and lysosomal fusion to the DV. They are all digested in DV-III when boiled algae are added to algae-free paramecia (Fig. 5b).

As mentioned above, the SGCs that appeared at 30 min after mixing with the algae-free paramecia and succeeded in establishing endosymbiosis in the host cell appeared to originate from either DV-IVa or DV-IVb. After 30 min after mixing with *Chlorella* sp., five kinds of cells appeared: those with no algae, those with digested algae only, those with DV-IVa, those with DV-IVb, and those with SGCs (Fig. 6a). The frequency of appearance of these cells was examined by fixing cells at various time points after a 1.5-min pulse label with isolated *Chlorella* sp. (Fig. 6b). When DVs of multiple types were present in the same cell, the cell was classified according to the DV that was oldest, following the scheme shown in Fig. 6a. As shown in Fig. 6b, the proportion of cells with SGCs was 0% at 0.5 h and increased as time elapsed. In contrast, the percentage of cells with DV-IVb decreased. At 72 h after mixing, all cells either contained SGCs or were without any

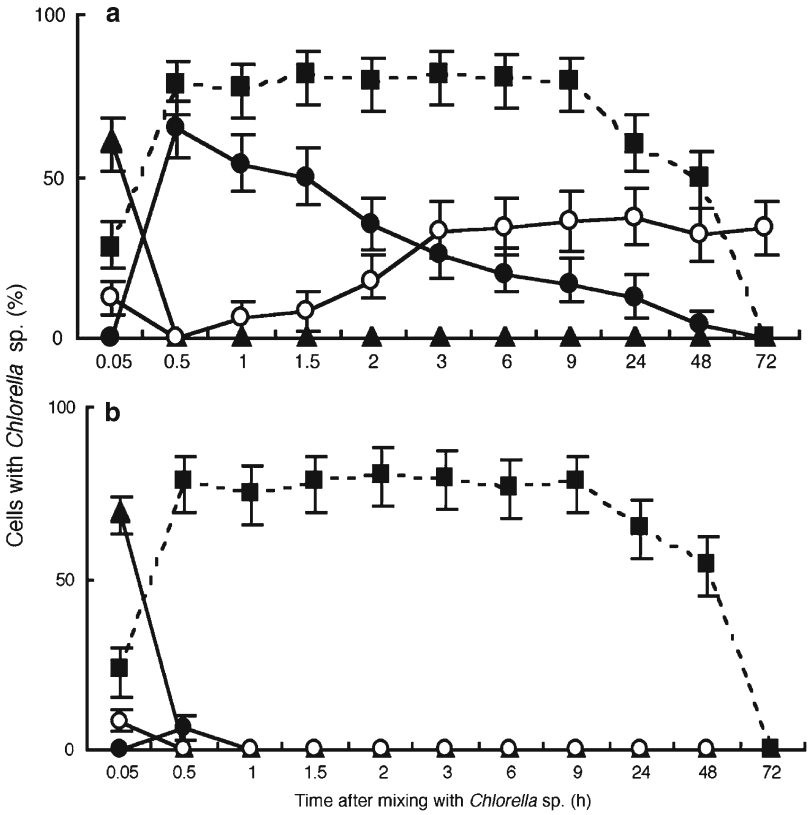

Fig. 5 Fates of living and boiled *Chlorella* sp. during the infection process. Isolated living (**a**) or boiled (**b**) *Chlorella* sp. and algae-free paramecia were mixed, washed, chased, and fixed at 0.05, 0.5, 1, 1.5, 2, 3, 6, 9, 24, 48, and 72 h after mixing. The percentages of cells with SGC, single digested *Chlorella* (SDC), DV-IIIa, DV-IIIb, DV-IVa, and DV-IVb were determined. All SGCs that appeared before 0.5 h after mixing were digested by 0.5 h. *Triangles* cells with DV-IIIa and DV-IIIb, *filled circles* DV-IVa or DV-IVb, *open circles* SGC, *squares* SDC. For each fixing time interval, 100–300 cells were observed. *Bar* 90% confidence limit. (From Kodama and Fujishima 2005)

algae. Dividing SGCs were observed at and after 24 h of mixing. At 72 h after mixing, about 35% of the cells contained SGCs. On the other hand, less than 5% of cells contained DV-IVa, if any. This indicates that the majority of the SGCs maintained in the host cell originated from DV-IVb. The reproducibility of these results was confirmed twice.

These observations suggest a new mechanism by which *Chlorella* sp. can avoid digestion. First, some of the algae show temporary resistance to the host lysosome enzymes in DV-III and DV-IV vacuoles. Second, the algae bud off from DV-IVb into the host cytoplasm. Finally, the algae lose their temporary resistance to the host lysosomal enzymes but are protected from lysosomal fusion by the PV membrane. The infection process of the symbiotic algae is depicted in Fig. 7.

