Biosynthesis of the Halogenated Auxin, 4-Chloroindole-3-Acetic Acid1[W][OA]

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Seeds of several agriculturally important legumes are rich sources of the only halogenated plant hormone, 4-chloroindole-3-acetic acid. However, the biosynthesis of this auxin is poorly understood. Here, we show that in pea (Pisum sativum) seeds, 4-chloroindole-3-acetic acid is synthesized via the novel intermediate 4-chloroindole-3-pyruvic acid, which is produced from 4-chlorotryptophan by two aminotransferases, TRYPTOPHAN AMINOTRANSFERASE RELATED1 and TRYPTOPHAN AMINOTRANSFERASE RELATED2. We characterize a tar2 mutant, obtained by Targeting Induced Local Lesions in Genomes, the seeds of which contain dramatically reduced 4-chloroindole-3-acetic acid levels as they mature. We also show that the widespread auxin, indole-3-acetic acid, is synthesized by a parallel pathway in pea.

The chlorinated auxin, 4-chloroindole-3-acetic acid (4-Cl-IAA), is found in certain higher plants and is more active in some bioassays than the most widespread endogenous auxin, indole-3-acetic acid (IAA; Reinecke, 1999). In seeds of some key legumes, such as Lens culinaris, Lathyrus latifolius, Vicia faba, and pea (Pisum sativum), the levels of 4-Cl-IAA are among the highest reported for any auxin in plant tissues (Reinecke, 1999). In pea, it has been suggested that 4-Cl-IAA moves from young seeds into the pod, where it is required for normal pod elongation (Reinecke, 1999; Ozga et al., 2009), but no mutant has been available to aid our understanding of the biosynthesis and roles of 4-Cl-IAA, and there is limited evidence on the origin of this compound (Reinecke, 1999).

Despite more than 70 years of intensive investigation (Normanly, 2010; Zhao, 2010), it is only recently that we have begun to fully understand auxin biosynthesis, even in Arabidopsis (Arabidopsis thaliana). In the past, four Trp-dependent IAA biosynthetic pathways have been proposed (Normanly, 2010; Zhao, 2010), but it now appears that one pathway, involving indole-3-pyruvic acid (IPyA), predominates in Arabidopsis (Mashiguchi et al., 2011; Stepanova et al., 2011; Won et al., 2011; Kriechbaumer et al., 2012). Until recently, it was thought that the enzymes involved in converting Trp to IPyA, the TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) family (Stepanova et al., 2008; Tao et al., 2008), acted in a separate pathway from the YUCCA (YUC) family (Zhao et al., 2001; Zhao, 2010). The YUCs were originally thought to function in the tryptamine pathway, converting tryptamine to N-hydroxytryptamine (Zhao et al., 2001; Expósito-Rodríguez et al., 2007; LeClere et al., 2010). However, the original biochemical function of the YUCs was called into question (Tivendale et al., 2010), and it has now been reported that YUCs act downstream of the TAA family, converting IPyA to IAA (Mashiguchi et al., 2011; Stepanova et al., 2011; Won et al., 2011; Kriechbaumer et al., 2012). Nevertheless, Arabidopsis is known to utilize Brassicaceae-specific biosynthetic pathways (Sugawara et al., 2009), and the IPyA pathway has yet to be demonstrated definitively in species other than Arabidopsis. A potential co-ortholog has been isolated from maize (Zea mays; Phillips et al., 2011), but its enzymatic activity has not been reported. In this study, we examine the biosynthesis of 4-ClIAA and IAA in pea seeds, presenting evidence for the importance of the pathway through IPyA and its chlorinated analog in these organs.

