CHAPTER VI

DISCUSSION

6.1 Establishment of Method for Evaluation of Paraquat Toxicity in C. reinhardtii

In order to develop paraquat resistant mutants from a corresponding wild type susceptible biotype for the consistency results in molecular studies of the paraquat resistance mechanisms, one important factor is to control the degree of resistance in those mutants used in the whole experiments.

The mode of paraquat action involves light, molecular oxygen and photosynthetic electron transfer (Markle et al, 1965 ; Brian, 1967 ; Summers, 1980b). If paraquat toxicity exceeds cellular protection systems, it will cause chlorophyll degradation and cell death. In order to construct cell lines of C. reinhardtii resistant to paraquat, it is of necessity to ascertain the herbicide toxic level. In this research two approaches were established and used for evaluation of the paraquat toxicity. One approach was based on the modification of spot test of Spreitzer and Mets (1981) which can be achieved conveniently for the rough estimate of the paraquat resistance extent in the green algae. Another approach was originally developed in our laboratory to quantitate the degree of resistance of the paraquat resistant cell lines in comparing to the wild type cell by determining the lethal dose-50 (LD_{50}) of para-

quat to C. reinhardtii. These two approaches gave different toxic level of paraquat that the latter was more sensitive. Such difference might deal with three reasons. First paraquat is highly water-soluble and rapidly fluxes into cells via polar entry route in cell wall (Suwanketnikom, 1982). Under lower water activity conditions such as in solid medium, paraquat therefore penetrates into cell at slower rate. Second, photosynthetic rate, one of the important factor for paraquat action, is diminished in Chlamydomonas cells grown on solid medium (Surzycki, 1971). Finally, it is believed to concern with the concentration of melecular oxygen in cells because liquid culture of the algae is thoroughly aerated.

It has been reported in Chlamydomonas eugametos that paraquat did not cause cell death when exposed to the herbicide concentration up to 100 mM for 90 min (Ensminger and Hess, 1985). Comparing to the developing method of this research which the LD_{50} value could be determined in C. reinhardtii when allowed to exposed to paraquat for 24 hours, the algal cells were completely killed by the herbicide at only 1 μM. With regardless to the algal species, it implies that besides cellular physiological status, an effective dose of paraquat depends on the period of exposure time to the herbicide. The shorter exposure to the herbicide, the higher concentration of the herbicide is required for killing cells. This highly sensitive and accurate method for assay of paraquat toxicity were importantly employed in screening and selection for the paraquat resistant mutants of C. reinhardtii in the present study.

6.2 Construction of Paraquat Resistant Mutants of C. reinhardtii

Paraquat resistant mutants of C. reinhardtii were constructed from the wild type strain 137c by two methods. They were selection under paraquat pressure and direct mutagenesis of the chloroplast DNA by 5-fluorodeoxyuridine (FdUd). Nontheless, whether either method was used, an important feature of the mutant isolation protocol used was that cells must be exposed to light (4,000 lux) in all steps of the construction and only mutants with green colonies presumably normal photosynthetic function were selected.

6.2.1 Construction by Selection under Paraquat Pressure The aim of construction of mutants by selection under paraquat pressure is to obtain mutants from sponteneous mutation of the cellular DNA. This could be achieved without awareness of gene recombination or gene segregation because vegetative cells of the Chlamydomonas possess haploid number of chromosome. The selections were undertaken by two stepwise procedure. Final mutant, PPQ-10/3, was obtained with about 2.0-2.4 folds higher resistant to paraquat (LD_{50} = 6.48 μM, Fig. 3.11) than its original mutant (PPQ-1) from the first selection (LD_{50} = 2.55 μM, Fig. 3.8). C. reinhardtii PPQ-10/3 was found to possess about 20 times higher resistance to paraquat than that of the wild type 137c.

Furusawa et al (1984) used this classical microbial selection method in developing tobacco calluses resistant to paraquat as high as 250 μM, from a clone leaf protoplast which was susceptible

to only 2 μM paraquat. However when those resistant calluses were subjected to biochemical analyses such as on some properties of the superoxide dismutase enzyme, the data so obtained were conflicted and could not lead to a clear conclusion of the paraquat resistance mechanisms. The significant disadvantage of this multicellular model system is that plant calluses in general possess very poor photosynthesis but they propagate by using carbon sources and other essential nutrients supplied in the synthetic medium. Hence the resulting calluses might be such kinds of false paraquat resistant populations. Although these paraquat resistant calluses were developed from a clone protoplast resistant to the herbicide, there had not been any appropriate tool to prove whether during callus development the resistance still existed or not.