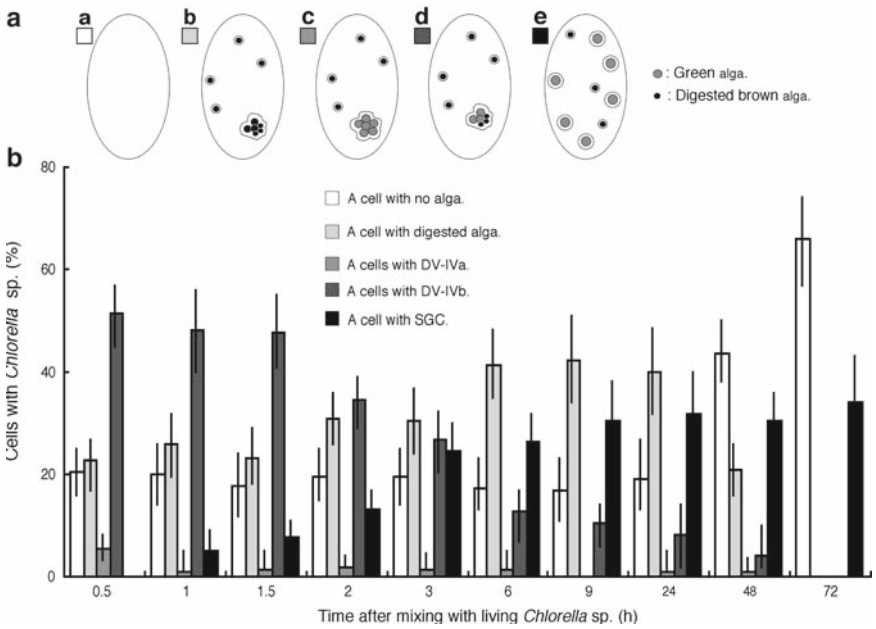

Fig. 6 Source of SGCs that can establish endosymbiosis. *Chlorella* sp.-free cells were mixed with isolated symbiotic algae, washed, chased, and fixed at 0.5, 1, 1.5, 2, 3, 6, 9, 24, 48, and 72 h after mixing. **a** Cells fixed at 0.5 h after mixing were classified into five types according to the stages of their DVs: **a** a cell with no algae, **b** a cell with digested algae, **c** a cell with DV-IVa, **d** a cell with DV-IVb, **e** a cell with SGC. When a cell had several types of DVs, i.e., types b-e are seen together, the cell was classified in the order b < c < d < e. For example, when a cell has DVs with digested algae and SGC, the cell was classified as type e. **b** Summary of DV types found during the infection process. At each fixing time interval, 100–230 cells were observed. *Bar* 95% confidence limit. (From Kodama and Fujishima 2005)

3.1 Acquisition of Temporal Resistance to Lysosomal Enzymes in the Host DVs

It is known that different types of algae infecting *P. bursaria* clones exhibit different infection ratios and host dependencies (Nakahara et al. 2003; Nishihara et al. 1999). This suggests a possibility that different *Chlorella* species or strains used together for infection might result in different algal fates in DV-IVb. To remove this possibility, a OS1g1N clone of *P. bursaria* that was produced by infection of algae-free *P. bursaria* OS1w cells with symbiotic algal clone 1 N cells was used in experiments (Kodama et al. 2007). This algal clone 1 N was obtained from isolated symbiotic algae from an OS1g cell and identified as *C. vulgaris* from observation of its morphological characteristics using light and electron microscopy and by performing a similarity search with the 18S ribosomal DNA sequence from the cells (M. Nakahara, unpublished data). All SGCs that can establish endosymbiosis arose from DV-IVb vacuoles, as

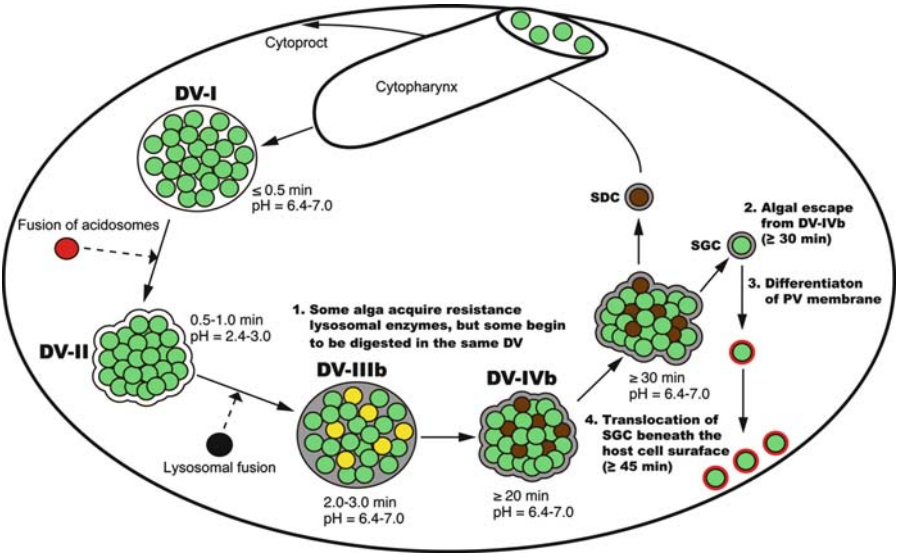

Fig. 7 The process of infection of algae-free *P. bursaria* cells by symbiotic *Chlorella* sp.. The spherical DV-I vacuole containing green algae differentiates to a condensed DV-II vacuole by fusion of acidosomes and acidification. Then the vacuole differentiates to a swollen DV-III vacuole by fusion of primary lysosomes. The AcPase activity-positive area by Gomori's staining is shown as a *gray* area in the DV. Some algae exhibit resistance to the lysosomal enzymes and can retain a green color and their original morphology. The remaining algae are partially digested and show a *yellow* color in the same DV. The internal pH of the DV-III vacuole increased. DV-III is further classified into three substages: DV-IIIa contains green algae only; DV-IIIb contains both discolored by partial digestion and green algae; and DV-IIIc contains discolored algae only. Then, the condensed DV-IV vacuole differentiates. DV-IV is classified into three substages: DV-IVa contains green algae only; DV-IVb contains both green and brown algae; DV-IVc contains brown algae only. SGCs and SDCs appear through DV-IVb vacuole membrane budding. This phenomenon occurs despite the fact that the alga is intact or partially digested. Algae in the buds remained covered by a *gray* thin layer by Gomori's staining. The SGCs translocate just beneath the host cell surface, anchor there, and initiate algal cell division to establish endosymbiosis. Algae attached beneath the cell surface have no AcPase activity, suggesting that the vacuole membrane wrapping the algae differentiates to the PV membrane (*red circle*) immediately after budding from the DV membrane. (Updated from Kodama and Fujishima 2005, 2007, 2009a)