RESULTS

In Vitro Analysis of PsTAR1 and PsTAR2

Since evidence for the involvement of Trp aminotransferases in auxin biosynthesis is accumulating (Stepanova et al., 2008; Tao et al., 2008; Chourey et al., 2008; Tivendale et al., 2008), and there is limited evidence on the origin of this compound (Reinecke, 1999).
2010; Phillips et al., 2011), we isolated from pea three Trp aminotransferase genes, which we named TRYPTOPHAN AMINOTRANSFERASE RELATED1 (PsTAR1), PsTAR2, and PsTAR3. Their inferred phylogenetic relationships are shown in Figure 1. The sequences of PsTAR1 and PsTAR3 are very similar to each other and 51% homologous to AtTAA1 at the protein level. However, PsTAR2 is more closely aligned to the AtTAR2 (56% identity).

To determine the biochemical function of PsTAR1 and PsTAR2, the corresponding coding sequences were expressed in Escherichia coli, and the resulting recombinant maltose-binding protein (MBP) fusion proteins were purified using affinity chromatography. As in Stepanova et al. (2008) and Mashiguchi et al. (2011), the MBP tag was not cleaved from the protein of interest prior to in vitro analysis. After incubation with potential substrates, analysis by ultra-performance liquid chromatography (UPLC)-mass spectrometry (MS) revealed that both PsTAR1 and PsTAR2 produced 4-chloroindole-3-pyruvic acid (4-Cl-IPyA) when 4-chlorotryptophan (4-Cl-Trp) was supplied and IPyA when Trp was supplied (Supplemental Fig. S1). The identities of the products were confirmed by comparison of retention times (RTs) and mass spectra with those of authentic 4-Cl-IPyA (Fig. 2) and IPyA (Supplemental Fig. S1). The novel compound, 4-Cl-IPyA, was synthesized in our laboratory. These results show that both proteins have aminotransferase activity for Trp and its chlorinated analog, 4-Cl-Trp.

4-Cl-IAA and IAA were also detected in the above 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 enzyme products, 4-Cl-IPyA and IPyA.

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

IPyA, like all compounds containing a ketone functionality, exists as an equilibrium between two tautomers: keto and enol (Supplemental Fig. S2; Schwarz, 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 auxin biosynthesis, we characterized it in vitro.

We analyzed authentic IPyA by 1H- and 13C-NMR and UPLC-MS. NMR analyses of IPyA in a variety of solvents (at concentrations greater than 10 mM), including CD3OD (1H-NMR, 300 MHz δ: 6.92 [s, 1H], 7.09 [m, 2H], 7.36 [d, J = 4.2 Hz, 1H], 7.68 [d, J = 7.5 Hz, 1H], 7.98 [s, 1H]; 13C-NMR, 75 MHz δ: 104.4 [CH], 110.2 [C], 111.2 [CH], 117.8 [CH], 119.5 [CH], 121.7 [CH], 127.1 [C], 127.4 [CH], 136.2 [C], 137.5 [C], 137.4 [C]), [D6]dimethyl sulfoxide (1H-NMR, 300 MHz δ: 6.76 [s, 1H], 7.08 [m, 2H], 7.38 [d, J = 8.1 Hz, 1H], 7.68 [d, J = 7.8 Hz, 1H], 7.84 [s, 1H], 8.73 [s, 1H], 11.39 [s, 1H]), and aqueous solutions with various pH values (neutral to 13) all indicated the presence of the IPyA enol tautomer only, along with small amounts of breakdown products. When synthetic IPyA was dissolved in methanol and analyzed by UPLC-MS (multiple reaction monitoring [MRM] mode; acetic acid program), one peak dominated the IPyA channels and the breakdown products IAA and indole-3-carboxaldehyde were observed in their respective channels (data not shown). The NMR data indicate that this peak was IPyA in the enol tautomer.

However, when we dissolved IPyA in KH2PO4/K2HPO4 buffer (greater than 10 mM; pH 6.5 or 8.5) and...
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 (1H-NMR, 400 MHz: 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: 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 in water) 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, top channel). 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).

