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The Acquisition of Gravisensitivity During the Development of Nodes of *Avena fatua*

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Abstract. Acquisition of gravisensitivity in the uppermost nodes of flowering stems of *Avena fatua* occurs during the period when the internode is extending from 30 to 100 mm. Within individual leaf sheath bases this event correlates with the development of 14–16 statocytes (cells containing sedimentable statoliths) in lateral association with each vascular bundle. This further correlates with the development of an ability by the leaf sheath base to control both the rate of transport of applied ^{3}H-IAA and the level of endogenous IAA in response to the gravity vector. Estimates of the level of endogenous IAA in pooled extracts were similar using either HPLC with coulometric detection or GC-MS measurement of the molecular ion.

Gravitropic growth of the flowering stems of grasses is localized in the nodal regions (Maeda 1958, Arslan and Bennet-Clark 1960, Bridges and Wilkins 1973, Dayanandan et al. 1976, Wright and Osborne 1977). It results from cell elongation of the leaf sheath base (Dayanandan et al. 1976, Wright and Osborne 1977), where it is accompanied both by changes in the level (Wright et al. 1978) and polarity of transport (Wright 1982) of endogenously produced auxin. These changes have been considered as an integral part of the gravitropic mechanism which restores normal orientation (Wright 1981), the auxin-promoting cell elongation, and the reduced polarity in accord with a reduced transport away from the region of elongation.

In *Avena fatua*, the uppermost node of the flowering stem acquires competence for gravitropic growth around the time panicle emergence begins. For about 1 week immediately prior to this, although nodes cannot grow in re-

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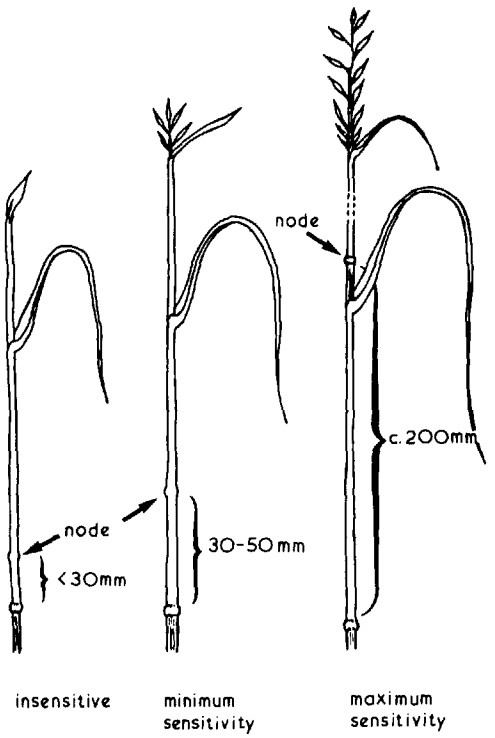

Fig. 1. Illustration of *Avena fatua* indicating the relationship between gravisensitivity and development of the uppermost node.

sponse to gravity, they are similar in size and appearance and may be compared with those that can. The stages of development spanning this period of acquisition of gravisensitivity are now described; they form the basis for the selection of nodes with a range of gravisensitivities which in turn can be quantified from the bending response. In the experiments reported here, nodes of known gravisensitivity have been excised immediately after bending and their endogenous IAA levels estimated by an HPLC coulometric detection method (Wright and Doherty 1985), the validity of this method being checked by GC-MS using deuterated IAA as an internal standard. In parallel experiments, nodes of similar gravisensitivities were used for measurement of auxin transport (during a further period of gravistimulation) and assessment of statocyte development. The results obtained are discussed in relation to the ontogeny of the graviperception mechanism.

Methods and Materials

Plants of *A. fatua* were grown as described previously (Wright 1981). Flowering stems were selected in which the panicles either had not quite emerged or were in the process of emerging from the leaf sheath (Fig. 1). The sheath of the second leaf immediately below the flag leaf was removed. This exposed the uppermost node which was cut out in a nodal segment (Wright and Osborne

1977) with 3 cm of leaf sheath plus peduncle above it and 3 cm of stem below it. These segments were supported with their basal ends in Oasis (hydrophilic phenolic foamed resin, available from florists) soaked in distilled water and placed horizontally at 25°C in the dark. Excess water loss was prevented by enclosure in clear plastic boxes. After 24 h the angle through which the nodes had bent was measured with a protractor. The nodes were then used for one of the following: (1) examination of statocyte tissue; (2) transport of ^{3}H-IAA; or (3) measurement of endogenous free IAA.

