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Effects of Auxin Transport Inhibitors on Gibberellins in Pea

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Abstract. The effects of the auxin transport inhibitors 2,3,5-triiodobenzoic acid (TIBA), 9-hydroxyfluorene-9-carboxylic acid (HFCA), and 1-*N*-naphthylphthalamic acid (NPA) on gibberellins (GAs) in the garden pea (*Pisum sativum* L.) were studied. Application of these compounds to elongating internodes of intact wild type plants reduced markedly the endogenous level of the bioactive gibberellin A_1 (GA_1) below the application site. Indole-3-acetic acid (IAA) levels were also reduced, as was internode elongation. The auxin transport inhibitors did not affect the level of endogenous GA_1 above the application site markedly, nor that of GA_1 precursors above or below it. When plants were treated with [^{13}C,^{3}H]GA_{20}, TIBA reduced dramatically the level of [^{13}C,^{3}H]GA_1 recovered below the TIBA application site. The internodes treated with auxin transport inhibitors appeared to be still in the phase where endogenous GA_1 affects elongation, as indicated by the strong response to applied GA_1 by internodes of a GA_1-deficient line at the same stage of expansion. On the basis of the present results it is suggested that caution be exercised when attributing the developmental effects of auxin transport inhibitors to changes in IAA level alone.

Key Words. *Pisum sativum*—Auxin transport inhibitors—Gibberellins—Auxin—Elongation—Transport—Metabolism

Auxin transport inhibitors, such as 2,3,5-triiodobenzoic acid (TIBA), 9-hydroxyfluorene-9-carboxylic acid (HFCA), and 1-*N*-naphthylphthalamic acid (NPA) are used widely in plant research (e.g. Jensen et al. 1998). These compounds are thought to block specifically the basipetal movement of indole-3-acetic acid (IAA) from its site of synthesis in the shoot apex to sites of action further down the stem (for a review, see Lomax et al. 1995). Thus the effects of auxin transport inhibitors on plant development are usually attributed to changes in the endogenous level of IAA (e.g. Fischer and Neuhaus 1996, Liu et al. 1993). In the garden pea, TIBA and HFCA inhibited stem elongation, and the use of mutant types provided strong evidence that this effect was at least partially attributable to reduced IAA levels (McKay et al. 1994). However, it is also important to investigate the effects of auxin transport inhibitors on the gibberellin (GA) economy of the pea shoot because GA_1 is a critical factor in the control of pea stem growth (Ross et al. 1997). An early report (Kentzer and Libbert 1961) provided some evidence that in sunflower the movement of exogenous GA may be inhibited by TIBA.

In the present paper the effects of TIBA, HFCA, and NPA on endogenous gibberellins in wild type (WT) tall pea plants are examined, as are the effects of TIBA on the metabolites recovered after feeds of labeled GA_{20} (GA_{20} is the immediate precursor of GA_1 in pea shoots; Fig. 1). The experiments involved applying the auxin transport inhibitors to an internode in a lanolin ring. At this stage internodes were typically less than 35% expanded, and it was relevant to study the responsiveness to GA_1 of internodes at this stage (or older). This was done by monitoring the response to applied GA_1 of the short, GA_1-deficient internodes of *le-1* plants. Mutation *le-1* blocks the step GA_{20} to GA_1 (Ingram et al. 1984), reducing the endogenous GA_1 content (Ross et al. 1992).

Materials and Methods

Plants

The lines used were the WT tall line, 205+, and the isogenic *le-1* line, 205−, developed by J. B. Reid from a cross originally made by I. C. Murfet (Ross and Reid 1989). Mutation *le-1* was previously referred to

Abbreviations: TIBA, 2,3,5-triiodobenzoic acid; HFCA, 9-hydroxyfluorene-9-carboxylic acid; NPA, 1-*N*-naphthylphthalamic acid; IAA, indole-3-acetic acid; GA, gibberellin; WT, wild type; HPLC, high performance liquid chromatography; GC-MS, gas chromatography-mass spectrometry; p.s.i., pound per square inch.

