PHOTOSYNTHETICA 39 (4): 557-563, 2001

The distinctive pattern of photosystem 2 activity, photosynthetic pigment accumulation, and ribulose-1,5-bisphosphate carboxylase/oxygenase content of chloroplasts along the axis of primary wheat leaf lamina

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Abstract

Wheat (*Triticum aestivum* L. cv. Sonalika) seedlings were grown in Hoagland solution. Primary leaves were harvested at 8, 12, and 15 d and cut into five equal segments. Contents of photosynthetic pigments and proteins, and photosystem 2 (PS2) activity increased from base to apex of these leaves. Chlorophyll (Chl) content was maximum at 12 d in all the leaf segments, but PS2 activity showed a gradual decline from 8 to 15 d in all leaf segments. In sharp contrast, the CO_2 fixation ability of chloroplasts increased from 8 to 15 d. CO_2 fixation ability of chloroplasts started to decline from base to apex of 15-d-old seedlings, where the content of ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RuBPCO-LSU) increased acropetally. RuBPCO-LSU content was maximum in all the leaf segments in 12-d-old seedlings. This shows a distinctive pattern of PS2, Chl, CO_2 fixation ability of chloroplasts, and RuBPCO-LSU content along the axis of leaf lamina during development and senescence. RuBPCO-LSU (54 kDa) degraded to fragments of 45, 42, 37, 19, and 16 kDa products which accumulated along the leaf axis during ageing of chloroplasts. Thus the CO_2 fixation ability of chloroplasts declines earlier than PS2 activity and photosynthetic pigment contents along the leaf lamina.

Additional key words: carotenoids; chlorophyll; CO_2 fixation ability; photosystem 2; protein; thylakoid; *Triticum aestivum*.

Introduction

Leaf growth in monocotyledonous plants is confined to their basal region, which is enclosed by the sheaths of subtending leaves (Boffey *et al.* 1980). Leaf primordia develop primarily by the action of a basal intercalary meristem. Therefore, the growth is largely unidirectional, with a basal meristem producing parallel files of cells (MacAdam *et al.* 1989). Cells within a file are displaced away from their origin (meristematic region) as a result of continued production and elongation of new cells within the same file. As cells are being displaced, they grow and differentiate. Thus the distance between a cell and its origin is a function of both its age and developmental stage. Therefore, the older cells and more developed plastids are located near the leaf tip, and progressively younger cells are located near the leaf base (Robertson and Laetsch 1974). Because of this unidirectional developmental gradient the leaf growth zone in monocots is well suited for the study of biophysical and biochemical process associated with growth and development (Boffey *et al.* 1980).

Cells in a rapidly growing barley leaf continue to enlarge for a period even after cell division stops. This creates a zone of cell elongation 2-3 cm long adjacent to the leaf basal meristem (Sharman 1942). In this region plastid number per cell increases steadily, plastid volume changes a little in part, because plastids are dividing. Plastid transcription activity increases during the lag period of Chl development. Following the lag phase, plastid volume per cell increases rapidly for 36-48 h. During this period plastid transcription and translation rates are high (Klein and Mullet 1987). This is also the phase in which

Received 22 May 2001, *accepted* 10 September 2001.
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Abbreviations: Car – carotenoids; Chl – chlorophyll; LSU – large subunit; MeV – methylviologen; PS2 – photosystem 2; RuBPCO – ribulose-1,5-bisphos-phate carboxylase/oxygenase.
Acknowledgement: This work was funded by a UGC Major Research Project No. F-3-55/94/Sr-II to ANM. We thank Dr. (Mrs.) J.K. Sainis, MBAD, BARC for the gift of RuBPCO antibodies. The encouragement and laboratory facilities of Dr. K.K. Ussuf, BTNA, BARC is thankfully acknowledged. The award of UGC-JRF to FD and CSIR-RAship to MM is gratefully acknowledged.

the bulk of the inner membrane of the chloroplast is synthesised. Once the phase of the rapid growth is completed, plastid transcription and translation rates, and mRNA and ribosome contents decline (Klein and Mullet 1987). This adjustment occurs as the chloroplast population shifts from the rapid synthesis and accumulation of new structures to the process required to maintain chloroplast function in the mature leaf.

