CHAPTER IV

MORPHOLOGY AND PHYSIOLOGY

4.1 Light Microscopy

Preliminary inspections of wild type and paraquat resistant C. reinhardtii strains under light microscope clearly indicated indistinguishable features between these strains (Fig. 4.1). The cell size of C. reinhardtii either PPQ-10/3 or UPQ-S1 was obviously bigger than that of 137c wild type strain. However, pretreatment of these strains with certain concentrations of paraquat before observations created some changes in size and shape of all the experimental strains.

4.2 Scanning Electron Microscopy

To elucidate more information especially on morphological aspects of the paraquat resistant mutants of C. reinhardtii, the specimens must be appropriately processed so that the electron microscopic observations reflect their in situ structures. A method was modified from the classical procedure for dehydration of living organisms and was carried out in the laboratory by which the algal cells were adsorbed on the surface of a Whatman #1 filter paper and then carefully dehydrated with a serial concentrations of graded ethanol (starting from 10% ethanol). The characteristics of the Whatman #1 paper under the scanning electron microscopic field

Figure 4.1 Light micrographs of Chlamydomonas reinhardtii wild type and paraquat resistant strains showing general morphology of cells when cultured in the absence and in the presence of paraquat.

Cells were grown in medium with and without paraquat. Exponential cultures at hour 6 of light period were observed by magnification x200.

