CHAPTER 6

DISCUSSION
The structure and function of chloroplast has been known to alter during leaf senescence. Instead of the active photosynthetic electron transport energy transduction in the thylakoid membranes and the carbon dioxide assimilation and other metabolic functions in the stroma, degradation of the chloroplast pigments, macromolecules such as proteins, membrane lipids and ribonucleic acid take place in senescing leaf chloroplasts. As results of these deteriorate processes some of the released metabolites and nutrients of the macromolecules can be translocated to the newly forming plant parts.

These ordered series of deteriorative events are under the controlled expression of genes. Like flag leaves in cereals, cotyledons of dicotyledon plants serve as a good test system to study the pattern of changes in senescence associated physiological, biochemical and molecular processes that occur at the cellular level. This is because, during their senescence period, the cotyledons serve as nutrient source for the seedling establishment, a transition from heterotrophic to autotrophic form of growth and development in plants. Since, the alterations associated with senescence process are under the control of gene expression (Gan and Amasino, 1997), the application of molecular biology techniques may help in delaying or arresting senescence process, thereby improving the crop yield.

Detailed understanding of senescence associated physiological and biochemical changes at the cellular and organelle level are however required, prior to the application of the tools of molecular biology. Therefore, we studied the alterations in the structural-functional relationships of thylakoid membranes in Cucumis sativus cotyledons during ageing or senescence. It is assumed that the changes that take place in the organelle (chloroplast) level during cotyledonary leaf senescence also occur in a programmed manner, in a temporal sequence under the controlled expression of both nuclear and plastid genes.

The Cucumis sativus plants, when grown in the specified growth conditions, under continuous illumination of light, supplemented with the mineral growth medium, the pigment, protein contents, the photosystem II and photosystem I electron
transport rates were maximum in 6-day old cotyledons, there after showed a gradual decline with time. Hence, we considered the 6th day cotyledons as reference control and 15th, 20th and 27th day grown cotyledons as senescing situations for comparison.

Loss of photosynthetic pigments is one of the most visible changes that occur during leaf senescence. The contents of both Chl a and Chl b declined at almost the same rate, indicating no significant change in the Chl a/b ratio during senescence of Cucumis cotyledons (Table 3.1). This suggests the losses of Chl a rich core antenna associated with PSII reaction centres and the Chl b containing peripheral antenna LHCII of PSII, could be parallel. However, Kura-Hotta et al., 1987 has observed a decline in the Chl a/b ratio in senescing rice seedlings. The authors suggested that the Chl a is more labile that Chl b during rice leaf senescence. It thus appears that in different test systems the core antenna or light-harvesting antenna degrades at differential rates. Differences in the degradation rates of Chl a, Chl b and carotenoids have been documented (Biswal and Mohanty, 1979; Grover et al., 1986a; Kura-Hotta et al., 1987 and Guiamet et al., 1991). In addition to the changes in the chlorophyll content, we have also noticed the decline in the leaf carotenoid content with the progress of the age of cotyledons (Table 3.2). We observed an increase in the carotenoid to chlorophyll ratio, reflecting the higher retention of carotenoid pigments over the chlorophylls. The significance of carotenoid pigments in the chloroplasts has been well studied when plants are grown under various stress conditions. They not only function in the light harvesting, but also quench the oxygen free radicals that are generated by excess light and also protect the chlorophylls against the photodamage. On the basis of over results, we suggest the carotenoids may involve in protecting the residual Chl molecules from further damage against excess light and against oxygen free radicals. Besides the loss in the pigment contents, we have also observed a gradual decrease in the total cotyledon protein content on the dry weight basis, with the progress of senescence (Table 3.3).

Besides the loss in photosynthetic pigments, we have observed changes in the electron transport carriers, differential changes in the steady-state levels of selected thylakoid proteins, changes in the characteristics of LHCII of photosystem II and also
the change in the structural organization of thylakoid membranes in *Cucumis* cotyledons during senescence.