The final phase of chloroplast development occurs during the leaf senescence, in which the plastid volume and plastid number per cell decline as the plastids translocate materials useful for development of new structures (Wardley *et al.* 1984). A conspicuous symptom of leaf senescence is the loss of Chl (Misra and Biswal 1980, 1981, Makino *et al.* 1984, Kutík *et al.* 1999). There is also a progressive deterioration of photosynthetic electron transport activity during chloroplast senescence (Misra and Biswal 1982).

The degradation of RuBPCO occurs in chloroplast (Huffaker 1990). During senescence in wheat and barley the amount of RuBPCO decreases much faster than that of the number of chloroplasts (Mae *et al.* 1984, Wardley *et al.* 1984). Bushnell *et al.* (1993) reported that a zinc protease from the stroma of the pea chloroplast was able to degrade the LSU to a smaller peptide fragment of about 36 kDa. Also irradiation or oxidative stress in leaves and chloroplasts (Mitsuhashi *et al.* 1992) accelerates the RuBPCO degradation. The involvement of active oxygen triggers the degradation of RuBPCO. The active oxygen species or oxy-radicals directly cleave the peptide bonds of the protein (Davies *et al.* 1989). Ishida *et al.* (1997) reported that degradation of RuBPCO-LSU to fragments of 37 and 16 kDa are due to active oxygen. The degradation of RuBPCO in intact chloroplast or in intact young leaves proceeds rapidly and smoothly, without showing any sign of detectable amount of their breakdown products. It is physiologically essential, due to their interference with a multitude of protein-protein interactions (Vierstra 1993). During the ageing of the leaf, further proteolysis of the fragments produces smaller peptides or amino acids. This process is accelerated under physiological condition. However, RuBPCO degradation *in vivo* during de-greening still requires further investigation.

Leaf senescence is initiated when the photosynthetic rate drops below a certain threshold (Šesták 1985, Čatský and Šesták 1997). That threshold may be at or near the CO_2 compensation concentration at which the leaf no longer contributes fixed carbon to the rest of the plant. The activity or content of RuBPCO decrease during senescence and a close correlation between the rate of photosynthesis and level of enzymes have been reported (Uchida *et al.* 1982, Makino *et al.* 1984). However, the photosynthetic electron transport property, PS2 in particular, is the most sensitive function during senescence (Misra and Biswal 1982). Still it is not resolved whether the loss of RuBPCO activity precedes that of PS2 activity. This could have been due to heterogeneity of the leaf samples in the senescence studies. However, the chloroplast developmental gradient along the wheat leaf axis provides an ideal system to overcome this problem.

In the present study, we studied the development of chloroplasts along the wheat leaf lamina and the ageing pattern of chloroplasts. We assessed (*1*) the changes in Chl, Car, and protein contents, (*2*) PS2 activity, and (*3*) CO_2 fixation ability of chloroplasts and RuBPCO quantity.

Materials and methods

Plants: Wheat (*Triticum aestivum* cv. Sonalika) seeds were germinated in Petri plates fitted with cotton as described by Misra and Biswal (1980) and supplied with 0.5 N Hoagland solution (Hoagland and Arnon 1938) at 26±1 °C. Seedlings were grown in continuous irradiation of 125 μmol(photon) m^{-2} s^{-1} (PAR) for 15 d. The primary leaves were harvested on 8, 12, and 15 d. The leaves were measured and cut into five segments. The basal segment was numbered one (1) and the apex one was numbered five (5). The age of the seedlings was counted from the day of root emergence.