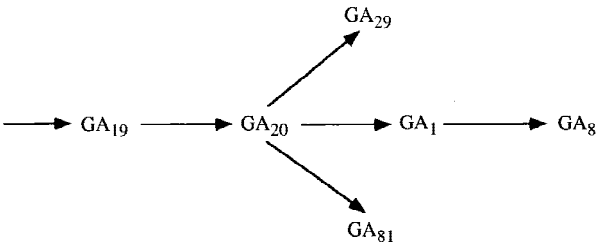

Fig. 1. Metabolic relationships of the GA_1 precursors and metabolites monitored in this study.

as *le.* Plants were grown in a heated glasshouse under an 18-h photoperiod (with natural light extended before dawn and after dusk) as described previously (Beveridge and Murfet 1996). Node counting began from the base of the plant, with the cotyledons as zero; internode x refers to the internode between nodes x and x+1.

Application of Chemicals

TIBA (58 mM), HFCA (50 mM), and NPA (32 mM) were applied to internodes in a ring of lanolin (hydrous lanolin for TIBA and NPA and anhydrous lanolin for HFCA); control plants received lanolin only. [17,17-^{13}C,$^{3}H_2$]Gibberellin A_{20} (88% ^{13}C, 15 mCi $mmol^{-1}$, provided by Dr. C. L. Willis, University of Bristol), was applied to expanded foliage leaves in a microdrop (5 or 10 μl) of ethanol. To investigate the effect of applied GA_1 on internodes that were already about 50% expanded, the dwarf line 205− was used. Data are presented from an experiment in which 36 plants with internode 6 approximately 50% expanded were selected: 18 plants were then decapitated at the top of that internode. GA_1 (2 μg of plant $^{-1}$, in 20 μL of ethanol) was then applied to nine intact and nine decapitated plants; the remaining (control) plants received ethanol only. Application was to leaf 6.

Extraction and Purification of GAs

Harvested material was immersed immediately in cold (−20°C) methanol, which contained butylated hydroxytoluene (1 mg/4 mL) if IAA was to be monitored.

Endogenous GAs and IAA were quantified using deuterated internal standards, as described previously (McKay et al. 1994, Ross et al. 1995). After extraction at 2–4°C for 24 h in 80% methanol and filtering, extracts were purified with Sep-Pak C18 cartridges (Ross et al. 1995) and subjected to HPLC using the system described by Potts et al. (1985). The solvent program ran from 20% to 75% methanol in 0.4% acetic acid over 25 min with a linear gradient. The flow rate was 2 mL min^{-1}, and 1-min fractions were collected. Fractions were pooled according to the retention time of tritiated GA tracers of high specific activity. IAA was pooled with GA_1. Pooled fractions were evaporated to dryness, methylated, and trimethylsilylated as before (Hasan et al. 1994, Ross and Reid 1989).

Plant material harvested after feeds of [^{13}C,^{3}H]GA_{20} was extracted at 2–4°C, as above. After filtering, aliquots were taken for radiocounting and for further analysis. The latter aliquots were purified with Sep-Pak C18 cartridges as before (Ross et al. 1995) except that GAs were eluted with 80% methanol in 0.4% acetic acid. The extract was then methylated and subjected to HPLC (Ross et al. 1995). The program ran from 30 to 60% methanol in distilled water over 35 min (exponential gradient) followed by isocratic (60%) elution. The flow rate was 1.6 mL min^{-1}, and 1-min fractions were collected. Aliquots were taken for radiocounting using a Beckman LS5801 scintillation counter.