Examination of Statocyte Tissue

Transverse sections of nodes (~2 cells thick) were cut freehand while maintaining the segments in the horizontal position. The sections were immediately immersed in I_2KI (4 g/l) containing 20% ethanol. Statocytes were identified as those cells having sedimented starch grains; they occurred in groups associated with the vascular bundles as previously reported (Wright and Osborne 1977, Dayanandan et al. 1976, Parker 1979). For each node the number of statocytes per bundle in each of three sections was measured. As there are approximately 30 bundles per node, a mean value was calculated based on counts for ~90 bundles.

Transport of ^{3}H-IAA

The nodal segments were placed vertically for a period of 1 h to allow for adjusting of endogenous auxin transport. The nodes were then excised, dissected, and used in the experimental system as described previously (Wright 1982). In the work described here two of the four leaf sheath base segments from each node were used for transport in the normal upright position and the other two for transport in the inverted position (also vertically oriented but with the apical end downward). In each of the two positions one segment was used for the acropetal and one for basipetal transport.

As before (Wright 1982) donor disks were prepared using 1 µl of 0.1 µM 5(n)-^{3}H-indole-3-acetic acid, specific activity 29 Ci/mM (CEA, Gif-sur-Yvette, France). However, some breakdown of the ^{3}H-IAA had occurred during storage, and it was purified as follows. The original solution was evaporated at 40°C under N_2 from ~400 µl to 50 µl. Two hundred microliters of 0.05 M phosphate buffer pH 8.5 was added and the volume reduced to 200 µl by further evaporation. This was made up to 3 ml in the same buffer and washed with 3 × 5 ml aliquots of redistilled ether. The pH was then adjusted to 3.0 using 3 M H_3PO_4 and the solution extracted with 3 × 5 ml aliquots of redistilled ether. These extracts were pooled and evaporated to dryness under N_2, and the residue was made up in 400 µl of 50% aqueous redistilled methanol.

Receiver disks were removed from the segments after 3 h and placed in miniature scintillation vials (1 disk/vial), each containing 0.3 ml of methanol. After 1 h at room temperature, 2.0 ml of K1-354 scintillant (Koch-Light Labs Ltd.) was added and radioactivity was measured with an efficiency of 45% in an LKB Rackbeta scintillation counter. As previously (Wright 1982), this was taken to be indicative of the ^{3}H-IAA content of the receiver disks.

Estimation of Endogenous IAA

Preparation of Plant Material

Immediately following measurement of bending, the nodes were excised and bisected longitudinally. The resulting two leaf sheath base segments (as described in Wright 1981) corresponding to their previous upper and lower positions were put into liquid N_2 then stored at −20°C. Sampling was repeated over a number of weeks until 50–60 upper plus 50–60 lower derived segments had been accumulated at each of the selected gravisensitivity ranges for each batch of plants (see Results).

Procedure for IAA Estimation

The methods for IAA extraction, sample preparation, and estimation by HPLC are as reported (Wright and Doherty 1985); an outline description is presented here.

Extraction and sample preparation. Half nodes were ground in liquid N_2 and extracted overnight in 20 ml of methanol at −20°C. Five milliliters of 0.1 M phosphate buffer pH 9.0 containing 200 g/l of sodium diethyldithiocarbamate (DDC) was added, and the mixture shaken and then left for 1 h at −20°C. Following centrifugation at 10,000*g* for 30 min the supernatant was reduced to 4 ml by rotary evaporation at 35°C and partitioned against 3 × 5 ml aliquots of redistilled ether containing 1 mg/10 ml of butylated hydroxytoluene (BHT). The remaining aqueous fraction was acidified to pH 2.5–3 with 1 M HCl then loaded onto a C^{18} Sep-Pak cartridge together with 5 ml of 0.01 M phosphate buffer containing 200 mg/l of DDC. The cartridge was eluted with 5 ml of the same phosphate buffer containing 10% methanol followed by 3 ml of the phosphate buffer containing 50% methanol. The latter 3 ml was reduced to ~1.5 ml by rotary evaporation at 35°C then extracted with 3 × 1 ml aliquots of redistilled ether containing BHT (1 mg/10 ml). The ether extracts were pooled and the aqueous components removed by freezing; the ether was evaporated to a residue under N_2, made up in 50 μl of methanol, and stored at −20°C.

