



EFFECT OF HALOGEN SUBSTITUTION OF INDOLE-3-ACETIC ACID ON BIOLOGICAL ACTIVITY IN PEA FRUIT

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Abstract—Auxins (a class of plant growth hormones naturally present in all plants) have been implicated in fruit growth of pea. Pea (*Pisum sativum* L.) fruit contain the auxins indole-3-acetic acid (IAA) and 4-chloroindole-3-acetic acid (4-C1-IAA). Fruits grow poorly and subsequently abscise when seeds are removed two days after anthesis, but 4-C1-IAA can substitute for the seeds in maintaining growth of deseeded fruit (pericarp) *in planta*. Applications of 4-C1-IAA promoted pericarp growth, the effect increasing with concentration from 1 to 100 μ M, but IAA was ineffective in stimulating growth when tested from 0.1 to 100 μ M. The effect of the position of the halogen on pericarp growth was examined by assaying the activities of 4-, 5-, 6- and 7-chloro- and fluoro-substituted IAA. The position and type of halogen dramatically affected auxin activity, with the natural product 4-C1-IAA being most effective. Of the other compounds tested, only 5-C1-IAA stimulated pea pericarp elongation, and then only moderately. Fluoro-substituted IAAs did not stimulate pericarp growth, and 4-F-IAA was inhibitory. This study is unique in that it reports the biological activity of 4-C1-IAA and halogen-IAA analogues in tissues of intact plants known to contain 4-C1-IAA. The relative activity of the compounds is discussed in reference to previous reports of auxin activity in other systems, and 4-C1-IAA's possible importance in pea fruit growth.

INTRODUCTION

The plant growth hormone indole-3-acetic acid (IAA) (Fig. 1) is ubiquitous in plants. 4-Chloroindole-3-acetic acid (4-C1-IAA) has been identified in extracts of plants of the Leguminosae tribe Viciae, and also in *Pinus sylvestris* [cf. 1]. No 4-C1-IAA was found by researchers examining members of other Leguminosae tribes, or several cultivated plants [2-4]. The biological role of 4-C1-IAA in plants is not known. Pless *et al.* [5] observed that *Vicia faba* seeds accumulated 4-C1-IAA (data expressed as nanograms per seed) during rapid seed development.

4-C1-IAA and its methyl ester were identified in immature seeds of *Pisum sativum* L. [6, 7]. 4-C1-IAA was also reported to occur in the shoot, the root and cotyledons of three-day-old etiolated *Pisum* seedlings [8], but 4-C1-IAA was not detected in 20- to 70-day-old light-grown stem and leaf tissues [9]. However, since no internal standard was used, a lower limit on levels could not be assigned. 4-C1-IAA was identified and quantitated by GC-mass spectrometry in six days after anthesis (DAA) pea pericarp and seeds (*P. sativum* L. line I₃ Alaska-type) using an internal standard method [10].

4-C1-IAA has been tested in many different bioassays and is as active as, or 10 to 100 times more active than, IAA as defined by the concentration required to reach maximum biological response. Biological assays generally measure 4-C1-IAA's stimulation of growth with excised tissue (e.g. pea epicotyl split curvature growth, pea epicotyl straight growth, oat and wheat coleoptile straight growth, and mung bean hypocotyl growth [11-13]). 4-C1-IAA treatment also increases rooting and ethylene evolution in pea shoot cuttings [14]. However, reports on 4-C1-IAA's effects on the growth of organs on intact plants are less frequent (e.g. mung bean root inhibition, tomato epinasty and tomato parthenocarp [13, 15, 16]). We now report on the growth promoting effects of 4-C1-IAA on tissues of intact plants known to contain 4-C1-IAA. These results may help in understanding the role of this unique auxin in the control of plant growth and development.

