Drought-Induced Increases in Abscisic Acid Levels in the Root Apex of Sunflower

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ABSTRACT

Abscisic acid (ABA) levels in 3-mm apical root segments of slowly droughted sunflower plants (Helianthus annuus L. cv Russian Giant) were analyzed as the methyl ester by selected ion monitoring gas chromatography-mass spectrometry using characteristic ions. An internal standard, hexadeuterated ABA (d6ABA) was used for quantitative analysis. Sunflower seedlings, grown in aeroponic chambers, were slowly droughted over a 7-day period. Drought stress increased ABA levels in the root tips at 24, 72, and 168 hour sample times. Control plants had 57 to 106 nanograms per gram ABA dry weight in the root tips (leaf water potential, –0.35 to –0.42 megapascals). The greatest increase in ABA, about 20-fold, was found after 72 hours of drought (leaf water potential, –1.34 to –1.47 megapascals). Levels of ABA also increased (about 7- to 54-fold) in 3-mm apical root segments which were excised and then allowed to desiccate for 1 hour at room temperature.

Apart from its widely recognized role as an agent of stomatal closure, ABA may have other roles to play in the adaptation of plants to drought stress (8, 17). The observations that ABA levels increase in the roots of water-stressed plants (7, 11, 12, 19, 22) and that this increase does not depend on transport from the shoot (19, 22) are particularly provocative. Here, any action of ABA would not directly involve stomatal closure.

There is evidence for the involvement of ABA in some drought-associated developmental responses; several studies (5, 9, 18, 23) have reported that the effects of exogenously applied ABA on plant development show similarities to the effects of water stress. Barlow and Pilet (1) have shown that exogenously applied ABA reduces cell division and DNA synthesis in the root apical meristem of corn. Ciamprova and Luxova (3) noted structural changes in the root apices of water-stressed maize plants. Earlier studies with sunflower seedlings found that drought stress inhibited root growth (5) and increased ABA levels in the root tissue (5, 7). Changes in the anatomy of the root apices accompanied reduced growth in the droughted seedlings; moreover, the application of ABA (10 μM) also inhibited root growth and induced anatomical changes at the root apices similar to those induced by drought (5).

These observations suggest that the increases in ABA levels in droughted sunflower roots (7) may directly affect growth and development at the root apex where the developmental pattern of the root unfolds. We report, herein, drought-induced increases in the amounts of endogenous ABA in the apical 3 mm of sunflower roots; this region includes the apical meristem proper and its immediate derivatives, the primary meristems and the root cap. The method used for analysis of ABA was selected ion monitoring GC-MS with an internal standard of d6ABA2,3 for quantification (20).

MATERIALS AND METHODS

Plant Material. Sunflower (Helianthus annuus L. cv Russian Giant) achenes were germinated in Terra Green (a baked clay material). Seven d after planting, seedlings were transplanted into aeroponic chambers (6) where they were maintained by 0.75 strength Hoagland solution. They were allowed to acclimate for a further 3 d before drought was initiated. The seedlings were illuminated (about 300 μE m2 s−1 PAR at the top of the plants) by Sylvania Gro-Lux lamps (F72T12-GRO-WS-VHO) for 16 h each day at 25°C, followed by a dark period of 8 h at 16°C.

Drought Regime. A time clock was attached to the electric motor which drives the spinner on one of the aeroponic chambers (7). Drought was imposed by withholding water for timed intervals; the seedlings were misted for 120 s h−1 in two 60-s bursts for the first 24 h of treatment, then for 90 s h−1 in one 60-s and one 30-s burst, thereafter. This droughting regime provided for a slow increase in water stress without subjecting the seedlings to a great initial shock at the beginning of the experiment. Control plants were continually misted. Leaf ψ, as an indication of water stress, was monitored by the dewpoint method with Wescor C 52 psychrometers attached to a Wescor HR-33T Dew Point microvoltmeter (Wescor Inc., Logan, UT).