(a.) 137c in paraquat free medium

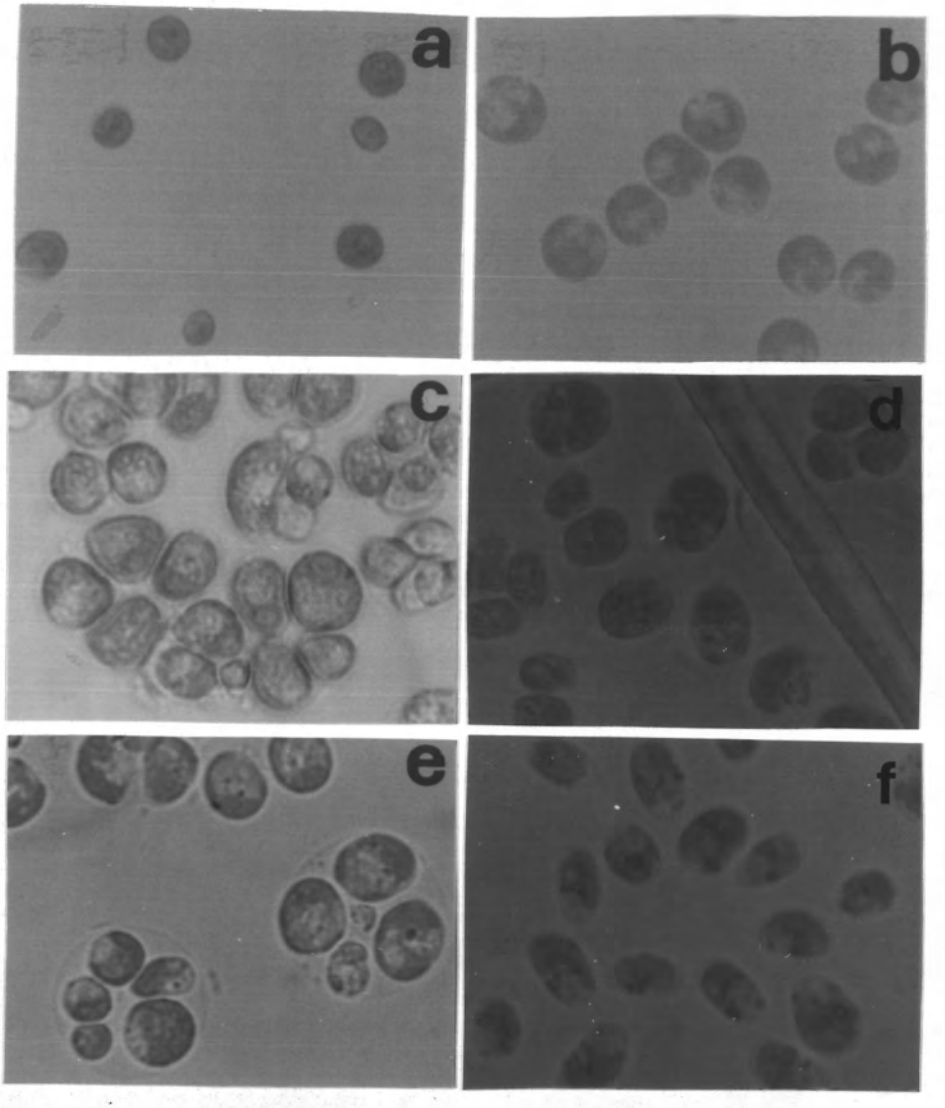
(b.) 137c in 0.5 μM paraquat

(c.) PPQ-10/3 in paraquat free medium

(d.) PPQ-10/3 in 1.0 μM paraquat

(e.) UPQ-S1 in paraquat free medium

(f.) UPQ-S1 in 1.0 μM paraquat


a
b
c
d
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appeared as cellulose fibril networks on which cells were adsorbed. Fig. 4.2 a and b illustrated the scanning electron micrographs of C. reinhardtii 137c cells. They could be seen as single ovoid-shaped cells dispersed on cellulose networks with an estimated size of 9x13 μ. Micrographs taken at higher instrumental magnification indicated that the outermost layer of a wild type is smooth and had a loose structure because electron beam could pass through it to a more electron dense structure inside. Treatment of wild type cells with paraquat at toxic level (0.5 μM) during growth caused abnormality of cells similar to that observed under a light microscope (Fig. 4.1b). Furthermore extensive cracks on the cell surface and the bigger in size (21x35 μ) were illustrated (Fig. 4.3 a and b).

Paraquat resistant mutants both PPQ-10/3 and UPQ-S1 exhibited distinct scanning electron microscopic morphologies. Both strains when cultivated in paraquat free medium established results corresponding to the light microscopic results (Fig. 4.1 c and e, Fig. 4.2 c to f) that cells appeared to form aggregation. These apparent aggregated cells were found to be a group of dense bodies packed together in an electron loose structure as previuosly described in the wild type. In case of the PPQ-10/3, although each individual body is still egg-shaped, its size (25x33 μ) was obviously larger than that of UPQ-S1 (16X24 μ) and of the wild type (9x13 μ) relatively.

Either PPQ-10/3 or UPQ-S1 which was grown in the presence of

Figure 4.2 Scanning electron micrographs of normal cells of Chlamydomonas reinhardtii wild type and paraquat resistant strains.

Cells cultured in paraquat free medium were subjected to analysis by scanning electron microscopy at different instrumental magnifications.

137c: (a) x500 (b) x3,500

PPQ-10/3: (c) x750 (d) x7,500

UPQ-S1: (e) x750 (f) x7,500

Wild type 137c cells were seen as single ovoid-shaped with an electron loose structure enveloped around each individual. PPQ-10/3 and UPQ-S1 appeared as aggregated cells packed together in a common electron loose structure.

