Materials & Methods
1. Plant material and growth conditions
Wheat seeds (*Triticum aestivum* L. cv. Kundan, IARI, New Delhi) were kept in tap water/herbicide solution (2 μM) for 12 h in dark. Turgid seeds were transferred to petriplates on moist germination paper and grown in complete darkness for 8 days at 25°C or seeds kept in tap were transferred to plant growth chamber maintained at 25±1°C, under the photoperiod of 14/10 h light dark cycle after treating with herbicide solution of 25 μM. Light flux density inside the growth chamber was 25 Wm⁻². For greening of dark grown seedlings, 8 day old etiolated plants were illuminated under a continuous light flux density of 5 Wm⁻². The middle 3 cm pieces of the leaves were used for the experimentation.

2. Herbicide treatment
The herbicide Norflurazon (SAN 9867) was obtained as a gift from, Sandoz Ltd., CH 4108, Witterwil, Switzerland. The herbicide was dissolved in acetone, as it is sparingly soluble in water. Then this solution was mixed in distilled water. However, the final herbicide solution always contained less than 1% acetone. Seeds were soaked in 2 μM herbicide solution as mentioned above or the two days old dark grown seedlings were treated with 25 μM norflurazon solution. The seedlings were treated with a fresh solution every alternative day after the earlier solution was washed out thoroughly with water.

3. Pigment estimation
Chlorophyll and carotenoid pigments were estimated spectrophotometrically according to the procedure of Lichtenthaler (1988) following their extraction in 80% (v/v) aqueous acetone. The following equations were used to calculate pigments in μg/ml of the extract:

\[
\text{Chl}a = 12.25A_{663.2} - 2.79A_{645.8}
\]
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\[
\text{Chlb} = 21.50A_{646.8} - 5.10A_{663.2} \\
\text{Chla+b} = 7.15A_{663.2} + 18.71A_{646.8} \\
\text{Carotenoids} = (1000A_{470} - 1.82\text{Chla} - 85.02\text{Chlb})/198
\]

4. Total leaf protein extraction

One g of leaf material was ground in a mortar and pestle with 20% (v/v) TCA filtered through two layers of gauge cloth to remove the debris. Ice-cold acetone was added to the extract and left on ice for 20 min for the proteins to settle down. It was centrifuged at 3000 rpm (Remi) for 10 min and the protein pellet was dissolved in 0.1N NaOH.

5. Isolation of etioplast inner membranes

Etioplasts were isolated according to Ryberg and Sundqvist (1982) with some modifications. All the experiments were done at 4°C under safe dim green light. 20 g of leaf material were homogenised with a blender (Philips India) in an ice-chilled isolation buffer containing 20 mM TES, 10 mM HEPES, 0.5 M sucrose, 1 mM MgCl₂, 1 mM EDTA and 5 mM β-Mercaptoethanol (pH 7.2 with KOH). The homogenate was filtered through four layers of nylon cloth and centrifuged at 2000 x g for 10 min, in a refrigerated centrifuge (Sorvoll RC5C, Beckman, USA).

The pellet was resuspended in the isolation medium and centrifuged at 100 x g for 5 min to remove the debris. The supernatant has etioplasts. It was mixed with 6-7 times the volume of sucrose-free medium and ground in a glass homogenizer with a teflon pestle, resuspended in 12.5% (w/v) sucrose medium and subjected to brief sonication (3 x, 5 s each) to break the connections between the tubules of PLBs and PTs and centrifuged at 42000 x g. The pellet contains the etioplast inner membranes.
6. Isolation of greening plastids and thylakoids

Greening plastids were isolated like etioplasts only. The plastids thus isolated were subjected to osmotic shock by suspending them in a hypotonic isolation buffer and centrifuged at about 40000 xg. The thylakoid pellet thus obtained was purified from other membrane impurities by a centrifugation over a discontinuous sucrose density gradient of 1.5 M, 1 M and 0.6 M. The pellet of thylakoids was used for further experimentation.

7. Room temperature chl a fluorescence emission spectrum

Room temperature chl a fluorescence emission of thylakoid suspension was measured using a spectrofluorimeter (Luminescence spectrophotometer, model L.S. 5, Perkin Elmer, USA). Thylakoid membranes equivalent to 5 μg/ml of chl were dark incubated for 5 min before measurements. The samples were excited at 440 nm and the emission was recorded between 650-800 nms.

