J Plant Growth Regul (1998) 17:107–114

Journal of Plant Growth Regulation

Morphogenetic Effect of the Herbicide Cinch on *Arabidopsis thaliana* Root Development

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Received August 8, 1997; accepted February 23, 1998

Abstract. Cinch is a morphogenetically active herbicide that inhibits primary root growth and induces abnormal ''nodule-like'' lateral roots on *Arabidopsis thaliana* seedlings. Using 200 nM Cinch, the early stages of lateral root formation occurred along the apical half of the root axis; but once emerged, they were inhibited from further growth. Second-order lateral roots formed at the base of stunted first-order lateral roots after 5 days of Cinch treatment. Results from Cinch experiments suggested that pericycle cells are determined in the meristem to be potential sites of lateral root formation, and the developmental transition point between emerged lateral roots and subsequent growth is inhibited. Results using 2,4-dichlorophenoxyacetic acid and 2,3,5-triiodobenzoic acid suggest that Cinch is not a chemical analog of auxin.

Key Words. *Arabidopsis thaliana*—Herbicide—Cinch—Root development—Lateral roots

The classical view of the root defines the root apex as having three regions: the zones of division, elongation, and maturation. Ivanov (1971) suggested a more accurate view in which there are transition points between cells that are dividing and cells that are elongating, and between cells that have elongated and are completing the maturation process. The relative location of the transition points is cell file specific. In *Pisum,* for example, the relative position of one transition point to the other is correlated with root length and growth rate (Rost and Baum 1988). In fast growing *Pisum* roots, the transition points are farther from the root tip than in slow growing ones.

Roots are ideal for studying the effects of growth-promoting and -inhibiting compounds and various stress factors because it is possible to measure the location of the differentiation events and their transition points (Ivanov 1971). Furthermore, the external symptoms of developmental interference (e.g. subapical swelling) are often diagnostic features (Rost and Hess 1993). Cinch (cinmethylin) is a cineole herbicide (E. I. duPont de Nemours and Co., Wilmington, DE) that was registered as a preemergence herbicide for use in soybeans (Technical Information, Shell Chemical Co.). Results from El-Deek and Hess (1986) investigating the growth-inhibitory effect of Cinch suggest that it inhibits entry of cells into mitosis. Its mode of action is unclear, however, and appears to be quite different from other herbicides known to inhibit mitosis.

Preliminary studies conducted at duPont showed that Cinch also induced morphogenetic effects in *Arabidopsis thaliana* (S. Russell, duPont, personal communication). When the *A. thaliana* plants were transferred to agar medium supplemented with 100–200 nM of Cinch, nodule-like appendages were induced along the root axis. In this study we will show that the nodule-like appendages are actually lateral roots. The results from this study will be discussed in the context of lateral root initiation and development in *A. thaliana* and the possible usefulness of Cinch as a chemical tool for studying root development.

Materials and Methods

Growing Conditions

A. thaliana cv. WS (seeds and medium recipe were provided by Sandra Russell, duPont) was grown on Murashige and Skoog medium (Life Science Technology, Inc.) supplemented with thiamine (final concen-

Abbreviations: Cinch, (7-oxabicyclo(2.2.1)heptane,1-methyl-4-(1-methylethyl)-2-(2-methylphenylmethoxy)-,exo-); DMSO, dimethyl sulfoxide; TIBA, 2,3,5-triiodobenzoic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; Pipes, 1,4-piperazinediethanesulfonic acid.

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tration 0.1 mg/liter), pyridoxine (0.5 mg/liter), nicotinic acid (0.5 mg/liter), and sucrose (1%). The pH of the medium was adjusted to 5.7 using 0.1 N KOH before adding agar (1%, Bacto-Agar, Difco Laboratories) followed by autoclaving for 18 min. Warm medium was poured into square 100- × 15-mm Petri plates (Falcon). Each plate contained 35 mL of medium. This will be referred to as germination medium.

To sterilize the seeds, uncovered glass Petri dishes containing approximately 1,000 seeds were placed 2–4 inches below a UV C lamp (Black-Ray lamp, model XX-15L, 115 volts, 60 HZ, 0.68 amps, San Gabriel, CA) for 8 min, shaking the Petri dish after 4 min to expose all sides of the seeds. The inside of the Petri dish cover was also sterilized. Once sterilized, the Petri dishes were sealed with Parafilm and stored at 4°C.

