Membrane Lipids in Senescing Flower Tissue of Ipomoea tricolor

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ABSTRACT

Rib segments excised from flower buds of Ipomoea tricolor Cav. pass through the same phases of senescence as the respective tissue on the intact plant. Such segments were used to correlate changes in lipid content with known symptoms of aging, such as rolling up of the ribs and ethylene formation. It was found that the level of phospholipid had already started to decline before visible signs of senescence were evident. As the segments began to roll up and to produce ethylene, the rate of phospholipid loss accelerated sharply. During the same period, the level of fatty acids esterified to phospholipids also fell by 40%. No qualitative changes in any lipid component could be detected during senescence. Labeling experiments using 32P as marker showed that the rate at which radioactivity was lost from phospholipids during aging was parallel to the rate at which the level of total phospholipids declined. Exogenously applied ethylene accelerated the loss of phospholipid and the senescence of rib segments while benzyladenine retarded both of these processes.

Ag⁺, which counteracts the effect of ethylene in many plants, inhibited rolling up of the rib segments but did not affect either spontaneous and ethylene-induced ethylene generation, or phospholipid loss. In contrast, Co⁺, a purported inhibitor of ethylene synthesis, reduced ethylene production, rolling up, and phospholipid loss. The inhibition of ethylene-induced rolling up by Co⁺ could not be overcome with exogenous ethylene, however.

Our results indicate that phospholipid loss is a marker for membrane degradation in rib segments. Changes in membrane integrity and in cellular compartmentation may be the basis for ethylene synthesis during aging of flower tissue.

Flowers of the morning glory Ipomoea tricolor Cav. produce ethylene during aging, and their senescence can be advanced by treatment with exogenous ethylene (8). Treating the flowers for 60 min with ethylene induces premature ethylene biosynthesis (8), a phenomenon which has been observed previously with other flower and fruit tissue and which has often been referred to as "autocatalysis." We suggested that the burst in ethylene production, either during natural aging or following ethylene treatment, was due to loss of cellular compartmentation which caused intermixing of previously sequestered components of the ethylene-generating system (8, 9). In a first attempt to test this hypothesis, we investigated cellular compartmentation during aging of segments excised from the ribs of the morning glory corolla (5). Such rib segments exhibit the same symptoms of senescence as the intact tissue. When aging and ethylene production commence, they start to roll up, just as the ribs of the intact corolla, and this process can be quantitated to assess the progress of senescence (5). The use of segments also permits measuring the release of cellular components into the bathing solution. Employing the technique of compartmental analysis with 35Cl and 86Rb (12, 13) as markers and measuring the rates of efflux of sucrose and organic acids, we were able to demonstrate an abrupt change in cellular compartmentation during natural and ethylene-induced aging (5).

Since a change in compartmentation most likely involves an alteration of membrane properties, we initiated a study on the composition of membranes of juvenile and senescing flower tissue. In addition, we also investigated the effects of cobalt and silver ions on phospholipid metabolism and senescence, since both of these metal ions have been reported to affect ethylene-induced processes (1, 10).

MATERIALS AND METHODS

Plant Material. Seeds of I. tricolor Cav. (cv. Heavenly Blue) were purchased from Agway Inc. (Syracuse, N.Y.), and plants were grown in an environmental chamber as described previously (5). Rib tissue of the corolla was prepared according to Kende and Hanson (9), except that in most experiments two 1-cm pieces were cut from each rib of the corolla. This permitted a more economic utilization of plant material and shortened the time needed to cut rib segments. The aging response of rib segments originating from the edge and from the inner part of the corolla was the same.

The following designations are used to describe the age of the flower tissue: day 0, day of opening and fading of the flower; day −1, 1 day before opening of the flower.

Incubation of Rib Segments. Usually, the rib segments were excised from flower buds between 3:00 and 6:00 PM on day −1. The segments were placed in Petri dishes containing three layers of Whatman No. 1 filter paper soaked with 5 mM KCl solution. The dishes were kept overnight in the same growth chamber in which the plants were grown. Between 7:30 and 8:30 AM, the segments were blotted on filter paper and transferred into 25-ml Erlenmeyer flasks, containing 1 ml 1% (w/v) agar in 5 mM KCl. Usually, 10 rib segments were used per flask. The flasks were sealed with serum vial caps, flushed for 2 min with ethylene-free air, and placed in a darkroom at 27 C.

Tissue treated this way passes through the same developmental stages as the respective tissue on the intact flower. On day −1, the segments are purple and slightly twisted, following the original shape of the bud. At one of the two longitudinal sides of the segment, the corolla tissue is folded over the rib. During overnight incubation the segments turn blue, unfold, and flatten. Around noon, the typical symptoms of senescence become evident; the rib segments become purple, they roll up, and begin to produce ethylene at a sharply increased rate. Treatment of the segments with various chemicals was usually carried out during the overnight incubation. The respective substances were

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dissolved in 5 mM KCl which was used to wet the filter paper. Only NaNO₃ was applied in water.

