Auxin Biosynthesis in Pea: Characterization of the Tryptamine Pathway

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One pathway leading to the bioactive auxin, indole-3-acetic acid (IAA), is known as the tryptamine pathway, which is suggested to proceed in the sequence: tryptophan (Trp), tryptamine, N-hydroxytryptamine, indole-3-acetaldoxime, indole-3-acetaldehyde (IAAld), IAA. Recently, this pathway has been characterized by the YUCCA genes in Arabidopsis (Arabidopsis thaliana) and their homologs in other species. YUCCA is thought to be responsible for the conversion of tryptamine to N-hydroxytryptamine. Here we complement the genetic findings with a compound-based approach in pea (Pisum sativum), detecting potential precursors by gas chromatography/tandem-mass spectrometry. In addition, we have synthesized deuterated forms of many of the intermediates involved, and have used them to quantify the endogenous compounds, and to investigate their metabolic fates. Trp, tryptamine, IAAld, indole-3-ethanol, and IAA were detected as endogenous constituents, whereas indole-3-acetaldoxime and one of its products, indole-3-acetonitrile, were not detected. Metabolism experiments indicated that the tryptamine pathway to IAA in pea roots proceeds in the sequence: Trp, tryptamine, IAAld, IAA, with indole-3-ethanol as a side-branch product of IAAld. N-hydroxytryptamine was not detected, but we cannot exclude that it is an intermediate between tryptamine and IAAld, nor can we rule out the possibility of a Trp-independent pathway operating in pea roots.

Auxin is a key plant growth hormone, involved in processes as diverse as branching, gravitropism, phototropism, and seed development (Davies, 2004). However, the biosynthetic pathways leading to the main auxin in plants, indole-3-acetic acid (IAA), are not well understood. Although there is good evidence that the amino acid Trp is an early precursor (Gibson et al., 1972; Wright et al., 1991; Tsurusaki et al., 1997), several routes from Trp to IAA have been proposed, and for any given species it is not clear which route or routes occur. The possible Trp-dependent pathways in higher plants are the indole-3-pyruvic acid (IPyA) pathway (Stepanova et al., 2008; Tao et al., 2008), the tryptamine (YUCCA) pathway (Zhao et al., 2001), the indole-3-acetaldoxime (IAOx) pathway (Bartel et al., 2001), and the indoleaceticamide pathway (Pollmann et al., 2002), on the basis of the first metabolite of Trp (Fig. 1). In addition, a possible Trp-independent pathway has been proposed (Normany et al., 1993), bypassing Trp completely, further complicating the process of IAA biosynthesis.

Since 2001, there has been renewed interest in the tryptamine route to IAA, after the discovery and functional analysis of the Arabidopsis (Arabidopsis thaliana) YUCCA gene, reported to encode the enzyme for converting tryptamine to N-hydroxytryptamine (Zhao et al., 2001, 2002). On the basis of the Zhao et al. (2001, 2002) reports, tryptamine pathways have generally been proposed in the sequence: Trp, tryptamine, N-hydroxytryptamine, IAOx, indole-3-acetaldehyde (IAAld), IAA (Fig. 1; Woodward and Bartel, 2005). Recently, however, Sugawara et al. (2009) suggested that IAOx be removed from the tryptamine pathway, and that IAOx-dependent IAA biosynthesis operates only in the Brassicaceae. In Arabidopsis, this pathway can be important, at least in some circumstances, because when a side branch is impaired, as in the sur1 mutant, IAA levels increase dramatically (Sugawara et al., 2009). On the other hand, the IAOx pathway is not the only pathway operating in Arabidopsis, because genetically blocking the step Trp to IAOx does not always reduce IAA content, compared with wild-type plants (Sugawara et al., 2009). This means that in Arabidopsis, the tryptamine and/or IPyA and/or indoleaceticamide pathways compensate for the loss of the IAOx pathway. Interestingly, Sugawara et al. (2009) do not include IAAld in their tryptamine pathway, and their model implies instead that N-hydroxytryptamine is directly converted to IAA.

Turning to other species, it has been reported that tryptamine is not present in pea (Pisum sativum; Schneider et al., 1972), despite being present in tomato (Solanum lycopersicum; Cooney and Nonhebel, 1991),
rice (*Oryza sativa*; Ishihara et al., 2008), Arabidopsis (*Sugawara et al., 2009*), and barley (*Hordeum vulgare*; Schneider et al., 1972). In tomato, although tryptamine is relatively abundant, and early metabolism studies indicated the conversion of tryptamine to IAA via IAAld (Schneider et al., 1972), Cooney and Nonhebel (1991) cast doubt on the role of tryptamine after studying patterns of labeling after incubation of plants with deuterated water. Again, in tobacco (*Nicotiana tabacum*), Songstad et al. (1990) showed that while tobacco plants overexpressing a Trp decarboxylase accumulated very high levels of tryptamine, IAA levels were unaffected. Although this result has been interpreted as evidence against the involvement of tryptamine (Bartel et al., 2001), another explanation is that excess tryptamine is converted to compounds via a side branch or side branches, although these are not well studied. Finally, the compound N-hydroxytryptamine is relatively unknown, with no reports of its presence in plants to date.