6.2.2 Construction by FdUd Mutagenesis FdUd mediated chloroplast gene mutation at about 24 hours after cells reach non-dividing stationary phase with no accumulative effect during exponential growth period (Wurtz et al, 1979). The preliminary attempts to mutagenize C. reinhardtii by growing cells in the presence of FdUd since initiation of the culture were not successful because prolonged effect of FdUd resulted in cell death. An effective mutagenesis process was resulted when pre-grown stationary phase culture was treated with FdUd for a period of 24 hours. Several mutants were recovered at very high frequency. However most mutants were leaky or were yellow mutants of photosynthetic pigment deficient

mutants. These yellow mutants were supposed to be the non-desired photosynthesis deficient mutants. The capability of these mutant to grow on the selective paraquat medium might result from that the herbicide could not act on the photosynthetic system which is the proposed site of the herbicide action in plants. Such mutants have been previously reported in another strains of C. reinhardtii undergoing mutation by FdUd to obtain mutants with abnormalities in photosynthetic apparatus Spreitzer and Mets, 1981).

Despite that several leaky paraquat resistant mutants were obtained, one mutant namely UPQ-2 (LD_{50} = 2.4 µM, Fig. 3.13) was subjected to second mutagenesis in order to obtain, if possible, mutants having high resistance to paraquat and to enhance the stability of the resistance phenotype. However the resulting mutant from double mutagenesis, UPQ-S1, responded to paraquat (LD_{50} = 3.07 µM, Fig. 3.16) at the considerably same level as its original cell line from the first mutagenesis (UPQ-2). Owing to the fact that FdUd directly mutagenized C. reinhardtii chloroplast DNA, and mutations in as many as 50 chloroplast genes can affect plant greening process (Arntzen and Duesing, 1983), it seemed to us that our criteria to select mutants with only normal green pigment should limit the probability to increase the degree of paraquat resistance in C. reinhardtii by FdUd mutagenesis. While in the former procedure of selecting paraquat resistant cell lines under the herbicide pressure, the resistance of PPQ-10/3 might be exerted in different mechanisms so that its degree of paraquat resistance was 2 folds

over UPQ-S1 mutant from the FdUd mutagenesis.

Both PPQ-10/3 and UPQ-S1 exhibited significantly resistant to paraquat 20 times and 10 times respectively when compared to the wild type 137c. The resistant ability of PPQ-10/3 and UPQ-S1 was tested to be stable throughout the research program with more than 60 subcultures on the maintenance medium. Such difference in the degree of resistance between the three cell lines made it possible to undertake investigation of the paraquat resistance mechanisms in C. reinhardtii.

6.3 General Remarks of C. reinhardtii 137c and Paraquat Resistant Strains

C. reinhardtii 137c, which is refered as a wild type strain in this research, is genetically a unicellular algae. Growth of the alga C. reinhardtii in liquid medium was followed by measuring cell density (number of cell/ml), turbidity at 540 nm, and the protein content. It was shown (Fig. 3.2, 3.9, and 3.14) that protein content of PPQ-10/3 and UPQ-S1 was higher than that of 137c. In all strains the decrease in protein in the stationary culture was suspected to due to the turn over of the algal cellular protein. During the stationary growth phase at which cell density was constant but the turbidity constantly raised. This could be explained by an observation that during stationary phase, cells formed clumping which caused the algal culture more turbid. Moreover, the declining in chlorophyll a content ($\mu g/10^7$ cells) at this

phase was concurrent with the yellowing of the culture. The value of cell density is routinely used to indicate the algal growth stage.

