Simultaneous Quantitation of Indole 3-Acetic Acid and Abscisic Acid in Small Samples of Plant Tissue by Gas Chromatography/Mass Spectrometry/Selected Ion Monitoring

Received for publication February 25, 1987 and in revised form June 10, 1987

JOHN H. VINE*, DOMINIQUE NOITON, JULIE A. PLUMMER, CRISTINA BALERIOLA-LUCAS, AND MICHAEL G. MULLINS

Department of Agronomy and Horticultural Science (D.N., J.A.P., C.B.-L., M.G.M.), and Department of Pharmacy, University of Sydney, N.S.W. 2006, Australia (J.H.V.)

ABSTRACT

Indole 3-acetic acid (IAA) was analyzed in apple, orange, and prune tissue by GC-MS by monitoring the protonated molecular ion of its methyl ester at m/z 261 and the corresponding ions from the methyl esters of either [3H]IAA (m/z 264, 250). Abscisic acid (ABA) was analyzed by monitoring the major fragment ions of its methyl ester at m/z 264 and m/z 247 and the corresponding ions from the methyl ester of [3H]ABA (m/z 264, 250). Detection limits for IAA and ABA were 1 and 10 picograms, respectively using standards and 1 nanogram per gram dry weight for both phytohormones in plant tissue.

A tenet of modern plant physiology is that growth and differentiation are coordinated by the interplay of phytohormones, and there has been massive investment in research on endogenous levels of auxins, gibberellins, cytokinins, ABA, and ethylene in relation to plant morphogenesis and in relation to the responses of plants to the environment. Most studies have involved separate determinations of the individual hormonal compounds and reports in which two or more classes of phytohormones are measured in the same extract are few. When there are no limitations on availability of the tissues of interest, phytohormone analyses on a 'one-at-a-time' basis are, of course, perfectly satisfactory, but experimental work with isolated apices or tissue cultures involves small amounts of tissue and the labor involved in obtaining samples for analysis is usually considerable. In these circumstances it is highly desirable that as much information as possible be obtained from each extract. Hitherto, analytical methods were generally inadequate for simultaneous determinations of trace amounts of different phytohormones, but this limitation has been overcome with the advent of GC-MS-SIM1 which makes determinations possible at the picogram level.

The method to be described here, for simultaneous quantitation by GC-MS-SIM of free IAA and free ABA in small samples (0.1–0.5 g dry weight), arose from research on morphogenesis in tissue cultures and intact plants of apple (9, 13), prune (1), and citrus (10). Of the few methods available for simultaneous measurement of IAA and ABA (2, 4–6, 8, 11) none was suitable for our purposes. Those based on chromatographic procedures alone (2, 5, 8, 11) require extensive purification of extracts and lack the specificity of GC-MS. Published methods involving GC-MS (4, 6) are primarily qualitative in nature and do not include use of deuterated internal standards and calibration curves. These methods lack the necessary precision for quantitative work. Accordingly, it was decided to modify a currently used quantitative GC-MS assay for ABA (12) to enable IAA to be measured in the same extract.

MATERIALS AND METHODS

Plant Materials, Extraction, and Derivatization. The plant materials were leafy microcuttings from aseptic proliferating shoot cultures of apple (cv Jonathan) and prune (cv D'Ente), 1-year-old extension shoots of Jonathan apple, and fully expanded mature leaves from adult cropping orange trees (cv Valencia).

All glassware was soaked in a 5% solution of Surfasil (Pierce Chemical Co., Rockford, IL) in hexane for 5 min, rinsed with methanol and oven-dried for 1 h at 100°C. All sample manipulations were carried out under dim light to minimize isomerization and photodecomposition. Samples and extracts were stored in the dark at −20°C when not in use. All solvents were of analytical reagent grade and were distilled before use.