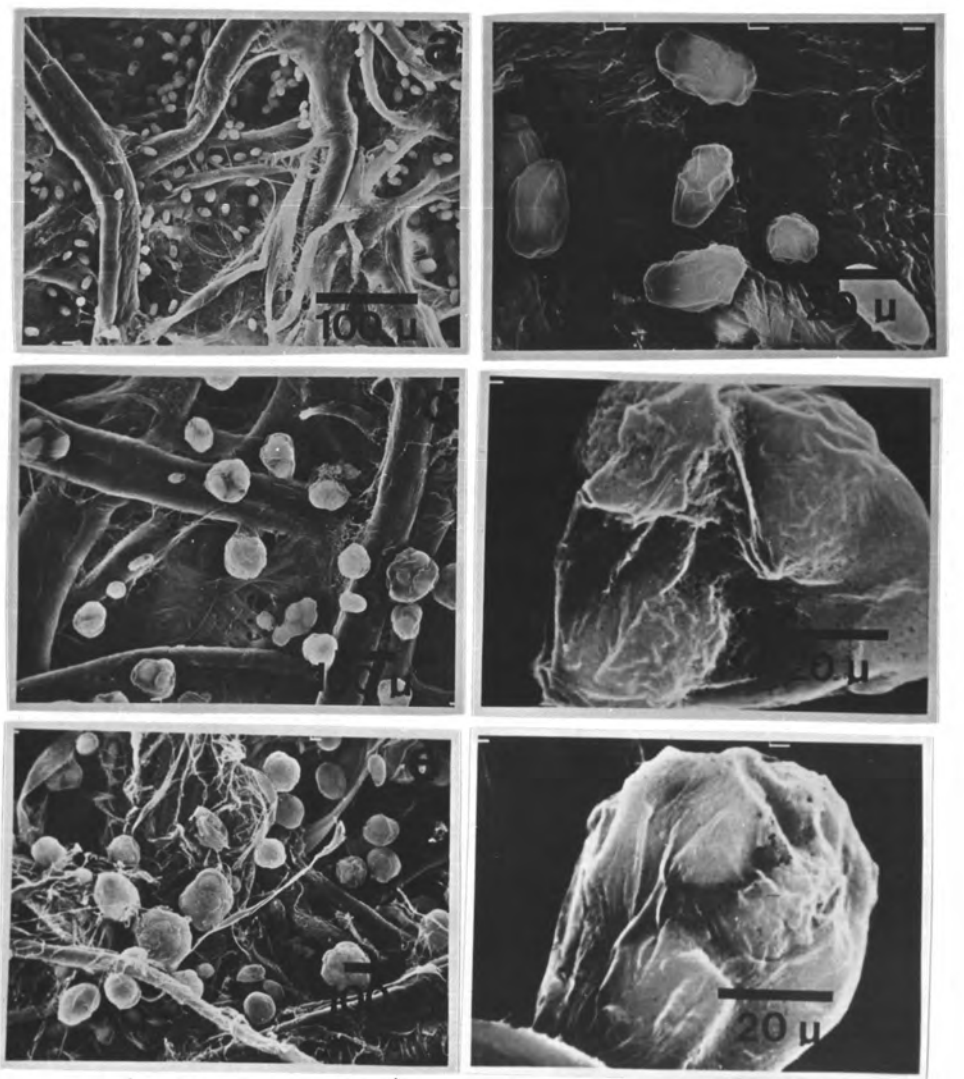
100 µ
µ
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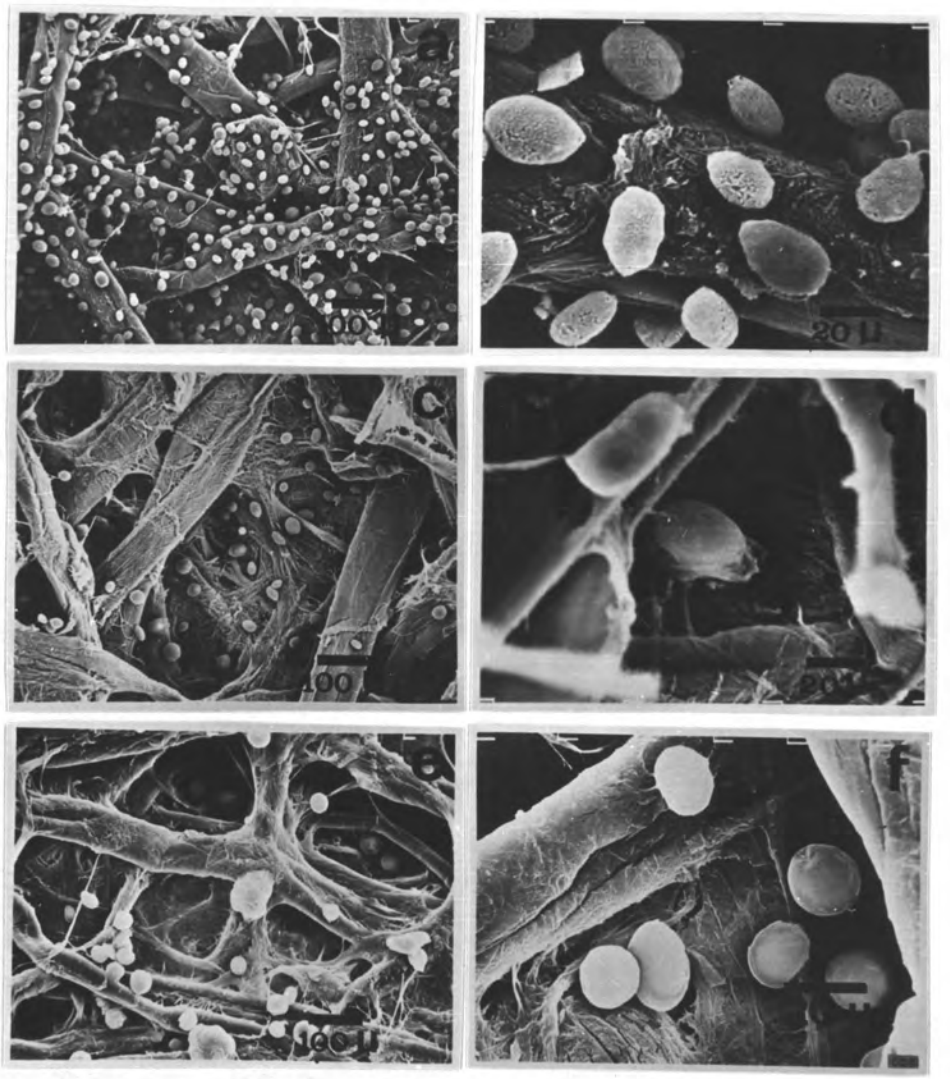
Figure 4.3 Scanning electron micrographs of paraquat treated cells of Chlamydomonas reinhardtii wild type and paraquat resistant strains.

