Biosynthesis of the Halogenated Auxin, 4-Chloroindole-3-Acetic Acid[1][W][OA]

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[illegible]

[illegible] *Pisum sativum*) [illegible]

tar2 [illegible]

[illegible] *Lens culinaris*, *Lath rus latifolius*, *Vicia faba*, [illegible] (*Pisum sativum*), [illegible]

[illegible] *Arabidopsis thaliana*). [illegible]

[illegible] (*Zea ma s*[illegible]

N-[illegible]

RESULTS

In Vitro Analysis of PsTAR1 and PsTAR2

[illegible]

[1] [illegible]

* [illegible]

[illegible]

[illegible]

TRYPTOPHAN AMINOTRANSFERASE RELATED1 (*PsTAR1*), *PsTAR2*, and *PsTAR3*.

Escherichia coli,

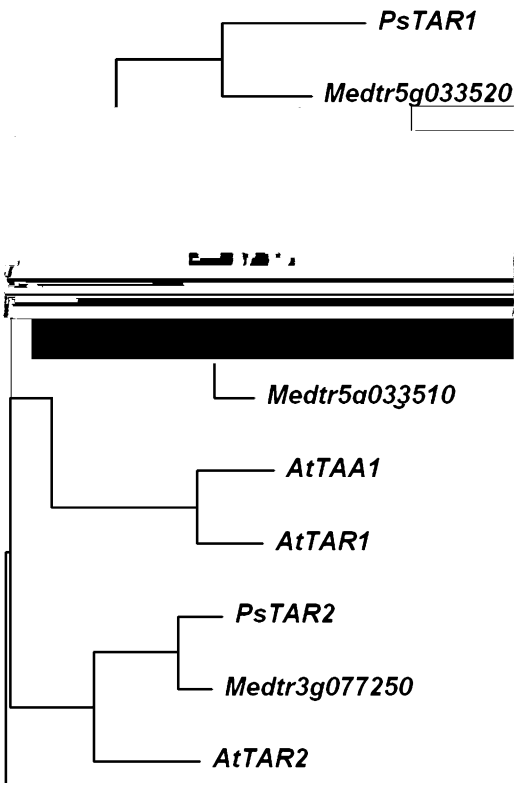

Figure 1. Inferred phylogenetic relationship of the three pea *TAR* genes, *PsTAR1* (JN990988), *PsTAR2* (JN990989), and *PsTAR3* (JN990990), with other *TAR*-like genes. The phylogram was generated as described previously (Tivendale et al., 2010). Included were *AtTAA1* (At1g70560), *AtTAR1* (At1g23320), 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 *M. truncatula* 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 MpM2D3* 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

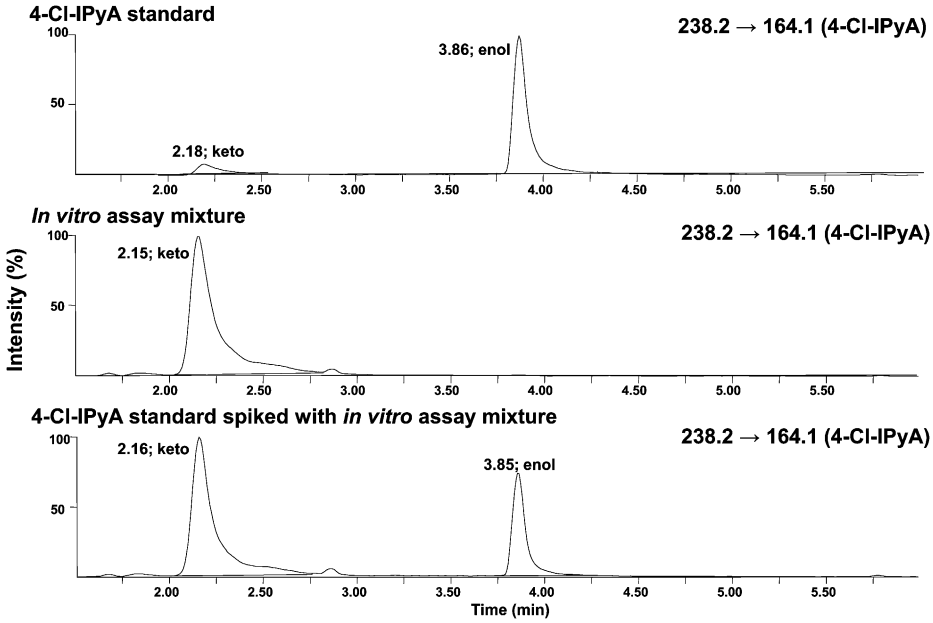

Figure 2. UPLC-MS chromatograms (MRM mode; acetic acid program) of 4-Cl-IPyA standard (top channel), an in vitro assay mixture using PsTAR1 as the enzyme and 4-Cl-Trp as the substrate (middle channel), and a mixture of the two samples (bottom channel). Mixing the two samples showed coelution of the product with the standard. Given the relative signal strengths of the keto and enol tautomers in the mixture, compared with the individual samples, the mixture represents approximately a 1:1 ratio of the two samples. Identical mass transitions and coelution of the product with the standard confirm its identity as keto 4-Cl-IPyA.

