Evidence That Chlorinated Auxin Is Restricted to the Fabaceae But Not to the Fabeae[1][OPEN]

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Auxin is a pivotal plant hormone, usually occurring in the form of indole-3-acetic acid (IAA). However, in maturing pea (*Pisum sativum*) seeds, the level of the chlorinated auxin, 4-chloroindole-3-acetic acid (4-Cl-IAA), greatly exceeds that of IAA. A key issue is how plants produce halogenated compounds such as 4-Cl-IAA. To better understand this topic, we investigated the distribution of the chlorinated auxin. We show for the first time, to our knowledge, that 4-Cl-IAA is found in the seeds of *Medicago truncatula*, *Melilotus indicus*, and three species of *Trifolium*. Furthermore, we found no evidence that *Pinus* spp. synthesize 4-Cl-IAA in seeds, contrary to a previous report. The evidence indicates a single evolutionary origin of 4-Cl-IAA synthesis in the Fabaceae, which may provide an ideal model system to further investigate the action and activity of halogenating enzymes in plants.

The chlorinated form of auxin, 4-chloroindole-3-acetic acid (4-Cl-IAA), is a highly active hormone that is thought to play a key role in early pericarp growth (Reinecke et al., 1995, 1999; Ozga et al., 2009). Exogenous 4-Cl-IAA, for example, has been shown to promote the pericarp elongation of deseeded pea (*Pisum sativum*) pods (Reinecke et al., 1999). Johnstone et al. (2005) reported that 4-Cl-IAA and bioactive GA (GA_3 or GA_1) act synergistically on pericarp growth when applied simultaneously, and a growth regulatory role has been proposed for 4-Cl-IAA through induction of GA biosynthesis and inhibition of ethylene action. In other species, e.g. tomato (*Solanum lycopersicum*), the nonchlorinated form of auxin, indole-3-acetic acid (IAA), also stimulates fruit growth via GAs (Serrani et al., 2008; Tang et al., 2015). The chlorinated auxin is mainly found in reproductive structures (Katayama et al., 1988), in which its levels often exceed those of the more widespread IAA (Tivendale et al., 2012). The chlorinated form is thought to be restricted to members of the leguminous tribe Fabeae (Reinecke 1999), which includes the genera *Vicia*, *Pisum*, *Lathyrus*, *Lens*, and *Vavilovia* (Schaefer et al., 2012). However, there is a curious exception: 4-Cl-IAA has been reported also from Scots pine (*Pinus sylvestris*; Ernstsen and Sandberg, 1986).

We previously published evidence that most 4-Cl-IAA in maturing pea seeds is synthesized from 4-Cl-tryptophan (4-Cl-Trp) via 4-Cl-indole-3-pyruvic acid (Tivendale et al., 2012, 2014). 4-Cl-Trp has been identified in extracts from pea and broad bean (*Vicia faba*) seeds (Kettner et al., 1992; Manabe et al., 1999), but whether the precursors of Trp can be chlorinated is unknown.

Virtually nothing is known about the enzymes that catalyze halogenation reactions in plants. In bacteria, fungi, and marine algae, there are six types of enzymes responsible for the addition of halogen atoms to organic molecules. These include heme haloperoxidases, vanadium-dependent haloperoxidases, mononuclear nonheme iron halogenases, flavin-dependent halogenases, *S*-adenosyl-L-Met-dependent chlorinases and fluorinases, and methyl halide transferases (Butler and Sandy, 2009; Wagner et al., 2009). However, in the genomes of angiosperms, the only type of halogenating enzyme that has been annotated are haloperoxidases, but very little is known about these enzymes. To further understand the activity and action of halogenating enzymes in plants, a comparative system is required.