For exogenous ethylene treatment, the appropriate amount of 0.1% (v/v) ethylene in air was injected into sealed Erlenmeyer flasks through serum vial caps.

Measurement of Rolling Up of Rib Segments. Angular measurements were taken during the rolling up process by aligning the sides of the segments in the flask alongside a protractor (5). When the segments were flat and unrolled, the angle was scored as 180°; as rolling up occurred, the angle increased to >360° with the segment ends overlapping.

Ethylene Determination. Ethylene was measured by gas chromatography as described earlier (9).

Determination of Total Phospholipid. Ten rib segments were ground in a 2-mL tissue grinder in 1 mL of boiling isopropyl alcohol. The extract was allowed to cool down, and 1 mL methanol and 4 mL of chloroform were added to it. This mixture was shaken for 90 min in the cold room at 4°C. After filtration and washing of the residue with a mixture of chloroform-methanol (2:1), the combined organic phase was extracted with 0.2 volumes of an aqueous 0.73% (w/v) NaCl solution and dried over anhydrous Na₂SO₄ (4). Aliquots were evaporated to dryness and subjected to phosphorus determination according to Rouser et al. (14).

Chromatography of Lipids. For extraction of total lipids, rib segments (usually 50) were ground in 5 mL boiling isopropyl alcohol; to this homogenate 5 mL methanol and 20 mL chloroform were added, and the mixture was shaken for 90 min at 4°C. After filtration and extraction with 0.2 volumes of 0.73% (w/v) NaCl solution, the organic phase was taken to dryness, redissolved in 3 mL chloroform, and applied to a silicic acid column. The column was prepared according to Rouser et al. (15) with minor modifications by suspending 1 g of silicic acid in 5 mL chloroform and pouring the slurry into a glass column of 1-cm diameter. After settling, the silicic acid was washed with 10 mL of chloroform before application of the sample. Fraction 1, containing mainly mono- and diacylglycerides, sterols and free fatty acids, was obtained by eluting the column with 13 mL of chloroform. Fraction 2, eluted with 13 mL acetone, consisted mainly of the glycolipids. A final wash with 13 mL of methanol (fraction 3) eluted the phospholipids. Fraction 3 was concentrated in vacuo to 1 mL, of which 50 to 100-μL aliquots were used for TLC analysis on silica gel plates (Brinkmann, precoated plastic sheets, Sil G, with a thickness of 0.2 mm). The plates were developed in two dimensions with chloroform-methanol-concentrated NH₄OH (18:14:3, v/v) in the first direction, and chloroform-acetone-methanol-acetic acid-water (6:8:2:2.1; v/v) in the second (14). The phospholipids were identified by co-chromatography with authentic standards and by group-specific reagents such as molybdenum, ninhydrin, Dragendorf and periodate-Schiff reagents (17). For quantitative determination, the phospholipid spots were localized with iodine vapor, scraped from the plate, and analyzed for phosphorus by the method of Rouser et al. (14).

Fatty Acid Analysis. Free fatty acids were extracted from the first column fraction with 1% Na₂CO₃ solution, following the method of Draper (2). Methyl esters of free and previously esterified fatty acids were produced according to Luddy et al. (11), using a methanolic base reagent, which was prepared by dissolving 1.45 g metallic potassium in 100 mL anhydrous methanol. Lipid samples, usually equivalents of 25 to 50 rib segments, were dissolved in 50 μL base reagent and 50 μL benzene and kept at 80°C for 20 min. Thereafter, 150 μL water and 150 μL diethyl ether were added. After thorough mixing, the phases were allowed to separate; the lower aqueous phase was removed, and the ether phase was washed with 100 μL water and dried over Na₂SO₄. Aliquots of 1 μL were injected into a Perkin-Elmer gas chromatograph model F 11, equipped with a column (½ inch × 6 ft) (10% DEGS-PS on 80–100 mesh Supelcoport purchased from Supelco, Bellefonte, PA). The chromatographic analyses were performed isothermically at 195°C.

Measurement of Radioactivity. The radioactivity of 32P was determined with a Packard Tri-Carb scintillation spectrometer, using the following scintillant: 3 liters toluene, 12 g PPO, 0.3 g dimethyl-POPPOP (Research Products International Corp., Elk Grove Village, Ill.), and 300 mL Bio-Solv-BBS-3 (Beckman Instruments, Fullerton, Calif.). The counting efficiency for 32P was 86%. Aliquots of lipid solutions were dried down in scintillation vials and redissolved in scintillation fluid. Radioactivity in chromatogram spots was determined by scraping the silica gel from the plate and suspending it in scintillation fluid.