A. Alterations in the electron transport carriers:

In several senescing test systems, decline in the efficiencies of PSII and PSI activities have been noted (Harnischfiger, 1974; Biswal and Mohanty, 1976; Jenkins and Woolhouse, 1981; Bricker and Newman, 1982; McRae *et al*., 1985; Sabat *et al*., 1985). In some senescing systems such as bean, cucurbits higher loss in PSI activity than PSII has been reported (Harnischfiger, 1974; Bricker and Newman, 1982). In contrast in other senescing systems loss in the PSII activity were greater than that of PSI electron transport rates (Biswal and Mohanty, 1976). Therefore, we monitored the changes in the electron transport rates of PSII and PSI in the thylakoid membranes isolated from *Cucumis* cotyledons with the progress of senescence. We observed a gradual decline in the activities of both the photosystems (Table 3.5). In the final stages of senescence (thylakoids from 27-day cotyledons) we noticed a greater loss by ~15% of PSII activity than that of PSI electron transport activity.

Since, the losses in the electron transport rates of photosystems were quite extensive, we studied the changes if any in the characteristics of electron transport carriers. For this purpose, we selected the inhibitors viz., DBMIB, atrazine, and KCN and studied the changes in the sensitivities of these inhibitors monitored as change in relative $I_{50}$ values, towards thylakoid membranes of senescing cotyledons with respect to control 6th day cotyledons. Dibromothymoquinone (DBMIB), which is an antagonist of PQH$_2$, known to block the electron transfer from PQH$_2$ to Cyt b$_{6/6'}$ complex. The experiments with DBMIB indicated an increase in the $I_{50}$ value in the 20th day senescing thylakoid samples as compared to 6th day thylakoid samples (Fig 3.4). From this experimental observation, we suggest that electron transfer from PQH$_2$ to Cyt b$_{6/6'}$ is hampered during cotyledon senescence. Further analyses of our experimental results also indicated that, the higher fraction of PQ remained in reduced form in senescing sample, which means that the rate of re-oxidation of PQH$_2$ by electron flow to PSII slowed down in senescing sample. This could result due to the
compositional changes in the Cyt b$_{6f}$/complex and/or the damage in the Q$_A$ to Q$_B$ electron transfer. The assumption of changes in the Cyt b$_{6f}$/complex is further supported by the western-blot analysis of Cyt f, where a gradual decline in the Cyt f steady state levels were observed (Fig 4.8). These results are in agreement with the earlier report of Jenkins and Woolhouse 1981, who has also observed the changes in the sensitivity of a similar inhibitor, trifluralin. Our experimental results are also in agreement with the Harnischfeger, 1974 findings, where he suggested the PQ pool in the chloroplasts of senescing Cucurbita cotyledons has been shown to remain in the reduced state. Sabat et al., 1989 has also been reported change in the sensitivity of DBMIB towards the senescing wheat thylakoids.

Experiments with atrazine which specifically blocks the electron transfer from Q$_A$ to Q$_B$ and KCN which inhibits the electron transfer from PC to PSI reaction centre (Fig 3.3), indicated an increase in the I$_{50}$ values of these inhibitors towards senescing thylakoid membrane (Fig 3.5 & 3.7). From the results obtained with the herbicide atrazine, we suggest the senescence induce damage in the Q$_A$ to Q$_B$ electron transfer (Fig 3.5). The KCN inhibition assay in the thylakoids isolated from 6 and 20-day cotyledons, suggest that the plastocyanin, the copper containing mobile electron carrier which feeds electrons to PSI also co-limits the electron transfer with PQ (Fig 3.7). Sabat et al., 1989 also changes in the KCN sensitivity towards senescing wheat thylakoid membranes and suggested an alteration in the site of PSI where plastocyanin feeds electrons to PSI.