Seeds were picked up on the end of a sharpened toothpick and transferred one seed at a time to the agar medium under a dissecting microscope. Once planted, the plates were sealed using Parafilm and placed vertically in a growth chamber set at 16-h light, 8-h dark, at 23°C. Unless noted, seeds were first grown on germination medium for 7 days before being transferred to Cinch treatments.

Chemical Preparation

Cinch (7-oxabicyclo(2.2.1)heptane,1-methyl-4-(1-methylethyl)-2-(2-methylphenylmethoxy)-,exo-) (provided by duPont) was diluted to 0.01 M using dimethyl sulfoxide (DMSO) (Fisher) and stored either for long periods of time (greater than 1 month) at −20°C or for short periods at 4°C. The appropriate amount of Cinch was added to autoclaved medium right before pouring into Petri plates. Plates were stored at 4°C and used within 2 weeks.

A 1 mM 2,3,5-triiodobenzoic acid (TIBA) (Sigma) and 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma) stock solution was prepared by first dissolving the TIBA in DMSO and 2,4-D in 100% ethanol before bringing them up to volume using glass-distilled water. TIBA was added to the medium in the same manner as Cinch; 2,4-D was added before autoclaving. Two controls were included in all experiments: no treatment and DMSO only. At the concentrations tested, DMSO was found not to interfere with normal root development. Each experiment consisted of four Petri plates/treatment, and all experiments were repeated at least twice.

Fixing, Embedding, and Staining

Tissue pieces were fixed overnight in 1.5% glutaraldehyde and 0.3% paraformaldehyde in 0.025 M Pipes buffer. The fixed tissue was rinsed three times for 15 min in buffer, postfixed in 1% osmium tetroxide for 30 min, dehydrated in a graded ethanol series (15 min/step), and then embedded in Historesin (1:1, 100% EtOH:Historesin, 1–2 h; two changes of pure Historesin, for 4 h and overnight, respectively). Plastic blocks were mounted on wooden dowels (with Duco cement) and sectioned on a Reichert-Jung 2050 Supercut microtome. Sections that were either 1.5 or 2 μm thick were mounted on gelatin-coated (subbed) slides. Subbed slides were made by first washing the slides for 15 min in a solution composed of 5% glacial acetic acid in 95% EtOH, rinsed three times in deionized water, and then dipped in a subbing solution composed of 0.5% gelatin in H_2O. Slides were allowed to dry before using. Sections were stained with either 0.05% toluidine blue O for 30 s or by a periodic acid-Schiff reaction (periodic acid, 40°C, 20 min; Schiff reagent, room temperature, 60 min) modified from O'Brien and McCully (1981) and counterstained with 1.0% fast green (room temperature, 90 s) and toluidine blue (0.05% room temperature, 30 s).

Sections were viewed and photographed using an Olympus Vanox AHBT photomicroscope, and measurements were made using a Scanarray-2 image analysis system (Galai Productions, Israel). Viewed images were captured and stored in RAM directly from the microscope using a CCD camera. Some images were saved for future use. Using the Scanarray software, the distance between lateral roots was measured directly from the microscope. Sections that were to be used for publication were photographed using Kodak 2415 technical pan film.

Root Clearing

Roots were cleared by placing them in test tubes containing 85% lactic acid (Fisher) (Baum et al. 1991). The test tubes were heated in a beaker of boiling water for 30–60 min and then allowed to sit overnight. Cleared roots were mounted in lactic acid and viewed using Nomarski optics.