Table 1. Effects of HFCA and NPA on endogenous GA_1 and IAA levels [ng · g (fresh weight)$^{-1}$] and on internode elongation. HFCA and NPA were applied at the top of internode 9. The ''apical'' portion was all material above the application site, and the ''internode below'' portion was between the application site and node 9. Material was harvested 26 h after application. $n = 8$ for length measurements. Internode 9 was 84% expanded at harvest.

Treatment and portion	GA_1	IAA	Length at treatment (mm)	Length at harvest (mm)	Fresh weight at harvest (g)
Control					
Apical	14.6	21.6	N.A.	N.A.	5.32
Internode below	12.2	91.4	30.7 ± 1.8	60.2 ± 2.7	1.82
HFCA					
Apical	9.6	29.9	N.A.	N.A.	5.46
Internode below	1.4	2.0	31.6 ± 1.4	41.5 ± 1.5	1.07
NPA					
Apical	14.4	36.1	N.A.	N.A.	5.64
Internode below	1.6	3.6	30.1 ±1.5	42.9 ± 1.6	1.15

Note. N.A., not applicable.

Gas Chromatography-Mass Spectrometry (GC-MS)

The GC-MS system consisted of a Hewlett-Packard 5890 GC coupled to a Kratos Concept ISQ mass spectrometer (Hasan et al. 1994). A Hewlett-Packard 25-m × 0.32-mm inner diameter × 0.17-μm film HP1 column was used. The oven temperature was programmed from 60°C to 230°C at 30°C min^{-1} and then at 3°C min^{-1} for GAs or from 60°C to 150°C at 30°C min^{-1} and then at 10°C min^{-1} for IAA, with a column head pressure of 15 p.s.i. For selected ion monitoring the mass spectrometer was operated at high resolution (R = 10,000). Calculations of endogenous levels were performed as described before (Lawrence et al. 1992, McKay et al. 1994), except that for GAs the contribution from naturally occurring isotopes was measured using standards rather than calculated.

Results

Effects of Auxin Transport Inhibitors on Endogenous GA and IAA Levels and on Internode Elongation

Application of TIBA, HFCA, or NPA to elongating internodes of the tall pea line 205+ reduced the level of GA_1 and IAA markedly, below the treatment site (Tables 1 and 2). The effects of TIBA on GA_1 and IAA levels in internode portions at successive stages of development are shown in Fig. 2. Levels are shown on a ng/g basis; the reduction in content is even greater on a ng/section basis because all three compounds reduced the fresh weight of internode sections below the application site. The auxin transport inhibitors did not affect the GA_1 content of the apical portion substantially, whereas the level of IAA

Table 2. Effects of TIBA on endogenous GA and IAA levels [ng · g (fresh weight)$^{-1}$]. TIBA was applied in a lanolin ring near the base of internode 7. Plant material was harvested after 47 h. Material from above the application site was partitioned into the apical portion and internode 7. The ''internode below'' portion was the basal part of internode 7 along with the top 25% of internode 6.

	Fresh weight (g)	GA_{19}	GA_{20}	GA_1	GA_{29}	GA_8	IAA
Control							
Above site							
Apical	3.06	7.4	29.1	26.0	16.3	60.9	17.7
Internode 7	1.38	10.4	6.5	29.8	8.8	36.8	80.7
Internode below	1.82	4.9	3.4	6.8	6.4	22.0	57.9
TIBA							
Above site							
Apical	2.99	5.8	18.4	23.7	19.8	69.1	23.2
Internode 7	1.43	4.4	4.3	29.3	11.7	57.2	72.3
Internode below	1.47	5.4	3.2	0.5	17.4	16.5	3.0

