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, A3, and A13 in the first fraction; ethyl indole-3-acetate and indole-3-aldehyde in the second fraction; and 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-La Roche Inc. Indole-3-acetic 
acid, ethyl indole-3-acetate (IAE), and indole-3-aldehyde 
(IALd) were from Calbiochem. Gibberellins A₃ and A₅ were 
from Abbott Laboratories and A₁, A₆, A₇, and A₈ 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 (dihydrozeatin), and 6-(4-hydroxy-3-methyl-2-trans-butenylamino)-9-β-d-ribo-
uranosylpurine (zeatin riboside) were from Sigma Chemical 
Company. The 6-(3-methyl-2-butenylamino)purine (2iP), 6-
(3-methyl-2-butenylamino)-9-β-d-ribорafuransylpurine 
(3iPA) were obtained from Dr. R. Hall, McMaster University.

Gas-Liquid Chromatography. One to 5 μl of each TMS de-
rivative was injected into a Varian Aerograph GLC (Model 
1200) equipped with a flame ionization detector, a tempera-
ture programmer, and a stainless steel column (3 mm × 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 tempera-
ture, 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 Goelke 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 μCi/mnmole (26); kinetin-8-14C, specific 
radioactivity 20 μCi/mnmole (Calbiochem); gibberellic acid-
14C, specific radioactivity 5.29 μCi/mnmole (gift from Dr. C. 
W. Coggins, Jr.); and indole-3-(acetic acid-1-14C), specific radio-
activity 183 μCi/mnmole (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 scintil-
lation counter was used and all counts were corrected for effi-
ciency, 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 14C-IAA, 14C-ABA, 14C-GA₃, or 14C-
kinetin. The AAA, GA₃, 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 GA₃ 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.

1 Percentage of calculated total counts/min originally added.
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 GA₁ and GA₃ peaks and the GA₁ and GA₃ 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>
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<tbody>
<tr>
<td>Fraction I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IAA[^3]</td>
<td>0.55</td>
<td>9.50</td>
</tr>
<tr>
<td>ABA[^3]</td>
<td>0.91</td>
<td>2.62</td>
</tr>
<tr>
<td>GA₁</td>
<td>0.99</td>
<td>554.60</td>
</tr>
<tr>
<td>GA₂</td>
<td>1.00</td>
<td>54.20</td>
</tr>
<tr>
<td>GA₃</td>
<td>1.11</td>
<td>9.15[^4]</td>
</tr>
<tr>
<td>GA₄</td>
<td>1.14</td>
<td></td>
</tr>
<tr>
<td>GA₅</td>
<td>1.19</td>
<td>2.70[^4]</td>
</tr>
<tr>
<td>GA₆</td>
<td>1.21</td>
<td></td>
</tr>
<tr>
<td>Fraction II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IAEt[^3]</td>
<td>0.88</td>
<td>65.10</td>
</tr>
<tr>
<td>IA1d[^3]</td>
<td>1.00</td>
<td>26.62</td>
</tr>
<tr>
<td>Fraction III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2iPA[^3]</td>
<td>0.89</td>
<td>99.30</td>
</tr>
<tr>
<td>Dihydrozeatin[^3]</td>
<td>0.93</td>
<td>13.47</td>
</tr>
<tr>
<td>Zeatin[^3]</td>
<td>1.00</td>
<td>12.19</td>
</tr>
<tr>
<td>2iPA[^3]</td>
<td>1.07</td>
<td>316.90</td>
</tr>
</tbody>
</table>

[^1] Retention time of GA₂, zeatin, and IA1d = 1.
[^2] Identity authenticated on computer-controlled GLC-MS.

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 GA₁ and GA₃, and for GA₁ and GA₃, 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 nonabsicising 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) GA₁, (d) GA₃, (e) GA₄ and GA₅, (f) GA₁ and GA₃, (g) IAEt, (h) IA1d, (i) 2iP, (j) Dihydrozeatin, (k) Zeatin, (l) 2iPA, and (m) Zeatin riboside.
On the other hand, Lyon et al. (20) and Bhardwaj and Abrol
(5) reported that young cotton fruits contained abscission-
retarding substances other than IAA.

Gibberellins. In addition to ABA and IAA, GLC analysis
indicated the presence of six gibberellins (Fig. 2; Table II).
All of the detected GAs have been separated and identified
previously (4, 18). However this is the first time GLC has been
used to identify so many GAs in a cotton extract. Bhardwaj
and Dua (6) located three areas of gibberellin activity from
paper chromatograms of cotton seed extracts. Using column
and TLC for separation, and dwarf maize bioassays to follow
activity, Smith (28) located two areas of gibberellin activity
from cotton fruit extracts. One was either GA3 or GA6, and
the other was either GA20 or GA30. Addicott and Lyon (3) used
GLC to confirm the presence of GA3 in cotton, and we were
able to confirm not only GA3 but also GA6, GA1, GA3, GA13,
and GA4. We found no evidence of GA5a, suggesting that
Smith’s (28) earlier extract probably contained GA6. Mitchell
et al. (23) indicated that growth-accelerating factors could be
detected in the eluates of unprocessed cotton fibers. They were
chromatographically unlike GA3, but resembled it on the basis
of induction of stem elongation of young bean plants.

PLANT HORMONES IN 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 IAlA, are present in cotton ovules.
The presence of IAEt and IAlA 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 IAlA and indole acetaldehyde
but these two compounds were not detected with GLC. Per-
haps IAlA is present in higher amounts in ovules than in
leaves and buds. We found 26.62 ng/g fresh weight in ovules,
although Weeks and Lane (32) suggested that IAlA 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 cyto-
kinins have been reported to be naturally occurring (25, 27).
To the best of our knowledge, ours is the first GLC-MS authenti-
cation 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. Bhardwaj 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, indole-
glyoxylic acid, indole-3-acetylaspartic acid, methyladenine,
and adenosine. However, we were able to identify adenoine, 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 endoge-
nous hormones. In the past, because of a variety of limitations,
morph 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 identified from a single extract.

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