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Comparison of the Outer and Inner Epidermis[1]

Inhibition of Auxin-Induced Elongation of Maize Coleoptiles by Glucan Antibodies

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ABSTRACT

Polyclonal antibodies, raised against β-D-glucans prepared from oat (*Avena sativa* L.) caryopses, cross-reacted specifically with (1→3),(1→4)-β-D-glucans when challenged in a dot blot analysis of related polymers bound to a cellulose thin layer chromatography plate. The antibodies suppressed indoleacetic acid (IAA)-induced elongation of segments from maize (*Zea mays* L.) coleoptiles when the outer surface was abraded. However, IAA-induced elongation of nonabraded segments or segments with abrasion restricted to the interior of the cylinder was not influenced by the antibodies. Fab fragments prepared from the antibodies gave similar results. The capacity for IAA to overcome outward curvature of split coleoptile segments was partially reversed by treatment of the segments with the antibodies. Fluorescence microscopy revealed that antibody penetration was largely restricted to the epidermal cell wall region. These results support the view that the degradation of (1→3),(1→4)-β-D-glucans in the outer epidermal cell wall serves an essential role in auxin-induced elongation of Poaceae coleoptiles.

The prevailing hypothesis that auxin-induced elongation of plant cells is initiated by cell wall loosening mediated by the modifications of certain wall components continues to be evaluated (1, 17, 18, 20, 25). Of those wall components that constitute Poaceae cell walls, the (1→3),(1→4)-β-D-glucans are the prominent polysaccharides undergoing auxin-induced changes. Glucan metabolism has been observed during auxin-induced elongation of *Avena* (16, 23), *Hordeum* (24), *Zea* (12), and *Oryza* (26) coleoptiles. Two enzymes, an endo- and an exoglucanase, are involved in the hydrolysis of the polysaccharide in maize (*Zea mays* L.) coleoptiles (2, 9, 10). Antibodies raised against (1→3),(1→4)-β-D-glucans (6) and glucanases (8, 12, 15, 22) are effective inhibitors of auxin-induced elongation, cell wall loosening, and the degradation of the glucans in maize coleoptile segments. The data support the concept that degradation of (1→3),(1→4)-β-D-glucans by cell wall glucanases is associated with cell wall loosening responsible for auxin-induced elongation.

Plant stems are composed of a load-bearing epidermis and inner tissues with walls that appear to bear a lesser physical stress. Auxin appears to induce elongation growth through events that mediate the loosening of the thick, growth-limiting outer epidermal cell wall (13, 18). The outer epidermal cell walls appear to serve as a target of auxin action even in coleoptile cylinders where both outer and inner epidermal layers exist (14, 19). However, the relationship between the degradation of (1→3),(1→4)-β-D-glucans and the critical roles of the outer epidermal cell wall in auxin-induced elongation growth of coleoptiles has not been clarified. The present study was undertaken to address this point in maize coleoptiles using antibodies raised against (1→3),(1→4)-β-D-glucans.

MATERIALS AND METHODS

Plant Material

Maize (*Zea mays* L. cv Golden Bantam T-51) seeds were soaked in running tap water for 24 h at 30°C and then grown in wet vermiculite at 25°C in the dark for 4 d.

Preparation of Antibodies and Fab Fragments

Antiserum was raised in rabbits in which an *Avena* (*Avena sativa* L.) caryopsis β-D-glucan fraction having an average molecular weight of 1.5×10^4 was injected (6). After the serum was collected, it was applied to a Protein A-Sepharose CL-4B column (Pharmacia). Adsorbed IgG[2] was eluted with 1 N acetic acid containing 0.15 M NaCl and then neutralized with 5 N NaOH. The recovered IgG was dialyzed against PBS (10 mM sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl) and stored in capped vials at −80°C. PIS, collected before the first antigen injection, was also purified and stored as described above.

Antibody solutions were concentrated by ultrafiltration (UCE-1, Toyo Roshi). IgG preparations were hydrolyzed with immobilized papain (ImmunoPure Fab preparation kit, Pierce) at 40°C for 5 h to generate Fab and Fc fragments. The Fab fragments were separated from Fc fragments and undigested IgG with a protein A-Sepharose CL-4B column and dialyzed against PBS.