Eeuwens and Schwabe [17] observed that synthetic auxin (naphthalene acetic acid) or gibberellin (GA, a plant hormone) treatments enhanced growth of pea pericarp. In these experiments, seeds were killed by needle-pricking and the terminal apical shoot was removed prior to treatments. We initially developed a split pericarp technique *in planta* to characterize the hormonal requirements for *Pisum* fruit development where fruit are

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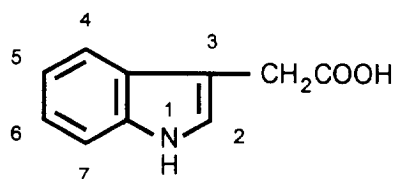


Fig. 1. Numbering system for indole-3-acetic acid and halogen analogues.

split down the dorsal suture, and pericarp and seeds continue to grow until maturity [18]. Removal of the seeds 2–3 DAA results in slowing of pericarp growth and abscission. Results obtained with the split pericarp method suggest that seeds are required to maintain GA metabolism in the pericarp (specifically, conversion of GA_{19} to GA_{20}) [18]. Preliminary data suggested that 4-Cl-IAA, as well as GA, stimulated deseeded pericarp growth [19, 20]. The current work examines the effect of IAA, 4-Cl-IAA and halogen-substitution of the indole ring (Fig. 1) on growth of pea pericarp *in planta*. This is the first report examining the activity of Cl- and F-IAA analogues on the growth of whole plant tissues that naturally contain the halogen containing auxin, 4-Cl-IAA.

RESULTS

Pea fruit growth, measured as an increase in pericarp length, was sigmoidal with respect to time and consisted of an initial slow growth phase, followed by a rapid linear growth phase and a slower final phase as the pericarp reached its final length (Fig. 2; intact pericarp). Splitting of the pericarp 2 DAA without disturbing the seeds (SP) reduced pericarp growth in the later growth phase, resulting in a lower final length when compared to the intact pericarp (Fig. 2). When seeds were removed (SPNS), the pericarp stopped growing after 24 hr, shrivelled, and subsequently abscised. Treatment with 4-Cl-IAA (50 or 100 μ M) following seed removal restored pericarp growth (Fig. 2).

The biological activities of three auxins that occur naturally in pea (IAA, 4-Cl-IAA and IBA [8, 21]), as well as seven synthetic halogen-substituted indoleacetic acids (5-, 6- and 7-Cl-IAA and 4-, 5-, 6- and 7-F-IAA; see Fig. 1) were compared using the same system. The effect of 4-Cl-IAA and the synthetic chloro-derivatives of IAA on pea pericarp growth varied with concentration and position of the chlorine on the indole ring (Fig. 3). The growth response to different levels of chloro-derivatives of IAA was log-linear ($P < 0.001$). A highly significant concentration by treatment interaction ($P < 0.0001$) indicated that the slopes varied among treatments. Pericarp length increased linearly with increasing log concentration of 4- or 5-Cl-IAA ($P < 0.0001$, $P < 0.01$, respectively), but the former (which is naturally occurring in pea fruit) was significantly more active. 4- and 5-Cl-IAA treated pericarp significantly increased in length when compared to the 0 μ M control (SPNS control: the

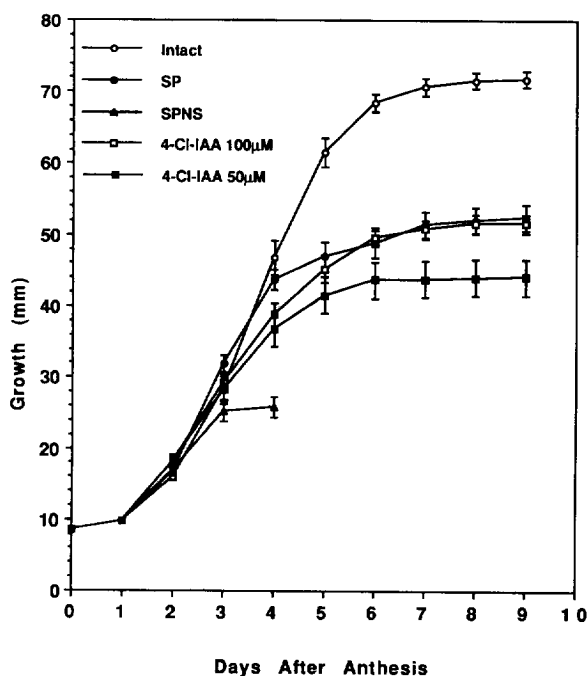


Fig. 2. Growth of pea pericarp *in planta* 0–9 DAA. For SPNS control (split pericarp seeds removed) and 4-Cl-IAA treatments, pericarps were split 2 DAA, seeds removed, and solutions applied to inner pericarp daily for 5 days. The SP treatment (pericarp split 2 DAA, seeds present) and SPNS control received 0.1% Tween 80 only. Error bars, \pm SE.

