Biosynthesis of the Halogenated Auxin, 4-Chloroindole-3-Acetic Acid\textsuperscript{[W][OA]}

Nathan D. Tivendale, Sandra E. Davidson, Noel W. Davies, Jason A. Smith, Marion Dalmais, Abdelhafid I. Bendahmane, Laura J. Quittenden, Lily Sutton, Raj K. Bala, Christine Le Signor, Richard Thompson, James Horne, James B. Reid, and John J. Ross*

School of Plant Science (N.W.D., J.H.), and School of Chemistry (N.W.D., J.A.S.), University of Tasmania, Sandy Bay, Tasmania, Australia 7005; School of Plant Science (N.D.T., S.E.D., L.J.Q., L.S., R.K.B., J.B.R., J.J.R.), Central Science Laboratory (N.W.D., J.H.), and School of Chemistry (N.W.D., J.A.S.), University of Tasmania, Sandy Bay, Tasmania, Australia 7005; and Unité de Recherche en Génomique Végétale, Evry, France 91018 (M.D., A.I.B.).

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is John J. Ross (john.ross@utas.edu.au).

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Corresponding author; e-mail john.ross@utas.edu.au.

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RESULTS

In Vitro Analysis of PsTAR1 and PsTAR2

Since evidence for the involvement of Trp amino-transferases in auxin biosynthesis is accumulating (Stepanova et al., 2010; Tao et al., 2010), it has been proposed that the enzyme involved in converting Trp to IPyA is the TRYPTOPHAN AMINOTRANSFERASE RELATED2 (TAR2) family (Stepanova et al., 2011; Won et al., 2011; Kriechbaumer et al., 2012). Until recently, it was thought that the enzyme involved in converting Trp to IPyA, the TRYPTOPHAN AMINOTRANSFERASE RELATED2 (TAR2) family (Stepanova et al., 2011; Tao et al., 2010), is located in vascular phloem from the YUCCA (YUC) family (Zhao et al., 2006; Tietz et al., 2006).

The YUCs were originally thought to function in the tryptamine pathway, converting tryptamine to 3-hydroxytryptamine (Yao et al., 2010; Expósito-Rodríguez et al., 2010; LeClere et al., 2010; Rodríguez et al., 2007). However, the enzymatic function of the YUCs has now been reported. In this study, we examine the biosynthesis of 4-Cl-I AA and IAA in pea seeds, providing evidence for the importance of the phloem through IPyA and its chlorinated analogs in these organs.

Despite more than 70 years of intensive investigation (Stepanova et al., 2010), the original biochemical function of the YUCs is still controversial (Stepanova et al., 2008; Tao et al., 2008; Chourey et al., 2008), but the IPyA pathway has yet to be definitively identified in species other than Arabidopsis. Nevertheless, the YUCs have been shown to utilize a variety of substrates, including Trp and IPyA (Yao et al., 2010; LeClere et al., 2010; Rodriguez et al., 2007). However, the enzymatic activity of the YUCs has not been reported. In this study, we examine the biosynthesis of 4-Cl-I AA and IAA in pea seeds, presenting evidence for the importance of the phloem through IPyA and its chlorinated analogs in these organs.
PsTAR1

Medtr5g035320

PsTAR2

PsTAR3

Figure 1. Inferred phylogenetic relationship of the three pea TAR genes, PsTAR1 (JN990888), PsTAR2 (JN990889), and PsTAR3 (JN990990), with other TAR-like genes. The phylogram was described as previously (Tivendale et al., 2010). Included were AtTAR1 (At1g70560), AtTAR2 (At1g23320), and AtTAR2 (At4g24670) from Arabidopsis and Medicago TAR-like sequences Medtr5g033510, Medtr5g033520, and Medtr3g077250. The Medicago sequences were obtained by a BLAST search of the International Medicago Genome Annotation Group (IMGAG) database version 3.5 at the Medicago sequencing Resources (www.medicagohapmap.org/?genome) and correspond to Mt5g034880, Mt5g034890, and Mt3g114550, respectively, from version 3.0 of the IMGAG database. Sequences from the gymnosperm Picea glauca BT106325 and the liverwort M. polymorpha Pmp2D3 AF542555_1 were chosen as the outgroup from the more comprehensive phylogram of Phillips et al. (2011).

