Levels of Indole-3-Acetic Acid in *Lemna gibba* G-3 and in a Large *Lemna* Mutant Regenerated from Tissue Culture

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**ABSTRACT**

Large changes in indole-3-acetic acid (IAA) levels occur during growth of *Lemna gibba* G-3 in sterile culture. The levels of IAA were measured in plants during a 45 day growth cycle using HPLC and isotope dilution analysis followed by selected ion current monitoring GC-MS analysis with 13C6-IAA as the internal standard. Even though the rate of plant growth remained constant over the entire growth period, IAA levels ranged from a high of 222 to a low of 6 nanograms per gram fresh weight. A *Lemna* mutant (jsR,) which has a giant phenotype was obtained by regeneration from primary callus cultures. Microspectrofluorometry of 2-phenylindole stained cells showed that jsR, has the same amount of DNA per nucleus as the parent line (PL). All jsR, cell types measured are about 1.5 times larger than in PL. The endogenous levels of IAA per gram fresh weight were higher in jsR, at several stages of the plant culture cycle as compared to PL. This difference ranged from 1.2 to over 100 times as much. While PL showed only one high peak at day 9, jsR, had IAA levels of 480 and 680 nanograms per gram fresh weight at days 9 and 45, respectively. Throughout the midculture stage of the growth cycle (20-28 days) both jsR, and PL had IAA levels in the range of 9 to 14 nanograms per gram fresh weight. In contrast to PL, at day 45, jsR, had no detectable ester or amide conjugates of IAA. These changes in IAA levels were determined in sterile plant cultures and thus cannot be attributed to bacterial or fungal activity.

The levels of the phytohormone indole-3-acetic acid have been measured in a number of plants using a wide variety of analytical techniques (8). Although such studies have provided us with a wealth of useful information on the presence and amounts of IAA in plant tissues, few studies have documented the changes in hormone levels throughout the life cycle of a plant. A number of studies, however, have attempted to correlate IAA levels with particular developmental events such as flowering (14) and senescence (1, 15), or with more rapid effects such as tropic curvature (5, 13). Changes in IAA levels seen in such experiments have usually been small, perhaps because of the small number of cells involved with such processes and the relatively large amount of tissue extracted. This has led to an assumption that large changes in IAA levels do not occur in higher plants (27).

Sterile cultures of the monocotyledonous plant, *Lemna gibba* G-3 (duckweed), offer several advantages for studies of IAA metabolism (25). Under short day conditions such cultures con-

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2 "The *Lemna* frond is a propagative structure consisting of a terminal leaf; a bud inclosed [sic] by a flattened bud scale, the base of which is fused to the base of the leaf and laterally to the stem; and an apical region from which new fronds are developed" (from Ref. 6).

3 Abreviations: FW, fresh weight; DAPI, diamidino-2-phenylindole; GC-SIM-MS, selected ion current monitoring GC-MS analysis; m/z, mass to charge ratio; PL, parent line of *L. gibba* G-3.
search Center, Rockville, MD) and were maintained on 'E media' (10) at 26 to 28°C. Illumination from a mixture of cool-white fluorescent and incandescent lamps was provided at 38 \mu\text{E} \text{m}^{-2} \text{s}^{-1} with a photoperiod of 8 h light, 16 h dark. Cultures were maintained in 2 L Erlenmeyer flasks containing 1 L of media which were 'planted' with 10 four-frond colonies at d 0. At harvest, plants were collected on a nylon screen, blotted dry with absorbent paper, weighed, and then frozen by immersion in liquid N\textsubscript{2}. Plants were stored at −80°C until analysis.

*L. gippa* G-3/jsR, was obtained as a spontaneous regenerant from tissue culture (23, 24). Growth conditions of the regenerated jsR line were identical to that of PL.

For genetic studies, flasks containing jsR, were placed under constant light conditions to induce flowering. Cultures were self-pollinated by gently rotating the flasks on a platform shaker for 1 h twice daily. Seed formed under these conditions were collected and germinated in fresh sterile E media.

Plants used for microspectrofluorometry and for morphological analysis were from 30 d cultures.

