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EFFECTS OF TEMPERATURE AND GIBBERELLIC ACID ON 4-DESMETHYLSTEROL COMPOSITION OF *AVENA SATIVA* STEM SEGMENTS

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Key Word Index—*Avena sativa*; Gramineae; oat; stem segments; temperature; phospholipids; fatty acids; 4-desmethylsterols; gibberellic acid.

Abstract—Of the three morphological subunits of *Avena sativa* stem segments (node, leaf-sheath and internode) examined, internodes constituted the richest source of phospholipids and sterols, yielding almost double the concentration of lipid found in the leaf-sheath. The phospholipid compositions of nodes and internodes were similar, comprising mostly phosphatidylcholine (PC) and phosphatidylethanolamine (PE), with linoleic, linolenic and palmitic acids as the predominant component fatty acids. Leaf-sheath tissue contained mainly PE, with equally high amounts of palmitic, linoleic and linolenic acids. β-Sitosterol and cholesterol were the major 4-desmethylsterols of the internode, while β-sitosterol was predominant in the node and leaf-sheath tissues. The growth temperature of segments prior to isolation produced its major effect on the concentration of stigmasterol, which decreased markedly with temperature. The sitosterol/stigmasterol ratio increased significantly as temperature decreased. Stem segments isolated from plants treated with gibberellic acid (GA_3) for 3 weeks, showed a significant reduction in the amounts of 4-desmethyl sterols on a dry wt basis when compared with control segments. However, when stem segments were incubated with GA_3 for 20 hr, no change in 4-desmethylsterol composition or concentration was observed, even though significant growth in response to GA_3 occurred.

INTRODUCTION

Avena sativa stem segments have been used extensively in studies of rapid growth responses to gibberellic acid (GA_3) [1–4]. These excised stem segments are known to be sensitive and specific in their response to exogenous gibberellin, especially GA_1 and GA_3 [5]. Segments consist of three distinct morphological units; the elongating internodal tissue, the surrounding leaf-sheath and the basal node-joint region [4]. Although the node and leaf-sheath portions do not grow in response to hormone treatment they are required for maximal growth of the internode [4]. Because of the differences in response of the node, sheath and internode to GA_3 [4], it is important to analyse any structural or biochemical differences between these units which may lead to an elucidation of the factors involved in the GA_3 response. Membranes have been implicated in the early mechanism or mode of GA_3 action [6–8] and the first part of this investigation aimed to isolate, separate and identify the major phospholipids, their component fatty acids, and sterols of the node, leaf-sheath and internode of *Avena* stem segments. It was anticipated that this analysis would determine where the majority of the lipid was to be found and establish whether the lipid compositions of these tissues differed significantly from each other.

The second part of this study was concerned with the way in which the 4-desmethylsterol content of the *Avena* stem segment responded to treatments, such as temperature and GA_3, which influence the growth of the segments. Observations of a dependence of growth [9, 10] and induction of flowering in certain plants [11–14] on sterol biosynthesis suggest that it may be a rate-limiting step in some plant growth and developmental patterns. This possibility is supported by the fact that tissues undergoing rapid growth and development contain more sterols than older, non-growing differentiated tissue [15].

It seems clear that growth temperature can influence the sterol composition of plants [16], yeast [17, 18], phytoflagellates [19], etc. Some workers have also reported effects of hormones on the sterol composition of plants [20–24]. The variation in the sterol composition of different types of membranes [25] may have important consequences for their physiological properties [26], and a possible mechanism of hormone action could involve alteration of sterol composition.

The results of the present investigation indicate that GA_3 can induce growth of oat stem segments without a concomitant change in 4-desmethylsterol composition.

