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4-CHLOROINDOLE-3-ACETIC AND INDOLE-3-ACETIC ACIDS IN *PISUM SATIVUM**

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Abstract—Endogenous IAA and 4-chloroindole-3-acetic acid (4-Cl-IAA) were analysed in vegetative and reproductive tissues of the garden pea (*Pisum sativum*) using GC-MS selected ion monitoring in the presence of stable-isotope labelled internal standards. In fruit collected 3–8 days after anthesis (DAA) conjugates of both auxins were more abundant than the free hormones. Auxin levels (ng g^{-1} fr. wt) in the seeds were higher by 1–2 orders of magnitude than in the pericarps. However, as seeds are small at this stage, the pericarp nevertheless contains a substantial fraction of the overall quantity of IAA and 4-Cl-IAA. While, in young fruit tissues, IAA was more abundant than 4-Cl-IAA, the opposite was true for seeds at the 'table-ready' stage and for roots of nine-day-old seedlings. Our data suggest that both IAA and 4-Cl-IAA are required to coordinate the vegetative and reproductive growth of pea plants. © 1997 Elsevier Science Ltd

INTRODUCTION

Immature seeds of the garden pea (*Pisum sativum* L.) were the first known biological source of the highly active, natural auxin, 4-chloroindole-3-acetic acid (4-Cl-IAA) and its conjugates [1–3]. The compound has now also been shown to occur in other members of the Fabaceae family [4] and in at least one gymnosperm: *Pinus sylvestris* L. [5]. For most species screened, only the seeds were analysed. *Vicia faba* L., however, contains 4-Cl-IAA in both the leaves and the seeds [6]. Contradictory results were reported for *Pisum*. Katayama *et al.* [7] were unable to find 4-Cl-IAA in vegetative tissues of cv. Aaska, but Schneider *et al.* [8] claimed its presence in seedlings (in particular, the radicle) of cv. Aaska. While differences between cultivars cannot be ruled out, 4-Cl-IAA may also decompose during the isolation procedure and thus evade detection. To recognize such losses, we performed extraction and work-up in the presence of a known amount of 4-chloro-[2,5,6,7-$^2H_4$]indole-3-acetic acid (4-Cl-IAA-d_4). This internal standard shares the chemical properties of the endogenous *protio*-4-Cl-IAA, but remains distinguishable by mass spectroscopy, thus permitting consistent low recoveries to be distinguished from low levels of the endogenous compound. Preliminary results obtained using this approach indicated the presence of 4-Cl-IAA in growing pea pericarps (pods) [9]. Here we describe the analysis of pea seeds, pericarps, and roots. All three tissues were confirmed to contain native 4-Cl-IAA, in addition to non-substituted indole-3-acetic acid (IAA).

RESULTS AND DISCUSSION

Analysis for free IAA and 4-Cl-IAA and, in some cases, for the conjugated auxins, was performed on the seeds and pericarps of developing pea fruits, and on roots of pea seedlings. The method used was based on a procedure by Chen *et al.* [10] in which sample purification for GC-MS was achieved in two steps: an anion exchange cartridge and HPLC on a C-18