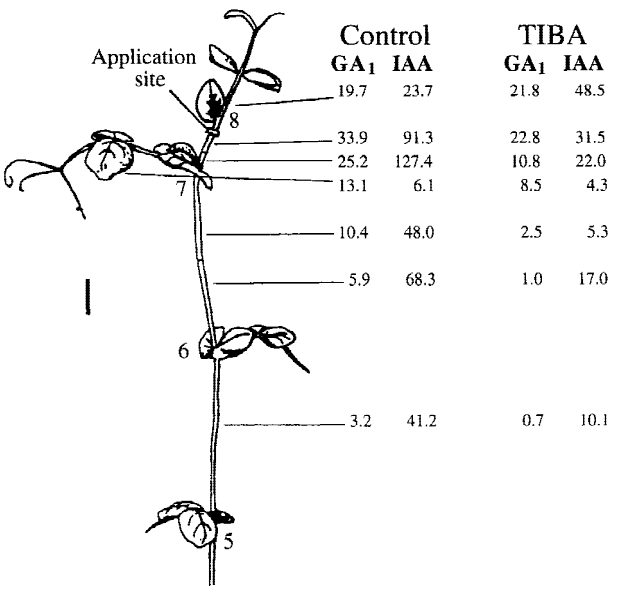

Fig. 2. Levels of GA_1 and IAA [ng · g (fresh weight)$^{-1}$] in various portions of 205+ (WT) plants with or without TIBA. The diagram represents control plants at harvest, drawn to scale; *bar* = 20 mm. The site of the TIBA application and node number are shown. Internodes 7 and 6 were about 8% and 62% expanded when TIBA was applied and 26% and 88% expanded (respectively) at harvest. Material was harvested 44 h after the TIBA application. The leaf at node 7 did not include the stipules.

was typically, but not always, elevated in this portion (up to twofold) compared with control plants. Similar results were obtained in repeat experiments.

The effects of TIBA and HFCA on the levels of endogenous GA_1 precursors and metabolites, both above and below the application site, were also monitored; typical data are shown in Table 2. Generally, effects on these GAs were small, although GA_{29} levels were typically elevated below the application site (2.7-fold in Table 2). Changes in GA_8 level below the site were variable: in one experiment there was a sevenfold reduction in TIBA-treated plants (data not shown), but in Table 2 the effect is very small.

The three auxin transport inhibitors tested all reduced elongation below the treatment site, as found previously for TIBA and HFCA (McKay et al. 1994). Typical data for HFCA and NPA are shown in Table 1.

Effects of TIBA on the Levels of Labeled GA_{20} Metabolites

The effects of TIBA on GAs in pea were even more obvious when the metabolites of [^{13}C,^{3}H]GA_{20} were monitored. These metabolites were [^{13}C,^{3}H]GA_1, [^{13}C,^{3}H]GA_{29}, [^{13}C,^{3}H]GA_8, [^{13}C,^{3}H]GA_{81}, as found before (Ross et al. 1995, Sherriff et al. 1994). Metabolite levels were determined by HPLC radiocounting (Table 3 and Fig. 3), and identities were confirmed by GC-MS (Table 4).

In TIBA-treated plants there was a dramatic reduction in the level of [^{13}C,^{3}H]GA_1 recovered below the TIBA application site (Table 3, Fig. 3). This occurred regardless of whether [^{13}C,^{3}H]GA_{20} was applied to a leaf below (Table 3) or above (Fig. 3) the TIBA site. The decrease in [^{13}C,^{3}H]GA_1 level caused by TIBA below the site was matched closely by an accumulation of [^{13}C,^{3}H]GA_1 in the apical portion above the TIBA application site (compared with control plants; Table 3 and Fig. 3). There was no evidence for a consistent effect of TIBA on the transport of [^{13}C,^{3}H]GA_{20} across the TIBA application site, which probably occurred in the phloem, as with exogenous GA_3 (McComb 1964).

Table 3. Metabolite levels after [^{13}C, ^{3}H]GA_{20} application to plants with and without TIBA. TIBA was applied to internode 10 (when it was about 25% expanded); 24 h later [^{13}C, ^{3}H]GA_{20} was applied (10^5 dpm plant^{-1}) to leaf 9. Portions were harvested after a further 28 h. The apical portion was all tissue above the lanolin ring; the internode below was between node 10 and the ring; and the mature internode was internode 9. Data are the total dpm for each GA recovered from the portion; HPLC fractions are indicated. Internode 10 was 84% expanded at harvest. Values in table are radioactivity in (dpm × 10^{-3}).