RESULTS

Phospholipid Levels during Normal Aging. Rib segments were cut on day −1, incubated overnight on 5 mM KCl, and transferred on day 0 into Erlenmeyer flasks containing agar. The phospholipid level of these rib segments declined slowly and steadily from day −1 until about noon of day 0 (Fig. 1). As rolling up of the rib segments and ethylene formation commenced, the rate of phospholipid loss increased, reaching at 5 PM a rate which was about six times higher than the rate during the previous night. In parallel experiments, segments excised on day −1 were incubated overnight on 5 mM KCl solution containing KH₂₃PO₄ (2 μCi/ml). In order to chase the radioactive phosphate, the ribs were floated on the morning of day 0 for 2 hr on 20 mM K-phosphate buffer (pH 6) before being placed on agar. The analysis of lipid phosphorus and of radioactivity indicated that total phospholipids and radioactivity in phospholipids declined in parallel (Fig. 2).

Phospholipid Ratios. In order to investigate whether or not all phospholipid species were lost at the same rate, phospholipids were analyzed by two-dimensional TLC. The analysis of tissue prior to senescence (day 0, 9:00 AM) and at an advanced stage of senescence (day 0, 9:00 AM) showed no striking change in the ratio of the major components PC, PE, and PI. The minor components, PA and PG, were present in such low concentrations that no changes could be detected with adequate accuracy using our methods. In tissue which was treated with 32P and chased as described above, we found that PC consistently retained relatively more radioactivity during aging than all other phospholipids (Fig. 3).

Analysis of Fatty Acids. The esterified fatty acids were converted to methyl esters from fraction 3 of the silicic acid column and were analyzed by gas chromatography. During senescence there was a 40% decrease in the level of fatty acids esterified to phospholipids which corresponded to the loss of phospholipids during the same period; no significant changes were found in the ratio of the fatty acid components (Table I). The level and composition of the free fatty acids which were extracted from fraction 1 of the silicic acid column remained unchanged during senescence.

Treatment with Exogenous Ethylene. Rib segments, cut on day −1 and incubated overnight on 5 mM KCl, were treated in the morning of day 0 with 10 μM/ethylene for 90 min. After this, the Erlenmeyer flasks were opened, flushed thoroughly with ethylene-free air, and recapped. The treatment with ethylene accelerated rolling up and endogenous ethylene production as described earlier (9). The rate of phospholipid loss was also significantly increased, but the effect of the gas on this process was less pronounced than that on rolling up and endogenous ethylene production (Fig. 4).

Abbreviations: PA: phosphatidic acid; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PI: phosphatidylinositol.
As transferred in the ethylene production the caused phosphorus lipid ids. The buffer segments rib production (Fig. 5). Three -1, incubated (0-0.4 m6 co 0-I-)

Incubation on Benzyladenine. Rib segments cut on day -1 were incubated on 5 μM BA in 5 mM KCl overnight and transferred in the morning of day 0 to agar in Erlenmeyer flasks. As observed earlier (9), treatment of the tissue with the cytokinin caused a delay in both rolling up and ethylene production (Fig. 5). Treatment with BA also reduced the loss of phospholipids. The effect of BA on phospholipid loss was already evident in the morning of day 0, when rolling up and the rise in ethylene production had not yet started. Similar results were obtained in three experiments.

Incubation on AgNO₃. Overnight incubation on 10 μM AgNO₃ solution prevented rolling up of the rib segments almost completely. Even incubation on 5 μM AgNO₃ inhibited rolling up by 80% (Fig. 6). However, phospholipid loss and ethylene production of silver-treated and control samples were almost identical (Fig. 6), and there was no difference in the time course of color change from blue to purple (data not shown). In the morning of day 0, silver-treated rib segments were always rolled slightly backward, i.e. in the opposite direction of the normal rolling up.

In experiments where rib segments were incubated on AgNO₃, it was interesting to note that Ag⁺ inhibited rolling up without affecting ethylene biogenesis (Fig. 6). For this reason, we investigated the effect of silver ions on another ethylene response, namely ethylene-induced ethylene synthesis. As in previous experiments, rolling up was severely inhibited in

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Table 1. Fatty Acid Composition of Phospholipids in Rib Segments prior to Senescence and at an Advanced Stage of Senescence

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>% of major esterified fatty acid in phospholipids¹</th>
<th>day -1, 6:00 PM</th>
<th>day 0, 9:00 PM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic acid (16:0)</td>
<td>30.3</td>
<td>26.7</td>
<td></td>
</tr>
<tr>
<td>Stearic acid (18:0)</td>
<td>2.9</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>Oleic acid (18:1)</td>
<td>1.5</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>Linoleic acid (18:2)</td>
<td>28.6</td>
<td>28.3</td>
<td></td>
</tr>
<tr>
<td>Linolenic acid (18:3)</td>
<td>36.7</td>
<td>38.9</td>
<td></td>
</tr>
</tbody>
</table>

¹Average values of three experiments which gave very similar results.
AgNO₃-treated segments, and exogenously applied ethylene did not overcome this inhibition. Neither the rate nor the time course of ethylene-induced ethylene formation was affected by preincubation on AgNO₃ (Fig. 7).

