

Localization of a Phytohormone Using Immunocytochemistry

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ABSTRACT The localization of cytokinins in corn root tips was investigated using antibodies or antibody fragments directed against dihydrozeatin riboside and labeled with rhodamine or colloidal gold. Roots were sectioned at -30° to -40° for immunofluorescence or freeze-substituted in ethanol or acetone and embedded in plastic for electron microscopy. Meristematic cells surrounding the quiescent center as well as root cap cells were specifically labeled using direct immunofluorescence techniques, whereas cells of the quiescent center did not bind label. Tissue sections treated with colloidal gold-labeled antibody fragments had gold particles widely distributed in the cytoplasm. The results show that the quiescent center is not the major site of cytokinin localization in root tips.

Phytohormones are low molecular weight substances having many important regulatory functions in plant growth and development. Their mode of action is not well understood and their site(s) of action are the subject of much speculation (1). The localization of plant hormones is difficult because they occur in low concentrations (2) and because they are highly soluble in solvents used in preparing samples for electron microscopy. Low temperature preparative techniques have been used for preserving the intracellular distribution of labile cellular components in plant and animal cells (3). We chose to couple immunocytochemical and low temperature preparative techniques to investigate the distribution of cytokinins in corn root tips. In this report we present results from our experiments on the localization of cytokinins in plant tissues.

MATERIALS AND METHODS

Plant Tissue: Corn seeds (Early Sunglow, W. Atlee Burpee Co., Warminster, PA) were surface sterilized with 5% NaClO for 8–10 min. They were rinsed at least three times with sterile water and placed on moistened filter paper in a covered dish. Germination dishes were covered with aluminum foil and placed in the dark. Seeds were allowed to germinate for 3–5 d. Roots, 2.5–3.5 cm long, were detached from the seed and root cap cells were gently teased off. Some root tips were cut longitudinally before being excised from the whole detached root. Root tips were cut from terminal 1.0–1.25 mm. They were kept on moist filter paper until 20 to 30 root tips were collected. All manipulations were carried out under green safe light.

Fixation: Root tips were fixed in freshly prepared 2% formaldehyde in 0.05 M phosphate buffer, pH 7.2, for 1 h. They were rinsed three times in buffer for a total of 15–30 min. Preliminary experiments showed that fixation with 2% formaldehyde produced acceptable morphological preservation and low levels of autofluorescence.

Freezing: Freshly excised root tips were gently wiped and placed on tapered wooden dowels or small copper rods. They were quench-frozen in Freon 22 or Freon 114 (DuPont Chemical Co., Wilmington, DE), cooled in a liquid nitrogen bath, and stored in liquid nitrogen until sectioned. Sectioning

was accomplished on a Sorvall MT-2 (DuPont Instruments-Sorvall, DuPont Co., Newtown, CT) equipped with a cryokit with a glass knife. Sections 0.25 or 0.5 μ m thick were cut at -30° C to -40° C.

Freeze Substitution: Root tips were placed on a cotton gauze support (4) and plunged into Freon slush in liquid nitrogen. Root tips were freeze substituted in ethanol or acetone at -85° C. Substitution solutions were changed every other day for 7–10 d. Infiltration was also conducted at -85° C until, in the final infiltration step, vials were brought to room temperature before 100% low viscosity resin (5) was added. Samples were polymerized at 70° C. Thin sections were cut with a diamond knife on a Sorvall MT-2, and were \sim 80–90 nm thick as judged by their silver to light gold appearance. Thick sections were cut at 0.25–0.5 μ m and stained with methylene blue.