Further, we analyzed the herbicide $^{14}$C-atrazine, binding characteristics of thylakoids isolated from 6-day non-senescing and senescing and 20-day senescing cotyledons, in order to examine the alteration at acceptor side of PSII, where Q$_A$ and Q$_B$ are involved. We observed an increased affinity for atrazine towards senescing thylakoid membranes (Fig 3.6). We propose from the $^{14}$C-atrazine binding studies, that senescence may induce the heterogenous populations of PSII units (i.e., some of the functional PSII units (Q$_B$-reducing centres) may be converting in to Q$_B$ non reducing centres (Cao and
 Govindjoe, 1990). To the best of our knowledge no reports are existing on the heterogeneity in PSII units during senescence.

In conclusion, from the inhibitor studies performed using the thylakoid membranes isolated from the control 6th day cotyledons and senescing 20th day cotyledons, we suggest that, the alterations of the PSII acceptor side and PSI donor sides occur, and these changes involve mobile electron carriers such as PQ and PC. The scrutiny of literature suggests that donor side of PSII be affected by senescence. Our studies provide information that acceptor side of PSII is also sensitive to senescence.

In addition to the damage in the mobile electron carrier molecules, light saturation studies indicated the decrease in the quantum yield of oxygen evolution, reflecting the decline in the functional antenna size and a loss in the maximum rate of electron transport. We interpret the decrease in the maximum rate of electron transport could be due to the loss in the functional PSII units as well as changes in the intermediate carriers involved in rate limiting steps of electron flow. The decrease in the quantum yield for PSII activity may ascribe to a quantitative loss of the functional antenna associated with PSII and/or the detachment of antenna from the PSII reaction centres. Further, we also assume possibly losses in PSII units occurred due to senescence in Cucumis cotyledons (Fig 3.2). Jenkins et al., 1981b suggested from the light saturation studies in the thylakoids isolated from senescing Phaseolus vulgaris primary leaves that, the decrease in the number of functional reaction centres per leaf was greater than the percentage decrease in the leaf chlorophyll content. This reflects the higher loss of the Chl a containing pigment-proteins (core antenna) of PSII than that of the LHCII. In contrast to their findings our results indicate, both the PSII functional units as well as the antenna decreases during senescence of Cucumis cotyledons. However, the quantitative loss of LHCII and/or the detachment of antenna from the PSII reaction centre results in the decreased quantum yield of electron transport, suggesting loss in the PSII functional units.

B) Analysis of Chl a fast fluorescence transients:

Monitoring of Chlorophyll a (Chl a) fast fluorescence transients, in vivo in dark adapted leaves at room temperature provides information on the redox changes of QA.
which acts as a quencher of Chl a. Since, the redox states of QA regulates electron transport between two photosystems, light utilization and a variety of other processes in the electron transport chain, the Chl a fluorescence transients provide information on structural and functional integrity of the photosystems. Therefore, we measured Chl a fast fluorescence transients in non-senescing 6-day cotyledons and senescing 20-day, 27-day cotyledons (Fig 3.9).

The analysis of Chl a fast fluorescence transients, showed an increase in the Fo value (the initial fluorescence yield when all the PSII reaction centres are open), which is an indicative of the detachment of antenna from the PSII reaction centre (Table 3.6 and 3.7). This experimental observation is in agreement with the light saturation studies, where we made an assumption of detachment of antenna from the reaction centre. This result is not in agreement with the existing report of Jenkins et al., 1981a, who has been observed the decrease in the Fo value in senescing Phaseolus vulgaris leaves.

In addition to an increase in the Fo value (may be due to the detachment of antenna from the reaction centre), we have found the decrease in the quantum yield of photchemistry of PSII. This is in consistence with the PSII catalyzed electron transport activity measurement where gradual decline in the PSII catalyzed oxygen evolution was observed due to ageing. We also suggest from the Chl a fast fluorescence transients that, i) Reduction in the quinone pool, ii) Decrease in the re-oxidation of reduced QA by QB (PQ) iii) Decrease in the density of the reaction centres, suggesting the loss in the functional PSII units per unit leaf area in senescing cotyledons. The results obtained from the Chl a fast fluorescence transients are in agreement with the experimental results obtained from inhibitor studies as well as the light saturation experiments.