The tissues were harvested, frozen by immersion in liquid N2, and freeze-dried. Although the possibility of losses of IAA and ABA during freeze drying cannot be completely ruled out, care was taken to ensure that the same technique and conditions were used for the freeze drying of all tissues. Samples of the freeze-dried material (100–500 mg) were ground in a mortar and extracted overnight (4°C) with 70% (v/v) acetone containing deuterated ABA (80 ng) and deuterated IAA (80 ng) as internal standards, i.e., [3H]ABA (12) and either [3H]IAA (7) (a gift from Professor K. S. Bandurski, Michigan State University) or [3H]IAA (purchased from MSD Isotopes, Montreal, Canada). The slurry was centrifuged (2 min) and the supernatant was retained. The pellet was reextracted with 70% acetone and centrifuged and the combined supernatants were then evaporated to the aqueous phase under a stream of N2. The pH of the aqueous phase was adjusted to 8.5 with a freshly prepared half saturated solution of NaHCO3. This alkaline aqueous extract was then washed (twice) with ethyl acetate and the organic phase, containing pigments, was discarded. The remaining aqueous solution was acidified (pH 2.5) with 1 N H2SO4 and extracted (2 × 5 ml) with ether. The ethereal extracts were combined and reduced to dryness (45°C) under N2. The dry residue was dissolved in 10% (v/v)
methanol in diethylether and was methylated by reaction with freshly prepared diazomethane as described by Rajasekaran et al. (12). The derivatized sample was then evaporated to dryness under nitrogen and stored in the dark (−20°C) until required. The methyl esters of ABA and IAA remained stable for more than 12 months under these conditions. The residue was dissolved in chloroform:methanol (4:1, v/v, 100 μl) and 1 μl aliquots were injected into the GC-MS.

Analysis by GC-MS-SIM. Analyses were made with a Finnigan TSQ46 triple stage quadrupole mass spectrometer interfaced to an Incon 2300 data system. The TSQ was operated as a conventional mass spectrometer using the Q1MS mode. The bonded phase fused silica capillary column (stationary phase BP10, 9 m × 0.3 mm i.d., 25 μm film thickness, S.G.E. Pty Ltd, Melbourne, Australia) was inserted directly into the ion source of the mass spectrometer. Samples were injected using the splitless technique onto the column at 80°C. After 1 min the column was programmed to 250°C at a rate of 20°C min−1 and held at 250°C for 5 min. The injection port and the GC/MS interface were maintained at 250°C. Helium was used as the GC carrier gas at a flow rate of 2 ml min−1 and methane was added through the make-up valve to give a source pressure of 133 Pa. Chemical ionization mass spectra were generated with an electron beam energy of 100 eV and the ion source was operated at 140°C. Under these conditions Me-IAA and Me-ABA had retention times of 6.8 and 7.9 min, respectively.

IAA was analysed by monitoring the protonated molecular ion of Me-IAA at m/z 190 together with the major fragment ion at m/z 130 and the corresponding ions from Me-[2H5]IAA at m/z 194 and 134 or m/z 195 and 135 from Me-[2H6]IAA. Abscisic acid was analyzed by monitoring the major fragment ions at m/z 261 (MH+−H2O) and 247 (MH+−CH3OH) and the corresponding ions from the [2H5]ABA at m/z 264 and 250. Peak areas were used in all calculations. On each day samples were to be analyzed, standard curves were constructed to cover the IAA and ABA concentration ranges anticipated in the samples. Aliquots of acetone containing IAA and ABA (0, 5, 10, 20, 40, 80, 160, and 320 ng) were placed in centrifuge tubes, the deuterated standards were added and the resulting mixtures were extracted in the same way as the plant extracts. Estimates of precision at high and low concentrations of IAA and ABA in the standards were obtained by replicate extraction and analysis of both 5 and 320 ng calibration standards. Estimates of recovery were made with respect to nonextracted standard solutions.

RESULTS AND DISCUSSION

The methane chemical ionization mass spectra of the methyl esters of IAA, [2H4]IAA and [2H5]IAA are shown in Figure 1. The protonated molecular ion peaks of Me-IAA and Me-[2H5]IAA or Me-[2H6]IAA were used for quantitation. As an additional check on specificity the fragment ions at m/z 134 or 135 were also monitored. The ratio of fragment ion abundance to protonated molecular ion abundance for each compound was used to ensure that there was no interference from other species which might give ions at these masses. The ratios were usually within ±5% of the mean value obtained from the calibration standards. Samples having values outside these limits were discarded.

Analysis of ABA was carried out as described previously (12) except that in the present work two fragment ions were monitored for ABA (m/z 247 and 261) and (2H3)ABA (m/z 250 and 264). The ratios of these ions were used as detailed above to ensure that there was no interference from other co-eluting compounds.