Immunological Reagents: (\pm) Dihydrozeatin and its riboside (DHZR)¹ were generously supplied by Dr. Joseph Corse (Western Regional Research Center). DHZR was conjugated (6) to BSA (Miles Laboratories, Elkhart, IN), yielding conjugates containing 7–14 mol/mol of protein, as indicated by UV absorbance spectra. Antibodies were raised in female New Zealand white rabbits, as described previously (7, 8). IgG, from normal or immune sera, was purified (9) by ammonium sulfate fractionation and chromatography on diethylaminoethyl-cellulose (DE-52; Whatman, Inc., Maidstone, Kent, UK). Purity of the fractions was determined by gel electrophoresis (10) and immunodiffusion. To maximize penetration and minimize background, antibody fragments were used for immunocytochemistry. Fab fragments from IgG were prepared according to Stanworth and Turner (11) and purified as described by Porter (12). Briefly, IgG was digested with papain (Sigma Chemical Co., St Louis, MO) and fractionated on carboxymethyl-cellulose (Cellex-CM; Bio-Rad Laboratories, Richmond, CA). Fractions were analyzed by gel electrophoresis and by immunodiffusion (Fig. 1), using antisera specific for Fab and Fc fragments of rabbit IgG (Miles Laboratories). Fab fragments were conjugated (13) with tetraethylrhodamine isothiocyanate (Sigma Chemical Co.). The purified product had $A_{555}:A_{280} = 1:2.3$ and was used without further fractionation. Colloidal gold was prepared (14) and Fab fragments directed against DHZR were adsorbed onto the gold particles (15). Particle diameter (7–10 nm) was checked by electron microscopy.

Immunofluorescence: Individual frozen sections or ribbons were placed on cooled coverslip pieces. After enough sections were cut, the coverslips were removed from the chamber of the cryokit and were allowed to thaw. Sections were rinsed briefly in PBS, and excess PBS was wicked away with a

¹ Abbreviations used in this paper: DHZR, dihydrozeatin riboside.

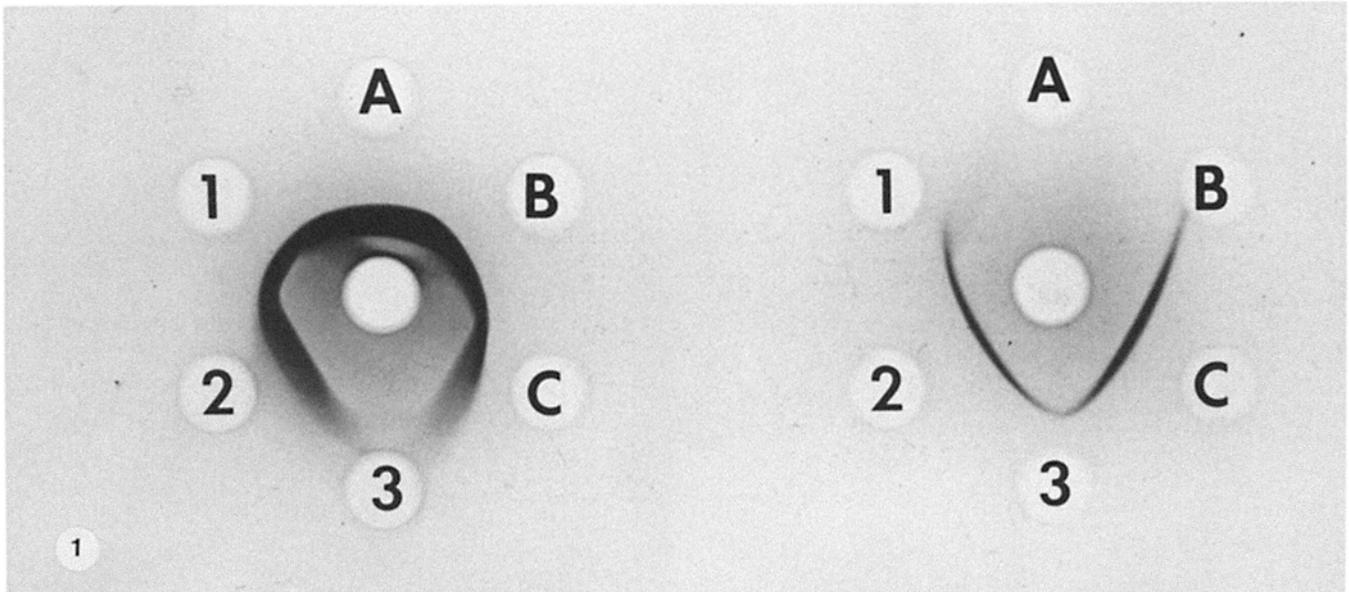


FIGURE 1 Fractions 1, 2, and 3 were obtained by chromatography of the papain digest of R230 IgG on diethylaminoethyl-cellulose in 0.01 M Tris-HCl, pH 8.0, and a NaCl gradient. Fraction 1 was rechromatographed on carboxymethyl-cellulose. The center wells contain antiserum specific for rabbit Fab (left) or Fc (right). Fractions A and B—free of Fc determinants—were used in the immunocytochemical studies.

piece of filter paper. Sections were exposed to rhodamine-conjugated anti-DHZR Fab (0.1 mg/ml) for 5–10 min, rinsed for 10 min in PBS and mounted in PBS. Controls included: (a) sections exposed only to PBS to monitor autofluorescence, and (b) a blocking experiment using 0.1 mM DHZR in the incubation medium. Untreated sections were stained with 0.05% toluidine in 0.05 M phosphate buffer, pH 4.4. Sections were observed with a Zeiss Photomicroscope III equipped for epifluorescence.