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column. The method was used essentially without modification with roots, and with immature seeds collected 6 and more days after anthesis (DAA). The putative IAA and the 4-Cl-IAA separated by HPLC were individually converted to their methyl esters and quantified by GC-mass spectrometry, based on the intensity ratios for corresponding characteristic ions derived from the endogenous auxins and from known amounts of [3a,4,5,6,7,7a-$^{13}C_6$]IAA ([$^{13}C_6$]IAA) and 4-Cl-IAA-d_4 added at the extraction stage. 4-Cl-IAA in very small seeds (3 DAA) and in the pericarp could not, however, be analysed without additional sample purification. The method finally adopted is summarized in Table 1. Recoveries estimated radiochemically (using [5-^{3}H]IAA which was routinely added as a tracer, and 4-chloro-[2,3,1′,2′-$^{14}C_4$]IAA which was used in a number of test runs) were reproducibly high, as long as the samples could be passed from step to step without evaporation to dryness. This was impracticable following the HPLC separation of IAA and 4-Cl-IAA (step 4) because the eluent contained acetic acid which would compete for the diazomethane used to convert the two auxins to their methyl esters (first operation in purification step 5). The samples were thus always taken to dryness before methylation. IAA (containing the routine amount of [5-^{3}H]IAA as a tracer) in standards without plant material, and in seed samples smaller than 0.5 g, tended to decompose to some extent, during this operation. This was reflected by variable recoveries in step 5 (Table 1). It is well known that commercial IAA labelled with ^{3}H or ^{14}C is subject to radiolytic decomposition and can only be stored (for limited periods of time) in solution. It was also reported [11] that [^{14}C]IAA decomposed when its solution was evaporated to dryness, unless anthracene was added to quench the free radicals formed by the β-radiation of the ^{14}C-label. We suspect that, in step 4 of our purification procedure, plant constituents eluting close to ^{3}H-labelled IAA (which is also a β-emitter) have a similar stabilizing effect. Thus, it may be best to collect the respective HPLC fraction as broadly as possible, and also to avoid overdrying the sample before methylation.

The required size of the cartridges used in purification steps 1a to 3 (see Experimental section for details) was determined by the amount of plant material processed and, more importantly, by the amounts of reagents (e.g. NaOH, imidazole buffer) added during extraction and work-up. The performance of the anion-exchange cartridge was particularly dependent on its commercial origin (compare protocols in the Experimental section and in ref. [10]).

For GC-mass spectrometry of IAA-OMe and 4-Cl-IAA-OMe (see Experimental for details) columns of medium polarity were required. The stationary phase DB-225 provided optimal peak shapes and separation from non-indolic plant constituents. DB-1701 was used for samples from roots and 'table-ready' seeds of cv. Sparkle, and from pericarps of snow peas, but was later abandoned because of inconsistent separation of IAA-OMe from non-indolic plant constituents. DB-5 was suitable for samples with high auxin levels, such as seeds collected 6 and more DAA, and was preferred in those cases, because IAA-OMe and 4-Cl-IAA-OMe were retained less than by the

Table 1. Isolation and purification of IAA and 4-Cl-IAA from seeds and pericarps of *P. sativum*

	Purification step	Recovery at individual steps* (%)
1.	Extraction into buffered aq. 2-propanol	94 ± 8 (n = 18;0)†
1a.	Desalting column (after hydrolysis of auxin conjugates)	78 ± 8 (n = 10; 2)†
2.	Anion-exchange cartridge	88 ± 3 (n = 2; 2)‡
3.	Reversed-phase cartridge	102 ± 15 (n = 40; 5)§
4.	HPLC separation of free IAA and 4-Cl-IAA	67 ± 8 (n = 33; 3)
5.	Methylation and HPLC purification of the methyl esteras of IAA and 4-Cl-IAA	48 ± 28 (n = 18; 3)¶
6.	Extraction of methyl esters into hexane	75 ± 11 (n = 30; 0)
7.	GC-MS, selected-ion monitoring	not determined

* Based on [5-^{3}H]IAA added as a tracer to step 1 unless explicitly stated otherwise. The same sample was then passed through the complete purification procedure, and recoveries were computed from the amounts of radioactivity present before and after the specified step. The number are arithmetic means ± s.d. (n = total number of replicates; number of replicates containing standards of IAA and 4-Cl-IAA, but no plant material).

† Estimated using a commercial batch of [5-^{3}H]IAA which contained about 35% of ^{3}H-labelled decomposition products (estimated by HPLC). These impurities accompanied the IAA in steps 1 and 1a, but were quantitatively removed in step 2.