In this study, we investigated the distribution of 4-Cl-IAA and 4-Cl-Trp in the Fabaceae by monitoring these compounds in the seeds of representative species spanning the phylogeny of this family. Most of these species have not been previously tested for the presence of the chlorinated compounds. In addition, we reexamined the reported occurrence of 4-Cl-IAA outside the Fabaceae, namely in Scots pine; several other *Pinus* species were investigated here as well. We also examined the endogenous levels of 4-Cl-IAA in both vegetative tissues and seeds of broad bean to address the question of whether 4-Cl-IAA is largely

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H.K.L. conducted the experiments and wrote part of the article; S.A.M.M. obtained the plant material, supervised the phylogenetic analysis, and wrote part of the article; E.L.M. supervised the hormone analyses and complemented the writing; J.J.R. initiated the project and wrote part of the article.

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restricted to seeds (Pless et al., 1984; Katayama et al., 1988).

RESULTS AND DISCUSSION

We screened 15 species from the Fabaceae for chlorinated auxin, selected to represent all major clades of the Papilionoid Fabaceae (Wojciechowski et al., 2004). We have extended the range of leguminous species that contain 4-Cl-IAA by detecting this compound in reproductive structures of *Trifolium repens*, *Trifolium subterraneum*, *Trifolium micranthum*, *Melilotus indicus*, and *Medicago truncatula*, all from outside the tribe Fabeae. Identification was based firstly on the correct