In functional assays of PsTAR1 and PsTAR2 with Trp or 4-Cl-Trp as the substrate, both keto and enol tautomers of IPyA and 4-Cl-IPyA were detected as products by MS, but the keto form predominated (Fig. 2; Supplemental Fig. S1). However, direct monitoring of endogenous 4-Cl-IPyA and IPyA extracted from pea seeds yielded evidence for the keto tautomers only (at low levels; Supplemental Fig. S4). The evidence presented above supports a role for keto IPyA in IAA biosynthesis in pea.

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 maximal, and PsTAR2 is strongly expressed at later stages (16–28 DPA), when 4-Cl-IAA levels are high, although past their peak (Fig. 3B).

Effects of the tar2 Mutation on Auxin Levels

To investigate the role of aminotransferases in 4-Cl-IAA biosynthesis in vivo, we utilized a Targeting Induced Local Lesions IN Genomes (TILLING) population to identify mutants affected in the PsTAR2 gene. A single tar2 knockout mutant was isolated on the genetic background Cameor. 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 \textit{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 \textit{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 at later stages of seed development, the extracts from mutant seeds contained significantly more 4-Cl-Trp and Trp than did extracts from wild-type seeds (Supplemental Fig. S5).

Taken together, the in vitro and in vivo analyses described above allow several conclusions to be drawn. In early seed development, pea seeds contain high levels of IAA and comparatively low levels of 4-Cl-IAA, and at this stage, \textit{PsTAR1} is far more strongly expressed than \textit{PsTAR2} (Fig. 3). In late seed development, the situation is reversed (high 4-Cl-IAA, low IAA, strong \textit{PsTAR2} expression, and comparatively weak \textit{PsTAR1} expression). Our evidence is consistent with a role for both \textit{PsTAR1} and \textit{PsTAR2} in maintaining levels of IAA and 4-Cl-IAA throughout the course of seed development.

**Labeled-Precursor Feeding Studies**

To investigate auxin biosynthesis in young pea seeds, where \textit{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 \(\text{[}\text{\textsuperscript{13}C}_6\text{]}\text{IAA} \ (20 \ \text{ng})\) and \(\text{[D}_5\text{]}\text{Trp} \ (5 \ \text{mg})\) into developing pea seeds (approximately 70 mg). The amount of \(\text{[}\text{\textsuperscript{13}C}_6\text{]}\text{IAA}\) injected was low to avoid a physiologically unrealistic situation. Incorporation of the D\(_5\) label from Trp into IAA was observed (Fig. 5A), and the IAA conjugate, indole-3-acetyl-Asp, contained \(\text{\textsuperscript{13}C}\) label (data not shown), indicating that IAA metabolism was occurring during the feeding period, but 4-Cl-IAA was not diluted with \(\text{\textsuperscript{13}C}\) label (Fig. 5B), indicating that it is not IAA itself that becomes chlorinated. In the same experiment, D\(_4\) label (from the [D\(_5\)]Trp) was detected in both 4-Cl-Trp and 4-Cl-IAA (Fig. 5, B 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 at a stage prior to Trp.

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

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**Figure 3.** IAA and 4-Cl-IAA levels (A) and \textit{PsTAR1}, \textit{PsTAR2}, and \textit{PsTAR3} transcript levels (B) over the course of seed development in line 107. Shown are means \(\pm \text{SE} \ (n = 3\) for auxin and \(n = 4\) for transcript levels). Mean seed fresh weights for each time point, in order of increasing age, were 3.8 \(\pm\) 0.4, 54 \(\pm\) 4, 199 \(\pm\) 10, 293 \(\pm\) 8, 432 \(\pm\) 27, and 459 \(\pm\) 12 mg. FW, Fresh weight.