‡ Numbers referring to recoveries determined with freshly prepared 4-chloro-[1′,2′,2,3-$^{14}C_4$]IAA verified to be radiochemically homogeneous. When commercial [5-^{3}H]IAA (radiochemical purity: 65%) was used instead, 57% ± 10% (n = 38; 5) of the label eluted as IAA which is about 90% of the radioactivity attributed to the [5-^{3}H]IAA in the sample applied.

§ Recovery also checked with 4-chloro-[1′,2′,2,3-$^{14}C_4$]IAA and found to be 102% (n = 1; 1).

¶ Seven replicates (comprising 3 standards and 4 seed samples derived from 0.12–0.54 g of plant material) gave recoveries between 4% and 31%, as opposed to 58–78% for the other 11 samples. See Results and Discussion for further comments.

above, more polar, stationary phases. More samples could thus be processed per unit of time.

The intensity ratios (peak area ratios in mass chromatograms) for ions derived from IAA and [$^{13}C_6$]IAA were used directly to compute the mass ratios of plant IAA to internal standard in the samples analysed. The 4-Cl-IAA-d_4 contained minor amounts of isotopomers with 0–3 equivalents of deuterium. Empirically determined calibration curves were thus used to convert ion-intensity ratios to mass ratios.

The conditions used to hydrolyse auxin conjugates (7 M NaOH, 100°, 3 hr) are sufficiently drastic to justify checking for nucleophilic displacement of the halogen in 4-Cl-IAA [12] and for base-catalysed deuterium-protium exchange in 4-Cl-IAA-d_4 [13]. A sample of 4-Cl-IAA (2.9 mg) was thus exposed to the above alkaline conditions, acidified, extracted into ether, and analysed by HPLC. The peak area corresponded to a recovery of 79% and no decomposition products were detected. In a second experiment, a mixture of 4-Cl-IAA (*ca* 3 μg) and 4-Cl-IAA-d_4 (*ca* 15 μg) in 0.2 M imidazole–HCl buffer, pH 7.0, was divided into three aliquots and: (a) diluted with iso-PrOH to 65% and processed like a plant sample analysed for free auxin, (b) mixed with 2 M NaOH (10 ml), kept at room temperature for 1 hr (conditions otherwise used to hydrolyse ester conjugates) and worked up like a plant sample analysed for bound auxin, or (c) mixed with solid NaOH (final concentration 7 mol l^{-1}), kept at 100° for 3 hr, under N_2 and processed as b. After the complete purification procedure outlined in Table 1, an identical 4-Cl-IAA/4-Cl-IAA-d_4 ratio of 0.17 was found for all three aliquots. In conclusion, major loss of halogen from 4-Cl-IAA, or of deuterium from its isotopomer, does not occur during alkaline hydrolysis under the conditions used in this work.

The use of internal standards ([$^{13}C_6$]IAA and 4-Cl[2,5,6,7-$^2H_4$]IAA) and of a radiolabelled tracer ([5-^{3}H]IAA) permitted the detection and quantification of IAA and 4-Cl-IAA, not only in pea seeds at the 'table-ready' stage, which are known to contain high levels of both auxins [7], but also in roots, in younger seeds, and in the pericarp, which contain increasingly lower concentrations. Particular attention was given to the auxin levels in pea fruit during the week in which the pericarp practically completes its growth while the seeds remain small. The auxin distribution in such fruit is illustrated in Table 2, for two replicates of material collected 3 and 6 DAA. It has already been shown that pea pods stop growing when deseeded, and exogenous 4-Cl-IAA was so far the only natural auxin which could restore pericarp development [14]. This would suggest that the seeds sustain pericarp growth by supplying the necessary 4-Cl-IAA. Indeed, at 3 and 6 DAA, the concentration (in ng g^{-1} fr. wt) of 4-Cl-IAA in the seeds was much higher than in the pericarp. At 6 DAA, the set of seeds also contained the major quantity of the 4-Cl-IAA present in an individual fruit (Table 2). At 3 DAA, however, approximately two-thirds of the 4-Cl-IAA (both free and conjugated) in a fruit was localised in the pericarp. The seeds could nevertheless be the most important source of auxin for a developing pea fruit, if their machinery for the biosynthesis of 4-Cl-IAA operates at the necessary capacity. This is not an unreasonable assumption, with respect to the fast accumulation of 4-Cl-IAA in the seeds (compare its levels at 3 and 6 DAA given in Table 2). Although a role for IAA in pericarp growth is uncertain [14], the extreme IAA levels in seeds at 6 DAA (Table 2) may indicate a role in seed development. Additional experiments, with due attention to auxin trafficking within the pea fruit, will be required to clarify these issues.