Growth characterisics of PPQ-10/3 and UPQ-S1 were not similar to that of wild type that their maximum growth yield was reduced. On the inspections under light microscope (Fig. 4.1), PPQ-10/3 and UPQ-S1 cultivated in the absence of paraquat pressure did not display unicellular feature but rather be a group of different sized cells remained encapsulated within a specialized structure throughout the cycle of cell growth. This morphology could not be linked to the division stage as described in wild type owing to the fact that zoospores are equal in sizes and closely impact. Unicellular phenotype of PPQ-10/3 and UPQ-S1 reappeared when paraquat was included in the medium. From light micrographs it was also remarkable that paraquat resistant cells, PPQ-10/3 and UPQ-S1 were much bigger than the wild type 137c. It might be suspected that the larger size of PPQ-10/3 cells resulted in 2 folds higher content of chlorophyll a comparing to 137c cells. However, it should be also considered that UPQ-S1 possessed chlorophyll a not distinct from 137c cells.

It has not been reported elsewhere before that the presence of paraquat in the medium essentially caused induction of normal division in Chlamydomonas. One investigation in Chlorella sorokiniana paraquat resistant cell line (up to 25 μM) revealed a morpho-

logy change suggesting incomplete cell division under the herbicide pressure (Rabinowitch et at, 1983).

6.4 Electron Microscopy and Physiological Implications in C. reinhardtii

Detailed in fine structures of wild type (137c), PPQ-10/3, and UPQ-S1 were investigated by electron microscopy. Our procedures for specimen preparations for scanning electron microscope (SEM) and transmission electron microscope (TEM) obtained cellular features resemble to those observed under light microscope except that the very fragile flagellas were slipped off. From SEM results, vegetative cells of wild type C. reinhardtii were measured to be 9x13 μ, without inclusion of the flagella portion, which was almost the same size (8x15 μ) as already described in the same algal strain (Levine and Ebersold, 1960).

It has been reviewed by Roberts et at (1985) that cell wall of C. reinhardtii consists of seven hydroxyproline rich glycoprotein lattice. Owing to this property made these alga possible to interact with cellulose fibre of Whatman #1 paper. The seventh layer (W7) is present as loose structures attached to the sixth layer (W6). Our results from TEM and SEM gave evidences enough to conclude that change in cell division in PPQ-10/3 and UPQ-S1 came from failure in deposition of W7 to W6. The expected W7 was suggested to accumulate around a group of cells when these cells were grown in paraquat free medium (Fig 4.4). In scanning electron

micrographs (Fig. 4.2), the electron loose structure resemble to W7, which encapsulated a group of cells, was shown in common with that structure around each individual of wild type cell together with each individual of PPQ-10/3 and UPQ-S1 themselves when growing in medium plus paraquat (Fig. 4.3). Our interpretations on assembly and disassembly of W7 should be warranted by immunological examination because this extracellular glycoprotein matrix are antigenically related (Smith et al, 1984).

Since W1 was reported to be specifically susceptible to hydrolysis by autolysin it should be noticed from the autolysin action on C. reinhardtii wild type and paraquat resistant cells (Fig. 5.2) that PPQ-10/3 was more tolerant to autolysin hydrolytic activity than UPQ-S1 and 137c respectively. This might imply that there was some alterations in the compositions or organization of the W1 layer of the paraquat resistant strains.

The extensive occurrence of large vacuoles in cytoplasm of PPQ-10/3 and UPQ-S1 (Fig. 4.4 and 4.5) is likely to be responsible for their apparently large size which is about 2-3 folds over the wild type. However the larger size of wild type appeared in cells treated with lethal dose of paraquat was subsequently a result of membrane destruction (both chloroplastic and cytoplasmic membrane) and disfunction of osmoregulatory systems. Such effect of paraquat on membrane function impairment was reported in other organisms, for example in Chlorella sorokiniana (Summers, 1980a) and Escherichia coli (Kohen and Chevion,1988). In case of E. coli cytoplasmic

membrane is the direct target site of paraquat action.

Highly stacking grana was extraordinaryily found to exist in PPQ-10/3 in accompanied with higher chlorophyll a content of this strian. It has been long known that thylakoid membrane of C. reinhardtii are not grouped into distinct grana but are continuously across the chloroplast (Levine and Ebersold, 1960). It is probable that super-foldings of the thylakoid membrane into stacking structures in PPQ-10/3 would allowed more sites of chlorophyll to be embeded on the membrane. Thylakoid with different degree of stacking or pigment compositions have been reported to show different photosynthetic activity (Park and Sane, 1971 ; Bazzaz and Govindjee, 1973). However PS I activity in PPQ-10/3 was not different from that of 137c strain (Table 4.1). Owing to the higher content of the major photosynthetic pigment (chlorophyll a), it is suggested that higher net photosynthetic capacity should be enhanced in C. reinhardtii PPQ-10/3 strain. In contrast to UPQ-S1 of which thylakoid organization was not distinct from that of wild type, its PS I activity substantially increased to 2 folds. Such increase in PS I activity of UPQ-S1 led to the suggestion of some alterations in electron acceptors or paraquat receptor site in PS I system in such a way to reduce the in vivo toxicity of paraquat on PS I. It was also illustrated that inclusion of paraquat at sublethal dose in the growth medium caused reduction in in vitro PS I activity of all strains tested. It was possible that paraquat even at the concentration which did not bring about cell death, could cause change in