portrayed in Fig. 6b. Approximately 50% of cells 30 min after mixing have DV-IVb vacuoles (Fig. 6b, Kodama and Fujishima 2005); most of the DVs at this time are AcPase activity-positive ones (Kodama and Fujishima 2009a), which shows that the DV-IVb vacuoles are AcPase activity-positive, and that some of the algae in DV-III and DV-IV vacuoles acquired lysosomal enzyme resistance in the DVs, although the remaining algae are digested in the same DV. Boiled algae are all digested in DV-III when they are added to algae-free paramecia (Fig. 5b). Furthermore, algae fixed with

2.5% (v/v) glutaraldehyde or with 5.0% (v/v) formaldehyde are also digested in DV-III (Kodama and Fujishima 2005, Kodama et al. 2007). Consequently, only living algae can develop temporary resistance to the lysosomal enzymes in the host DV. To date, what factor determines their fates in the host DV remains unknown. However, the following have been revealed (Kodama et al. 2007). Although genetically identical symbiotic algal cells, strain 1 N, are used for the infection, DV-IVb vacuoles appear. Light microscopy shows that the algal fate does not depend on the cell cycle stage or the location in the DV. Electron microscopy shows that the nondigested algae are not protected using a preexisting PV membrane in the DV-IVb vacuoles (Fig. 8). Moreover, this phenomenon is observed in the presence of cycloheximide and puromycin, which are known to inhibit algal and host protein synthesis, respectively.

3.2 Escape from the Host DVs by Budding of the Membrane

Some algae are freed from the DV-IVb vacuoles by budding of the DV membrane 30 min after mixing with the host cells. This phenomenon occurred even in cases where the alga was intact or partially digested. Furthermore, the budding is induced not only by living *Chlorella* sp., but also by boiled algae or fixed algae (Kodama and Fujishima 2005). Yeasts also escaped from the *P. bursaria* DVs (Suzaki et al. 2003; Y. Kodama and M. Fujishima, unpublished results). However, India ink, polystyrene latex spheres of 0.81-μm diameter, and the food bacterium *Klebsiella pneumoniae* do not induce this phenomenon (Kodama and Fujishima 2005). The bacterium *Pseudomonas* sp. and the yeasts *Rhodotorula rubra* and *Yarrowia lipolytica* ingested by *P. bursaria* can be maintained in host PVs, indicating that these organisms also induce budding of the host DVs (Görtz 1982; Suzaki et al. 2003). Molecular mechanisms for the budding and escape from the DV are not known.

3.3 Differentiation of PV Membrane

To understand the timing of differentiation of PV from the host DV, algae-free *P. bursaria* cells were fed symbiotic *C. vulgaris* cells for 1.5 min, washed, chased, and fixed at various times after mixing. Then, AcPase activity in the vacuoles enclosing the algae was detected using Gomori's staining (Fig. 3). This activity appears in 3-min-old vacuoles; all DVs containing algae demonstrate the activity at 30 min. Algal escape from the DVs begins at 30 min by budding of the DV membrane (Fig. 3m). In the budded membrane, each alga is surrounded by a layer of Gomori's thin positive staining (Fig. 3n, red arrowhead). The vacuoles involving a SGC move quickly and attach immediately beneath the host cell surface (Fig. 3p, arrow). Such vacuoles are Gomori's staining-negative (Fig. 3q, arrow; Kodama and

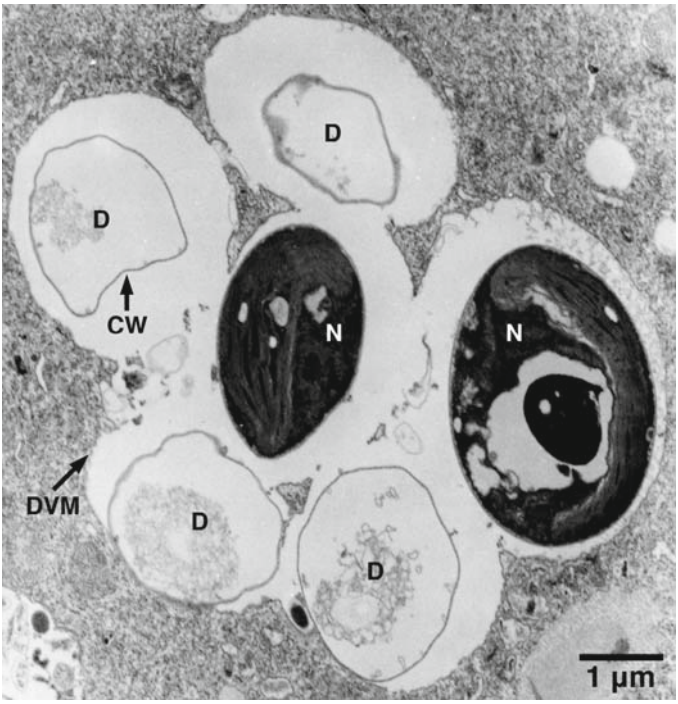

Fig. 8 Transmission electron micrograph of a DV-IVb vacuole. Three hours after mixing with *C. vulgaris*, algae-free *P. bursaria* were fixed for TEM observation. Note that the DV-IVb vacuole is condensed. Partially digested (*D*) and nondigested (*N*) algae are present together in the same DV. The nondigested algae are not separated from the digested algae by a membrane representing a PV membrane. *DVM*, DV membrane; *CW,* cell wall. (Provided by Y. Kodama and I. Inouye, University of Tsukuba)

Fujishima 2009a). These observations indicate that the PV membrane differentiates soon after the algal escape from the host DV, as presented in Fig. 7.