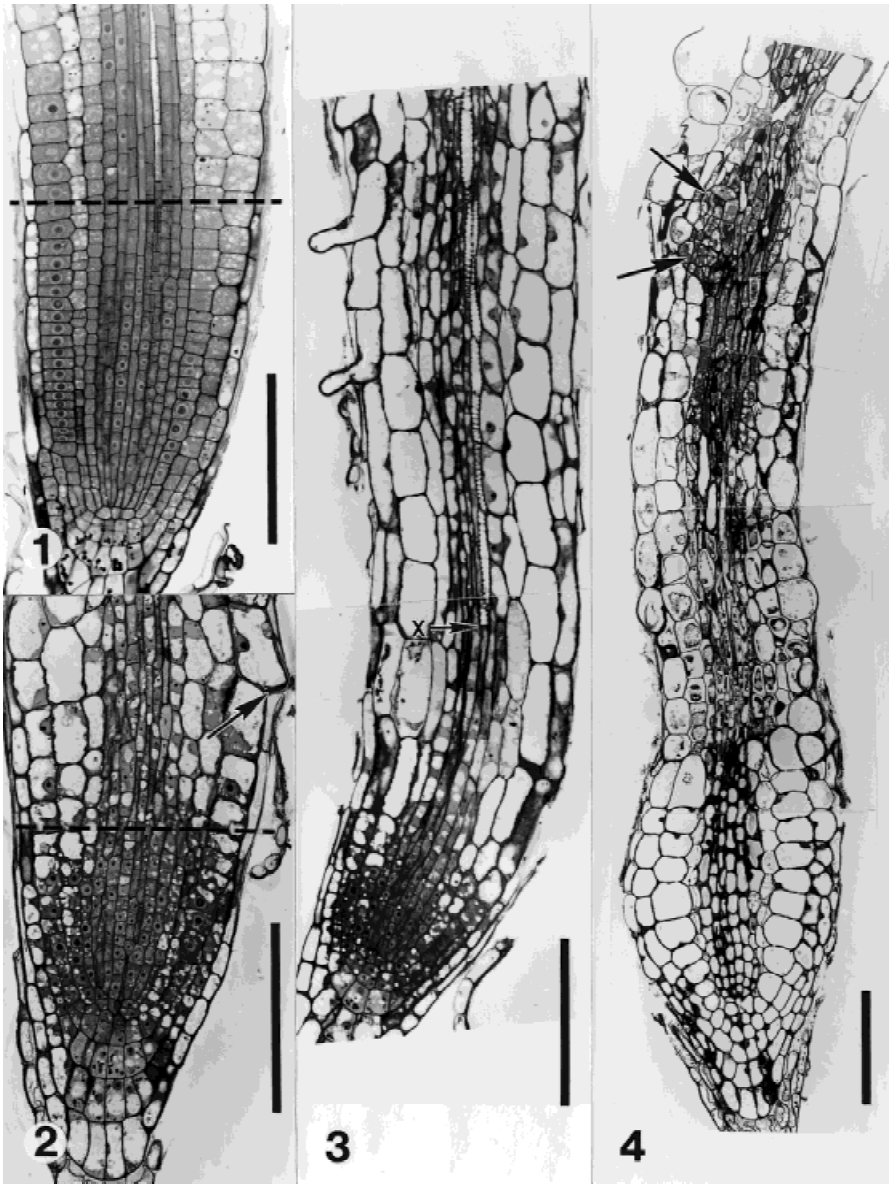
Results

Primary Root Meristem Anatomy

Root apical meristems from wild type *A. thaliana* plants grown on agar are composed of small densely cytoplasmic cells with relatively large nuclei. These relatively unvacuolated cells extend about 200 μm basal to the root cap boundary (Fig. 1). Transferring 1-week-old plants either to 100 or to 200 nM Cinch-treated plates caused the primary root to stop growing followed by changes in the root apical meristem. After 2 days on either treatment, epidermal cells located proximal to the meristem base became swollen in the radial direction, and some began to show signs of early root hair formation (*arrow*, Fig. 2). By day 4 of the Cinch treatment, cortical cells vacuolated prematurely and continued to elongate while the size of the root apical meristem decreased. Xylem tracheary elements were observed closer to the meristematic zone than those observed in the controls (Fig. 3). By day 5 on Cinch, the region proximal to the reduced root apical meristem was radially swollen. During days 6 and 7, a continuation of premature maturation of xylem and epidermal cells continued, and the root apical meristem became very small. By day 7, roots that were treated with 200 nM Cinch exhibited both extreme vacuolation of all meristematic cells plus subapical swelling (Fig. 4).