the thylakoid membrane integrity at some extent such that the function of photosynthetic electron carrier system on the membrane was lowered in its activity in transfering electrons along the thylakoid membrane.

6.5 Uptake of paraquat in C. reinhardtii

Owing to the fact that biological membrane is composed of lipid bilayers generating highly non-polar environments, paraquat which has a nature of positvely charge compound should not be freely diffuse across lipid bilayers of membrane. Any change in membrane structures or components possibly causes reduction of the paraquat uptake into cells or its target sites. This has been supposed to be a basic characteristic of the herbicide resistance. In this research, we first established a reliable method of the extraction and applied it to the measurement of intracellular [^{14}C]paraquat in oder to verify the involvement of membrane structures and components to the uptake of paraquat into C. reinhardtii .

Studies in protoplast of C. reinhardtii (Fig. 5.10) and from the effect of dinitrophenol on the uptake (Fig. 5.8) indicated that the uptake of paraquat was energy independent and considerably saturable which further implies active transport across membrane of paraquat. The only other works to support this discovery were reported in E. coli (Kao and Hassan, 1985) that energy from glycolysis is needed to facilitate paraquat uptake in this bacterial cell. In the present study, we did not exert in any inhibitors of

aerobic and anaerobic glycolysis since C. reinhardtii cell has abundant glycogen storage in pyrenoid and starch granules.

The kinetic constants of paraquat uptake directly across C. reinhardtii membrane by the function of membrane protein carrier system were investigated in the algal protoplasts to eliminate the effect of cell wall portion. The strikingly higher K_m of uptake in PPQ-10/3 (0.156 µM) than in wild type 137c (0.022 µM) together with UPQ-S1 (0.038 µM) (Fig. 5.13 and Table 5.4) suggested probable alterations in specific sites for paraquat entry on cytoplasmic membrane or alterations in membrane protein involving the uptake bringing about less affinity to the herbicide. However, dealing with the so high V_{max}, it seemed that once interaction between paraquat and its specific sites on PPQ-10/3 membrane occurred, the herbicide was rapidly carried into cytoplasmic side. The simplest explanation for the apparent discrepancy in kinetic constants between uptake in cells and protoplasts of C. reinhardtii may reside in the interference of cell wall moiety. It has been reported that paraquat molecule can freely penetrates through cellulosic layer of the higher plant cell wall and that binding of [^{14}C]paraquat to the purified cell wall from leaf cells of a weed Conyza bonariensis paraquat resistant biotype was not different from that wall of the susceptible biotype. However the components of C. reinhardtii cell wall are not similar to the higher plant cell wall in such a way that the C. reinhardtii cell wall is consisted of hydroxyproline rich glycoproteins. At physiological pH

these glycoprotein molecules might interact with the positively charged molecules of paraquat.

Furthermore it was also noticed (Fig. 5.9) that exposure of cells to sublethal dose of paraquat for a period of time could itself induce *in situ* uptake of the herbicide in PPQ-10/3 and UPQ-S1. Whereas in wild type 137c paraquat pregrown cells exhibited somewhat abnormality of the membrane function. The mechanism is not quite understood. It could be explained at some certainty that paraquat treatment during growth caused induction of carrier system for active transport across membrane of paraquat.