It is clear, therefore, that the tryptamine pathway to IAA remains poorly understood. In this article, we further characterize the pathway, using the garden pea as a model species. We report on the presence/absence and levels of the putative endogenous intermediates, as determined by gas chromatography/tandem mass spectrometry (GC/MS/MS), and investigate their metabolic fates using $^{[14]}$C and deuterated versions of the compounds. Our evidence indicates that key elements of the tryptamine pathway are operative in pea roots.

**RESULTS**

**Chemical Synthesis of IAA Precursors**

We synthesized labeled forms of L-Trp, tryptamine, IAOx, and indole-3-acetonitrile (IAN), as confirmed by GC/MS/MS, all with up to five deuteriums on the indole ring, as well as a deuterated form of indole-3-ethanol (IEt). For the $^{[2]}$H$_5$-labeled compounds, over 50% of the material was $^{[2]}$H$_5$ labeled, as shown in Supplemental Table S1. However, IAAld was too unstable to allow the production of a labeled form, and in fact, spontaneously converted to IAA when left at room temperature (as previously suggested by Ernstsen et al., 1986). Therefore, it was necessary to use sodium borohydride to convert IAAld to IEt for quantification, making the $^{[2]}$H$_2$IEt invaluable as an internal standard. We also synthesized unlabeled N-acetyltryptamine and unlabeled N-hydroxytryptamine.

**Quantification of IAA Precursors**

The identification and quantification of putative IAA precursors provides insight into both their involvement within the pathway and their distribution in the plant. The putative IAA precursors Trp, tryptamine, IAAld, and IEt were identified and quantified in pea shoot and root extracts. The identifications were based on GC/MS/MS analyses, using synthesized standards for reference (as shown for tryptamine in Fig. 2). The putative precursor IAN was not detected in pea tissue (the $^{[3]}$H$_5$IAN internal standard added to the extract was not diluted by endogenous IAN), nor was IAOx, despite both being present in Arabidopsis seedling extracts (IAN: 9,720 ng g fresh weight $^{[FW]}$; IAOx: 1.7 ng g $^{[FW]}$; Sugawara et al., 2009).

In pea, Trp levels ranged from 6,000 to 30,000 ng g $^{[FW]}$, with a pattern of distribution very similar to that of IAA itself (10–75 ng g $^{[FW]}$). Levels of both these compounds were higher in internode and apical bud tissue than in root and leaf tissue ($P < 0.05$; Table I). After IAAld was converted to IEt, and total IEt...
quantified, IAAld levels were calculated by subtracting from that total the IEt level found in extracts not treated with sodium borohydride. The level of IAAld was between 2 and 21 ng g \(\text{FW}^{-1}\), higher in the roots compared with the apical bud, leaf, and internode tissue \((P < 0.001;\) Table I). The levels of IEt, and particularly tryptamine (as shown in Fig. 2, in root tissue), were very low in all tissues.

\([\text{H}_5]\)Tryptamine Metabolism Studies

The labeled forms synthesized in this study were essential also for metabolism studies. Roots of sterile whole pea seedlings were incubated with \([\text{H}_5]\)Trp for 24 h, and both the products and the substrates were quantified using appropriately labeled internal standards. A time-course study found that \([\text{H}_5]\)Trp moved into the roots by 6 h, and the compound was detected in roots at all subsequent time points (every 6 h for 24 h). In the experiment represented in Table II, endogenous IAA was diluted with \([\text{H}_5]\)IAA, showing that \([\text{H}_5]\)Trp was taken up and converted to IAA; the deuterium incorporation was approximately 8.3% of the total root IAA pool (Fig. 3). The percentage of labeling with \([\text{H}_5]\) material for each putative IAA precursor is shown in Table II. The percentage of \([\text{H}_5]\)tryptamine was 160% of the endogenous tryptamine pool and that of IAAld, 61.5%. Interestingly, IEt had a relatively high (190%) \([\text{H}_5]\) incorporation (Table II). In no case was deuterium found when extracts were tested for IAN, nor was any endogenous IAN found within the plant material. Similarly, no conversion of \([\text{H}_5]\)Trp to \([\text{H}_5]\)IAOx was found after 24 h. Furthermore, in none of the experiments was any deuterium-labeled compound found in the control cases (i.e. tubes with plants but no substrate), and in selected experiments no label was found in shoot material. Moreover, \([\text{H}_5]\)Trp left in tubes without plant material showed no conversion to any of the compounds analyzed.

\([\text{H}_5]\) and \([^{14}\text{C}]\)Tryptamine Metabolism Studies

For this study, it was important to determine whether tryptamine can be converted to subsequent intermediates within the pathway. After conducting a dose-response study, it was found that a \([\text{H}_5]\)tryptamine concentration of 7 to 10 \(\mu M\) was sufficient to allow the roots to take up and metabolize tryptamine to intermediates in the pathway. Quantitation of \([\text{H}_5]\)tryptamine in roots using a \([^{14}\text{C}]\)-labeled internal standard indicated that the substrate was taken up rapidly (within 6 h), and this compound was detected