Harvest. In the first experiment (trial 1), droughted and control samples (about 1000 root tip sample−1) were harvested at 24, 72, and 168 h after the initiation of drought. The experiment was repeated (trial 2), since not enough material could be harvested at one time to provide for more than one sample. The 3-mm apical segments were cut from the main root and from elongated primary laterals. Short laterals were rejected. The root tips were cut on ice-cold plates and placed immediately into liquid N2. All work was performed in reduced light. After freezing in liquid N2, the samples were freeze-dried and weighed. The dry weight of the samples ranged from 9 to 16 mg.

In a second experiment, 3-mm apical root segments were harvested from 11-d-old plants and allowed to dry in the air for

2 Abbreviations: ABA-Me, ABA methyl ester; d6ABA, hexadeuterated cis, trans ABA; Rt, retention time; ψ, water potential.

3 Hexadeuterated ABA was a kind gift to R.P.P. from L. Rivier, Institute of Plant Biology and Physiology of the University, 6 Place Riponne, 1005 Lausanne, Switzerland.

1 Supported in part by grants from the Natural Sciences and Engineering Research Council of Canada: A-2585 (R.P.P.), A-6704 (E.C.Y.), and A-5727 (D.M.R.).
1 h in darkness at room temperature, then frozen in liquid N₂ and treated as the samples from experiment 1, thereafter. Root tips were harvested in small batches that were timed separately. The individual small harvests were pooled to make up sample sizes of about 1000 root tips. The appropriate controls for this experiment are the 24-h control samples. The experiment was repeated 2 more times from another harvest of plants. In the repeat experiments, the degree of stress on the root tips was monitored as water potential. Two methods were used: the dew point method had proven unreliable for measuring ψ in the root tips from droughted plants. For that reason, an isopiestic method, Chardokov’s dye drop method (2), was used as a check on the accuracy of the dew point readings. In repeated tests, the dew point values fell within the upper and lower limits of ψ determined by Chardokov’s method. For estimation of the ψ of the samples, batches of about 50 root tips were taken from the sample, after treatment, for dew point readings. Three readings were taken from each sample. Root tips from each sample were tested against sucrose solutions of known ψ (from -1.56 to -2.69 MPa) for determination of ψ by Chardokov’s method; for each test, one ml of solution was placed in a sealed Pasteur pipet, to this about 50 root tips from the sample were added.

Two further control samples were done for experiment 2. Root tips were excised and then placed in 2 ml of bathing solution (0.75 strength Hoagland solution) for 1 h. The root tips and the solution in which they were kept hydrated were analyzed separately for ABA.

**Extraction Procedure.** The tissue was extracted 3 times in 13.3 ml of methanol:water:acetic acid (80:19:1 v/v) by grinding in a cold mortar and pestle. Approximately 150 ng of d6ABA at an internal standard (d6ABA was taken from a single dilution and the volume was kept constant) and 50,000 dpm of [3H]ABA (Amersham 24 Ci mmol⁻¹) were added at the beginning of the extraction procedure. The 80% methanol filtrate (40 ml) was then passed through a preparatory column of C18 material (10) to remove nonpolar compounds. The column was then washed with an additional 20 ml of extraction solvent. The 60 ml of eluate was then taken to the aqueous phase in vacuo and partitioned 3 times against equal volumes of diethyl ether at pH 2.8. The organic phase containing the ABA was taken to dryness in vacuo, at 35°C, resuspended in absolute methanol, filtered through a 0.5-μm film Millipore filter, taken to dryness under a N₂ stream, and resuspended in 500 μl of 32.5% methanol:1% acetic acid.