Treatment and portion	[^{3}H]GA_{20} Fns[a] 42, 43	[^{3}H]GA_1 Fns 25–27	[^{3}H]GA_{29} Fns 19, 20	[^{3}H]GA_{81} Fns 22, 23	[^{3}H]GA_8 Fns 12–14	Total
Control						
Apical	89.2	28.1	16.1	0.3[b]	10.6	157.1
Internode below	3.15	6.3	1.94	0.20	1.89	14.4
Mature internode	1.40	6.9	0.88	1.05	3.29	15.4
TIBA						
Apical	99.8	40.7	24.6	0.46[b]	14.5	195.8
Internode below	1.73[c]	0.18	3.66	0.20	1.07	7.1
Mature internode	3.12[c]	0.73	3.95	2.50	5.3	18.6

[a] Fns, fractions.
[b] Estimates only.
[c] Includes some [^{3}H]GA_{29}-catabolite

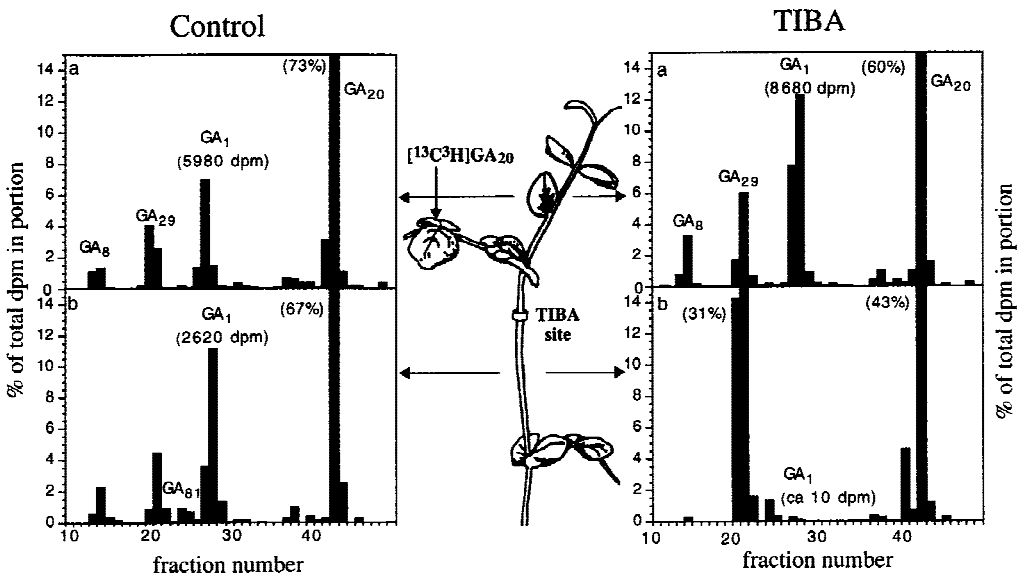

Fig. 3. Effects of TIBA on the distribution of metabolites of [^{13}C,^{3}H]GA_{20}. TIBA was applied to internode 6, and 48 h later, [^{13}C,^{3}H]GA_{20} was applied (1.5×10^5 dpm/plant) to leaf 7, which had just completed expansion. Portions were harvested after a further 24 h. *a*, apical portion (all tissue above the TIBA application site, apart from the leaflets and petiole at node 7); *b*, internode section immediately below the TIBA application site. Radioactivity in each HPLC fraction is shown as a percentage of the radioactivity recovered in that plant portion; where off-scale, this percentage is shown in *parentheses*. For each portion the level of radioactivity corresponding to [^{13}C,^{3}H]GA_1 is shown. Total recoveries of radioactivity were 41,607 dpm for TIBA apical, 61,400 dpm for control apical, 4,582 dpm for TIBA internode, and 16,380 dpm for control internode.

