Wounding *Nicotiana tabacum* Leaves Causes a Decline in Endogenous Indole-3-Acetic Acid

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**ABSTRACT**

We have previously observed that auxin can act as a repressor of the wound-inducible activation of a chimeric potato proteinase inhibitor II-CAT chimeric gene (pin2-CAT) in transgenic tobacco (*Nicotiana tabacum*) callus and in whole plants. Therefore, this study was designed to examine endogenous levels of indole-3-acetic acid (IAA) in plant tissues both before and after wounding. Endogenous IAA was measured in whole plant tissues by gas chromatography-mass spectrometry using an isotope dilution technique. 13C-Labeled IAA was used as an internal standard. The endogenous levels of IAA declined two- to threefold within 6 hours after a wound. The kinetics of auxin decline are consistent with the kinetics of activation of the pin2-CAT construction in the foliage of transgenic tobacco.

When plant foliage is mechanically wounded a series of specific and nonspecific plant defenses are induced (9, 14). One group of the well studied defense products are the proteinase inhibitors of solanaceous plants. These proteinase inhibitors are activated at the transcriptional level following wounding (13). However, their kinetics of induction (mRNA begins to appear 2 to 4 h after wounding) makes them distinct from other wound-inducible genes from the lignin and phytoalexin biosynthetic pathways (9). Genes encoding the proteinase inhibitors from both tomato and potato have been isolated and characterized (6, 12, 19, 23, 27).

Transgenic plants have been made with both proteinase inhibitor I and II and the expression of these genes in plants has been well studied (15, 23, 27). When tobacco plants bearing the pin2-CAT gene were examined for their expression of CAT protein, they expressed CAT protein in a manner which was identical with the wild-type expression of proteinase inhibitor II in young tomato plants (26, 28). Therefore, these plants make a good model system for the study of the wound-inducible genes. Further, when introduced into potato, the same chimeric gene confers both wound-induced and tuber specific expression on the transgenic plants (17).

Because these transgenic plants are a good model system for the study of wound-inducible genes, we have previously investigated the role of the five classical plant hormones on the induction of the pin2-CAT gene. These studies revealed that auxin, when present at near physiological levels, can repress the synthesis of CAT protein in the transgenic callus and also in plants (18).

This specific repression of the inhibitor II gene activation led us to address the question of IAA levels in whole plants following wounding. In this study we have measured the levels of endogenous IAA in the foliage of whole tobacco plants by an isotope dilution technique coupled with a GC-MS detection system.

**MATERIALS AND METHODS**

Plants

The plants used in this study were tobacco, *Nicotiana tabacum* cv Xanthi. Both wild-type and transformed progeny plants were used. The transformed plants were previously described (28). They were an R4 homozygous line of transgenic plants (four self-pollinations after regeneration, R0) containing a pin2-CAT construction which was also previously described (27). All plants were used once and then never again, so that multiple wounds did not occur on the same plant.

**Materials**

[13C]IAA (3a,4,5,6,7,7a-hexa[13C]indole-3-acetic acid) was a gift of Dr. Jerry D. Cohen (USDA Agricultural Research Center) to Dr. C. E. LaMotte of the Iowa State University Plant Hormone Analysis Facility. Unlabeled IAA was from Sigma Chemical Co. Butylated hydroxytoluene was obtained from Fisher Chemical Co. Diazomethane was generated according to the method of Feiser and Feiser (11) and used within 2 months of generation. The diazomethane was stored in 50 mL aliquots at −20°C. All other materials were obtained locally.

Wounding

Only large fully expanded leaves of the transgenic tobacco plants were used in this study. The leaves were wounded by repeated pinching with a pair of surgical hemostats which caused a large, severe wound (2 cm wide and extending from the leaf edge to the midvein) across the central portion of the leaf as previously described (28). To avoid interplant and interleaf variability in the IAA assays, we utilized a half-leaf assay. In this assay, one-half of the leaf lamina (from one edge up to but not including the midvein) was removed at the start.