The entire sample was then passed onto a Waters reverse-phase C18 μ-bondapak analytical column (4 mm x 30 cm). The HPLC conditions were: 32.5% methanol:1% acetic acid isocratic for 55 min, followed by a 5-min gradient to absolute methanol and 20 min of absolute methanol at a flow rate of 1 ml min⁻¹. Under these conditions, the Rt for ABA was 44 to 46 min. The UV-absorbing substances in the extract were monitored in-line by UV absorbance at 254 nm. The internal standard of [3H]ABA was monitored by a Berthold model LB503 HPLC radioactivity detector (Labserco Ltd. Oakville, Ontario, Canada) equipped with a homogeneous flow-through scintillant cell. The radioactive fractions were collected and taken to dryness under N₂ in a Reacti-vial, then methylated in a few drops of methanol with 300 μl of cold ethereal diazomethane. The reaction mixture was taken to dryness under N₂, then resuspended in 500 μl of 32.5% methanol:1% acetic acid and again subjected to reverse-phase C18 HPLC under the same conditions noted above. The Rt for ABA-Me was 65.5 min. The radioactive ABA-Me fraction was collected, taken to dryness under N₂, and resuspended in 25 μl of methylene chloride.

**GC-MS-SIM Analysis.** For quantitative GC-MS analysis, we used a method similar to that of Rivier and Filet (20). However, our stock of d6ABA could not be weighed with confidence. Therefore, a constant amount, about 150 ng of d6ABA, was used in each extract. This was accomplished by making an approximate dilution from which a constant volume was taken for each extract. Endogenous ABA levels were quantified from a standard curve, which established the ratio of the base ion (m/z 194) peak height of the constant amount of d6ABA to the base ion (m/z 190) peak height of precisely known quantities of authentic ABA (Sigma grade IV). The following ratios of ABA and d6ABA were used for the standard curve: a constant amount of d6ABA and 50,000 dpm of [3H]ABA were mixed with 0, 0.5, 2.5, 5.0, and 25.0 ng of ABA. Standards were handled in the same way as samples. Quantification of the samples was done by entering the ratio of m/z 190:m/z 194 into the standard curve.

**GC-MS Conditions.** For GC-MS analysis, a Hewlett-Packard model 5790A series GC with a 15-m DB5 capillary column (Capillary Specialty Materials) (Chromatographic Specialties Ltd.) connected to Hewlett-Packard 5970 MSD was used. The selected ion monitoring mode was used with the Autotune program: m/z 190 and m/z 194 were monitored, window size 0.1, ionizing potential 1800 eV, dwell time 50 ms.

ABA was identified from the Rt of ABA and of ABA-Me on HPLC, and from the Rt of ABA-Me on the GC column. A full-spectrum analysis on the most active sample (72 h drought) was also attempted. The following ion fragments, characteristic of ABA-Me (20), were found at the correct Rt: m/z 278, m/z 222, m/z 190, m/z 162, m/z 134, m/z 125, m/z 106, m/z 91.

The advantage of the internal standard technique is that it allows for the trace analysis of ABA from very small amounts of tissue (20). The fact that quantitation is accomplished from a ratio greatly reduces error from calculating losses and from machine variability. Variability between samples was about ±3% based on repeated injections of known standards.

**RESULTS**

The standard curve yielded the following relationship: $a = 0.0063; b = 0.0290$; where:

$$y = \frac{m/z 190 \text{ peak height}}{m/z 194 \text{ peak height}} x = \text{ng ABA}; \text{ when } y = ax + b.$$

The $b$ term accounts for any contribution from d6ABA or from [3H]ABA to the m/z 190 peak. The plot of this curve was linear through the concentrations tested (0 to 25 ng): $r = 0.9986$.

