Plant Physiol. (1997) 115: 683–692

A Secreted Factor Induces Cell Expansion and Formation of Metaxylem-Like Tracheary Elements in Xylogenic Suspension Cultures of Zinnia[1]

Alison W. Roberts*, Shannon G. Donovan, and Candace H. Haigler

Department of Biological Sciences, University of Rhode Island, 10 Ranger Road, Kingston, Rhode Island 02881 (A.W.R, S.G.D.); and Department of Biological Sciences, Texas Tech University, Lubbock, Texas 79409 (C.H.H.)

Conditioned medium from mesophyll cell-suspension cultures of *Zinnia elegans* L. has striking effects on cell expansion and tracheary element differentiation when applied to cultures of freshly isolated mesophyll cells. These effects include (a) induction of early cell expansion, (b) delay in differentiation by 48 h or more, (c) reduction in the synchrony of differentiation, and (d) early formation of very large, metaxylem-like tracheary elements. Like reduced osmotic potential and buffering at pH 5.5, conditioned medium appears to have its primary effect on cell expansion. Partial characterization of the expansion-inducing factor indicates that it is heat stable, of low molecular mass, and is resistant to protease. It also binds reversibly to concanavalin A but is not adsorbed by charcoal. We suggest that the secreted factor may be an oligosaccharide involved in the coordination of cell expansion and differentiation and the regulation of the protoxylem-like to metaxylem-like transition in xylogenic suspension cultures.

In apical meristems cell expansion and cell differentiation are coordinated to give rise to highly organized tissues. For example, protoxylem TEs, which develop while surrounding cortical cells are expanding, deposit secondary cell walls in annular or helical patterns that allow the TEs to expand longitudinally in spite of the inelasticity of the secondary wall. As cell expansion ceases, developing metaxylem TEs deposit reticulate or pitted secondary cell walls that resist stretching and are also better able to withstand the pressure differential that develops between TEs and surrounding tissues during transpiration.

Correlations between patterns of cell expansion and cell and tissue differentiation have been noted in differentiating culture systems, including embryogenic carrot (*Daucus carota* L.) cell-suspension cultures (van Engelen and de Vries, 1992), tobacco (*Nicotiana tabacum* L.) thin-layer cultures (Tran Thanh Van and Mutaftschiev, 1990), and xylogenic suspension cultures (Roberts and Haigler, 1994). In developing xylem the abundance of TEs (Torrey et al., 1971) and pattern of secondary-cell-wall deposition (Brower and Hepler, 1976; O'Brien, 1981; Roberts and Haigler, 1994) are both influenced by cell expansion.

It is likely that intercellular communication plays an important role in the coordination of cell expansion and cell differentiation. In carrot suspension cultures a secreted glycoprotein with peroxidase activity both inhibits cell expansion and promotes somatic embryogenesis (Cordewener et al., 1991; van Engelen and de Vries, 1992), whereas a secreted chitinase restricts cell expansion, leading to proper development of the somatic embryo epidermis (de Jong et al., 1992). In tobacco thin-layer cultures cell expansion and organogenesis are altered by pectic fragments derived from sycamore cell walls (Tran Thanh Van et al., 1985; Eberhard et al., 1989). Hydrogen ions also have been shown to alter the patterns of cell expansion and differentiation in culture (Cousson et al., 1989, 1992; Smith and Krikorian, 1992; Roberts and Haigler, 1994).

Biologically active oligosaccharides termed "oligosaccharins" appear to play an important role in intercellular communication (for review, see Hahn et al., 1989; Aldington et al., 1991; Darvill et al., 1992; Fry et al., 1993; Cote and Hahn, 1994). Among their many activities, oligosaccharins from at least three classes are known to modulate cell expansion (Fry et al., 1993). For example, xyloglucan oligosaccharins, produced by partial hydrolysis of xyloglucan with β-(1–4)-D-glucanase (York et al., 1984) and identified in CM from spinach suspension cultures (Fry, 1986), can inhibit or stimulate cell expansion, depending on the structure and concentration (York et al., 1984; McDougall and Fry, 1989, 1990). Pectic oligosaccharides, released from cell walls by endopolygalacturonase, antagonize auxin-induced growth (Lo Schiavo et al., 1991; Filippini et al., 1992). The GalA-rhamnose disaccharide lepidimoide, which was isolated from the secretions of *Lepidium sativum* L. roots, induces hypocotyl elongation in the seedlings of *Amaranthus caudatus* L. (Hasegawa et al., 1992). Finally, an *N*-glycan isolated from CM of *Silene alba* (Miller) E.H.L. Krause has been shown to induce hypocotyl elongation at low concentrations and inhibit growth at higher concentrations in synergy with auxin (Priem et al., 1990, 1994).

Xylogenic suspension cultures derived from the mesophyll of *Zinnia elegans* L. have provided an important

[1] This work was supported by grants from the U.S. Department of Agriculture/National Research Initiative (no. 94-37311-1116) to A.W.R., the National Science Foundation (no. DCB-87-10243) to C.H.H., and the University of Rhode Island Council for Research to A.W.R.

* Corresponding author; e-mail awrobrts@uriacc.uri.edu; fax 1–401–874–5974.