<table>
<thead>
<tr>
<th>Compound</th>
<th>Root</th>
<th>Internode</th>
<th>Leaf</th>
<th>Apical Bud</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp</td>
<td>6.514 ± 287</td>
<td>29.701 ± 2.403</td>
<td>14.140 ± 1.293</td>
<td>21.176 ± 2.053</td>
</tr>
<tr>
<td>Tryptamine</td>
<td>0.424 ± 0.21</td>
<td>0.021 ± 0.006</td>
<td>0.038 ± 0.004</td>
<td>0.024 ± 0.006</td>
</tr>
<tr>
<td>IAAld</td>
<td>20.35 ± 2.18</td>
<td>0.68 ± 0.04</td>
<td>1.15 ± 0.12</td>
<td>3.94 ± 0.08</td>
</tr>
<tr>
<td>IEt</td>
<td>1.59 ± 0.14</td>
<td>0.18 ± 0.01</td>
<td>0.23 ± 0.01</td>
<td>1.60 ± 0.14</td>
</tr>
<tr>
<td>IAA</td>
<td>17.49 ± 0.66</td>
<td>76.27 ± 1.22</td>
<td>9.68 ± 0.50</td>
<td>57.70 ± 0.72</td>
</tr>
</tbody>
</table>

Table I. The level of IAA precursors in pea tissue

The mean levels (ng g \([\text{FW}^{-1}]\) of Trp, tryptamine, IAAld, IEt, and IAA in the root, internode, leaf, and apical bud of 3-week-old pea tissue as determined by GC/MS/MS. Data are from three separate experiments in which environmental conditions were similar. Shown are means ± SE \((n = 2 \text{ for IAAld and IEt [experiment 1] and 3 for Trp and IAA [experiment 2, and tryptamine [experiment 3]}}\).
in roots at all subsequent time points (every 6 h for 24 h). The extent of uptake was also monitored by using \([^{14}\text{C}]\)tryptamine as the substrate (7 \(\mu\text{M}\)). For example, in one experiment, 88% of the label was taken up by the roots after 24 h, as determined by scintillation counting. Relative to endogenous levels, this value was very high: Endogenous levels of \([^{2}\text{H}_5]\)triptamine were approximately 0.4 ng g \((\text{FW})^{-1}\).

After incubation of sterile roots with \([^{2}\text{H}_5]\)tryptamine, GC/MS/MS analysis showed that approximately 5% of the total IAA pool (Fig. 4), and 32% of the IAAld pool was \([^{2}\text{H}_5]\) labeled (Table III). A high degree of deuteration labeling was also detected in IEt, with levels of \([^{2}\text{H}_5]\)IEt 30.2 times higher than endogenous IEt, at 4.98 ng g \((\text{FW})^{-1}\) (Table III). No evidence for the conversion from \([^{2}\text{H}_5]\)tryptamine to \([^{2}\text{H}_5]\)IAOx was found. However, a HPLC profile of \([^{14}\text{C}]\)triptamine-incubated sterile roots (Fig. 5) showed strong conversion of \([^{14}\text{C}]\)triptamine to an initially unidentified product (fraction 20; Fig. 5). On the basis of first principles MS evidence—the loss of 59 mass units \((\text{acetamide})\) from \([\text{M} + \text{H}]^{+}\) 205 to mass-to-charge ratio \((m/z)\) 146—this was postulated to be \([^{14}\text{C}]\)N\(_2\)-acetyltryptamine. This was ultimately confirmed by coelution with synthetic N\(_2\)-acetyltryptamine and an identical MS/MS spectrum (allowing for the mass difference between \([^{14}\text{C}]\) and \([^{12}\text{C}]\); as shown by liquid chromatography (LC)/MS analysis in Fig. 6). The \([^{14}\text{C}]\)N\(_2\)-acetyltryptamine product, which had a \([^{14}\text{C}]\)-to-\([^{12}\text{C}]\) ratio identical to that of the \([^{14}\text{C}]\)triptamine substrate, was then used as an internal standard to quantify, if possible, endogenous N\(_2\)-acetyltryptamine in an extract from 4 g of pea root material. No dilution of the internal standard was detected, indicating that the endogenous compound is either absent or present at very low levels (less than 0.1 ng g \((\text{FW})^{-1}\)).

Analyses by LC/MS and GC/MS showed that the conversion of \([^{14}\text{C}]\)triptamine to \([^{14}\text{C}]\)N\(_2\)-acetyltryptamine far exceeded the conversion of the substrate to \([^{14}\text{C}]\)IAA or \([^{14}\text{C}]\)IEt. N\(_2\)-acetyltryptamine is not thought to be involved in IAA biosynthesis. Again, tubes containing \([^{2}\text{H}_5]\)triptamine, but no plant material, showed no evidence of metabolism of the substrate; the compound appeared stable when left in distilled water. After feeds of \([^{14}\text{C}]\)triptamine, there was no evidence from LC/MS of \([^{14}\text{C}]\)N\(_2\)-hydroxytryptamine as a metabolite (this compound elutes approximately 1 min later than tryptamine with the LC/MS method used, as shown with a standard synthesized in our laboratory) and it was therefore concluded that \([^{14}\text{C}]\)triptamine was not converted to \([^{14}\text{C}]\)N\(_2\)-hydroxytryptamine in pea roots, or if it was, that the levels were too low to detect. Endogenous N\(_2\)-hydroxytryptamine was likewise not detected (detection limit approximately 0.5 ng g \((\text{FW})^{-1}\)), although the absence of a labeled internal standard and the instability of N\(_2\)-hydroxytryptamine made the detection of this compound difficult.