3.4 Translocation and Attachment Beneath the Host Cell Surface

The SGCs that are able to escape from DV-IVb vacuoles by budding of the DV membrane translocate beneath the host surface and are embedded among the trichocysts. This phenomenon is observed only with living and not with boiled algae (Kodama and Fujishima 2005). A similar phenomenon has also been reported for the yeasts *Rhodotorula rubra* and *Yarrowia lipolytica* ingested by *P. bursaria* (Suzaki et al. 2003) and also in symbiotic algal cells of various protists (Reisser 1986). These observations indicate that the PV membrane functions not only for protection of the symbiont from lysosomal fusion, but also for stable attachment of the symbiotic algae to the host cell.

Tonooka and Watanabe (2002, 2007) collected a natural aposymbiotic strain of *P. bursaria*. Infection experiments revealed that this strain showed unstable symbiosis with *Chlorella* sp. The algae aggregated at the posterior region of the host, resulting in aposymbiotic cell production after cell division. Crossbreeding analyses between this strain and a normal strain showed that all F1 progenies showed stable symbiosis with the algae, but some F2 progenies, through sibling crosses between symbiotic F1 progenies, showed unstable symbiosis resembling that of the original aposymbiotic strain. These results indicate that the attachment of the PVs beneath the host cell surface involves a genetically controlled unknown host factor.

3.5 Cell Division of Algae After Establishment of Endosymbiosis

The timing of algal cell division in the infection process remained unknown for a long time. Algae-free paramecia at the stationary phase of growth were pulse-labeled by isolated symbiotic algae for 1.5 min, chased, and fixed at 1, 3, 6, 9, 24, and 48 h after mixing; the mean number of green algae per *Paramecium* cell was counted (Kodama and Fujishima 2005). The mean number of green algae was 9.4 algae/cell at 1 h, decreasing to 4.0 algae/cell at 3 h, and remaining constant until 9 h. However, it began to increase to 5.3 algae/cell at 24 h. In addition, dividing algae were frequently observed beneath the host cell surface at 24 h. Thus, algae begin cell division at about 24 h after mixing with the host cells. On the other hand, *Paramecium* cells apparently did not multiply by binary fission until 72 h after mixing the algae.

4 Different Behaviors in Infection Between Infection-Capable and Infection-Incapable *Chlorella* Species

In *P. bursaria*, only three free-living *Chlorella* and *Parachlorella* species can establish endosymbiosis: *C. vulgaris*, *P. kessleri*, and *C. sorokiniana*. Actually, *C. ellipsoidea*, *C. saccharophila*, *C. luteoviridis*, *C. zofingiensis*, and *C. mirabilis* are infection-incapable species (Takeda et al. 1998). Symbiotic *Chlorella* species derived from *Stentor polymorphus* or *Spongilla fluviatilis* were digested (Bomford 1965). What is the difference between the infection behaviors of these *Chlorella* species?

4.1 Infectivity of Various Symbiotic and Free-Living *Chlorella* Species

Takeda et al. (1998) reported that “infection-capable” *Chlorella* species, including symbiotic ones, are distinguishable by the presence of glucosamine as a chemical

component in their rigid walls (alkali-insoluble part of the cell wall), whereas the rigid walls of "infection-incapable" species contained glucose and mannose. They suggested that the presence of glucosamine in the rigid wall of the alga seems to be a prerequisite for determination of the symbiotic association between *P. bursaria* and *Chlorella* species. Algae-free *P. bursaria* cells were mixed with 15 strains of cultivated *Chlorella* species and observed for the establishment of endosymbiosis at 1 h and 3 weeks after mixing to determine the relationship between the infectivity of various *Chlorella* species and the nature of their cell wall components. Only two free-living strains – *C. sorokiniana* strain C-212 and *P. kessleri* strain C-531 – were maintained in the host cells. In contrast, free-living *C. sorokiniana* strain C-43, *P. kessleri* strain C-208, *C. vulgaris* strain C-27, *C. ellipsoidea* strains C-87 and C-542, *C. saccharophila* strains C-183 and C-169, *C. fusca* var. *vacuolata* strains C-104 and C-28, *C. zofingiensis* strain C-111, *C. protothecoides* strains C-150 and C-206, and a cultivated symbiotic *Chlorella* sp. strain C-201 derived from *S. fluviatilis* could not be maintained. Therefore, it appears that the establishment of endosymbiosis is not only algal-species-specific but is also algal-strain-specific. It is noteworthy that these infection-incapable strains were able to escape from the host DV by budding of the host DV membrane, but they failed to localize beneath the host cell surface and were eventually digested (Fig. 9) (Kodama and Fujishima 2007). Consequently, algal attachment beneath the host cell surface is an indispensable phenomenon for establishment of endosymbiosis.