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

Effects of the *tar2* Mutation on Auxin Levels

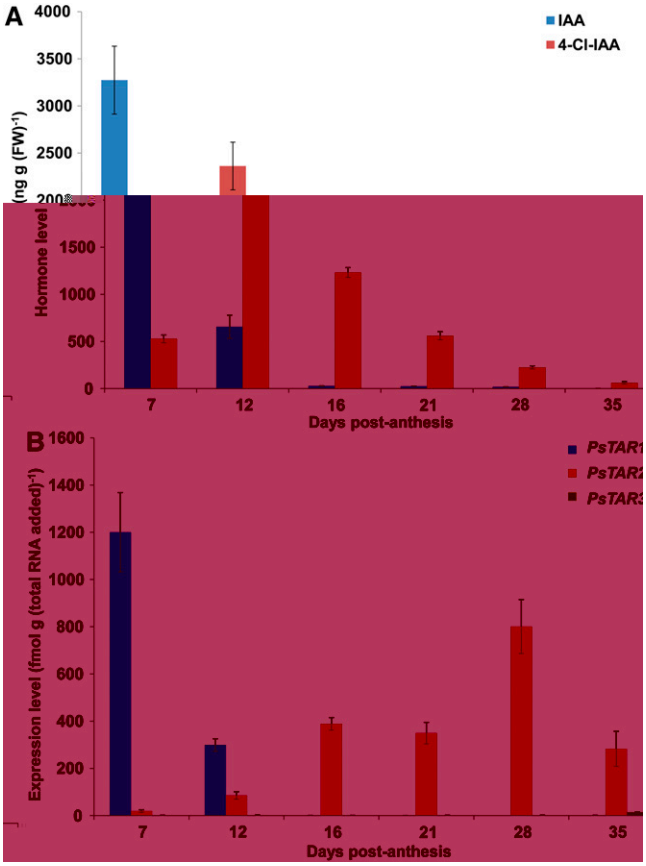

Figure 3. IAA and 4-Cl-IAA levels (A) and *PsTAR1*, *PsTAR2*, and *PsTAR3* transcript levels (B) over the course of seed development in line 107. Shown are means ± SE ($n = 3$ for auxin and $n = 4$ for transcript levels). Mean seed fresh weights for each time point, in order of increasing age, were 3.8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

tar2

$P <$

tar2

PsTAR1

PsTAR2 *PsTAR1*

Labeled-Precursor Feeding Studies

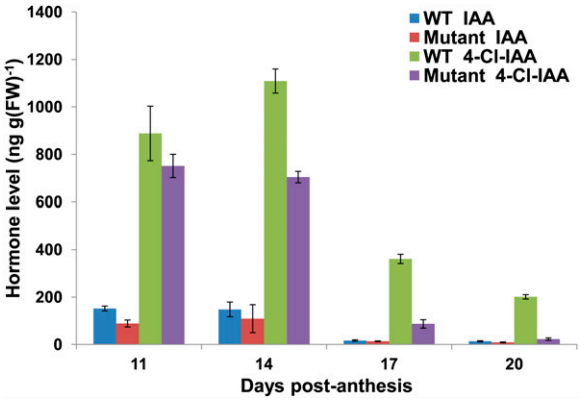

Figure 4. IAA and 4-Cl-IAA content of wild-type (WT) and *tar2* mutant seeds. Shown are means ± SE ($n = 3$). Wild-type seed fresh weights were as follows: 30 ± 1 mg (11 DPA), 114 ± 5 mg (14 DPA), 252 ± 17 mg (17 DPA), and 339 ± 7 mg (20 DPA); *tar2* seed fresh weights were as follows: 41 ± 8 mg (11 DPA), 125 ± 5 mg (14 DPA), 260 ± 5 mg (17 DPA), and 339 ± 13 mg (20 DPA). All plants were on a tall background. FW, Fresh weight.

