

# Comparative Indole-3-Acetic Acid Levels in the Slender Pea and Other Pea Phenotypes<sup>1</sup>

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## ABSTRACT

Free indole-3-acetic acid levels were measured by gas chromatography-mass spectrometry in three ultra-tall 'slender' *Pisum sativum* L. lines differing in gibberellin content. Measurements were made for apices and stem elongation zones of light-grown plants and values were compared with wild-type, dwarf, and nana phenotypes in which internode length is genetically regulated, purportedly via the gibberellin level. Indole-3-acetic acid levels of growing stems paralleled growth rates in all lines, and were high in all three slender genotypes. Growth was inhibited by *p*-chlorophenoxyisobutyric acid, demonstrating the requirement of auxin activity for stem elongation, and also by the ethylene precursor 1-aminocyclopropane-1-carboxylic acid. It is concluded that the slender phenotype may arise from constant activation of a gibberellin receptor or transduction chain event leading directly or indirectly to elevated levels of indole-3-acetic acid, and that increased indole-3-acetic acid levels are a significant factor in the promotion of stem elongation.

In many pea genotypes internode length is correlated with the content of GA<sub>1</sub> in the growing region (23). This, however, is not the case in ultra-tall 'slender' peas. The slender phenotype in pea (*Pisum sativum* L.) is conferred by the gene combination *la cry*<sup>s</sup> (17). This phenotype has been described as strongly resembling a dwarf plant treated with a saturating dose of active GA<sup>2</sup>, e.g. plants display thin elongated internodes, lighter green shoots, parthenocarpy, and reduced leaflet size (22). However, internode elongation is not inhibited by GA-biosynthesis inhibitors (18), and indeed the phenotype is not altered by insertion of the recessive alleles *le* or *na*, which inhibit conversion of GA<sub>20</sub> to GA<sub>1</sub>, and the synthesis of all GAs, respectively (7, 8, 22). It has been postulated that the gene combination *la cry*<sup>s</sup> may affect the GA receptor site or some other early step in GA-induced elongation (22). Thus, even in the absence of endogenous GAs, growth and morphogenesis may be affected as if GAs were present and nonlim-

iting. In a mutant like slender, then, one might not find a good correlation between stem growth and the levels of extractable hormone(s) which regulates stem elongation (5).

Auxin levels are often higher in apices and elongating shoots of tall plants than in dwarfs (e.g. 12, 15, 16, 20). Treatment with GAs also frequently increases IAA levels in growing stems (6, 10, 15, 21, 26) and there is considerable evidence that GA<sub>3</sub> can induce increased IAA biosynthesis (10, 11, 13, 26, 28). Thus, if the slender phenotype arises from an activated GA-receptor then it is possible that free IAA levels might be elevated through a similar process to GA-increased auxin biosynthesis. We examined IAA contents of the stem elongation region and of apical buds with enclosed meristems (the presumed sites of IAA synthesis) in three light-grown slender pea lines differing dramatically in active GA content. Results were compared with individual lines of three other stem length phenotypes in a genetic background closely related to the slender lines. These included a tall line (allele *Le*) with a high GA<sub>1</sub> level, a dwarf (allele *le*) with a low GA<sub>1</sub> level, and the extreme dwarf 'nana' (allele *na*) in which GAs are virtually absent.

## MATERIALS AND METHODS

### Plant Material

Pea (*Pisum sativum* L.) seeds of each line were from stock in the Hobart collection. All genotypes were homozygous (22, 23). Line 133 is a slender selection with a block in GA<sub>20</sub> to GA<sub>1</sub> conversion (*le la cry*<sup>s</sup> *Lh Ls Na Lm Lk*). The nana phenotype used was Line 1766 (*Le La Cry Lh Ls na Lm Lk*). Slender lines 188 (*Le la cry*<sup>s</sup> *Lh Ls na Lm Lk*) and 197 (*Le la cry*<sup>s</sup> *Lh Ls Na Lm Lk*) were derived from crosses of lines 133 and 1766. The wild type tall line was 1769 (*Le La Cry Lh Ls Na Lm Lk*) and the dwarf line was 203 (*le La Cry Lh Ls Na Lm Lk*) (22, 23).