RESULTS

Distribution of phospholipids, fatty acids and sterols between node, internode and leaf-sheath

The results indicated that internodes were the richest source of phospholipids (Fig. 1). The total phospholipid contents of internodes, nodes and sheaths were 21.5, 7.6

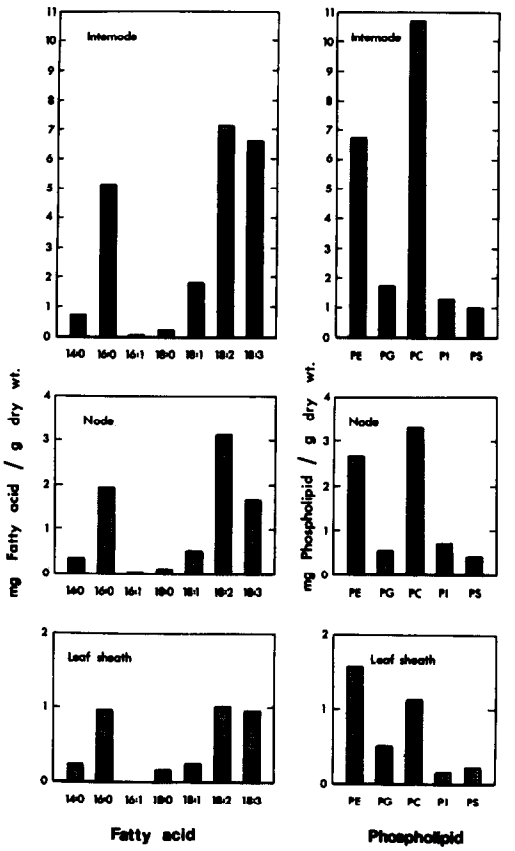

Fig. 1. Distribution of fatty acids and phospholipids in the internode, node and leaf-sheath of *Avena* stem segments.

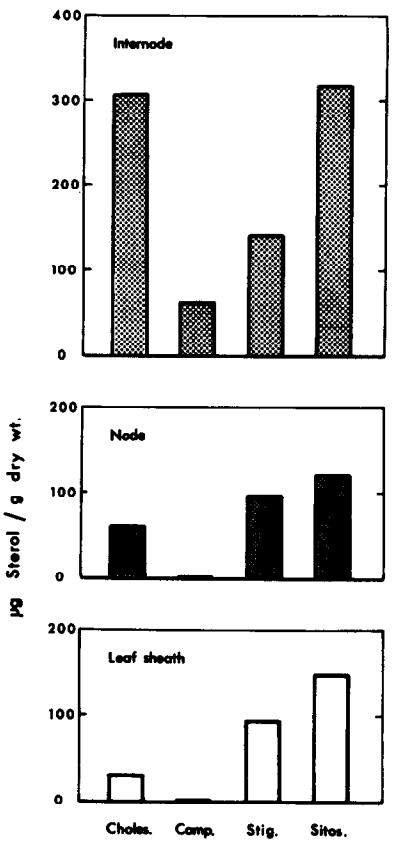

Fig. 2. Distribution of 4-desmethylsterols in the internode, node and leaf-sheath of *Avena* stem segments. Choles. = cholesterol, Camp. = campesterol, Stig. = stigmasterol, Sitos. = sitosterol.

and 3.5 mg/g dry wt, respectively. Similarly, internodes were the richest source of sterols (Fig. 2). The total 4-desmethylsterol contents of internodes, nodes and sheaths were 0.83, 0.28 and 0.27 mg/g dry wt, respectively.

Although the sheath made the greatest dry wt contribution to the segment, and the internode the least, the internode contained almost double the amount of lipid found in the sheath (Table 1). While *ca* 20% of the total lipid in the node and sheath consisted of phospholipid, the internode contained *ca* 30% phospholipid. Total 4-desmethylsterols in each tissue did not vary markedly, constituting *ca* 1% of total lipids in the node and internode, and *ca* 1.5% of total lipids in the sheath.

The phospholipid composition of nodes and internodes were similar (Fig. 1). The most abundant phospholipids were phosphatidylcholine (PC) and phosphatidylethanolamine (PE). The major fatty acid was linoleic acid (18:2), although these tissues also contained large amounts of linolenic acid (18:3) and palmitic acid (16:0). Leaf-sheath tissue, on the other hand, was richest in PE, and contained almost equal amounts of 16:0, 18:2 and 18:3.