**Figure 4.** IAA and 4-Cl-IAA content of wild-type (WT) and \textit{tar2} mutant seeds. Shown are means \(\pm \text{SE} \ (n = 3)\). Wild-type seed fresh weights were as follows: 30 \(\pm\) 1 mg (11 DPA), 114 \(\pm\) 5 mg (14 DPA), 252 \(\pm\) 17 mg (17 DPA), and 339 \(\pm\) 7 mg (20 DPA); \textit{tar2} seed fresh weights were as follows: 41 \(\pm\) 8 mg (11 DPA), 125 \(\pm\) 5 mg (14 DPA), 260 \(\pm\) 5 mg (17 DPA), and 339 \(\pm\) 13 mg (20 DPA). All plants were on a tall background. FW, Fresh weight.
putative intermediate, indole-3-acetamide (IAM; data not shown); endogenous IAM was similarly below the limit of detection (Supplemental Fig. S6). These results indicate that auxin biosynthesis does not occur via this intermediate in these organs.

**DISCUSSION**

In this study, we provide evidence for the biosynthesis of 4-Chloroindole-3-Acetic Acid via the 4-chlorinated version of the IPyA pathway. We have isolated three new genes from pea, *PsTAR1* to 3 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 aminotransferase activity using either Trp or its 4-chlorinated analog. The novel intermediate, 4-Cl-IPyA, was detected as a product of 4-Cl-Trp, and its identity was confirmed by comparison with 4-Cl-IPyA synthesized in our laboratory. In
Chlorate

Trp synthase α

Trp synthase β

Halogenase

tryptophan

4-chlorotryptophan

PsSTAR1/2

PsSTAR1/2

indole-3-pyruvic acid

4-chloroindole-3-pyruvic acid

Figure 6. Proposed parallel IAA and 4-Cl-IAA biosynthetic pathways in pea seeds, based on this study and previous evidence (Manabe et al., 1999; Reinecke, 1999; Quittenden et al., 2009; Tivendale et al., 2010; Zhao, 2010; Mashiguchi et al., 2011; Stepanova et al., 2011; Won et al., 2011; Kriechbaumer et al., 2012). IAA does not become chlorinated, and Trp is a point of chlorination.

In 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 RTs relative to that of IAA, previous aminotransferase assays appear to have yielded mainly the enol form of IPyA (He et al., 2011). Tao et al. (2008), Stepanova et al. (2008), and He et al. (2011) all report the production of IPyA from in vitro Trp aminotransferase assays, but not the phenomenon of tautomerization. However, an unidentified broad peak is visible in the UV chromatograms presented by Tao et al. (2008) and He et al. (2011), which might represent the keto tautomer of IPyA. 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 aminotransferases remains to be resolved, but our in vivo evidence indicates that in pea seeds, both 4-Cl-IPyA and IPyA 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, PsSTAR1 is a key enzyme during the early stages of seed development, whereas the dramatic effect of tar2 on 4-Cl-IAA levels in maturing seeds is consistent with an important role for PsSTAR2 in the later stages. We found no evidence that IAA itself becomes chlorinated, and metabolism studies indicated that Trp (and possibly earlier precursors) become(s) chlorinated and that the resulting 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 (and, by implication, 4-Cl-Trp) is not converted to auxin in pea seeds via tryptamine, IAM, or indole-3-acetaldoxime.

There is currently a 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 the IPyA pathway (Mashiguchi et al., 2011; Stepanova et al., 2011; Won et al., 2011; Kriechbaumer et al., 2012), as suggested previously by Strader and Bartel (2008). However, Mano and Nemoto (2012) recently noted that functional activity for Trp aminotransferases from the IPyA pathway has been demonstrated only for Arabidopsis genes and implied that the IPyA pathway might be restricted to the Brassicaceae. Our evidence from metabolism and genetic studies indicates that the IPyA pathway does operate, and indeed can predominate, in other species.

MATERIALS AND METHODS

Chemicals

The following compounds were obtained from commercial sources: [13C₆]IAA (Cambridge Isotope Laboratories), 4-Cl-Trp (Amatek Chemical), Trp (Sigma-Aldrich), and IPyA (Sigma-Aldrich). The deuterated 4-Cl-IAA was
supplied by Prof. Jerry Cohen (Department of Horticultural Science, University of Minnesota). All other compounds were synthesized in our laboratory as described previously (Quittenden et al., 2009) or below.