4.2 Lectin Binding Ability of Symbiotic and Free-Living *Chlorella* Species

To confirm the relationship between the infectivity of various *Chlorella* species and the nature of their rigid walls, as suggested by Takeda et al. (1998), various *Chlorella* species were mixed with Alexa Fluor 488-conjugated wheat germ agglutinin (WGA), a lectin derived from *Griffonia simplicifolia* (GS-II) or concanavalin A (Con A), with or without pretreatment with 0.4 N NaOH. As presented in Table 1, however, no relationship was found between their infectivity and stainability with these lectins (Kodama and Fujishima 2007). Our results suggest that the infectivity of *Chlorella* species for *P. bursaria* is not based on sugar residues on their cell wall and on the alkali-insoluble part of the cell wall components, but on their ability to localize immediately beneath the host cell surface after escaping from the host DV, as described in Sect. 4.1. Reisser et al. (1982) and Weis (1980) reported that the infection ratio of algae-free cells decreased if the algae were pretreated with Con A. However, we were unable to observe a statistically significant difference in the infection ratio between the lectin-treated and the untreated *Chlorella* sp. cells (Kodama and Fujishima 2007).

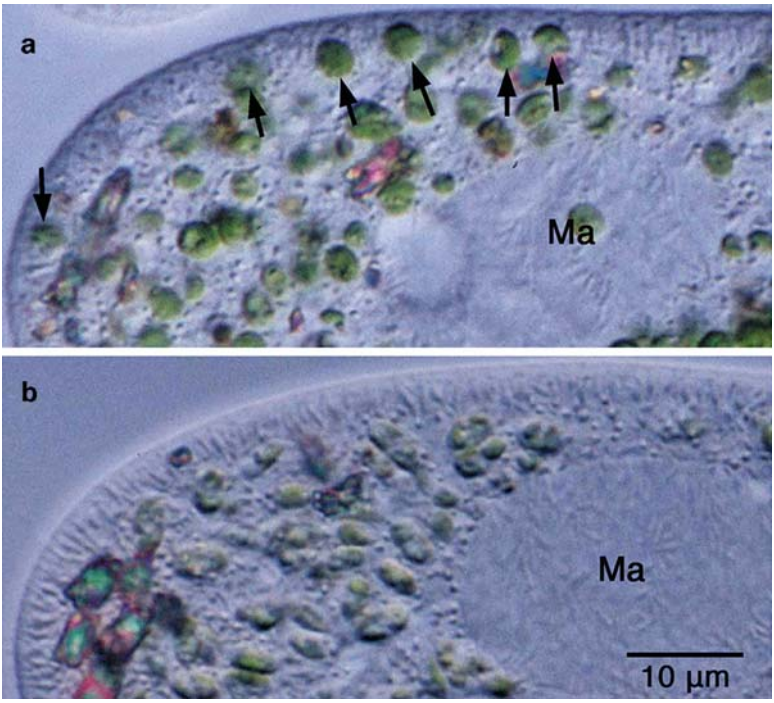

Fig. 9 Photomicrographs of *P. bursaria* OS1w cells pulse-labeled with symbiotic *Chlorella* sp. cells isolated from *P. bursaria* OS1g cells (**a**) and with *C. saccharophila* strain C-169 cells (**b**). Paramecia were observed 3 h after mixing. Note that the symbiotic *Chlorella* sp. cells are localized beneath the host cell surface (*arrows*), whereas *C. saccharophila* C-169 cells are not. *Ma,* macronucleus. (From Kodama and Fujishima 2007)

5 Characteristics of PV Membrane

To date, the following qualitative differences are known to exist between the DV and the PV membrane:

1. The PV encloses a single algal cell (Gu et al. 2002; Karakashian and Rudzinska 1981).
2. The gap separating the algal cell wall and the PV membrane is about 0.05 μm, so the PV membrane is hardly observable under a light microscope (Reisser 1986).
3. The PV diameter does not vary much (2.5–4.5 μm), except during the division of the enclosed alga (Reisser 1992).
4. The PV does not participate in cyclosis, but localizes beneath the host cell surface (Kodama and Fujishima 2005; Reisser 1986).
5. Particle density and its distribution of the PV show few signs hinting at any endocytotic or exocytotic activity (Meier et al. 1984).

Table 1 Lectin-binding activity of the NaOH-treated and untreated cell walls of *Chlorella* species and their infectivity for algae-free *Paramecium bursaria*

				Lectin labeling of[a]					
		Infectivity		Nontreated cells			NaOH-treated cells		
Species	Strain (alternative name)	T316w	OS1w	WGA	GS-II	Con A	WGA	GS-II	Con A
C. vulgaris	C-27[b]		−	−	−	−	+	+	+
C. sorokiniana	C-212 (211-8k)[c]	+	+	+	−	−	±	+	±
	C-43[b]		−	+	−	−	+	+	+
Parachlorella kessleri (formerly called *C. kessleri*)	C-208 (211-11g)[c]	+	−	−	−	−	−	±	−
	C-531 (211-11h)[c]	+	+	−	−	−	−	±	−
C. ellipsoidea	C-87 (211-1a)[c]	−	−	−	−	+	−	+	+
	C-542 (211-1a)[c]	−	−	−	−	+	−	+	+
C. saccharophila	C-183[b]		−	−	+	+	−	+	+
	C-169[b]		−	−	−	+	−	−	+
C. fusca var. *vacuolata*	C-104 (211-8b)[b]		−	−	−	+	−	−	+
	C-28[b]		−	−	−	+	−	−	−
C. zofingiensis	C-111 (211-14)[b]		−	−	−	+	−	−	+
C. prototheocoides	C-150 (211-11a)[b]		−	−	−	+	−	−	+
	C-206[b]		−	−	−	+	−	−	+
Symbiotic *Chlorella* sp.	C-201[b]		−	−	−	+	−	+	+
	OS1g[b]		+	−	−	−	+	+	+
	Dd1g[b]		+	−	−	−	+	+	+
	KM2g[b]		+	−	−	−	+	+	+
	Bwk-16 (C^+)[b]		+	±	−	±	+	+	+
	1N[b]		+	−	−	−	+	+	+