Isolation of chloroplasts and thylakoids: Chloroplasts were isolated from wheat leaves according to Cockburn *et al.* (1968). All operations were done at 0-4 °C. The leaf segments were ground in a pre-chilled mortar and pestle with grinding buffer containing 330 mM sorbitol, 5 mM $MgCl_2$, 2 mM iso-ascorbate, and 10 mM $Na_4P_2O_7 \times 10$ H_2O adjusted to pH 7.5. The slurry was filtered though 4 layers of muslin cloth and the filtrate was centrifuged at 5 000×*g* for 5 min. The supernatant was discarded. The pellet was washed with chilled suspending buffer containing 330 mM sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM ethylene diamine tetraacetic acid-disodium salt (EDTA), and 50 mM N-[2-hydroxyethyl]piperazine-N'-2-ethanesulphonic acid (HEPES) adjusted to pH 7.6, and centrifuged at 500×*g* for 5 min. The pellet was discarded. The supernatant was centrifuged at 5 000×*g* for 5 min. The chloroplast pellet was washed once again in the suspending medium and finally suspended in the same medium.

Thylakoids were isolated from wheat leaf segments as described by Misra *et al.* (1997). The leaf segments were ground in pre-chilled mortar and pestle with grinding

buffer containing 0.3 M NaCl, 1 mM EDTA, 30 mM Tricine, pH 7.8. The slurry was filtered though 4 layers of muslin clothe and the filtrate was centrifuged at 5 000×*g* for 5 min. The supernatant was discarded, and the pellet was washed with washing buffer containing 200 mM sucrose, 30 mM NaCl, 200 mM 3-[N-morpholino]-propanesulfonic acid (MOPS), pH 7.4, and centrifuged at 500×*g* for 5 min. The supernatant was discarded and the pellet that contained the thylakoids was retained. The pellet was then suspended in the suspension buffer containing 30 mM NaCl and 20 mM MOPS, pH 7.2. All these operations were done at about 0-4 °C.

Extraction and estimation of pigments: Each segment was ground with 80 % (v/v) chilled acetone in a chilled mortar and pistil. The homogenate was centrifuged at 10 000×*g* at 4 °C and the supernatant was taken as the pigment source. The pellet was again washed with acetone and centrifuged. The supernatant was pooled and taken for pigment estimation. Chl was estimated according to Arnon (1949), and Car according to Liaaen-Jensen and Jensen (1971).

Extraction and estimation of proteins: The pellet, remaining after pigment extraction, was washed once with 10 % TCA and twice with ethanol and ether in the ratio of 2 : 1 (v/v). Then the pellet was digested with 1 M NaOH at 90 °C in a boiling water bath for 15 min and centrifuged at 10 000×*g* for 10 min. The supernatant was used for the estimation of protein. Protein estimation was done by Folin-Ciocalteu reagent method as described by Lowry *et al.* (1951).

Photosynthetic electron transport activity of thylakoid membranes was measured polarographically using a Clark type oxygen electrode (*Gilson Medical Electronics Oxygraph*, Ohio, U.S.A) at 21 °C in a rate-saturating red radiation (Misra *et al.* 1997). The basic assay buffer (1.5 cm^3) consisted of 20 mM MOPS, pH 7.2, 30 mM NaCl, thylakoid membranes equivalent to 10 g(Chl) m^{-3}, 2.5 μM gramicidin, and 0.2 mM 2,6-dichloro-*p*-benzoquinone (DCBQ) as PS2 electron acceptor.

CO_2 fixation ability of chloroplasts: The method described by Walker *et al.* (1968) was followed for the determination of $^{14}CO_2$ fixation as a measure of RuBPCO activity. The reaction mixtures consisted of 330 mM sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 7.5 mM $NaH^{14}CO_3$ (1.6×10^5 Bq), 50 mM HEPES, pH 7.6, and chloroplast suspension equivalent to 20 μg Chl in a total volume of 1.5 cm^3.