Seeds were sown in pots of a soil mix and plants were grown in a chamber at 18°C ± 1°C under an 18 h photoperiod (mixed incandescent and fluorescent lamps, 181.4 ± 1.1 μmol m<sup>-2</sup> s<sup>-1</sup> PAR, with only incandescent lights [3.3 ± 0.2 μmol m<sup>-2</sup> s<sup>-1</sup> PAR] on during the first and last 0.5 h of the photoperiod). At 14 to 16 d, the youngest visible bud (at nodes 5 or 6, counting the cotyledons as zero) and the subtending stem elongation zone were separated with a razor blade and were immediately immersed in liquid nitrogen. Frozen samples were quickly weighed and were stored at -80°C until analyses.

Plants used in inhibitor application experiments were

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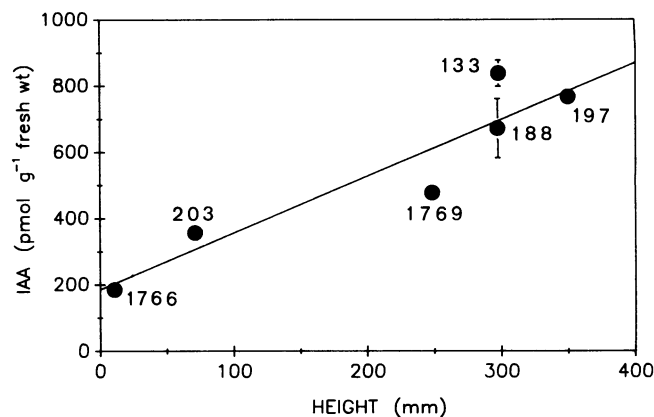
<sup>2</sup> Abbreviations: GA, gibberellin; ACC, 1-aminocyclopropane-1-carboxylic acid; PCIB, *p*-chlorophenoxyisobutyric acid; SIM, selective ion monitoring; TIBA, triiodobenzoic acid; BHT, butylated hydroxytoluene; R<sub>t</sub>, retention time.

grown in vermiculite under the same conditions except at  $125 \pm 5 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAR. At 7 to 9 d, seedlings showing 20 to 60% total elongation of the upper internode were selected and handled as described below.

### IAA Purification

Each frozen sample (0.39–1.60 g for stems, 1.15–7.46 g for apices) was plunged into boiling 80% aqueous methanol with  $0.1 \text{ mg L}^{-1}$  BHT (volume to fresh weight  $\geq 5$ ) for 3 min (14). Appropriate amounts of [ $^{13}\text{C}_6$ ]IAA (Cambridge Isotope Laboratories, Woburn, MA) ( $77\text{--}1,180 \text{ pmol g}^{-1}$ ) and 1-[ $^{14}\text{C}$ ]IAA (Amersham, specific activity  $55 \mu\text{Ci } \mu\text{mol}^{-1}$ ) ( $89\text{--}673 \text{ pmol g}^{-1}$ , or  $4.9\text{--}37.0 \text{ nCi g}^{-1}$ ) were added and the tissue was ground with a mortar. After extraction for 0.5 h on a shaker (75 rpm) the extract was filtered (Whatman No. 1) and the residue reextracted for 20 min with 80% methanol/BHT. Extracts were combined and the volume was reduced to about 5 mL *in vacuo* on a rotary evaporator. Samples were neutralized with  $0.1 \text{ N NH}_4\text{OH}$  and 10 to 20 mL hexanes (Baker 85% *n*-hexane, HPLC-grade) were added. The aqueous and hexane phases were mixed for 5 to 10 min and then the volume was reduced *in vacuo*, precipitating most Chl in the extract. The 1 to 3 mL aqueous extract was neutralized with  $0.1 \text{ N NH}_4\text{OH}$  and centrifugally filtered through glass fiber prefilters (Whatman GF/D) and then through a Nylon-66 filter (Rainin,  $0.45 \mu\text{m}$  pore size). Samples were either stored at  $-20^\circ\text{C}$  or taken immediately to HPLC purification.