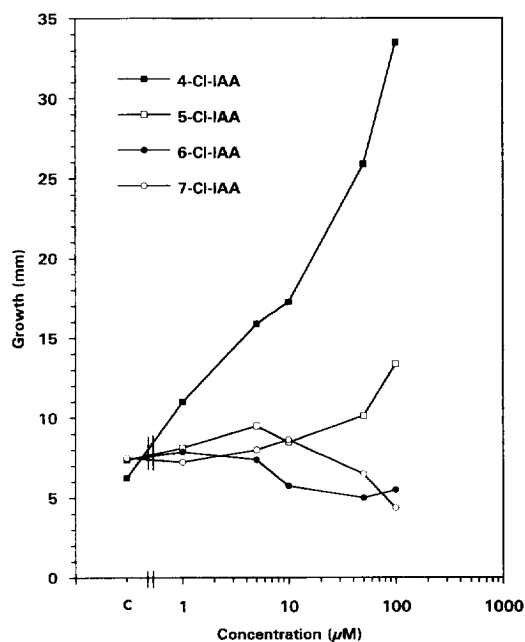


Fig. 3. The effect of 4-, 5-, 6- and 7-Cl-IAA on pea pericarp elongation. Two DAA deseeded pericarps *in planta* were treated with auxin solutions daily for 5 days; C = control (0 μ M); growth = final length (9 DAA) minus initial length (2 DAA).

results obtained using single degree of freedom [SDF] contrasts, with $P < 0.0001$ and $P < 0.05$, respectively). 6- and 7-C1-IAA were not active in this bioassay (the slope obtained was not different from zero as tested by regression analysis, and the treatment means were not significantly different from the $0 \mu\text{M}$ control as tested by SDF contrast analysis). The mean values obtained for pericarp length following treatment with 4- and 5-C1-IAA (20.6 and 9.9 mm, respectively) were significantly lower than SP treatment means (34.6 and 29.3 mm, respectively; $P < 0.0001$).

Pericarp width, pedicel and peduncle diameter, and fresh and dry weight increased linearly with increasing log concentration of 4-C1-IAA ($P < 0.001$, except peduncle diameter $P = 0.05$; Table 1). The 5-C1-IAA treatment linearly increased pericarp width and pedicel and peduncle diameter with increasing log concentration ($P < 0.01$), but did not significantly affect fresh or dry weight. 4-C1-IAA was significantly more active in stimulating pericarp width, pedicel diameter, fresh weight and dry weight than was 5-C1-IAA (mean of 4-C1-IAA versus 5-C1-IAA, Table 1). Pericarp width and fresh and dry weight of intact and SP treatments were significantly greater than in deseeded pericarp treated with 4- or 5-C1-IAA (Table 1). However, pedicel and peduncle diameters in 4-C1-IAA-treated pericarp, and peduncle diameter in 5-C1-IAA-treated pericarp, were similar to those in controls (Table 1). No comparisons are made with the $0 \mu\text{M}$ control because pericarp in this treatment shrivel-

led, and subsequently abscised prior to harvest of tissue 9 DAA.

The effect of the fluoro-derivatives of IAA on pea pericarp growth varied with concentration and position of the fluorine on the indole ring (Fig. 4). A significant concentration by treatment interaction ($P < 0.0001$) indicated that the fluoro-IAAs responded differently over the concentration range. Pericarp length decreased linearly with increasing 4-F-IAA log concentration ($P < 0.0001$), whereas the 5-, 6- and 7-F-derivatives of IAA were not active in this bioassay as tested by SDF contrast analysis ($0 \mu\text{M}$ controls versus fluoro-IAA treatments). Variation in control ($0 \mu\text{M}$) values was a result of determining control growth values at the time of each compound-series application.

IAA linearly decreased pericarp growth with increasing log concentration ($P < 0.05$; Fig. 5), with the pericarp usually shrivelling and abscising during the experiment. IAA treatments at 0.1 and $1000 \mu\text{M}$ were also not effective in stimulating pericarp growth (data not shown). In contrast, IBA moderately promoted growth (log-linear trend, $P < 0.05$; SDF contrast, $0 \mu\text{M}$ control versus IBA treatment, $P < 0.01$; Fig. 5). The ethyl ester of IAA did not promote pericarp elongation at 5, 10, 50 or $100 \mu\text{M}$ (5.8 ± 0.4 , 5.3 ± 0.8 , 3.9 ± 0.5 , 3.8 ± 0.3 mm, respectively) when compared to the $0 \mu\text{M}$ control (7.1 ± 1.1 mm).

