Age dependent alterations in photosystem II acceptor side in Cucumis sativus cotyledonary leaf thylakoids: Analysis of binding characteristics of herbicide \[^{14}\text{C}^-\text{atrazine}\]

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Senescence induced temporal changes in photosystems can be conveniently studied in cotyledonary leaves. We monitored the protein, chlorophyll and electron transport activities in Cucumis sativus cv Poinsett cotyledonary leaves and observed that by 20th day, there was a 50%, 41% and 30-33% decline in the chlorophyll, protein and photosystem II activity respectively when compared to 6th day cotyledonary leaves taken as control. We investigated the changes in photosystem II activity \((O_2\) evolution) as a function of light intensity. The photosystem II functional antenna decreased by 27% and the functional photosystem II units decreased by 30% in 20-day old cotyledonary leaf thylakoids. The herbicide \[^{14}\text{C}^-\text{atrazine}\] binding assay to monitor specific binding of the herbicide to the acceptor side of photosystem II reaction centre protein, D1, showed an increase in the affinity for atrazine towards D1 protein and decrease in the Qa binding sites in 20th day leaf thylakoids when compared to 6th day leaf thylakoids. The western blot analysis also suggested a decrease in steady state levels of D1 protein in 20th day cotyledonary leaf thylakoids as compared to 6th day sample which is in agreement with \[^{14}\text{C}^-\text{atrazine}\] binding assay and light saturation kinetics.

Senescence is an integral phase of plant life and it induces the decline in the photosynthetic yield, which ultimately leads to the decrease in crop yield\(^1\). Study of leaf-senescence related changes in structure and function in chloroplasts is of physiological significance in crop plants. Senescence is a physiologically programmed process during which metabolites are remobilized from older to young plant parts\(^2,3\). In several senescing systems, a drastic decline in photosystem II (PS II), photosystem I (PS I) and whole chain electron transport activities have been reported\(^4,7\). The mobile electron carriers such as plastoquinone (PQ) and plastocyanin (PC) seem to be involved in the senescence induced decline in the photosystem activities\(^8-10\). Recently, we have reported the changes in the sensitivity of the photosynthetic membranes towards the herbicides, atrazine and dibromothymo quinone (DBMIB) which inhibit electron transport at the acceptor side of PS II and the plastocyanin inhibitor potassium cyanide on PS I donor side during senescence in Cucumis sativus cotyledonary leaves and observed that, during leaf senescence, the alterations in PS II acceptor side and PS I donor sides occur and these changes involve mobile electron carriers such as PQ and PC\(^11\). In the present communication, we provide further evidence on the alterations at the acceptor side of PS II by using \[^{14}\text{C}^-\text{atrazine}\] binding assay\(^12\), to characterize the nature of alterations at the PS II acceptor side. Our results suggest that, acceptor side of PS II is a sensitive indicator of foliar senescence in Cucumis cotyledonary leaves.

Materials and Methods

Cucumis sativus cv. Poinsett seedlings were raised in a growth chamber maintained at 25± 2 °C under continuous white fluorescent radiation of 35-40 \(\mu\text{mol} \text{m}^{-2} \text{s}^{-1}\) and relative humidity of 85-90%.

Estimation of chlorophyll and protein contents

Chlorophylls (Chl) were extracted in chilled 100% dimethyl formamide in dark and estimated as described earlier\(^13\). Total leaf proteins were precipitated with 10% trichloroacetic acid, dissolved in \(1\%\) sodium hydroxide and the total amount of protein was estimated according to Lowry's method\(^14\) using bovine serum albumin (Sigma Co., Fraction V) as standard.

Isolation of thylakoid membranes and measurement of photosystem II activity

Thylakoid membranes were isolated from 6th and 20th day Cucumis cotyledonary leaves as described
by Nakatani and Barber\textsuperscript{3}. Oxygen evolution under varying light intensities 1100-82.5 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) was assayed in 1 ml of reaction mixture containing 1 mM ammonium chloride 100 \( \mu \text{M} \text{DCBQ} \), thylakoids equivalent to 10 \( \mu \text{g} \) of chlorophyll and 50 mM Hepes-KOH (pH 7.5) buffer at 25\(^\circ\)C using Clark type oxygen electrode\textsuperscript{16}. Irradiance was measured using LI-COR model LI 189 Quantum meter.