The presence of 4-Cl-IAA in seeds and pericarps collected 6 DAA was confirmed by mass spectra showing the full (seeds) or the most characteristic (pericarp) range of ions. The data for the pericarps are presented in Fig. 1. The ion clusters corresponding to the $[M]^+$ (223–226; 4-Cl-IAA – OMe) and 5-chloroquinolinium (164–167) ions showed the intensity pattern expected with regard to the natural distribution of chlorine (^{35}Cl and ^{37}Cl) and carbon (^{12}C and ^{13}C) isotopes, as confirmed by the mass spectrum of an authentic standard obtained under identical conditions. The lower-mass ions omitted from the figure were of weak intensity (not more than 15% of the ion at 164 a.m.u.) and mostly lacked chlorine; they were thus of limited diagnostic value.

Free 4-Cl-IAA was also detected in the pericarp of commercial snow (sugar) peas (3–16 ng g^{-1} fr. wt; $n = 7$) of unknown variety. The cultivar 'Sparkle', which is grown for its immature seeds, contained the following quantities of free 4-Cl-IAA (IAA): 3840 (48) ng g^{-1} fr. wt ($n = 1$) in 'table-ready' seeds and 250–1040 (17–185) ng g^{-1} fr. wt ($n = 4$ for both auxins) in the roots of 9-day-old seedlings. The pericarps of commercial snow peas were from fruit of various age and postharvest history. This may explain the variability in the auxin levels found. In a similar fashion, the germination of peas is not an absolutely synchronous process, and the root samples analysed included variable amounts of material from seedlings 1–2 days younger than the nominal 9 days. Indeed, the comparison of our data with those for the radicles of three-day-old seedlings (20 ng g^{-1} fr. wt IAA, up to 7 ng g^{-1} fr. wt 4-Cl-IAA) published by Schneider *et al.* [8], suggests that auxin levels in the root change rapidly as the seedlings develop. The latter authors did not have a deuterium-labelled internal standard of 4-Cl-IAA at their disposal. It was thus important to corroborate their results by methods that give definitive quantitative values such as provided by the stable-isotope dilution-analysis GC-mass spectrometry techniques we describe here.

The garden pea has been widely used in plant hormone laboratories, and the results have mostly been interpreted in terms of IAA being the only endogenous auxin. Here we demonstrate that 4-Cl-IAA is present as second, comparably abundant, auxin in the fruit and the roots of several cultivars of *Pisum sativum*. Its