As with endogenous GA_8, the effects of TIBA on the level of [^{13}C,^{3}H]GA_8 below the application site were variable. For example, there was a large reduction in Fig. 3 and little change in Table 3. This might be related to the differing sites of treatment with [^{13}C,^{3}H]GA_{20} in these two experiments.

Evidence That Relatively Mature Internodes Can Respond to GA_1

Application of GA_1 to 205− (dwarf) plants whose uppermost internode was already 50% expanded strongly promoted the subsequent elongation of that internode

Table 4. Examples of mass spectra obtained to identify GAs after the application of [^{13}C, ^{3}H]GA_{20}. Metabolites were analyzed as the methyl ester trimethylsilyl ethers, and full scan mass spectra were obtained in all cases. Kovats Retention Index (KRI) values are shown, as are data from authentic standards. Spectra are from the experiment reported in Table 3, except for that of [^{13}C,^{3}H]GA_1, which is from the experiment represented in Fig. 3. Fn, fraction.

GA identified, tissue, and HPLC fractions	KRI	Characteristic ions m/z (% relative intensity of base peak)
[^{13}C,^{3}H]GA_1		
Control, internode below, Fn 28	2,673	507(M^+,100), 506(42), 492(12), 449(28), 448(31), 378(18), 377(29), 376(24), 314(19), 236(12), 208(57)
Standard [$^{2}H_2$]GA_1	2,671	508(M^+,100), 493(12), 450(31), 449(17), 379(21), 378(27), 377(19), 315(24), 237(15), 209(51)
[^{13}C,^{3}H]GA_{29}		
TIBA, apical, Fn 19	2,685	507(M^+,100), 506(49) 492(11), 478(5), 448(7), 390(8), 376(11), 304(18), 208(10).
Standard [$^{2}H_2$]GA_{29}	2,683	508(M^+,100), 493(11), 479(5), 449(7), 391(8), 377(11), 305(19), 209(21).
[^{13}C,^{3}H]GA_{81}		
TIBA, mature intern ode, Fns 22 and 23	2,674	507(M^+,89), 506(23), 492(10), 460(15), 448(18), 432(12), 417(16), 390(16), 376(52), 348(16), 304(100), 236(12), 208(44)
Standard [$^{2}H_2$]GA_{81}	2,673	508(M^+,95), 493(9), 461(16), 449(19), 433(14), 418(17), 391(16), 377(47), 348(15), 305(100), 237(10), 209(47).
[^{13}C,^{3}H]GA_8		
TIBA, apical, Fn 13	2,819	595(81), 594(100), 580(6), 536(8), 505(5), 449(21), 380(9), 376(17), 269(8), 239(11), 208(17)
Standard [$^{2}H_2$]GA_8	2,818	597(47), 596(100), 581(7), 537(10), 506(6), 450(31), 381(14), 377(14), 270(10), 240(15), 209(25)

(Fig. 4). The GA_1 appeared to act (directly or indirectly) in the internode tissue itself because decapitated plants also responded strongly (Fig. 4).

Discussion

Auxin transport inhibitors are thought to block specifically auxin transport, but the present results show that these compounds, when applied to elongating internodes of intact WT pea plants, also reduce markedly the level of endogenous GA_1 below the application site. An even more dramatic reduction was observed when the level of [^{13}C,^{3}H]GA_1 below the TIBA application site was monitored after application of [^{13}C,^{3}H]GA_{20} to a mature leaf. The reductions in endogenous GA_1 (at least sixfold) were sufficiently large to be physiologically significant because they were comparable in magnitude to the effects of certain GA biosynthesis mutations, which confer a dwarf habit (e.g. *le-3*, Ross et al. 1995). Futhermore, the internodes monitored in these experiments were not fully expanded and comparable internodes of dwarf plants responded strongly to applied GA_1 (Fig. 4).