Updated from Kodama and Fujishima (2007)

WGA wheat germ agglutinin, *GS-II Griffonia simplicifolia*, *Con A* concanavalin A

[a] Algal cells were labeled with Alexa Fluor 488-conjugated Con A, WGA, or GS-II. +, 100% of cells with fluorescence; ±, less than 100%; −, 0%. For each experiment, more than 100 algal cells were observed.

[b] Strain used only in Kodama and Fujishima (2007)

[c] Strain used in Kodama and Fujishima (2007) and by Takeda et al. (1998)

6. The PV has no AcPase activity (Karakashian and Rudzinska 1981; Kodama and Fujishima 2009a).
7. The PVs show synchronous swelling by treatment with cycloheximide in the presence of algal photosynthesis (Kodama and Fujishima 2008).

5.1 Protection from Lysosomal Fusion

The timing of differentiation of the PV membrane from the host DV membrane is presumed to occur soon after the algal escape by budding of the host DV membrane and before the PV's attachment beneath the host cell surface, as explained in Sect. 3.3. However, no direct evidence demonstrates that the PV membrane does not allow lysosomal fusion to the membrane. The PVs containing SGC of about 3–4-μm diameter are embedded among trichocysts beneath the host cell surface; the surface area of 5–10-μm depth is AcPase activity-negative by Gomori's staining (Kodama and Fujishima 2008, 2009a, 2009b). These observations raise the possibility that the PV membrane has no capability for protection from lysosomal fusion, but can avoid lysosomal fusion by localization at the primary lysosome-less area of the cell. This AcPase activity-negative area can be reduced to less than 3-μm depth if the trichocysts are removed through treatment with 1mg/ml lysozyme. In such cells, some of the PVs were exposed to the AcPase activity-positive area, although such PVs were not stained with Gomori's staining, and the algae in each PV were not digested (Y. Kodama and M. Fujishima 2009b). These results demonstrate that the PV membrane requires no trichocysts for localization beneath the host cell surface, and that the PV membrane, unlike the DV membrane, provides protection from host lysosomal fusion, although molecular mechanisms that would enable the PV membrane to give protection from lysosomal fusion are unknown.

5.2 Synchronous Swelling of PVs

Cycloheximide is known to inhibit protein synthesis of symbiotic *Chlorella* of *P. bursaria* preferentially, but it only slightly inhibits host protein synthesis (Kodama and Fujishima 2008; Kodama et al. 2007; Weis 1984). Treatment of algae-bearing *Paramecium* cells with cycloheximide induces synchronous swelling of PVs in the host cell at about 1 day after treatment (Fig. 10b; Kodama and Fujishima 2008). This phenomenon is tentatively designated as "synchronous PV swelling (SPVS)". The space between the symbiotic algal cell wall and the PV membrane expands to about 25 times its usual width 24 h after the treatment. Then, the vacuoles left from beneath the host cell surface become stained with Gomori's staining, and the algae in the vacuoles are digested. Although SPVS is induced only under a constant-light (LL) condition, not under a constant-dark (DD) condition, even under a LL condition, this phenomenon is not induced in paramecia treated with cycloheximide in the presence of the photosynthesis inhibitor DCMU. These results indicate that the algal proteins synthesized in the presence of the algal photosynthesis serve some important function to prevent expansion of the PV and to maintain the ability of the PV membrane for localization beneath the host cell surface and for protection from host lysosomal fusion. The possible mechanisms of induction of the SPVS and digestion of the symbiotic algae are presented in Fig. 11. To date, little is known about qualitative characteristics of the PV membrane.