Lateral Root Development

Cinch (200 nM) stimulated the production of first-order lateral roots, but their further growth was arrested once the primordia emerged through the epidermis. Plants that were on Cinch-treated medium for 24 h exhibited early signs of lateral root initiation; anticlinal and periclinal divisions were observed in pericycle cells opposite the protoxylem poles. By days 2 and 3 of Cinch treatment, lateral root primordia were visible (Figs. 5 and 6). By day 4 or 5 the lateral roots had an abnormal appearance; they were less than 1 mm long, and their surface cells were swollen (Fig. 7). Xylem tracheary elements differentiated very close to the lateral root tip (Fig. 8). Roots



Figs. 1–4. Median longitudinal sections of *A. thaliana* root tips treated with Cinch for varying periods of time. **Fig. 1,** a control root grown on agar for 7 days. The *dashed line* marks the approximate basal boundary of the root apical meristem. Note the small, densely cytoplasmic cells of the root apical meristem. **Fig. 2,** root treated for 2 days with 200 nM Cinch. The size of the root apical meristem has decreased. The *arrow* points to an epidermal cell that is starting to form a root hair prematurely. **Fig. 3,** root treated for 4 days with 200 nM Cinch shows a decrease in root apical meristem size along with the concomitant premature vacuolation of cortical cells and maturation of xylem vessel members (×) near the root tip. **Fig. 4,** root treated for 7 days with 200 nM Cinch. All cells of the meristem have become vacuolated, and a lateral root primordium is visible (*arrows*). All *bars* represent 100 μm.

treated with 100 nM Cinch responded the same way except with a slight increase in lateral root growth.

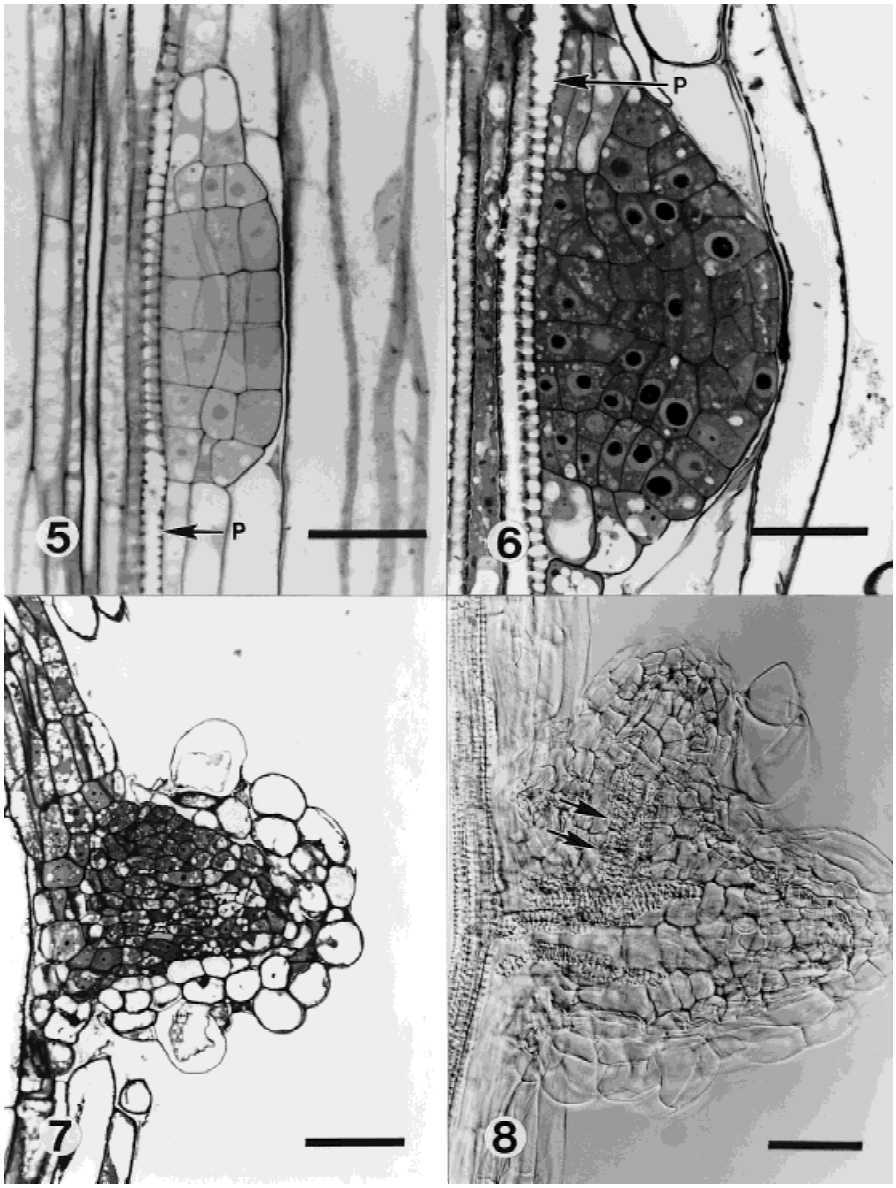
After day 5, second-order lateral roots emerged from the base of the short first-order lateral roots (Fig. 8). Plants that remained on Cinch-treated plates for longer than 2 weeks produced clusters of small second-order laterals. The result of this abnormal growth is that the root appeared to have nodule-like structures along the root axis (*arrowheads,* Fig. 9).