The reaction was carried out for 5 min at 21 °C under nitrogen atmosphere in presence of saturating "white light" from a tungsten lamp (*Sylvania* 150 W projector lamp (*Photophone*, Mumbai, India) and focused by a *Prado* slide projector. Acidifying the reaction mixture with 0.4 M formic acid stopped the reaction. Then the reaction mixture was thoroughly vortexed and filtered with *Whatman* No. *1* paper. The clear aliquot was quantified for the amount of ^{14}C using a *Beckman* liquid scintillation counter and Bray's scintillator medium (10 % naphthalene and 0.5 % PPO in dioxane).

Western blotting of RuBPCO: Chloroplasts (equivalent to 20 μg Chl) were solubilised in a solubilisation (2×) buffer containing 50 mM Tris-HCl (pH 6.8), 10 % glycerol, 10 % sodium dodecyl sulphate (SDS), 0.1 % bromophenol blue, and 10 % mercaptoethanol, and loaded on to each elecrophoresis well. Proteins separated by SDS-PAGE using 12 % (m/v) acrylamide (Misra *et al.* 1999) were blotted on a nitrocellulose filter (*Millipore*) using a *Biorad* miniblotter system. This blot was incubated with primary antibody of RuBPCO. Primary antibody was prepared against RuBPCO from spinach purified according to Paulsen and Lane (1966). The rabbit was injected with 1 mg of RuBPCO protein in Freund's complete adjuvent. Booster was given after 1 month comprising of 1 mg RuBPCO in 1 cm^3 Freund's incomplete adjuvent. Antisera were collected with the booster. The nitrocellulose blots were blocked with bovine serum albumin, incubated with 1 : 1 000 dilution of RuBPCO antibody (antirabbit – IgG) linked with alkaline phosphatase in the presence of chomogenic substrate NBT and BCIP as described by Sambrook *et al.* (1989).

Results

Photosynthetic pigment contents are a measure for the development and senescence of chloroplasts (Misra and Biswal 1980, 1981): The Chl content increased from base (1) to apex (5) of the leaf lamina (Fig. 1). The Chl content of basal segment (1) was 0.65, 0.72, and 0.44 g per kg fresh leaf in 8-, 12-, and 15-d-old seedlings, respectively. The other segments also showed similar trend in the Chl content, with a peak at 12 d and decreasing at 15 d.

The changes in Car content of leaf segments of 8-, 12-, and 15-d-old seedlings followed a similar trend as Chl (Fig. 1). The Car content increased from base to apex in all the seedlings starting from 8 d to 15 d. The Car content in the segment 1 of 8-d-old seedling was 0.240 g per kg fresh leaf compared to that of 0.209 g at 12 d, and 0.178 mg at 15 d. The Car contents of apical leaf segments were maximum at 12 d and subsequently decreased in 15-d-old seedlings. The Chl/Car ratio of the developing leaves (Fig. 1) increased gradually in 8- and 15-d-old

seedlings, but remained stable at 12 d. The seg-ments 1 to 5 showed Chl/Car peaks up at 12 d, suggest-ing relatively faster synthesis of Chl compared to that of Car up to 12 d, and less accumulation and/or degradation at 15 d (Fig. 1).

Protein content increased gradually from base to apex of the leaves (Fig. 2). The increase was from 7.4 g per kg fresh leaf in segment 1 to 12.7 g in segment 5 of 8-d-old seedlings. Similar trend in the leaf protein content was observed in the 12- and 15-d-old seedlings. The protein content of the basal two leaf segments (1 and 2) increased from 8 to 12 d and was minimum at 15 d. On the contrary, the protein content of the apical leaf segments starting from segment 3 decreased with age of the seedling.

The protein/Chl ratio decreased gradually from base to apex in 12- and 15-d-old leaves. The decrease in the protein/Chl ratio with a concomitant increase in Chl and protein from base to apex of 12- and 15-d-old seedlings suggested a more rapid increase in Chl content than in protein content. However, the ratio also increased in all the segments of 15-d-old seedling compared to that of 8- and 12-d seedlings. Looking at the degradative pattern of change in both Chl and protein in 15-d-old seedlings, the ratio indicates a rapid breakdown of Chl compared to that of proteolysis.