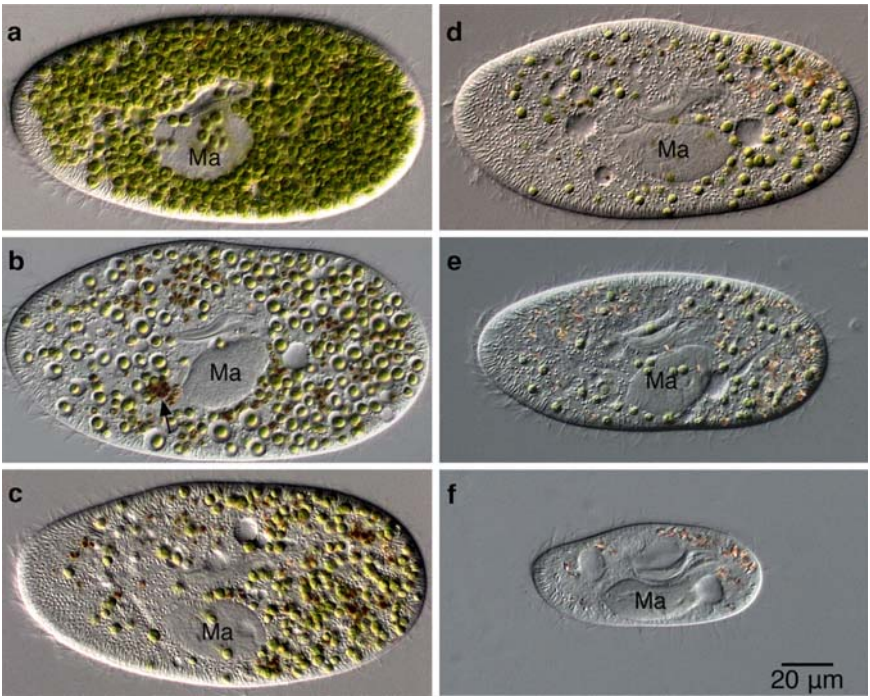

Fig. 10 Photomicrographs of algae-bearing OS1g1N cells suspended in fresh culture medium containing 10 µg ml^{-1} cycloheximide at 5×10^3 paramecia/ml at 25 ± 1°C under a constant-light (LL) condition: **a** Before treatment; **b** 1 day after mixing with cycloheximide. All PVs containing green algae swelled synchronously. Furthermore, digested algae appeared in the cytoplasm (*arrow*). **c** 2 days after mixing with cycloheximide, the green algae were numerically reduced; **d** 3 days after mixing; **e** 5 days after mixing; and **f** 7 days after mixing. All algal cells disappeared from the host cytoplasm. The *Paramecium* cells became small. *Ma*, macronucleus. (From Kodama and Fujishima 2008)

The intramembrane particles of the PV membrane are fewer than those of the DV membrane (Meier et al. 1984); lysosomes do not fuse with the PV membrane (Gu et al. 2002; Karakashian and Rudzinska 1981). Identification of SPVS will contribute to studies of PV membrane properties.

6 Protection of Symbiotic Algae from *Chlorella* Virus by Endosymbiosis

Chlorella virus has been identified as a lytic virus in symbiotic *Chlorella* cells of *P. bursaria* (Kawakami and Kawakami 1978). Later, Van Etten et al. (1982, 1983) found similar viruses in *P. bursaria* and established a laboratory infection system for these viruses using an exsymbiotic algal strain as the host that was originally isolated

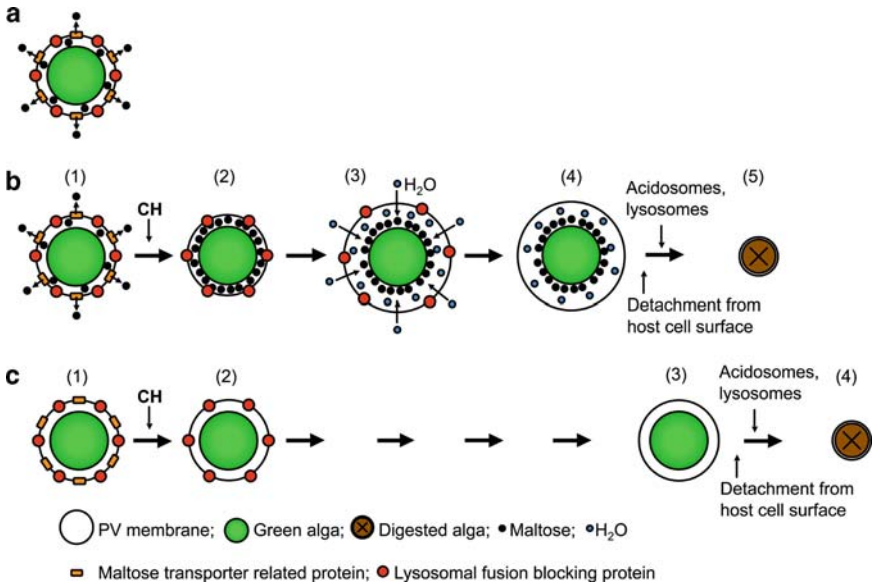

Fig. 11 Some hypotheses related to induction of synchronous PV swelling (SPVS) and digestion of symbiotic alga after treatment of algae-bearing *P. bursaria* cells with 10 μg ml^{-1} cycloheximide under LL or constant-dark (DD) conditions. As molecules responsible for functions of the PV membrane, two proteins that are synthesized by the algae, excreted outside the algae and localized on the PV membrane, are postulated. The first is a hypothetical maltose-transporter-related protein (*orange squares* on the PV membrane) (Willenbrink 1987), which is synthesized by the alga during photosynthesis and transports maltose from inside the PV membrane to the outside. Loss of this protein in the LL condition induces accumulation of photosynthesized carbohydrates, mainly maltose. The second is a hypothetical lysosomal fusion blocking protein (*red circles* on the PV membrane) that is synthesized by the algae and which has abilities to block lysosomal fusion to the PV membrane and also to attach to unknown structures immediately beneath the host surface, so loss of this protein induces detachment of the PV from the host cell surface and induces fusion with the host lysosomes. The maltose-transporter-related protein disappears rapidly from the PV membrane when algal protein synthesis is inhibited by cycloheximide. On the other hand, the lysosomal fusion blocking protein has a longer turnover time than that of the former; for this reason, this protein remains for some time on the PV membrane when the algal protein synthesis is inhibited by cycloheximide. Under the LL condition (**a**), the symbiotic alga synthesizes mainly maltose by photosynthesis in the host cell (Muscatine et al. 1967) and excretes it into a lumen between the algal cell wall and the PV membrane. The maltose is then transferred outside the PV membrane through the maltose-transporter-related protein on the PV membrane. The maltose-transporter-related protein disappears from the PV membrane (**b**-*2*) when the algal protein synthesis is inhibited by treatment with cycloheximide under the LL condition (**b**). Ayala and Weis (1987) reported that, by treatment with 100 μg ml^{-1} cycloheximide, the rate of carbohydrate secretion by symbiotic algae under the LL condition showed no significant difference between the treated and untreated groups. Consequently, the concentration of the carbohydrates including maltose increases inside the PV membrane, and outside water flows into the PV and induces the SPVS (**b**-*3*). Later, the lysosomal fusion blocking protein disappears from the swollen PV membrane (**b**-*4*). Therefore, the vacuole containing an alga detaches from the host surface; then the host acidosomes fuse to the swollen vacuole and the vacuole contracts by membrane replacement between the acidosomal membrane and the swollen vacuole (Fok et al. 1982; Kodama and Fujishima 2005). Thereafter, lysosomal fusion occurs to the contracted vacuole and the alga is digested (**b**-*5*). As presented in Fig. 10c, the PVs that can avoid the lysosomal