Previously reviewed data on the comparative examinations of paraquat uptake to clarify its relationship to the resistance has been reported in only a few species of plants. Among these plants were resistant biotypes of weed from the weed controlling area, i.e perennial ryegrass (Harvey *et al*, 1978), *Erigeron philadelphiaes* and *E. canadensis* (Tanaka *et al*, 1986), *Hordeum glaucum* (Powles and Cornic, 1987), *Conyza bonariensis* (Shaaltiel and Gressel,1987) and gametophyte of a fern *Ceratopteris richardii* (Carrol *et al*, 1988). In all cases, no work had been done on the determination of the degree of paraquat resistance in those sources of resistant biotypes. The cellular basis for paraquat resistance do not lie on the diminished permeability of paraquat through the symplasm nor plasmalemma and chloroplast envelope membrane. Except for one case study in *C. bonariensis* (Fuerst *et al*, 1985) which it was proposed from quenching effect of paraquat on chlorophyll fluo-

rescence that the mechanism of resistance to paraquat is the exclusion of paraquat from its site of action in the chloroplast.

Although paraquat has been proposed to *in vitro* act on PS I in plants (Markle *et al*, 1965 ; Brian, 1967 ; Summers, 1980b), it was also found that the herbicide syphons electron streams from aerobic respiratory chain (Hassan and Fridovich,1978). Our efforts on the studies of the distribution of paraquat into chloroplast (Fig. 5.14) indicated that in *C. reinhardtii* 137c the herbicide extensively fluxes into chloroplast. The lower amount of paraquat detected in PPQ-10/3 and UPQ-S1 chloroplasts interestingly affirmed a restricted migration of the herbicide to its proposed target sites of action which made these two strains less susceptible to paraquat toxicity. The altered compartmentation of paraquat in PPQ-10/3 and UPQ-S1 could be explained by two mechanisms; (a) alteration in uptake route of paraquat on their chloroplast membrane, or (b) entrapment of the herbicide in vacuoles or in combinations of these mechanisms. Due to the fact that in plant cells vacuoles displays a key storage site for most toxic materials (Malite,1978), it is possible that this special organelle in the vacuolated PPQ-10/3 and UPQ-S1 might involve in precluding the exposure of chloroplasts to the herbicide.

6.6 Correlation of Enzyme Defense System and Paraquat Resistance in *C. reinhardtii* PPQ-10/3 and UPQ-S1

Paraquat toxicity involves the production of destructive

reactive oxygen species, i.e superoxide, hydrogen peroxide, and hydroxyl radical (Hassan and Fridovich, 1979 ; Winterbourn and Sutton,1984), then the resistance in any organism could be involve in the increased ability to detoxify the reactive oxygen species formed.

The superoxide dismutase, catalase and peroxidase have been intensively investigated for the correlation of the detoxification mechnism of those excited oxygen molecules and the resistance of paraquat.

Paraquat resistance of several plant species is confered by a high content of superoxide dismutase (Harper and Harvey, 1978; Rabinowitch et al, 1983; Furusawa et al,1984) or by a high content of superoxide dismutase and ascorbate peroxidase (Shaaltiel and Gressel, 1986; Powles and Cornic, 1987). Our clear investigations of the enzyme activity content in PPQ-10/3 and UPQ-S1 help strengthen the evidence that paraquat resistance was dealt with the constitutive augmentation of cellular content of superoxide dismutase, catalase, and ascorbate peroxidase (Table 5.5, 5.6, and 5.7). The catalase enzyme has been found very concentrated in peroxisome where its active catalytic degradation of hydrogen peroxide has been demonstrated (Asada and Takahashi, 1987). It is more likely that catalase scavenges hydrogen peroxide which diffuses to the peroxisomes. On another hand, ascorbate peroxidase has been supported by ample evidences to have a role in scavenging hydrogen peroxide localized in chloroplast (Nakano and Asada, 1980; Nakano

and Asada, 1981; Jablonski and Anderson, 1982). Moreover, superoxide dismutase and ascorbate peroxidase co-operation has been described in Chlamydomonas (Asada, 1987). In the present study, constitutively higher level of catalase and ascorbate peroxidase as well as their activities which were found in association with the constitutively higher level of superoxide dismutase in PPQ-10/3 and UPQ-S1 strongly demonstrated co-operation between superoxide dismutase-catalase and superoxide dismutase-ascorbate peroxidase in detoxification of superoxide and hydrogen peroxide in these strains of C. reinhardtii. The higher content of superoxide dismutase was determined to be correlated with the degree of paraquat resistance in these strains.