**[\([^{2}\text{H}_5]\)IAN Metabolism Studies**

Despite the lack of labeling found in the IAN pool after incubation with \([^{1}\text{Trp}]\), and failure to detect IAN in pea root extracts, it was important to determine whether the machinery needed for the production of IAA from IAN was present within pea. It was formally possible that IAN was being created (albeit at an undetectable level) via another pathway, and subsequently converted to IAA. However, after incubation with \([^{2}\text{H}_5]\)IAN, no detectable amount of \([^{2}\text{H}_5]\)IAA was found in any case, indicating that the compound is not, in fact, involved. This finding was repeated and the plants analyzed for the presence of \([^{2}\text{H}_5]\)IAN itself; the compound was, indeed, taken up by the roots.

**Table II. Metabolism of \([^{2}\text{H}_5]\)Trp in pea roots**

Levels (ng g \((\text{FW})^{-1}\)) of endogenous and \([^{2}\text{H}_5]\)-labeled compounds, and percentage of \([^{2}\text{H}_5]\) incorporation for tryptamine, IAAld, IEt, IAA, and IAOx in pea roots after incubation with \([^{1}\text{Trp}]\). Data are representative of repeated experiments. Shown are data from two experiments; in one, internal standards were used to quantify endogenous and \([^{2}\text{H}_5]\)-labeled compounds. Both experiments yielded percentage incorporation data, and the means \(\pm\) SE are shown.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Endogenous</th>
<th>([^{2}\text{H}_5]) Labeled</th>
<th>% Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptamine</td>
<td>0.88</td>
<td>1.29</td>
<td>160 (\pm) 13.2</td>
</tr>
<tr>
<td>IAAld</td>
<td>26.4</td>
<td>17.0</td>
<td>61.5 (\pm) 2.5</td>
</tr>
<tr>
<td>IEt</td>
<td>0.49</td>
<td>1.02</td>
<td>190 (\pm) 20.5</td>
</tr>
<tr>
<td>IAA</td>
<td>9.60</td>
<td>0.37</td>
<td>8.31 (\pm) 3.15</td>
</tr>
<tr>
<td>IAOx</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>
As for IAN, although no metabolism of $[2H_5]$Trp to $[2H_5]$IAOx nor evidence of an endogenous pool of the compound was found in pea (Supplemental Fig. S1), we assessed the ability of the plants to metabolize $[2H_5]$IAOx. After incubation of pea roots with IAOx, a dilution of the IAA and IEt pools by their $[2H_5]$versions was found (Supplemental Figs. S2 and S3). The IAA pool was diluted with 10.46% $[2H_5]$, and the IEt pool more than 500%. No evidence of conversion of $[2H_5]$IAOx to $[2H_5]$IAN was observed.

DISCUSSION

Recently, there has been renewed focus on the tryptamine pathway as a route to IAA biosynthesis, based on genetic studies with Arabidopsis (for review, see Zhao, 2008). The present study provides clear physicochemical evidence that key elements of the tryptamine pathway (tryptamine, IAAld, IAA) can operate in the garden pea.

First, Trp, tryptamine, IAAld, and IEt were identified and quantified in pea tissues. It appears that in this species the tryptamine pathway does not involve high endogenous levels of intermediates: Although Trp was present at $\mu g g^{-1}$ levels, and IAA itself is reasonably abundant, the intermediates were present at lower levels, comparable to those of other hormone pathways, e.g. gibberellins (Ross, 1998), with the level of tryptamine particularly low. These endogenous levels indicate that the Trp-to-tryptamine conversion might be rate limiting in pea, and/or that tryptamine is metabolized rapidly. This is an interesting finding, as the putative next step in the tryptamine pathway to IAA (from tryptamine to $N$-hydroxytryptamine) is thought to be a rate-limiting step in Arabidopsis (Zhao et al., 2001).

Second, metabolism studies with labeled compounds indicated that tryptamine is an intermediate in peas, with $[2H_5]$Trp converted to $[2H_5]$tryptamine, and $[2H_5]$tryptamine to $[2H_5]$IAA. It has been reported previously that labeled Trp can be converted to IAA in pea roots (Mitchell and Davies, 1972), although in that case GC/MS analysis was not used, and the intermediates were not monitored. Here, monitoring the intermediates has shown a reduction of labeling in the sequence tryptamine, IAAld, and IAA, consistent with this being their sequence in the pathway (Nonhebel et al., 1993). Our findings differ somewhat from those of Cooney and Nonhebel (1991), who suggested that in tomato, tryptamine is derived from a different Trp pool to that used for IAA, and by implication that tryptamine is not converted to IAA. Our study clearly shows that tryptamine can be converted to IAA in pea roots. That tryptamine was metabolized to $N\text{-}\text{acetyltryptamine}$ is evidence that, like Trp itself, tryptamine can play a role in other pathways as opposed to a solely linear pathway from tryptamine to IAA.

It appears that a pathway including IAN and/or IAOx is not operative in pea, consistent with the findings of Sugawara et al. (2009), who found no evidence for either compound in rice, maize (Zea mays), and tobacco. Not only were the compounds not present within peas, but we could find no labeled IAN or IAOx after incubation with labeled L-Trp, or subsequent labeled IAA after incubation with IAN. Although IAOx did appear to be converted to IAA, this result may demonstrate a situation where although the enzymes required for this process are present, the conversions may not occur naturally. This result provides evidence that the pathway to IAA through IAOx is not widespread, but may produce IAA as a second-

![Conversion of $[2H_5]$tryptamine to $[2H_5]$IAA in pea roots. GC/MS/MS chromatograms showing the presence of label in IAA from roots of whole peas incubated with $[2H_5]$tryptamine (A) and its absence in untreated plants (B). The percentage incorporation of deuterium is shown.](image)

![Table III. Metabolism of $[2H_5]$tryptamine in pea roots](table)
ary metabolite of indole glucosinolate production specifically within the Brassicaceae family, and “play a more restricted role in plant growth and development...,” as suggested by Sugawara et al. (2009, p. 5434).