C. Changes in the ultra-structure of chloroplasts:

We studied the ultra-structure of chloroplasts by transmission electron microscopy, since any organizational changes in the thylakoid membranes may results in the declined efficiency of photosystems. Hence, we studied the ultrastructure of chloroplasts isolated from 6th day control cotyledons and 27th day senescing cotyledons. As we expected the control 6th day chloroplasts contained properly stacked grana with
stroma exposed lamellae (Fig 4.1 A&B; Fig 4.2 A). Where as the senescing chloroplasts showed the loosely stacked large granum like structures (Fig 4.2B; Fig 4.3A&B). We suggest that, LHCII may be involved in the formation of giant grana in senescing chloroplasts. As stated earlier, Bricker and Newmann 1982, observed higher loss in the PSI activity than PSII activity. The authors suggested that the destruction of stroma lamellae occur prior to the damage of the grana during senescence. Incontrast, our findings suggest that, these grana like structures contain possibly both PSII and PSI. The greater loss of PSII electron transport rates than the PSI electron transport in the final stages of senescence (in the thylakoid samples of 27-day cotyledons) could be linked to an extensive damage in the components of PSII (i.e., loss in D1 proteins, LHCII, over all loss of functional PSII units, alteration in the QA to QB electron transport) than that of PSI components. In addition to these extened grana, we have also observed the appearance of plastoglobuli in the senescing chloroplasts (Fig 4.3A&B). These extended grana and presence of plastoglobuli have been reported in several other senescing systems (Lichtenthaler, 1969; Gil and Schaedle 1973; Tevini and Steinmuller, 1985; Biswal and Biswal 1988; Hashimoto, 1989). Tevini and Steinmuller, 1985 analysed the composition of isolated plastoglobuli during Fagus sylvatica leaf senescence and reported that carotenoid esters oxidized plastoquinones and the liberated fatty acids were the main components of plastoglobuli. As suggested by Tevini and Steinmuller (1985) we assume that the plastoglobulie appeared in the chloroplasts of senescing Cucumis cotyledons too contain the fatty acids, carotenoid esters and the degradative products of the chloroplast proteins (thylakoid proteins stromal proteins of chloroplast..?) We suggest that, these break down products released due to the programmed deteriorative events may stored in the plastoglobulie prior to their translocation into the newly forming plant parts.

D. Differential changes in the specific thylakoid proteins:

The protein complexes of the thylakoid membrane function co-ordinately in transferring the electrons from water to NADP and thus, generating ATP and NADPH in active chloroplasts. Since, each of these proteins play an unique and specific role in mediating electron transfer, changes in the steady-state levels of these proteins may
results in the altered rates of electron transport. Therefore we analysed the steady state levels of some of the specific proteins from each protein complex of the thylakoid membrane and analysed by western-blot method. We observed differential changes in the levels of these proteins with the progress of senescence. We have observed Cyt b$_{559}$ of PSII remained nearly constant in the thylakoids isolated from the 6th day control as well as senescing thylakoid samples (Fig 4.5). The Cyt f was observed as most sensitive to senescence, reflecting the intersystem electron transfer components gets affected due to senescence earlier than the components of either photosystems (Fig 4.8). Functional analyses involving linear electron flow and PSII electron flow also reflects that the extent of loss in linear electron flow is more that PSII (Harnischfeger, 1973; Holloway et al., 1983; Ben David et al., 1983; Roberts et al., 1987; Hiderjia et al., 1991). This may be ascribed to susceptibility of Cyt f to senescence.