Dot Blot Analysis on a TLC Plate

(1→3),(1→4)-β-D-Glucans were purified as described previously (6). Xyloglucans were extracted from the cell wall of

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[2] Abbreviations: IgG, immunoglobulin G; PIS, preimmune serum.

etiolated epicotyls of azuki bean (*Vigna angularis* Ohwi et Ohashi) with 24% KOH and purified with a Sepharose CL-4B column (Pharmacia) followed by iodine precipitation. These polysaccharides as well as lichenan (from *Cetraria islandica*, Sigma), laminarin (from *Laminaria digitata*, United States Biochemical), pachyman (Calbiochem), and carboxymethylcellulose (Wako Pure Chemical) were applied as dots to a cellulose-coated TLC plate (Merck). The plate was incubated in a solution of PBS containing 100 μg/mL antibodies and slowly shaken for 1 h at 25°C in the dark. The plate was then washed with PBS containing 1% (v/v) Tween 20 and stained with 0.5% Coomassie brilliant blue G250 (Fluka AG) solution containing 45% methanol and 0.5% acetic acid (pH adjusted to 6.0 immediately before use) for 10 min at 25°C. In separate experiments, the plate, after incubation with the β-D-glucan antibodies, was washed and subsequently treated with horseradish peroxidase conjugates of goat anti-rabbit IgG antibodies (1:500, Sigma) and 1% BSA for 30 min at 25°C in the dark. The plate was rinsed with PBS containing 1% (v/v) Tween 20 and then stained with nitrotetrazolium blue reagent (POD Immunostain, Wako) for 10 min in the dark.

Measurement of Growth

Coleoptiles were selected when they achieved a length of 30 to 40 mm, and the first leaves were removed. The cuticle of the outer surface of some coleoptiles was abraded with a Carborundum slurry by rubbing the surface between the thumb and forefinger. The cuticle of inner surface of coleoptiles was abraded with a soft polyester filament (1-mm core with an expanded total diameter of approximately 2 mm) embedded with a Carborundum slurry. The filament was threaded through the coleoptile core and moved forward for 10 cm and then in reverse for an equal distance. Then, coleoptile segments of 15 mm were excised and floated on 10 mM Mes-KOH buffer, pH 6.0, with or without 400 μg/mL antibodies or 100 μg/mL Fab fragments for 1 h at 25°C in the dark. Antibodies or Fab fragments were injected with a syringe into the lumen of those coleoptiles subjected to inside abrasion. The segments were washed with water, and then 10-mm segments were cut from the central region. The 10-mm segments were floated on the Mes buffer with or without 10^{-5} M IAA at 25°C in the dark. The length of segments was measured with the aid of a binocular microscope (×6.3) equipped with an ocular micrometer. Each growth experiment was repeated at least twice.

Split Test

The cuticle of the outer surface of coleoptiles was abraded as described above. Subapical segments (15 mm) were excised and split longitudinally with a sharp blade for a distance of approximately 10 mm through the upper elongating region. The segments were then floated on Mes buffer with or without 10^{-5} M IAA and in the presence and absence of 400 μg/mL antibody at 25°C in the dark. The angle of outward or inward bending was measured from a photocopy of the segments taking the tangents at the extreme tips of the split halves to describe the angle with the main axis of the segments.

Fluorescence Microscopy

Abraded or nonabraded coleoptile segments were incubated in PBS containing 100 μg/mL antibodies for 1 h at 25°C in the dark and then thoroughly washed with PBS. The central region of the segments was transversely cut into thin sections with a sharp blade. In some experiments, thin sections of coleoptiles cut with a blade were incubated in the antibody solution and then washed. The thin sections were treated with fluorescein isothiocyanate-labeled goat anti-rabbit IgG antibodies (Sigma) at 100 μg/mL for 10 min. After the sections were washed with PBS, they were mounted on a glass slide and examined with the aid of an Olympus BH microscope fitted with an epifluorescence incident light attachment, as described previously (5, 7). A band-pass filter (peak at A_{490}) served as the excitation filter with a long-wave barrier filter at A_{530}.

