Lipid Transformations in Greening and Senescing Leaf Tissue

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ABSTRACT

Analyses were made of chlorophyll a and b and fatty acids (18:3, 18:2, 18:1, 18:0, 16:2, 16:1, and 16:0) of greening and senescing leaf tissue. Those dark-grown tissues given a prior treatment of red, far red, or red followed by far red light showed similar increases in chlorophylls and linolenate (18:3) when exposed to continuous white light. In contrast, green barley (Hordeum vulgare L.) leaves placed in the dark lost chlorophylls and fatty acids, especially 18:3. Senescing cocklebur (Xanthium strumarium L.) leaf tissue showed a decline in chlorophyll and fatty acids, especially again 18:3. Abscisic acid, but not sucrose, accelerated these senescent changes. Radioactive acetate incorporation into the galactolipids and phospholipids of senescing cocklebur leaf tissue increased and then the radioactivity of the lipids decreased in senescent tissues.

An effort has been made to study the lipid transformations, especially acyl groups, during two important changes in leaves. These two changes were greening and senescence. Since no one single tissue, in general, lends itself to such a study, two kinds of leaf tissue were selected—barley (Hordeum vulgare L.) and Cocklebur (Xanthium strumarium L.). These two changes are of interest since one is a phase in which photosynthesis is initiated and the other is a phase during which photosynthesis shuts down. To follow changes in the fatty acid composition during greening, barley leaf tissue was used. An effort was made to alter the lag phase in the chlorophyll and fatty acid changes by treating the tissue with red, far red, or red followed by far red light prior to a continuous white light treatment. As opposed to following the greening process, several experiments were conducted to follow lipid changes in the senescent phase of photosynthetic tissues. For example, the changes in pigments and fatty acids of green barley leaf tissue placed in the dark were followed. Placing the barley leaf tissue in the dark causes a simulated senescence effect. To continue the study of senescence, cocklebur leaf discs were floated on an aqueous medium. Cocklebur is an excellent choice since the excised leaf discs senesce rapidly without a concomitant necrosis. We also followed the rate of acetate-14C incorporation into senescing cocklebur leaf lipids such as MGDG2, DGDG, and the phospholipids.

Unser and Mohr (11) have found that phytochrome medi-

1 Supported by National Science Foundation Grant GB-17635.

2 Abbreviations: MGDG: monogalactosyl diglyceride; DGDG: digalactosyl diglyceride; DEGS: diethylene glycol succinate; HMDS: hexamethyldisilazane.

We wondered if a red light treatment might reduce the lag effect in chlorophyll synthesis as well as in the fatty acid changes which are associated with greening. We have found that if greening is prevented red light did not reduce the lag period for the synthesis of linolenate (8). However, no attempt was made to allow chlorophyll synthesis to occur while determining the effect of a prior red light treatment on the subsequent fatty acid synthesis. Akoyunoglou (1) found that there was a pronounced effect of red and far red light treatment on chlorophyll synthesis in old leaves; no effect was observed in young leaves. We therefore allowed the plants to green while studying the effect of a prior red light treatment on the fatty acid changes.

Concerning the senescent phase, Draper (2) found that the final stages of this phase in cucumber cotyledons are marked by a loss of all classes of lipids. We have attempted to make a rough estimate of acetate incorporation and carbon retention in the lipids in senescing cocklebur leaf discs treated with either sucrose or abscisic acid. Khudairi (4) found that leaf discs floated on sucrose showed a more rapid loss of chlorophyll than those floated on water. We tried the same experiment using RNase-free sucrose. We wondered if the sucrose would also hasten changes in the lipids which were characteristic of the senescence phase. The abscisic acid was used to accelerate the senescence process.