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of the experiment and immediately frozen in liquid nitrogen. The opposite half-leaf was wounded as above and left on the plant for the incubation period. After the indicated time, the remaining wounded half of the leaf lamina (up to but not including the midvein) was removed and also frozen in liquid nitrogen. Sometimes several leaves from a single plant were processed together by this half-leaf assay to provide sufficient material for analysis. The tissues were stored at −70°C until analysis. Both the unwounded sample and the wounded sample from the same plant were processed for IAA analysis on the same day as described below.

CAT assays were conducted as previously described (18) and used to confirm that the wounding process was sufficient to induce this gene system.

**IAA Analysis**

These analyses were conducted at the Iowa State University Plant Hormone Analysis Facility. IAA analysis was conducted by an isotope dilution technique (20) using the modified methods of Cohen et al. (8). Three grams of leaf tissue were used for each assay of IAA in the plant tissues. The leaf tissue previously stored at −70°C, was ground with 3 g of washed sea sand in a mortar and pestle precooled with liquid nitrogen. Ten milligrams of butylated hydroxytoluene per gram of tissue was added during this grinding step. A quantity of acetone (9.6 mL) was added to bring the final concentration of acetone to 80%, assuming that fresh leaves contain 80% water. An additional 20 to 25 mL of 80% acetone was added to facilitate grinding. A known amount of [13C]IAA was added to each sample at the beginning of extraction. Following this extraction, the ground material was rapidly filtered through two layers of Whatman No. 1 paper. The extraction was repeated (2 times, 20 min) with 80% (v/v) acetone:H2O. The filtrate and washings were pooled into a 100 mL pear-shaped flask and reduced to the aequous phase by rotary evaporation at 35°C under vacuum. The pH was adjusted to 3.0 with HCl. The IAA was then partitioned into ethyl acetate and the ethyl acetate was removed at 35°C under reduced pressure. A highly viscous, pigmented, oily residue remained. The pigments were removed by dissolving the residue in 15 mL (3 × 5 mL) of 70% methanol and passing the methanolic solution through a C-18 Sep-Pak in small aliquots. The Sep-Pak was prerased with 10 mL of 100% methanol followed by 10 mL of 70% methanol. The pigments bound to the column, while the IAA eluted freely. The eluents were pooled and roto-evaporated to dryness at 35°C. The residue which contains IAA was dissolved in a total of 1.2 mL of 20% methanol/80% 0.1 M acetic acid. The entire sample was applied to a 1 × 25 cm Phenomenex HPLC column containing a 5 μm Apex octadecyl (C-18) silica. A gradient was developed from 20% methanol/80% 0.1 M acetic acid to 100% methanol. In this gradient, IAA elutes between 63 to 73% methanol. Those fractions containing IAA were pooled and concentrated in a roto-evaporation flask to remove the methanol and acetic acid.

The remaining material contained the free IAA. It was dissolved in 50 μL of methanol and methylated with diazomethane for 30 min at room temperature. The partially purified IAA-methyl ester was concentrated to a small volume (10 μL) and 2 μL aliquots were injected into a Hewlett-Packard 5890 gas chromatograph. We utilized a splitless injection so that 100% of the GC effluent was bled into a Hewlett-Packard 5970 quadrupole mass spectrometer. This spectrometer utilized an electron impact method to generate ionization. Ions with mass to charge ratios of 189 and 195 (the molecular ion) and 130 and 136 (the major fragment, IAA minus the carboxymethyl group) were selectively monitored and the data were stored in digital form. GC-MS parameters were set according to Cloud (7). The recovery of IAA by this method was estimated by two methods. With this system, Cloud reported a recovery of approximately 50% using radiolabeled IAA. We have also monitored the total ion count for [13C]IAA. By comparison with ion counts from known amounts of [13C]IAA, we calculate an average recovery of [13C]IAA that ranges from 47 to 67% for the experiments described herein.