Keto-Enol Tautomerization of IPyA and 4-Cl-IPyA

IPyA, like all compounds containing a ketone function, exists as an equilibrium between two tautomers: keto and enol (supplemental fig. S1; Schau, 1961). In the case of IPyA, it might be predicted that the enol tautomer would be more stable, as it increases the conjugation of the molecule. To better understand the behavior of this compound in vivo, we characterized it in vitro.

We analyzed authentic IPyA by 1H- and 13C-NMR and UPLC-MS. NMR analysis of IPyA in a variety of solvents (at concentrations greater than 10 mM) indicated the presence of the IPyA enol tautomer, as evidenced by comparison of retention times (RTs) and mass spectra with those of authentic 4-Cl-IAA and IAA. UPLC-MS analysis of reaction mixtures showed no difference in 4-Cl-IAA or IAA production between the tests and controls, thereby indicating that the 4-Cl-IAA and IAA detected after feeds of 4-Cl-Trp and Trp, respectively, were due to physicochemical, rather than enzymatic, conversion of the initial enzym products, 4-Cl-Trp and IAA.

4-Cl-IAA and IAA were also detected in the hone in vitro assays, and to determine if this was due to enzymatic conversion or physicochemical breakdown, we conducted in vitro assays using 4-Cl-IPyA and IPyA as substrates. UPLC-MS analysis of reaction mixtures showed no difference in 4-Cl-IAA or IAA production between the tests and controls, thereby indicating that the 4-Cl-IAA and IAA detected after feeds of 4-Cl-Trp and Trp, respectively, were due to physicochemical, rather than enzymatic, conversion of the initial enzym products, 4-Cl-Trp and IAA.

In vitro assays using 4-Cl-IPyA and IPyA as substrates confirmed the presence of the IPyA enol tautomer and suggested the potential for IPyA metabolism. When synthetic IPyA was dissolved in methanol and a byproduct by explosion of IPyA was detected by HPLC-MS, multiple reaction monitoring (MRM) mode, a peak at the IPyA channel on the break-down products A & B in cold-cast aqueous solutions with various pH values (neutral to basic) indicated the presence of the IPyA enol tautomer and suggested the potential for IPyA metabolism. When synthetic IPyA was dissolved in methanol, a byproduct by explosion of IPyA was detected by HPLC-MS, multiple reaction monitoring (MRM) mode, a peak at the IPyA channel on the break-down products A & B in cold-cast aqueous solutions with various pH values (neutral to basic) indicated the presence of the IPyA enol tautomer and suggested the potential for IPyA metabolism.
heated to 40°C for 3 h (roughly mimicking the in vitro assay conditions), water-suppression NMR revealed primarily the keto tautomer of IPyA with a small amount of the enol form (H-NMR, 400 MHz d: 4.1 [s, 2H] 7.0–7.2 [m, 3H], 7.4 [d of d, J = 8.23 Hz, 8.45 Hz 2H]; 13C-NMR, 100 MHz d: 40.9 [CH₂], 110.9 [C], 117.1 [CH], 123.7 [CH], 124.6 [CH], 127.1 [CH], 130.5 [CH], 132.0 [C], 141.4 [C], 175.7 [C], 209.5 [C]). This treatment of IPyA also changed its behavior on UPLC-MS (acetic acid program). Under these conditions, IPyA (shown by NMR to be the keto form) eluted earlier than IAA, whereas the enol tautomer eluted later than IAA. There were substantial similarities in the tandem MS spectra produced from the solution in CD₃OD and the solution in KH₂PO₄/K₂HPO₄ buffer (pH 8.5), both consistent with IPyA (keto: mass-to-charge ratio [m/z]: 115 [8%], 118 [8%], 130 [100%], 142 [8%], 158 [63%]; enol: m/z: 103 [6%], 130 [100%], 144 [7%], 158 [82%]). As further evidence that the early peak represented IPyA, we performed a multiplicity-edited 13C-1H heteronuclear single quantum coherence (HSQC) NMR experiment on an IPyA sample prepared in KH₂PO₄/K₂HPO₄ buffer (pH 8.5) and heated to 40°C for 3 h. This revealed the presence of five aromatic CH groups and one aliphatic CH₂ (Supplemental Fig. S3). These findings indicate that the early peak was the keto tautomer of IPyA. Interestingly, at lower concentrations, mixtures of the tautomers were observed by UPLC-MS, sometimes approximating to 1:1 (Supplemental Fig. S4). Our analyses show that the relative proportions of the IPyA tautomers can be controlled using temperature and pH, and the two forms are readily resolved by UPLC; the same was found for 4-Cl-IPyA (Supplemental Fig. S4).