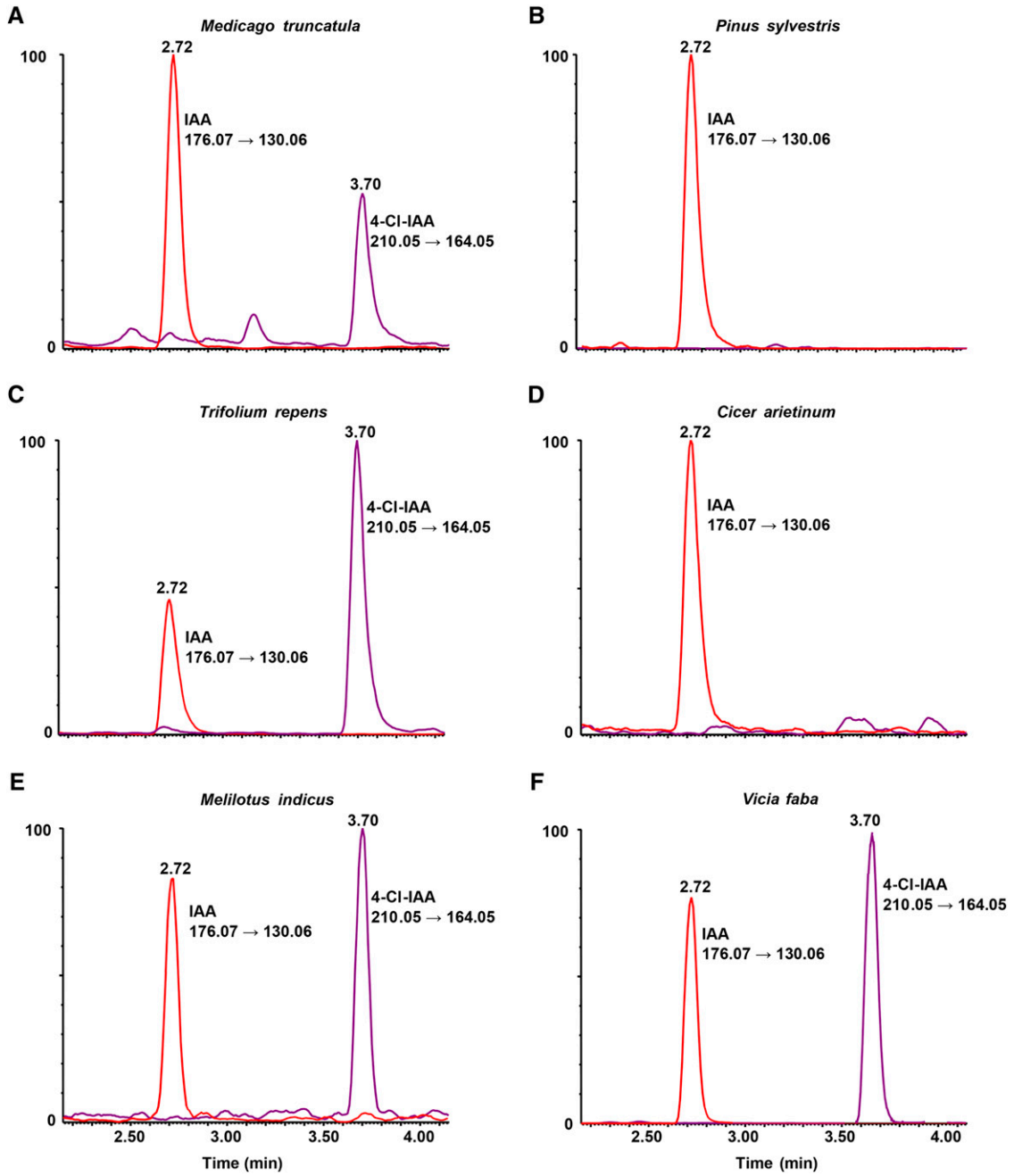

Figure 1. Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS) chromatograms (Multiple Reaction Monitoring [MRM] mode) obtained from *M. truncatula* (A; dry seeds, after hydrolysis), Scots pine (B; dry seeds), *T. repens* (C; fresh pods, containing seeds), *C. arietinum* (D; young seeds), *M. indicus* (E; fresh pods, containing seeds), and broad bean (F; young seeds), showing the presence of endogenous IAA (red channel, MRM transition mass-to-charge ratio, 176.07–130.06; retention time = 2.72 min) and 4-Cl-IAA (purple channel, MRM transition mass-to-charge ratio, 210.05–164.05; retention time = 3.70 min). Retention times were subjected to minor adjustment to compensate for run-to-run variation.

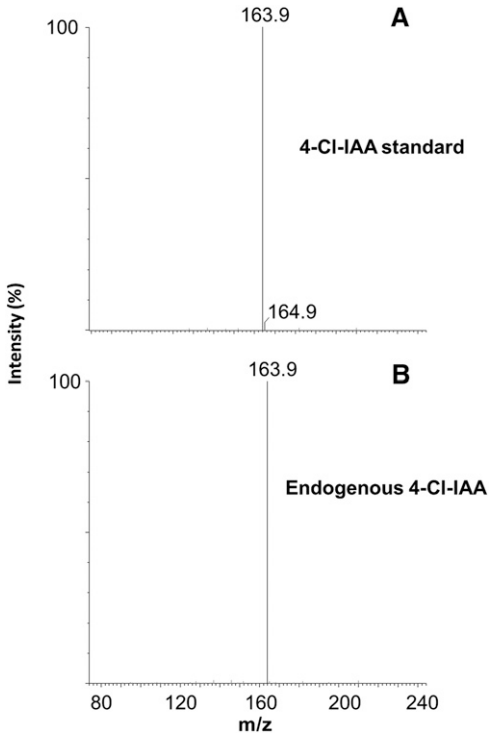

Figure 2. Product ion spectrum from ^{35}Cl 4-Cl-IAA. A, Product ions of $[M+H]^+$ (mass-to-charge ratio, 210) from 4-Cl-IAA standard. B, Endogenous 4-Cl-IAA from *T. repens*.

liquid chromatography retention time (Fig. 1) and fragmentation patterns (Fig. 2), as indicated by comparison with standard forms of 4-Cl-IAA. Secondly, peaks for the expected mass transitions, 210 to 164 and 212 to 166, were observed, with the latter comprising approximately 33% of the former (data not shown). This ratio of peak areas is diagnostic for compounds containing one chlorine atom (Gross, 2011). The peak corresponding to the transition for 211 to 165 was only 10% of the 210 to 164 peak, as expected from naturally occurring isotopes. In some cases, dry seeds were analyzed (Table I), and in these cases, the extract was hydrolyzed to release 4-Cl-IAA and IAA from their conjugated forms.