Abbreviations: Con A, concanavalin A; CM, conditioned medium; EIF, expansion-inducing factor; PVPP, polyvinylpolypyrrolidone; TE, tracheary element.

model for investigating the process of TE differentiation (for review, see Fukuda, 1992). These cultures mimic many aspects of xylogenesis in planta, including the formation of protoxylem-like TEs in the early phase of culture and metaxylem-like TEs later (Falconer and Seagull, 1985). Here we describe the partial characterization of a compound, possibly an oligosaccharide, that is present in CM from *Z. elegans* suspension cultures and induces cell expansion and early formation of metaxylem-like TEs from freshly isolated mesophyll cells.

MATERIALS AND METHODS

Plant Material and Culture Technique

Isolated mesophyll cells from *Zinnia elegans* L. var Envy were cultured in 50-mL Erlenmeyer flasks or shell vials as described previously (Roberts and Haigler, 1990). Growth regulators in the xylogenic medium included 0.5 μM NAA and 0.9 μM BA. For nonxylogenic medium, the BA concentration was reduced to 0.0045 μM.

Preparation of CM

CM was prepared from cultures grown in 50-mL Erlenmeyer flasks as follows: (a) low-speed (200*g*) centrifugation or filtration of cell suspension to remove cells, (b) higher-speed centrifugation (5000*g*) of the supernatant to remove insoluble cell secretions, (c) sterile filtration (0.2 μm), and (d) storage at −20°C. For some experiments, medium was heat-treated by autoclaving (121°C) or by immersing tubes containing whole CM in a 100°C water bath for 10 min, followed by slow cooling.

Bioassay

Culture medium consisting of 1 part fresh culture medium and 1 part sterile, filtered CM was inoculated with freshly isolated *Z. elegans* mesophyll cells and incubated in 25- × 95-mm shell vials, each containing 1.75 mL of cell suspension, on a rotary shaker at 27°C in the dark for 7 d.

TE-projected cell areas were measured using a computer image analysis system consisting of a microscope (Vanox, Olympus), a video camera (Hitachi, Danbury, CT), an IBM PCAT computer, a digitizing tablet, and Microcomp planar morphometry software (Southern Micro Instruments, Atlanta, GA). Fifty cells were measured from each of three replicate cultures for each treatment. Error bars on graphs represent 95% confidence intervals. All experiments were repeated at least twice with similar results, and data were analyzed using a Student's *t* test or Duncan's multiple range test.

Secondary-cell-wall patterns were classified as described previously (Roberts and Haigler, 1994). Briefly, helical patterns were defined as having transverse bands with no apparent connecting bands, helical-reticulate patterns generally had helical patterns with forks and anastomoses, reticulate patterns were netted with no obvious transverse bands, and pitted TEs had secondary wall material covering the entire cell, except for small, nearly round pits. At least 200 cells were counted from each treatment, including three separate batches of CM. Treatments were ranked according to the percentage of cells that had metaxylem-like (reticulate or pitted) secondary cell wall patterns and analyzed statistically using the Mann-Whitney *U* test.

Molecular Mass Determination

CM was fractionated using centrifugal concentrators (Amicon, Beverly, MA) with molecular mass cutoff points of 1, 3, and 10 kD, according to the manufacturer's instructions. The retentate from each concentration run was restored to the original volume by adding fresh culture medium.

Adsorption Experiments

Con A-conjugated agarose beads (Sigma) were washed extensively with 1 M NaCl to remove thimerosol, which was used as a preservative. Beads were poured into a Pasteur pipette column (1 mL) and equilibrated with fresh culture medium. Ultrafiltered (molecular mass cutoff of 3 kD) CM (5 mL) was loaded onto the column and the eluate was filter-sterilized before the cells were added. Alternatively, we used a batch method in which CM (5 mL) was incubated with 1 mL of beads in a 12-mL conical centrifuge tube for 10 min with gentle agitation and collected by centrifugation. The Con A pellet was eluted with fresh culture medium containing 0.2 M α-methylmannopyranoside in place of mannitol. The supernatant, eluate, and medium containing α-methylmannopyranoside (as a control) were each filter-sterilized and mixed 1:1 with fresh medium for cell culture.

CM was also adsorbed (batch method) with activated charcoal or PVPP (Sigma). Charcoal was pretreated by washing extensively with distilled water and equilibrating with fresh culture medium. PVPP was pretreated by boiling in 10% HCl for 10 min (Olsson and Samuelson, 1974) followed by extensive washing with distilled water and equilibration with fresh culture medium.

Enzyme-Sensitivity Experiments

Stock solutions (100×) of enzymes were prepared in water and low-molecular-mass components, which had been determined to interfere with differentiation, were removed using centrifugal concentrators (molecular mass cutoff of 3 kD). The stock solutions were then divided into two aliquots, and one aliquot was inactivated by incubating in a boiling water bath for 30 min. Aliquots of CM, from which high-molecular-mass components had been removed using centrifugal concentrators (molecular mass cutoff of 3 kD), were incubated with active or heat-inactivated pectolyase (2 units/mL, Sigma), cellulase (1 unit/mL, Worthington, Freehold, NJ), α-mannosidase (0.2 unit/mL, from jack bean, Sigma), or trypsin (10 units/mL, Sigma) at 37°C for 0.5 to 3 h. Trypsin-treated medium was titrated to pH 7.0 with NaOH before the addition of enzyme and titrated back to pH 5.5 with HCl after enzyme treatment. Cellulase, pectolyase, and mannosidase hydrolysis was carried out at pH 5.5. Following hydrolysis, en-

zymes were removed from CM using centrifugal concentrators (molecular mass cutoff of 3 kD). Cells were cultured in enzyme-treated CM mixed 1:1 with fresh medium.