Initial purification was performed by HPLC using a radioactivity detector (model 7140, Packard) to detect the [ $^{14}\text{C}$ ] IAA and trigger a fraction collector to collect the total IAA in the sample (column:  $25 \text{ cm} \times 4.6 \text{ mm } 5 \mu\text{m}$  Spherisorb ODS-2 (Phase Sep, Norwalk, CT); load loop volume = 5 mL; gradient: A = water with  $2 \text{ mL L}^{-1}$  glacial acetic acid, B = methanol: 0% B for 2 min; to 55% B over 15 min; to 100% B over 2 min; IAA,  $R_t = 15.8 \text{ min}$ ). The IAA fraction was neutralized with  $0.1 \text{ N NH}_4\text{OH}$ , dried *in vacuo*, and taken up in  $200 \mu\text{L}$  methanol for methylation with ethereal diazomethane. Diethyl ether was removed under a gentle stream of nitrogen, the flask was washed twice with  $500 \mu\text{L}$  methanol, and the washings were added to 4 mL water for a second HPLC purification under the same conditions except that at 2 min the methanol content was increased to 65% over 15



**Figure 1.** Comparison of IAA contents of stem elongation zones with heights (above node 2) at harvest for light-grown pea phenotypes. Correlation coefficient = 0.944. Where not shown, se bars are smaller than the symbol.

min ( $R_t$  methyl IAA = 17.8 min). The methyl IAA fraction was neutralized with  $0.1 \text{ N NH}_4\text{OH}$ , dried under  $\text{N}_2$  and taken up in 10 to  $20 \mu\text{L}$  methanol. Samples were stored at  $-20^\circ\text{C}$  until GC-MS analyses.

### IAA Measurements

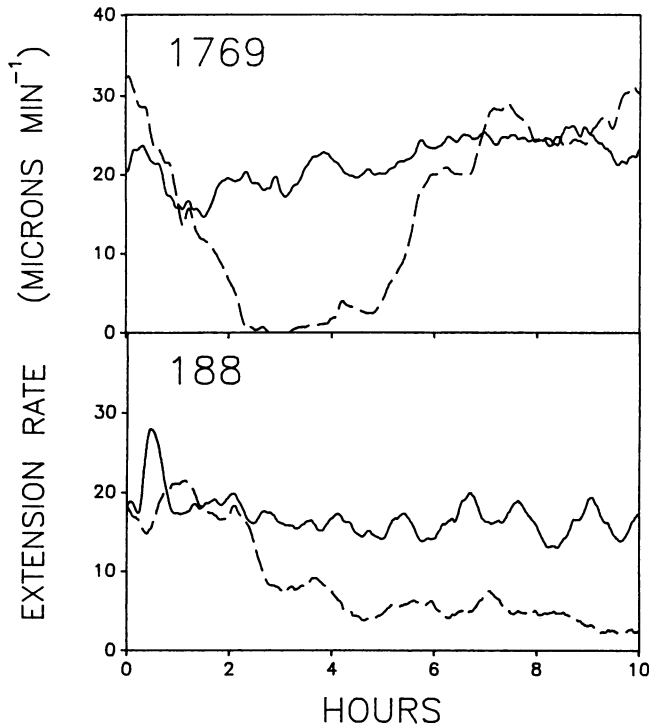
GC-MS was carried out on a Hewlett-Packard gas chromatograph (model 5890A) with a bonded methyl silicone capillary column (HP-1,  $12 \text{ m} \times 0.2 \text{ mm} \times 0.33 \mu\text{m}$  coating thickness), connected to a model 5970B mass selective detector. Both scanning and SIM ( $m/z$  130, 136, 189, 191, and 195) modes were employed with a temperature regime of  $105^\circ\text{C}$  for 1 min, then increasing by  $30^\circ\text{C min}^{-1}$  to  $210^\circ\text{C}$  followed by  $4^\circ\text{C min}^{-1}$  to  $270^\circ\text{C}$ , with a carrier gas flow rate of 25 to  $30 \text{ cm s}^{-1}$  ( $R_t$  methyl IAA = 8.2 min). Sample injection sizes were 1 to  $2 \mu\text{L}$ . Endogenous IAA was measured by the isotope ratio based upon the relative magnitude of 130 (endogenous [ $^{12}\text{C}$ ]IAA) and 136 ([ $^{13}\text{C}$ ]IAA) ion signals with subtraction of the contribution of [ $^{14}\text{C}$ ]IAA to the 130 peak, calculated from the proportional amount of [ $^{14}\text{C}$ ]IAA added at the start of the extraction (3). Three to five replicate samples were extracted and analyzed for each value reported.