β-Sitosterol and cholesterol were the major sterols of

Table 1. Weights of node, sheath and internode of *Avena* stem segments and total lipid extracted from each

Tissue	Fr. wt per segment (mg)	Dry wt per segment (mg)	Total lipid per g dry wt (mg)	Total lipid per segment (μg)	Total phospholipid per segment (μg)	Total 4-desmethyl-sterol per segment (μg)
Node	17.7	2.9	35.0	100.2	21.9	0.8
Sheath	19.2	3.2	18.1	58.1	11.5	0.9
Internode	16.5	1.4	74.5	104.0	30.2	1.2

Table 2. *F*-values for the effects of growth temperature and *in vivo* GA_3 treatment on sterol composition of *Avena* stem segments

Sterol	Source of variation		
	Temperature	GA_3	Temperature × GA_3
Cholesterol	12.5**	9.3*	1.7
Campesterol	4.8	21.8**	10.7*
Stigmasterol	9.4*	13.5*	3.3
Sitosterol	1.6	31.4**	6.3*
Total 4-desmethylsterols	3.0	23.7**	5.3*

Significance levels: $*P = 0.05$, $**P = 0.01$.

the internode, occurring in *ca* equal amounts (Fig. 2). β-Sitosterol was the most abundant sterol in the node and sheath, but in these tissues the amount of cholesterol was only 50 and 20% of that of sitosterol in the node and sheath, respectively. Campesterol was found predominantly in the internode, while only trace amounts were detected in the node and sheath.

Application of GA_3 to plants before segment excision (long term)

A. sativa plants grown at 10, 20 or 30° were treated with GA_3 for 3 weeks, (p − 1) stem segments were harvested, total lipids extracted and sterols analysed. The most significant effects of growth temperature were on the amounts of cholesterol and stigmasterol (Table 2). β-Sitosterol was the major sterol, followed by stigmasterol, cholesterol and campesterol. Both the weight and proportion of stigmasterol decreased as temperature decreased (Figs. 3A and 4). Cholesterol, on the other hand, was maximal at 20°, and significantly lower at both 10 and 30°. Sterol content of each segment followed the pattern of dry wt. As temperature decreased, so dry wt increased and total sterol per segment increased (Fig. 3B). The increase at 10° was largely attributable to the increase in β-sitosterol. The ratio of sitosterol/stigmasterol increased almost linearly as growth temperature was lowered from 30 to 10° (Fig. 5A). While segments grown at 30° contained *ca* equal proportions of stigmasterol and sitosterol, 10° segments contained 18 and 57%, respectively (Fig. 4).

GA_3 pre-treatment of plants produced a significant reduction in the concentration of 4-desmethylsterol (Fig. 3A; Table 2). Segments from plants kept at 20° had the highest sterol content on a dry wt basis, while plants grown at 10 or 30° generally had smaller amounts in their segments. Plants treated with GA_3 did not display this pattern, and total 4-desmethylsterols increased only slightly between 10 and 20°, while 30° segments contained