Calibration curves for the range 5 to 320 ng showed excellent linearity; typical data are given in Table I. Variations in calibration data were small and the same calibration curve could be used for several days. Nevertheless, a new calibration curve was prepared each day samples were to be run. Recoveries based on comparisons of extracted and nonextracted standard solutions were approximately 60% for IAA and 80% for ABA. These high recoveries were achieved by ensuring that all glassware coming into contact with samples was thoroughly silanized without the need to filter solvents through cotton fibers (3). Up to 30 plant extracts could be analyzed in a day.

The use of a bonded phase fused silica capillary column gave an approximately 10-fold increase in sensitivity for ABA (detection limit 10 pg) compared to that obtained on the packed columns used previously (12), as a result of greatly improved peak shape. Me-IAA showed an even greater improvement in chromatographic properties on the capillary column and 1 pg was easily detectable. These detection limits could not be achieved in practice, however, due to interference by other co-eluting compounds present in the plant extracts. For both IAA

![Fig. 1. Methane chemical ionization mass spectra of the methyl esters of IAA (a), [2H4]IAA (b), and [2H5]IAA (c).](image-url)
QUANTITATION OF IAA AND ABA BY GC-MS-SIM

### Table I. Calibration Curve Data for IAA and ABA

<table>
<thead>
<tr>
<th>Phytohormone</th>
<th>Equation of the regression line</th>
<th>Correlation Coefficient</th>
<th>Coefficient of Variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IAA</td>
<td>$y = 0.009502x - 0.007472$</td>
<td>0.9991</td>
<td>8.9</td>
</tr>
<tr>
<td>ABA</td>
<td>$y = 0.003920x + 0.03499$</td>
<td>0.9998</td>
<td>3.8</td>
</tr>
</tbody>
</table>

Table II. Simultaneous Quantitation of IAA and ABA by GC-MS-SIM

Typical values (high and low range) for IAA and ABA in some woody perennial fruit plants.

<table>
<thead>
<tr>
<th>Species</th>
<th>Origin of Extract</th>
<th>IAA High value</th>
<th>ABA Low value</th>
<th>IAA Low value</th>
<th>ABA High value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sweet orange (Citrus sinensis) cv Valencia</td>
<td>Mature leaves</td>
<td>76</td>
<td>7</td>
<td>1360</td>
<td>38</td>
</tr>
<tr>
<td>Apple (Malus pumilla) cv Jonathan</td>
<td>Tissue culture (leafy microcuttings)</td>
<td>200</td>
<td>50</td>
<td>250</td>
<td>33</td>
</tr>
<tr>
<td>Prune (Prunus domestica) cv D'Ente</td>
<td>Tissue culture (leafy microcuttings)</td>
<td>395</td>
<td>200</td>
<td>2000</td>
<td>1000</td>
</tr>
</tbody>
</table>

FIG. 2. Ion chromatograms of m/z 190 (Me-IAA) (above) and m/z 195 (Me-[2H₅]IAA) (below): a, 20 ng standard; b, extract from axillary shoots of Jonathan apple produced in vitro calculated to contain 23.4 ng IAA.

and ABA, detection at a level of 1 ng g⁻¹ of plant tissue was usually possible.

This technique has been applied to the determination of IAA and ABA in a variety of tissues in citrus, prunes, and apples. Some typical results are shown in Table II and Figures 2 and 3.

LITERATURE CITED

5. Knegt E, E Vermeer, J Brunsena 1981 The combined detection of indoly-
Fig. 3. Ion chromatograms of m/z 261 (Me-ABA) (above) and m/z 264 (Me-[^3]H3]ABA (below): a, 20 ng standard; b, extract from axillary shoot of Jonathan apple produced in vitro calculated to contain 18.6 ng ABA.

2-acetic and abscisic acids in plant material. Anal Biochem 114: 362–355
7. MAGNUS V, RS BANDURSKI, A SCHULZE 1980 Synthesis of 4,5,6,7 and 2,4,5,6,7-deuterium-labeled indole-3-acetic acid for use in mass spectrometric assays. Plant Physiol 66: 775–781
10. PLUMMER JA 1987 Shoot growth and flowering in *Citrus sinensis* (L) Osbeck) and related species, PhD thesis. University of Sydney