Table 2. Auxin distribution in fruit of *P. sativum* cv. Alaska

	3 DAA		6 DAA	
	rep. 1	rep. 2	rep. 1	rep. 2
*Seeds**				
Total fr. wt (g)	0.154	0.124	0.400	0.640
Number of fruit per sample	38	54	5	6
Number of seeds per fruit	7.05	7.15	6.8	4.8
Fr. wt per seed (mg)	0.575	0.321	11.77	22.07
IAA				
ng g^{-1} fr. wt				
Free IAA	423	362	1420	1570
Total IAA	2610	10300	39500	16400
ng/seed				
Free IAA	0.243	0.116	16.7	34.6
Total IAA	1.50	3.31	465	362
ng/set of seeds in 1 fruit				
Free IAA	1.71	0.831	114	167
Total IAA	10.6	23.7	3160	1750
4-*Cl-IAA*				
ng g^{-1} fr. wt				
Free 4-Cl-IAA	112	177	223	239
Total 4-Cl-IAA	158	182	834	1111
ng/seed				
Free 4-Cl-IAA	0.0644	0.0568	2.62	5.27
Total 4-Cl-IAA	0.0909	0.0584	9.82	24.5
ng/set of seeds in 1 fruit				
Free 4-Cl-IAA	0.454	0.406	17.8	25.5
Total 4-Cl-IAA	0.640	0.418	66.8	119
Pericarps†				
Total fr. wt (g)	7.833	11.34	8.56	8.27
Number of fruit samples	38	54	5	6
Fr. wt per 1 pericarp (g)	0.206	0.210	1.71	1.38
IAA				
ng g^{-1} fr. wt				
Free IAA	30	28	18	16
Total IAA	59	62	56	56
ng/pericarp				
Free IAA	6.18	5.88	30.8	22.1
Total IAA	12.2	13.0	95.8	77.3
4-*Cl-IAA*				
ng g^{-1} fr. wt				
Free 4-Cl-IAA	4	3	7	5
Total 4-Cl-IAA	6	4	12	18
ng/pericarp				
Free 4-Cl-IAA	0.824	0.630	12.0	6.9
Total 4-Cl-IAA	1.24	0.840	20.5	24.8

* Additional determinations for 4-Cl-IAA (IAA) in seeds: 6 DAA, free auxins: 130 (1440), 134 (1990); 6 DAA, total auxins: 967 (19000), 1300 (—); 8 DAA, free auxins: 233 (289), 214 (1550); 8 DAA, total auxins: 1160 (8700), 1610 (7200) ng g^{-1} fr. wt. Missing values for one of the two auxins are indicated as —. Total fr. wts analysed were in the same range as for the samples detailed in the Table. Fr. wt per seed and seed numbers per fruit were not determined.

† Additional determinations for 4-Cl-IAA (IAA) in pericarps: 6 DAA, free auxins: 3 (19), — (22) ng g^{-1} fr. wt. Specifications in conformance with the previous footnote.

role in the life cycle of a pea plant may thus be quite as important as that of IAA.

EXPERIMENTAL

General. Solns were concd *in vacuo* using a Speed Vac evaporator at room temp., unless specified otherwise. $NaHSO_4$ buffer (pH 1) contained, per litre, 55 ml (1.98 mol) conc H_2SO_4 and 55 g (1.38 mol) NaOH. Solvents for HPLC contained 0.1 mol l^{-1} HOAc, unless specified otherwise.

Materials and equipment for chromatography. Solid-phase extraction chromatography was performed in plastic cartridges (Varian Mega Bond Elut) packed

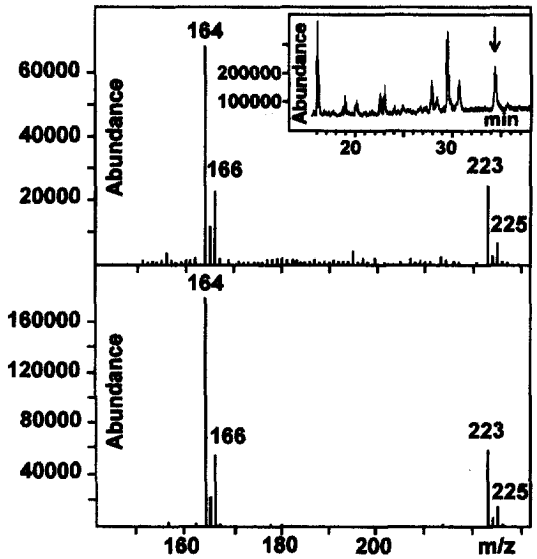

Fig. 1. GC-MS identification of 4-chloroindole-3-acetic acid in pea pericarps. After conversion to its methyl ester, the native compound (marked by an arrow) appeared as a well separated peak in the total ion chromatogram (insert), at the same R_t as the authentic methyl ester of 4-chloroindole-3-acetic acid. The mass spectra of the authentic standard (bottom) and of the material isolated from pea pericarps (top) showed the same pattern of characteristic ions. GC was performed in the same way as in the quantitative analyses.