MATERIALS AND METHODS

Growth of Plants and Light Treatments. The barley (Hordeum vulgare L. var. Harrison) that was used for the red, far red, or red followed by far red light treatments was grown in the dark for 7 days at 23 C. The dark-grown barley was irradiated for 5 min on each of 4 successive days with red, far red, or red followed by far red light. The plants were placed in a growth chamber and illuminated with red light transmitted from Sylvania F20 T12-R red fluorescent tubes through a one-fourth inch layer of red Plexiglas (light intensity, 530 μW/cm²). Those irradiated with far red light were exposed to incandescent light filtered through a one-fourth inch piece of black Plexiglas (84 μW/cm²). Plants irradiated with red and far red light were given 5 min of each, one immediately after the other. Twenty-four hr following the final light treatment, all plants were placed in continuous white light and samples were taken for lipid and chlorophyll analyses at 0, 6, 24, 57, 78, and 126 hr.

The barley plants used in the dark treatments were grown in continuous white light for 7 days and then placed in the dark. Tissue was harvested after having been in the dark for 0 hr, 67 hr, 7 days, and 10 days. The top 7 cm of leaf tissue was used; no coleoptile material was included in the sample.

As a preliminary study leaf discs of cocklebur (Xanthium strumarium L.) from the second node of 7-week-old cocklebur plants were floated on a phosphate buffered (pH 7.4, 0.2 M)
solution containing 30 \(\mu\)moles of sodium bicarbonate per ml.
The leaf discs were exposed to constant illumination, and samples were taken for chlorophyll analyses after 6, 30, 54, and 102 hr.

There was some question as to whether senescence of cocklebur leaf discs could be accelerated by abscisic acid or sucrose (4). Discs were cut from second node, 5-week-old cocklebur leaves and sterilized with 2% calcium hypochlorite for 5 min, washed with sterile distilled water, and placed on the indicated sterile solution. The sucrose solutions used were RNAase free. The leaf discs were incubated under constant white light illumination at approximately 23 C. Samples were harvested after 0, 2, 4, and 6 days of treatment. Chlorophyll and fatty acid analyses were made on the samples.

Next, cocklebur leaf discs were floated on distilled water containing acetate-2-\(^{14}\)C (10 \(\mu\)c/40 ml; 52.5 mc/m mole) for 1, 2, 4.25, and 6 days under constant illumination. Analyses of the labeling in the neutral lipids, MGDG, DGDG, and the phospholipids were made. Chlorophyll analyses were also made on these samples.

**Lipid Extraction and Chlorophyll Analyses.** The leaf tissue was boiled for 3 min in chloroform-methanol (2:1, v/v). The tissue was homogenized in the chloroform-methanol, filtered, and the filter washed clean with the solvent. Where chlorophyll and lipid analyses were made, 10% of the sample was taken for the chlorophyll analysis, and 90% was saved for the lipid analysis—either fatty acid or whole lipid analysis. For the chlorophyll analyses, the samples were taken to dryness with a rotary evaporator (40 C) and then immediately taken up in 10 ml of acetone, and the flasks were washed with 10 ml of diethyl ether. The chlorophyll extract was then transferred to diethyl ether in a separatory funnel and the ethereal layer washed several times with distilled water. The ethereal layer was dried with anhydrous sodium sulfate. The extract was made to volume and the absorbancies determined at 644 and 663 nm (6).

**Fatty Acid Analyses.** The aliquot for fatty acid analysis was washed through an acidified aqueous layer and dried with anhydrous sodium sulfate. The fatty acids were transsterified with methanolic-HCl (2.5% HCl, w/w) under reflux for 1.5 hr in an atmosphere of \(N_2\) (3). The methyl esters were purified by passing through silicic acid columns. The fatty acid methyl esters were resolved by gas chromatography on a 10 ft column of 10% DEGS on HMDS treated, acid-washed Chromosorb W. Separations were made isothermally at 185 C.

**Column Chromatography of Glycerolipids.** The aliquot used for lipid analysis was similarly washed and dried and was then placed on a 10-g column of UniSil silicic acid (Clarkson Chemical Company, Williamsport, Pa.). Neutral lipids were eluted with 500 ml of chloroform; MGDG was eluted with 100 ml of chloroform-acetone (1:1, v/v); DGDG was eluted with 100 ml of acetone; and the phospholipids were eluted with 100 ml each of chloroform-methanol (9:1, v/v), chloroform-methanol (1:1, v/v), and methanol.