It has been reviewed (Asada et al, 1977; Asada, 1987) that most eukaryotic algae, including Chlamydomonas sp., contains iron type and manganese type of superoxide dismutase (Fe-SOD and Mn-SOD) and lack copper/zinc type enzyme (Cu/Zn-SOD) of which is found only in green algae in the groups of Chara, Nitella, and Spirogyra and in higher plants. Very strikingly, we could detect Cu/Zn-SOD in C. reinhardtii, in addition to the abundant cellular Fe-SODs, based on cyanide inhibition (Fig. 5.16 and 5.17c) and hydrogen peroxide stimulation (Fig. 5.17b). More specifically, the augmentation of Cu/Zn-SOD content in PPQ-10/3 and UPQ-S1 (Table 5.9 and 5.10) emphasized the role of this isozyme in paraquat resistance of these strains. Although Mn-SOD was not visualized in slab gel electrophoresis with the activity staining of both wild type 137c and the

paraquat resistant PPQ-10/3 strains, at least two Mn-SODs were consistently detected in UPQ-S1 superoxide dismutase electrophoregrams when stimulated by hydrogen peroxide (Fig. 5.19b). It still remained to be clarified whether these novel superoxide dismutases constitutively biosynthesized in UPQ-S1 represented a new gene products or were post-translationally modified forms of Fe-SOD since both genes of Fe- and Mn-SOD has been reported to code for a high amino acid sequence homology to each other while no homology with Cu/Zn-SOD (Asada, 1987).

With regard to the finding that hydrogen peroxide enhanced detection of Cu/Zn-SOD in all strains of C. reinhardtii and especially Mn-SOD in UPQ-S1 strian, it was reasonably suggested to be due to the localization of high concentration of superoxide generating from sponteneous decomposition of hydrogen peroxide. Hence, it seemed that both Cu/Zn-SOD and Mn-SOD essentially function at high concentration of superoxide. It has been reviewed (Asada, 1987) that spinach Cu/Zn-SOD has higher K_m to superoxide (0.35 mM) than the Fe-SOD (0.08 mM). This means that in higher plants Cu/Zn-SOD are fully active at the higher concentration of superoxide than the Fe-SOD.

Fe-SODs are chloroplastic enzymes (Bridges and Salin,1981; Rabinowitch et al, 1983 ; Sevill et al , 1984 ; Kwiatowski et al, 1985 ; Asada et al, 1987). While soluble Cu/Zn-SOD has been found in intrathylakoid space of spinach chloroplast (Hayakawa et al, 1984) and maize chloroplast (Baum et al, 1983). The chloroplastic

Cu/Zn-SOD from both sources were reported to be dimeric haloenzymes with the subunit molecular weight of approximately 14,500 -16,000. In addition, the dimeric Cu/Zn-SODs (MW 31,000-33,000 were found in the cytosol of plant cells with distinct amino acid compositions and immunological properties from the chloroplastic enzyme (Baum and Scandalios, 1981). Generally, tetrameric Mn-SOD was detected in the mitochondria of eukaryotic cells with a molecular weight of 85,000 (Asada and Takahashi, 1987), however, one dimeric enzyme (MW 52,000) was found binding to spinach thylakoid membrane (Hayakawa et al, 1985). It is not yet proved whether the novel isozymes of UPQ-S1 superoxide dismutase, Cu/Zn-SODs (SOD-11 and SOD-12) and Mn-SODs (SOD-13 and SOD-14) being detected in our research, are chloroplastic enzymes or not. However it could be deduced from their requirements of high hydrogen peroxide environment that they are more likely to be chloroplastic isozymes and co-function with Fe-SOD in scavenging superoxide.

The experimental results showed that band of SOD-7 which was identified to be Fe-SOD could be always detected as residual band in either cyanide treatment alone or along with hydrogen peroxide treatment. It was possible that this SOD-7 band might be a comigration band of Fe-SOD and Mn-SOD. Moreover, it was reported a dimeric hybrid composing of one molecule of Fe-SOD and one molecule of Mn-SOD in E. coli (Dougherty et al, 1978). The Fe-SOD and Mn-SOD were demonstrated to be homologous in molecular weight, subunit hybridization, amino acid compositions, and amino acid

sequences (Asada and Takahashi, 1987).