Cells cultured in paraquat containing medium were subjected to analysis by scanning electron microscopy at different instrumental magnifications.

137c in 0.5 μM paraquat:	(a) x500	(b) x3,500
PPQ-10/3 in 1.0 μM paraquat:	(c) x500	(d) x3,500
UPQ-S1 in 1.0 μM paraquat:	(e) x500	(f) x2,000

Extensive cracks of the outermost layer of 137c cells were observed in celss treated with 0.5 μM paraquat. When PPQ-10/3 and UPQ-S1 were cultivated in medium containing 1 μM paraquat, unicellular characteristic was seen as described for normal cells of 137c strain.


10 µ
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c
50
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e
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1 μM paraquat exhibited different characteristics from those cells in paraquat free medium. The morphologies observed under light microscope (Fig. 4.1d and f) and scanning electron microscope (Fig. 4.3 c to f) were similar to those cells undergoing unicellular feature as usually seen in the wild type. Nevertheless no change in size could be demonstrated (25x35 μ for PPQ-10/3 and 16x28 μ for UPQ-S1).

4.3 Transmission Electron Microscopy

A comparison of the fine structure of the two paraquat resistant strains to the wild type cell C. reinhardtii was studied by a transmission electron microscope. Fig. 4.4a showed a unicellular feature of the wild type taken from culture in paraquat free medium. Compartmentation of subcellular organelles could be seen in thin sections of the specimen, for instance nucleus (n), pyrenoid structure (py), starch granules (s), and vacuoles (v). The electron micrograph emphasizing on a part of the chloroplast of C. reinhardtii wild type (137c)(Fig. 4.4d) pointed out that thylakoid discs appeared fused into a very long grana of two to five discs (g2 to g5) while unfused discs (d) were rare. The chloroplast envelope was marked as "ce",the cell membrane as "cm", and the cell wall as "cw". Not similar to the 137c strain, the PPQ-10/3 and UPQ-S1 paraquat resistant mutants of this unicellular algae apparently were groups of cells assembled together by surrounded wall-like matrix (Fig. 4.4 b and c). These findings were well related to the results in

scanning electron microscopy that the aggregated cells were surrounded by an electron loose structure. Besides the remarkable cell aggregation characteristics, the electron micrograph clearly demonstrated the large amount of huge vacuoles existing in both types of paraquat resistant cells. In addition, the grana of the PPQ-10/3 thylakoid discs were observed to be more tightly packed than that of the wild type, which could be identified as electron dense areas in the chloroplast sac (symbol "g" in Fig. 4.4e). This occurred with respect to the high content of photosynthetic pigment in PPQ-10/3. On the other hand, grana of UPQ-S1 was not distinct from that found in wild type, of which the grana were built by two to four discs (g2 to g4) with unfused discs (d) interconnecting between adjacent grana (Fig. 4.4f).