RESULTS

Specificity of Antibodies

Polyclonal antibodies raised against *Avena* β-D-glucans specifically cross-reacted with the glucans and to some extent with lichenan dotted onto a cellulose-coated TLC plate (Fig. 1). The antibodies did not exhibit binding with laminarin,

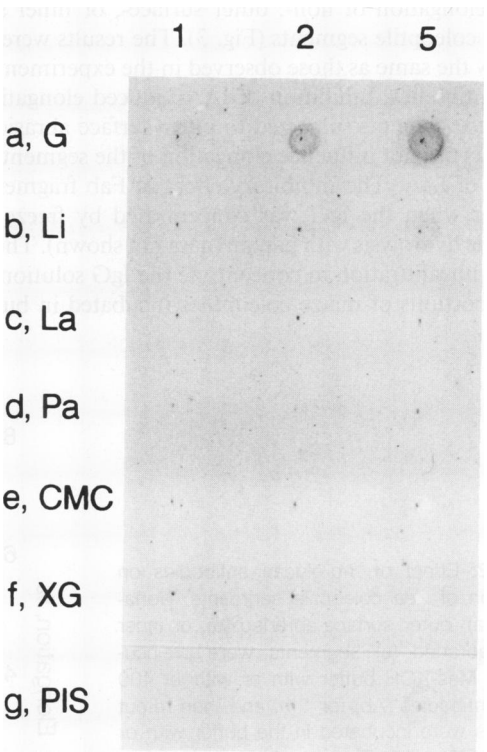

Figure 1. Dot blots for evaluating the specificity of β-D-glucan antibodies when challenged with different cell wall polysaccharides. Dots of 1, 2, and 5 μg of *Avena* β-D-glucan (a [G] and g [PIS]), lichenan (b [Li]), laminarin (c [La]), pachyman (d [Pa]), carboxymethylcellulose (e [CMC]), and azuki bean xyloglucan (f [XG]) were applied to a cellulose-coated TLC plate and then reacted with the β-D-glucan antibodies (a–f) or PIS (g). In this instance, the plate was stained with Coomassie brilliant blue solution.

pachyman, carboxymethylcellulose, or xyloglucan. Some PIS bound to the matrix when exposed to the highest concentration of β-D-glucan, but PIS did not bind to the glucan in cell walls. Antibodies used in the present study were specific for (1→3),(1→4)-β-D-glucans.

Effect of Antibodies on Elongation and Bending of Coleoptile Segments

Pretreatment of nonabraded segments of maize coleoptiles with β-D-glucan antibodies at 400 μg/mL did not influence subsequent elongation in the presence of absence of 10^{-5} M IAA (Fig. 2a). However, when the cuticle of the outer surface of coleoptiles was abraded followed by incubation of the segments in the antibody solution, IAA-induced elongation was suppressed (Fig. 2b). The suppression of elongation by the antibodies was imposed without an apparent lag period. Antibodies do not influence elongation of nonabraded coleoptile segments in the absence of IAA, and no significant suppression of elongation was observed in the presence or absence of IAA when the inner surface-abraded segments were pretreated with antibodies (Fig. 2c).

To facilitate antibody penetration into coleoptile tissues, the IgG was hydrolyzed with papain. After fragment purification, we examined the inhibitory effect of the Fab fraction, the immunological binding region of the β-D-glucan antibodies, on elongation of non-, outer surface-, or inner surface-abraded coleoptile segments (Fig. 3). The results were fundamentally the same as those observed in the experiments using intact antibodies. Inhibition of IAA-induced elongation was limited to segments subjected to outer surface abrasion. Fab fragments did not influence elongation of the segments in the absence of IAA. The inhibitory effect of Fab fragments was less clear when the IgG was concentrated by freeze drying before the hydrolysis with papain (data not shown). Therefore, we used ultrafiltration to concentrate the IgG solution.

Split portions of maize coleoptiles incubated in buffer extended outward (Fig. 4). The bending was rapid during the first hour and then continued as a function of time up to 4 h. IAA at 10^{-5} M counteracted the outward bending. The split portions responded by bending inward after 2 h in the presence of IAA (Fig. 4). The glucan antibodies did not influence the outward bending in the absence of IAA. However, antibodies at the concentration used partially reversed the effect of IAA on the bending.

Binding of Antibodies to the Cell Wall

There was no detectable PIS binding to maize coleoptile sections (Fig. 5, a and c). When thin sections of coleoptiles were incubated in the antiglucan IgG solution, the antibodies were bound to the cell wall, and fluorescence was distributed almost evenly throughout the tissue cell walls (Fig. 5b).