Endogenous ABA levels showed an increase with drought at

**Table 1. Levels of ABA in the 3-mm Apical Segment of Sunflower Roots, after 24, 72, and 168 h of Drought**

<table>
<thead>
<tr>
<th>Duration of Drought</th>
<th>Trial 1</th>
<th>Trial 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>h</td>
<td>ABA</td>
<td>Leaf ψ</td>
</tr>
<tr>
<td></td>
<td>ng g⁻¹ dw⁻¹</td>
<td>MPA ± SD</td>
</tr>
<tr>
<td>24 Control</td>
<td>65</td>
<td>-0.38 ± 0.08</td>
</tr>
<tr>
<td>Drought</td>
<td>418</td>
<td>-0.76 ± 0.15</td>
</tr>
<tr>
<td>72 Control</td>
<td>106</td>
<td>-0.42 ± 0.12</td>
</tr>
<tr>
<td>Drought</td>
<td>2048</td>
<td>-1.47 ± 0.18</td>
</tr>
<tr>
<td>168 Control</td>
<td>275</td>
<td>-0.63 ± 0.08</td>
</tr>
<tr>
<td>Drought</td>
<td>1630</td>
<td>-2.07 ± 0.24</td>
</tr>
</tbody>
</table>

*dw, dry weight.*
Table II. Levels of ABA in 3-mm Apical Root Segments Which Had Been Excised from 11-d-Old Seedlings and Then Dessicated or Kept Hydrated at Room Temperature for 1 h

<table>
<thead>
<tr>
<th>Trial</th>
<th>Treatment</th>
<th>ABA (ng g⁻¹ dw⁻¹)</th>
<th>Dew point (±</th>
<th>Chardakov</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>sd) MPa</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Dessicated</td>
<td>1225</td>
<td>Not measured</td>
<td>Not measured</td>
</tr>
<tr>
<td>2</td>
<td>Dessicated</td>
<td>4977</td>
<td>Not measured</td>
<td>Not measured</td>
</tr>
<tr>
<td>3</td>
<td>Dessicated</td>
<td>700</td>
<td>−1.79 ± 0.37</td>
<td>−1.56 &gt; Ψ &gt; −2.11</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>94</td>
<td>0.00</td>
<td>Ψ &gt; −0.08</td>
</tr>
<tr>
<td></td>
<td>Effluxed into bathing solution</td>
<td>59</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>4</td>
<td>Dessicated</td>
<td>1654</td>
<td>−2.33 ± 0.41</td>
<td>−2.11 &gt; Ψ &gt; −2.69</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>104</td>
<td>−0.09 ± 0.05</td>
<td>Ψ &gt; −0.08</td>
</tr>
<tr>
<td></td>
<td>Effluxed into bathing solution</td>
<td>81</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

* dw, dry weight; N.A., not applicable.

all sample times in experiment 1 (Table I). After 24 h of drought, the increase was 6.4-fold in trial 1 and 3.1-fold in trial 2 relative to control values. After 72 h, the increases were much greater: 19.3-fold in trial 1 and 24.6-fold in trial 2 relative to control values. After 168 h, the increases relative to controls declined: 5.9-fold in trial 1 and 3.5-fold in trial 2. Overall, the trends were similar in trials 1 and 2: initially ABA levels increased rapidly with drought, but then tended to level off or decline as stress continued and became more severe. Leaf Ψ, although not an accurate monitor of stress at the root tip, does give some indication of the increasing degree of drought stress encountered by the plant. At 24 h, the sunflower seedlings showed few visible signs of stress. By 72 h, the droughted plants were suffering noticeably: chlorosis of leaves was evident, and growth was obviously reduced relative to control plants. After 168 h of drought, plants were severely stressed; some tissue necrosis had occurred in both root and shoot tissue. Care was taken to avoid harvesting necrotic tissue. The ABA levels in the root tips of control plants increased about 2- to 4-fold by 168 h relative to earlier control samples. There is no obvious explanation for this, although by h 168, the plants had begun to outgrow the capacity of the aerenchyma chambers and were under mild stress, as indicated by leaf Ψ (Table I).