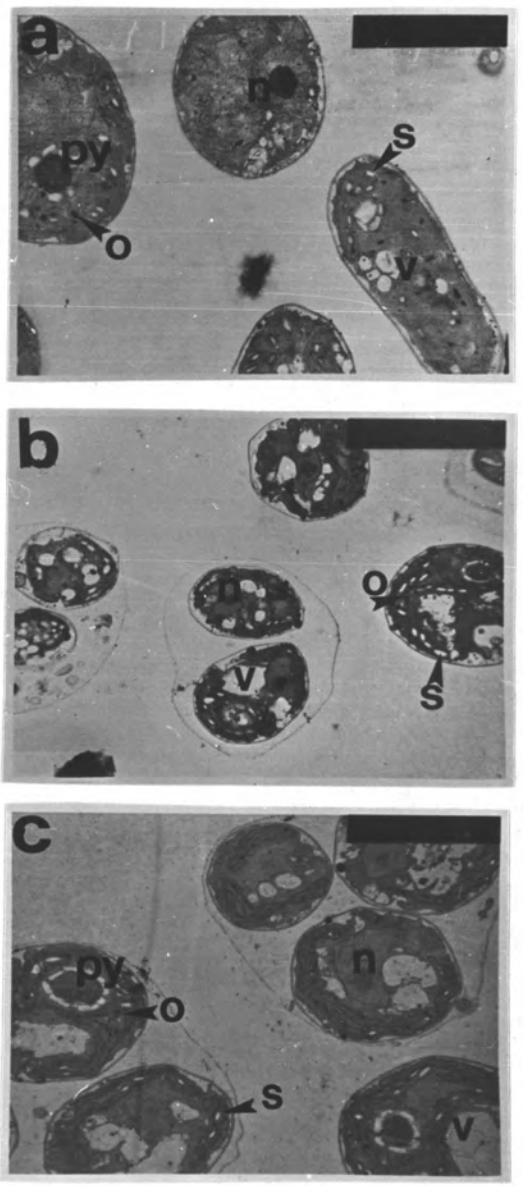
Cell wall of C. reinhardtii 137c, which was used as a wild type strain in the present research, has been reported to consist of seven impact layers (Roberts et al, 1985) whereby wall layer 2-6 (W2-W6) forms a sandwitch structure so called a central triplet. For the paraquat resistant mutant PPQ-10/3 being constructed, electron micrograph in Fig. 4.4e revealed that cell wall very close to the plasmalemma boundary contains six layers (W1-W6). The outermost layer which lied around at some distance away was suggested to be W7 that had not yet deposited to complete cell wall formation in each individual. Despite that all seven layers underwent synthesis and deposition in UPQ-S1 growing in paraquat free medium (Fig. 4.4f), from the outlook feature it was reasonable enough to interprete that

Figure 4.4 Transmission electron micrographs of normal cells of Chlamydomonas reinhardtii wild type and paraquat resistant strains.

Cells cultured in paraquat free medium were subjected to analysis by transmission electron microscopy at different instrumental magnifications.

137c:	(a) x3,700	(d) x59,000
PPQ-10/3:	(b) x2,000	(e) x59,000
UPQ-S1:	(c) x3,000	(f) x30,000