To determine if 4-F- or 5-C1-IAA would affect the activity of 4-C1-IAA in stimulating growth, these analogues were applied simultaneously with 4-C1-IAA at

Table 1. Effect of 4- and 5-C1-IAA on pericarp width, pedicel diameter, peduncle diameter and pericarp fresh and dry weight*

Treatment	Concentration (μM)	Pericarp width (mm)	Pedicel diameter (mm)	Peduncle diameter (mm)	Pericarp fr. wt (mg)	Pericarp dry wt (mg)
4-C1-IAA	5	7.8 bc†	1.4 cd	1.4 b	269 c	25 c
	10	8.5 b	1.4 bdf	1.4 b	352 bc	32 bc
	50	11.0 a	1.7 ab	1.5 ab	609 ab	55 ab
	100	12.8 a	1.8 a	1.5 ab	876 a	80 a
5-C1-IAA	5	5.9 cf	1.3 cef	1.4 b	100 c	8 c
	10	6.1 cde	1.3 cef	1.5 ab	93 c	5 c
	50	7.0 bef	1.5 bd	1.7 a	252 c	9 c
	100	7.9 bd	1.5 bde	1.6 ab	241 c	17 c
Mean of concn						
	5	6.8 r	1.3 r	1.4 r	185 r	16 r
	10	7.3 r	1.4 r	1.5 r	222 r	19 r
	50	9.0 s	1.6 s	1.6 s	431 s	32 rs
	100	10.3 s	1.6 s	1.6 rs	559 s	49 s
Chemical X concn		‡	ns	ns	‡	‡
Mean of 4-C1-IAA		10.0 y	1.6 y	1.5 z	527 y	48 y
Split pericarp with seeds		13.4¶	1.5	1.4	1201¶	155¶
Intact		18.6¶	1.6	1.6	2331¶	343¶
Mean of 5-C1-IAA		6.7 z	1.4 z	1.5 z	171 z	10 z
Split pericarp with seeds		13.3¶	1.6§	1.6	1344¶	170¶
Intact		20.0¶	1.7	1.7§	2766¶	421¶

*Measured at harvest (9 DAA), except for dry wt.

†Means followed by the same letter are not significantly different from one another within columns among all treatments (a–f), concentrations (r, s), and chloro-IAAs (y, z) by LSD ($P < 0.01$).

‡,ns Interaction significant at the 5% level or not significant (F test), respectively.

§||¶ Significantly different from 4- or 5-C1-IAA means at $P < 0.01$, 0.001 or 0.0001, respectively (F test).

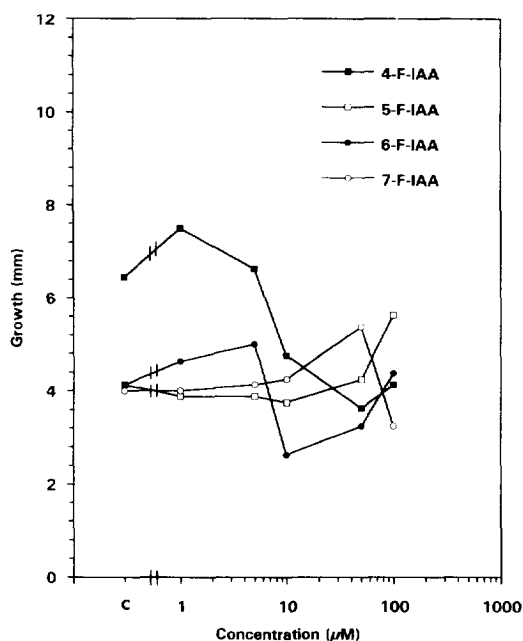


Fig. 4. Effect of 4-, 5-, 6- and 7-F-IAA on pea pericarp elongation. Pericarps were treated and growth determined as in Fig. 3; C = control (0 μ M).

