Meristematic Activity during Adventitious Root Primordium Development

INFLUENCES OF ENDOGENOUS AUXIN AND APPLIED GIBBERELLIC ACID

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ABSTRACT

Intact brittle willows (Salix fragilis L.) were treated so that developing adventitious root primordia in the stems would be subjected to elevated gibberellic acid or reduced endogenous auxin levels. Observations were made of primordia that were initiated during the experiments and of primordia that were established before the experiments began. The results indicated that as primordia became older and contained more cells, auxin basipetally transported in the stem seemed to be of less importance in determining cell number per primordium. Thus, established primordia depended upon this auxin to a lesser extent than primordia which were being initiated. These observations were explained on the basis of differential contributions during primordium development of cell division in the cambium of the stem and in the primordia themselves. As opposed to the effects of reduced auxin levels, applied gibberellic acid reduced the cell number per primordium most in established primordia. Initiating primordia were least affected by gibberellic acid treatment. Gibberellic acid treatment seemed mainly to reduce intraprimerordium cell division, on which continued development of established primordia most depends. Seemingly, at least in brittle willow, applied gibberellic acid blocks the action of auxin in primordium development subsequent to the initiation phase.

Although the role of hormones in the development of adventitious root primordia has been the subject of intense study, their influence on meristematic activity during and after initiation needs further clarification. It is generally true that applied auxins increase, whereas applied gibberellins and cytokinins decrease, the number of macroscopically visible root primordia in cuttings that maintain some natural ability for root regeneration. Clearly, however, it would be helpful to know how hormonal action influences cell division in primordia at defined stages of development.

The literature concerning adventitious root primordium development is particularly confusing because it has proven difficult to identify the cells which ultimately generate primordia in cuttings and tissue cultures. Frequently no such attempts have been reported in support of experimental results and, understandably so, because initiation is asynchronous (32). The cells involved have been described in only a few species (4, 5, 17, 20, 30–32); the locale of initiation may be fixed in a specific tissue (4, 8, 9, 20, 21, 25, 29, 31, 32, 35), but within that tissue likely areas of initiation cannot always be accurately predetermined.

Because of these problems in identification, many data concerning primordium development cannot be specifically related to particular aspects of development such as differentiation, cell division, and cell enlargement. Although development cannot occur without initiation, initiation may freely proceed even though subsequent development is checked or slowed, for example, by an inhibition of cell division within the primordium. Similarly, initiation and growth do not yield a functional root when differentiation (histogenesis) fails.

In order to clarify the role of hormones in primordium development it is essential that individual developmental phases be identified. The necessary identification can be accomplished with brittle willow (Salix fragilis L.), a unique plant within which the experimental modification of any phase of adventitious root primordium development can be exactly studied (6, 14).

Brittle willow develops an adventitious root primordium adjacent to each of the two lateral leaf traces of a node, except the uppermost ones, during normal development. The position of these primordia within a node is invariant, and they are regularly initiated (6). Under controlled environmental conditions, the first microscopically detectable stages of primordium initiation in sectioned tissue occur only in node 4 of plants with 15 nodes (14). Thus, plants in the same stage of development contain in their nodes identical, discrete stages of primordium development from initiation through histogenesis. With such material, the assessment of treatment responses during any stage of primordium development is unambiguous.

The present work thus employed brittle willow in a further clarification of the histological responses attributable to auxin and gibberellin action during initiation and two subsequent developmental stages.

MATERIALS AND METHODS

Plant Materials. Plants of a single clone were grown to a height of 15 nodes as previously described (14). Nodes were numbered from the top to the bottom of the plant, beginning with node 1 whose leaf was next in succession to separate from the terminal leaf cluster. Experiments were begun when leaf 2 was 5.0 to 5.5 cm long.

In such plants primordia develop adjacent to each of the two lateral leaf traces of a node, from node 4 downward. Initiation of two primordia per node nearly always occurs (14). Thus,
at the beginning of an experiment, node 3 did not contain primordia, node 4 held the initiation stage, and node 5 held a subsequent developmental stage. Control plants grew at a rate of about three plastochrons per week, the duration of an experiment, resulting in the initiation of primordia in node 3, and further development of primordia in nodes 4 and 5. It was thus possible to compare accurately initiation and development of primordia in untreated controls with the same events in treated plants.

In order to avoid confusion, nodes 3, 4, and 5 were designated 3', 4', and 5', respectively, at the termination of an experiment because their numerical position had changed.

**Experiment 1. Inhibition of IAA Transport by Triiodobenzoic Acid.** Two levels of TIBA; Eastman Organic Chemicals, (0.1 and 1% in lanolin) were used. The TIBA was dissolved in 95% ethanol, placed into heated lanolin (70 C), and the ethanol was evaporated under vacuum. Control lanolin preparations without TIBA were made in the same way.