Titration and pH Measurements

Culture medium pH was measured over the time course of differentiation using an Accumet H^+/ion meter (model 925, Fisher Scientific) and a Sure-Flow semimicroelectrode (Orion Research, Boston, MA). For these experiments, 42 mL of mesophyll suspension was distributed to 24 culture tubes and the pH of two tubes was measured at each time. Aliquots (10 mL) of fresh xylogenic medium and CM were titrated with 0.1 N NaOH using the same meter and electrode.

Analysis of Total IAA

Batches of CM from independently initiated 12-mL xylogenic or nonxylogenic cultures were harvested and combined. Specifically, CM was collected from the following: three separate 4-d-old xylogenic cultures (112 mL total volume), six separate 9-d-old xylogenic cultures (265 mL total volume), and five separate 9-d-old nonxylogenic cultures (158 mL total volume). Total IAA (free IAA plus conjugated IAA) was quantitated using [^{3}H]IAA as a radioactive tracer and [$^{13}C^6$]IAA as an internal standard, as previously described (Chen et al., 1988; Cohen et al., 1992). Briefly, after evaporating in vacuo the medium was subjected to alkaline hydrolysis (3 h, 7 N NaOH, 100°C) then purified on a Prep Sep C_{18} disposable column (Fisher Scientific) and by HPLC on a C_{18} reverse-phase column. The IAA fraction was methylated and analyzed by GC-selected ion-monitoring MS (5890 series II GC/5971A MS, Hewlett Packard), according to published methods (Chen et al., 1988; Cohen et al., 1992).

RESULTS

When freshly isolated *Z. elegans* mesophyll cells were cultured in medium supplemented 1:1 with CM from 7- to 9-d-old cultures, the resulting TEs were very large compared with TEs from control cultures (Fig. 1). CM-supplemented cultures also contained a higher percentage of TEs with metaxylem-like reticulate and pitted secondary cell wall patterns (Fig. 2). Furthermore, CM delayed differentiation for up to 3 d and reduced its synchrony. The effect of CM on the appearance of TEs and the timing of differentiation is identical to that of fresh medium buffered at pH 5.5, the primary action of which is to stimulate cell expansion (Roberts and Haigler, 1994). These data indicate that CM contains an EIF that is secreted by cultured cells.

In preparation for characterizing EIF, we developed a quantitative bioassay to measure its activity. The most striking characteristic of CM-supplemented cultures is the enormous size of the TEs, so we measured the projected cell areas of TEs using computer image analysis. A size-frequency diagram for TEs from control and CM-supplemented cultures is shown in Figure 3. The data shown are representative of more than 50 experiments that included both control and CM-supplemented cultures as treatments. The mean projected cell areas of TEs in control cultures varied from 1000 to 2000 μm^2. TEs in CM-supplemented cultures were always larger than controls; the maximum mean projected cell area recorded was nearly 7000 μm^2.

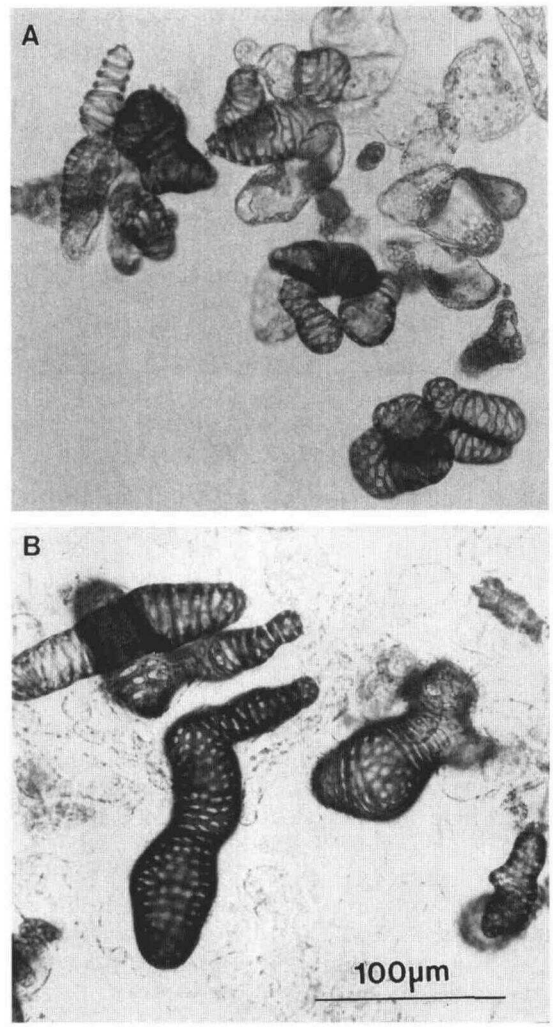

Figure 1. Comparison of TEs from 7-d-old cultures of *Z. elegans* mesophyll cells incubated in fresh xylogenic medium (A) or fresh xylogenic medium supplemented 1:1 with CM (B) from 7-d-old xylogenic cultures showing differences in size and secondary-cell-wall pattern.