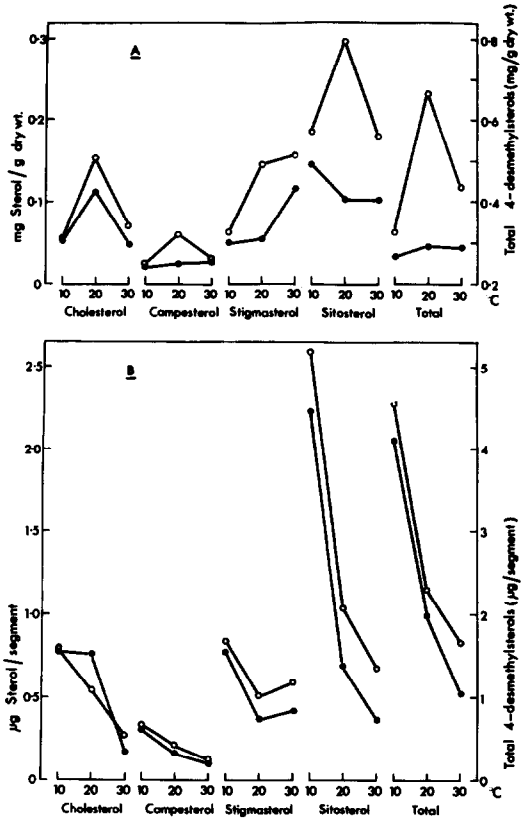

Fig. 3. Effect of growth temperature and *in vivo* GA_3 treatment on sterol composition of *Avena* stem segments. A, Sterol on dry wt basis. B, Sterol per segment. ○ = control; ● = 100 µg GA_3/plant.

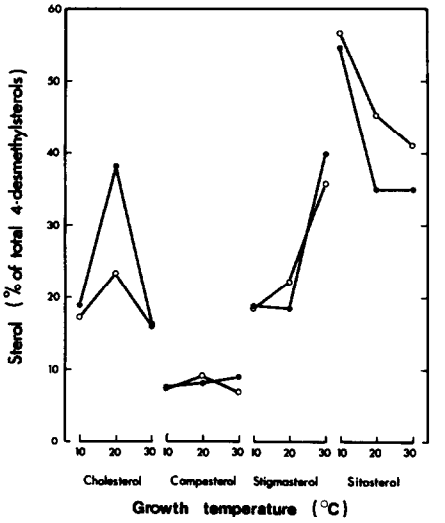

Fig. 4. Percentage (by wt) of 4-desmethylsterols in segments from *Avena* plants grown at different temperatures and treated with GA_3. ○ = control; ● = 100 µg GA_3/plant.

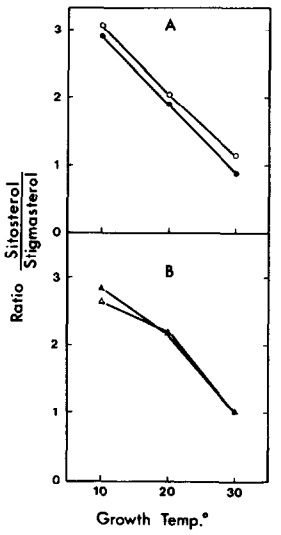

Fig. 5. Sitosterol: stigmasterol ratio of segments from *Avena* plants grown at different temperatures. A, Plants treated with GA_3. B, Excised stem segments treated with GA_3. ○ = control; ● = 100 μg GA_3/plant; △ = segments incubated for 20 hr with 0.1 M sucrose; ▲ = segments incubated for 20 hr with 0.1 M sucrose + 10^{-6}M GA_3.

the same concentration of sterol as 20° segments. GA_3 treatment significantly decreased the amounts of all sterols (Fig. 3A), and a small but consistent decrease in the sitosterol/stigmasterol ratio was also observed at each growth temperature (Fig. 5A). On a per segment basis, GA_3 also lowered the sterol content (Fig. 3B). This was evident at all three growth temperatures and for each of the sterols from 20°-grown plants except cholesterol.

GA_3 increased the amount of cholesterol and decreased the amount of β-sitosterol as a percentage of total 4-desmethylsterols. In contrast, the relative contents of campesterol and stigmasterol were little affected (Fig. 4). Once again the largest differences were evident in segments from 20°-grown plants.

Application of GA_3 to segments after excision (short term)

In this experiment, (p − 1) internode segments from plants grown at 10, 20 or 30° were incubated in solutions of 10^{-6} M GA_3 in 0.1 M sucrose for 20 hr prior to extraction and analysis of sterols. Over this period, GA_3-treated segments produced significantly more growth than control segments (Table 3). Segments from 20°-grown plants demonstrated a 100% stimulation of growth due to GA_3, 30° segments a 73% increase, while 10° segments exhibited a 47% increase due to GA_3 treatment.