The control, 0.1 and 1% TIBA mixtures were each used to ring five plants between nodes 2 and 3. All plants were returned to the growth chamber, and after 24 hr 50 μl of 50% ethanol containing 2 μc of IAA-2-14C (Calbiochem) (specific radioactivity 14.2 mc/mmol, purity at time of use greater than 95% by standard methods) was pipetted into the terminal leaf cluster of each plant above node 1. This experiment was replicated three times.

After 6 days from the time of IAA application, nodes 2' through 5' were removed from each plant, and the individual nodes were immediately placed into separate scintillation vials containing 5 ml of absolute methanol. After 48 hr the capped vials were opened to allow slow evaporation of the methanol. The nodes were further individually extracted for 1 week at 55 C in another set of scintillation vials containing 1 ml of strong base (Soluene, Packard Instruments Co.). Subsequently, the Soluene extracts were neutralized with 250 μl of glacial acetic acid. The amount of radioactivity in each methanol and Soluene extract was determined with equipment and methods described elsewhere (15). Count data were quantified with an electronic computer (16) such that the total of all errors in estimating the mean amount of 14C per sample was determined as less than 5% of the calculated dpm.

**Experiment 2. Influence of TIBA Treatment on Primordium Development.** A ring of lanolin with 0.1 or 1% TIBA or without TIBA (control) was applied to each of five plants between nodes 2 and 3. The plants were returned to the growth chamber for 1 week during which the lanolin rings (with and without TIBA) were periodically examined and repaired if they showed signs of deterioration. The experiment was replicated four times.

Nodes 3', 4', and 5' from control and treated plants were prepared for microscopic examination after they had been fixed for 24 hr in Craf III and embedded in paraffin (15). The 10 μ thick transverse serial sections were scored for the presence or absence of primordia. Absence of primordia in node 3' of treated plants indicated a failure in initiation, whereas absence of primordia in nodes 4' and 5' indicated a complete regression in the former primordium cells of those cytological characteristics (6, 14) which signal their meristematic nature (as illustrated in ref. 15, p. 30).

Primordia were also scored for number of cells in order to ascertain what amount of cytological regression, if any, had occurred in remaining primordia. Remaining primordia are those which were initiated before (nodes 4' and 5') or after (node 3') an experiment began, and which were still detectable at its termination. The number of cells was counted in the largest cross section of each primordium. Preliminary counts indicated that this method gave a good index of the total number of cells in a primordium.

An estimate of cambial cell division was made by counting the number of fibers produced in files from the interfascicular cambium above the leaf gap of the lateral leaf traces centripetal to the possible sites of primordium development. Cells in one file consisting solely of fibers were counted in one section from node 3', 4', and 5' of each plant.

**Experiment 3. Influence of GA Treatment on Primordium Development.** Plants were placed with their roots in covered styrofoam cups, each containing about 200 ml of nutrient solution (controls) or nutrient solution plus GA (Calbiochem) (1 μM, 10 μM, or 100 μM). Five plants were used per treatment in each of four replications. The nutrient solutions and cups were replaced every 48 hr in order to avoid microbial contamination. Nodes 3', 4', and 5' from control and treated plants were harvested after 1 week and examined as described for experiment 2.

**RESULTS**

**Experiment 1. Inhibition of IAA Transport by TIBA.** Preliminary experimentation established with reasonable certainty that TIBA reduces the basipetal transport of applied IAA in brittle willow, as it does in other species (Fig. 1). Data obtained from the Soluene extracts showed the same relative differences among treatments and nodes which are illustrated for the methanol extracts. However, on an absolute basis, up to twice