Biswa, 1997 have been proposed that the disassembly of PSII proceeds in the sequence, beginning with disorganization of its oxygen-evolving complex, followed by damage of RC complex and finally loss of LHCs. In contrast, our results suggest that in Cucumis sativus cotyledons, the damage in the PSII reaction centre, D1 and D2 (Fig 4.6 A&B) occurs faster than that of MSP (Fig 4.7C) and the structural apoprotein of LHClI (Fig 4.7 B; Fig 4.10 A,B and C). The loss in the PSII reaction centre proteins D1, D2 are in consistence with the $^{14}$C-atrazine binding studies and the light saturation studies where we suggested that, the decline in the number of Q$_{B}$ binding sites on D1 (i.e., decrease in the D1 protein) and the decrease in the functional PSII units respectively. Ageing induced loss in the D1 protein has been shown in Festuca praeensis (Biswal et al., 1994). The loss in the Chl b and quantitative loss of the apoprotein of light harvesting complex II have been reported by Mae et al., (1993). Our results with Cucumis cotyledons show a similar nature of changes in the D1 and LHCII proteins with the progress of senescence. In contrast to our findings Choudhury and Imaseki, (1990) and Nock et al., (1992) reported the loss of 33 KDa Mn stabilizing protein during leaf senescence prior to the loss in reaction centre proteins of PSII. Further, Camp et al., (1982) and Roberts et al., (1987), working with wheat and been respectively observed the loss in the photophosphorylation ATP synthase and the loss in the components of the ATP synthase complex. We also observed a gradual decline
in the β-subunit of ATP synthase with the progress of senescence (Fig 4.9B). Coming to the PSI complex, the steady state level of PSI reaction centre declined only in the final stages of senescence. However, when compared to the PSII reaction centre proteins, the PSI reaction centre is quite stable (Fig 4.9A).

From our experimental observations, we present the comparative losses of these intrinsic membrane proteins of thylakoid membrane in the following order:
Cyt f > D1 > D2 > LHCII > MSP > CP47 > PSIRC > β-subunit ATPase > Cyt b559
This order represents the temporal sequence of loss of these membrane proteins.

E. Characterization of LHCII and PSI holo complex:

Since, senescence is associated with loss of Chls, we characterized the structural and functional organization of LHCII during senescence in *Cucumis* cotyledons. For this purpose, we have isolated the LHCII macroaggregates in control 6th day cotyledons and senescing 27th day cotyledons and studied the absorption, fluorescence and CD spectral properties of LHCII. No change in the Chl α/β ratio of isolated LHCII of 27th was observed when compared to the 6-day sample. The isolated LHCII readily form macroaggregates in the addition of salts. This salt induced aggregation is due to the lipids that surround the isolated LHCII. We observed the 6-day LHCII readily aggregated to in the addition of salts as described in Fig 5.1, whereas, we found difficulty in the aggregating the LHCII by salts in 27-day sample. This change in the aggregation property of 27-day LHCII sample is attributed to the changes in the lipid environment that surrounds the LHCII as a result of senescence.

In addition, the absorption spectrum of 27-day LHCII sample showed the peak shifts, with reference to the 6 day LHCII control (Fig 5.3), which may be due to change in the lipid environment.

Apperently no peak shift in the Chl α fluorescence emission spectrum of 27-day sample was observed when compared to 6-day sample (Fig 5.5). Analysis of circular dichroism spectra of the isolated LHCII of control 6th day and senescing 27th day cotyledons suggest that, the changes in the pigment orientation leading to the
decreased efficiency of energy transfer among the Chls of LHCII in senescing 27th day cotyledons (Fig 5.7).

In our isolation procedure for LHCII, the PSI holocomplex was obtained near 1.0 M region as a distinct green band. The Chl a/b ratio of this PSI holocomplex was about 5 to 5.5 in the 6th day sample. Where as the Chl a/b ratio was declined in 27th day PSI sample indicating the presence of LHCII in PSI holocomplex. In the room temperature absorption spectrum of 27-day PSI holocomplex (Fig5.4), we observed a 653 nm shoulder to the 680nm peak (characteristic to the Chl b of LHCII). This 653nm shoulder was not seen in the 6th day PSI sample. In addition the liquid nitrogen Chl a fluorescence emission spectra of 27-day PSI holocomplex exhibited a 680nm Chl a fluorescence emission peak characteristic to LHCII, which was absent in the Chl a fluorescence emission spectrum of 6-day PSI holocomplex (Fig 5.6).

