Identification of Plant Hormones from Cotton Ovules

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ABSTRACT

An extract from 8-day-old cotton ovules (Gossypium hirsutum L.) was partitioned into three fractions and each fraction was derivatized and analyzed separately. Gas-liquid chromatography and computer-controlled gas-liquid chromatography-mass spectrometry were used to separate, measure, and identify the naturally occurring plant hormones. A single extract contained abscisic acid, indoleacetic acid, and gibberellins A1, A2, A3, A5, and A6 in the first fraction; ethyl indole-3-acetate and indole-3-aldehyde in the second fraction; and the cytokinins 6-(3-methyl-4-hydroxybutylamino)purine (dihydrozeatin), 6-(4-hydroxy-3-methyl-2-trans-butenylamino)purine (zeatin), 6-(3-methyl-2-butenylamino)purine (2iP), 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (2iPA), and 6-(4-hydroxy-3-methyl-2-trans-butenylamino)-9-β-D-ribofuranosylpurine (zeatin riboside) in the third fraction.

Many research workers have attempted to identify and measure hormone levels in cotton plants by using various bioassays (1, 6, 7, 9, 24, 25, 28). Because plant hormones are present in microgram quantities, the usefulness of bioassays has been limited because of interference of excessive quantities of impurities in extracts containing the hormones (2, 14).

Recently, development of GLC has provided a physical-chemical assay of ABA (11, 19), auxins (13, 29), cytokinins (30), and gibberellins (8), demonstrating the feasibility of qualitative as well as quantitative measurements of naturally occurring plant hormones. Gas-liquid chromatography-mass spectrometry has been used for further identification of some of these hormones (15, 21, 22, 31).

Computer-controlled GLC-MS has been used for identification of cytokinins (31), gibberellins, and ABA (21). Abscisic acid (11) and IAA (32) have been identified from cotton tissues by GLC but we have found no report in which ABA, gibberellins, cytokinins, and auxins were measured in a single extract by physical-chemical assays.

The objective of this investigation was to utilize GLC and computer-controlled GLC-MS to separate, identify, and measure individual plant hormones of four major groups from a single extract of ovules from 8-day-old cotton fruit.

MATERIALS AND METHODS

Cotton plants (Gossypium hirsutum L., cv. Acala SJ-1) were grown in a greenhouse. Cotton flowers were tagged at anthesis and bolls were harvested 8 days thereafter. The developing ovules were separated from the fruits, frozen immediately on Dry Ice, and stored at −20°C until the time of extraction.

Extraction Procedure. In different experiments, 2 to 5 g of frozen ovules were ground in cold 80% (v/v) aqueous methanol with a mortar and pestle. The macerate was transferred to a flask with fresh methanol and the volume was adjusted to 20 ml of methanol for each g fresh weight of ovules. The tissue was allowed to extract for 24 hr at 0°C and then was vacuum filtered through Whatman No. 42 paper. The filter paper and the residue were returned to the flask with a fresh volume of methanol, shaken 30 min on a wrist-action shaker, and filtered again. The procedure was repeated once more and the combined extracts were evaporated to the aqueous phase in a rotary flash evaporator. The aqueous phase (10 to 30 ml) was adjusted to pH 8.6 with 1% NaOH and partitioned three times with equal volumes of ethyl acetate. The combined ethyl acetate fraction was evaporated to dryness and held for further purification. The aqueous phase was adjusted to pH 2.8 with 1% HCl and partitioned three times with equal volumes of ethyl acetate. The remaining aqueous phase was discarded. The combined acidic ethyl acetate phase was reduced in volume (Fraction I) to be used for GLC determination of acidic hormones such as IAA, ABA, and GAs.

The dried basic ethyl acetate fraction was dissolved in 80% methanol. The methanol was evaporated under vacuum, leaving an aqueous phase which was adjusted to pH 2.8 with 1% HCl and partitioned three times with 25 to 50 ml of ethyl acetate. The ethyl acetate phases were combined (Fraction II), reduced to 5-ml volume, and stored at −20°C until GLC analysis for neutral auxins. The remaining aqueous phase was adjusted to pH 5.5 with 1% NaOH and partitioned three times with 50 to 100 ml of water-saturated l-butanol. All butanol phases were combined (Fraction III), reduced to 5-ml volume, and stored at −20°C until GLC analysis for cytokinins (Fig. 1).

Silylation. Aliquots (0.5 to 1 ml) from each fraction or 1-mg samples of each standard compound were placed in 1-ml
reacti-vials, dried under nitrogen, and then dissolved in 100 µl of N, O-bis(trimethylsilyl)acetamide (BSA) (17) to prepare trimethylsilyl derivatives. The vials were immediately capped and heated over a hot plate (50 C) for 30 min before GLC analysis.