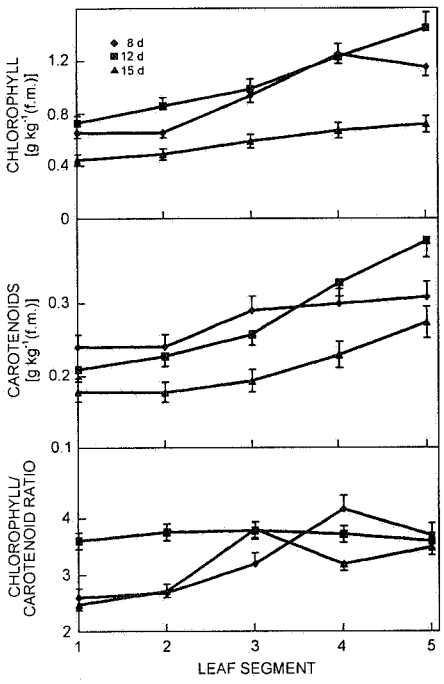

Fig. 1. Changes in contents of chlorophylls and carotenoids, and chlorophyll/carotenoid ratio of wheat leaf segments from 8-, 12-, and 15-d old seedlings. Segment 1 represents base and segment 5 apex of the leaf lamina. Means ± S.E. of 3 separate experiments.

Photochemical activity (Fig. 2) of chloroplasts mediated by DCBQ increased from base to apex of leaf in 8-, 12-, and 15-d-old seedlings.

CO_2 fixation ability of chloroplasts and RuBPCO content: The CO_2 fixing ability of RuBPCO increased from base to apex at 8 and 12 d, but decreased acropetally in 15-d-old seedlings. Therefore the activity was maximum in segments 3 to 5 at 8 d and minimum at 15 d. PS2 activity increased from segment 1 to segment 5 in leaves of all kinds of seedlings, but the increase was fairly small in 15-d seedlings.

RuBPCO-LSU 54 kDa polypeptide undergoes during senescence a proteolytic attack that comes from the vascular protease (Huffaker 1990, Vierstra 1993). We therefore studied the degradation of RuBPCO in wheat leaf lamina from 8 till 15 d. The Western blot analysis of RuBPCO-LSU showed that at 8 d the 54-kDa protein showed similar pattern of accumulation from base to apex of 8- to 15-d-old seedlings, peaked at 12 d and decreased at 15 d seedlings. The degradation products of RuBPCO-LSU of 45, 42, 37, 19, and 16 kDa were detected in segments 3, 4, and 5 of the 8-d-old seedlings. In 12- and 15-d-old seedlings all these degraded protein fragments were present from base to apex. Due to a decreased protein concentration in 15-d-old leaves, the fragments were not distinctly visible.

Discussion

The changes in Chl and Car contents of the developing leaf segments showed a uniform pattern of accumulation at 8, 12, and 15 d (Fig. 1). Chl and Car contents of the seedlings were highest at 12 d and lowest at 15 d, which suggests that chloroplasts in the leaf segments develop up to 12 d, and at 15 d they show symptoms of senescence, irrespective of their position in the leaf lamina. The developmental signal for the chloroplast along the leaf lamina is uniform from base to apex along the leaf lamina. So under normal leaf development in the seedlings grown in light, the chloroplasts develop simultaneously along the leaf lamina and also start senescing at the same period. However, the pattern of pigment accumulation starting from base to apex of wheat leaves showed an increase in pigment contents, suggesting a developmental gradient along the lamina. This gradient, however, could be rather due to a disproportionate synthesis of pigments in different zones of the leaf than to the presence of a gradient of developing chloroplasts. The developmental pattern of the chloroplast, however, parallels leaf development in a uni-

form manner and thus facilitates the study of development, maturation, and senescence. The proper assembly of the thylakoid pigment-protein complexes requires Chl in precise stoichiometric amounts (Thomas 1997). Chl has a constructional and stabilising role in addition to photon harvesting and photosynthetic function. During senescence Chl is removed from that complex, making it photodynamically harmless and preserving the vitality of the senescing cells (Matile *et al.* 1999).