with silica gel modified with a C-18 coating for reversed-phase sepns, or with an aminopropyl ligand (NH_2) functioning as an anion exchanger. The latter was substituted by diethylaminoethyl-silica gel, in some experiments, with no major differences in performance. C-18 cartridges containing 1 g ('column volume' 2.5 ml) or 10 g ('column volume' 15 ml) of sorbent were conditioned immediately before use, by rinsing with 2 column vols each of (1) hexane, (2) MeOH, (3) H_2O, (4) 1% (v/v) aq. HOAc. NH_2 cartridges (10 g; 'column volume' 15 ml) were conditioned by rinsing with 2×15 ml each of (1) hexane, (2) MeCN, (3) H_2O, (4) 0.2 M imidazole–HCl buffer, pH 7.0, and finally with 5×15 ml H_2O. The hexane rinse was performed by gravity. In all other operations, a flow rate of *ca* 3 drops min^{-1} was maintained by applying pressure at the top of the cartridge or by collecting the effluent under a weak vacuum.

HPLC was on Spherisorb S5 ODS2 (C-18, 25 cm × 4 mm i.d., particle size 5 μm), at 30°. All solvents contained 0.1 mol l^{-1} HOAc. The effluent was continuously monitored for absorbance at 280 nm, and for radioactivity using a flow-through cell packed with yttrium silicate.

Chemicals. [3a,4,5,6,7,7a-$^{13}C_6$]IAA was prepd as described previously [15]. Unlabelled 4-Cl-IAA was synthesized after Engvild [16]. 4-Cl-IAA-d_4 was a custom-synthesized commercial product. To prepare 4-Cl-[2,3,1′,2′-$^{14}C_4$]IAA [17], commercial sodium 2-oxo-[U-^{14}C]glutarate (50 μCi, 171 nmol), unlabelled 2-oxo-glutarate (140 μg, 829 nmol), and 3-chlorophenylhydrazine hydrochloride (3.6 mg, 20 μmol), freshly dissolved in pyridine (150 μl), were mixed with conc HCl (200 μl) and 85% H_3PO_4 (50 μl), in a threaded 10 ml round-bottom culture tube which was flushed with N_2, closed with a teflon-lined screw cap, and heated in an oven, at $115 \pm 3°$ for 22 hr. The cooled reaction mixt. was diluted with $NaHSO_4$ buffer (8 ml) and extracted with Et_2O (5×10 ml). The extract was layered on to H_2O (5 ml) and the organic solvent was removed in a stream of N_2. The aq. residue was passed through a C-18 cartridge (1 g, previously rinsed with MeOH and 20% aq. MeOH) which was eluted with 10 ml each of 20, 30, 40 and 50% aq. MeOH. The expected mixt. of radiolabelled 4-Cl-IAA and 6-Cl-IAA emerged with 30%–50% aq. MeOH and was sepd by HPLC (gradient from 24 to 25% MeCN in 45 min). The frs containing the pure isomers (chemical yield: 19% each; sp. act 12–14 μCi μmol^{-1}) were stored at 4° in the dark.

Plant material. Pisum sativum L. cv. Alaska, line I_3, was grown as described previously [14]. In brief, plants were raised in a growth chamber illuminated by cool-white fluorescent lamps, with an average photon flux density of 470 μE m^{-2} sec^{-1} (measured at the top of the plant canopy), and a light-to-darkness (temp) cycle of 16 hr (19°) to 8 hr (17°). One fruit per plant was grown for 3, 6, or 8 DAA; further flower buds appearing during that period were removed. Seeds and pericarps were sepd and, if not analysed immediately, stored at −80° or in liquid N_2. Root material from 9-day-old seedlings and 'table-ready' (*ca* 20 DAA) seeds were from cv. Sparkle. Snow (sugar) peas of unknown variety were purchased locally.