PPQ-10/3 and UPQ-S1 were observed as aggregated cells within a common wall-like matrix. At low magnification, the nucleus was marked at n, the pyrenoid at py the starch granules at s, the osmiophilic granules at o, and the vacuoles at v. Large vacuoles were extensively found in the cytoplasm of PPQ-10/3 and UPQ-S1. High magnification illustrated a part of the chloroplast. Thylakoid discs of 137c and UPQ-S1 fused into very long grana of two to five discs (g2 to g5), unfused discs (d) were rare. The grana (g) of PPQ-10/3 were packed with more than five discs. Note the typical situation of osmiophilic granules (o) in the chloroplasts. The chloroplast envelope was seen at ce, the cell membrane at cm, and the cell wall at cw. Explanatory drawings of cell wall were used to described the seven wall layers of the algal cells.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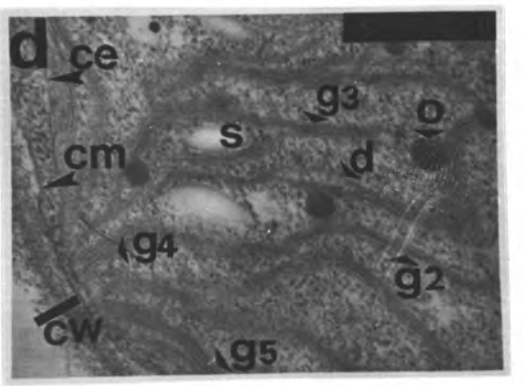
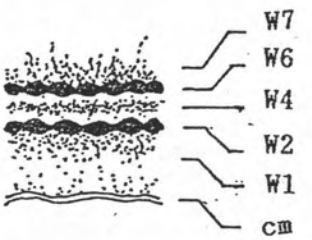
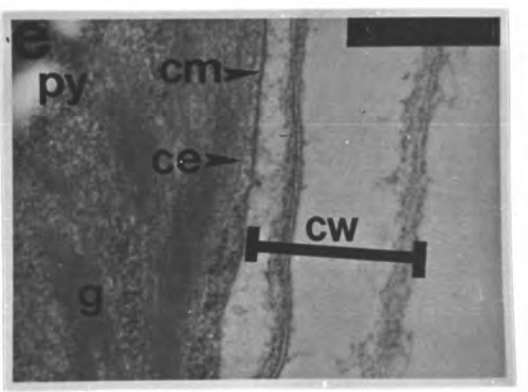
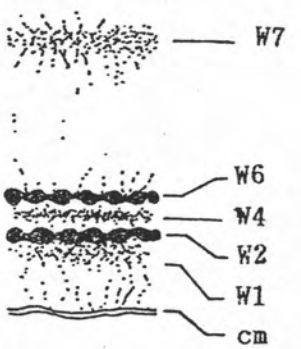


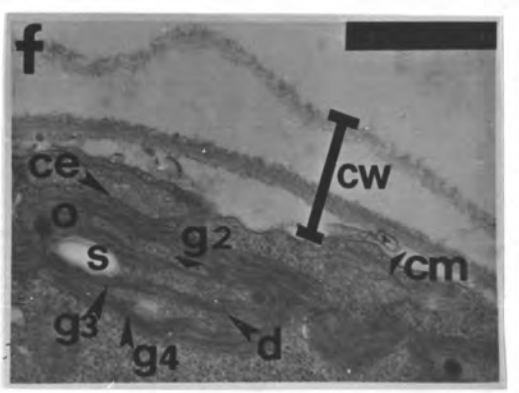
Explanatory Drawing

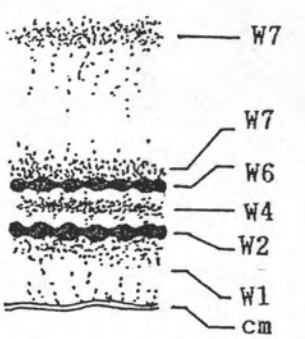
of Cell Wall











the extra lattice surrounding the whole aggregated cells had been developed to be as a part of the W7.

In all of the three cell types, 137c, PPQ-10/3, and UPQ-S1 respectively, a number of small size osmiophilic granules, symbol "o" in the figures, were found widely distributed in chloroplastic stroma of the paraquat unaffected cells. The transmission electron microscopy of 137c cells growing in the presence of 0.5 µM paraquat indicated that cells had completely lost their complex cellular organization. Fig. 4.5a illustrated electron density of osmiophilic granules which formed in unusually large sizes and moved from chloroplastic stroma to the cytoplasmic area indicating the destruction of chloroplast components. Other concurrent abnormalities were also observed such as leakage of cell membrane and the disapperance of well-organized wall lattices (Fig. 4.5d). The paraquat toxicity at 0.5 µM on wild type could be even detected under a light microscope that cells became bleached (Fig. 4.1b).

When exposed to 1 µM paraquat, the only significant structural change of the two paraquat resistant mutants was that cells exhibited a unicellular characteristic of which the sheded wall still remained in some cells as indicated by arrows in Fig. 4.5 b and c. The sheded wall in those remarked cells at higher magnifying parameter (Fig. 4.5 e and f) was identified to be a broad zone of the W7 layer.

Figure 4.5 Transmission electron micrographs of paraquat treated cells of Chlamydomonas reinhardtii wild type and paraquat resistant strains.