Root Growth

A series of experiments was conducted to investigate the effect of Cinch on primary root growth and development. Plants were first grown on germination medium and then transferred to a treatment medium containing either 25, 50, 100, or 200 nM Cinch for 5 days. Increasing the concentration of Cinch decreased primary root elongation (Fig. 10*A*). At 200 nM Cinch, the primary root only grew 1–2 mm. Germinating seeds directly on increasing concentrations of Cinch decreased root growth.

Lateral Root Production

Increasing concentrations of Cinch had a promotive effect on the number of lateral roots formed along the primary root axis (Fig. 10*B*). In this particular experiment, plants that were transferred to 200 nM Cinch plates formed an average of 13 lateral roots after 5 days. The absolute number of lateral roots produced for each treat-



Figs. 5–8. Development of lateral roots on 200 nM Cinch-treated *A. thaliana* roots. **Figs. 5** and **6,** median longitudinal sections of lateral root primordia from roots treated for 2 days **(Fig. 5)** and 3 days **(Fig. 6)** with Cinch showing the early stages of lateral root development. *p,* protoxylem vessel; *bar,* 25 μm. **Fig. 7,** longitudinal section of a lateral root from a plant treated for 4 days with 200 nM Cinch showing swollen surface cells. *Bar,* 50 μm. **Fig. 8,** a cleared abnormal first-order lateral root from a plant treated for 6 days with Cinch. Xylem maturation is close to the lateral root apex, and a second-order lateral root has formed with accompanying xylem tissue (**double arrows**). *Bar,* 50 μm.

ment fluctuated among individual experiments, but the trend was consistent. As higher concentrations of Cinch were used, the length of lateral roots decreased, so much so that the lateral roots induced using 400 nM Cinch barely broke through the epidermis during the duration of the experiment.

Lateral Root Spacing

Treating plant roots with various concentrations of Cinch altered the spacing of lateral roots along the primary root axis (Fig. 11). In the control without Cinch, the acropetal half of the root lacked lateral roots, whereas the basipetal half of the root contained lateral roots spaced at least 2 mm apart. Roots that were treated with Cinch had lateral roots all along the root axis, and in particular, they appeared grouped together near the tip. Roots treated with 200 or 400 nM Cinch had the largest number of lateral roots, many of which were located within 1 cm from the root tip. Roots treated with 200 or 400 nM Cinch commonly exhibited two and three lateral roots next to each other or directly opposite each other.

Experiments were designed in an attempt to mimic the Cinch treatment effect. Plants were grown on various concentrations of 2,4-D (100 nM, 1 μM, or 10 μM), TIBA (100 nM, 1 μM, or 10 μM), or the root tip was decapitated (Fig. 11). Auxin inhibited primary root growth and induced lateral roots all along the primary root axis with the acropetal end often exhibiting fasciated roots. TIBA inhibited both lateral root formation and primary root growth. Root decapitation induced lateral root formation,

Fig. 9. *A. thaliana* root treated for 2 weeks with 200 nM Cinch showing clusters of abnormal lateral roots mainly near the root apex. The *arrowheads* point to examples of abnormal (nodule-like) lateral roots along the root axis. The *arrow* points to the swollen primary root tip and accompanying cluster of lateral roots. *Bar,* 1 mm.