Isolation and Cloning of PsTAR1, PsTAR2, and PsTAR3

PsTAR-MBP fusion and MBP were purified as follows. The column was poured and then rinsed with water (120 mL), SDS (45 mL; 0.1%, v/v), cold water (60 mL), and finally column buffer (75 mL; 100 mM K$_2$HPO$_4$/KH$_2$PO$_4$ [pH 8.5]) at a flow rate of 5 mL min$^{-1}$. The crude extract, diluted 1.6 with lysis buffer, was loaded onto the column at a flow rate of 3 mL min$^{-1}$. The column was then washed with cold washing buffer (50 mL K$_2$HPO$_4$/KH$_2$PO$_4$ buffer [pH 8.5], 0.2 mM PLP, and 0.5 mM phenylmethylsulfonyl fluoride [added just before use]; 180 mL, flow rate of 5 mL min$^{-1}$). The protein was eluted with elution buffer (10 mL, 0.1 M, in washing buffer; 15 mL) followed by column buffer (15 mL). Fractions (2.5 mL) were collected, and the total protein concentration was determined by spectrophotometry (Thermo Scientific NanoDrop 8000); the fractions were stored at −70°C until needed.

PsTAR1 was obtained using the same procedure.

In Vitro Assays Using PsTAR2 and PsTAR1

The following procedure was adapted from Stepanova et al. (2008) and Tao et al. (2008). Fusion protein tags are not typically required to be cleaved from the protein of interest for in vitro assays (Zhao et al., 2001; Stepanova et al., 2008; Mashiguchi et al., 2011).

For in vitro assays where Trp or 4-Cl-Trp was the substrate, each reaction tube contained purified recombinant protein (0.20 μg mL$^{-1}$ substrate) (34 ng μL$^{-1}$), sodium pyruvate (4.8 μg μL$^{-1}$) PLP (1.2 μmol), and maltose (3.8 μmol) in K$_2$HPO$_4$/KH$_2$PO$_4$ buffer (10 mM; pH 8.5). For in vitro assays where IPyA or 4-Cl-IPyA was the substrate the reaction tube contained recombinant protein (0.20 μg μL$^{-1}$ substrate) (34 ng μL$^{-1}$), sodium pyruvate (43 μmol) PLP (1.2 μmol), and maltose (4.3 μmol) in K$_2$HPO$_4$/KH$_2$PO$_4$ buffer (10 mM; pH 6.5). For all functional assays, the total reaction volume was 174 μL. “No-protein” controls were prepared in a similar manner, except that the protein solution was replaced with buffer. All samples were incubated at 37°C for 3 h, after which the reaction was stopped by the addition of acetic acid in methanol (5%, v/v) to give an 80:19:1 buffer:methanol:acetic acid mixture. These mixtures were then analyzed by UPLC-MS.

Expression and Purification of Recombinant PsTAR2 and PsTAR1

The following procedure (adapted from the NEB pMAL Protein Fusion and Purification Instruction Manual) was used for protein expression and purification.

A starter culture was prepared by inoculating superoptimal broth with competent E. coli (10 μL) containing ampicillin (0.29 μmol) with E. coli, known to contain pTAR2 in frame in the vector pMAL. A “vector-only” starter culture was also prepared. The two starter cultures were incubated at 37°C for 16 h with shaking, after which the optical density at 600 nm (OD$_{600}$) was determined. The column was held at 35°C, and the sample compartment was at 6°C. Approximate RTs using solvent combination 1 were 0.89 min for Trp, 1.0 min for keto-IPyA, 1.3 min for enol-4-Cl-IPyA, 2.0 min for enol-4-Cl-IPyA, and 2.2 min for 4-Cl-IAA and [D$_3$]4-Cl-IAA. RTs using solvent combination 2 were approximately 1.7 min for keto-IPyA, 1.3 min for enol-4-Cl-IPyA, 2.0 min for enol-4-Cl-IPyA, and 2.2 min for 4-Cl-IAA and [D$_4$]-CH$_2$-IAA. RTs using solvent combination 2 were approximately 1.7 min for keto-IPyA, 3.5 min for enol-IPyA, 2.7 min for 4-Cl-IPyA, 3.9 min for enol-4-Cl-IPyA, and 3.3 min for 4-Cl-IAA.