The time course of accumulation of EIF activity is shown in Figure 4A. The results of four separate experiments, each of which involved the collection of CM from single batches of cultures on successive days, are summarized. Although we noted variation in activity among batches, the EIF activity usually reached a maximum at about 7 d. The time course of accumulation of EIF activity for nonxylogenic cultures (Fig. 4B) shows that secretion of the expansion inducing activity is not restricted to differentiating cultures. To gain a perspective on the range of EIF activity for CM from xylogenic and nonxylogenic cultures, we summarized the results of 26 experiments in which the mean projected cell area had been determined for control and

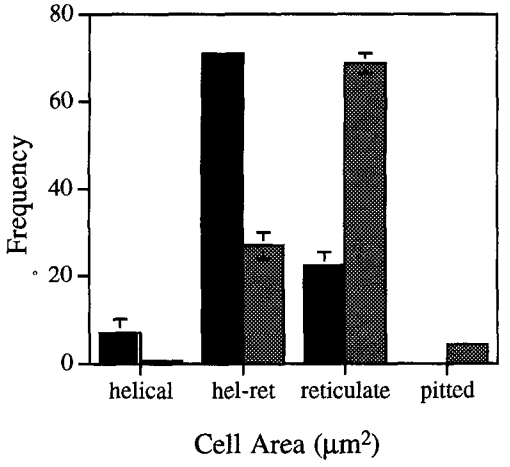

Figure 2. Percentage of TEs having each class of secondary-cell-wall pattern in 7-d-old cultures of *Z. elegans* mesophyll cells incubated in fresh xylogenic medium (black bars, $n = 4$) or fresh xylogenic medium supplemented 1:1 with CM from 7-d-old xylogenic cultures (shaded bars, $n = 5$). Error bars represent SES. CM treatments differed significantly from controls ($P < 0.01$) based on the Mann-Whitney U test.

CM-supplemented cultures (Table I). Although the mean for all experiments is smaller for CM from nonxylogenic cultures, the range of the means is strikingly similar for CM from xylogenic and nonxylogenic cultures, and Duncan's multiple range test revealed no significant difference between the two types of CM ($P < 0.01$).

Boiling or autoclaving CM did not inhibit the EIF activity (Fig. 5). In some experiments the mean projected area of TEs cultured in medium supplemented 1:1 with heat-treated CM was not significantly different from that of TEs cultured in medium supplemented 1:1 with untreated CM (Fig. 5A). In other experiments the EIF activity was enhanced by heat treatment ($P < 0.01$, Fig. 5B). This effect was observed when the untreated CM had relatively little EIF activity.

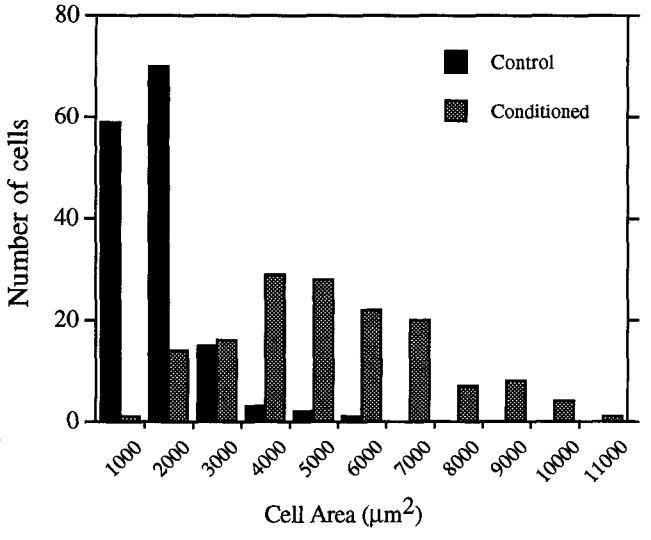

Figure 3. Frequency of occurrence of TEs according to size class in mesophyll suspension cultures containing fresh xylogenic medium or fresh xylogenic medium supplemented 1:1 with CM from 7-d-old xylogenic cultures.

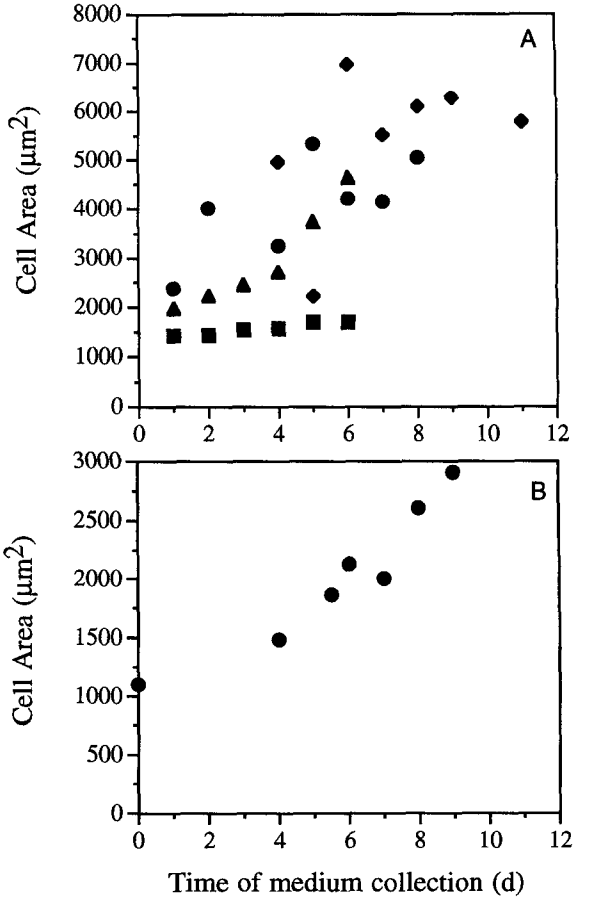

Figure 4. A, Mean projected areas of TEs from *Z. elegans* mesophyll suspensions cultured for 7 d in xylogenic medium supplemented 1:1 with CM from 1- to 11-d-old xylogenic cultures. B, Mean projected areas of TEs from *Z. elegans* mesophyll suspensions cultured in xylogenic medium supplemented 1:1 with CM from 1- to 9-d-old nonxylogenic cultures.