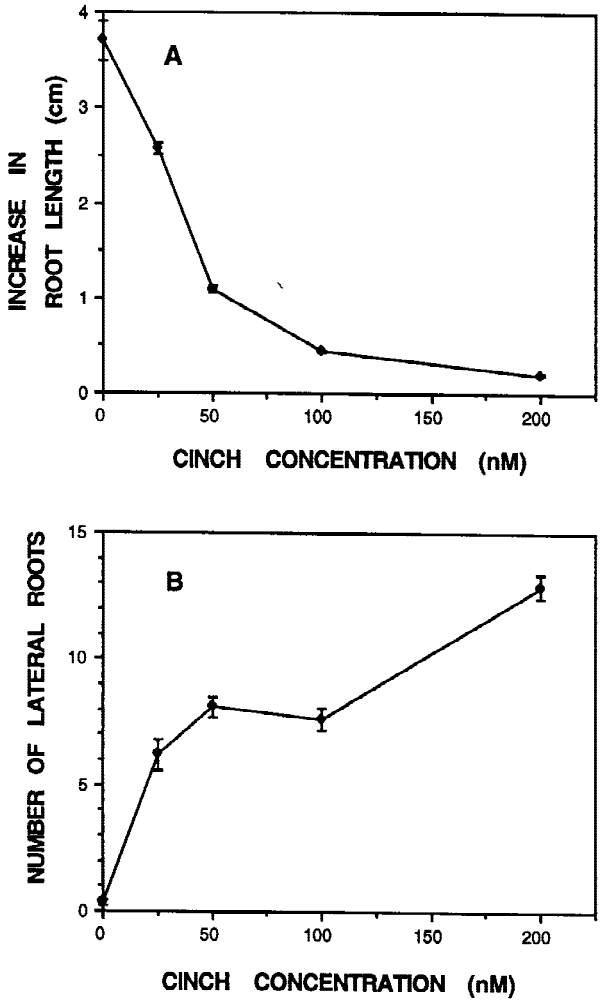


Fig. 10. Effects of various concentrations of Cinch on *A. thaliana* primary root growth and lateral root production. **Panel A,** effect of Cinch on new primary root growth. *A. thaliana* plants were first grown on agar medium for 7 days before being transferred to Cinch-supplemented agar Petri plates. **Panel B,** effect of Cinch on lateral root production. *Error bars* equal the S.E. of the mean.

although the number of laterals produced was less than that by Cinch.

Placing agar blocks supplemented with 1 μM 2,4-D on *A. thaliana* roots growing on TIBA-supplemented medium produced a localized region of lateral roots. Roots treated in the same way but with agar blocks supplemented with 200 nM Cinch showed no effect.

Location of Youngest Lateral Root

On plants treated with Cinch, the location of the youngest lateral root occurred closer to the apex than roots not treated with Cinch. Roots that were treated with concentrations of Cinch greater than 50 nM produced lateral roots that were within 2 mm of the root tip. Roots treated with 200 and 400 nM Cinch produced lateral roots 400–700 μm from the tip (Figs. 4 and 11).

Recovery from Cinch Treatment

Plants that were growing on 100 and 200 nM Cinch for 7 days were transferred to Cinch-free plates and allowed to grow for 4 days. After 24 h of growth on Cinch-free plates, one or two secondary lateral roots from each cluster resumed normal growth. In more than 50% of the roots that had been treated with 100 nM Cinch, the primary root also resumed normal growth. Primary roots