Standards used in GLC analysis were obtained as follows: ABA was provided by Hoffman-LaRoche Inc. Indole-3-acetic acid, ethyl indole-3-acetate (IAEt), and indole-3-aldehyde (IAld) were from Calbiochem. Gibberellins A1, A2, A3, A5, and A7 were from Abbott Laboratories and A6, A7, A8, A9, and A10 were from Imperial Chemical Industries, Ltd, England. Samples of 6-(4-hydroxy-3-methyl-2-trans-butenylamino)purine (zeatin), 6-(3-methyl-4-hydroxybutylamino)purine (dihydrotezeatin), and 6-(4-hydroxy-3-methyl-2-trans-butenylamino)-9-β-D-ribofuranosylpurine (zeatin riboside) were from Sigma Chemical Company. The 6-(3-methyl-2-butenylamino)purine (2iP), 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (3iPA), and 6-(3-methyl-3-butenylamino)-9-β-D-ribofuranosylpurine (3iPA) were obtained from Dr. R. Hall, McMaster University.

Gas-Liquid Chromatography. One to 5 µl of each TMS derivative was injected into a Varian Aerograph GLC (Model 1200) equipped with a flame ionization detector, a temperature programmer, and a stainless steel column (3 mm x 150 cm) packed with 3% QF-1 on 60/80 mesh Chrom-Q. The GLC was operated under the following conditions: hydrogen flow, 30 ml/min; air flow, 200 ml/min; nitrogen carrier gas flow, 30 ml/min; injector temperature, 200 C; and detector temperature, 275 C. Temperature programming started at 100 C and increased linearly to 250 C at the rate of 4 C/min. Retention time and temperature for each peak were recorded and compared to those of TMS derivatives of authentic standards. Co-chromatography of unknowns and standards was also done to facilitate identification.

Computer-controlled GLC-MS. Analyses of TMS derivatives of authentic standards or extract fractions were carried out with a Systems 150 output control module on a Finnigan mass spectrometer (Model 1015C) interfaced to a Varian Aerograph GLC (Model 1400) with a Goekle all-glass separator. The mass spectra were taken at 70 ev and 300 µamp ionizer current. The mass spectrometer scanned at 5-sec intervals under the control of the computer, and all data were recorded on magnetic tape and printed out on a teletype.

Radioactivity Studies. The following radioactive hormones were used for "spiking" the plant tissue: ABA-2-^14C, specific radioactivity 26.3 mCi/mmol (26); kinetin-8-^14C, specific radioactivity 20 µCi/mmol (Calbiochem); gibberelic acid-^14C, specific radioactivity 5.29 µCi/mmol (gift from Dr. C. W. Coggins, Jr.); and indole-3-(acetic acid-1-^14C), specific radioactivity 183 µCi/mmol (gift from Dr. L. N. Lewis).

In separate experiments, radioactive solutions (5-100 µl) of each hormone were added to 1 to 2.5 g of tissue during grinding. The separation procedure (Fig. 1) was then followed. An aliquot (0.5 to 1 ml) out of every fraction and the discarded tissue were transferred to vials containing approximately 14 ml of scintillation fluid (18.9 g of PPO and 378 g of naphthalene in 3.78 l of dioxane). A Beckman LS 100 scintillation counter was used and all counts were corrected for efficiency, background, and quenching.

RESULTS AND DISCUSSION

The effectiveness of the separation scheme presented in Figure 1 was tested by fortifying extracts at the time of the initial grinding with either ^4C-IAA, ^4C-ABA, ^4C-GA, or ^4C-kinetin. The IAA, GA, and ABA were expected to appear in the acidic ethyl acetate fraction (Fraction I) and the kinetin in the neutral l-butanol fraction (Fraction III). We realize that kinetin is not a naturally occurring hormone; however, its similarity in structure provided information on the usefulness of the separation procedure for cytokinin-type compounds. We did not include a labeled representative of the compounds expected to appear in Fraction II.

As shown in Table I, approximately 80% each of the labeled IAA, ABA, and GA, were recovered in Fraction I and 70% of the labeled kinetin was recovered in Fraction III. Although the recovery of kinetin was lower than we desired, it was repeatable in three separate experiments. The unrecovered 30% radioactivity was randomly lost during the separation. The actual detection of cytokinins (Table II) in Fraction III supports the feasibility of this system. It is quite possible that some other hormones may have been lost in the two aqueous fractions which were not analyzed by GLC. Therefore, the present study deals only with those hormones extracted with ethyl acetate and with l-butanol.