**Microspectrofluorometry.** Root and frond sections obtained from the jsR, line and from PL were compared as to the amount of DNA per cell. Plants were treated with the fluorochrome DAPI, essentially as described by Hammerschlag (17) and Kapuscinski and Skoczylas (18) except that the intact plant was floated on the dye for 2 h. Fluorescence measurements of random chosen nuclei from comparable tissue sections of PL and jsR, were made using a Zeiss\textsuperscript{a} Universal microscope with epifluorescence. A Zeiss BP 365/10 excitation filter and an LP 395 barrier filter were used with a 200 W HBO high pressure mercury lamp. The photomultiplier was standardized against the emission of a Zeiss GG17 broad spectrum fluorescing filter. Fluorescence emission was measured using a R 777 Hamamatsu photomultiplier. From each tissue section examined, 7 to 11 cells were measured and at least 5 readings were made per cell. Measurements from 14 roots and 19 fronds were recorded and a total of 755 and 770 readings each were made from jsR, and PL, respectively.

**Cell Size Measurement.** Cell size measurements were made on free hand sections of fresh plant material using an American Optical Phase Star 110 microscope equipped with an ocular micrometer calibrated against a Bausch & Lomb stage micrometer. Measurements were made using normal contrast at \times 100−400. Only plant material of similar age and location within the tissue were used for comparison.

Frond area was determined by tracing the frond and a standard scale in photographs projected onto paper, then cutting and weighing the paper image. Nail polish peels of the upper surface of the fronds were made following photography and the approximate cell number in the entire epidermis determined by placing the peel on a hemacytometer and counting the cells in a given area. At least eight fields of 1.6 mm\textsuperscript{2} each were counted per frond and all fields counted were from the central region of the frond.

**Culture Cycle.** Whole plant cultures of both PL and of jsR, were initiated and grown as described above. At about 4 d intervals, flasks were harvested, frond numbers counted, fresh weight determined, and the tissue then frozen in liquid N\textsubscript{2} for later analysis. Samples taken 8 to 11 d apart were used for IAA analysis. Each data point for the growth curve and for IAA analysis is an average of two or more replicate flasks each analyzed individually.

**IAA Analysis.** IAA was determined by GC-SIM-MS (12). Briefly, plants were homogenized in 70% acetone containing 0.5 \mu\text{C}\textsubscript{13C}-[benzene ring]IAA (99% enrichment, synthesized as in Ref. 12) and 30,000 dpm \textsuperscript{14}C-[methylene]-IAA tracer (59 mCi/mmol, Amersham). Sample size varied from 100 mg to over 20 g F.W. The extraction volume was adjusted such that the final acetone concentration was greater than 60%. After evaporation of the acetone and partitioning with ether at pH 7.0 and 2.5, the sample was purified by C\textsubscript{18}-HPLC on a 4.6 mm \times 25 cm Whatman Partisil ODS-3 column using 1 ml/min of 35% methanol/water containing 1% acetic acid for elution. The IAA fraction (retention time of 19–20 min) was methylated using diazomethane (11) and analyzed by GC-SIM-MS using a four ion program monitored at m/z 130, 136, 189, and 195. Each sample was injected three times and the average of the peak areas from the final two injections were used. GC-MS was done on a Hewlett Packard 5992 quadrupole machine equipped with a 18740B capillary inlet system and an open-split capillary connection to the analyzer unit. GC was on an 11 m Chrompack CPSil 19 CB 0.32 mm o.d. bonded phase midpolarity fused silica column. The injector was at 260°C and the oven was programmed with a 2 min isothermal hold at 140°C then increased at 5°C/min. Ultra-pure He, after passage through an oxygen trap and through a molecular sieve (Linde 4A), was used as carrier gas at a flow rate of 1 ml/min.

Ester and amide conjugates were determined by using selective base hydrolysis as previously described (12). Data presented are the average of four replicate experiments.

**RESULTS**

**Growth Characteristics.** *L. gippa* G-3 is an aquatic angiosperm that grows well in sterile culture on minimal media. Under short day conditions propagation is entirely vegetative. Using E media, which consists of salts and contains 1% sucrose, flasks planted with *Lemna* support plant growth at a logarithmic rate for over 45 d, with no apparent lag phase or plateau (Fig. 2). Growth rate does not account for the difference in size of PL and jsR,. *Lemna* fronds exhibit a determinant growth characteristic. Each frond will produce approximately 15 daughter fronds and then the mother frond senesces. In our cultures, the first senescent fronds (yellow) are usually apparent after about 20 d of growth; however, they account for only a minor fraction of the total plant population even up to 45. After this time yellow fronds occur more frequently and the plants become crowded on top of one another, no longer forming just a single layer of plants on the surface of the medium. At all stages of the culture cycle fronds of all nonsenescent stages of development are present. During the culture cycle the pH of the media gradually rose from an initial value of 4.6 to approximately 5.8.