Paraquat treatment has been demonstrated to cause induction of some isozymes in Chlorella sorokiniana (Rabinowitch et al, 1983) and in E. coli (Gregory and Fridovich ,1973). Unexpectedly, our attempts on investigating enzyme induction by paraquat at sublethal dose clearly demonstrated that such paraquat treatment resulted in reduction of total content of superoxide dismutase in all strains (Table 5.8, 5.9, 5.10). These results were supported by the evidences from activity staining of the superoxide dismutase (Fig. 5.17 , 5.18, and 5.19). In wild tpe 137c, there was suppression of an Fe-SOD (SOD-5) and the Cu/Zn-SOD (SOD-10), whereas in PPQ-10/3 strain paraquat treatment caused significant suppression of an Fe-SOD (SOD-5) and noticable induction of Cu/Zn-SOD (SOD-10). In UPQ-S1, about 20% decrease in total content of superoxide dismutase was measured with a slight reduction in one major Cu/Zn-SOD (SOD-10) and two minor Cu/Zn-SODs (SOD-11 and SOD-12) together with the two minor Mn-SODs (SOD-13 and SOD-14), nevertheless a slight induction of one minor Fe-SOD was observed. This suppression of superoxide dismutase was concurrent with increase in level and activity of ascorbate peroxidase especially PPQ-10/3 and UPQ-S1 strains.

Hydrogen peroxide can freely diffuse through chloroplast membrane to effect other organelles and high cellular concentration of hydrogen peroxide can non-enzymatically react with superoxide and paraquat radical as well forming a more reactive molecule, hydroxyl radical (Winterbourn and Sutton, 1984). It is therefore

postulated that when C. reinhardtii is affected by paraquat, it is more crucial for the organism to rapidly degraded hydrogen peroxide to oxygen and water. Suppression of Fe-SOD and Mn-SOD might generate chloroplastic environment of hydrogen peroxide appropriate for fully catalytic activity of ascorbate peroxidase. Alternatively, it is possible to be a protective adaptation to Fe-SOD itself since higher activity of the enzyme produces higher concentration of chloroplastic hydrogen peroxide which in turn inhibits activity of Fe-SOD found abundant in chloroplast. In addition, results from patterns of cellular proteins (Fig. 5.20) affirmed that paraquat treatment consistently caused losses of some proteins in all strains, although it could not be identified which proteins were influenced.

6.7 Molecular Approach of C. reinhardtii UPQ-S1: Emphasis on Chloroplast DNA

5-Fluorodeoxyuridine (FdUd) specifically affected chloroplast DNA replication. The compound is phosphorylated by a chloroplast specific thymidine kinase (Wurtz et al, 1979). Then the resulting 5-fluorodexyuridylate, acting as an analogue of deoxyuridylate, becomes covalently bound to thymidylate synthase which therefore inhibits de novo thymidylate biosynthesis. This effect of FdUd can be reversed by adding excess thymidine source. In the present study, one paraquat resistant mutant, C. reinhardtii UPQ-S1 , was constructed by FdUd direct mutagenesis. Hence it was of

interest to investigate the restriction patterns of the UPQ-S1 chloroplast DNA in comparing to the wild type 137c chloroplast DNA. If the paraquat resistance correlating restriction fragments could be identifid, it will be much valuable for genetic manipulation of the paraquat resistance gene.

As other plant cells, C. reinhardtii contains three distinct genetic systems located in the nucleus, chloroplast and mitochondria , respectively. Owing to the difference in the buoyant density of chloropllast DNA (1.696 $g.cm^{-3}$) and nuclear DNA (1.724 $g.cm^{-3}$), these two DNA can be absolutely separated from each other (Grant et al, 1980). However, chloroplast DNA fraction has been reported to be regularly contaminated by mitochondrial DNA (buoyant density in cesium chloride = 1.706 $g.cm^{-3}$)(Rochaix, 1978). In Fig. 5.22, the chloroplast DNA preparations always showed a band of DNA fragment at 14.1 kb (less than 1% contamination), which was correspondingly related to mitochondrial DNA (15 kb as reported by Grant and Chiang, 1980). Physical maps of chloroplast DNA of C. reinhrdtii 137c, wild type strain in the present study, has already been established by sequencing of DNA fragments from digests of BamH I, Bgl II, and EcoR I (Rochaix, 1978). However our strategy to identify UPQ-S1 chloroplast DNA could not be resolved by BamH I, Bgl II ,EcoR I, together with Xho I restriction fragments because of the unclear and incompletely separated discrete bands. The difference between the DNA from both strains were interestingly exhibited in Pst I digests (Fig. 5.22) of which its restriction sites on C.