**Calculations**

The amount of IAA in leaves was calculated by the isotope dilution equations (20) as follows:

\[ Y = X/R \left( \frac{C_i}{C_f} - 1 \right) \]  

where:

\[ Y = \text{amount of naturally occurring IAA (in ng),} \]
\[ X = \text{known amount of [13C]IAA added as internal standard (in ng),} \]
\[ R = \text{the ratio of the fraction of endogenous IAA that has a peak at m/z 130 to the fraction of the internal standard that is fully substituted and has an ion at m/z 136 (8).} \]
\[ C_i = \text{initial concentration (% of [13C]IAA [before extraction]} \]
\[ C_f = \text{final concentration (% of [13C]IAA [after extraction]} \]
\[ X/R = \text{methyl-[13C]IAA peak area} \]
\[ \times 100\% \]
\[ + \text{methyl-[13C]IAA peak area} \]

The method was always determined in parallel for each set of determinations. A known amount of [13C]IAA (Y) was added to an estimated amount of [13C]IAA (X/R, the exact amount was unknown). The [13C]- and [13C]IAA were mixed and methylated, then processed through GC-MS. X/R was then calculated by rearranging Equation 1 to obtain the following:

\[ X/R = \frac{Y}{\left( \frac{C_i}{C_f} - 1 \right)} \]

Therefore, with the amount of [13C]IAA (X/R) known, the amount of naturally occurring IAA in leaf sample (13C]IAA can be calculated with Equation 1.

**RESULTS AND DISCUSSION**

Tobacco foliage has been previously examined for levels of IAA and found to contain approximately 10 to 20 ng of free IAA per gram of leaf tissue (2). Therefore, to determine whether our IAA assay was sufficiently sensitive to measure...
these low concentrations of IAA in tobacco leaf tissues, we constructed a calibration curve by adding a standard amount of \([{}^{13}\text{C}]\text{IAA}\) to samples containing varying amounts of \([{}^{12}\text{C}]\text{IAA}\) ranging from 2 to 30 ng. These samples were extracted and IAA isolated according to the method outlined above. \([{}^{13}\text{C}]\)- and \([{}^{12}\text{C}]\text{IAA}\) was quantitated with the GC-MS assay. We obtained a linear standard curve over the range of interest, and we were able to accurately detect low amounts (2 ng) of IAA. Thus, the isolation and quantitation of IAA in our hands was sufficiently sensitive to determine the amounts of IAA in tobacco foliage.

Our initial studies to measure IAA in plant tissues found high variability in the levels of IAA in different leaves. This variability may relate to the positioning of the leaf on the plant as has been previously observed (24); however, even among adjacent leaves, we observed sufficiently high variation in IAA levels of the unwounded state that we had little confidence in the reproducibility of the assay when different leaves were used for the wounded and unwounded samples. Because of this variability, we began to use an assay in which half of the leaf was removed for a time zero control, and the other half of the leaf was treated to determine wounded levels of IAA (Fig. 1).

When transgenic plants were assayed, a small portion of the wounded and unwounded leaf material was utilized for CAT assays to determine the activity of the pin2-CAT gene. Routinely, we found that in the time zero sample there was no CAT activity, after 3 h there was low level of induction, and at later timepoints, CAT activity was well induced (28). Because we found that there are significant differences in IAA content of unwounded leaves between plants and among different leaves on the same plant, the data are expressed as percent decline in IAA content in the wounded halves of leaves relative to the unwounded leaf halves. Data expressed in this manner utilizing an “internal” time zero control are much more consistent between replicates even though the levels of IAA in unwounded leaves varies between replicates.

These analyses indicate that the levels of IAA found in the foliage declines following wounding of the foliage (Table 1). This decline is maximal between 3 and 6 h after the wound. Eventually, the levels of IAA decline by two- to threefold in the 24 h following the wound. The kinetics of this decline in IAA levels is very similar to the kinetics of activation of the proteinase inhibitor gene system in either normal tomato plants (13), or in transgenic tobacco plants (28). This decline in IAA levels in wounded tobacco foliage is consistent with a role of IAA in the regulation of the inhibitor II gene system.

In other systems, such as sweet potato tubers, IAA levels are low in unwounded tissues (1–5 ng/g fresh weight) and increase dramatically following wounding reaching a maximum after 18 h (25). This observation raises an interesting point about the tuber specific regulation of proteinase inhibitors. In \(\text{Solanum tuberosum}\) tubers the proteinase inhibitor genes are normally expressed at high levels, then following wounding, transcription of the inhibitor genes are shut off (17). While sweet potato tubers arise from distinctly different structures than potato tuber tissue, this finding does raise the question of whether IAA can also participate in the regulation of expression of the inhibitor genes in potato tubers as well as in the foliage.