Our results clearly indicate that IAAld can be formed from tryptamine in pea. This is significant because a recent model includes N-hydroxytryptamine as the intermediate between tryptamine and IAA in Arabidopsis (Sugawara et al., 2009). Interestingly, in pea, IEt accumulated markedly more label than the other compounds analyzed, possibly consistent with its putative role as a storage product for IAAld (Brown and Purves, 1980; Ludwig-Müller and Hilgenberg, 1989). However, it should be noted that IEt was present at relatively low endogenous levels. Certainly the high accumulation of label in IEt indicates that the metabolic fate of IAAld is regulated in some way, with relatively more converted to IEt than to IAA when the formation of IAAld is high, as when tryptamine is provided exogenously. Another way in which high levels of tryptamine might be diverted from IAA synthesis in pea roots is the formation of Nv-acetyltryptamine, detected here in large amounts after feeds of [14C]tryptamine.

The conversion of [14C]tryptamine to IAA has been previously reported in tobacco apices (Phelps and Sequeira, 1967), and to IAAld, IEt, and IAA in both tomato and barley (Schneider et al., 1970; Gibson et al., 1972). Furthermore, the conversion of [14C]Trp to tryptamine was noted in Nicotiana callus (Liu et al., 1978) and terminal buds (Phelps and Sequeira, 1967), and also in tomato and barley shoots (Schneider et al., 1970). However, none of these studies used the definitive techniques of GC/MS. Furthermore, endogenous tryptamine has not previously been detected in pea (Schneider et al., 1972). In addition, virtually all previous metabolism studies have used parts of plants or plant extracts, as opposed to undamaged whole plants. It has been reported that wounding and/or stress of plant tissues can have a major impact on the IAA biosynthetic route used by plants (Ljung et al., 2002).

Our results are consistent with previous evidence that IAA is produced in the roots. Deuterated IAA precursors fed to pea roots in metabolism studies were not transported to the shoots, but were metabolized within the roots themselves; that is, no deuterated compound was found in shoot extracts after the metabolism studies carried out herein. This is consistent with the findings of Ljung et al. (2001), who found that the shoot is not the only IAA-producing area in the plant, as was once thought.

It cannot be ruled out that IPyA is a pathway constituent in peas. Because IAAld is an intermediate to IAA on both the tryptamine and IPyA pathways, it could be said that some accumulation of labeled IAAld after Trp incubation occurred via the IPyA route. However, our studies show that at least a portion of the IAAld pool can be formed from tryptamine, indicating that this pathway may be active in vivo. Recently, Tao et al. (2008) and Stepanova et al. (2008) discovered allelic genes that encode an Arabidopsis...
Trp aminotransferase (TAA1). When TAA1 was expressed in Escherichia coli, the induced protein converted Trp to IPyA (confirmed by LC/MS/MS analysis). These recent findings brought IPyA back into the IAA biosynthesis spotlight; however, our studies on IPyA, for both quantitation and metabolism purposes, were rendered difficult by the instability of this compound, which spontaneously degraded to IAA itself.

The renewed focus on the tryptamine pathway stemmed from the discovery of the Arabidopsis YUCCA gene, which encodes a monoxygenase-like enzyme that appears to oxidize tryptamine to N-hydroxytryptamine (Zhao et al., 2001). YUCCA genes have also been isolated in rice (Yamamoto et al., 2007) and Medicago (Cheng et al., 2006), and orthologs found in petunia (Petunia hybrida; FLOOZY: Tobera-Santamaria et al., 2006), tomato (ToFZY: Exposito-Rodriguez et al., 2007), and maize (spi1: Gallavotti et al., 2008). These findings suggest the widespread nature of the tryptamine pathway in a number of both monocotyledonous and dicotyledonous species. However, to establish that a pathway is active within the plant, the compounds should be isolated and the conversion of intermediates to downstream products should be demonstrated. If the pathway is active within the plant, the compounds should be isolated, and the conversion of intermediates to downstream products should be demonstrated.

For metabolism purposes, pea seeds were sterilized in 70% ethanol for 1 min, followed by a sodium hypochlorite solution containing 0.2% available chlorine for 5 min, before being rinsed several times in sterile distilled water. Seeds were placed in 10 mL of autoclaved revised Murashige and Skoog (1962) medium (Sigma) containing 1.2% agar and 20 g·L⁻¹ Suc as additives, in 15 mL Falcon tubes. Tube lids were screwed half on to maintain the tube sterility while permitting gas exchange, and then placed in a growth cabinet in the dark at 20°C for 7 d. The germination rate for this technique was approximately 75%. Seedlings were then transplanted (in a laminar flow hood) into tubes containing sterile distilled water that had been aerated for 1 h. Control plants were placed solely in distilled water, whereas treated plants were placed in distilled water containing the compound of interest. A dose-response study was conducted to judge an appropriate level of [²H₅]tryptamine required for root incubation. Treated plants were separated into three groups in this study—those incubated with 1, 10, and 100 µM [²H₅]tryptamine. Thereafter, 10.8 µM was used, as this amount of substrate enabled the detection of labeled products. For studies involving [¹⁴C]tryptamine metabolism, plants were incubated in a 7 µM solution. For [²H₅]L-Trp the concentration was 8.5 µM, for [²H₅]IAN the concentration was 2 µM, and for [²H₅]IAOX the concentration was 1 µM, with three to four seedlings placed in each 15 mL tube. Plants were maintained under these conditions for 24 h, and then harvested from below the seed—i.e., the roots only.