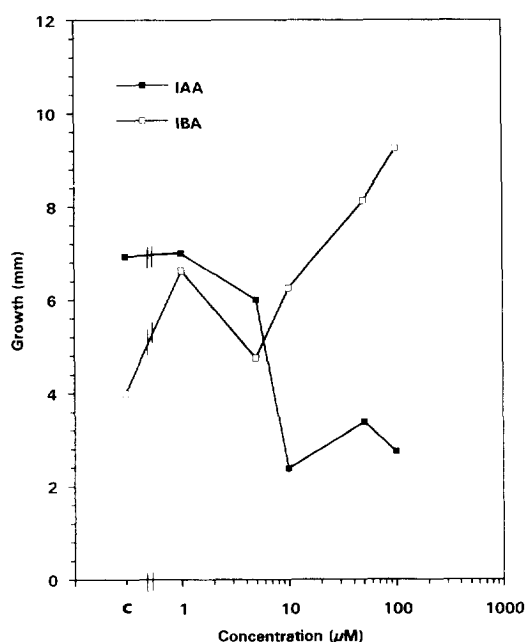


Fig. 5. Effect of IAA and IBA on pea pericarp elongation. Pericarps were treated and growth determined as in Fig. 3; C = control (0 μ M).

concentrations 0, 1 and 10 times that of 4-C1-IAA (Table 2). 4-F-IAA reduced 4-C1-IAA-stimulated pericarp growth only when applied in the ratio 50 μ M/5 μ M, respectively, resulting in a 27% decrease in growth over the 4-C1-IAA control (Table 2), whereas

5-C1-IAA increased 4-C1-IAA-stimulated pericarp growth only when applied in the ratio 500 μ M/50 μ M, respectively, resulting in a 19% increase in growth over the 4-C1-IAA control (Table 2).

DISCUSSION

Both IAA and 4-C1-IAA are naturally occurring auxins in pea fruit. However, their biological activities differed, with only 4-C1-IAA application stimulating pea pericarp growth. These results are unusual since the growth promotive effect of IAA is the standard comparison for an auxin growth assay. The split pericarp assay differs from most auxin assays in two regards: the tested tissue remains attached to the plant, and 4-C1-IAA is tested in a tissue known to contain 4-C1-IAA. The duration for many auxin assays is 24 hr, while the split pericarp method requires 48 hr to observe significant growth over the control.

The lack of activity of IAA on pea pericarp tissue could reflect its biochemical or chemical instability. To minimize such effects, fresh IAA solutions were prepared for each experiment and applied once every 24 hr. In unfertilized tomato fruit, the ethyl and methyl esters of IAA are 100 times more active than IAA when applied as a lanolin paste [16]. The tomato data suggested that IAA was less active due to metabolic instability. However, both IBA and the ethyl ester of IAA, auxins reportedly more biochemically stable than free IAA, had low or no growth promotive activity, respectively, in the pea split pericarp assay. The methyl ester of 4-C1-IAA was as active as 4-C1-IAA in the pea pericarp assay (data not shown). Uptake, conjugation, oxidation or decarboxylation [22] all potentially reduce the availability of IAA for growth promotion and the relative rates of such processes with regard to IAA and 4-C1-IAA need to be determined *in planta*. Horseradish peroxidase oxidizes IAA more rapidly than 4-C1-IAA *in vitro* [13], but no other reports on relative stability are known to the authors.

When halogen-substituted IAAs were assayed on pea pericarp, 4-C1-IAA, and to a lesser extent, 5-C1-IAA, promoted growth. The activity of halogen-substituted auxins on pea pericarp differs from other systems in previous studies. In such studies, IAA, 4-, 5-, 6- and 7-C1-IAA and 5-F-IAA were all active, although maximum activity was observed at different concentrations [12, 15, 16]. For *Pisum* stem and *Triticum* coleoptile assays, the level of activity was: 4-C1-IAA > IAA \geq 5-C1-IAA \geq 6-C1-IAA > 5-F-IAA > 7-C1-IAA (recalculated from ref. [12]). For the pea pericarp assay the level of activity was: 4-C1-IAA > 5-C1-IAA, with all other derivatives being inactive or inhibitory. There are no previous data available on the auxin activity of the 4-, 5- 6- and 7-F-IAA substitution series; previously, only 5-F-IAA had been tested for auxin activity. Exchange of fluorine for chlorine at position 4 resulted in a dramatic decrease in auxin enhanced growth; however, 4-F-IAA only moderately reduced 4-C1-IAA activity when added at a 10-fold excess (500 μ M). One of several possible explana-

Table 2. Effect of 4-F- and 5-Cl-IAA on 4-Cl-IAA stimulated pea pericarp growth

4-Cl-IAA (μM)	4-F-IAA (μM)	Length (mm)*	5-Cl-IAA (μM)	Length (mm)
5	0	16.7 b†	0	17.9 a
	5	16.4 b	5	16.6 a
	50	12.1 a	50	19.9 a
50	0	29.3 c	0	30.8 b
	50	29.1 c	50	28.9 b
	500	27.0 c	500	36.6 c

*Final length (9 DAA) minus initial length (2 DAA).