These two observations (absorption and Chl a fluorescence emission spectral changes) are attributed to the senescence induced association of a fraction of LHCII with the PSI holocomplex. Further, from the resolution of polypeptides by gradient gel electrophoresis, indicated the presence of a 27-29KDa polypeptide (LHCII) in 27 day PSI sample, which was absent in the 6-day PSI holocomplex (Fig 5.9 B). Since, the presence of LHCII in PSI holocomplex invariably occurred in all the preparations from the 27-day cotyledons, we tend to believe that this is likely to be senescence induced association of a fraction of LHCII to PSI in 27-day cotyledonary samples. At present we have no idea whether the observed LHCII associated with PSI holocomplex of 27-day cotyledons, functions in harvesting the light energy and transferring to PSI.

These results are further supported by the invitro phosphorylation studies, using γ-labelled 32P-ATP. We have observed an initial increase in the 32P incorporation in the LHCII of 15-day sample and there after showed a gradual decline (Fig 5.10). The activation of protein kinase is known to depends on the oxidation-reduction state of plastoquinone and the b type hemes of Cyt b6/f complex (Allen et al., 1981). Since, we have observed the alteration in the electron transfer...
from PQH\textsubscript{2} to Cyt \textsubscript{b_{56}} complex (slower reoxidation of PQH\textsubscript{2}) we deduced that the membrane bound protein kinase that phosphorylates LHCII, becomes activated with the progress of senescence. The diffusion of phospho-LHCII could have resulted in the formation of loosely stacked extended grana like structures as discussed in section 4.2(Fig. 4-1 A&B). Unlike in the reversible phosphorylation of LHCII by protein kinase and phosphatase under high light intensities (Allen, 1995), we assume that the phosphorylation of LHCII seen here is possibly irreversible in senescing condition. However, to the best of our knowledge no reports are available on the membrane phosphatases during leaf senescence.

Based on the findings of the present investigations and on the existing literature we present a schematic sketch as shown in Fig 6.1. The numerical numbers in the Fig 6.1 indicate the results observed for the present investigations with the Cucumis sativus cotyledons. The details of the sketch are given below:

1. Alteration in the Q\textsubscript{A} to Q\textsubscript{B} electron transfer at the acceptor side of PSII as inferred from the atrazine/\textsuperscript{14}C-atrazine binding studies and analysis of Chl \textsubscript{a} fast fluorescence transients.

2. Alteration in the electron transfer from PQH\textsubscript{2} to Cyt b_{56} complex. Slower rate of reoxidation of PQH\textsubscript{2} by PSII (analysis from the Chl \textsubscript{a} fast fluorescence transients and the DBMIB inhibition studies) This was also suggested by Jenkins \textit{et al.}, 1981a; Harnischfiger, 1974 and Sabat \textit{et al.}, 1989.

3. Alterations in the electron transfer from the plastocyanin to PSI reaction centre as deduced from the KCN inhibition studies.

4. Evidence of Uncoupling of antenna from the PSII reaction centre as deduced from the light saturation studies, Chl \textsubscript{a} fast fluorescence transients, ultrastructural studies, and the \textit{In vitro} phosphorylation studies.

5. Differential changes in the steady-state levels of specific thylakoid proteins evaluated by western-blot analysis.

6. Conformational changes in the LHCII of photosystem II as derived from CD spectral analysis.
Fig 6.1 Proposed scheme for the sequential advancement of Cucumis sativus cotyledon senescence showing structural and functional alterations in thylakoid membrane. The width of open arrow marks corresponds to the extent of loss or changes of respective proteins.
7. Association of LHCII to photosystem I as observed from the spectral properties and polypeptide profiles of isolated PSI holocomplexes. Senescence induced association of LHCII with PSI may or may not be functional?

8. Activation of membrane bound protein kinase and the diffusion of phospho-LHCII through the thylakoid membrane ascribed from ultra-structural studies of chloroplasts, F₀ measurements, Invitro phosphorylation studies and the characterization of PSI holocomplex.

9. Appearance of plastoglobulie in chloroplasts of senescing Cucumis cotyledons as observed in other systems.

This scheme thus provides a working model on the alterations in the structure and function of thylakoid membranes of Cucumis cotyledons during senescence.