Analysis of PsTAR1 and PsTAR2 Expression Patterns and Auxin Levels during Seed Development

We next measured auxin levels and the expression of PsTAR1, PsTAR2, and PsTAR3 over the course of seed development. UPLC-MS analyses showed that the level of 4-Cl-IAA in developing seeds increased dramatically from 7 to 12 d post anthesis (DPA) and then steadily declined until the completion of seed development (Fig. 3A). IAA levels were initially high but decreased markedly from 7 to 16 DPA and remained low thereafter (Fig. 3A). Quantitative real-time PCR showed that PsTAR1 is strongly expressed early in seed development (7 DPA), when IAA levels are initially high but decreased markedly from 7 to 16 DPA and remained low thereafter (Fig. 3A). Similarly, PsTAR2 is strongly expressed early in seed development (7 DPA), when IAA levels are high, although past their peak (Fig. 3B).

Effects of the tar2 Mutation on Auxin Levels

To investigate the role of PsTAR1 and PsTAR2 in 4-Cl-IAA biosynthesis in vivo, we utilized a targeting-induced local lesioning in genome (TILLING) population to identify null mutants in PsTAR2 (Fig. S4). A single tar2 null mutant was isolated on the genetic background and characterized. The stop codon in this
mutant results in a protein truncated prior to the catalytic Lys that is common to all pyridoxal-5-phosphate (PLP)-dependent aminotransferases. The tar2 mutation did not substantially affect auxin levels during early stages of seed development (Fig. 4). However, seeds of the mutant contained much less 4-Cl-IAA than the wild type at the later stages ($P < 0.001$); by 20 DPA, the reduction was approximately 90%. Effects of the tar2 mutation on the content of IAA, the minor auxin at later stages, were relatively small. The large reduction in 4-Cl-IAA at the later stages indicates the importance of the 4-Cl-IPyA pathway for 4-Cl-IAA biosynthesis in seeds. 4-Cl-Trp was identified by UPLC-MS in seed extracts, and we found that extracts from mutant seeds contained significantly more 4-Cl-Trp and Trp than did extracts from wild-type seeds (Supplemental Fig. S5).

Labeled-Precursor Feeding Studies

To investigate auxin biosynthesis in young pea seeds, where PstAR2 does not strongly affect auxin levels, we injected labeled intermediates into seeds at the liquid endosperm stage. We first tested the possibility that 4-Cl-IAA is synthesized directly from IAA by injecting a mixture of [13C6]IAA (20 ng) and [D5]Trp (5 μg) into developing peas seeds (approximately 70 mg). The mutant of 4-Cl-IAA injected was allowed to avoid any physiologically unrealistic situation. Incorporation of the D5 label from Trp into IAA was observed (Fig. 5A), and the IAA conjugate, indole-3-acetyl-Asp, contained 13C label (data not shown), indicating that IAA metabolism occurring during the feeding period, but 4-Cl-IAA was not diluted with 13C label (Fig. 5B), indicating that it is not all IAA itself that becomes chlorinated. In the same experiment, D4 label (from the [D5]Trp) was detected in both 4-Cl-Trp and 4-Cl-IAA (Fig. 5B and C), indicating that Trp is a point of chlorination and that the biosynthesis of 4-Cl-IAA continues parallel to that of IAA (Fig. 6). Consistent with the theory that Trp is a point of chlorination, when deuterated 4-Cl-Trp was injected, label incorporation into 4-Cl-IAA was observed (Fig. 5D). It cannot be excluded, however, that chlorination also occurs before Trp.