**SDS-urea PAGE and immunoblot analysis**

Samples for urea-SDS denaturing polyacrylamide electrophoresis were prepared by mixing thylakoid membranes in sample buffer containing 6 \( \text{M} \text{urea, 2}\% \text{ w/v sodium dodecyl sulphate, 2.5}\% \text{ w/v 2-mercapto} \) ethanol, 62.5 mM Tris-HCl (pH 6.8) and 10\% w/v glycerol. The mixture was incubated for 30-60 min at room temperature with intermittent shaking. The sample was then centrifuged at high speed for 2 min prior to loading. Thylakoids equivalent to 15 \( \mu \text{g} \) of protein was loaded per lane. Electrophoresis was carried out using Bio-Rad’s Mini-Protein gel electrophoresis system. Gels were made according to Laemmli\textsuperscript{17}. Western blot analysis of D1 protein was carried out according to Towbin et al.\textsuperscript{18}

\[ ^{14}\text{C} \] atrazine binding assay

\[ ^{14}\text{C} \] Atrazine binding assays were carried out essentially by following the standard procedures\textsuperscript{12,19}. Binding reactions were initiated by vortex mixing 1 ml suspensions of 50 \( \mu \text{g} \text{ Chl/ml} \) with different concentrations of \( ^{14}\text{C} \] atrazine (25 Ci/mol) dissolved in ethanol\textsuperscript{12}. After 2 min incubation, the samples were centrifuged for 2 min at 15,600 \text{g} in an eppendorf centrifuge. Aliquots (0.5 ml) of the supernatants were removed and added to 3 ml of scintillation fluid (8 g PPO, 1 \text{I Triton x-100, 2 I toluene, 400 ml water}). Radioactivity of the samples was measured by a liquid-scintillation counter. Binding reactions were carried out in dark to avoid changes in the Q\textsubscript{B} redox-states due to illumination of thylakoids.

**Results and Discussion**

During foliar senescence, the loss of photosynthetic pigments is one of the most visible changes and measurement of Chl loss is commonly used as a measure of progress of senescence. We monitored the chlorophyll, total protein content and PS II activity at 2-day intervals from the day of seed germination (data not shown). The total Chl, total protein and PS II activities were maximum in 6th day old cucumber cotyledonary leaves and later showed a gradual decline. By 20th day there was about 50% loss in total Chl, 40% loss in total protein and 30-33% loss in PS II activity. Hence, we considered 6th day cucumber cotyledonary leaves as control and 20-day cucumber cotyledonary leaves as senescing situation (Table 1).

We attempted to determine the relative functional antenna sizes of 6-day old and 20-day old \textit{Cucumis} cotyledonary leaf thylakoids, since, changes in the functional antenna reflect the changes in the photosystem II activity\textsuperscript{20}. Fig. 1 shows photosystem II electron transport measured in terms of rate of \( \text{O}_2 \) evolution as a function of light intensity. The double reciprocal plot (1/\( \text{l}\text{ight intensity vs 1/PS II activity} \) shown in inset, for 6th and 20th day grown cotyledonary leaf thylakoids provides the relative PSII functional antenna size. The intercept on abscissa represents the maximal rate of electron transport which is related to the functional PS II units\textsuperscript{20}. Our result indicates that ca. 27% loss in the functional PS II units in senescing (20-day old) thylakoids. The slope value of each plot represents a relative quantum yield\textsuperscript{20} which in turn reflects the functional antenna size. There was a 30% decrease in the functional antenna size in 20-day thylakoids, compared to the control.

Fig. 2 shows the steady state level of the PS II reaction centre protein (Q\textsubscript{B} binding protein) D1\textsuperscript{21}. It is clear from the immunoblot analysis that the number of D1 polypeptides declined upon senescence in 20th day sample as compared to the 6th day control.

The herbicide, atrazine, like diuron inhibits the electron transfer at the acceptor side of PS II\textsuperscript{22}. This herbicide molecule competes with a secondary quinone acceptor Q\textsubscript{B}, for the binding site of D1 protein at the reaction center of PS II\textsuperscript{12}. Earlier we reported a small increase in \( I_{50} \) value for atrazine i.e., 0.22 \( \mu \text{M} \) for 6th day thylakoids to 0.25 \( \mu \text{M} \) for 20th day thylakoids, compared to the control.

**Table 1**—Changes in total chlorophyll (Chl), total leaf protein and uncoupled photosystem II electron transport activity in thylakoids isolated from \textit{Cucumis sativus} cotyledonary leaves as a function of leaf age. [PSII activities were expressed as \mu mol \( \text{O}_2 \) evolved \text{mg} \textsuperscript{-1} \text{Chl} \text{hr} \text{-1}]. Chlorophyll content was estimated in 100% DMF according to the method of Porra \textit{et al.} and expressed as \text{mg} \text{g} \textsuperscript{-1} (d.w.). Total leaf protein content was estimated according to the method of Lowry and expressed as \text{mg} \text{g} \textsuperscript{-1} (d.w.). Values are means of three independent experiments with standard deviation.