Immunogold: Thin sections of freeze-substituted plastic embedded root tips were collected on grids and incubated with 1% normal rabbit serum in PBS for 10 min. Sections were stained specifically with Fab adsorbed on colloidal gold for 10 to 20 min. They were rinsed in PBS and then in glass-distilled water. PBS and distilled water were filtered through 0.22- μ m filters. Controls for the immunogold experiments included (a) incubation with anti-DHZR Fab before staining with colloidal gold-Fab, and (b) staining with colloidal gold adsorbed onto Fab fragments prepared from nonimmune serum. For electron microscopy, a JEOL 100 CX operated at 80 kV was used.

RESULTS

Roots of this cultivar had typical corn root morphology. Removing the root cap improved freeze preservation of excised root tips. At the light microscope level, preservation of roots either freeze sectioned on freeze substituted was excellent (Fig. 2, *a* and *b*). At the electron microscope level some ice crystal damage was evident (Fig. 3*a*), especially in basal regions and at the center of the tissue mass. One major problem with sectioning frozen material was collecting enough sections for experimental and control slides before the sections freeze-dried in the chamber. Since freeze-dried

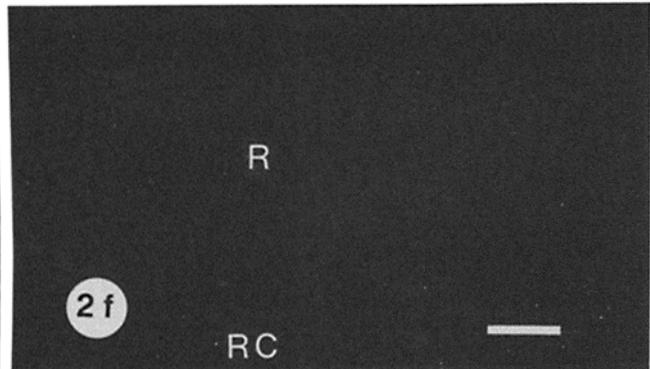
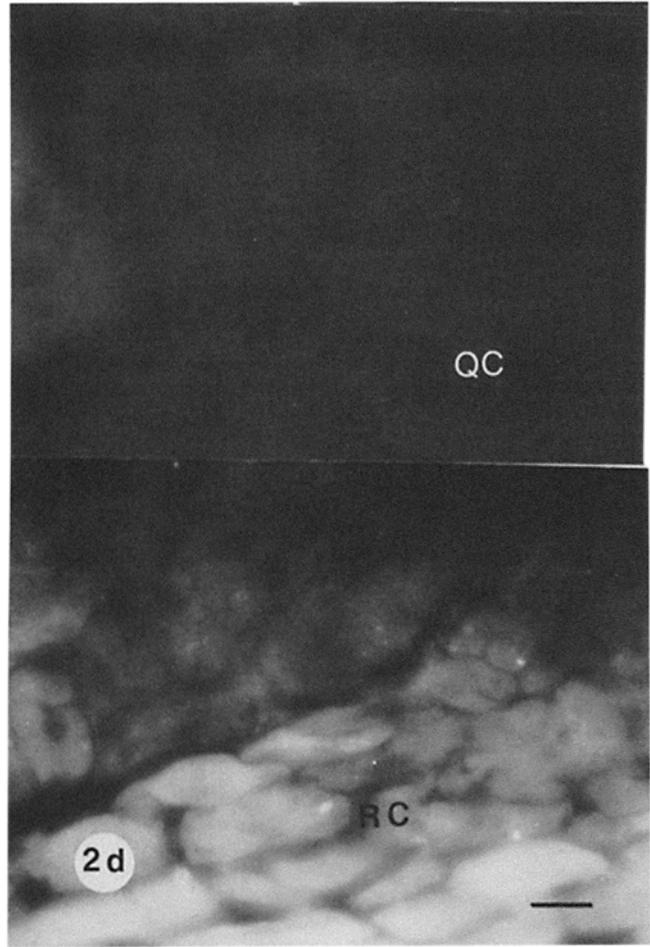
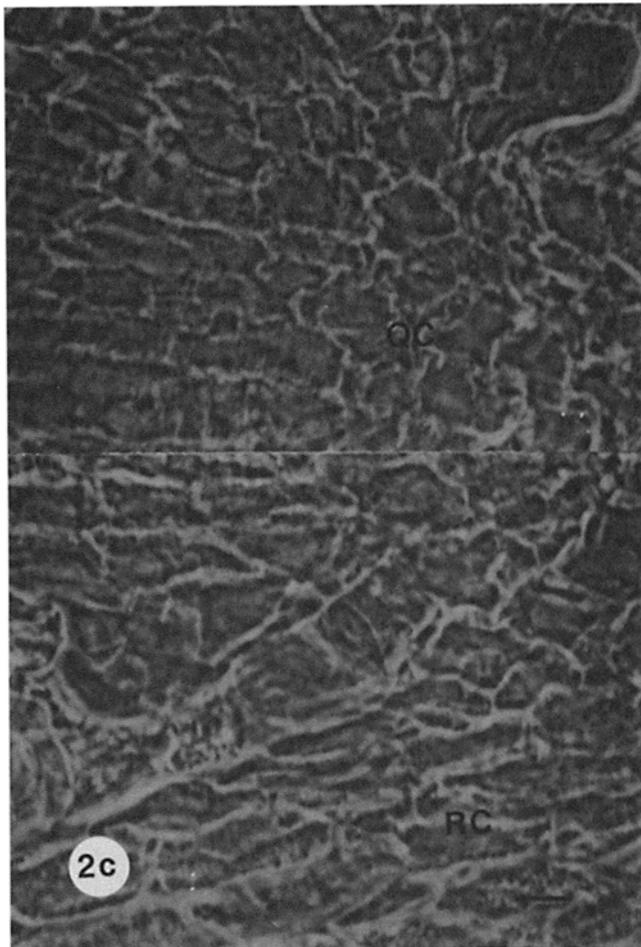
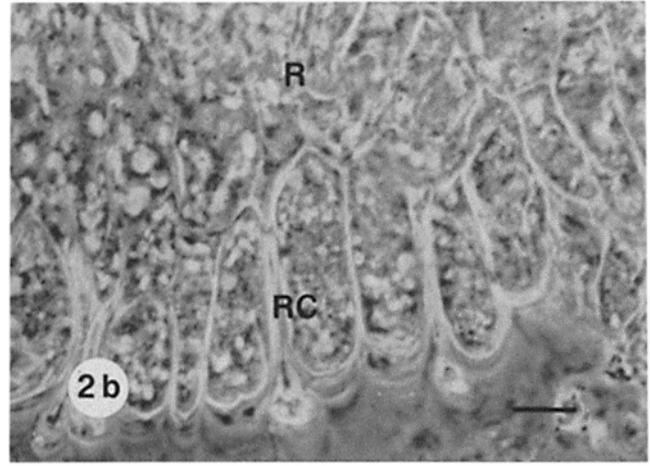
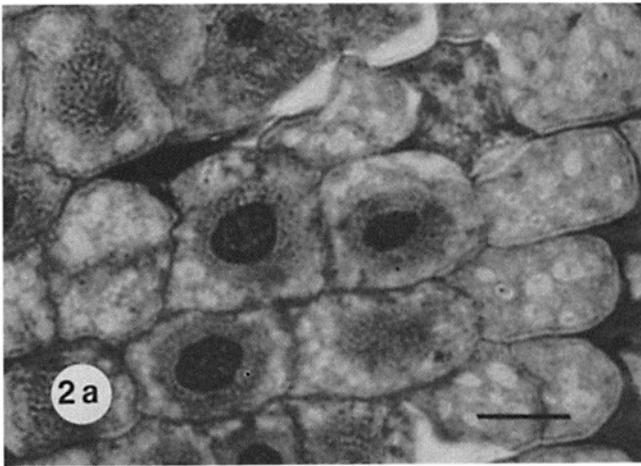
sections tended to blow off the coverslips, sectioning had to be done rapidly once the desired area of the root was reached. Sectioning was optimal between -30 and -40°C . At lower temperatures roots would chip or the whole root would become dislodged from the holder. At higher temperatures sections were irregular and surfaces looked melted. Cellular integrity was lost within 2 h of thawing and more rapidly if cells were vigorously washed.