**Table 1.** IAA Contents of Elongating Stems and Apices of Light-Grown Pea Phenotypes

Each value is based upon 3 to 5 replicate extractions and analyses. Heights are an average of 20 plants above node 2 at time of harvest, which was when internodes 5 or 6 had emerged from the apical bud.

Line	Phenotype	Genotype	GA Content	Height mm	IAA Content			
					Stem		Apex	
					$\text{pmol g}^{-1} \pm \text{SE}$		$\text{pmol organ}^{-1} \pm \text{SE}$	
197	Slender	<i>Le la cry<sup>s</sup> Na</i>	High $\text{GA}_1$	350.0	$766 \pm 6a^*$	$250 \pm 40b$	$22.6 \pm 0.8$	$26.5 \pm 1.2$
133	Slender	<i>le la cry<sup>s</sup> Na</i>	Low $\text{GA}_1$	298.1	$837 \pm 40a$	$201 \pm 32b$	$21.3 \pm 0.8$	$27.8 \pm 0.8$
188	Slender	<i>Le la cry<sup>s</sup> na</i>	GAs lacking	297.4	$671 \pm 90a$	$206 \pm 9b$	$19.5 \pm 1.1$	$34.7 \pm 1.3$
1769	Wild type	<i>Le La Cry Na</i>	High $\text{GA}_1$	248.1	$476 \pm 11c$	$252 \pm 39b$	$14.1 \pm 0.4$	$35.0 \pm 1.5$
203	Dwarf	<i>le La Cry Na</i>	Low $\text{GA}_1$	71.2	$356 \pm 21d$	$179 \pm 13b$	$8.5 \pm 0.8$	$37.1 \pm 0.7$
1766	Nana	<i>Le La Cry na</i>	GAs lacking	10.8	$184 \pm 9e$	$53 \pm 3f$	$0.6 \pm 0.2$	$4.0 \pm 0.3$

\* Different letters indicate significant differences at the 5% level using *t*-test comparisons.



**Figure 2.** Effect of PCIB on elongation rates of tall (line 1769) and slender (line 188) pea plants. PCIB (broken line) or H<sub>2</sub>O (solid line) was applied as a 1 mm soil drench at 0 h. Results are means of six determinations. Other slender lines (133 and 197) responded similarly to line 188.

### Inhibitor Studies

Seedlings were grown in 80 mL cups of vermiculite. At 6 to 8 d of age, internode lengths were measured and inhibitors were applied. Lanolin alone or containing TIBA (20 mM) or PCIB (5 mM) was applied as a 2 mm diameter ring around the elongation zone of slender plants. PCIB was also applied to the soil (1 mm until drainage occurred) or as a 10  $\mu$ L drop (1 mm) between the stipules of the apical bud. A concentration series was studied with the tall wild-type line. ACC (1 mM) was also applied as a soil drench. To record growth kinetics plants were connected to angular position transducers (Gould, Pittsburgh, PA) interfaced with an AT&T 6300 computer, allowed to equilibrate under fluorescent lights ( $74 \pm 5 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAR) for several hours, and were then treated with water or 1 mM PCIB as a soil drench (100 mL added to 80 mL volume cups). Growth rates were determined for 10 h.