Growth temperature had a significant effect on the sterol composition of segments (Table 4). Both the absolute amount and the percentage of stigmasterol increased markedly as growth temperature increased, while the proportion of β-sitosterol decreased, resulting in an overall decrease in the sitosterol/stigmasterol ratio (Fig. 5B). Segments from 20 and 30° plants had double the weight of campesterol found in 10° segments while cholesterol was not significantly affected by growth temperature. Total 4-desmethylsterols increased on a dry wt basis as temperature increased. On a segment basis, as temperature increased and dry wt of segments decreased, so sterol content decreased (Fig. 6).

GA_3 treatment, on the other hand, produced no significant differences in the composition of 4-desmethylsterols (Table 4; Figs. 5B, 6, 7), even though significant growth of the internode in response to GA_3 occurred over this treatment period (Table 3). A consistent effect was seen with segments grown at 10°, in which GA_3 increased the concentration of individual sterols, both on a dry wt and on a segment basis. The percentage distribution of sterols, however, remained unchanged.

DISCUSSION

In many plant tissues, the most abundant phospholipids are PC and PE, and the most abundant fatty acids are the C_{18}-unsaturated and the C_{16}-saturated acids [27–31]. These relationships were also evident in *Avena* stem segments used in the present investigation. The major sterol components of most plant tissues are β-sitosterol and stigmasterol [15, 32–34], and they were the major ones in the present tissue system. However, the large cholesterol content of internodal tissue (Fig. 2) is unusual for most plant tissues. In *Triticum aestivum* [16] and *Hordeum vulgare* shoot tissue [32], cholesterol accounted for less than 1% of the total sterol extracted. Similarly in tobacco leaves [34, 35], mung bean seedlings [15] and *Phaseolus vulgaris* leaves [36], cholesterol was the least abundant 4-desmethylsterol, accounting for no

Table 3. Growth of segments from *Avena* plants grown at different temperatures

Plant growth temperature	Segment growth after 20 hr (mm)		L.S.D. ($P = 0.05$)
	Control	+GA	
10°	3.0	4.4	0.2
20°	2.8	5.6	0.3
30°	2.6	4.5	0.4

Segments were incubated in flasks containing 0.1 M sucrose + 10^{-6} M GA_3 or 0.1 M sucrose alone, and length of internode was measured after 20 hr. Solutions were continuously bubbled with O_2. Incubation temperatures were 20° for segments from 10 and 20° plants, and 30° for segments from 30° plants.

Table 4. *F*-values for the effects of growth temperature and GA_3 (short-term treatment) on sterol composition of excised *Avena* stem segments

Sterol	Source of variation		
	Temperature	GA_3	Temperature × GA_3
Cholesterol	0.1	2.1	1.0
Campesterol	22.9**	0.3	1.3
Stigmasterol	36.5***	1.0	2.1
Sitosterol	9.9*	0.7	3.2
Total 4-desmethylsterols	17.4**	0.4	2.5

Significance levels: * $P = 0.05$, ** $P = 0.01$, *** $P = 0.001$.

more than *ca* 12% of the sterol fraction. Thus, by comparison, *Avena* stem segment tissue (particularly the internode) was a relatively rich source of cholesterol. Campesterol in this tissue was the least abundant of the 4-desmethylsterols.

Of the three types of tissue analysed, the internode provided the major portion of the lipid. It was difficult to demonstrate (nor was it the intention of this report to do so) whether the extracted lipid was predominantly membrane or storage lipid. However, storage lipid consists mainly of triacylglycerols and neutral lipids [37], while phospholipids [38] and 4-desmethylsterols [34] are more important as membrane components than as storage products. In *Avena* root tips, 38.2% of the total membrane lipid consisted of phospholipid [39]. Thus, in the present tissue system one would expect all the phospholipid extracted to be membranous. Neutral lipids, glycolipids, steryl esters and glycosides are storage forms of lipid, and possibly make up the remainder of the lipid extract.