Immunocytochemistry

Rabbit anti-DHZR antibodies were analyzed for specificity by radioimmunoassay, as shown in Table I. Labeled anticytokinin stained experimental samples with apparent specificity. Rhodamine Fab stained cells adjacent to the quiescent center (Fig. 2, *c* and *d*), but cells within the quiescent center were not stained. The quiescent center is a lens shaped region near the extreme tip of the root (2). The cytoplasm of meristematic and root cap cells was fluorescent; nuclear regions were not. Fluorescence was blocked in cells incubated with DHZR in the medium, indicating that the bound antibody was associated with cytokinin (Fig. 2, *e* and *f*).

Immunogold label was clearly limited to cytoplasmic regions. Label was rarely found in nuclei, vacuoles, or cell walls. Particles were associated with intracellular membranes which might be endoplasmic reticulum (Fig. 3, *a* and *b*). The plasma

FIGURE 2 (a) Light micrograph of corn root freeze substituted in ethanol and stained with methylene blue. Longitudinal section. Symbols used on micrographs: root cap, RC; root, R; quiescent center, QC; ice damage, I; and cell wall, CW. Bar, 10 μ m. $\times 1,250$. (b) Light micrograph of root fixed in formaldehyde, sectioned at -40°C , thawed, stained with toluidine blue, and observed with phase optics. Longitudinal section. Bar, 10 μ m. $\times 800$. (c) Light micrograph of corn root fixed in formaldehyde, sectioned at -40°C , thawed, and incubated with rhodamine labeled Fab anti-DHZR as observed with phase optics. Longitudinal section. Bar, 10 μ m. $\times 800$. (Montage.) (d) Fluorescence micrograph of section shown in c. Note that fluorescence is present in the root cap but not in the quiescent center nor in the nuclear regions of other root tip cells. Bar, 10 μ m. $\times 800$. (Montage.) (e) Cross-section through corn root tip obtained as in c. Incubated in rhodamine labeled Fab anti-DHZR + 0.1 mM DHZR. Bar, 10 μ m. (f) Fluorescence micrograph of section shown in e. Fluorescence is not observed. Bar, 10 μ m. $\times 800$.