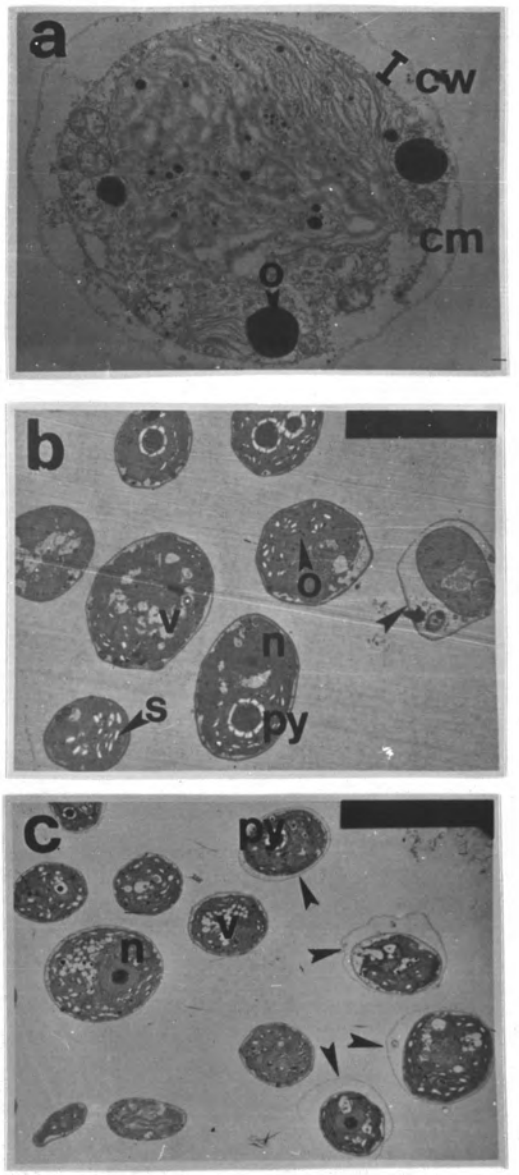
Cells cultured in paraquat containing medium were subjected to analysis by transmission electron microscopy at different instrumental magnifications.

137c in 0.5 μM paraquat: (a) x3,700 (d) x13,000

PPQ-10/3 in 1.0 μM paraquat: (b) x2,000 (e) x30,000

UPQ-S1 in 1.0 μM paraquat: (c) x1,500 (f) x30,000

PPQ-10/3 and UPQ-S1 were observed as single cells. At low magnification, fine structure of the resistant cells treated with 1 μM paraquat was illustrated with the extensive starch granules (s) and large vacuoles (v). The nucleus was marked at n, the pyrenoid at py, the osmiophilic granules at o, the cell membrane at cm, and the cell wall at cw. In wild type cells treated with 0.5 μM paraquat, abnormal large osmiophilic granules appeared in the cytoplasm together with the destruction of the cell membrane and cell wall. High magnification illustrated a part of the cell wall (cw). The explanatory drawings of cell wall described a change in the organization of cell wall of PPQ-10/3 and UPQ-S1 treated with 1 μM paraquat comparing to the normal cells grown in paraquat free medium (Fig. 4.4). The nucleus was marked at n, the stacking grana of PPQ-10/3 at g, the two discs and three discs grana of UPQ-S1 at g2 and g3, and the unfused discs at d.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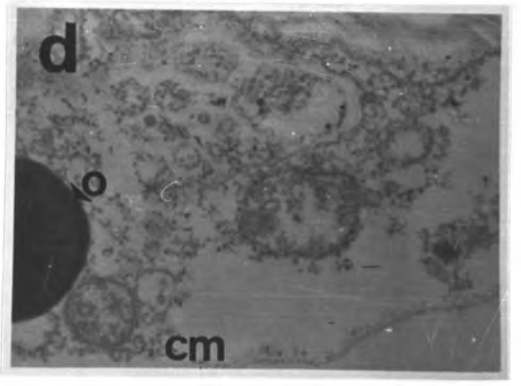
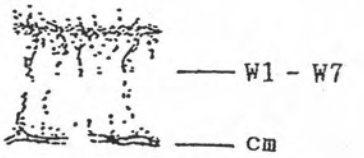
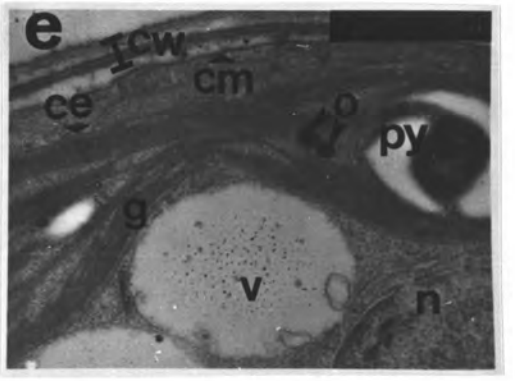
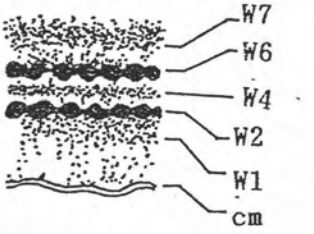