Alteration of sterol concentrations in plant tissue by growth temperature has been reported before [16]. The major effect observed is a decrease in stigmasterol and an increase in β-sitosterol as temperature was decreased.

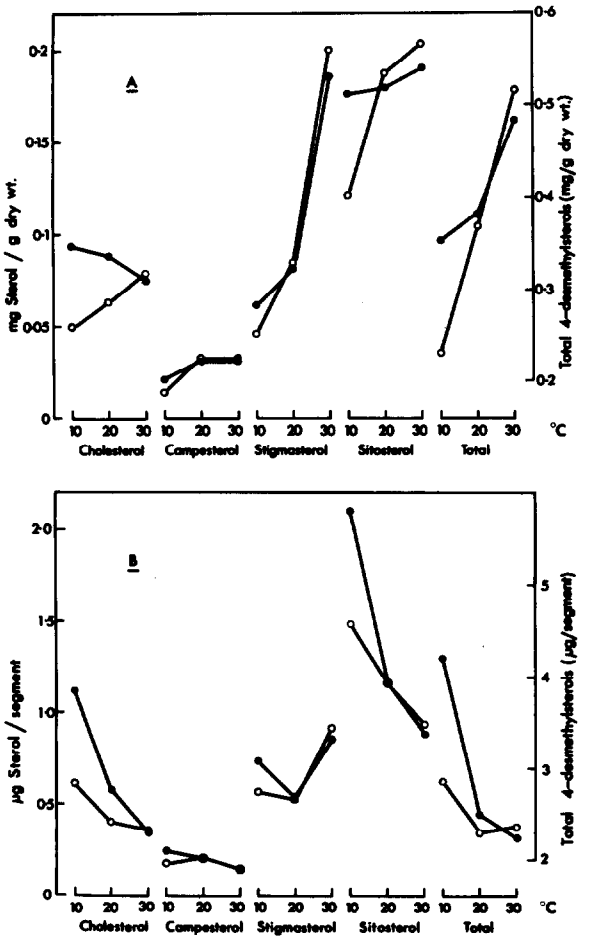

Fig. 6. Effect of growth temperature and GA_3 treatment of excised *Avena* stem segments on sterol composition of segments. A, Sterol on dry wt basis. B, Sterol per segment. ○ = segments incubated for 20 hr with 0.1 M sucrose; ● = segments incubated for 20 hr with 0.1 M sucrose + 10^{-6} M GA_3.

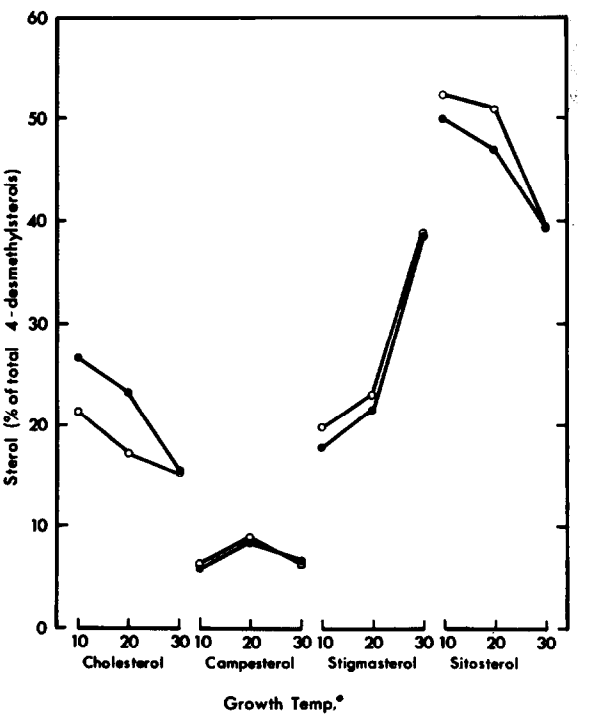

Fig. 7. Sterols (as percentage of total 4-desmethylsterols) in *Avena* stem segments treated with GA_3. ○ = segments incubated for 20 hr with 0.1 M sucrose; ● = segments incubated for 20 hr with 0.1 M sucrose + 10^{-6} M GA_3.