Furthermore, after injections of deuterated Trp, we did not detect, by UPLC-MS, a labeled form of another...
DISCUSSION

In this study, we provide evidence for the biosynthesis of 4-Cl-IAA, which is identical to the proposal of Tao et al. (2008). We have isolated three new genes from pea, PsTAR1, which are homologous to the AtTAA family (Stepanova et al., 2008; Tao et al., 2008), and vt2 and ZmTAR1 from maize (Chourey et al., 2010; Phillips et al., 2011). We have shown, by in vitro assays, that they have an aminotransferase activity using either Trp or its 4-chlorinated analog.

Figure 5. UPLC-MS chromatograms (MRM mode; acetic acid program) obtained from extracts of pea seeds that had been previously injected with a mixture of [D$_4$]Trp and [13C$_6$]IAA (A–C) or [D$_4$]4-Cl-Trp (D). On UPLC, the RT of a deuterated species is earlier than the RT for the endogenous species; $^{13}$C-labeled species have the same RT as their endogenous counterparts (on the UPLC-MS system used, within 0.01 min). A, IAA became enriched with D$_4$ label (middle channel; RT = 4.08) after injection of [D$_4$]Trp; the endogenous IAA (bottom channel; RT = 4.11) and the injected [13C$_6$]IAA (top channel; RT = 4.10) were also detected. B, 4-Cl-IAA also became enriched with D$_4$ label (middle channel; RT = 4.89) from the injected [D$_4$]Trp (one deuterium is replaced with a chlorine atom), but the $^{13}$C$_6$ label from the injected [13C$_6$]IAA was not incorporated into 4-Cl-IAA (top channel); the peaks observed in this channel did not have the correct RT for [13C$_6$]4-Cl-IAA. C, 4-Cl-Trp became enriched with D$_4$ label (top channel; RT = 2.18) from the injected [D$_4$]Trp. D, In a separate experiment, 4-Cl-IAA became enriched with deuterium label (top two channels) after deuterated 4-Cl-Trp was injected; endogenous 4-Cl-IAA was also detected (bottom channel). The [D$_4$]4-Cl-IAA signal detected in this experiment contained a small contribution from [D$_4$]2Cl$_2$4-Cl-IAA.

PyK pHwy. We have isolated three new genes from pea PsTAR1, which are homologous to the AtTAA family (Stepanova et al., 2008; Tao et al., 2008), and vt2 and ZmTAR1 from maize (Chourey et al., 2010; Phillips et al., 2011). We have shown, by in vitro assays, that they have an aminotransferase activity using either Trp or its 4-chlorinated analog. The novel intermediate, 4-Cl-IAA, was detected as a product of 4-Cl-Trp. Our identification was confirmed by comparison with 4-Cl-IAA synthesized in our laboratory.
These assays, 4-Cl-IAA and IAA were also detected, but further analysis showed that this was merely due to nonenzymatic conversion from the initial products, 4-Cl-IPyA and IPyA to 4-Cl-IAA and IAA, respectively. However, the conversion of IPyA to IAA is reportedly enzymatic in Arabidopsis (Mashiguchi et al., 2011; Stepanova et al., 2011; Won et al., 2011; Kriechbaumer et al., 2012). Interestingly, in the functional assays, both the enol and keto tautomers of IPyA and 4-Cl-IPyA were detected as products, but the keto form predominated. In contrast, on the basis of liquid chromatography, previous non-enzymatic assays appeared to have yielded mainly the enol form of IPyA (He et al., 2011; Stepanova et al., 2011; He et al., 2012) and IAA, which might represent the keto tautomer of IAA. Nevertheless, the in vitro assays were conducted under conditions that favor the keto form, as indicated by our NMR analyses. The issue of which form is predominantly produced by non-enzymatic reactions remains to be resolved, but our in vivo evidence indicates that in pea seeds, both 4-Cl-IPyA and IAA are present primarily as their respective keto tautomers.