The pigment ratio analysis showed that both relative rate of synthesis and degradation of Car were faster compared to Chl during leaf development and senescence. These data corroborate the earlier reports on the relative rate of synthesis and degradation of these pigments during development and senescence of chloroplasts (Misra and Biswal 1980, 1981). Chl content increases from base to apex in *T. aestivum* (Boffey *et al.* 1980) and *Lolium perenne* (Davies *et al.* 1989). Within the leaf blade the distribution of chloroplast differs in different phases of development. During chloroplast development, Chl amount increases and content of pigments of light-harvesting complexes decreases in *T. aestivum* (Webber *et al.* 1984).

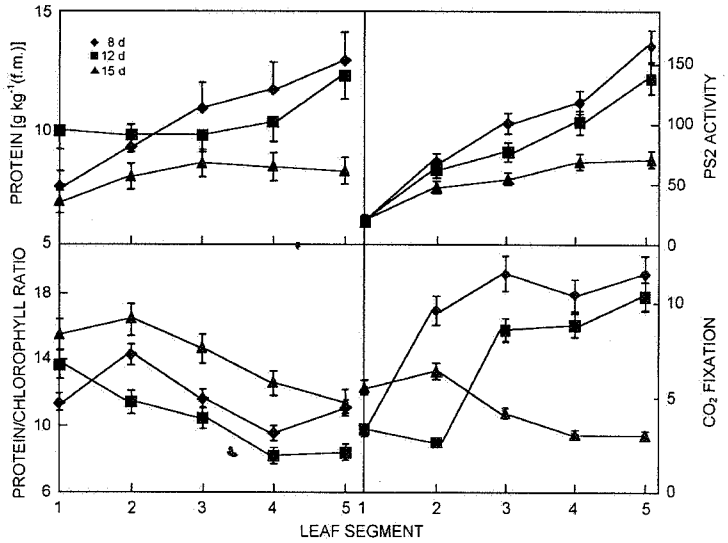

Fig. 2. Changes in protein content, protein/chlorophyll ratio, PS2 activity [mmol(O_2 evolved) kg^{-1}(Chl) s^{-1}] and CO_2 fixation ability [mmol(CO_2 fixed) kg^{-1}(Chl) s^{-1}] of wheat leaf segments from 8-, 12-, and 15-d old seedlings. Segment 1 represents base and segment 5 apex of the leaf lamina. Means ± S.E. of 3 separate experiments.

The changes in leaf protein contents, however, were not parallel with the leaf pigment content. The protein content of the basal two segments increased up to 12 d, but that of the other segments decreased gradually till 15 d. The metabolite contents of the leaves are a net result of the synthesis and degradation of the system. Proteolysis in the leaves is a criterion for the onset of cellular catabolic phase, *i.e.*, senescence (Misra and Biswal 1982, Mae *et al.* 1984, Huffaker 1990). The increase in protein content of the basal leaf segments at 12 d suggests that the protein synthesis is more active in these two segments. Also the segments 3, 4, and 5 probably attained cellular maturation phase, *i.e.*, maximum protein synthesis, at 8 d and then started senescing up to 15 d, although chloroplasts still developed as shown by Chl accumulation up to 12 d in all the segments. So the protein synthesis in leaves can be separated temporarily and spatially from that of the intrinsic pigment content of the thylakoids during chloroplast development along the leaf lamina.

The functional integrity of thylakoid membranes as measured by the oxygen evolving capacity of PS2 declined with leaf age in all the leaf segments. Decrease in the PS2 activity during chloroplast senescence was reported in wheat (Misra and Biswal 1982). The 33 kDa oxygen evolving complex (OEC) is degraded by proteolysis during chloroplast senescence (Kuwabara and Hashimoto 1990). This leads to the loss of Mn^{+2} ions in the OEC and the decline in the O_2-evolving capacity of PS2. So in terms of functional ability of thylakoid membranes chloroplast senescence started at 12 d in the whole leaf. However, similar to Chl, Car, and protein contents, PS2 activity showed an acropetal developmental gradient across the leaf lamina.