To estimate the molecular mass of EIF, whole CM was fractionated by sequential ultrafiltration with 10-, 3-, and 1-kD molecular mass cutoff centrifugal concentrators. Cells were cultured in fresh medium supplemented 1:1 with retentate that had been restored to its original volume with fresh medium or in fresh medium supplemented 1:1 with filtrate from each of the concentrators. As shown in Figure 6A, the mean projected cell areas of TEs from cultures supplemented with 10-, 3-, or 1-kD retentate were not significantly different from that of unsupplemented cultures. The TEs from cultures supplemented with 10- or 3-kD filtrate were not significantly different in mean projected cell areas from TEs in cultures supplemented with whole CM. A small but significant difference was noted between 1-kD filtrate and whole CM. These data indicate that the EIF most likely has a molecular mass of slightly less than 1 kD. In some experiments the filtrate enhanced cell expansion relative to whole CM (Fig.

Table I. *Summary of data from 26 experiments in which control and CM-supplemented cultures were compared*

For each treatment within an experiment, the mean projected cell area for 150 cells (50 cells from each of three replicate cultures) was calculated. Results are the means calculated from the treatment means from all experiments. Treatments with the same lowercase letter are not significantly different from each other (P >0.05) based on Duncan's multiple range comparison.

Treatment	*n*	Mean Projected Cell Area for 150 Cells			
		Mean	SE	Minimum	Maximum
			μm^2		
Control[a]	26	1778	98.2	995	2977
CM[b] (xylogenic cultures)	30	3258	249.6	1632	6189
CM[b] (nonxylogenic cultures)	10	2850	491.8	1901	7168

6B). As noted for heat-stability experiments, this enhancement was apparent when the whole CM had relatively low EIF activity.

Adsorption tests were carried out to further characterize EIF. Adsorption of CM with activated charcoal did not reduce the EIF activity (Fig. 7A). As a control, fresh medium was adsorbed with charcoal and mixed 1:1 with nonadsorbed, fresh medium. A slight increase in the mean projected cell area of TEs resulted from this treatment (data not shown). In contrast, adsorption of CM with PVPP and Con A-conjugated agarose nearly eliminated the EIF activity. In the experiment shown in Figure 7, TEs from cultures supplemented 1:1 with Con A-adsorbed CM were slightly, but significantly, larger than TEs from unsupplemented control cultures. In some replicate experiments we found no significant difference between these treatments. Con A adsorption was carried out using the column method in early experiments. We became concerned that the column was retaining the EIF by the gel-filtration effect. However, later experiments in which the batch method was used yielded similar results. When adsorbed Con A-agarose was eluted using culture medium in which mannitol had been replaced by α-methylmannopyranoside, the eluate contained the EIF activity (Fig. 7B). The α-methylmannopyranoside alone did not induce cell expansion when added to fresh culture medium. To observe the effect of eluate on cell size, it was necessary to reduce the volume of eluent to one-half the volume of CM with which the Con A had been adsorbed.

The sensitivity of EIF activity to enzymes that hydrolyze proteins and polysaccharides was tested by culturing cells in fresh medium supplemented with CM that had been incubated with active or heat-inactivated enzymes. In initial experiments both active and inactive enzymes inhibited cell differentiation. However, removal of the low-molecular-mass components of the enzyme mixtures using centrifugal concentrators eliminated much of this inhibitory activity. As shown in Figure 8, EIF was insensitive to hydrolysis by cellulase and trypsin. Some reduction in the effectiveness of EIF was seen with mannosidase, but longer incubations of CM with the enzyme resulted in inhibition of differentiation. When cultures were supplemented 1:1 with CM that had been hydrolyzed with pectolyase, differentiation was inhibited and the proportion of dead cells increased. A toxic factor was also produced by pectolyase hydrolysis of the high-molecular-mass fraction (molecular mass cutoff of 3 kD) of CM (data not shown).

The similarity between the effects of CM and medium buffered with Mes (Roberts and Haigler, 1994) raises the possibility that the EIF acts as a buffer. To test this, three batches of fresh medium and four batches of CM from independent 7-d-old cultures were titrated with NaOH over a pH range of 4.0 to 9.0. As shown in Figure 9, CM has less buffering capacity than fresh medium, probably because of a reduction in the concentration of phosphate, which is the predominant buffer in fresh medium. Furthermore, pH fluctuations in control and CM-supplemented