reinhardtii DNA have not been ever examined elsewhere before. The explanation of the reduced amount of Pst I fragments at 15.5 kb, 14.8 kb, 13.5 kb, 12.4 kb, and 12.0 kb of UPQ-S1 chloroplast DNA with respect to the corresponding DNA fragments of wild type is that there might be some alterations of base sequences at those restriction sites for Pst I or around those sites in such a way that they could not be no more recognized by the enzyme. Nevertheless, according to the high copies of chloroplast DNA in a single chloroplast (approximately 50 copies) (Rochaix, 1983), the reason why residual signals of those Pst I fragments could still be detected is on the basis that not all chloroplast DNA, saying remarkably 50% (Fig. 5.22), has been mutated.

Such mutation in UPQ-S1 chloroplast DNA might be point mutation or frameshift mutation. The most well known and well clarified circumstance is the resistance to a group of herbicides, i.e. atrazine, bromacil, diuron , and DCMU, in C. reinhardtii (Galloway and Mets, 1984; Erickson et al, 1984). The resistance concerns an alteration in 32 kilodalton oxidoreductase enzyme of photosystem II resulting in less affinity to the herbicide. From sequencing analysis of a C. reinhardtii chloroplast DNA, psb A which is the coding region for the 32 kilodalton protein, it was found a single transversion of T-A base pair to G-C base pair. This mutation caused amino acid change of serine in the wild type protein to alanine in that of the mutant. It led to a suggestion that the molecular basis for such herbicides resistance in C. reinhardtii. The serine

residue was proposed to involve directly in the binding of the herbicide or might have a conformational effect on the herbicides binding sites.

On the view of UPQ-S1, it was shown the increase in PS I activity of this strain (Table 4.1) which might be implicated probable alteration in the electron carrier in PS I system. Despite of the fact that components of the photosynthetic apparatus has been long concluded to be encoded in nuclear DNA (Levine and Goodenough, 1970), recent researches of the chloroplast genome in many plant species, including C. reinhardtii, has been declared that about twenty genes of chloroplast DNA encode components of the chloroplast photosynthetic electron transport system, those are genes for components of PS I, PS II, cytochrome b/f complex, for examples, (Ohyama et al, 1988). In order to identify chloroplast genomic DNA corresponding to the 15.5 kb, 14.8 kb, 13.5 kb, 12.4 kb, and 12.0 kb Pst I fragments, Pst I physical map of UPQ-S1 chloroplast DNA must be constructed in accompanied with that of wild type. The responsible gene for the paraquat resistance in UPQ-S1 could then be found out from the standard physical and genetic map of C. reinhardtii (Rochaix, 1978). Along with the analysis of chloroplast genomic DNA involving paraquat resistance in C. reinhardtii, it is also much significant to identify specific proteins on the photosystem I that directly bind to paraquat. These two lines of joint evidences will conclusively explain the paraquat resistance down to the molecular basis.

However with regard to the enhanced biosynthesis of superoxide dismutase in C. reinhardtii UPQ-S1, it is notable that paraquat resistance in UPQ-S1 possibly resulted from a combination of nuclear DNA and chloroplast DNA mutation. This is because all forms of superoxide dismutase has been found to be encoded in nuclear DNA (Scandalios, 1969 ; Baum et al, 1983). However, in this research work no attempts had been done on the characterization of the restriction patterns of nuclear DNA comparing to that of 137c strain. Since C. reinhardtii is an eukaryotic alga having 11 linkages of the nuclear DNA (Sager, 1974), it needs a more complicated instrument of pulse electrophoresis to investigate the DNA restriction patterns.

In case of PPQ-10/3 , although inheritance of resistance has not yet genetically characterized in this present work, its physiological and biochemical resulted evidences help confirm it to be a model of paraquat resistance via nuclear DNA mutation. The strongest evidences were (1) enhancement of the biosynthesis of enzyme detoxicification system (2)enhancement of degree of stacking of thylakoid membrane (refered to Sager, 1974) and (3) enhancement of chlorophyll a content (Levine and Goodenough, 1970).