HPLC. Prior to HPLC, the extracts were evaporated to 20 μl, 80 μl of 10 mM tetrabutylammonium phosphate (TBAP) pH 6.5 was added, and the resulting 100-μl mixture was filtered using a BAS centrifugal filter (0.2 μm). The sample was run on a linear gradient from 80% 10 mM TBAP pH 6.5/20% methanol (V:V) to 100% methanol over 2 h at 0.1 ml/min using a 25-cm Spherisorb ODS2 column. IAA was detected at ~108 min using a Kratos FS.970 fluorimeter (excitation 280 nm, emission 375 nm band pass) and collected in a fraction of ~100 μl. This fraction was rerun isocratically in 60% 10 mM TBAP pH 6.5/40% methanol (V:V), its composition having been adjusted to that of the eluent. A 25-cm Spherisorb ODS2 column was again used, and IAA was detected at 9–10 min using a model 5100A Coulochem electrochemical detector

fitted with a model 5020 guard cell (potential +0.75 V) and model 5011 dual electrode analytical cell (potentials +0.45 V and +0.65 V).

GC-MS analysis. The 100-μl fractions from the initial gradient HPLC were used as the starting point for the GC-MS analysis. Ten-microliter aliquots from each of two sets of eight pooled fractions were made up to 50 μl with 28 μl of 10 mM TBAP (pH 6.5) and 12 μl of methanol. The IAA levels were then estimated electrochemically. Equal levels of (2,4,5,6,7-$^2H_5$)IAA (Merk, Sharp and Dohme, Canada) were added to the remainder of the fractions. These were reduced to their aqueous components by evaporation under N_2, made up to 1.5 ml in 40 mM acetic acid, and partitioned against 3 × 1 ml aliquots of redistilled ether. The ether fractions were frozen in liquid N_2 to remove their aqueous components (Wright and Doherty 1985), evaporated to residues under N_2, then made up in 50-μl aliquots of methanol.

The samples were converted to the methyl ester trimethylsilyl derivatives (Me TMS) as follows: methylation was by the dropwise addition of diazomethane in ether and standing for 10 min at room temperature. Following evaporation to dryness under N_2, transfer in methanol to glass ampuls, and drying *in vacuo*, trimethylsilylation was by the addition of N-methyl-N-trimethylsilyltrifluoroacetamide (4 μl), sealing the ampuls and heating at 90°C for 30 min. GC-MS analysis was performed using a Kratos MS80 RFA double-focusing mass spectrometer combined with a Carlo-Erba MFC500 gas chromatograph. One-microliter aliquots were injected into a 25 m × 0.2 mm WCOT BP-1 quartz silica capillary column (SGE) by the Grob splitless injection method. The GC oven was maintained at 50°C for 1 min, then programmed at 15°C min^{-1} to 180°C and then at 4°C min^{-1} to 250°C. Helium inlet pressure was 0.8 bar; the inlet split of 50:1 was opened after 0.5 min. Mass spectrometer conditions were as follows: electron impact-positive ion mass spectra were acquired from 600-50 amu at 1 s per mass decade, source temperature 200°C, electron energy 60 eV, and emission current 1.3 mA.

Results

Morphology of Flowering Stems and Development of Statocytes

The morphological parameter that most consistently could be related to development of gravisensitivity was found to be the distance between the uppermost and second nodes. As shown (Figs. 1, 2), during the insensitive and early sensitive stages the first node is concealed in the second leaf sheath. The quantitative relationship between internode length and gravisensitivity as judged by the bending response is illustrated in Fig. 2. The relationship is approximately linear between 100 and 200 mm. However, only a certain proportion of nodes from plants of internodes less than 100 mm long will bend: those of 30 mm, where only 3% of nodes bend, are generally considered insensitive (Fig. 1). It was therefore necessary to measure the bending ability of nodes prior to further experimentation where this was taken as the primary criterion for gravisensitivity, internode length being used as an initial selection criterion. Stato-