8. Estimation of proteins

Protein contents in the suspensions were estimated by Coomassie method (Bradford 1976). The reagent has 0.01% (w/v) coomassie brilliant blue (G), 5% (v/v) 10% (v/v) H3PO4. A standard curve was constructed against BSA by mixing, 10-50 μg of BSA form 1 mg/ml stock, with 5 ml of reagent. The colour developed after 2 min was read spectrophotometrically at 595 nm.

9. SDS-PAGE analysis of membrane proteins

Polypeptide analysis of the membrane proteins was done by SDS-PAGE according to Lamelli et al. The composition of the stacking and resolving gels were as following:

Resolving gel: 7-14% acrylamide gradient, 6 M-3 M urea gradient, 0.8 M Tris-HCl (pH 8.8) and 0.1% SDS
Stacking gel: 4% acrylamide, 3 M urea, 0.12 M Tris-HCl (pH 6.8) and 0.1% SDS

The gels were polymerized by addition of 0.075% ammonium persulphate and 0.05% TEMED.

Sample preparation: Membrane aliquots containing 150 µg of proteins were resuspended in 100 µl sample buffer. The sample buffer has 0.125 M Tris-HCl (pH 6.8), 4% SDS, 10% β-mercaptoethanol, 20% glycerol, and 4 mg/ml bromophenol blue. Proteins equivalent to 50 µg were loaded in each slot.

The electrophoresis was done at room temperature, initially at a constant voltage of 75 V and then raised to 100 V when the samples entered resolving gel. The reservoir buffer has 0.192 M glycine, 0.1% SDS and 25m M Tris (pH 8.3). The separated proteins were either used for western transfer or stained with 0.125% coomassie brilliant blue R-250 (Sigma, USA), 50% methanol and 10% acetic acid for few hours. The destaining was carried out initially in 50% methanol and 10% acetic acid for 30 min. and in 10% methanol and 7% acetic acid till the polypeptide bands were clearly visible.

10. Western blotting for immunodetection of membrane polypeptides

The electroblotting of the SDS-PAGE resolved polypeptides, onto nitrocellulose membrane was carried out for 13 h at 30 V in Towbin tank buffer (25 mM Tris, 192 mM glycine, 0.05% SDS, 20% methanol, pH 8.3). Prior to this the gels were equalibriated with the same buffer. The transfer was started at 4°C and maintained below 20°C throughout.

The transfer was checked using PonceauS stain (PonceauS in 1% acetic acid). The membrane was washed with distilled water to remove the stain and the standard molecular weights marked. It was kept in the blocking solution (5%
notfat milk in Tris Buffer Saline, TBS: 25mM Tris, 150 mM NaCl, pH 7.4) for 2 hours. Excess blocking agent was washed three times, 5 min. each with TBST (TBS with 0.05% Tween 20), and incubated for 30 min. in the primary antibody diluted with TBST containing.

Blots were washed three times of 5 min each with TBST and incubated with anti-rabbit IgG antibodies conjugated to horse radish peroxidase for 30 min. Blots were washed again for three times with TBST and developed by a chemiluminiscence kit.

11. Native gel assay of pigment protein complexes
The chlorophyll binding proteins of the thylakoid membranes were seperated electrophoretically in their native pigment binding state as described by Delepelaire and Chua (1979) after some modifications. The procedure was same as SDS-PAGE except a Lithium Dodecyl Sulfate (Sigma) was used as a detergent instead of SDS and the crosslinker concentartion was 0.33%. The resolving and stacking gels have 10% and 4% acrylamide respectively. The sample buffer has LDS instead of SDS and the samples equivalent to 300 µg proteins were treated with the buffer on ice for 30 min and mixed by gentle tapping. The samples were centrifuged at 2500 xg for 1 min and the clear supernatant was loaded onto the gel. The gels were run, at a constant voltage of 90 V, at 4°C for 90 min in a mini-gel apparatus.