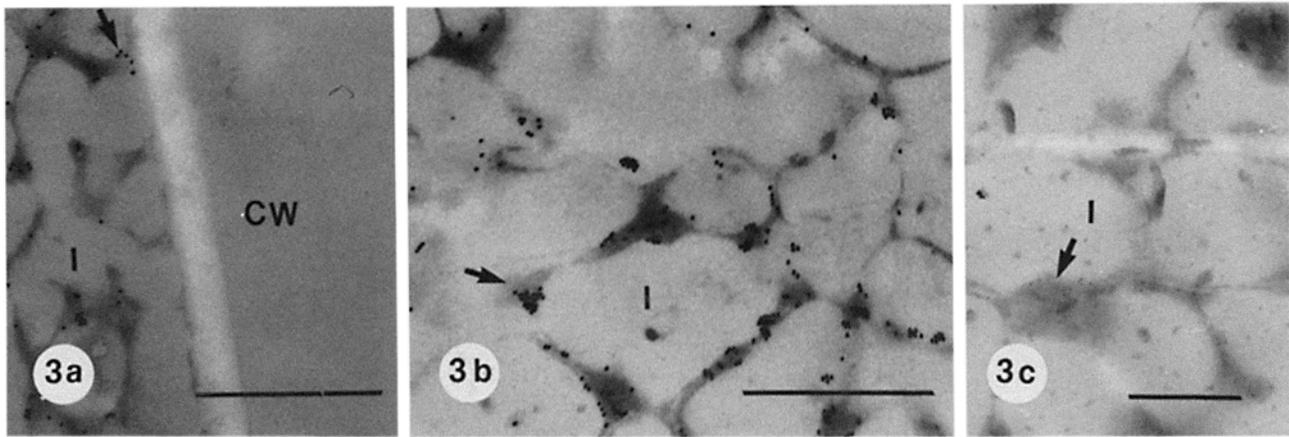


FIGURE 3 (a and b) The sample was freeze substituted at -85°C . Plastic-embedded thin section of a root incubated with anti-DHZR Fab adsorbed onto colloidal gold (arrow points to gold particles). Bar, $1.0\ \mu\text{m}$. $\times 25,000$. (c) The section was obtained as in a and b, but was preincubated with unlabeled anti-DHZR Fab. The section was then treated with anti-DHZR Fab adsorbed onto colloidal gold. Binding of particles is greatly decreased, indicating that unlabeled and gold-labeled antibodies bind to the same sites (arrow points to gold particles). Bar, $1.0\ \mu\text{m}$. $\times 15,000$.

TABLE I
Inhibition of Isopentenyladenosine Binding to Anticytokinin Antibody

Compound	Concentration for 50% inhibition (M)
(\pm)DHZR	10^{-8}
(\pm)Dihydrozeatin	10^{-7}
Kinetin	5×10^{-5}
Adenosine	2×10^{-2}
Ribose	$\gg 10^{-2}$
1-Methylguanosine	$\gg 10^{-2}$
6-Dimethylaminopurine	$\gg 10^{-2}$

In brief, the assay involves incubating $20\ \mu\text{l}$ of [^3H]isopentenyladenosine ($0.05\ \mu\text{Ci}$, $10\ \text{Ci/mmol}$, Moravek Biochemicals, Los Angeles, CA), with $80\ \mu\text{l}$ of test sample, and $100\ \mu\text{l}$ of R230 serum (1:500). After 30 min the IgG fraction is precipitated with ammonium sulfate, and the precipitate is dissolved in Formic acid and assayed on a liquid scintillation counter.

membrane, which could not always be positively identified, was not densely labeled (Fig. 3b). Labeling by gold anti-DHZR was decreased when sections were first treated with unlabeled anti-DHZR. Sections adsorbed few particles when exposed to control Fab-colloidal gold (Fig. 3c). A high background of gold aggregates occurred if samples were not first blocked with normal rabbit serum and rinsed well with filtered PBS and water.

DISCUSSION AND CONCLUSION

These results indicate that immunocytochemical techniques can be used together with low temperature histological techniques to determine the localization of phytohormones by light and electron microscopy. Labeling could be blocked by unlabeled antibody and by hapten (DHZR), demonstrating that antibodies are not adsorbed by the tissue nonspecifically. However, preincubation of sections with 1% normal rabbit serum was required to reduce background (nonspecific) labeling.

The regional specificity of the rhodamine-conjugated Fab is surprising. The unlabeled region corresponded morphologically to the quiescent center. That is, few mitotic figures are observed in this region, and it occupies an area basipetal to

the root cap initials. We conducted pilot experiments using ^{125}I -labeled antibody (data not shown). The electron microscopic autoradiographs showed silver grains predominantly in cells of the root cap and meristematic areas surrounding the quiescent center. The quiescent center contained few grains, confirming the results obtained with the fluorescent and gold-labeled probes.

Torrey (16) proposed that the quiescent center was involved in the biosynthesis of substances that promote cell division. Specifically, he suggested that the quiescent center might contain supraoptimal levels of a growth regulator which inhibited cell division. Feldman (2) has shown that the terminal 1 mm of corn roots (cv. Kelvedon 33) contains cytokinin-like substances. Since his preparations included meristematic regions surrounding the quiescent center, it is possible that these regions were the source of the cytokinin-like material. Moreover, Feldman (2) noted that cytokinin levels decreased following decapping. This and other indirect evidence (17) indicate that cytokinin biosynthesis occurs outside the quiescent center during this stage of development. Cells of the quiescent center rarely divide (18), and cytokinins are generally considered to be hormones that stimulate division (19). Thus our observations are consistent with the biology of the quiescent center. Since the association of the quiescent center with cytokinin biosynthesis may change during root development, it would be of particular interest to correlate cytokinin localization with physiological and developmental events.

The sensitivity of immunological reagents used in conjunction with low temperature histological techniques demonstrates the utility of these methods in investigating the mechanisms of plant growth regulators. To our knowledge this is the first demonstration of a cytokinin localization in situ using these techniques.

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