†Means followed by the same letter (a, b, c) are not significantly different from one another within columns among treatments by LSD at $P < 0.05$.

tions for the result is that 4-F-IAA does not bind tightly to a postulated auxin receptor site or enzyme.

4-Cl-IAA and to a lesser extent 5-Cl-IAA affect several parameters of pericarp growth, including length, width, fresh weight, dry weight, and pedicel and peduncle diameter. Maintenance of a healthy pedicel and peduncle (tissues which attach the pod to the plant and contain an abscission zone at the pedicel-peduncle junction) is probably affected by 4-Cl-IAA. Other seed and pericarp factors such as GA [17, 19] likely interact with 4-Cl-IAA in modulating pericarp growth in terms of length, width, fresh weight and dry weight.

Our results suggest a biologically significant role for 4-Cl-IAA in pea fruit growth, since this auxin occurs naturally in pea fruit, and is the only halogen-derivative of IAA that substantially stimulated deseeded pericarp development in our tests. 4-Cl-IAA may be a seed transmissible factor [17, 20] that is required for pericarp growth and coordination of pea pericarp and seed development. The specificity of the location and type of halogen substitution on the indole ring suggests either differing chemical stability, different routes of metabolism, or different receptor sites for IAA and 4-Cl-IAA in developing pea fruit. IAA may have other functions in pea fruit development or an ancillary role in stimulating pericarp elongation.

EXPERIMENTAL

Plant material and treatments. Pea (*P. sativum* L.) line I₃ (Alaska-type) seeds were germinated in 20 cm pots in a potting mixture of Metro-mix-sand (1:1). Three seedlings were selected for uniformity and grown in a Conviron growth chamber with a 16 hr photo-period and 19°/17° day/night cycle. The plants were illuminated with cool-white fluorescent plus incandescent lights 30 cm above the canopy with an average photon flux density of 470 $\mu\text{E m}^{-2} \text{sec}^{-1}$.

The pea split pericarp method [18] was modified as follows. One fruit per plant (at the third to fifth flowering node) was treated; earlier and later fruits were removed as they developed. The terminal apical shoot meristem of the plant was intact and the pericarp remained attached to the plant during the entire experiment. The pericarps of ovaries were cut along the dorsal suture 2 DAA (15–21 mm), either without disturbing the seeds (SP) or,

alternatively, the seeds were removed (SPNS). Test compounds in 0.1% aq. Tween 80 were applied to the inner-pericarp wall of deseeded pericarp daily for 5 days (total 180 μl). The purity of the test compounds was > 97% as determined by HPLC monitored at 280 nm. The SP treatment and SPNS control (0 μM control) received 0.1% Tween 80 only. The pericarps were enclosed in clear plastic bags to maintain high humidity, and pericarp growth was monitored *in planta* daily for 1 week. As little as 60 pmol of 4-Cl-IAA stimulated pericarp growth when compared to the 0 μM control during the first 2 days of treatment.

Statistical analysis. Treatments reported in Figs 3–5 and Tables 1 and 2 were arranged in a randomized complete block design blocked over time. Control treatments were not included in the 2 factor (treatment \times concentration) factorial ANOVAs (Figs 3–5 and Table 1). The appropriate control within each block was used for treatment vs control SDF contrasts. The effect of log treatment concn on pericarp growth was subjected to trend analysis. The IAA and IBA experiments presented in Fig. 5 were performed and analysed separately. Statistical analyses were performed using the program SAS (SAS Institute). The experimental unit for these analyses was the plant. The experiment in Fig. 2 was repeated twice over time with a minimum of 4 pericarps per replication; the data presented are the means for the two experiments.