For improved separation of interfering peaks for IAM analyses, solvent combination 2 and a slower gradient were employed; this was a linear gradient from 99% A:1% B to 80% A:20% B at 6 min, followed by a linear gradient to 40% A:60% B at 12 min, followed by immediate reequilibration to starting conditions for 5 min.

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UPLC-MS

Samples were analyzed using a Waters Acquity H-series UPLC device coupled to a Waters Xevo triple quadrupole mass spectrometer. A Waters Acquity UPLC BEH C$_{18}$ column (2.1 mm × 100 mm × 1.7 μm) was used. There were two solvent combinations: (1) 5 mol ammonium acetate (pH 5.4; solvent A) and acetonitrile (solvent B), and (2) 1% (v/v) acetic acid in water (solvent A) and acetonitrile (solvent B).

All experiments were performed in a negative ion mode with electrospray ionization. The mass spectrometer was operated in positive ion electrospray mode with electrospray ionization. The mass spectrometer was operated in negative ion mode with electrospray ionization. The mass spectrometer was operated in positive ion electrospray mode with electrospray ionization. The mass spectrometer was operated in negative ion mode with electrospray ionization. The mass spectrometer was operated in positive ion electrospray mode with electrospray ionization. The mass spectrometer was operated in negative ion mode with electrospray ionization.
channel), the second window from 0 to 6 min was for 4-Cl-IPyA (dwell time, 66 ms per channel), and the third window from 1.6 to 6 min was for 4-Cl-IAA and [D-4]35Cl-IAA (dwell time, 66 ms per channel). MRM channels for the chlorinated species were as follows: 4-Cl-IPyA, m/z 239.2 to 222.2; 4-Cl-CTrP, m/z 241.2 to 224.2; [D-4]J-Cl-IAA, m/z 242.2 to 225.2 and m/z 242.2 to 224.2 (cone voltage, 19 V; CE, 12 V); 4-Cl-IPyA, m/z 238.2 to 164.1 and 238.2 to 192.1; 4-Cl-IAA, m/z 240.2 to 166.1 (cone voltage, 20 V; CE, 24 V); 4-Cl-CT- IAA, m/z 220.05 to 164.05; [D-4]J-35Cl-IAA and 4-Cl-35Cl-IAA, m/z 212.05 to 166.05; [D-4]J-35Cl-IAA, m/z 213.05 to 167.05 (cone voltage, 20 V; CE, 18 V). When tandem MS was required, the cone voltage and CE were 20 V.

Plant Material

Wild-type (TAR2) pea plants used for this study were the Hobart tall (LE) line 107 (derived from cv Torsdag) and the dwarf (le-1) line Camero. All plants were grown as described previously (Jager et al., 2005).

Quantitative Real-Time PCR

At a range of developmental stages, three or four seeds from multiple pods were harvested and immersed in liquid N2 for each of four replicates. Comparable seeds from the same pods were harvested for analysis of auxin content (Fig. 3). The seeds were ground frozen, and total RNA was extracted from approximately 100 mg of ground tissue (RNaseasy plant mini kit; Qiagen) with on-column DNase digestion (RNase-free DNase set; Qiagen). For later-stage seeds, the ground tissue was divided among four or five QIAshredder columns and the flow-through was combined to one RNA column, to avoid overloading one shredder with contaminants that can reduce yield and damage the RNA.