The phenotypes of the F\textsubscript{1} generation obtained by self pollination of cultures indicated that jsR, is a genetic mutant. About half of the progeny exhibited the large phenotype or intermediate phenotypes, indicating that the trait is probably polygenic. Backcross data is not yet available.

The large and dark green frond phenotype of jsR, suggested that these plants might be polyploid. Measurement of cell sizes of several different tissues showed that, in general, the cells of jsR, were about 1.5 times the size of comparable cells in PL (Table I), also indicating the possibility of jsR, being polyploid. This possibility was discounted, however, by comparing the nuclear DNA content of PL and jsR,. Nuclei were labeled with DAPI and the fluorescence emission of individual nuclei was measured with a microspectrofluorometer (Table II). Both PL and jsR, contained approximately the same amount of DNA per nucleus.

The approximate number of cells in the upper epidermis of jsR, is not significantly different from the number determined for PL (Table III).

**Levels of IAA in *Lemna*.** At the earliest time sampled, 9 d, the
levels of IAA in *Lemna* are very high (Fig. 3). At this time point, the IAA content of the large mutant, 420 ng/g FW, is approximately twice that found in PL, 222 ng/g FW. By d 20, the amount of IAA in both plant lines decreased to less than 5% of the amount at d 9. For the remainder of the culture cycle PL maintained a level of about 10 ng/g FW; a value similar to those reported for other plant tissues (2, 4, 13, 14, 21). In contrast, the level of IAA in jsR, increased dramatically by the end of the culture cycle (Fig. 4). The identity of the IAA was confirmed by full scan mass spectral analysis (Fig. 4).

The primary bound forms of IAA in PL occur as ester conjugates, which account for 75% of the total IAA in the PL plants. Amide conjugates comprise 19% of the total IAA. In contrast, IAA conjugates were not detected in jsR, (Table IV).

Variation between replicate samples for IAA analysis did not exceed 12% for any of the samples; the analytical precision of the GC-MS assay technique is better than 4% (12). The greatest variability was seen in the samples from periods where plants had a high IAA content.

**DISCUSSION**

A number of techniques have been used for IAA analysis in biological materials. Most recent approaches have utilized high performance chromatographic techniques with various selective detection systems. The problems inherent in such measurements due to contamination by the myriad of plant aromatic acids present in extracts, the lability of the indole moiety, and changes in indole fluorescence intensity upon exiplex formation (7) have been eliminated by using the 13C-[benzene ring]IAA internal standard for quantitative GC-MS (12).

At specific times during the culture cycle both PL and jsR, show levels of IAA that are over an order of magnitude higher than has been reported for vegetative tissues of a variety of plant genera other than *Lemnaceae* (2, 4, 13, 14, 21). For example, seedlings of pea, maize, and oat all contain between 16 to 35 ng/g FW free IAA (2, 4). Whether the differences among plant types are due to developmental stage or to the state of growth of the plants remains to be determined. High auxin levels have previously been reported for members of the *Lemnaceae* (26, 29).

While other mutant plants with increased levels of IAA have been isolated (14, 16, 28), the levels of IAA reported are much lower than that found in 45 d cultures of jsR, High accumulation variants are rare and, in some cases, the analytical information provided was limited. A genetic line of *Sorghum bicolor* with a late maturing characteristic shows an increase in IAA level during the late phase of the preflowering period (14), but the highest level of IAA in such plants was about an order of magnitude less than the peak level found in jsR,. Large changes in the IAA content of individual plant parts over short time intervals have also been reported (19). To our knowledge, however, this is the first report of such large changes in IAA content occurring in a population of whole plants.

In contrast to recent assertions that large changes in phytohormone levels do not occur in plants (27), it is clear from our results that IAA levels change dramatically in *Lemna*. These changes occur at defined time intervals during a period of logarithmic growth. Alterations in the level and timing of the ob-

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**Table I. Size of Pollen, Stomata, and Cells in Selected Tissues of Lemna PL and jsR,**

Mean values ± se are reported; 9 to 24 cells of each type were measured.