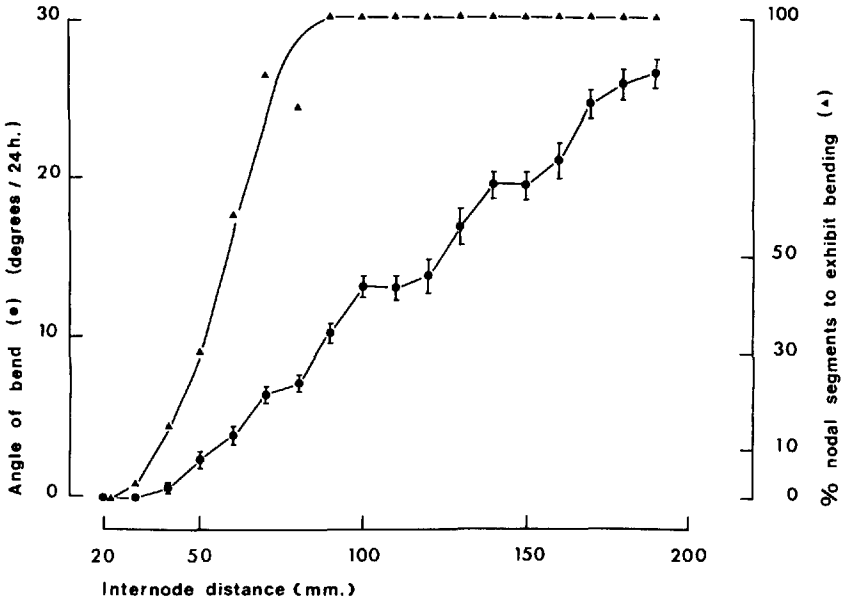

Fig. 2. The relationship of gravisensitivity (bending angle) to the uppermost internodal distance. Bars indicate standard errors, the data shown being derived from measurements made on 660 nodes.

cytes were present in small numbers some considerable time before nodes developed the ability to bend (Fig. 3). However, only when 15 or more statocytes were associated laterally with each bundle did bending occur. As this result was consistently found in nonuniform greenhouse grown plants over a period of 3 months, it would suggest that 14–16 statocytes represent a true threshold level for the gravitropic response. Following the onset of gravitropic sensitivity the ability of nodes to bend could be related to the increasing number of statocytes.

Transport of ^{3}H-IAA

Segments of leaf sheath base excised from gravisensitive nodes had a predominantly basipetal mode of auxin transport (Table 1). The absence of a correlation between the previous bending experience for angles of less than 20° and the degree of polarity of segments in the normal, upright position confirms previous findings that changes in transport are reversible (Wright 1982). This suggests that the 1-h period following the quantification of gravisensitivity by bending is sufficient for reestablishment of normal basipetal polarity. Thus the reversal of polarity to a predominantly acropetal mode (Table 1) can be attributed to the inversion of the excised nodal segments as found previously (Wright 1981, 1982). The reduced polarity of segments from nodes that had bent more than 20° could, however, be a residual effect of the bending experience. With this proviso, it appeared that the degree of reversal was greatest in

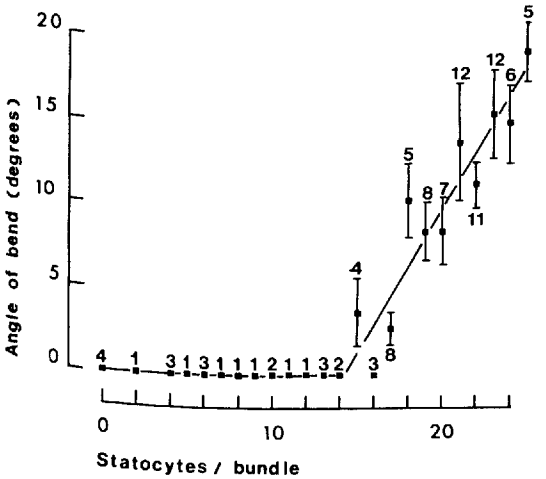

Fig. 3. The relationship of gravisensitivity (bending angle) to the number of statocytes. For each point, the bars and accompanying figures indicate the standard errors and the number of nodes measured. The nodes were taken from three separate batches of plants harvested over a period of 3 months.

Table 1. The relationship of gravisensitivity (bending angle) to the polarity of auxin transport during inversion of leaf sheath base (lsb) segments.