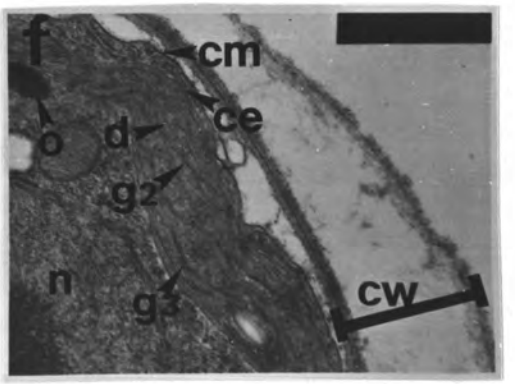
Explanatory Drawing

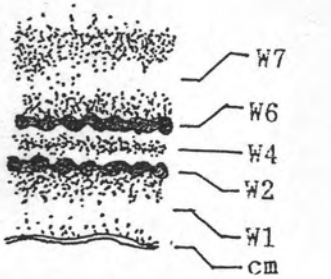
of Cell Wall











4.4 Activity of Photosystem I (PS I)

The activity of PS I in this research project was defined in terms of specific activity (activity per mg chlorophyll a (CHL a)). The value was measured in broken algal cell suspensions by the photoreduction of methyl red (MR). The results presented in Table 4.1 indicated that PS I specific activity of C. reinhardtii PPQ-10/3 was not correlated with the higher degree of stacking of the thylakoid membrane and the higher content of chlorophyll a which is the major photosynthetic pigment found in those cells 2-3 folds higher than UPQ-S1 and 137c. In PPQ-10/3 grown in paraquat free medium, PS I activity (404.0 ± 51.8 nmoles MR reduced/min/mg CHL a) was almost the same as that of the wild type (332.52 ± 23.6 nmoles MR reduced/min/mg CHL a). But in contrast, the activity of PS I of the UPQ-S1 normal growing cells obviously increased to about 2 folds comparing to those of the two strains (620.0 ± 16.2 nmoles MR reduced/min/mg CHL a).

Effect of paraquat to in situ PS I activity of the three Chlamydomonas strains was investigated by including the herbicide at sublethal dose in the culture medium, 0.1 μM for 137c, 1.0 μM for PPQ-10/3 and UPQ-S1. The above paraquat levels neither caused bleaching of the algal culture nor destruction of the chlorophyll a. However the presence of 1 μM paraquat during growth of PPQ-10/3 could reduce the PS I activity of PPQ-10/3 down to about one-third

of the original value (173.3 ± 57.9 nmoles MR reduced/min/mg CHL a) , whereas the PS I activity of UPQ-S1 and 137c was only slightly affected by the presence of 1.0 μM and 0.1 μM paraquat in the culture medium respectively.

Table 4.1 Photosystem I (PS I) activity and chlorophyll a (CHL a) content of Chlamydomonas reinhardtii wild type and paraquat resistant strains.

Cells (exponential phase) were disrupted through a French pressure Cell. The whole crude homogenate was used as a component for PS I activity measurement by the photoreduction of methyl red (MR) at the light intensity of 17,000 lux.

Strain	Growth condition	CHL a content ($\mu g/10^7$ cells)	PS I activity (nmoles MR reduced) min/mg CHL a
137c	paraquat free	20.4	332.5 ± 23.6
	0.1 µM paraquat	18.3	270.0 ± 30.0
PPQ-10/3	paraquat free	59.1	404.0 ± 51.8
	1.0 µM paraquat	59.7	173.3 ± 57.9
UPQ-S1	paraquat free	27.6	620.0 ± 16.2
	1.0 µM paraquat	29.6	530.0 ± 33.0