**Incubation on CoCl₂.** Overnight incubation of rib segments on 500 μM CoCl₂ reduced the total amount of ethylene produced during day 0 and also rolling up of segments by over 80% (Fig. 8). As observed in earlier experiments with AgNO₃, the rib segments showed first "reversed rolling" (α=180°) which turned to values above 180° during the day. The rate of phospholipid loss was markedly reduced by Co²⁺, but a significant difference.
loss is a consequence of other ethylene-controlled catabolic processes which affect membrane integrity. Rib segments are composed of several cell types, and the earliest ethylene response appears to be confined to relatively few cells in the two ridges of each rib. The rolling up of the ribs is driven by those two ridges, at least in part through loss of turgor on the adaxial side and possibly also through turgor-mediated growth on the abaxial side. It is doubtful that we could have observed phospholipid loss in a small fraction of the total population of rib cells during the earliest response to applied ethylene. In the course of natural aging, as opposed to premature ethylene-induced aging, there may be a far greater synchrony in the metabolic state of all cells of the rib tissue which may explain the close temporal coincidence of phospholipid loss and other symptoms of flower aging.

The level of free fatty acids, glycolipids, and steroids remained unchanged during aging of the rib segments (unpublished data).

In quantitative terms, our results are in agreement with those of other investigators (2, 3, 18) who also found decreasing levels of membrane lipids in senescing tissue. However, in contrast to these authors, we did not find significant qualitative changes in lipid composition during aging of flower tissue. This is most likely because earlier experiments (2, 3, 18) were performed with yellowing leaf tissue where degradation of chloroplast membranes with distinct lipid composition predominated during the early phases of senescence. The effect of ethylene on lipid metabolism was investigated by Irvine and Osborne (7) who found a decreased incorporation of 14C-glycerol into neutral lipids and phospholipids of pea stem sections following ethylene treatment. They concluded that ethylene inhibited biosynthesis of all lipids which contained glycerol. Since possible changes in the pool sizes of glycerol were not taken into account, these experiments cannot be interpreted unequivocally.

The results of our experiments with AgNO3 were surprising inasmuch as silver ions inhibited one ethylene response in rib segments (rolling up) while not affecting others (ethylene synthesis and phospholipid loss). Beyer (1), who first showed that silver ions abolished the effect of ethylene in a number of plant systems, postulated that Ag+ interfered with the action of ethylene at its receptor site. If this were true, one would have to assume the existence of at least two sites of ethylene action: in flower tissue of Ipomoea, one of which is Ag+-sensitive and one which is not. Alternatively, Ag+ may inhibit rolling up of rib segments at a step subsequent to the initial interaction of ethylene with its binding site.

Lau and Yang (10) found that Co2+ inhibited the production of ethylene in mung bean hypocotyls and in apple tissue, presumably by blocking the conversion of methionine to ethylene. Our experiments also showed an inhibition by Co2+ of ethylene synthesis and of other symptoms of aging. However, exogenously applied ethylene did not overcome the Co2+-mediated inhibition of rolling up. In view of this, Co2+ cannot be regarded as a specific inhibitor of ethylene biosynthesis in flower tissue of Ipomoea, even though methionine serves as precursor of ethylene in this system as well (6).

Our experiments were undertaken to elucidate further the chain of events which leads to senescence of the morning glory flower. According to our hypothesis, the sharp increase in ethylene generation is not the event that triggers senescence, but is the consequence of earlier metabolic reactions, probably degradative in nature, which lead to increased membrane permeability. The resultant loss in cellular compartmentation (5) is thought to cause mixing of previously sequestered components
of the ethylene-generating system, thereby stimulating ethylene evolution. Once formed, ethylene enhances its own synthesis and accelerates aging by further increasing membrane permeability. Support for the view that ethylene generation is a fairly late event in the senescence process comes from two lines of evidence presented in this communication. (a) Loss of phospholipids consistently preceded ethylene evolution and was already detectable during the night between day -1 and day 0. (b) BA, which delays senescence of rib segments when given during the night between day -1 and day 0, retarded the loss of phospholipid, an effect which was already evident early in the morning of day 0. We suggest that phospholipid loss is a marker for degradative processes which affect membrane integrity and which ultimately lead to a breakdown of cellular compartmentation, a sharp increase in ethylene synthesis, and senescence of the flower tissue. While ethylene undoubtedly increases membrane permeability (5), it is still an open question whether it does so by directly affecting phospholipid metabolism.

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