### RESULTS

IAA levels were much higher in the growing regions of slender stems than in the wild type, dwarf, and nana lines (Table I). Auxin levels did not vary significantly among stems or apices of the different slender genotypes and thus do not correlate with endogenous GA activity in these lines. However, a good correlation exists when the IAA concentration in the elongation zones is compared with the stem length of the different genetic lines at the time of harvest (Fig. 1). IAA

levels in the apices, the presumed sites of synthesis, were equivalent in all lines except the extreme dwarf line 1766 (Table I). Since sizes and weights of stem sections and apices vary, data were also calculated on a per organ basis (Table I). The correlation between heights and IAA contents was very similar to that based upon weight.

When TIBA was applied as a lanolin ring to eight slender plants (line 133) within the elongation zones, a diageotropic growth was observed above the ring within 24 h; however, total elongation as well as growth above and below the ring were not inhibited in comparison to control plants receiving lanolin only. Within 48 h epinasty was pronounced above the point of application in all treated plants. In 90 h, lateral bud growth was present at the lowest three nodes in half of the treated plants.

PCIB inhibited elongation by all methods of application in both tall and slender plants. When applied as a soil drench, inhibition, as great as 70%, was apparent within 2 h (Fig. 2) followed by a gradual recovery. Trial treatments of wild type peas with different concentrations of PCIB showed no obvious toxic effects even 10 d after application, though lateral root initiation was inhibited in that time, as might be expected (29) (application as a soil drench to slender seeds at planting inhibited emergence of the epicotyl from the soil surface). The ethylene precursor ACC induced epinasty and inhibited the stem elongation rate within 24 h (Table II).

### DISCUSSION

In general, there is a good correlation between endogenous IAA contents in elongation zones and the stem growth of all the various lines.

In the slender phenotype, IAA contents did not vary significantly regardless of the presence of GA<sub>1</sub> (line 197), the virtual absence of GA<sub>1</sub> (line 133), and the near absence of all GAs (line 188) (22). This phenotype, conferred by the genetic pair *la cry*<sup>s</sup>, acts as if it has an activated GA receptor or a promotion of the GA signal transduction pathway so that it grows as if GA-saturated.

Recent work has shown that tallness in peas correlates with the content of GA<sub>1</sub> (7, 23, 24). Tall plants possessing the allele *Le*, have high levels of GA<sub>1</sub> because *Le* regulates production of a 3- $\beta$ -hydroxylase which converts GA<sub>20</sub> to GA<sub>1</sub> (7). Dwarf

**Table II.** Effect of ACC on Stem Elongation

ACC (1 mM) or H<sub>2</sub>O was applied as a soil drench (100 mL to 80 mL cups) and growth rates were determined by ruler measurements at 24 and 48 h after treatment. Values are means of 8 to 10 replicates. Slender lines 133 and 188 responded similarly to line 197.

Line	Phenotype	Treatment	Growth Rate at h after Application	
			24	48
<i>mm h</i> <sup>-1</sup>				
1769	Wild type	ACC	0.3 $\pm$ 0.1	1.1 $\pm$ 0.1
		H <sub>2</sub> O	0.8 $\pm$ 0.1	1.0 $\pm$ 0.1
197	Slender	ACC	0.5 $\pm$ 0.1	0.3 $\pm$ 0.1
		H <sub>2</sub> O	1.2 $\pm$ 0.1	1.2 $\pm$ 0.1

plants possess the homozygous recessive allele *le* which largely blocks the conversion of GA<sub>20</sub> to GA<sub>1</sub>; often GA<sub>1</sub> cannot be detected in dwarf plants, though the presence of the 2- $\beta$ -hydroxylation product GA<sub>8</sub> and its metabolite, GA<sub>8</sub>-catabolite, provide evidence that some GA<sub>1</sub> must be produced (8). In plants possessing different alleles affecting the conversion of GA<sub>20</sub> to GA<sub>1</sub> (*Le*, *le*, *le<sup>d</sup>*) there is a direct correlation between the level of GA<sub>1</sub> and internode elongation (24). Thus, it appears that GA<sub>1</sub> may be the specific GA regulating internode elongation in *Pisum*, while all other GAs are either precursors or metabolites of GA<sub>1</sub>. In very dwarf plants (*nana*, determined by the presence of the homozygous recessive allele *na*) there is a block in GA synthesis prior to GA<sub>12</sub>-aldehyde (the first GA) so that the plants are deficient in all GAs, including GA<sub>1</sub> (9).