Chlorinated auxin was not detected in representative species from the Galegeae tribe (*Clianthus puniceus*), the Robinioids (*Lotus japonicus*), the Indigoferoids (*Indigofera australis*), the Millettioids (*Glycine clandestine* and *Hardenbergia comptoniana*), the Mirbelioids (*Pultenaea juniperina*), the Dalbergioids (*Arachis hypogaea*), or the Genistoids (*Lupinus angustifolius*; Table I). Previously, 4-Cl-IAA was not detected in a number of Dalbergioid species, including *Phaseolus vulgaris* (Hofinger and Böttger, 1979), *Glycine max*, *Vigna catiang*, and *Dolichos lablab* (Katayama et al., 1987). Furthermore, we confirmed a previous finding that 4-Cl-IAA is not detectable in *Cicer arietinum* (Engvild, 1994; Fig. 1).

In additional experiments, chlorinated auxin was quantified using a deuterated internal standard. We found very high levels of 4-Cl-IAA in fruits of *T. repens* (which consisted mainly of seeds; Fig. 3) and in immature seeds of broad bean (Table I). In the case of *M. truncatula*, the most convincing evidence for chlorinated auxin was obtained after hydrolysis of dry seeds. For the five species shown, to our knowledge, for the first time here to contain 4-Cl-IAA (*M. truncatula*, *M. indicus*, *T. repens*, *T. subterraneum*, and *T. micranthum*), the level of the chlorinated compound in reproductive structures of these species exceeded that of IAA, as is the case for pea (Tivendale et al., 2012) and broad bean (Table I).

Table I. *Endogenous levels of 4-Cl-IAA and IAA in 15 representative species from the family Fabaceae*
n.d., Not detected.

Species	Phylogenetic Clade	Tissue Type	IAA	4-Cl-IAA
			ng g^{-1}	
M. truncatula	Trifoleae	Dry seeds	26[a]	85[a]
T. repens	Trifoleae	Fresh pods, containing seeds	36	1,388
T. micranthum	Trifoleae	Fresh pods, containing seeds	47	569
T. subterraneum	Trifoleae	Fresh pods, containing seeds	100	243
M. indicus	Trifoleae	Fresh pods, containing seeds	7	39
Broad bean	Fabeae	Young seeds	175	885
		Old seeds	73	91
		Young leaves	16	3
		Old leaves	9	n.d.
C. arietinum	Cicereae	Young seeds	14	n.d.
C. puniceus	Galegeae	Young seeds	343	n.d.
I. australis	Indigoferoid	Fresh pods, containing seeds	11	n.d.
L. japonica	Robinioid	Dry seeds	98[a]	n.d.[a]
G. clandestine	Millettioid	Young seeds	340	n.d.
H. comptoniana	Millettioid	Young seeds	2,066	n.d.
P. juniperina	Mirbelioid	Young seeds	274	n.d.
A. hypogaea	Dalbergioid	Dry seeds	137[a]	n.d.[a]
L. angustifolius	Genistoid	Dry seeds	30[a]	n.d.[a]

[a]Total level of IAA or 4-Cl-IAA, including both free acids and conjugated forms.

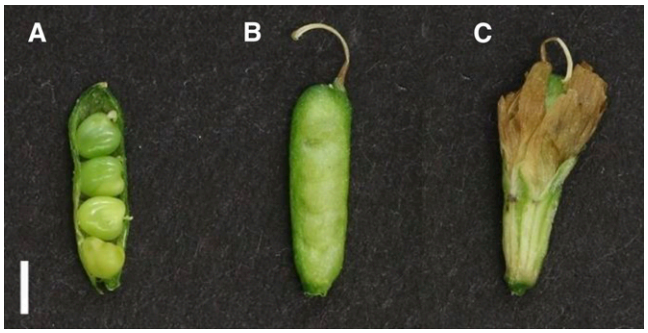

Figure 3. Fruit of *T. repens*. A, Seeds in a bisected seed pod. B, Whole seed pod. C, Floret covering a single seed pod. Bar = 1 mm.