It appears that in pea seeds, IAA and 4-Cl-IAA are synthesized in parallel via the IPyA pathway and its 4-chlorinated version (Fig. 6). On the basis of gene expression patterns, PsTAR1 is a key enzyme during the early stages of seed development, whereas the dramatic effect of tar2 on 4-Cl-IAA levels in mature seeds is consistent with an important role for PsTAR2. In the later stages, we found evidence that IAA itself becomes chlorinated, and metabolism studies indicate that Trp becomes chlorinated, with the result that 4-Cl-Trp is subsequently converted to 4-Cl-IAA. Compound-based studies reported here and previously (Quittenden et al., 2009; Tivendale et al., 2010) indicate that Trp is not converted to auxin in pea seeds via tryptamine, IAM, or indole-3-acetamide.

There is currently renewed focus on auxin biosynthesis, due to reports that the YUC proteins do not operate in the tryptamine pathway (Tivendale et al., 2010) but rather in the IPyA pathway (Mashiguchi et al., 2011; Stepanova et al., 2011; Won et al., 2011; Kriechbaumer et al., 2012), a suggested previously by Strader and Botrel (2011). However, Mano and Nemoto (2012) recently noted that functionally, Trp and non-enzymatic IPyA might be restricted to the Brassicaceae. Our evidence from metabolismo genic studies in Arabidopsis indicates that Trp becomes chlorinated, and indeed co-predominates in other species.

**MATERIALS AND METHODS**

**Chemicals**

The following compounds were obtained from commercial sources: [35] IAA (Cambridge Isotope Laboratories), 4-Cl-IAA (Amatek), 4-Cl-Trp (Sigma-Aldrich), and IAA (Sigma-Aldrich). The deuterated 4-Cl-IAA was prepared at the BBSRC NMR facility.
supplied by Prof. Jerry Cohen (Department of Horticultural Science, University of Minnesota) and other companies as described previously (Quittenbaum et al., 2011). Below.

**Isolation and Cloning of PsTAR1, PsTAR2, and PsTAR3**

_Faba_ Sativum (L) var. (4×) was grown in a greenhouse, and the seed was collected. The seeds were ground in liquid nitrogen and extracted with 95% ethanol. The supernatant was then heated to 60°C for 10 min and centrifuged for 10 min at 5000 g. The supernatant was concentrated and used for cloning.

**Expression and Purification of PsTAR2 Fusion Protein**

The following protocol was adapted from NEB pMAL Protein Fusion and Purification Instruction Manual for protein expression and purification. The culture was grown at 30°C in a shaken flask. The plasmid was transformed into E. coli competent cells and plated on LB agar plates containing ampicillin (100 μg/mL). The colonies were grown at 37°C for 24 h and then transferred to 30°C for further growth. The culture was concentrated by centrifugation and used for protein expression and purification.

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**In Vitro Assays Using PsTAR2 and PsTAR1**

The following procedure was adapted from the NEB pMAL Protein Fusion and Purification Instruction Manual for protein expression and purification. The culture was grown at 30°C in a shaken flask. The plasmid was transformed into E. coli competent cells and plated on LB agar plates containing ampicillin (100 μg/mL). The colonies were grown at 37°C for 24 h and then transferred to 30°C for further growth. The culture was concentrated by centrifugation and used for protein expression and purification.