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1. Abbreviation: TIBA: 2,3,5-triiodobenzoic acid.
2. Mention of trade names does not constitute endorsement of the products by the United States Department of Agriculture Forest Service.
The Development portion abnormal, much contained standard plants. bars, right half) by fibers divisions sites two left bars, were (TIBA) 888 Experiment 2. Influence of TIBA Treatment on Primordium Development. The portion of plants above the 1% TIBA ring appeared abnormal, much as if they had been treated with auxin. Such plants showed pronounced swelling of the internode immediately above the TIBA ring. Plants treated with 0.1% TIBA appeared normal, and neither they nor the control plants had swellings. These observations generally support the results from experiment 1 which indicated a concentration-dependent blockage of polar auxin transport by TIBA. Thus, in the remainder of this presentation, it will be assumed: (a) that below a TIBA ring, auxin levels were suboptimal in comparison with control plants; (b) that the level of auxin below a TIBA ring was an inverse function of the level of TIBA applied. The auxin deficiency promoted by 0.1% TIBA resulted in a negligible reduction in the number of primordia in nodes 3', 4', and 5' (Fig. 2A). The effect on cell number per remaining primordium was slight. The greater auxin deficiency induced by 1% TIBA substantially reduced the number of primordia in node 3, while resulting in a lesser reduction in the number of primordia in nodes 4' and 5' (Fig. 2B). Substantial decreases also occurred in the number of cells per remaining primordium (Fig. 2B). The reduced level of auxin resulting from the 1% TIBA treatment depressed cambial cell division in the region of primordium development about equally in nodes 3', 4', and 5' (Fig. 3). The reduction in number of cells per remaining primordium was related to the lessened cambial cell division (compare Figs. 2B and 3). As percentages, using control values as the base, the number of cells per remaining primordium decreased with age of the primordium from 73% (node 3') to 49% (node 4') and 52% (node 5'). The percentage decrease in cambial cell division was fairly constant in nodes 3' (74%), 4' (67%), and 5' (80%). It thus seems that primordium initiation, which occurred in node 3' during the course of this experiment, depended most upon sustained cambial cell division. Experiment 3. Influence of GA Treatment on Primordium Development. Neither the number of primordia found in node 3' of plants treated with 1 mM GA, nor their mean number of cells differed appreciably from that of the controls (Fig. 4A).
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Fig. 4. Influence after 1 week of 1 \( \mu M \) (A), 10 \( \mu M \) (B), and 100 \( \mu M \) (C) GA on the development of root primordia in brittle willow. Each set of histograms shows the numbers of remaining root primordia expressed as percentage of the controls (open bars, left half) and the number of cells per remaining root primordium (solid bars, right half) by node in untreated (C) and GA-treated (GA) plants. Standard errors are indicated for cell numbers where two or more root primordia were found. Individual bars represent data obtained from 20 plants, each of which contained two sites of primordium development per node.

The number of primordia in node 3' was reduced at higher levels of applied GA, and, as a correlate, remaining primordia had fewer cells (Fig. 4,B and C).

Even the lowest concentration of applied GA reduced the number of primordia and the number of cells per remaining primordium in nodes 4' and 5' (Fig. 4A). This reduction became more pronounced at higher GA levels (Fig. 4B and C, nodes 4' and 5'). Regardless of the level of GA, the number of primordia and the number of cells which the primordia contained were lowered most in nodes 4' and 5'.

The relation between number of remaining primordia and their mean number of cells became clouded with GA at 10 \( \mu M \) and 100 \( \mu M \) because so few of the possible primordia were present in treated plants. It seems, however, on the basis of data obtained with GA at 1 \( \mu M \) (Fig. 4A), that loss of primordia under the influence of GA occurs through gradual attrition of the cell population, rather than by a sudden, general loss of meristematic activity. Remaining primordia lost cells even though cambial activity leading to increased xylem production was stimulated by GA treatment (Fig. 5,A and B).
With GA at 1 μM, a gradient of increased cambial cell division is evident, with older cambial cells having shown the greatest total response (Fig. 5A). A complete interfascicular cambium is present at about the stage of development reached in node 4 of plants similar to those with which this experiment was started (14). Thus, nodes 4 and 5 had a fully formed, active interfascicular cambium when GA was applied. The interfascicular cambium of node 3 matured only during the course of the experiment. High dosages of GA were so stimulatory that differential rates of cambial cell division among nodes, if they occurred, went undetected (Fig. 5B).

**DISCUSSION**

**Influence of Auxin.** The results of the present experiments support earlier findings obtained with brittle willow (15) which suggest that the number of cells per root primordium declines when there is a reduction in the basipetal transport of auxin from the expanding leaf zone. As primordia become older and contain more cells, basipetally transported auxin seems to be of less importance in determining cell number per primordium. These observations are readily explained by the differential contributions during primordium development of cell division in the interfascicular cambium and the primordia themselves.

Root primordia in brittle willow are originated by division of cells of the interfascicular cambium (6, 14). Cambial cell division in brittle willow and other woody plants (10–13, 22, 23, 33, 34) depends in part upon auxin. As a consequence, the greatest reduction is cell number per primordium might be expected during the initiation of primordia if the auxin supply were limiting. In accord with this expectation, limiting the auxin supply to cambial cells (1% TIBA treatment) resulted in a 74% decrease in cambial cell division, and this decrease was accompanied by a 73% reduction in the mean number of cells per primordium in node 3', the node in which primordia were initiated during the experiment. In older nodes (4' and 5') where primordia were established at the start of the experiment, before the auxin supply was reduced, the direct relation between reduced cambial cell division and number of cells per root primordium was less apparent.

Established primordia thus seem to depend upon basipetally transported auxin in the stem to a lesser extent than primordia which are being initiated. As primordia contain more cells, division of these cells supplants interfascicular cambial cell division as the source of new primordium cells. Clearly, established primordia require basipetally transported auxin to support normal rates of cell division, but cell division in established primordia does not completely cease even in plants whose expanding leaves and axillary buds have been removed (15). Continued cell division in established primordia which are deprived of basipetally transported auxin suggests that established primordia generate sufficient auxin to maintain a reduced rate of cell division. If so, established primordia might import less auxin than they did during initiation, and such a shift in uptake of label from ³C-IAA has been found (15).