<table>
<thead>
<tr>
<th>Tissue</th>
<th><em>Lemna</em> Line</th>
<th>Size</th>
<th>jsR,/PL</th>
<th>jsR,/PL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length</td>
<td>Width</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frond, mesophyll</td>
<td>27.5 ± 4.2</td>
<td>2.03</td>
<td>28.4 ± 4.2</td>
<td>1.86</td>
</tr>
<tr>
<td></td>
<td>jsR,</td>
<td>56.0 ± 2.3</td>
<td>52.8 ± 2.3</td>
<td>1.51</td>
</tr>
<tr>
<td>Stomata</td>
<td>27.2 ± 1.8</td>
<td>1.34</td>
<td>13.4 ± 0.4</td>
<td>1.45</td>
</tr>
<tr>
<td></td>
<td>jsR,</td>
<td>36.5 ± 1.6</td>
<td>20.3 ± 0.6</td>
<td>1.24</td>
</tr>
<tr>
<td>Root, epidermal</td>
<td>64.3 ± 5.3</td>
<td>1.50</td>
<td>26.3 ± 0.9</td>
<td>1.45</td>
</tr>
<tr>
<td>Pollen (diameter)</td>
<td>96.2 ± 2.9</td>
<td>38.2 ± 6.0</td>
<td>25.0 ± 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>jsR,</td>
<td>31.0 ± 0.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table II. Fluorescence Emission of Nuclei in Cells of Lemna PL and jsR, Measured after Staining with DAPI
Values are the mean ± SE of the microspectrofluorometer measurements.

<table>
<thead>
<tr>
<th>Plant Tissue</th>
<th>PL</th>
<th>jsR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root</td>
<td>1.09 ± 0.02</td>
<td>1.15 ± 0.02</td>
</tr>
<tr>
<td>Frond</td>
<td>0.97 ± 0.02</td>
<td>0.99 ± 0.03</td>
</tr>
</tbody>
</table>

Table III. Approximate Number of Cells in the Upper Epidermal Layer of Two Lines of L. gibba G-3
Values are the mean ± se, n = 8.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>PL</th>
<th>jsR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frond area</td>
<td>15.5 ± 1.7 mm²</td>
<td>27.9 ± 1.6 mm²</td>
</tr>
<tr>
<td>Number of cells (× 10⁴)</td>
<td>3.4 ± 0.3</td>
<td>3.9 ± 0.4</td>
</tr>
</tbody>
</table>

Fig. 3. IAA content of L. gibba at different times during the plant culture cycle under short day conditions as measured by quantitative GC-MS analysis.

Fig. 4. Mass spectrum of the methylated IAA sample isolated from Lemna jsR line at 45 d in culture. Ions at m/z 195 and 136 are from the added ¹⁴C₂⁻IAA internal standard and ions at m/z 189, 130, 103, and 77 are characteristic of methyl IAA.

Table IV. Free and Conjugated (Ester and Amide Linked) IAA in PL and jsR, L. gibba G-3 in 45 d old Cultures Grown under Short Day Conditions Determined by Selective Hydrolysis and Quantitative GC-MS Analysis

<table>
<thead>
<tr>
<th>Compound</th>
<th>PL</th>
<th>jsR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free IAA</td>
<td>6.6</td>
<td>681</td>
</tr>
<tr>
<td>Ester IAA</td>
<td>79</td>
<td>ND</td>
</tr>
<tr>
<td>Amide IAA</td>
<td>20</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Below the level of detection (4% of the free IAA level).

L. gibba is a particularly suitable organism for studies of plant metabolism and especially phytomorphogenesis, because of the ease in maintaining sterile cultures and the lack of a cuticular barrier on the lower leaf surface. These features make Lemna especially attractive for pulse-chase and other labeling studies (see, for example, Ref. 25). The mutant line described in this report accumulates IAA in large amounts relative to the amount of phytomorphogenesis usually found in higher plants. Regardless of whether the large phenotype of jsR is directly correlated with increased IAA levels, future experiments using stable and radioisotope tracer techniques to study IAA metabolism should be greatly enhanced by the use of this genetic mutant, because the high levels of free IAA should facilitate such analyses.

Acknowledgments—Dr Robert J. Griesbach (USDA/ARS Florist and Nursery Crops Laboratory, Beltsville, MD) graciously allowed us the use of his facilities and also provided helpful advice for the microspectrofluorescence studies. We thank Jo Eta Hubbard and Suzanne Fisher for their assistance with Lemna plant and tissue culture.

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