Spectrophotometer measurements (Thermo Scientific NanoDrop 8000) showed the RNA to be of good quality, with a clear peak at 260 nm, 260:280 ratio of 2, and 260:230 ratio above 1.5. (260:230 mean of 2.17, median of 2.30, and sto of 0.30).

In a single batch, cDNA was synthesized from 0.5 μg of total RNA using reverse transcriptase primer mix (QuantiTect reverse transcription kit; Qiagen) with integrated removal of genomic DNA. Controls lacking reverse transcriptase enzyme were prepared to monitor for contamination with genomic DNA.

First-strand cDNA was diluted 6-fold, and 3 μL was used in each quantitative real-time PCR using SYBR Green chemistry (SensiMix SYBR; Bioline). In a single batch, cDNA was synthesized from 0.5 g of total RNA using reverse transcriptase primer mix (QuantiTect reverse transcription kit; Qiagen) with integrated removal of genomic DNA. Controls lacking reverse transcriptase enzyme were prepared to monitor for contamination with genomic DNA.

Quantification of IAA, 4-Cl-IAA, Trp, and 4-Cl-CTrP

Seeds were harvested, weighed, homogenized, and extracted as before (Tivendale et al., 2010). After filtering, internal standards ([12C]3I-IAA and [D-4]35Cl-IAA) were added, and aliquots were purified using a Sep-Pak cartridge. Smaller aliquots were taken for the determination of Trp and 4-Cl-CTrP; the internal standards were [D-4]J-Cl-IAA and [D-4]J-Cl-IAA. All samples were analyzed by UPLC-MS (MRM).

TILLING

PsTAR2 mutants were identified from an ethyl methanesulfonate mutant collection of 4,800 pea lines using TILLING screening as described by Dalmais et al. (2008). Details of all primer sequences are given in Supplemental Table S2. The nature of the mutations was identified by sequencing. PsTAR2 genomic sequence and TILLING mutations were integrated in U TillLD (http://urgv.evry.inra.fr/UTILdb).

The tar2 mutant (line 918), with a stop codon encoding a protein truncated prior to the catalytic Lys common to all LPL-dependent aminotransferases, was obtained among 15 mutations identified in the TILLING screen and was backcrossed four times to the parental cv Camero (dwarf; le-1). Line 918 was also crossed to the Hobart tall line 107, and single plant selection was carried out for three generations to give tall TAR2 and tar2 plants on a similar background. Seeds from these plants were harvested for the quantification of auxins and auxin precursors.

Genotyping

DNA from plants at the seedling stage was amplified with PsTAR2-specific primers (Phire Plant Direct kit; Finnzymes); the restriction enzyme BseRI (NEB) cut the mutant allele. Alternatively, when they failed to emerge from the soil, the genotype was determined by digging up the seed, removing theesta, and extracting DNA from the cotyledons/embryo (DNasey plant kit; Qiagen). PCR amplification (Taq DNA polymerase with Thermopol buffer; NEB), and BseRI (NEB) digestion.

Synthesis of Deuterated 4-Cl-CTrP

A suspension of [1,4-d3]-Cl-CTrP (69 mg) in deuterated HCl in D2O, prepared by the careful addition of thionicl chloride (0.5 mL; Riedel-de Haën) to D2O (4.5 mL), was stirred at room temperature for 4 weeks in a sealed vial. The solvent was reduced under reduced pressure, and a fresh portion of deuterated HCl in D2O was added and the suspension was stirred for a further 4 weeks. This process was repeated a second time, before evaporation under reduced pressure, to yield deuterated [1,4-d3]-Cl-CTrP as the hydrochloride salt. The product was checked for deuterium incorporation by UPLC-MS (D4, 15%; D3, 25%; D2, 33%; D1, 23%; unlabeled, 5%).