Extraction. The plant material was frozen in liquid N_2. Samples above *ca* 1 g in wt were ground in a prechilled mortar and dropped into 65–80% iso-PrOH prepd with 0.2 M imidazole/HCl buffer, pH 7.0 (4 ml g^{-1} fr. wt). Smaller plant samples were homogenized with a Polytron type homogenizer, in the same solvent (15 ml + 2 × 5 ml for rinsing), maintained at a temp. slightly above its freezing point (dry ice/MeOH bath). To the homogenate were added: [5-^{3}H]IAA (*ca* 400 000 dpm, sp. act 15–30 Ci $mmol^{-1}$), [$^{13}C_6$]IAA (same amount as expected IAA level), and 4-Cl-[2,5,6,7-$^2H_4$]IAA (*ca* 5 × the expected 4-Cl-IAA level). The mixt. was stirred for at least 3 hr at 0–4° in the dark, for isotope equilibration, and centrifuged (10 000 *g*, 20 min, 3°).

Hydrolysis of auxin conjugates. An aliquot of the supernatant was concd at 40° and *ca* 1 mm Hg, using a rotary evaporator. The aq. residue was transferred to a threaded round-bottom culture tube (capacity of 20–25 ml) and diluted with H_2O to 10 ml. NaOH pellets (2.8 g) were added to yield a 7 M soln; this was purged with N_2 for 10 min. The tubes were then tightly closed with new, temp.-resistant, teflon-lined screw caps and kept for 3 hr in an oven preheated to 100°. The cooled samples were diluted with H_2O (150 ml), adjusted to pH 2.5 with conc HCl, and immediately passed through a conditioned C-18 cartridge (10 g) to

desalt. The column was rinsed with H_2O (2 × 15 ml), and the free auxins were eluted with MeCN (4 × 15 ml). The radioactive ([5-^{3}H]IAA) frs were concd to the aq. residue and diluted with 0.02 M imidazole/HCl, pH 7.0 (150 ml, pH readjusted).

Anion exchange cartridge. The original plant extract diluted with a five-fold vol. of H_2O, or the desalted samples obtained after hydrolysis of auxin conjugates, were passed through a conditioned NH_2 cartridge. Air was then aspirated through the column for 2 min, and the following sequence of eluents was applied: (1) hexane (2 × 15 ml), (2) EtOAc (2 × 15 ml), (3) MeCN (2 × 15 ml), (4) MeOH (2 × 15 ml), (5) freshly prepd 5% HOAc in MeOH (4 × 15 ml). The radioactive frs eluted with the last solvent were added, in appropriate aliquots, into a conical centrifuge tube (capacity: 12 ml) containing initially 2 ml of H_2O (level marked) and re-concentrated to the 2 ml mark. The residue (soln in aq. HOAc) was immediately diluted with H_2O (6 ml), stored at 0–4° in the dark, and further processed within 3–4 hr.

Reversed-phase cartridge. The above sample was passed through a conditioned C-18 cartridge (1 g). The centrifuge tube in which the soln had been stored was rinsed with H_2O (2 × 1 ml) which was added to the sample. Elution was by H_2O (5 ml), followed by 5% aq. MeOH (5 ml), and 60% aq. MeOH (4 × 5 ml). The radioactive frs eluted with the latter solvent were concd to an approximate vol. of 100 μl.

HPLC separation of IAA and 4-Cl-IAA. The above sample was diluted with 20% aq. MeCN (0.1 mol l^{-1} HOAc) to yield the chromatographic sample (*ca* 800 μl). The eluents were (1) 20% aq. MeCN (20 min), (2) gradient to 60% aq. MeCN (10 min), (3) 60% aq. MeCN (5 min), (4) gradient to 100% MeCN (5 min), (5) MeCN (5 min), (6) gradient to 20% aq. MeCN (10 min), (7) 20% aq. MeCN (12 min). The R_ts were 18 min (IAA) and 30 min (4-Cl-IAA). The frs containing the title compounds were evapd just to dryness and redissolved in MeOH (0.5 ml).