Endogenous 4-Cl-Trp was also detected in *M. truncatula* burrs as well as the fresh pods (containing seeds) of *T. repens* and *M. indicus*, providing further evidence that Trp or a Trp precursor can be chlorinated (Tivendale et al., 2012; Fig. 4). Again, chlorinated Trp was identified on the basis of retention times and fragmentation pattern (Fig. 4), as well as the diagnostic ratio (approximately 3 to 1) of peak areas for the chlorine isotopes of the $[M+H]^+$ cluster (Gross, 2011). Chlorinated Trp was not detected in the Fabaceae spp. *C. arietinum*, *C. puniceus*, *I. australis*, *G. clandestine*, and *H. comptoniana* or the *Pinus* spp. *Pinus flexilis* or *Pinus parviflora*.

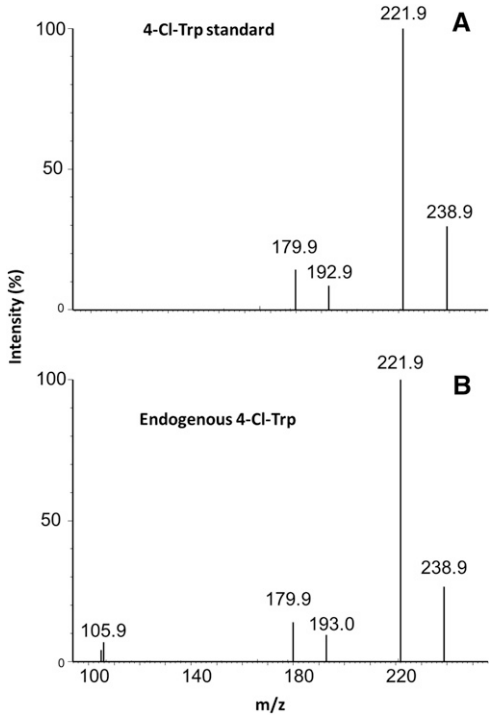

Figure 4. Product ion spectrum from ^{35}Cl 4-Cl-Trp. A, Product ions of $[M+H]^+$ (mass-to-charge ratio, 239) from 4-Cl-Trp standard. B, Endogenous 4-Cl-Trp from *T. repens*.

Scots pine is the only species outside the Fabaceae previously reported to contain chlorinated auxin, although the levels reported (only from seeds) were low (Ernstsen and Sandberg, 1986). In our investigation, UPLC-MS/MS failed to detect any trace of 4-Cl-IAA in dry seeds of Scots pine (Fig. 1; Table II). Furthermore, we did not detect 4-Cl-IAA in young seeds of a diversity of species from the genus *Pinus*, including *P. flexilis*, *P. parviflora* 'Glauca,' and *P. parviflora* 'Shikoku-goyo,' or in mature seeds of *Pinus pinea* or *Pinus radiata* (Table II). Our results cast serious doubt over the reported presence of 4-Cl-IAA in Scots pine seeds.

In another intriguing report, very high levels (16,000 ng g^{-1} fresh weight [FW]) of 4-Cl-IAA were documented for young but fully developed leaves of field-grown broad bean plants (Pless et al., 1984). This is inconsistent with the hypothesis that 4-Cl-IAA is largely restricted to seeds (Katayama et al., 1988). Moreover, the figure reported by Pless et al. (1984) exceeds their reported seed content (up to 15,000 ng g^{-1} FW). To investigate these findings, we determined the distribution of 4-Cl-IAA in broad bean. We detected only very low levels in young leaves (approximately 3 ng g^{-1} FW), but none in mature leaves. We found that the levels of 4-Cl-IAA in young and mature seeds of broad bean were 886 and 90 ng g^{-1} FW, respectively. These data do not support the reported levels of Pless et al. (1984) and confirm, instead, previous evidence (Katayama et al., 1988) that seeds contain much higher levels of chlorinated auxin than vegetative tissues.