Selected experiments included a harvest of the whole shoot tissue above the seed, separately, to examine possible uptake of labeled compounds from the root to the shoot tissue. In two studies, involving the assessment of uptake of [²H₅]tryptamine and [²H₅]Trp, both sterile root material and the distilled water incubation medium, were sampled every 6 h for 24 h, and the subsequent extracts tested for the substrates themselves. Apart from those experiments involving Trp quantification, once harvested and weighed the solutions were homogenized, and filtered as described previously for IAA (Jager et al., 2005). For those experiments involving Trp quantification, plant material was harvested and extracted in distilled water at 4°C, and all filtration was carried out in distilled water. The filtrate was stored at −20°C. For quantification studies, after filtration, a known amount of appropriate internal standard was added to the extracts. In some metabolism experiments, internal standards were also added to quantify metabolites. For the quantification of IAA, [¹³C]IAA (Cambridge Isotope Laboratories) was used as an internal standard; for Trp, [⁰⁷C]Trp (Radiolabeled Chemicals, Inc.) was used; and for tryptamine, either [²H₅]tryptamine or [²H₅]L-Trp (Cambridge Isotopes Inc.) or [⁰⁷C]tryptamine (Vitras) was used. For all other compounds, the labeled forms shown in Supplemental Table S1 were used as internal standards. All internal standards apart from [²H₅]L-Trp and [²H₅]IAOX were added at the level of 10 ng·g⁻¹ (FW)⁻¹ into raw extracts after filtration. [²H₅]L-Trp was added at 500 ng·g⁻¹ (FW)⁻¹, and [²H₅]IAOX at the level of 2 ng·g⁻¹ (FW)⁻¹.

**Preparation of Extracts for the Isolation and Quantification of Compounds**

Unless otherwise stated, extracts were reduced under vacuum at 30°C to a small volume (1–4 mL) using a rotary evaporator. Extracts used for IAA and IAA biosynthesis purposes were purified with Sep-Pak C₁₈ cartridges (VAC RC 500 mg; Waters) and prepared as described for IAA previously (Jager et al., 2005).

Before drying, extracts for the quantification of IAAld, including the [²H₅]IEt internal standard, were stirred on a block heater, with 1 g of sodium borohydride per 50 mL extract (Sigma) added twice over a 9 h period to

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**MATERIALS AND METHODS**

**Chemicals**

Labeled forms used for metabolism studies and internal standards were synthesized in our laboratories (see Supplemental Data), apart from [²H₅]I-tryptamine (α-[²H₅]-β-[⁰⁷C]-Trp: Medical Isotopes Inc.), [¹³C]IAA (Cambridge Isotope Laboratories), [⁰⁷C]Trp (sidechain ³⁻⁷C; American Radiolabeled Chemicals, Inc.), and [⁰⁷C]tryptamine (α-[⁰⁷C]; Vitras). In our laboratories, the compounds [²H₅]IEt (Supplemental Protocol S1), [²H₅]Trp (Supplemental Protocol S2), [²H₅]tryptamine (Supplemental Protocol S3), Nα-acetyltryptamine (Supplemental Protocol S4), [²H₅]IAN (Supplemental Protocol S5), and [²H₅]IAOX (Supplemental Protocol S6) were synthesized, as well as an unlabeled form of N-hydroxytryptamine (Supplemental Protocol S7; Schwartz et al., 1986).

All deuterated compounds were checked for both the level of [²H₅] incorporation as determined by GC/MS/MS (Supplemental Table S1), as well as for target products of metabolism studies to eliminate the possibility that any observed positive results were due to contamination from the deuterium-fed treatments. After purification, no such contamination was found in the compounds. The conditions of metabolism were also tested—deuterated compounds (tryptamine, L-Trp, and IAN) were left in the same conditions without plant material and tested for spontaneous degradation to the target compounds; none was found. Nor was any loss of deuterium observed, and the labeled compounds were as such considered stable.

**Plant Material**

Pea (Pisum sativum) plants of line 107 were used throughout. Line 107 (Torsdag) is wild type with respect to internode length genes. For quantification purposes, nicked seeds were grown, two per pot, in experimental and glasshouse conditions as described previously (Jager et al., 2008). Three-week-old pea tissue was used for quantification experiments. Internode segments included the uppermost two expanding internodes. The leaf material included the two uppermost expanding leaves, and the apical tissue included the apical bud only. Root material (excised at the seed) was washed in distilled water, and blotted before weighing.