Chemicals. 5-F-IAA was purchased from Sigma. The following compounds were prep'd from commercial haloindoles via the corresponding substituted gramines [3-(*N,N*-dimethylaminomethyl)indoles] and indole-3-acetonitriles [23]: 4-F-IAA [24], 6-F-IAA [25], 4-Cl-IAA [replacement of 4-chlorogramine by 4-chloro-3-(*N,N*-diethylaminomethyl)indole improved yields] [26–28], 5-Cl-IAA and 6-Cl-IAA [26, 28]. 7-Cl-IAA [26, 28] was prep'd in a manner similar to the procedure described below for its 7-F analogue.

7-F-IAA. A soln of L-glutamic acid (7.47 g, 60 mmol) in 0.5 M NaOH (120 ml) was stirred into freshly prep'd [29] 1 M NaOCl (60 ml; containing 0.1 M NaOH), at 0–5° [30, 31]. When complete formation of *N*-chloro-L-glutamic acid was indicated by a negative test with aniline water (after *ca* 1 min), 3 N HCl (21 ml) was added and the mixture (pH 5) was immersed in a 50° water bath

until non-reactive towards KI/starch paper (ca 20 min). The resulting soln of 4-oxobutyric (bulk) and 2-oxoglutaric (trace) acids was slowly mixed with a soln of 2-fluorophenylhydrazine hydrochloride in 30% (w/w) HOAc (21 ml, final pH 4) and kept at 4° overnight. After collecting the pptd hydrazone derivative (crystals and syrupy product) the mother liquors were acidified to pH 2, newly formed crystals collected, and the remaining hydrazones extracted with EtOAc. TLC (Silica gel GF₂₅₄) developed with CH₂Cl₂-MeOH-HOAc (90:10:1) showed a major, light yellow, UV fluorescence-quenching spot (R_f = 0.7) in all crops of the crude hydrazone; the overall yield was 6.3 g. An aliquot [2.1 g, 9.9 mmol assuming pure 4-(2-fluorophenylhydrazone)-butyric acid] was dissolved in dry pyridine (24.3 ml). Conc HCl (32.4 ml) and 85% H₃PO₄ (8 ml) were added and the mixture was boiled (115°) under reflux for 16 hr [32]. The soln was diluted with ice water (30 g) and, after raising the pH to 2.0 (20% NaOH), extracted with Et₂O (6 × 80 ml). The organic phase was washed with NaHSO₄ soln (3 × 25 ml; pH 1.5) and brine, evapd, and passed through a column (22 × 1.5 cm) of Sephadex LH-20, which was eluted with *iso*-PrOH-H₂O (1:1), to yield the crude title compound (708 mg, 37%; elution vol. 175–240 ml). Recrystallization from H₂O (filtering off insoluble tars) and repeatedly from CHCl₃-cyclohexane afforded leaflets, mp 161–162°. EIMS (probe) 70 eV, m/z (rel. int.): 193.0539 [M]⁺ (38)—C₁₀H₈FNO₂ requires 193.0539; 148.0559 [M - COOH]⁺ (100)—C₉H₇FN requires 148.0563; 128.0486 [M - COOH - HF]⁺ (15)—C₉H₆N requires 128.0500. ¹H NMR spectrum (90 MHz, Me₂CO-*d*₆, Me₄Si, c = 6 mg ml⁻¹): δ 10.50 (1H, broad, s, H-1), 7.38 (1H, *m*, H-2), 7.41 (1H, *m*, H-4), 7.00 (1H, *td*, $J_{4,5} = J_{5,6} = 8$ Hz, $J_{5,F} = 5$ Hz, H-5), 6.86 (1H, *ddd*, $J_{4,6} = 1.5$ Hz, $J_{6,F} = 11$ Hz, H-6), 3.76 (2H, *d*, $J_{2,CH_2} = 0.9$ Hz, CH₂). ¹³C NMR spectrum (broad band ¹H-decoupled, 22.5 MHz, Me₂CO-*d*₆, Me₄Si, c = 120 mg ml⁻¹): δ 125.0 (s, C-2), 109.5 (*d*, $^4J_{C,F} = 3$ Hz, C-3), 131.8 (*d*, $^3J_{C,F} = 6$ Hz, C-3a), 115.0 (*d*, $^4J_{C,F} = 4$ Hz, C-4), 119.3 (*d*, $^3J_{C,F} = 5$ Hz, C-5), 106.2 (*d*, $^2J_{C,F} = 17$ Hz, C-6), 149.8 (*d*, $^1J_{C,F} = 242$ Hz, C-7), 30.6 (s, CH₂), 172.8 (s, COOH).

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