Angle of bend of nodal segments	Orientation of excised (¼ lsb)	Polarity of ^{3}H-IAA transport (Ba/Ac)	
0	Normal	3.95 ± 0.37	(12)
	Inverted	2.1 ± 0.3	
1–10	Normal	4.56 ± 0.3	(9)
	Inverted	0.62 ± 0.1	
11–20	Normal	4.55 ± 0.8	(6)
	Inverted	0.37 ± 0.003	
>20	Normal	2.7 ± 0.2	(4)
	Inverted	0.24 ± 0.002	

Figures in brackets indicate the number of replicates in each of the normal and inverted treatments. Ac = acropetal d min^{-1}; Ba = basipetal d min^{-1}.

segments from nodes that had bent between 11° and 20°. It was slightly less from nodes that had bent 10° or less. Segments from insensitive nodes kept in the normal position had the same degree of basipetal polarity as sensitive nodes. However, in contrast to sensitive nodes, they did not display a reversal of polarity on inversion; although polarity decreased somewhat, basipetal polarity was still predominant.

Levels of Endogenous IAA

IAA levels in nodal segments were estimated electrochemically on fractions that had undergone an initial HPLC separation (Methods and Materials).

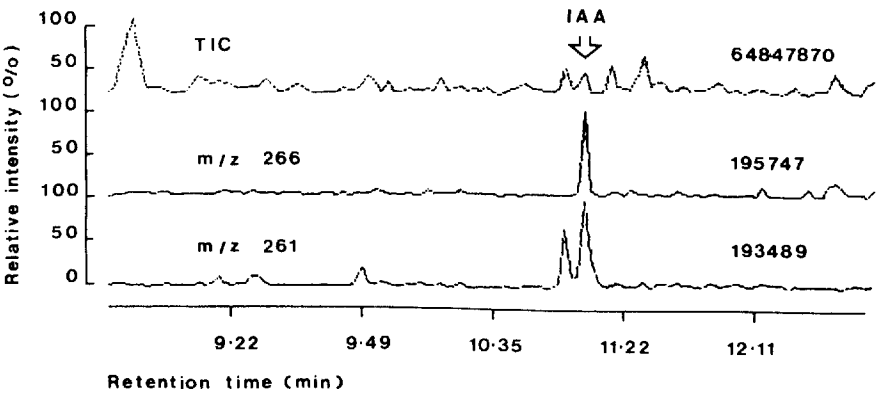

Fig. 4. Total ion current (TIC) and mass chromatograms from GC-MS of a purified extract of nodes of *Avena fatua* spiked with ($^2H_5$)IAA. The extract was first methylated and trimethylsilylated. The values to the right are absolute ion intensities of the most intense ion in each of the chromatograms.

Table 2. Relationship of gravisensitivity (bending angle) to the level of endogenous auxin in horizontally placed segments.

Orientation of nodal segments	Angle of bend	Position of ½ lsb	IAA levels, pg/½ lsb Exp. 1 (May 1983)	Exp. 2 (Aug. 1983)	Exp. 3 (July 1984)
Horizontal	0	Upper	54	78	41
		Lower	58	74	51
	1–10	Upper	73	67	44
		Lower	94	78	67
	11–20	Upper	93	55	50
		Lower	145	182	115
	>20	Upper	95	79	71
		Lower	122	127	139
Vertical	0	Right	—	82	58
		Left	—	88	68

GC-MS analysis of these fractions revealed the presence of major ions at m/z 261 and 202 (due to IAA Me TMS) and at m/z 266 and 207 (due to the deuterated internal standard), all at the retention times of authentic IAA Me TMS. Mass chromatograms for the ions at m/z 266 and 261 showed them to be of equal intensity (Fig. 4). Thus IAA from *Avena* extracts and ($^2H_5$)IAA were present in equal amounts, confirming the electrochemical estimation of IAA.

Segments of leaf sheath base excised from the upper and lower sides of gravitropically sensitive horizontally placed nodes had marked differences in the level of auxin (Table 2). The greatest differences, where the levels from lower sides were 1.5–3 times those from the upper sides, occurred in segments

from nodes that had bent 11–20°; differences were smaller in nodes that had bent more than 20° and markedly less in nodes that had bent 1–10°. The segments from insensitive nodes showed no marked differences when compared to controls.

Discussion

Changes in the level of endogenous auxin in the leaf sheath base of horizontally placed nodal segments of *Avena fatua* compared very closely with those previously found in similarly placed excised segments of leaf sheath base (Wright et al. 1978). It is therefore assumed that the mechanism responsible for the changes in auxin production of the excised tissue also operates and is responsible for the changes in levels in the intact nodal segments; thus, although auxin transport could (as will be discussed) contribute to these changes in levels in intact stems, it is not considered to be their prime cause.