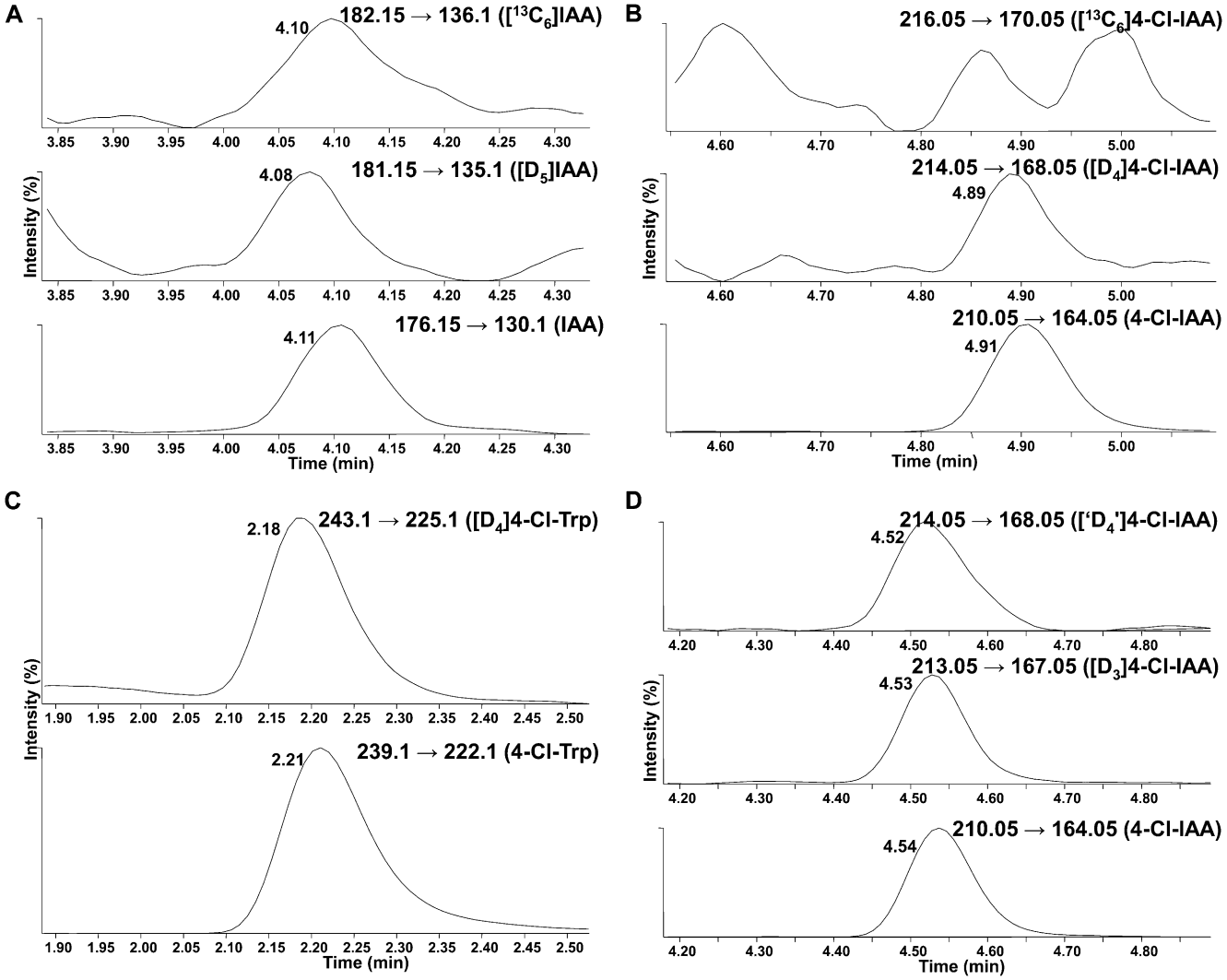

Figure 5. UPLC-MS chromatograms (MRM mode; acetic acid program) obtained from extracts of pea seeds that had been previously injected with a mixture of $[D_5]$Trp and $[^{13}C_6]$IAA (A–C) or $[D_4]$4-Cl-Trp (D). On UPLC, the RT of a deuterated species is earlier than the RT for the endogenous species; ^{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_5 label (middle channel; RT = 4.08) after injection of $[D_5]$Trp; the endogenous IAA (bottom channel; RT = 4.11) and the injected $[^{13}C_6]$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 $[D_5]$Trp (one deuterium is replaced with a chlorine atom), but the $^{13}C_6$ label from the injected $[^{13}C_6]$IAA was not incorporated into 4-Cl-IAA (top channel; the peaks observed in this channel did not have the correct RT for $[^{13}C_6]$4-Cl-IAA). C, 4-Cl-Trp became enriched with D_4 label (top channel; RT = 2.18) from the injected $[D_5]$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 $[^{37}Cl,D_2]$4-Cl-IAA.