For metabolism purposes, pea seeds were sterilized in 70% ethanol for 1 min, followed by a sodium hypochlorite solution containing 0.2% available chlorine for 5 min, before being rinsed several times in sterile distilled water. Seeds were planted in 10 mL of autoclaved revised Murashige and Skoog (1962) medium (Sigma) containing 1.2% agar and 20 g·L⁻¹ Suc as additives, in 15 mL Falcon tubes. Tube lids were screwed half on to maintain the tube sterility while permitting gas exchange, and then placed in a growth cabinet in the dark at 20°C for 7 d. The germination rate for this technique was approximately 75%. Seedlings were then transplanted (in a laminar flow hood) into tubes containing sterile distilled water that had been aerated for 1 h. Control plants were placed solely in distilled water, whereas treated plants were placed in distilled water containing the compound of interest. A dose-response study was conducted to judge an appropriate level of [²H₅]tryptamine required for root incubation. Treated plants were separated into three groups in this study—those incubated with 1, 10, and 100 µM [²H₅]tryptamine. Thereafter, 10.8 µM was used, as this amount of substrate enabled the detection of labeled products. For studies involving [¹⁴C]tryptamine metabolism, plants were incubated in a 7 µM solution. For [²H₅]L-Trp the concentration was 8.5 µM, for [²H₅]IAN the concentration was 2 µM, and for [²H₅]IAOX the concentration was 1 µM, with three to four seedlings placed in each 15 mL tube. Plants were maintained under these conditions for 24 h, and then harvested from below the seed—i.e., the roots only.

Selected experiments included a harvest of the whole shoot tissue above the seed, separately, to examine possible uptake of labeled compounds from the root to the shoot tissue. In two studies, involving the assessment of uptake of [²H₅]tryptamine and [²H₅]Trp, both sterile root material and the distilled water incubation medium, were sampled every 6 h for 24 h, and the subsequent extracts tested for the substrates themselves. Apart from those experiments involving Trp quantification, once harvested and weighed the solutions were homogenized, and filtered as described previously for IAA (Jager et al., 2005). For those experiments involving Trp quantification, plant material was harvested and extracted in distilled water at 4°C, and all filtration was carried out in distilled water. The filtrate was stored at −20°C. For quantification studies, after filtration, a known amount of appropriate internal standard was added to the extracts. In some metabolism experiments, internal standards were also added to quantify metabolites. For the quantification of IAA, [¹³C]IAA (Cambridge Isotope Laboratories) was used as an internal standard; for Trp, [⁰⁷C]Trp (Radiolabeled Chemicals, Inc.) was used; and for tryptamine, either [²H₅]tryptamine or [²H₅]L-Trp (Cambridge Isotopes Inc.) or [⁰⁷C]tryptamine (Vitras) was used. For all other compounds, the labeled forms shown in Supplemental Table S1 were used as internal standards. All internal standards apart from [²H₅]L-Trp and [²H₅]IAOX were added at the level of 10 ng·g⁻¹ (FW)⁻¹ into raw extracts after filtration. [²H₅]L-Trp was added at 500 ng·g⁻¹ (FW)⁻¹, and [²H₅]IAOX at the level of 2 ng·g⁻¹ (FW)⁻¹.
reduce IAAld to IEt (as previously noted by Moore and Shaner, 1968).

Subsequently, the extract was dried on a rotary evaporator. Extracts for analysis of IAAld and IEt were taken up in 30 mL of KHSO₄ (0.3 N) and distilled water, respectively, and partitioned three times with 10 mL chloroform. The organic phase was then dried under rotary evaporation, transferred to a tapered-bottom vial, and taken to complete dryness in a sample concentrator. Trimethylsilylation was then performed by adding 40 mL N/O-bis (trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) to the dry sample with 10 mL pyridine in aid for dissolution, and heating to 80°C for 30 min. Subsequently, the extract was dried under nitrogen and 15 µL BSTFA (1% TMCS) was added. The sample was then placed in an oven at 80°C for a further 30 min.

For analysis of tryptamine, extracts in 80% methanol were dried and then taken up in 2 mL of 2% acetic acid in distilled water, and partitioned three times with 1 mL chloroform. The organic phase was discarded, and the aqueous phase taken to pH 11, using 1 N NaOH, and partitioned with chloroform again. In some tryptamine quantification or metabolism experiments (Figs. 2 and 5), extracts were subjected to HPLC as previously described (Ross et al., 1993), but with solvents 1% acetic acid (A) and acetonitrile (1% acetic acid, B), at 1 mL min⁻¹ over a gradient of 10% to 60% B in A over 20 min, followed by 60% to 100% B over the next 10 min. Tryptamine eluted in fractions 15 to 16. For quantification, tryptamine-containing fractions were dried and acylated using 100 µL of both pentafluoropropionic anhydride (Alltech) and acetonitrile in a 60°C oven for 90 min (as described previously by Martinez and Gelpi, 1979; Sobolevsky et al., 2003).

For LC/MS analyses of N-acetyltryptamine, N-hydroxytryptamine, and tryptamine, a Waters Alliance 2690 HPLC coupled to a Finnigan CQ classic ion trap mass spectrometer were used; the HPLC column was a µBondapak C₁₈, 10 µm, 8 mm Radial-Pak cartridge. Solvent specifications were as previously described for acetonitrile-based HPLC except that the flow rate was 0.8 mL min⁻¹; retention times differed between LC systems. The mass spectrometer was operated in atmospheric pressure chemical ionization mode using a vaporizer temperature of 360°C and a capillary temperature of 165°C, with nitrogen sheath gas at 240 kPa. For full-scan MS/MS studies, either data-dependent MS or MS/MS was monitored and the compound was found to elute approximately 1 min after tryptamine. This information enabled us to monitor for [¹⁴C]N-acetyltryptamine after treatment of roots with [¹⁴C]tryptamine, monitoring ion m/z 179.