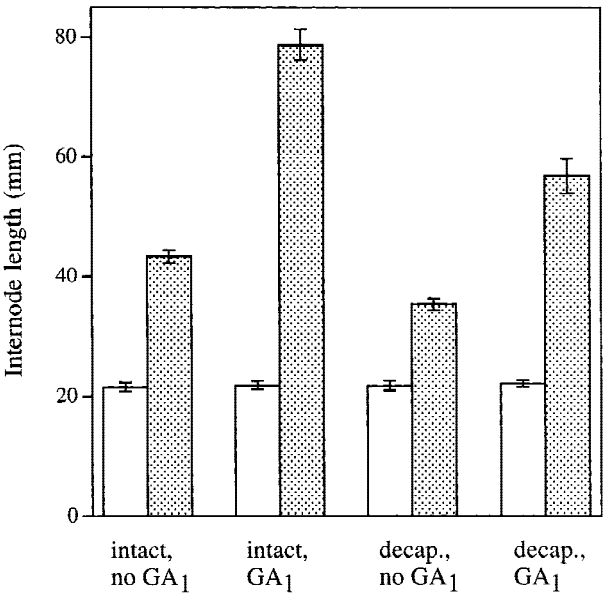

Fig. 4. Effect of GA_1 on the length of internode 6 of dwarf (205−) plants. When internode 6 was about 50% expanded, plants were decapitated or left intact before application of GA_1 (2 μg). *Clear bars*, initial length; *stippled bars*, final length. Data are means ± S.E., $n = 9$).

It is unlikely that the effects of auxin transport inhibitors on GA_1 content are the result of a direct inhibition of the step GA_{20} to GA_1, because this would not account for the accumulation of [^{13}C,^{3}H]GA_1 observed above the TIBA application site (Table 3 and Fig. 3). However, two hypotheses are suggested which might account for the observations.

The first hypothesis is that GA_1 is synthesized mainly in the expanding leaves and/or apical bud and then transported basipetally to the elongating internodes and that this transport is blocked by auxin transport inhibitors. This would explain why Sherriff et al. (1994) found that removal of the apical bud of WT plants reduced dramatically the recovery of [^{13}C,^{3}H]GA_1 (after feeds of [^{13}C,^{3}H]GA_{20}) in the remaining stem tissue; decapitation would have removed the main site of GA_1 production. This hypothesis is difficult to test directly because of problems involved in introducing labeled GA_1 into the intact apical bud. Grafting results (e.g. Reid et al. 1983) do not preclude basipetal movement of endogenous GA_1,

although they provide no evidence for acropetal movement.

The second hypothesis is that even though the conversion of GA_{20} to GA_1 occurs in internodes, it only proceeds when the level of IAA is normal. Auxin transport inhibitors (Table 1) and decapitation (Beveridge et al. 1994) both reduce the level of IAA in the stem. It may be relevant also that a related auxin, 4-chloro-IAA, is though to regulate the expression of another GA synthesis gene, a 20-oxidase, in pea pods (van Huizen et al. 1997).

In conclusion, it is clear that TIBA, HFCA, and NPA dramatically reduce the level of GA_1 below the site at which they are applied to the pea stem. This appears to result from effects on either GA_1 transport from the apical bud or on GA_{20} metabolism in the stem. Both possibilities have important implications for attempts to understand how GA_1 and IAA regulate stem elongation (e.g. Yang et al. 1996). The present results suggest that caution be exercised when attributing the developmental effects of auxin transport inhibitors entirely to changes in IAA content. The molecular basis of the effects reported here is currently under investigation utilizing our recent cloning of the GA_{20} 3β-hydroxylase gene, *LE* (Lester et al. 1997).

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