The penetration of antibodies into tissues was examined following incubation of coleoptile segments in the antibody solution after either the inner or outer surface was abraded. There was no evidence for the antibody penetration into nonabraded segments, although some antibodies did migrate into vascular bundles (Fig. 5d). Penetration of the antibodies into epidermal cell wall regions was clearly observed in outer surface-abraded (Fig. 5e) or inner surface-abraded segments (Fig. 5f) as a function of the appropriate abrasion treatment. Some migration of antibodies into vascular bundles was also observed in these segments.

DISCUSSION

β-D-Glucan antibodies used in the present study cross-reacted with only (1→3),(1→4)-β-D-glucans, *Avena* β-D-glucans, and lichenan bound to a cellulose-coated TLC plate (Fig. 1). These same polysaccharides were capable of precipitating the appropriate antibodies in solution, whereas laminarin, carboxymethylcellulose (6), or pachyman (our unpublished data) was ineffective. Thus, the antibodies are specific

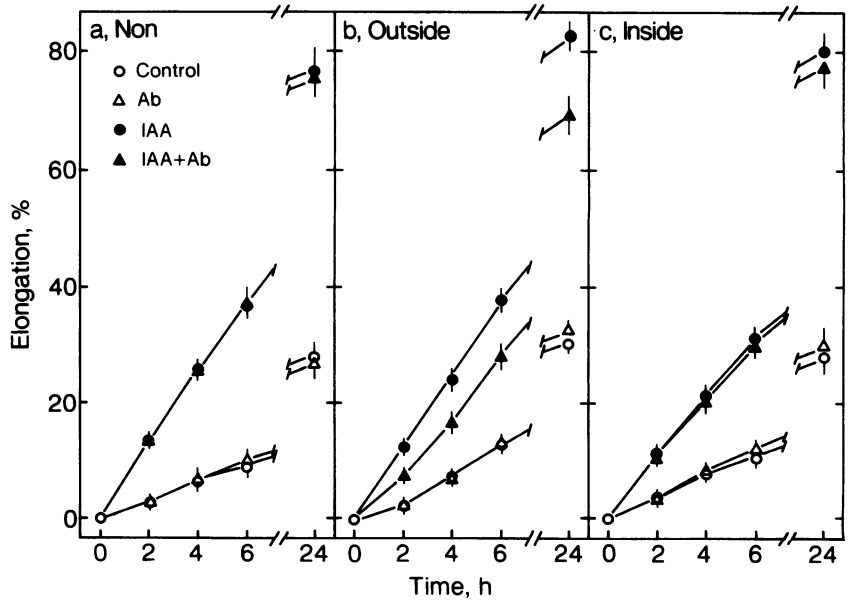

Figure 2. Effect of β-D-glucan antibodies on elongation of *Zea* coleoptile segments. Nonabraded (a), outer surface-abraded (b), or inner surface-abraded (c) segments were preincubated in Mes-KOH buffer with or without 400 μg/mL antibodies (Ab) for 1 h, and then re-cut segments were incubated in the buffer with or without 10^{-5} M IAA. Elongation is expressed as the percentage increase in segment length. Values are means ± SE (n = 20).

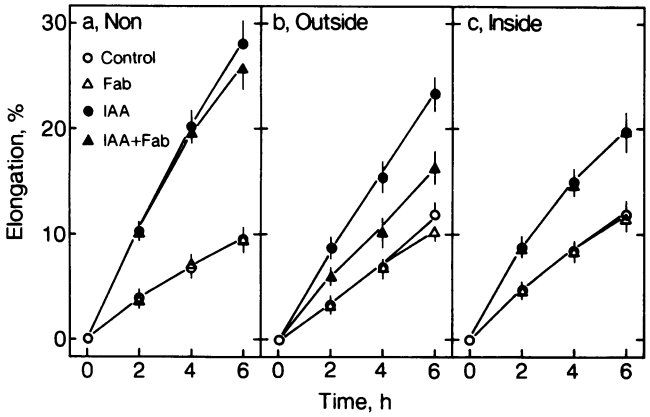

Figure 3. Effect of Fab fragments prepared from β-D-glucan antibodies on elongation of *Zea* coleoptile segments. Nonabraded (a), outer surface-abraded (b), or inner surface-abraded (c) segments were preincubated in the buffer with or without 100 μg/mL Fab fragments and then incubated as described in Figure 2. Otherwise, the experimental protocol is indicated in Figure 2.