While there is thus a good agreement between the level of GA<sub>1</sub> (or its metabolic products) in many tall and dwarf plants produced by known gene changes, slender, which is ultra-tall regardless of the presence or level of GA<sub>1</sub> (22), provides a notable exception. Here it is shown that slender plants contain a high level of IAA regardless of their GA content. Thus, it would seem that IAA is involved in the regulation of stem elongation in the slender phenotype and the presence of GA<sub>1</sub> may be substituted by the action of the genes *la cry<sup>s</sup>*. As our results demonstrate, at least part of the action of *la cry<sup>s</sup>*, either directly or indirectly, is to raise the level of auxin.

For many years evidence has accumulated for a GA-induced increase in auxin levels (*cf.* references in 13). Applied GA<sub>3</sub> dramatically increased free IAA levels in a dwarf pea (15). Furthermore, the GA biosynthesis inhibitor uniconazole decreased both IAA levels and stem growth in a wild-type pea; subsequent addition of GA<sub>3</sub> to uniconazole treated plants increased both growth and IAA, and there was a very good correlation between stem growth and IAA content of these young stems (16). More specifically, Kutacek (11) has presented evidence for GA-enhanced indoleacetaldehyde oxidase activity and Law (13) has argued in favor for a regulatory role of GA in generation of D-tryptophan as an IAA precursor. Others have found evidence for GA<sub>3</sub>-enhanced D-tryptophan aminotransferase activity (19). A striking feature of the slender phenotype is the lack of effect of endogenous GA content on growth (18, 22) and on IAA content, as demonstrated here. However, these results are consistent with the hypothesis that this phenotype arises from a mutation at the GA receptor (or another early step in the transduction sequence of GA-induced events) and further implicate a role for auxin in the mediation of GA-induced growth. That auxin activity is required for pea stem elongation is implied by the inhibition produced by PCIB (assuming PCIB acts specifically as an antiauxin). The auxin transport inhibitor TIBA caused epinasty above the point of application and lateral bud release below this point. The latter suggests that IAA levels in the lower portion of the stem declined due to transport inhibition (2, 6). Auxin may have accumulated above the lanolin ring to a sufficient extent to increase ethylene production and thus induce epinasty, though elongation was not inhibited as was the case following ACC treatment through the roots. In 'decapitated' pea stems in which growth may be promoted by

exogenous GA<sub>3</sub> or low levels of applied IAA, TIBA inhibited the elongations induced by both hormones (4).

IAA concentrations found in elongating green slender pea stems are quite high and exceed those in the tall line used in these analyses, though they fall within the range reported for other tissues (1), and much higher endogenous levels have been reported in rapidly elongating liverwort setae (27). While IAA contents of apices (the presumed site of synthesis) do not vary significantly except in the *nana* line, the range of stem concentrations may arise from differences in degradation, conjugation, or transport rates out of the region capable of elongation. Furthermore, it is possible that IAA biosynthesis occurs to a significant extent *in vivo* in the stem itself, since excised stem segments are capable of IAA biosynthesis from precursors (13). Peas also contain auxins other than IAA (25) which may play a role in stem elongation, and growth may also be modulated by the presence of other hormones (2, 6).

The high IAA content does not seem to trigger ethylene induced growth inhibition, however, since stem growth is inhibited by exogenous ACC. This may arise from an impairment in ACC synthesis, though the epinasty induced by TIBA would seem to argue for normal ethylene biosynthesis. It seems more likely that auxin levels do not pass a threshold for induction of ethylene synthesis until TIBA causes an accumulation of auxin above the point of application.

These results indicate that IAA plays a central role in stem elongation and also support the hypothesis that GAs increase auxin levels, both directly and indirectly through an activated GA receptor. Thus GA(s) appear to enhance pea stem elongation, at least in part, through an increase in IAA levels, though there is no reason to conclude that GAs themselves do not also have a direct effect on growth. Work on the slender pea phenotype is continuing with the hope of clarifying the effects on auxin metabolism.

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