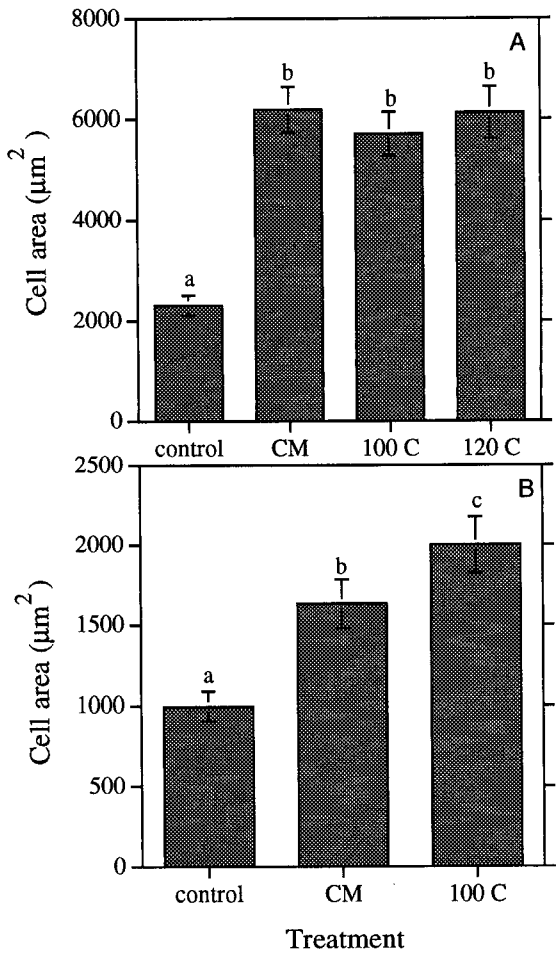

Figure 5. Results of two experiments showing mean projected areas of TEs in *Z. elegans* mesophyll suspensions cultured for 7 d in fresh xylogenic medium (control), xylogenic medium supplemented 1:1 with CM, xylogenic medium supplemented 1:1 with CM heated to 100°C in a boiling water bath (100 C), or xylogenic medium supplemented 1:1 with CM heated to 120°C in an autoclave for 10 min (120 C). Note that the CM used for the experiment shown in B had reduced expansion-inducing activity compared with that used for the experiment reported in A and that heating appears to have activated the EIF. Means of treatments with the same lowercase letter are not significantly different from each other (P > 0.01) based on a Student's *t* test.

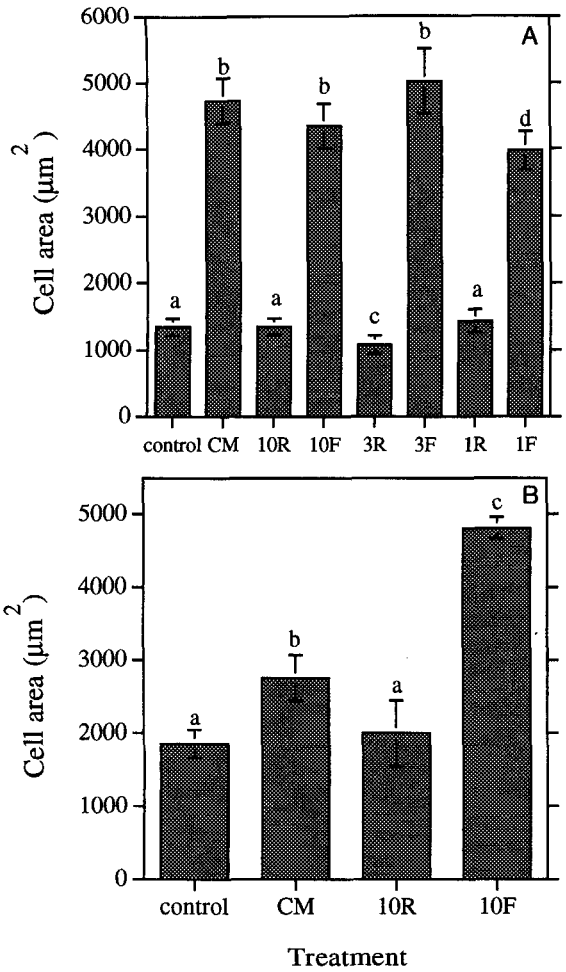

Figure 6. Mean projected areas of TEs from *Z. elegans* mesophyll suspension cultures containing fresh xylogenic medium (control) or xylogenic medium supplemented 1:1 with whole CM or with the filtrate (F) or volume-adjusted retentate (R) from sequential ultrafiltrations using membranes with molecular mass cutoffs of 10 kD (10), 3 kD (3), and 1 kD (1) (A) or ultrafiltration using a membrane with a 10-kD molecular mass cutoff (B). Note that the CM used for the experiment shown in Figure 5B had reduced expansion-inducing activity compared with that used for the experiment reported in Figure 5A and that removal of high-molecular-mass components appears to have activated the EIF. Means of treatments with the same lowercase letter are not significantly different from each other ($P > 0.01$) based on a Student's *t* test.

cultures were very similar, including an initial increase in pH, followed by a decrease in pH prior to the onset of differentiation and a subsequent increase in pH (Fig. 10). Delay of the final increase in pH corresponds with delayed onset of differentiation in CM-supplemented cultures.

We have also tested the possibility that the secreted IAA is responsible for the effect. Addition of IAA to fresh medium at concentrations ranging from 1 to 50 μM did not mimic the effect of CM and higher concentrations were toxic (data not shown). Analysis of the IAA content of CM by GC-selected-ion-monitoring MS showed that the concentration of total IAA (free and conjugated) in 4-d xylogenic, 9-d xylogenic, and 9-d nonxylogenic cultures was 0.18, 0.38, and 5.75 ng/mL, respectively.