from a *P. bursaria* cell. The virus infects the algal cell by attaching to the algal cell wall. Subsequently, it degrades the cell wall at the attachment point, and the algae are lysed within about 6 h (Meints et al. 1984, Reisser et al. 1988). Reisser et al. (1988) also showed that algae-bearing paramecia grown in the presence of viruses show virus particles only in DVs but never in symbiotic *Chlorella* nor in PVs or in the cytoplasm of the paramecia, indicating that the viruses cannot leave the DVs. Although freshly isolated algae from the host cells do not lyse spontaneously within 14 days, the algae are lysed rapidly by mixing with the viruses. This fact demonstrates that the algae are not kept in a lysogenic status in the host cell, but are instead protected from viral infection. The reason that virus particles cannot enter into PVs with algae when the algae escape from the DV by budding of the DV membrane has not yet been clarified.

It is noteworthy that all the experimental host strains used so far for viral infection were originally endosymbionts of paramecia or hydras, and the virus cannot infect any of the free-living *Chlorella* strains tested to date (Van Etten et al. 1991). The *Chlorella* viruses can be collected in high densities in various countries (Van Etten et al. 1985; Yamada et al. 1993; Zhang et al. 1988). However, the symbiotic algae are protected from the viral infection in the host PVs, and no cells have so far been found which can be regarded as natural hosts (Yamada et al. 1993). Yamada et al. (1993) suggested that unknown free-living *Chlorella* species, which are natural hosts of the viruses, are ubiquitously distributed, and such natural host cells must share some common properties with the infection-capable symbiotic algal strains in the cell wall composition. If this is the case, we can expect that free-living *Chlorella* species become expressed receptors needed for the viral infection on their cell wall when the algae establish endosymbiosis with the *P. bursaria* cell, and that monoclonal antibodies specific for the symbiotic algal cell wall should be obtained. We recently succeeded in developing such a monoclonal antibody, which can react with the symbiotic algal cell wall of all strains of *P. bursaria* examined but not with the cell walls of free-living algal strains (A. Nishijima, Y. Kodama, and M. Fujishima, unpublished results).

7 Concluding Remarks and Further Perspectives

About 50 years have passed since infection experiments involving *P. bursaria* cells and the symbiotic *Chlorella* sp. were described by Siegel and Karakashian (1959). However, since that time, *P. bursaria* and its symbiotic algae have come to be used

Fig. 11 (continued) fusion in the presence of cycloheximide are recontracted. Such vacuoles might be produced by evasion of lysosomal fusion after acidosomal fusion. Under the DD condition (**c**-*1*), cycloheximide treatment induces loss of the maltose-transporter-related protein from the PV membrane (**c**-*2*), but no morphological change is induced. Later, the lysosomal fusion blocking protein disappears from the PV membrane (**c**-*3*). The vacuole detaches from the host cell surface and fuses with acidosomes and lysosomes; then the algae are digested (**c**-*4*). Under the DD condition, the fate of the PV is the same as that in **c**, irrespective of the presence or the absence of cycloheximide. *CH,* cycloheximide. (From Kodama and Fujishima 2008)

by many researchers for studies of endosymbiosis because both can grow separately and establish endosymbiosis soon after their mixing. Nevertheless, the infection process has not been revealed over those many years. Recently, through pulse-labeling with isolated symbiotic algae from *P. bursaria* for 1.5 min and chasing for various times by Kodama and Fujishima (2005), differentiation processes of the DVs, the route of infection, and four important cytological phenomena induced for establishing endosymbiosis were found, as summarized in Fig. 7. Although molecular analyses of these four phenomena have just begun, the results of such analyses will dramatically promote the study of endosymbiosis control mechanisms in the near future. How can the symbiotic algae acquire temporal resistance to lysosomal enzymes in the host DV-III and DV-IV vacuoles? How can algae escape from the host DV by budding of the DV membrane? What are substantial differences between the DV and the PV? What is the transportation system of the PV beneath the host cell surface? What is the signal for induction of algal cell division after localization beneath the host cell surface? Can endosymbiosis be established when the symbiotic algae are not taken up through DVs, but are instead inserted by microinjection? Why, among all of the various *Paramecium* species, can only *P. bursaria* establish endosymbiosis with *Chlorella* species? How can the PV membrane segregate an old cell wall, which is discarded after the algal cell division, and daughter algal cells? Can a single *Paramecium* cell maintain plural *Chlorella* species or plural strains of the same *Chlorella* species in a single *P. bursaria* cell? These problems remain to be elucidated.

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