Since stigmasterol has been shown to be a dehydrogenation product of sitosterol [40, 41], the decrease in stigmasterol and the increase in sitosterol may be indicative of the biosynthetic relationship of these sterols. The sitosterol/stigmasterol ratio has also been found to change during germination [42], aging of tissue [15] and in response to growth hormone application [23]. In the present experiments, short-term treatment of *Avena* stem segments with GA_3 did not significantly alter this ratio, although the effect of long-term treatment, or of temperature, was significant.

GA_3 pre-treatment of the intact *Avena* plant for 3 weeks significantly affected 4-desmethylsterol composition of stem segments at each of the three growth temperatures studied. At all temperatures, GA_3 also markedly stimulated internodal elongation well above that of control plants.

In summary, it was found that long-term (3 weeks) treatment of *Avena* plants with GA_3 produced marked effects on 4-desmethylsterol parameters, while short-term treatment (20 hr) of stem segments, although promoting growth significantly, left the 4-desmethylsterol composition essentially unaltered.

EXPERIMENTAL

A. sativa cv Avon seeds were germinated in Petri dishes and grown (10 per pot) in black plastic pots containing a peat/sand/mineral nutrients mixture, in controlled environment cabinets at either 10, 20 or 30° with a photon flux density of 500 μE/m^2/sec and a photoperiod of 20 hr.

Preparation of segments for node/internode/leaf-sheath study. Stem segments from 20°-grown plants were harvested when plants were 30–33 days old, at which time the internode immediately below the peduncular node [(p − 1) internode] was *ca* 2.0 ± 0.5 cm long. One cm segments were cut with a 2-bladed cutting device so as to include the node immediately below the peduncular node [(p − 1) node], and the (p − 1) internode together with its surrounding leaf-sheath [43]. Each segment was divided into node,* sheath and internode tissues by cutting transversely through the segment immediately above the joint, and sliding the internode out from the leaf-sheath with the aid of a needle. The node portion therefore contained a very small amount of internodal tissue, primarily meristematic, enclosed by the encircling joint. A total of 253 segments was dissected in this way, resulting in 4 g each of node and sheath tissue, and 3.5 g of internode tissue for lipid extraction. This expt was not replicated.

Preparation of segments for temperature/GA_3 studies. In expts involving long-term GA_3 treatment of *Avena* plants, GA_3 (100 μg per plant) was applied in a 20 μl drop to a small needle scratch at the base of the uppermost fully expanded leaf. Treatments began *ca* 3 weeks before harvest of stem segments, and continued at 6-day intervals. Stem segments from 10, 20 and 30°-grown plants were harvested at *ca* 65–70 days, 30–33 days, and 23–25 days respectively, at which stage the (p − 1) internode was *ca* 2.0 ± 0.5 cm long. After weighing, segments were frozen in liquid N_2, prior to lipid extraction.

In the second set of expts, involving short-term treatment of stem segments with GA_3, segments from untreated plants grown at 10, 20 or 30° were harvested as before, and were then incubated in 500 ml flasks containing either 200 ml 0.1 M sucrose, or 200 ml 10^{-6} M GA_3 in 0.1 M sucrose soln, continuously bubbled with industrial grade O_2. Temp. control was achieved by immersing the lower half of the flask in a H_2O bath at the required temp. (± 1°). Segments from 20 and 30° plants were incubated at 20 and 30° respectively, while segments from 10° plants were incubated at 20°. The 20 hr incubation was carried out under diffuse room light. Since the leaf-sheath portion and the node did not grow in response to GA_3 [4], growth was measured from the top of the leaf sheath to the top of the internode, i.e. not including the original 1 cm segment length. Total lipids were then extracted from these segments.