DISCUSSION

In a growing plant the pattern of secondary cell wall deposition in developing primary xylem TEs "reflects the rate of extension taking place" within the organ (O'Brien, 1981), i.e. protoxylem TEs with annular or helical secondary cell wall thickenings differentiate while the organ is still expanding, whereas metaxylem TEs with reticulate or pitted secondary-cell-wall thickenings differentiate after organ expansion has ceased. This coordination of cell expansion and TE development has important functional

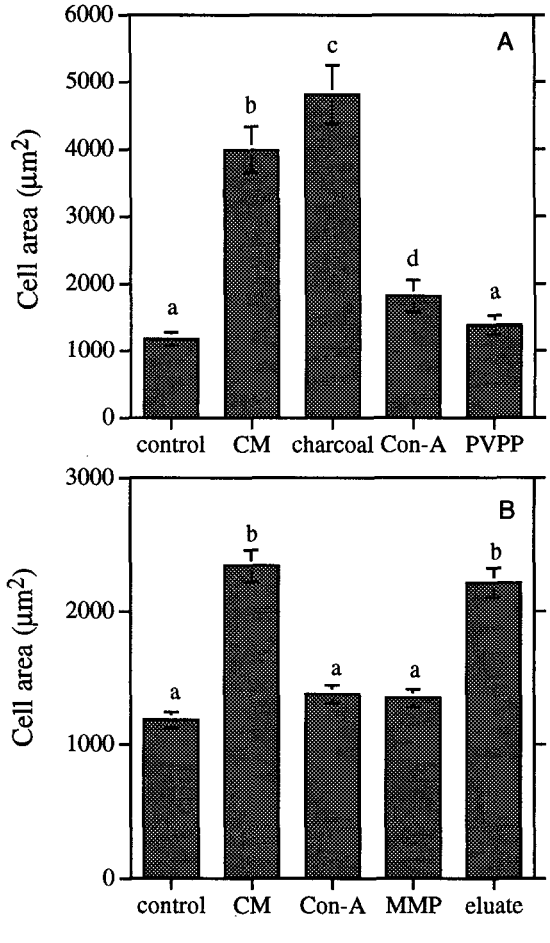

Figure 7. A, Mean projected areas of TEs from *Z. elegans* mesophyll suspension cultures containing fresh xylogenic medium (control) or xylogenic medium supplemented 1:1 with CM or CM adsorbed with activated charcoal, con-A-conjugated agarose beads, or PVPP. B, Comparison of mean projected areas of TEs from *Z. elegans* mesophyll suspension cultures containing xylogenic medium supplemented 1:1 with CM adsorbed with Con A-agarose, xylogenic medium in which mannitol is replaced by α-methylmannopyranoside (MMP), and xylogenic medium supplemented 1:1 with the α-methylmannopyranoside eluate from the adsorbed Con A-agarose. Means of treatments with the same lowercase letter are not significantly different from each other ($P > 0.01$) based on a Student's *t* test.

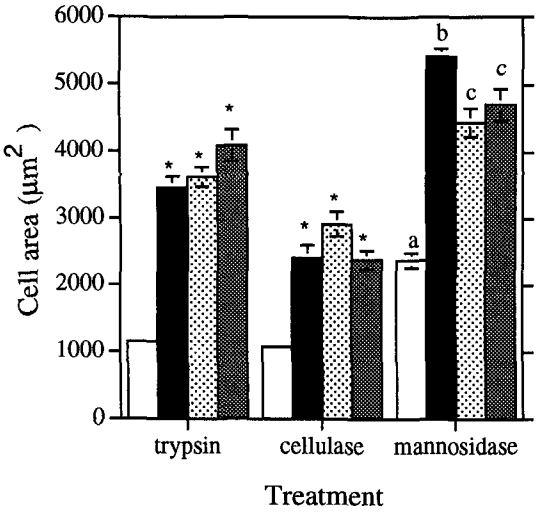

Figure 8. Mean projected areas of TEs from *Z. elegans* mesophyll suspension cultures containing xylogenic medium (open bars), xylogenic medium supplemented 1:1 with CM (black bars), or xylogenic medium supplemented 1:1 with CM treated with active (stippled bars) or heat-denatured (shaded bars) trypsin, cellulase, or mannosidase. Means of treatments marked with an asterisk (*) differ significantly from the control but not from each other (trypsin and cellulase). Means of treatments with the same lowercase letter are not significantly different from each other ($P > 0.01$) based on a Student's *t* test (mannosidase).

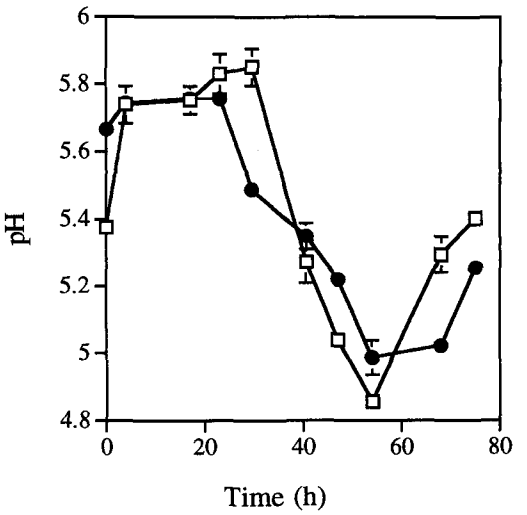

Figure 10. Time course of pH changes in *Z. elegans* mesophyll suspensions cultured in xylogenic medium (open symbols) or xylogenic medium supplemented 1:1 with CM from 9-d-old cultures (closed symbols). Each point represents the mean of two independent cultures from the same isolate and error bars represent the range. This experiment was repeated four times with similar results.