**UPLC-MS**

Samples were analyzed using Waters Acquity UPLC-MS system. The Waters Acquity UPLC BEH C18 column (1.7 μm, 2.1 mm × 150 mm) was used. The mobile phase was water (A) and acetonitrile (B) at a flow rate of 0.35 mL/min. The column temperature was 35°C. The mass spectrometer was operated in positive ion electrospray mode with cone voltage of 18 V. The ion source temperature was 100°C. Data acquisition was performed using Waters MassLynx software.

The columns were eluted using three gradients. The first gradient was 0% B to 100% B over 2.0 min, followed by a linear gradient to 100% B over 3.0 min, and then a linear gradient to 0% B over 2.0 min. The second gradient was 0% B to 100% B over 5.0 min, followed by a linear gradient to 100% B over 3.0 min, and then a linear gradient to 0% B over 2.0 min. The third gradient was 0% B to 100% B over 2.0 min, followed by a linear gradient to 100% B over 3.0 min, and then a linear gradient to 0% B over 2.0 min. The mass spectrometer was operated in positive ion electrospray mode with cone voltage of 18 V. The ion source temperature was 100°C. Data acquisition was performed using Waters MassLynx software.

**References**

chopped, the second was frozen from 6 mg water, 4-Cl-I-Pyk (well t im., 50 mg per channel). The third was frozen from 4-Cl-I-Pyk (well t im.), 50 mg per channel. All samples were frozen for 4-Cl-I-AK and 4-Cl-I-4AA (well t im., 65 mg per channel). RNAs for the chloroplast and mitochondrial species were isolated using Qiagen. The samples were subsequently eluted in Ni-NTA column (Qiagen), PCR amplification, and restriction enzyme digestion. The PCR products were amplified in the TILLING screen and was characterized by UPLC-MS for comparison with the product of PsTAR2. 

**Plant Material**

Wild-type (TAR2p) peas used for this study were the Rostov tall (LE line 27) derived from a tobacco line (LE line 27) obtained from the Rostov. The seeds were used to determine the RNA content and were prepared for analysis (Fig. 3).

**Quantitative Real-Time PCR**

A total of 20,000 cDNA copies, three for each sample, were used for the analysis. The cDNA was synthesized from 0.5 g of total RNA using the TaqMan Gold 2× Master Mix (Roche). The cDNA was amplified using a LightCycler 480 II (Roche) with the following conditions: 1 cycle at 95°C for 10 min, followed by 45 cycles at 95°C for 10 s and 60°C for 1 min. The temperatures for the PCR products were modified to match the primer specificity and the results were analyzed by DNA sequencing.

**Synthesis of Deuterated 4-Cl-Trp**

A suspension of 4-Cl-Trp (16 mg) in deuterated HCl (8 ml) was prepared by the careful addition of 10 ml of 1 M NaOH to 4-Cl-Trp (16 mg). The suspension was stirred at room temperature for 4 h. This process was repeated until the desired concentration of deuterium incorporation was achieved. The product was then purified using HPLC (VWR, Phenomenex, C18, 5 μm, 250 × 4.6 mm). The product was then characterized by UPLC-MS for comparison with the product of PsTAR2.

**Application of Heavy-Isotope-Labeled Intermediates**

The seeds were harvested, weighed, homogenized, and extracted before (T 1), after freezing (T 2), and after freezing and thawing (T 3). The seeds were then extracted with 80% ethanol, and the extracts were analyzed by UPLC-MS for comparison with the product of PsTAR2.
Supplemental Data

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

Supplemental Figure S1. UPLC-MS chromatogram of 3-indolepyruvic acids.

Supplemental Figure S2. Total ion chromatograms of IPyA.

Supplemental Figure S3. IPyA NMR spectrum for IPyA.

Supplemental Figure S4. UPLC-MS chromatogram showing gerade auxin.

Supplemental Figure S5. UPLC-MS chromatograms showing endogenous IAM in pea seeds.

Supplemental Figure S6. UPLC-MS chromatogram showing GluTryptophan.

Supplemental Table S1. αIPyA-specific primers.

Supplemental Table S2. TILLING-specific primers.

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LITERATURE CITED