For the analysis of IAOx, extracts were loaded on to Sep Pak C₁₈ cartridges in 0.4% acetic acid and eluted with 50% methanol (0.4% acetic acid). IAOx samples were then subjected to HPLC as described for tryptamine, but with solvent A being 0.4% acetic acid in distilled water and solvent B (methanol, on a gradient of 20% to 75% B over 25 min; flow rate 2 mL min⁻¹), IAOx eluted in fractions 14 to 15. Samples were then trimethylsilylated as described for IEt.

Aqueous Trp-containing extracts were acidified to pH 2 using 1 N HCl, 400 µL penta-fluoro-propanol (Fluka) and 100 µL pyridine were added, and the samples were vortexed for 5 s. After this, 100 µL of ethyl chloroformate (Merck) was added and the sample was vortexed for 10 s and left to stand for 10 min (as described previously by Perrin et al., 2004). The sample was then partitioned with 200 µL of chloroform, three times, and the organic phase dried under N₂ and taken to complete dryness in a sample concentrator. The samples were then trimethylsilylated using BSTFA (1% TMCS) as described for IEt.

All samples were then dried under N₂ and taken up in 50 µL of chloroform to transfer to an autosampler vial for subsequent GC/MS/MS analysis.

**GC/MS/MS Analysis**

Identification and quantification of the compounds were performed using GC/MS/MS. A Varian 3800 GC coupled to a Varian 1200 triple quadrupole MS was employed, with quantitation via Varian Star software. The column used was a 3.5 mm x 30 m, 0.25 mm internal diameter and 0.25 micron film, using a Varian 1177 split/splitless injector in splitless mode. The ion source was held at 220°C, the injector 250°C, and the line temperature 290°C, with the typical injection volume being 1 µL. The helium carrier gas was supplied at a constant flow of 1.4 mL per min. For tryptamine and IEt the column oven temperature program was 50°C for 2 min, then to 190°C at 30 degrees per min, then to 220°C at 10 degrees per min, and finally to 270°C at 30 degrees per min. For Trp the column oven temperature program was 50°C for 2 min, then to 270°C at 10 degrees per min. The cycle time was 0.3 s. The GC/MS conditions and specifications for IAA were as described (Jones et al., 2005), as shown in Supplemental Table S2.

For tryptamine, IEt, IAN, and Trp full-scan MS/MS spectra of standards were acquired to determine suitable MS/MS transitions for quantitation. The molecular ion (or in some cases a prominent fragment ion) were selected as the precursor ions. Once relevant product ions were observed, the conditions for quantitation were optimized, including the collision energy for each transition. For tryptamine, IEt (including IAAld converted to IEt using sodium borohydride), Trp, IAOx, and IAN, the GC/MS conditions and conditions were as shown in Supplemental Table S2.

Compounds were all separable by GC/MS/MS, and eluted in the order tryptamine, followed 71 s later by IAN, then (5 s) IEt, (5 s) IAA, (52.6 s) IAOx, and finally (213.4 s) Trp.

Compounds were identified on the basis of retention times and by monitoring a selected transition (or two transitions when possible) characteristic of the compound. Calculations of endogenous levels were performed by comparing peak areas of a transition derived from the endogenous hormone and the corresponding stable-isotope-labeled internal standard. To calculate the endogenous hormone level, the corrected endogenous product ion intensity was divided by the internal standard product ion intensity, and this figure was multiplied by the amount of internal standard added, divided by the FW of the tissue, to give results in ng g⁻¹ (FW)⁻¹. Corrections were made for the small amount of unlabeled material in the deuterated standards, and for contributions from natural isotopes in unlabeled material to peaks corresponding to internal standards. For measurement of endogenous IAAld after its conversion to IEt, the endogenous actual IET was subtracted from the total IEt (including IAAld), after the conversion was complete.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Endogenous IAOx was not detected in extracts from pea tissues.

**Supplemental Figure S2.** Conversion of [⁶H₅]IAOx to [⁶H₅]IAA in pea roots.

**Supplemental Figure S3.** Conversion of [⁶H₅]IAA to Trp in pea roots.

**Supplemental Table S1.** The composition of synthesized IAA precursors.

**Supplemental Table S2.** GC/MS/MS specifications for unlabeled and labeled versions of Trp, tryptamine, IAN, IEt, IAOx, and IAA.

**Supplemental Protocol S1.** Procedures for the synthesis of IAAld and IAOx-2,4,5,6,7-[^³H₅].

**Supplemental Protocol S2.** Procedures for the synthesis of IET-2,4,5,6,7-[^³H₅].

**Supplemental Protocol S3.** Procedures for the synthesis of tryptamine-2,4,5,6,7-[^³H₅].

**Supplemental Protocol S4.** Procedures for the synthesis of Nu-acetyltryptamine-2,4,5,6,7-[^³H₅].

**Supplemental Protocol S5.** Procedures for the synthesis of IAAld-2,4,5,6,7-[^³H₅].

**Supplemental Protocol S6.** Procedures for the synthesis of IAOx-2,4,5,6,7-[^³H₅].

**Supplemental Protocol S7.** Procedures for the synthesis of unlabeled N-hydroxytryptamine.

**ACKNOWLEDGMENTS**

We thank Ian Cummings and Tracey Winterbottom for technical assistance, and the Australian Research Council for financial assistance. We also thank Hiroyuki Kasahara for providing a sample of N-hydroxytryptamine.

Received May 18, 2009; accepted August 24, 2009; published August 26, 2009.
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