The 0.1% TIBA treatment did not diminish the number of cells per primordium in nodes 3', 4', and 5', although experiment 1 indicated that this treatment hindered the basipetal transport of auxin. It appears that primordium development may be insensitive to reduced auxin levels until a critical minimum is reached, below which cell division subsides in the interfascicular cambium and in the established primordia. The supply of basipetally transported auxin apparently exceeds the need of primordia for cell division under normal conditions, and, therefore, auxin supply would not be a factor which determines the developmental rate of primordia in brittle willow.

It has been established with certain plant systems that TIBA effectively blocks the polar transport of both applied and endogenous auxin (1, 26, 27) without itself being an auxin (19). In addition, it has been possible to reduce adventitious root formation by blocking the polar transport of auxin with TIBA (7, 18). However, the observed differences in number of cells per primordium which were noted in these experiments might be attributed to an effect of the 1% TIBA treatment other than an effect on basipetal auxin transport. Although such a possibility cannot be completely discounted, it seems of limited validity. Surgical removal of expanding leaves and axillary buds of brittle willow in order to reduce basipetal transport of auxin to developing primordia has produced results very similar to those reported here (15). In addition, application of IAA to
surgically treated plants has overcome the effect of surgical treatment in diminishing the number of cells per primordium (15). Apparently there was a similar effect of surgical and TIBA treatment on primordium development, and the effect similarly resulted from decreases in basipetal transport of auxin.

**Influence of GA.** The results suggest that applied GA did not affect the number of cells in primordia which were originated during the course of the experiment (node 3') until interfascicular cambial cell division was markedly influenced. Primordia which were established at the start of the experiment (nodes 4' and 5') were far more sensitive to applied GA. For example, when GA was applied at 1 μM, the percentage decrease in number of cells per primordium in node 3' (0%) was substantially less than that of primordia in nodes 4' and 5' (about 35% each). At higher levels of applied GA, the number of cells in primordia of node 3' declined but not as much as in primordia of nodes 4' and 5'.

As noted above, during the course of this experiment, division of the interfascicular cambium cells initiated the primordia found in node 3'. Thus, if applied GA were to have had little effect on the number of cells in root primordia during their initiation, GA should not have markedly influenced cambial cell division. In this experiment, GA at 1 μM neither markedly influenced cell number per primordium nor cambial cell division. However, as the level of GA was increased, both cambial cell division and number of cells in root primordia of node 3' were affected.

In older primordia (nodes 4' and 5') whose development depends more upon intraprimordium cell division than upon cambial cell division, all levels of applied GA markedly reduced the number of cells per root primordium. Seemingly, the data indicate that applied GA inhibited intraprimordium cell division, and thereby limited primordium development. The effect of applied GA became more pronounced with each advancing developmental stage of the primordia because older primordia gain cells mainly by intraprimordium cell division.

It is doubtful that at any level of applied GA the observed influences on cell number per primordium could be attributed to a concentration gradient within the brittle willows. Dwarf peas accumulated applied gibberellins in those regions of the plant where organ enlargement occurred as a result of treatment (24). If brittle willow performed similarly, the concentration of GA would have decreased in the order: node 3', 4', 5'. Growth of leaves and axillary buds associated with node 3' exceeded that of the same organs at nodes 4' and 5'. Internodes above all three nodes were insensitive to GA treatment.

Results of the present experiments agree with many previous reports which have shown that GA treatment reduced root formation, although in those studies it was frequently unclear that the early stages of root primordium development were distinguished. Previously, Brian et al. (3) found that GA was most effective in inhibiting root formation in beans that were treated with GA before cuttings were taken. In another instance, early GA treatment of tissues was also most effective in limiting root formation (28). In addition, Bigot and Nitsch (2) have shown that budding was best inhibited in tissue cultures when the explants were exposed to GA within minutes of excision. The results of other investigators in conjunction with the present ones support a hypothesis that the deleterious action of applied GA is manifest at some very early stage of organ formation.

**Auxin-GA Interactions.** It has been proposed that gibberellins block the auxin-induced responses of cells in the process of root primordium initiation (3). The present results do not fully support that hypothesis. The most obvious result of GA treatment was reduced cell division in established primordia. Contrariwise, the manipulation of auxin levels in brittle willow in the present and other experiments (15) has shown that initiation is most dependent upon auxin. It may reasonably be assumed from these observations that, at least for brittle willow, applied GA blocks the action of IAA in some process subsequent to the initiation of primordia.

**LITERATURE CITED**