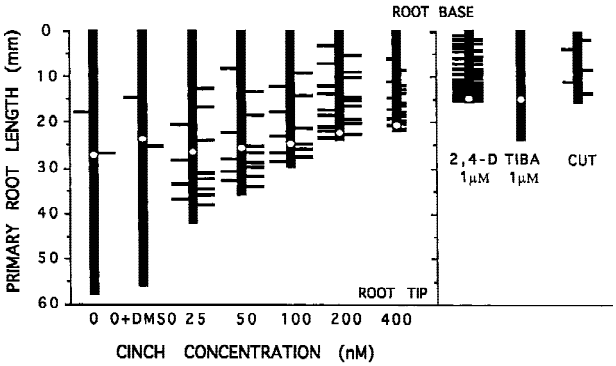


Fig. 11. Effects of various concentrations of Cinch as well as 1 μM 2,4-D, 1 μM TIBA, and root decapitation on primary root growth, production, and spacing of lateral roots. The number and spacing of lateral roots on each root schematic drawing were taken from one root from each treatment. Note that the schematized lateral roots in the 400 nM treatment were purposely made smaller to illustrate their small size compared with those from other treatments. The *white dot* indicates the average root length before the treatments began.

treated with 200 nM Cinch did not show any signs of recovery growth after 4 days on Cinch-free medium.

Discussion

Cinch has a unique morphogenetic effect on agar-grown *A. thaliana* root development: root growth is inhibited, and abnormal (nodule-like) lateral roots are induced along the root axis in a predictable pattern. Lateral roots that were induced using 200 nM Cinch emerged through the epidermis but then arrested, followed by the growth of second-order lateral roots. If the plants remained on Cinch-supplemented agar for more than 2 weeks, these lateral roots appeared along the root axis as nodule-like appendages mainly at the acropetal end. The inhibitory affect of Cinch on lateral root growth demonstrated the existence of a developmental transition point between emerged lateral roots and their continued growth.

Although the term *nodule-like* is used to describe the outgrowths that occur because of Cinch treatment, they should not be confused with nitrogen-fixing nodules or pseudonodules. It has been shown that auxin transport inhibitors (e.g. *N*-(1-naphthyl)phthalamic acid and TIBA) produce nodule-like (pseudonodules) structures that resemble *Rhizobium*-induced nitrogen-fixing root nodules (Hirsch et al. 1989, Wu et al. 1996). Pseudonodules and nitrogen-fixing root nodules develop from cortical cell divisions. The anatomy of pseudonodules does not resemble the anatomy of the nodule-like appendages that are formed along root axes treated with Cinch for extended periods of time. Pseudonodules from *Melilotus alba* (white sweetclover) lack a clearly defined apical meristem and contain a proximal and central region of tracheary elements surrounded by small cells exhibiting amyloplasts (Wu et al. 1996). Cinch-induced nodule-like appendages are the result of successively higher order lateral roots being formed on lower order lateral roots whose elongation has been severely inhibited. When viewed topographically, these structures resemble pseudonodules.

Chemical and physical stresses that disrupt mitosis are known to have an adverse effect on meristem structure. Tissue differentiation and maturation continue acropetally even after mitosis has stopped, and some cells enlarge under stress (Hess 1982, Rost 1977). After 3 or 4 days of Cinch treatment, the meristematic region was reduced in size and tracheary elements differentiated close to the tip. High concentrations of Cinch caused vacuolation of all meristematic cells within the root tip, resulting in a swollen root tip. Bayer et al. (1967), using the microtubule-inhibiting herbicide trifluralin, observed similar swelling in cotton root tips. Scopoletin, a natural plant product, elicited the same response on *Phleum pratense* roots (Avers and Goodwin 1956).

Lateral Root Development

There is a predictable pattern associated with lateral root development and their position along the main axis. Lateral roots arise in an acropetal series from pericycle cells often adjacent to the protoxylem poles (Esau 1965). Based on experiments in which auxins and cytokinins were used to substitute either for the cotyledons or root tips, Hinchee and Rost (1986) concluded that there are three stages to lateral root formation: initiation, organization, and emergence. The observation that there is a developmental difference between the initiation and organization phases was demonstrated further by Laskowski et al. (1995). They observed that not all auxin-induced lateral root initiation sites formed organized meristems, indicating that there is a developmental transition between the two events. In the current study, high concentrations of Cinch inhibited emerged lateral root meristems from further growth, suggesting that a developmental transition point exists between these two phases of development.