In conclusion, our evidence extends the range of species that produce chlorinated auxin beyond the Fabeae but restricts it to the Fabaceae. We have obtained unequivocal evidence for 4-Cl-IAA and its precursor, 4-Cl-Trp, from *M. truncatula* and from genera between *Medicago* and the Fabeae in phylogenetic terms. However, we were not able to repeat the finding that chlorinated auxin occurs in Scots pine. Hence, there is no longer an indication that the capacity to chlorinate arose more than once in the evolutionary history of plants.

We suggest that the capacity of plants to produce chlorinated auxin evolved only once and that that event occurred in the Fabaceae. As with previous researchers (Engvild, 1994), we did not detect 4-Cl-IAA

Table II. *Endogenous levels of 4-Cl-IAA and IAA of six Pinus spp.*
n.d., Not detected.

Species	Tissue Type	IAA	4-Cl-IAA
		ng g^{-1}	
Scots pine	Dry seeds	320[a]	n.d.[a]
P. flexilis	Immature seeds	74	n.d.
P. pinea	Mature seeds	38	n.d.
P. radiata	Mature seeds	3 to 6	n.d.
P. parviflora 'Glauca'	Immature seeds	71	n.d.
P. parviflora 'Shikoku-goyo'	Immature seeds	121	n.d.

[a]Total level of IAA or 4-Cl-IAA, including both free acids and conjugated forms.

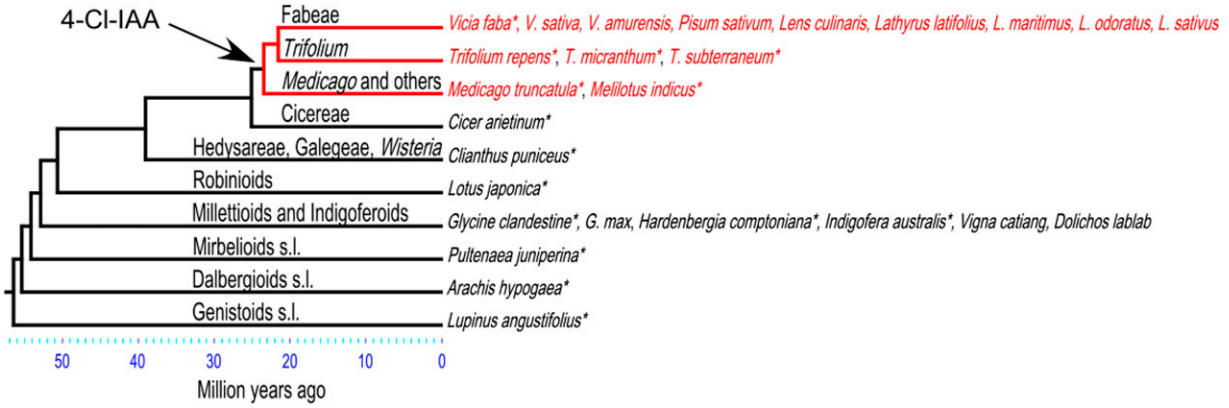

Figure 5. Phylogeny of the major lineages of the Meso-Papilionoideae clade of the family Fabaceae and closest lineages to the tribe Fabeae. The ability to produce 4-Cl-IAA appears to have evolved after the divergence of the genus *Cicer*, approximately 25 million years ago (indicated by arrow and red branches). Species with detectable 4-Cl-IAA are shown in red; species that do not have detectable 4-Cl-IAA are shown in black. Species investigated in this study are indicated by an asterisk. Phylogenetic relationships and divergence dates are taken from Choi et al. (2004), Lavin et al. (2005), Schaefer et al. (2012), and Wojciechowski et al. (2004). s.l., Sensu lato.

in *C. arietinum*. Also, Lulsdorf et al. (2013) did not report this compound when they studied endogenous hormone profiles during early seed development in *C. arietinum* and *Cicer anatolicum*. Therefore, there is no evidence from this or previous studies that species of *Cicer* produce chlorinated auxin. If that is the case, we can suggest that the chlorination capacity arose approximately 25 million years ago, after the divergence of the genus *Cicer* from the common ancestor of the Fabeae and Trifoleae tribes (which include the genera *Trifolium*, *Melilotus*, and *Medicago*; Fig. 5; Choi et al., 2004; Wojciechowski et al., 2004; Lavin et al., 2005; Schaefer et al., 2012).