**Liquid Scintillation Counting.** The radioactive glycerolipid samples were dissolved in a mixture of toluene-2,5-bis-[2-(5-tert-butylbenzoxazolyl)]-thiophene (4 g/liter) and counted with a liquid scintillation counter. Quench corrections were made to bring the counting efficiency to 90%.

**RESULTS AND DISCUSSION**

**Red, Far Red Effect on Greening and Fatty Acid Composition.** Dark-grown barley leaf tissue showed a progressive increase in chlorophylls when placed in the light (Table I). Those tissues given a treatment of red, far red, or red followed by far red light showed a similar increase in chlorophylls when exposed to continuous white light.

Dark-grown plants showed a relative increase in 18:3 with increased exposure to continuous white light (Table II). Others have found a similar result (7, 9). The relative amount of 18:2 and 16:0, the other major components, decreased. Oleate (18:1), 18:0, and 16:1 were minor constituents. It is apparent that a prior red light treatment had little effect on the expected changes in relative fatty acid composition during the continuous white light treatment. Perhaps there was a slightly greater relative amount of 18:3 after 6 hr of continuous white light in those plants given a prior red light treatment, but this trend certainly did not continue with increased white light treatments. So that, for example, after 126 hr of continuous white light all plants, regardless of the prior light treatment, contained approximately the same relative concentrations of 18:3, 18:2, and 16:0, the most abundant fatty acids. The data would suggest that the greening process is marked not only by an accumulation of chlorophylls but also by the insertion of double bonds in the fatty acid population of the cell.

The fatty acid composition of the leaves per unit tissue of the plants given the different treatments is given in Table III. Regardless of the prior light treatment the total fatty acid amount per unit weight of tissue increased upon exposure to continuous white light and then tended to decrease with continued exposure to white light, so that the tissue given about 57 to 78 hr of continuous white light contained the greatest total amount of fatty acid. This points up an interesting fact too often disregarded. That is, the total fatty acid accumulation in the tissue is a function of the developmental stage of the tissue. Further, the amounts of the different fatty acids relative to one another are also functions of the developmental stage of the tissue (Table II; see also ref. 5). Again, the prior
red light treatment did not cause any marked changes in the developmental changes in the fatty acid population of the cell as compared to the other prior light treatments. One might conclude from this that the gross changes in the total fatty acid population and in the relative fatty acid population are not phytochrome mediated if the tissue is allowed to synthesize chlorophylls. The rate of fatty acid accumulation did not necessarily follow the rate of chlorophyll synthesis, especially during the later periods of the experiment. During the early stages of greening there was an increase in both the chlorophylls and total fatty acids but the amount of 18:3 in most samples declined later upon further greening (Table III).

A major share of the 18:3 was probably esterified to MGDG and therefore probably reflects the relative change in MGDG. Trémolieres and Lepage (10) found that in greening pea leaves there was an increase in galactolipid and 18:3. An interesting observation is evident from the data in that in all prior treatments except red light, there was a relative decline in 18:3 after 6 hr of continuous white light (Table II). Trémolieres and Lepage (10) made no attempt to identify the influence of red light treatment, nor did they find this relative decline in 18:3 after a short period (6 hr) of illumination.

Pigment and Lipid Changes in Green Barley Leaves in the Dark. Green barley plants placed in the dark showed a progressive loss of chlorophylls with increased time in the dark, so that after 10 days in the dark the leaves contained only a trace of chlorophyll (Table IV). The most changes in the fatty acid composition were in the relative amounts of 16:0 and 18:3 (Table IV). With increased time in the dark there was a relative increase in the amount of 16:0 and a relative
decline in 18:3. This simulated type of senescence apparently causes a rapid change in the 18:3 level (see also ref. 12). Draper (2) found that senescing intact cucumber cotyledons contained a relatively lower percentage of 18:3 in the intact acyl lipids. We also found that with increased senescence there was a concomitant reduction in 18:3, most of which is esterified in MGDG. In terms of 18:3 and 16:0 the changes which occur during senescence are just the opposite to those which occur during the greening phase. Apparently, the catabolic environment of the senescent tissues favors a more rapid degradation and/or decreased synthesis of 18:3 as compared to that of 16:0. There seems to be relatively little change in 18:2 during an exposure to darkness and of course the minor fatty acids did not show significant changes in relative percentages during the dark exposure.
Table V. Fatty Acid Content of Green Barley Leaves Placed in the Dark for the Specified Periods