Other investigators have implicated a variety of other biochemical compounds in the regulation of the inhibitor II gene system. Among these compounds are endogenous plant cell wall oligosaccharide fragments (3, 4), fungal cell wall fragments (29), sucrose (16), abscisic acid (21), methyl jasmonate

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**Figure 1.** Before wounding with hemostats, one-half of each leaf was removed and immediately frozen in liquid nitrogen for a time zero control. At various times postwounding, the remaining leaf blade halves minus the central midvein were harvested, frozen, and analyzed on the same day as the time zero samples.

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**Table 1. Levels of IAA following Wounding of Tobacco Leaves**

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>Unwounded</th>
<th>Wounded</th>
<th>Percentage of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>h</td>
<td>ng IAA per g fresh wt*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>18.9 ± 2.0</td>
<td>18.6 ± 3.0</td>
<td>98.1 ± 7.5</td>
</tr>
<tr>
<td>3</td>
<td>10.5 ± 0.5</td>
<td>8.8 ± 1.1</td>
<td>84.3 ± 9.0</td>
</tr>
<tr>
<td>6</td>
<td>12.7 ± 3.4</td>
<td>8.2 ± 0.2</td>
<td>68.0 ± 17.9</td>
</tr>
<tr>
<td>12</td>
<td>18.4 ± 7.6</td>
<td>8.9 ± 2.6</td>
<td>54.9 ± 28.1</td>
</tr>
<tr>
<td>24</td>
<td>16.2 ± 5.9</td>
<td>7.5 ± 3.9</td>
<td>44.4 ± 8.0</td>
</tr>
</tbody>
</table>

* One-half of the leaf lamina (from one edge up to but not including the midvein) was removed at time 0 and immediately frozen in liquid nitrogen. The opposite half was wounded by repeated pinching with a pair of surgical hemostats in a 2 cm wide band of tissue. These wounded half-leaves were incubated on the plant for the indicated times, and then the wounded half of the leaf lamina (up to but not including the midvein) was removed and frozen in liquid nitrogen. For each time point, the two samples were processed sequentially and analyzed on the same day. All data are expressed as percentage of IAA present in the wounded leaf halves divided by that present in the control unwounded leaf halves (n = 3).
(10), and IAA (18). All of these compounds, with the exception of IAA, have inducing effects on the expression of the inhibitor II gene system, whereas IAA represses the expression of the genes. We are currently trying to determine the hierarchy of the inductive and repressive effects played by these compounds in inducing the inhibitor II gene within the plant.

Further, we are interested in determining the effect that each of these positive regulating compounds has on the levels of IAA within the plant. There are several reports (1, 5, 22) that exogenous application of ABA to plant tissues can cause a decrease in the endogenous levels of IAA within those tissues. These effects are thought to occur through either influencing auxin transport or through direct effects on the auxin metabolism. Thus, some of the many positive regulating compounds indicated above, especially ABA, may act to induce inhibitor gene activation by decreasing the levels of IAA within the responding tissues. Experiments to examine this are currently underway.

Our working hypothesis is that endogenous levels of IAA in unwounded plant tissues are sufficient to maintain the inhibitor II gene system in a repressed state so that the genes are not expressed. However, following a wound, the levels of IAA in bulk tissues decline by two- to threefold, allowing a derepression of the gene system with concomitant expression of the CAT protein. The exact role of IAA in the regulation of the inhibitor II gene system is unknown and will require further elucidation. The roles of the other positive regulators will also need to be determined within the framework of the IAA derepression of the inhibitor II gene system.

Also, the mechanism which is responsible for the decline in IAA levels is not yet understood. The IAA pool size could be influenced at one of several points, including the biosynthesis of IAA, its degradation, or the formation of amide or ester storage forms of IAA. Indeed, the decrease in IAA pools in leaves could even be influenced through IAA transport.

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