In *A. thaliana* there are two ranks of lateral roots corresponding to the diarch xylem pattern (Laskowski et al. 1995). The spacing of lateral roots within a rank is not influenced by lateral roots from other ranks, at least in tomato (Barlow and Adam 1988). Using auxin to stimulate lateral root formation reduces the spacing between lateral roots (Barlow and Adam 1988, Blakely et al. 1982, Laskowski et al. 1995). Roots treated with Cinch show a distinct change in polarity of lateral root formation; more lateral roots were formed near the tip compared with the number formed along the basal half of the root. Appropriate concentrations of Cinch initiate lateral

root formation from pericycle cells located abnormally close to the root apex.

Lateral Root Initiation Sites are Determined in the Root Apical Meristem

As part of the cell division and differentiation process within the root apical meristem, pericycle cells are specified as potential lateral root initiation sites (Barlow and Adam 1988, Charlton 1991). Within the vicinity of the root tip, lateral root initiation is inhibited, and it is believed that cytokinins and/or abscisic acid are responsible for this inhibition (Charlton 1991). The pattern of acropetal lateral root initiation has been shown to be rather stable and resistant to experimental modification in excised tomato roots (Barlow and Adam 1988). Clowes (1958), using radiolabeled adenine, demonstrated that the initiation of lateral roots in *Pistia* and *Eichhornia* occurred in the meristem of the primary root.

In *Pisum sativum*, lateral root initiation can be inhibited by temperature changes (Gladish and Rost 1993). Growing peas at 25°C supports normal acropetal lateral root formation along the primary axis. Transferring the plants to 32°C for 1 day and then returning them to 25°C produces a zone in which the acropetal induction of lateral roots is highly reduced. The zone without lateral roots can be interpreted as originating from cells that were within the meristem at the time the plant was subjected to 32°C. Normal lateral root formation resumes and is believed to arise from pericycle cells that originated within the meristem at a time when the root was not exposed to 32°C.

Concentrations of Cinch greater than 100 nM stimulate lateral root production mainly near the acropetal end of the root. High concentrations of Cinch induce lateral root formation within 700 μm of the apex. In addition, second-order lateral roots are formed on growth-inhibited 4-day-old first-order lateral roots. The polarized induction of lateral root formation along the main axis and the induction of second order lateral roots are evidence that sites of lateral root initiation are determined within or very near the root apical meristem.

Cinch Mode of Action

Growth can be defined as ''a combination of cell division and cell enlargement which leads to an irreversible increase in size'' (Hess 1982). Inhibiting one or both of these processes will terminate growth. Herbicides are categorized according to which component of growth is disrupted or inhibited, cell enlargement or cell division. An important component associated with herbicidal action is whether the effect is caused by a primary influence or is the result of a secondary one. Distinguishing between the two involves a time course analysis and then comparison of the results with herbicides characterized previously. El Deek and Hess (1986) have shown that 10^{-7} M Cinch interferes with oat root tip growth by reducing the mitotic activity of cells when treated for 12 h. They concluded that the mitotic arrest using Cinch was different from other known mitosis-inhibiting herbicides, suggesting that it may be a secondary effect.

The mode of action of Cinch is not similar to auxin, i.e. it is not a chemical analog to auxin. This is because 2,4-D and indoleacetic acid IAA (Laskowski et al. 1995) stimulate lateral root formation all along the root axis including fasciated roots near the apex, whereas Cinch induced lateral roots mainly along the acropetal end of the root with no occurrences of fasciated roots. In addition, 2,4-D stimulated a localized region of lateral root formation on roots growing on TIBA-supplemented medium whereas Cinch did not. Root decapitation did not stimulate the production of lateral roots with the same polarity or frequency as those initiated in response to Cinch treatment, therefore eliminating the possibility that the observed pattern of lateral root formation was strictly the result of chemically wounding the root tip. We suggest that lateral root induction by Cinch is a secondary effect possibly mediated by auxin. Further experimentation will be needed to elucidate the mode of action of Cinch.

Acknowledgments. We thank members of the S.F.B. dissertation committee (Profs. John Harada and Lew Feldman) for helpful comments on the manuscript. This work was supported by a duPont weed science fellowship (to S.F.B.), a Henry A. Jastro graduate fellowship (to S.F.B.), and a University of California, Davis faculty research grant (to T.L.R.).

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