<table>
<thead>
<tr>
<th>Time in Dark</th>
<th>18:3</th>
<th>18:2</th>
<th>18:1</th>
<th>18:0</th>
<th>16:1</th>
<th>16:0</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>μmoles/1.5 g fresh wt</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 hr</td>
<td>6.75</td>
<td>1.56</td>
<td>0.14</td>
<td>0.21</td>
<td>0.18</td>
<td>0.87</td>
<td>10.25</td>
</tr>
<tr>
<td>67 hr</td>
<td>5.66</td>
<td>1.04</td>
<td>0.09</td>
<td>0.12</td>
<td>0.20</td>
<td>1.25</td>
<td>8.34</td>
</tr>
<tr>
<td>7 days</td>
<td>2.10</td>
<td>0.59</td>
<td>0.12</td>
<td>0.12</td>
<td>0.18</td>
<td>4.13</td>
<td>3.33</td>
</tr>
<tr>
<td>10 days</td>
<td>1.38</td>
<td>0.48</td>
<td>0.11</td>
<td>0.12</td>
<td>0.23</td>
<td>1.32</td>
<td>3.33</td>
</tr>
</tbody>
</table>

Table VI. Chlorophyll Content of Cocklebur Leaf Discs Floated on an Aqueous Solution for the Indicated Periods of Time

<table>
<thead>
<tr>
<th>Time Floated</th>
<th>Chl a/b</th>
<th>Total Chl</th>
</tr>
</thead>
<tbody>
<tr>
<td>hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2.76</td>
<td>1.83</td>
</tr>
<tr>
<td>30</td>
<td>2.62</td>
<td>1.35</td>
</tr>
<tr>
<td>54</td>
<td>2.23</td>
<td>0.98</td>
</tr>
<tr>
<td>102</td>
<td>1.94</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Table VII. Chlorophyll Content of Cocklebur Leaf Discs Floated on the Indicated Solutions for the Indicated Periods of Time

The leaf discs were exposed to continuous white light illumination.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time Floated on Solution</th>
<th>Chl a/b</th>
<th>Total Chl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>2.92</td>
<td>1.65</td>
</tr>
<tr>
<td>Water control</td>
<td>2</td>
<td>2.05</td>
<td>0.70</td>
</tr>
<tr>
<td>Abscisic acid, 10 mg/l</td>
<td>2</td>
<td>1.82</td>
<td>0.43</td>
</tr>
<tr>
<td>Sucrose, 10 mM</td>
<td>2</td>
<td>1.89</td>
<td>0.67</td>
</tr>
<tr>
<td>Water control</td>
<td>4</td>
<td>1.80</td>
<td>0.28</td>
</tr>
<tr>
<td>Abscisic acid, 10 mg/l</td>
<td>4</td>
<td>1.37</td>
<td>0.10</td>
</tr>
<tr>
<td>Sucrose, 10 mM</td>
<td>4</td>
<td>1.36</td>
<td>0.25</td>
</tr>
<tr>
<td>Water control</td>
<td>6</td>
<td>1.32</td>
<td>0.13</td>
</tr>
</tbody>
</table>

The fatty acid composition per unit weight of tissue for plants placed in the dark for varying periods of time is given in Table V. The total fatty acid amount decreased markedly with increased time in the dark; most of this seemed to be a result of a decline in the 18:3 content. The actual amount of 18:2 also declined. There was no change in 18:2 relative to the other fatty acids, but there was an actual change in 18:2 per unit weight. The saturated fatty acids, even though in a catabolic environment, did not seem to show a consistent change in the actual amount during the dark treatment. Again, the senescent phase seems to be characterized by a more chemically reduced state.