Lipid extraction. Total lipids from node, internode, leaf-sheath or total segment tissues were extracted using the method of ref. [44]. Half of the final extract was used for phospholipid/fatty acid analysis, the remainder for sterol analysis.

TLC of phospholipids. All prep. TLC was performed on glass plates coated with a 0.3 mm thick layer of Si gel G. The 5 major classes of phospholipids [phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylserine (PS)] were separated from the total lipid extract by developing plates in $CHCl_3$–MeOH–H_2O (14:6:1) [45, 46]. Plates were dried and bands visualized by spraying with Rhodamine 6G (0.005% in H_2O) and viewing under UV light. Phospholipid classes were identified by co-chromatography with authentic standards, and by comparison of R_f values with documented values for the same solvent system [46].

TLC of sterols. Total lipid extract TLC plates were developed twice in CH_2Cl_2–Et_2O (24:1), allowing the plates to dry under N_2 between developments. Sterols were separated into 3 major bands: the 4-desmethyl-, 4-methyl- and 4,4′-dimethylsterols. These bands were identified using cholesterol (4-desmethylsterol) and β-amyrin or lanosterol (4,4′-dimethylsterols) as standards for the outer two bands. Plates were sprayed with 0.05% (w/v) berberine HCl in MeOH, and viewed under UV light.

GC of fatty acids. Fatty acid Me esters were prepared by scraping the relevant phospholipid band into a glass vial containing 5 ml 5% H_2SO_4 in MeOH. Me heptadecanoate was added as int. standard and vials were sealed and heated for 3 hr at 70° with periodic agitation. Fatty acid Me esters were extracted by adding H_2O (5 ml) and 5 ml petrol (40–70°) and shaking vigorously. The upper petrol layer was removed to another vial, H_2O (2 ml) was added and again shaken vigorously. The petrol layer was removed and dried (1–2 g, Na_2SO_4). The petrol was decanted and Me esters were analysed using a GC with dual FID detectors. Glass columns (1.8 m × 3.2 mm) were silanized by rinsing × 3 with 3% (v/v) HMDS in C_6H_6, and heating to 60° for 5 min between rinses. Columns were packed with 15% DEGS Operating temps were: column 175°, detector 220°, injection port 240°, outlet 240°. Flow rates for N_2 carrier gas, H_2 and air were 50, 60 and 600 ml/min, respectively.

Fatty acid Me esters were identified by comparing their R_t with those of authentic compounds and published values obtained under similar conditions [47], as well as by their ECL values [48].

GC–MS of sterol. The 4-desmethylsterol band (containing cholesterol, campesterol, stigmasterol and β-sitosterol) was scraped from the TLC plate into a funnel containing Whatman No. 1 filter paper. Sterols were extracted from the Si gel with several washes of Et_2O (2 + 2 + 1 + 1 ml). The Et_2O was evapd and the residue redissolved in 50 μl MeCN, 50 μl *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA), and 1 drop TMCS reagent. Cholestane was added as int. standard and the mixture heated at 60° for 1 hr, then taken to dryness. TMSi ethers were

* The term (node) will be used to include both the node and the joint tissues [4].

redissolved in BSTFA and separated by GC on a prepacked glass column (2m × 2mm) containing 1% OV-101. The operating temps were column 260°, FID 300° and injection port 290° Carrier gas (N_2) flow rate was 25 ml/min. Peaks were tentatively identified by comparison of R_t values with those of authentic sterol TMSi ethers. Mass measurements of each sterol peak were determined using MS interfaced to the GC via a single stage jet separator and operated at 30 eV. Mass measurements always agreed with calculated mass numbers for each TMSi ether [49], providing positive identification. Sterol data were subjected to analysis of variance to test the significance of the temp., GA_3 and interaction effects. These expts were replicated × 3, and the data presented are the means of the 3 replicates.

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