DISCUSSION

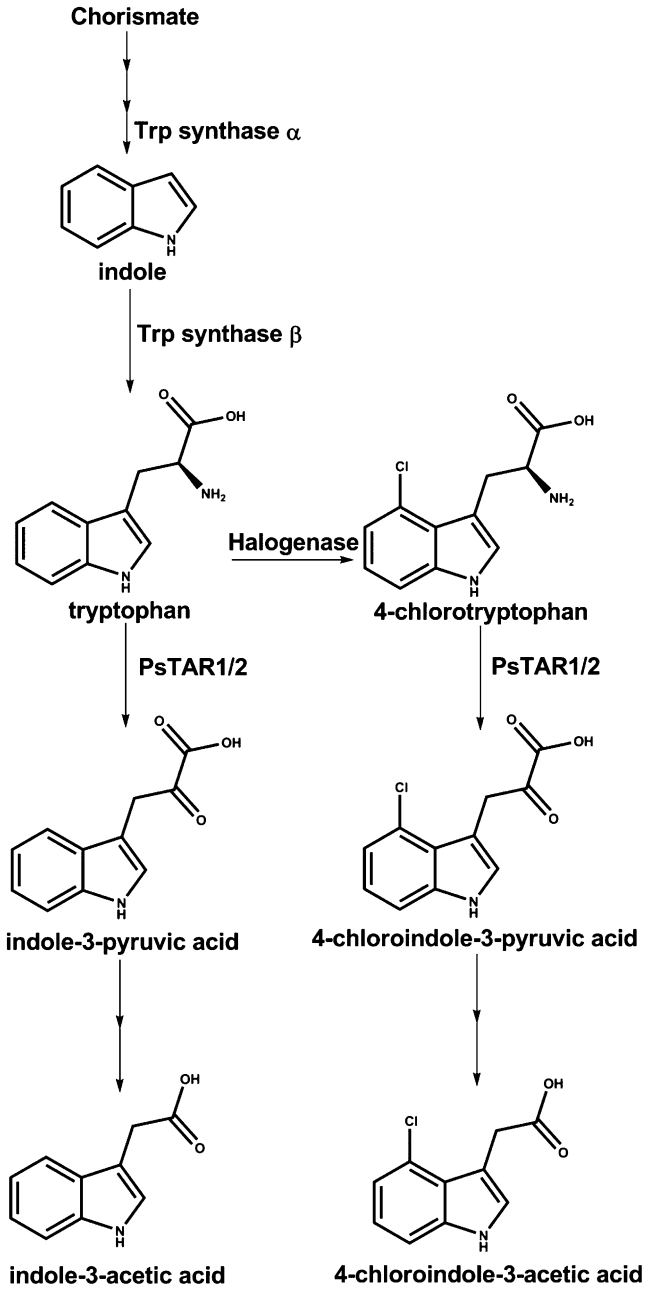

Figure 6. Proposed parallel IAA and 4-Cl-IAA biosynthetic pathways in pea seeds, based on this study and previous evidence (Manabe et al., 1999; Reinecke, 1999; Quittenden et al., 2009; Tivendale et al., 2010; Zhao, 2010; Mashiguchi et al., 2011; Stepanova et al., 2011; Won et al., 2011; Kriechbaumer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

MATERIALS AND METHODS

Chemicals

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

Pisum sativum) *Escherichia coli*

(*Arabidopsis thaliana*), *Medicago truncatula*, *Oryza sativa*, *Zea mays*, *Marchantia polymorpha*, and *Physcomitrella patens*. *TAR*-like *M. truncatula*

M. truncatula

Escherichia coli

PsTAR2 *Not*I

PsTAR1 *Sbf*I *Xmn*I *Sbf*I

E. coli

Expression and Purification of Recombinant PsTAR2 and PsTAR1

E. coli, *PsTAR2*

β-D-

β-D-

g

In Vitro Assays Using PsTAR2 and PsTAR1

UPLC-MS

Plant Material

Quantitative Real-Time PCR

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

TILLING

Genotyping

Synthesis of Deuterated 4-Cl-Trp

Synthesis and Characterization of 4-Cl-IPyA

Application of Heavy-Isotope-Labeled Intermediates

PsTAR1), *PsTAR2*), *PsTAR3*). *PsTAR1*), *PsTAR2*), *PsTAR3*).

Supplemental Data

Supplemental Figure S1.

Supplemental Figure S2.

Supplemental Figure S3.

Supplemental Figure S4.

Supplemental Figure S5. *tar2*

Supplemental Figure S6.

Supplemental Table S1.

Supplemental Table S2.

ACKNOWLEDGMENTS

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