Chlorophyll Changes in Senescing Cocklebur Leaf Discs. Discs excised from the leaves of some plants such as cocklebur will senesce when floated on an aqueous medium. As a preliminary trial to identify how rapidly this senescence occurs we floated leaf discs on an aqueous medium. Those floated for 4 days exhibited a progressive decline in chlorophyll content (Table VI). There seemed to be a more rapid reduction of chlorophyll a than of chlorophyll b during the incubation period.

Effects of Abscisic Acid and Sucrose on the Chlorophyll and Fatty Acid Composition of Senescing Cocklebur Leaf Discs. Sucrose and abscisic acid supposedly accelerate senescence, or yellowing, in excised cocklebur leaf discs. We found that this was true for abscisic acid but not true for sucrose (Table VII). Again, there was a decline in the chlorophyll a to chlorophyll b ratio with increased senescence. There was a change in the fatty acid composition of these cocklebur leaf discs with senescence. Those leaf discs floated on water showed a decline in the relative percentage of 18:3, little change in 18:2, and a relative increase in 16:0 with increased senescence. The addition of sucrose to the incubation medium did not accelerate the change in those fatty acids; whereas the addition of abscisic acid slightly accelerated the fatty acid changes associated with senescence.

The data concerning the total quantity of fatty acids per unit leaf material are very revealing (Table IX). With increased incubation time on water, there was a decline in the total fatty acid content, most of which was caused by a decline in the level of 18:3. But here is the interesting point—the sucrose-treated leaf discs retained higher concentrations of the fatty acids than did the water controls; whereas the abscisic acids may have slightly accelerated the decline in fatty acids as compared to that in the water controls. Perhaps the sucrose had a sparing effect on the fatty acids—the provision of an

Table VIII. Relative Percentages of Fatty Acids from Cocklebur Leaf Discs Floated on the Indicated Solutions for the Indicated Periods of Time

The leaf discs were exposed to continuous white light illumination. Maximum variations were about ±4%.

Table IX. Fatty Acid Composition of Cocklebur Leaf Discs Floated on the Indicated Solutions for the Indicated Periods of Time

The leaf discs were exposed to continuous white light illumination.
Alternate substrate for oxidation. As is the case with dark-treated leaves, senescing cocklebur leaf discs showed a more rapid decline in the actual amount of 18:3 than in that of 16:0. It appears that most green leaves which senesce and yellow exhibit this relative change in the fatty acid composition as well as a total decline in the fatty acids, most of which is a decline in the amount of 18:3. The minor fatty acids remained about the same and are not affected by the yellowing process.

**Acetate Incorporation into Lipids of Senescing Cocklebur Leaf Discs.** We thought that we would try to get a rough estimate of acetate incorporation into the various lipids in senescing tissues as well as carbon retention therein. Again, we used cocklebur leaf discs but this time we floated the discs on labeled acetate-2^14C. Following incubation with the labeled acetate, the various glycerolipid classes were separated and the radioactivity counted. Even though there was a progressive yellowing of the tissue, the neutral lipid fraction containing the pigments became increasingly more labeled with time of incubation (Table X). The labeling in the polar lipid fractions (MGDG, DGDG, and phospholipids) increased and then decreased with time of incubation, indicating a comparatively rapid turnover. This probably reflects a decreased rate of synthesis with a normal or above normal rate of degradation of these lipids in the senescent tissues.

The greening and senescence phases seem to exhibit opposite changes in the pigment and lipid compositions. The greening phase is characterized not only by increased chlorophyll accumulation but also by an increase in lipids as well as a change in the saturation level of the acyl moieties of these lipids. That is, the fatty acids become more unsaturated; especially, there is a large increase in 18:3. This increase in 18:3 is not phytochrome-mediated. In contrast, senescent tissues exhibit a decrease in the lipids and a trend toward more saturated acyl groups due to a decline in 18:3.

**LITERATURE CITED**