Preparation and purification of methyl esters. To the above samples was added a freshly prepd soln (2 ml) of CH_2N_2 in Et_2O, and the mixt. was left at room temp. for 30 min. The reagent was removed in a stream of N_2, and the methylation was repeated. H_2O (400 μl) was added, and the samples were concd to *ca* 200 μl. They were then diluted with 20% aq. MeCN (600 μl, containing 0.1 mol l^{-1} HOAc) and subjected to HPLC, eluting as follows: (1) gradient from 20 to 35% aq. MeCN (10 min), (2) 35% aq. MeCN (19 min), (3) gradient to 100% MeCN (15 min), (4) 100% MeCN (5 min), (5) gradient to 20% aq. MeCN (5 min), (6) 20% aq. MeCN (15 min). R_ts were 25 min (IAA-OMe) and 36 min (4-Cl-IAA-OMe). The respective frs were combined and concd to *ca* 200 μl. After neutralizing the remaining HOAc with solid $NaHCO_3$, the methyl esters were extracted with hexane (6 × 1 ml). The extract was cooled (dry ice/MeOH) to freeze out the carried-over aq. phase, decanted, and concd to 20–50 μl, depending on the expected level of IAA-OMe or 4-Cl-IAA-OMe.

GC-MS-selected ion monitoring. The carrier gas was He at 0.8 ml min^{-1}. Samples were injected, at 35°, directly into a pre-column (deactivated silica, 1 m × 0.53 mm), using a two-minute delay before the temp. gradient was applied. Sepn was usually on DB-225 (J & W Scientific: 50% methyl silicone + 50% cyanopropyl phenyl silicone) coated, to a thickness of 0.15 μm, on to an open silica capillary (30 m × 0.32 mm i.d.). The temp. gradient was: 70° min^{-1} to 190° followed by 1° min^{-1} to 220°, resulting in the following average R_t-values: IAA-OMe, 19.6 min, 4-Cl-IAA-OMe, 33.6 min. Samples from roots, seeds at the 'table-ready' stage, and pericarps of snow peas were analysed on a column (15 m × 0.25 mm i.d. × 0.25 μm film) of DB-1701 (14% cyanopropyl phenyl + 86% methyl silicone) with a temp. gradient of 70° min^{-1} to 170° followed by 1° min^{-1} to 230°, for IAA-OMe (R_t = 14.6 min), and of 70° min^{-1} to 210°, followed by 1° min^{-1} to 230°, for 4-Cl-IAA-OMe (R_t = 12.0 min). DB-1 (methyl silicone; 15 m × 0.25 mm i.d. × 0.25 μm film) gave unsatisfactory sepn and DB-5 (5% phenyl + 95% methyl silicone; 30 m × 0.25 mm i.d. × 0.1 μm film) was only suitable for samples containing more than *ca* 100 ng g^{-1} fr. wt of IAA or 4-Cl-IAA, such as seeds 6 and 8 DAA.

Ion intensities were monitored at *m/z* 130 (136) and 189 (195) a.m.u. for IAA-OMe ([$^{13}C_6$]IAA-OMe) and at *m/z* 164 (168) and 223 (227) a.m.u. for 4-Cl-IAA-OMe (4-Cl-IAA-OMe-d_4). The calibration curves used for 4-Cl-IAA-OMe quantification (obtained from a series of mixts containing various ratios of protio- and d_4-4-Cl-IAA) were $y = 5.3795x + 0.0031$ ($r = 0.9999$, $s_{y,x} = 0.0755$) for the 164/168 (5-chloroquinolinium ion) pair and $y = 5.6618x - 0.0498$ ($r = 0.9997$, $s_{y,x} = 0.1334$) for the 223/227 (molecular ion) pair, wherein x stands for the mass ratio, and y for the corresponding ion intensity (integrated peak areas on ion chromatograms) ratio.

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