Gas-Liquid Chromatography. Our studies show that TMS derivatives of authentic standards as well as endogenous hormones can be separated from each other (Table II). Retention times of individual hormones changed slightly from day to day but the elution order did not change. In preliminary

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Table I. Recovery of ^14C-IAA, 2-^14C-ABA, 4^14C-kinetin, and 14C-GA from Extracts of 8-Day-Old Cotton Ovules

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Added</th>
<th>Recovered</th>
<th>Percentage recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>^14C-IAA</td>
<td>1.24 X 104</td>
<td>9.64 X 104</td>
<td>77.0</td>
</tr>
<tr>
<td>2-^14C-ABA</td>
<td>7.15 X 104</td>
<td>5.97 X 104</td>
<td>83.5</td>
</tr>
<tr>
<td>4^14C-kinetin</td>
<td>5.71 X 104</td>
<td>4.11 X 104</td>
<td>70.2</td>
</tr>
<tr>
<td>14C-GA</td>
<td>2.23 X 104</td>
<td>1.77 X 104</td>
<td>80.0</td>
</tr>
</tbody>
</table>

1 Percentage of calculated total counts/min originally added.

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experiments, we tested various percentages of column-coating materials, including SE-30, OV-17, and QF-1. Among these, 3% QF-1 was selected as the most satisfactory for separating individual hormones, especially the cytokinins. Column lengths from 75 to 300 cm were compared, with the best separation and sharpest peaks being obtained from a 150-cm column. The 4 C/min linear temperature program was selected because the GA<sub>1</sub> and GA<sub>3</sub> peaks and the GA<sub>1</sub> and GA<sub>3</sub> peaks did not completely mask each other.

Preparation of TMS derivatives by BSA was selected after

Table II. Relative Retention Times and Amounts of TMS Derivatives of Plant Hormones Detected in Extracts of 8-Day-Old Cotton Ovules

<table>
<thead>
<tr>
<th>Plant Hormone</th>
<th>Relative Retention Time&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Amount Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>ng/g</td>
</tr>
<tr>
<td>Fraction I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IAA&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.55</td>
<td>9.50</td>
</tr>
<tr>
<td>ABA&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.91</td>
<td>2.62</td>
</tr>
<tr>
<td>GA&lt;sub&gt;13&lt;/sub&gt;</td>
<td>0.99</td>
<td>554.60</td>
</tr>
<tr>
<td>GA&lt;sub&gt;5&lt;/sub&gt;</td>
<td>1.00</td>
<td>54.20</td>
</tr>
<tr>
<td>GA&lt;sub&gt;1&lt;/sub&gt;</td>
<td>1.11</td>
<td>9.15&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>GA&lt;sub&gt;3&lt;/sub&gt;</td>
<td>1.14</td>
<td></td>
</tr>
<tr>
<td>GA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1.19</td>
<td>2.70&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>GA&lt;sub&gt;5&lt;/sub&gt;</td>
<td>1.21</td>
<td></td>
</tr>
<tr>
<td>Fraction II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IA&lt;sub&gt;1&lt;/sub&gt;&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.88</td>
<td>65.10</td>
</tr>
<tr>
<td>IA&lt;sub&gt;1d&lt;/sub&gt;</td>
<td>1.00</td>
<td>26.62</td>
</tr>
<tr>
<td>Fraction III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2iPA&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.89</td>
<td>99.30</td>
</tr>
<tr>
<td>Dihydrozeatin&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.93</td>
<td>13.47</td>
</tr>
<tr>
<td>Zeatin&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.00</td>
<td>12.19</td>
</tr>
<tr>
<td>2iPA</td>
<td>1.07</td>
<td>316.90</td>
</tr>
<tr>
<td>Zeatin riboside&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.18</td>
<td>13.40</td>
</tr>
</tbody>
</table>

<sup>1</sup> Retention time of GA<sub>3</sub>, zeatin, and IA<sub>1d</sub> = 1.
<sup>2</sup> Identity authenticated on computer-controlled GLC-MS.
<sup>3</sup> Value for GA<sub>4</sub> + GA<sub>5</sub>.
<sup>4</sup> Value for GA<sub>1</sub> + GA<sub>3</sub>.

preliminary trials with TRI-SIL, TRI-SIL'/Z', TRI-SIL/BSA, BSTFA, and BSA. The BSA process was fast and direct and only single peaks were formed from the authentic hormone standards.

The computerized GLC-MS system was used to verify identification of some isolated hormones. Our system was particularly desirable because the two gas chromatographs were compatible, allowing the same column to be moved from one instrument to the other. The fragmentation patterns of the TMS derivatives of authentic hormones were compared to those of the extracts. Even though the retention times for GA<sub>1</sub> and GA<sub>3</sub> and for GA<sub>1</sub> and GA<sub>3</sub> were very close, the continuous scan capability of the system allowed successful identification of each gibberellin. Also, there was no problem in separating ABA from IAA or the gibberellins in the acidic ethyl acetate fraction. Separation of these hormones from a single extract is important since they tend to be antagonistic when applied together in a number of bioassay systems.