Our results may provide the basis for a unique model system to investigate halogenating enzymes in plants. A comparison of haloperoxidase sequences across the genera *Pisum*, *Trifolium*, *Medicago*, and *Cicer* may well be instructive with regard to discovering the genes encoding the halogenating enzymes.

MATERIALS AND METHODS

Plant Material

Medicago truncatula 'Jemalong,' broad bean (*Vicia faba*), and *Cicer arietinum* were grown in greenhouse conditions as described previously (Jager et al., 2007). Young seeds or fresh seed pods of other legume species, including *Trifolium repens*, *Trifolium micranthum*, *Trifolium subterraneum*, *Melilotus indicus*, *Indigofera australis*, *Clianthus puniceus*, *Glycine clandestine*, *Hardenbergia comptoniana*, and *Pultenaea juniperina* were collected from plants grown in the field (Hobart and Kingston, Tasmania). Dry seeds of *M. truncatula* 'Jemalong,' *Lotus japonica*, *Arachis hypogaea*, *Lupinus angustifolius*, and Scots pine (*Pinus sylvestris*) were obtained from commercial sources. Mature seeds of *Pinus radiata* were harvested from cones collected from wild plants (Kingston, Tasmania). Immature seeds were extracted from young cones of *Pinus flexilis*, *Pinus pinea*, *Pinus parviflora* 'Glauca,' and *P. parviflora* 'Shikoku-goyo' grown in the Royal Tasmanian Botanical Gardens (Hobart, Tasmania).

Extract Preparation for the Detection and Quantification of Compounds

For the extraction and quantification of IAA and 4-Cl-IAA from young, fresh tissues, 0.3 to 2.5 g of tissue was weighed (±0.0001 g FW) and placed into a falcon tube with 4 volumes of cold (–20°C) extraction solvent (80% [v/v] methanol in water with butylated hydroxytoluene [BHT; 250 mg L^{-1}]). The tissue was then homogenized and held at 4°C overnight to extract. For each species, the supernatant was then divided in half to conduct two separate analyses: one for detection of the compounds of interest and one for quantification of these compounds. For the former, no labeled internal standards were added; and for the latter, [$^{13}C_6$] IAA (Cambridge Isotope Laboratories) and [$^{2}H_4$] 4-Cl-IAA (supplied by Jerry Cohen, Department of Horticultural Science, University of Minnesota) were added as internal standards. The samples were reduced under vacuum at 35°C, taken up in 2% (v/v) acetic acid in water, and partitioned twice against diethyl ether (2/3 volumes). After drying the ether, samples were taken up in 1% (v/v) acetic acid in water and centrifuged for 5 min at 13,000*g*. Aliquots were then taken for analysis by UPLC-MS/MS as described previously (Tivendale et al., 2012).

In viable, dry seeds, the total levels of each of IAA and 4-Cl-IAA were monitored, including both free acids and conjugated forms. IAA and 4-Cl-IAA were extracted as described above, but 65% (v/v) isopropanol in water with BHT (250 mg L^{-1}) was used as the extraction solvent. From the supernatant, hydrolysis of conjugated IAA and 4-Cl-IAA was carried out using the method described by Symons et al. (2002).

For the extraction of Trp and 4-Cl-Trp, tissue was weighed, homogenized, and extracted as described above, but distilled water with BHT (250 mg L^{-1}) was the usual extraction solvent.

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