for (1→3),(1→4)-β-D-glucans. We considered the possibility that antibodies were inadvertently raised against the cell wall proteins contaminating the polysaccharide preparation and that these antibodies contributed to suppression of IAA-induced elongation. As a precaution, proteins were removed from the antigen fraction with a hydroxyapatite column (6). Furthermore, the cell wall proteins extracted from maize coleoptile cell walls with 3 M LiCl did not precipitate the antibodies (data not shown). These results support the idea that the antibodies exhibit physiological effects by reacting with (1→3),(1→4)-β-D-glucans present in the cell wall.

The specificity of the reaction of the antibodies with polysaccharides was examined by a dot blot test on a cellulose-coated TLC plate in the present study (Fig. 1). Similar tests have been conducted using nitrocellulose papers (21) suitable for proteins. However, the binding of polysaccharides to nitrocellulose is not always reliable. At least for polysaccharides showing strong binding to cellulose, such as (1→3),(1→4)-β-D-glucans and xyloglucans, a cellulose-coated TLC plate appears to be more appropriate. The reaction of antibodies with polysaccharides is detected easily (with protein-staining dyes) or more sensitively (by immunostaining) on the plate. Cellulose papers were found to be inappropriate for the purpose, because of strong backgrounds.

Inhibition of IAA-induced elongation by (1→3),(1→4)-β-D-glucan antibodies was only effective when the antibodies were administered through the outer epidermis (Fig. 2). No significant inhibition was observed when the antibodies were introduced from the interior. Fab fragments prepared from the antibodies gave similar results (Fig. 3). These antibodies were also effective in suppression of IAA-induced cell wall loosening and degradation of (1→3),(1→4)-β-D-glucans in outer surface-abraded segments (6). Furthermore, inhibition by IAA of the outward curvature of split coleoptile segments was partly reversed by treatment with the antibodies (Fig. 4). With abrasion, antibody penetration was largely restricted to the epidermal cell walls (Fig. 5, e and f). Migration into vascular bundles does not appear to be involved in the antibody action, because migration was observed irrespective of abrasion treatment. These results support the view that the degradation of (1→3),(1→4)-β-D-glucans in the outer epidermal cell wall plays an essential role in auxin-induced elongation of Poaceae coleoptiles.

The results of fluorescence microscopy with β-D-glucan antibodies indicate that (1→3),(1→4)-β-D-glucans are present in the cell wall of not only epidermis but also parenchyma and vascular bundles of coleoptiles (7; Fig. 5b). Endo- and exoglucanases involved in degradation of the polysaccharides are also distributed throughout the tissue of *Avena* coleoptiles (8). The fluorescence due to the binding of the antibodies to the cell wall was decreased by IAA treatment almost evenly in the walls of different tissues of coleoptiles (7). Thus, the ultimate degradation of (1→3),(1→4)-β-D-glucans induced by auxin appears to occur throughout the tissue of coleoptiles. However, it is the degradation within the epidermal cell wall that may be fundamental to the initiation of auxin-induced cell elongation.

Because the inhibitory effect of antibodies on IAA-induced cell wall loosening persisted in segments even after methanol fixation before stress relaxation analysis, we conclude that residual antibodies were not responsible for directly influencing the physical properties of the tissues. Cross-linking of glucan polymers by antibodies would have been disrupted by methanol fixation. More likely, the inhibition is due to a