The changes in photosynthetic pigment contents and photosystem activities could lead to a change in the ultimate carboxylation efficiency of the leaves. There was a rapid increase in the CO_2 fixation ability of chloroplasts from 8 to 15 d in basal leaf segments. The CO_2 fixation ability of chloroplasts increased from base to apex at 8 and 12 d, but decreased acropetally at 15 d. Mae *et al.* (1984) reported that RuBPCO activity increased in the primary wheat leaves prior to chloroplast maturation. Development of photochemical and RuBPCO activities was reported to be similar during wheat leaf ontogeny. Similar

developmental responses are observed in the wheat leaf axis for PS2 oxygen evolution efficiency and CO_2 fixation ability of chloroplasts. However, the decrease in the CO_2 fixation ability of chloroplasts along the wheat leaf lamina at 15 d could be due to degradation and/or inactivation of RuBPCO. This is taken as a measure for chloroplast senescence. The degradation product of the LSU of RuBPCO might be due to the proteolysis and oxidative damage (Huffaker 1990, Vierstra 1993, Ishida *et al.* 1998). During the senescence of wheat leaf, production of superoxide increases almost four-fold during the early stage of degreening (McRae and Thompson 1983). Under physiological conditions such as leaf senescence the scavenging capacities becomes insufficient, and as a result the LSU fragmentation might occur.

Thylakoid membrane fragments are required but stroma components are not obligatory for the fragmentation of RuBPCO-LSU (Ishida *et al.* 1998). DCMU completely inhibits the fragmentation indicating the electron transport process beyond PS2 in thylakoid membranes is involved in the process of RuBPCO-LSU turnover. This involves superoxide radical generation, its disproportion to H_2O_2, and hydroxyl radical generation by the Fenton reaction in presence of metal ions (Asada 1999). Methylviologen (MeV) decreases the production of hydroxyl radical in the thylakoid membranes and suppresses the fragmentation of RuBPCO-LSU (Ishida *et al.* 1998). However, during *in vivo* ageing of chloroplasts, MeV reduction decreases faster than DCPIP Hill reaction (Misra and Biswal 1982). We found a gradual loss of PS2 activity in all the leaf segments at 12- and 15- compared to 8-d-old seedlings (Fig. 2) suggesting disorganisation of thylakoid membranes and loss of reductant generation in the irradiated thylakoids. Considering the recent mechanism proposed by Ishida *et al.* (1998) on the fragmentation of RuBPCO-LSU by hydroxy-radicals by the irradiated thylakoids, a rapid loss of PS1 activity in ageing leaves could have accounted for such non-enzymatic degradation of RuBPCO-LSU to 45, 42, 37, 19, and 16 kDa fragments in the senescing wheat leaf segments. These fragmentation patterns corroborate the reported pattern of fragmentation of RuBPCO-LSU in isolated irradiated chloroplasts (Ishida *et al.* 1998). However, the absence of any detectable amount of smaller fragments in 15-d-old seedlings can be attributed to a rapid turnover of RuBPCO-LSU fragments in senescing chloroplasts. Although the protein content of RuBPCO-LSU increased from base to apex in 15-d-old leaves, the CO_2 fixation ability of chloroplasts decreased significantly from base to apex at 15 d (cf. Figs. 2 and 3). The changes in functional aspects of RuBPCO could be the cumulative effect of protein structure and function of the RuBPCO holozyme. We found that PS2 activity declined with leaf age (8, 12, and 15 d). But along the developmental gradient in the leaf axis, PS2 activity increased and CO_2 fixation ability decreased at 15 d. So we conclude that CO_2 fixation ability of chloroplasts declines earlier than PS2 activity and photosynthetic pigment contents along the wheat leaf lamina. There may be a developmental signal with seedling age and a specific chloroplast signal for the gradient across leaf lamina. This is because there is a general age-related change in all the leaf segments that varies along the leaf axis.

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