In the second experiment, where excised root tips were allowed to dessicate at room temperature, the increases relative to controls were remarkable: up to about 50 times control levels (Table II). Control plants for this experiment are the same as for 24 h in experiment 1. There was an appreciable disparity between absolute levels of ABA (4-fold) in the two trials of experiment 2. This was due, perhaps, to variability in the method of stressing. In the repeat trials (trials 3 and 4, Table II), root tip Ψ was monitored as an indicator of stress. In separate tests, the Ψ of root tips dessicated for 1 h at room temperature varied from −1.5 to −2.8 MPa. The Ψ of the sample root tips varied considerably as did the ABA levels in the samples (Table II). It would seem that variation in the levels of stress accounts at least partially for the disparity in absolute levels of ABA in the dessicated samples.

Nevertheless, in all four trials, the trend was the same: ABA levels increased dramatically in the excised root tips subjected to rapid dessication. This experiment shows that the apical 3 mm possesses the capacity to produce ABA in large quantity and to produce it rapidly in response to drought. ABA levels in the control samples which had been excised and kept hydrated were in the same range as aeroponically grown control samples. This indicates that the dessication treatment, not excision, was responsible for the large increase in ABA levels in the dessicated root tips. The Ψ of the hydrated control samples was near 0.00 MPa (Table II). This compares with 0.11 ± 0.04 MPa for the root tips of aeroponically grown control plants. The bathing solution used to hydrate excised control root tips contained measurable amounts of ABA; this indicates some efflux of ABA from well-watered root tips. Efflux may be a means of keeping ABA levels low in well-watered roots.

The data from experiment 1 were tested by three factor analysis of variance comparing treatments (droughted versus control), h of treatment, and trials. First-order interactions were not significant at the p < 0.05 level. Of the main effects, only treatment was significant at the p < 0.05 level. This indicates that the chief factor involved in the differences between readings was the drought treatment. No statistical analysis was possible for experiment 2.

**DISCUSSION**

There are two noteworthy observations from these results: (a) levels of ABA in the root apices of sunflower increase with drought stress; and (b) the apical 3-mm segment of the root is capable of producing ABA in response to drought.

Watts et al. (23) have suggested that ABA mediates drought responses indirectly through its effect on solute transport. However, the fact that ABA levels rise dramatically in the root apices suggests that, in sunflower at least, ABA may have a more direct effect on root development.

ABA has been variously reported to both promote and inhibit root growth. Most studies found ABA inhibitory to root growth and extension (for a full discussion, see 14). The effects may be concentration related; in lower concentration, ABA may promote growth (15, 23). In sunflower seedlings, ABA (10 μM) inhibits overall root growth (5) and extension (J. M. Robertson, unpublished data). Other studies show that exogenously applied ABA will affect developmental processes at the root apex (1, 5).

In most cases, water stress reduces root growth (3, 5, 7, 16, 23), although mild stress can increase extension (4, 21, 23). This is thought (4, 21) to be the result of an osmoregulation process which preferentially maintains root growth during periods of water stress. It is unlikely, however, that vigorous root growth could continue under severe or prolonged drought; osmoregulation places a high carbon demand on the shoot (13), and this demand would likely become prohibitive with higher levels of stress. As mentioned above, ABA inhibits root growth at higher
concentrations, but may promote growth at lower concentrations. Controlling root growth through changes in the endogenous levels of ABA may provide the plant with a mechanism to balance the need for expansion of the root system in droughty soils with the restrictions placed upon it by reduced carbon supplies from the shoot. If so, then increasing ABA levels at the root apices could act as an adaptive mechanism which serves to optimize available reserves within a changing environment.

If changes in ABA levels at the root apex do indeed serve to regulate root development during periods of drought stress, then it would seem that the root apex, including the apical meristem and its immediate derivatives, has the capacity to perceive and respond directly to the stress (Table II).

To date, the evidence that ABA acts as a developmental regulator of drought adaptive responses is slight. It would be rash to expect that variations in the levels of one plant hormone could account for all developmental changes in droughted sunflower roots; yet, large increases in ABA at the root apices are almost certain to affect root growth and development. We are currently engaged in comparative studies on the effects of drought and ABA on the root apical meristem.

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