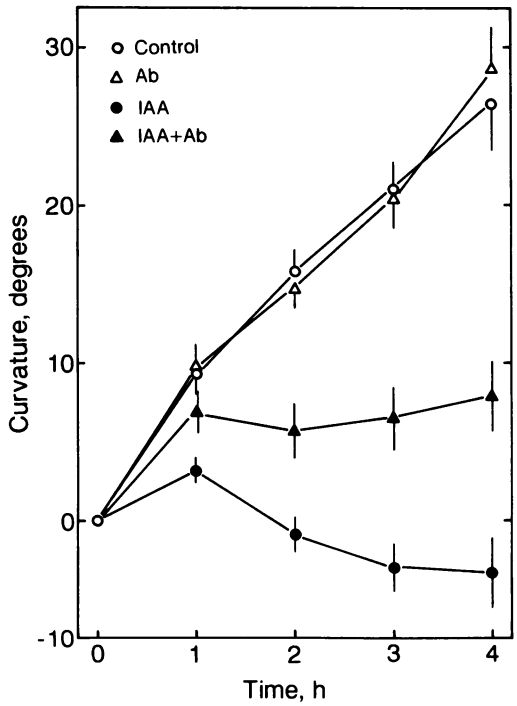

Figure 4. Bending of split *Zea* coleoptile segments as affected by β-D-glucan antibodies. Outer surface-abraded segments were split longitudinally and then incubated in the buffer with or without 10^{-5} M IAA or 400 μg/mL antibodies (Ab). Bending was measured from a photocopy, taking the tangents at the extreme tips of the split halves and determining the angle with the main axis of the segments. Values are means ± SE (n = 15).

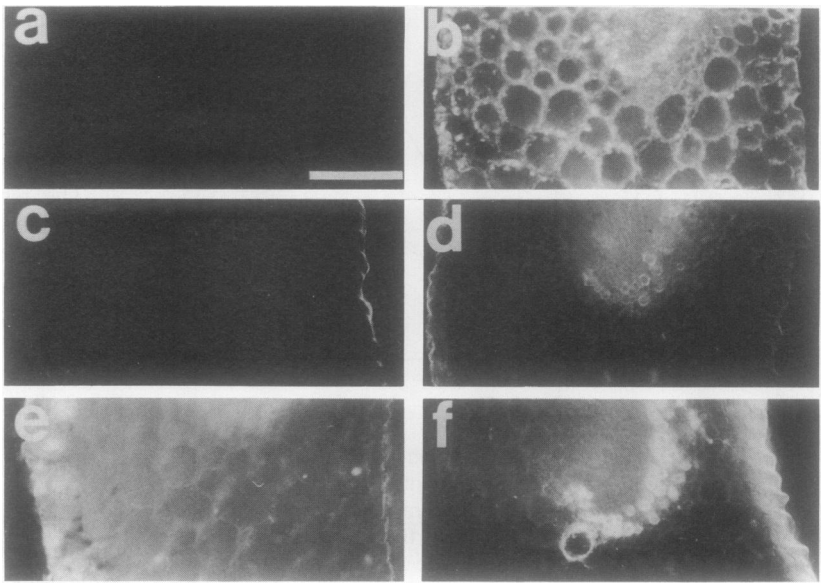

Figure 5. Fluorescence micrographs of coleoptile sections treated with β-D-glucan antibodies. Thin sections of coleoptiles incubated in PIS (a) or antibody solution (b); c through f, segments treated with antibodies after the appropriate abrasion treatment and then sectioned: c, outer surface abraded but treated with PIS; d, not abraded; e, outer surface abraded; f, inner surface abraded. All sections were treated with fluorescein isothiocyanate-labeled secondary antibodies and examined under a fluorescence microscope. The outer epidermis is located to the left side of each micrograph. Bar, 100 μm.

physical masking of the glucan, thereby suppressing its metabolism during the incubation period with IAA (6). This conclusion was supported by the observation that Fab fragments also suppressed IAA-induced elongation (Fig. 3). Fab fragments are monovalent and would not be expected to form cross-links between cell wall polymers.

Neither the β-D-glucan antibodies nor Fab fragments inhibited IAA-induced elongation of nonabraded coleoptile segments (Figs. 2 and 3). In the treatment protocol, segments are subjected to antibodies or Fab fragments before IAA treatment, and elongation of recut segments is examined in the absence of the antibodies (3, 22). Therefore, any direct action of antibodies on the physiological effects of auxin due to prevention of auxin uptake by coating of the tissue surface seems unlikely. In fact, the presence of antibodies does not inhibit [^{14}C]IAA uptake into maize coleoptile segments (data not shown).

The cause-effect relationship between auxin-induced changes in chemical structure of cell wall polysaccharides and elongation growth has been examined using lectins (4, 5), antibodies raised against cell wall polysaccharides (6), and proteins (3, 8, 11, 12, 15, 22). The results obtained in the present study further confirm the usefulness of antibodies specific for cell wall components for understanding the mechanism by which auxin regulates elongation growth of higher plants.

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