Synthesis and Characterization of 4-Cl-IPyA

The 4-CIPyA synthesis method was adapted from Politi et al. (1996). Triethylamine (76 μL) was added to a suspension of [1,4-d3]-Cl-IPyA (102 mg) in methanol (1.7 mL) under an atmosphere of N2 at room temperature. After stirring for 10 min, pyridine-4-carboxaldehyde (80 μL) was added and the mixture was stirred for a further 10 min, after which ZnCl2 (anhidrous, 42 mg) was added to the mixture, which was stirred for a further 10 min. Total dissolution was achieved when 1,6-diazabicyclo[5.4.0]-undec-7-ene (164 μL) was added to the mixture, which was then stirred for a further 3 h under N2, after which it was quickly drop-wise to HCl (2 M, 5 mL) preheated to 50°C. After 10 min, the temperature was increased to 55°C and the mixture was left for another 25 min, after which the mixture was cooled to room temperature and stood for 16 h. The resulting brown precipitate was collected by vacuum filtration, air dried overnight, and then dried in a desiccator. The precipitate was identified as the target compound (47 mg, 46% yield) by 1H- and 13C-NMR (H-NMR, CD3OD, 300 MHz δ: 7.06 [m, 2H], 7.33 [doublet of doublets, f = 2.4 Hz, 3.9 Hz, H1], 7.72 [d, J = 21.2 Hz, H2], 11.18 [broad singlet, H3]; 13C-NMR, CD3OH, 75 MHz δ: 105.4 [CH], 110.5 [C], 110.4 [CH], 120.9 [CH], 122.1 [CH], 122.9 [C], 125.9 [C], 129.4 [CH], 137.1 [C], 137.8 [C], 167.3 [C]) of the 3Cl-IPyA. This compound was characterized by UPLC-MS for comparison with the product of our PsTAR1 and PsTAR2 in vitro assays using 4-Cl-CTrP as the substrate (Fig. 2).

Application of Heavy-Isotope-Labeled Intermediates

Solutions of 1- or 13C-labeled substrates in water were injected into excised immature pea seeds (30-135 mg), which contained liquid endosperm, using a sterile syringe, as described previously (Tivendale et al., 2010), and left up to 3 h before harvesting. After the appropriate incubation time, seed extracts were prepared in one of two ways: (1) the seeds were quickly frozen in liquid N2, and ground into a powder, which was immersed in methanol at 4°C, for 1 h; (2) the seeds were immersed in ~20°C 4.1 methanol/water containing butylated hydroxytoluene (1.13 mM) and subsequently extracted as before (Tivendale et al., 2010). After removal of impurities using Waters Sep-Pak Vac RC C18 cartridges, all extracts were analyzed by UPLC-MS.
Sequence data for pea cv Torsdag mRNA can be found in the GenBank/EMBL data libraries under accession numbers JN909088 (PsTAR1), JN909089 (PsTAR2), and JN909090 (PsTAR3). The GenBank accession numbers for genomic nucleotide sequences from cv Cameor are JQ002582 (PsTAR1), JQ002584 (PsTAR2), and JQ002583 (PsTAR3).

### Supplemental Data

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

**Supplemental Figure S1.** UPLC-MS chromatograms of in vitro assay mixtures.

**Supplemental Figure S2.** Keto-enol tautomerization of IPyA.

**Supplemental Figure S3.** HSQC NMR spectrum for IPyA.

**Supplemental Figure S4.** UPLC-MS chromatograms showing endogenous IPyA and 4-Chl-IPyA in pea seeds.

**Supplemental Figure S5.** Trp and 4-Cl-Trp levels of wild-type (WT) and tar2 seeds.

**Supplemental Figure S6.** UPLC-MS chromatograms showing lack of endogenous IAm in pea seeds.

**Supplemental Table S1.** qPCR-specific primers.

**Supplemental Table S2.** TILLING-specific primers.

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### LITERATURE CITED


### Biosynthesis of 4-Chloroindole-3-Acetic Acid