12. Determination of ANS binding sites in the thylakoid membranes
The number of binding sites and dissociation constant of ANS bound to the thylakoid membrane fragments were determined from Scatchard plots constructed according to Azzi (1975) as described by Andley et al. (1981). 5 µl aliquots of a stock solution of ANS (900 µM) were added to the sample cuvette
containing membrane fragments (60 μg/ml protein) in a total volume of 3 ml of suspension medium (250 mM sucrose, 2 mM Tris-Cl pH 7.4). From the fluorescence intensity F (at 480 nm, excitation at 390 nm) developed after each addition of ANS was subtracted the fluorescence value F₀ obtained by adding ANS in the absence of the membrane. The fraction of \([\text{ANS}]_{\text{bound}}\) (μM) is

\[
[\text{ANS}]_{\text{bound}} = \frac{(F - F_0) \times 3}{F_{\text{max}}}
\]

where \(F_{\text{max}}\) represents the limiting fluorescence intensity determined in the presence of 3 μM ANS under the same experimental conditions. For the determination of \(F_{\text{max}}\), varying concentrations of membrane protein were added to the 3ml suspension containing 3 μM ANS. A double reciprocal plot of the fluorescence intensity (arbitrary units) vs the reciprocal of the concentration of the membrane protein (mg/ml) was constructed. Extrapolation to the ordinate gave the reciprocal of the limiting fluorescence enhancement, \(F_{\text{max}}\), of ANS in the membrane fragments.

The concentration of free ANS was calculated by subtracting \([\text{ANS}]_{\text{bound}}\) from the concentration of the ANS added. A plot of \([\text{ANS}]_{\text{bound}}\) per mg protein vs \([\text{NAS}]_{\text{bound}}\) per mg protein/[ANS]_{free} gave a straight line. Extrapolation at the zero ordinate gave the number of binding sites (n) per unit protein in the membrane and extrapolation to zero abscissa gave the value \(n/k_d\) from which \(k_d\) was calculated.

13. Estimation of ANS fluorescence

Fluorescence spectra of ANS were measured according to Azzi (1975). 3 ml mixture of 60 μg/ml protein, 3 μM ANS were incubated for 10 min at room temperature. The fluorescence emission spectrum was measured between 400-700 nm after exciting at 390 nm.
14. Assay of lipid peroxidation
Stress-induced lipid peroxidation was determined by the production of thiobarbituric acid (TBA) reactive compounds which signify the formation of polyunsaturated fatty acid peroxides. The TBA reactivity was determined according Mishra and Singhal (1992) by adding equal aliquots of 0.5% TBA in 20% TCA and incubation mixture containing 175 mM NaCl, 50 mM Tris (pH 8.0) and membranes equivalent to 750 µg/ml of protein. The solution was heated at 95°C for 25 min and then centrifuged for 1 min at 250xg in a table top centrifuge (Remi, India) to make the solution clear. Absorbency was measured at 532 nm as $A_{532-600}$ nm on a Shimadzu UV160 spectrophotometer operated in dual wavelength mode. The subtraction was done inorder to correct for the non-specific turbidity (Heath and Packer 1968). The amount of malondialdehyde (MDA) formed was calculated by using an extinction co-efficient of 155 mM$^{-1}$cm$^{-1}$.

15. Assay of enzyme activities

**Preparation of samples:** Activities of enzymes were assayed in leaf homogenates prepared from control and norfluorazon treated greening plants according to Schoner and Krause (1990). The leaves were homogenized under ice-cold condition in 50mM potassium phosphate (pH 7.0), 0.25% Triton x-100 and 1% PVPP and filtered through eight layers of cheese cloth. The filtrate was then used for enzyme assays.

**Catalase:** Catalase (EC 1.11.1.6) was assayed at 25°C in a reaction mixture containing 50mM potassium phosphate, pH 7.0, 11mM H$_2$O$_2$ and leaf homogenate equivalent to 20µg protein. Activity was determined spectrophotometrically from the decrease in absorbance from at 240 nm as
described by Aebi (1983). The extinction coefficient is 0.04 cm\(^2/\mu\text{M}\) for H\(_2\)O\(_2\) at 240nm.

**Superoxide dismutase (EC 1.15.1.1):** SOD was assayed according to Marklund and Marklund (1974) with some modifications which is an assay based on the ability of SOD to inhibit autooxidation of pyrogallol. The assay mixture of 1 ml has 50mM phosphate buffer (pH 7.8), 0.1mM EDTA and 0.48mM pyrogallol and tissue homogenate equal to 20 µg protein. The change in absorbance at 420 nm was measured for 90sec at 25°C against a blank that contained all the ingredients except the tissue homogenate. The enzyme activity is expressed as Units/mg protein/min \(\times 10^3\) and was calculated by using the formula:

\[
\Delta\text{OD} \times \text{Total volume} \times \text{Dilution}
\]

\[
\text{Volume of sample} \times \varepsilon \times \text{Time}
\]

Where ΔOD is change in optical density per minute, \(\varepsilon\) is the extinction coefficient 0.04 cm\(^2/\mu\text{M}\).