In this study, gravisensitivity has so far been considered to be synonymous with the graviresponse (bending ability) of nodes. During development, acquisition of gravisensitivity can be correlated with the number of statocytes (Fig. 3) and the gravicontrol of auxin transport (Table 1) and auxin production (Table 2). The graviresponse may therefore result from the integration of a number of gravicontrolled processes. Thus gravisensitivity could be considered to reside in other individual processes (auxin transport/production) as well as within statocytes. Alternatively, gravisensitivity may reside exclusively within statocytes, which are then responsible for the gravicontrol of the other processes.

The importance of making a distinction between these alternatives and their possible combinations is necessary to relate the structural and biochemical changes in developing nodes to the development of the graviresponse mechanism. This can be illustrated by statocyte development in which sedimentation of statoliths occurs long before nodes are able to bend (Fig. 3). Three possibilities therefore exist: (1) Although statoliths can sediment, other components that contribute to the gravisensitivity of statocytes have not yet developed, so the cells are unable to effect gravicontrol of other processes. (2) The sedimentation of statoliths is indicative of functionally mature statocytes. However, there are too few of these to control other processes. (3) There are enough mature statocytes, but the other processes with which they integrate have not developed either gravisensitivity or receptivity to control.

The first possibility is consistent with the situation in epicotyls of *Asparagus officinalis* (Perbal and Rivière 1980), where statolith sedimentation is followed by a number of other events within the statocytes before the graviresponse occurs. However, the finding in nodes (Fig. 3) that the number of statocytes is critical both for the threshold response and for the subsequent degree of response tends to suggest that individual statocytes are mature but in insufficient numbers to control other processes.

This situation may be comparable to the development of the graviresponse in roots of *Asparagus officinalis* seedlings, where the volume of amyloplasts can be correlated with the rate of root curvature (Perbal and Rivière 1976). In decapped roots the onset of the response coincides with the first appearance of sedimentable statoliths (Hillman and Wilkins 1982); the apparently reduced

need for large numbers of statocytes could reflect the presence of other, important gravitropic processes unaffected by decapping. In addition to highlighting the importance of the event of statolith sedimentation, this suggests that large numbers of statocytes may not be necessary for the graviresponse in this system. However, the relative contribution of the other processes may be different from that during primary development. The evidence presented here for the nodal system indicates that the processes involved in the graviresponse have a high degree of synchrony during development and that their relative contributions may be critical for the *initial* graviresponse.

The gravicontrol of auxin transport correlates very closely with that of auxin production, being absent in nonbending nodes and maximal in nodes that bend between 11° and 20° (cf. Tables 1 and 2). This may be indicative of a gravitropic mechanism involving an integration of these processes; in a horizontal node the elevated levels of auxin, proposed to be responsible for cell elongation (Wright et al. 1978), would be more easily maintained with the greatly reduced total flux of auxin (Wright 1981). During subsequent bending, the higher rate of basipetal movement in the more vertically oriented apical part of the node would lead to an accumulation of auxin and a corresponding enhanced cell elongation in the adjacent, more horizontally oriented basal part. Thus although additional control mechanisms must operate to produce cessation of bending, the gravicontrol of auxin transport and auxin production reported here is consistent with the view that these processes are integrated components of the graviresponse.

The correlation between reversal of auxin transport and the previous bending experience of the node may differ slightly from the relationship that would have existed 24 h earlier, before bending. However, the existence of the relationship following bending suggests that it does operate during bending and could contribute to the response.

The trend for auxin transport to be in the direction of the gravity vector might also operate in the radial plane of the tissue; it would then follow that lateral transport could contribute to bending. The enhancement of lateral transport in gravitropically stimulated coleoptiles has already been demonstrated (Gillespie and Thimann 1963). Its occurrence in nodal tissue could explain why in *Echinochloa colonum*, although growth of leaf sheath base segments occurs independently according to their orientation, it is always less than in the intact node (Wright and Osborne 1977). This may be another way whereby auxin transport could be linked with auxin production to form an integral part of the gravity control system of plant growth.

The purpose of this study has been to understand something of the mechanisms of gravity perception and response through their development in nodes. From this has emerged the finding that the patterns of development in various processes relate to the graviresponse. Of these, the development of gravicontrol of auxin production and transport appears to coincide with the acquisition of gravisensitivity whereas the development of functionally mature statocytes could precede this. It is hoped that more detailed studies at the level of a single node will reveal how these processes are integrated in the gravitropic mechanism.

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