PLANT HORMONES IN FRACTION I

Abscisic Acid. Abscisic acid was detected by both GLC and GLC-MS in Fraction I. All of the most significant ions of TMS-ABA, including those at m/e 190 (base peak), 183, 170, 162, 147, 134, 106, 91, 83, 75 and 73, were readily apparent in the spectra. This result is in agreement with previous reports in which either whole or dissected cotton fruit were extracted. Davis and Addicott (10) examined ABA levels in various parts of the fruit and showed that ABA could be detected in seeds and lint and in fruit walls. They showed higher levels in abscising than in nonabscising fruit. Even though ABA was detected in fruits, we observed that fruit abscission was minimal because of the controlled environmental conditions and continuous removal of flowers. We did not attempt to detect or identify other substances which have been isolated from cotton fruits such as phaseic acid (12), bound (hydrolyzable) ABA, phthalic acid, and β-bisabolol (20).

Indoleacetic Acid. Various bioassays and chemical methods have been used to tentatively identify IAA in cotton extracts (1, 3, 9). Our results (Fig. 2; Table II) and those of Weeks and Lane (32) confirmed the presence of IAA in cotton with GLC.

![Fig. 2. Gas-liquid chromatograms of Fractions I, II, and III from cotton ovules extracted as shown in Figure 1. Identified peaks are: (a) IAA, (b) ABA, (c) GA<sub>13</sub>, (d) GA<sub>5</sub>, (e) GA<sub>4</sub> and GA<sub>5</sub>, (f) GA<sub>1</sub> and GA<sub>3</sub>, (g) IA<sub>1</sub>d, (h) IA<sub>1d</sub>, (i) 2iP, (j) Dihydrozeatin, (k) Zeatin, (l) 2iPA, and (m) Zeatin riboside.](image-url)
PLANT HORMONES IN COTTON FRACTION II

This fraction was examined for indole auxins other than IAA. Our data (Fig. 2; Table II) indicated that two neutral auxins, IAEt and IAlD, are present in cotton ovules.

The presence of IAEt and IAlD in cotton extracts has been reported before (1, 32). Addicott et al. (1) found IAEt, IAA, and an unidentified auxin in extracts of ovulary walls. Their experiments indicated that neither indolebutyric acid nor tryptophan were present in the extracts. Weeks and Lane (32) used TLC and GLC to identify IAEt, IAA, and tryptamine from extracts of cotton leaves and buds. Their TLC results also suggested the presence of IAlD and indole acetaldehyde but these two compounds were not detected with GLC. Perhaps IAlD is present in higher amounts in ovules than in leaves and buds. We found 26.62 mg/g fresh weight in ovules, although Weeks and Lane (32) suggested that IAlD was present in too low a quantity to show up in GLC.

PLANT HORMONES IN FRACTION III

Five cytokinins were identified as 2iP, dihydrozeatin, zeatin, 2iPA, and zeatin riboside (Table II). Based on bioassays and various chromatographic techniques, all of these cytokinins have been reported to be naturally occurring (25, 27). To the best of our knowledge, ours is the first GLC-MS authentication of zeatin, 2iP, and dihydrozeatin in extracts of higher plants. Recently, Horgan et al. (16) used GLC-MS to identify zeatin riboside in sycamore sap. Bharadwaj and Dua (6) determined the total cytokinin activity in pericarp and seeds of three cotton varieties, but they made no attempt to identify individual cytokinins. Based upon a soybean callus bioassay and paper chromatography, Sandstedt (25) suggested the presence of zeatin and zeatin riboside in cotton.

We realize that cotton fruit may contain more endogenous hormones than we separated and identified. Several unknown peaks were found in every fraction, but positive identification was limited to available authentic standards. In addition to the compounds reported in Table II we looked for, but found no evidence for, indoleacetonitrile, indoleglycolic acid, indoleglyoxylic acid, indole-3-acetylaspartic acid, methyldenamine, and adenosine. However, we were able to identify adenyne, guanine, and guanosine in Fraction III by GLC.

Many physiological functions can be influenced by the presence or absence of several or perhaps all types of endogenous hormones. In the past, because of a variety of limitations, many studies have involved measuring changes in level of a single hormone. More useful, and perhaps more meaningful interpretations could have been made if knowledge of the presence or absence of all hormones in the extracted tissue had been known. The technique used in this study demonstrates that a variety of hormones can now be separated, isolated, and confirmed from a single extract.

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