<table>
<thead>
<tr>
<th>Plant age (d)</th>
<th>Total Chl</th>
<th>Total leaf protein</th>
<th>PS II activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 (control)</td>
<td>28.58±0.97</td>
<td>344±12.7</td>
<td>241.5±6.36</td>
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<tr>
<td>10</td>
<td>21.67±0.58</td>
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<td>20</td>
<td>14.26±0.57</td>
<td>202±8.7</td>
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Fig. 1—Rate of photosystem II electron transport (water to DCBQ) of thylakoids isolated from 6th, (+•) and 20th, (−•) day cucumber cotyledonary leaves, as a function of light intensity. [Inset: Shows the double reciprocal plot (1/intensity vs 1/PS II activity) of both 6th and 20th day cotyledonary leaf thylakoids. The maximum light intensity used for the experiments was 1100 μE m⁻² s⁻¹. PS II activity was measured at varying light intensities in 1 ml assay mixture containing 1 mM ammonium chloride, 100 μM DCBQ, thylakoids equivalent to 10 μg of chlorophyll and 50 mM Hepes-KOH (pH 7.5) buffer using a Clark type electrode. Vertical bars represent standard deviation of the mean value of three different experimental observations].

Fig. 2—Western-blot analysis of D1 protein of thylakoids isolated from 6th and 20th day cucumber cotyledonary leaves. [Thylakoid samples were loaded on equal protein basis (15 μg) on a 12.0 % acrylamide gel containing 2 M urea. Lane 1, 6th day; lane 2, 20th day steady state levels of D1 protein respectively].

Fig. 3—Binding of [¹⁴C]-atrazine to 6th day, (+•) and 20th day, (−•) cucumber cotyledonary leaf thylakoids. [Double reciprocal plots are shown that allow for calculation of the number of binding sites and its dissociation constant. Binding reactions were initiated by vortex mixing 1 ml suspensions of 50 μg Chl/ml buffer with various (50-300 nM) of [¹⁴C]-atrazine dissolved in ethanol. After 1 min incubation the samples were centrifuged and 0.5 ml of supernatants were added to 3 ml of scintillation fluid. Radioactivity of samples was measured by a liquid scintillation counter].

day thylakoids. The increase in the Iₕ₀ value was small but significant (about 13.5%). Since titration of thylakoids with non-radio labelled atrazine in PS II activity vs inhibitor on assay may not differentiate the specific binding of the inhibitor. Experiments were done with radiolabelled [¹⁴C]atrazine in order to determine the alterations in specific binding characteristics on D1 protein and to estimate the changes in the dissociation constant (Kd) of the inhibitor. Following the method of Jursinic and Stemler, we present in Fig. 3, a double reciprocal plot (1/free atrazine vs 1/bound atrazine) for thylakoids isolated from 6th day and 20th day cotyledonary leaves. Reciprocal of intercept on abscissa of the plot indicates the specific binding sites for atrazine on D1 protein, whereas, the slope of the plot relates to herbicide dissociation constant. Specific binding sites in thylakoids isolated from 6th day old cotyledonary leaves and 20th day old cotyledonary leaves were estimated to be 4.8 nmol bound atrazine mg⁻¹ Chl and 2.1 nmol bound atrazine mg⁻¹ Chl respectively which indicates the decrease in the specific binding sites (QB binding sites on D1 protein) in 20th day thylakoids. This is in agreement with the western-blot analysis of D1 protein (Fig. 2). The calculated dissociation constants were 0.16 μM and
0.08 μM for 6th day and 20th day cotyledonary leaf thylakoids respectively. This indicates an increased affinity for atrazine in 20th day thylakoids. The binding affinity of Qb is known to change with the level of reduction of QB. In its semiquinone form QB has higher affinity to the D1 site than the hydroquinone form. Increased affinity for atrazine in 20th day sample can be attributed to a decrease in the semiquinone form of QB in senescing cotyledonary leaf thylakoids. A decrease in the functional antenna size, as inferred from light saturation curve (Fig. 1) and decreased level of QB binding protein might have caused the conversion of some of the PS II functional units (QB reducing units) into the PS II non-functional (QB non reducing centres) units. As stated earlier, QB has lower affinity towards D1 in PS II non-functional units, due to its high redox potential. This could be a cause for increased affinity for atrazine towards D1 protein (see slope in Fig. 3). Although added atrazine concentration in both 6th and 20th day thylakoids remained same (see Materials and Methods) its relative concentration over the semiquinone form of QB, as well as the total number of binding sites, seemed to be more in 20th day old leaf thylakoids when compared to 6th day old thylakoids of cucumber cotyledonary leaves. Further work is necessary to prove the senescence induced alterations in PS II heterogeneity.

In summary our results show that acceptor side of photosystem II is a sensitive indicator of foliar senescence. In Cucumis cotyledonary leaves, senescence induces decrease in functional antenna and QB binding protein of PS II. These alterations affect the herbicide binding site in the acceptor side of PS II. Thus PS II acceptor site among other sites is sensitive to ageing.

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