implications and is likely to be tightly regulated. Xylogenic *Z. elegans* mesophyll suspension cultures provide a unique model for investigating the relationship between cell expansion and the pattern of secondary-cell-wall deposition. TE differentiation in these cultures normally occurs in two "bursts" (Falconer and Seagull, 1985). The first burst at 48 h is fairly synchronous and produces small TEs with protoxylem-like secondary cell walls. The second burst at 6 to 7 d is less synchronous and produces large TEs with metaxylem-like secondary cell walls. Here we have shown that supplementing cultures with CM promotes cell expansion, suppresses the first burst of protoxylem-like TE differentiation, and accelerates the formation of metaxylem-like TEs. The data presented here indicate that a CM component, which we have designated EIF, is a potential candidate for a novel intercellular signal involved in the coordination of cell expansion and the protoxylem-to-metaxylem transition. Specifically, we propose that (a) EIF stimulates expansion of incipient TEs and, as shown previously (Roberts and Haigler, 1994), (b) cell size, particularly cell diameter, at the time of TE differentiation affects the pattern of secondary-cell-wall deposition.

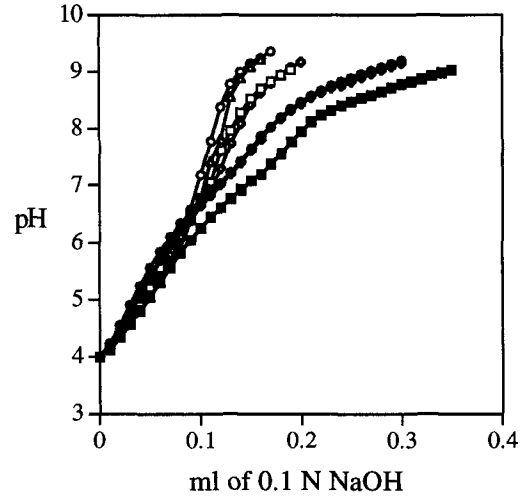

Figure 9. Titration curves for three batches of fresh medium (closed symbols) and four batches of CM from independent 7-d-old cultures (open symbols) titrated with NaOH.

Realizing its potential importance for the coordination of protoxylem and metaxylem development, we were initially intrigued by the possibility that a factor secreted by developing TEs could influence the morphology of TEs that differentiate subsequently. However, EIF is apparently not a product of developing TEs because (a) CM from nonxylogenic cultures contains the activity; (b) EIF activity increases markedly after 4 d, and at this point the first-formed TEs have autolyzed and the remaining living cells are dividing and expanding rapidly; and (c) we have noted that cultures with exceptionally high differentiation percentages in the first burst have lower EIF activities (these cultures contain fewer living cells after the first 72 h of culture, which may result in the production of less EIF). These observations lead us to conclude that EIF is most likely a product of dividing or expanding cells. This conclusion does not weaken the argument that EIF is involved in the protoxylem-to-metaxylem transition. Instead, it implies that chemical signals controlling TE development may be passed to TE initials from adjacent parenchyma cells. These signals may coordinate protoxylem and metaxylem development by relaying information related to the rate of organ expansion.

Whereas the transition from protoxylem formation to metaxylem formation must be coordinated with longitudinal expansion if organ development is to be optimized, regulation of the radial expansion of TE initials has important implications with regard to efficiency and stress resistance of water conductance. Aloni (1992) attributed the increase in vessel diameter from leaves to roots, the large diameters of earlywood vessels in ring-porous wood, and the small diameters of vessels in auxin-overproducing plants to gradients in auxin concentration. According to this hypothesis, the higher auxin concentrations occurring in the upper portions of trees in the late part of the growing season and in overproducing plants induce rapid differentiation and the formation of small TEs. When auxin concentrations are low, differentiation is delayed and TE initials enlarge prior to depositing a secondary cell wall. It is generally (although not universally) true that metaxylem vessels have greater diameters than protoxylem vessels. However, the hypothesis of Aloni (1992) does not adequately explain this relationship. Although metaxylem initials are separated from the shoot apex by a greater distance than protoxylem initials at their time of differentiation, an additional mechanism must be invoked to explain why the differentiation of metaxylem elements is delayed relative to protoxylem elements, since incipient protoxylem and metaxylem TEs would occupy the same position in the shoot-to-root auxin gradient while protoxylem elements differentiate. A potential explanation is that metaxylem initials remain sensitive to EIF, resulting in continued expansion, delayed differentiation, and larger final TE diameters. In many plants active control over the diameters of incipient TEs is implied, since xylem vessels have much smaller or larger diameters than adjacent parenchyma cells. Positive control over cell enlargement via EIF may augment the role of auxin, which is proposed to influence TE differentiation directly and play a more passive role with respect to radial cell enlargement (Aloni, 1992).

As we have described, a secreted factor in CM has dramatic effects on cell expansion and TE differentiation in *Z. elegans* mesophyll suspension cultures. Determining whether EIF is present or plays an active role in xylogenesis in planta will require further investigation. To guide future research, we propose the following model for a dual role of auxin and EIF in coordinating cell expansion and the pattern of secondary-cell-wall deposition: (a) TE initials are stimulated to expand by positive regulatory factors such as EIF; concentration of and sensitivity to these factors determine the extent of expansion of a particular TE initial. (b) TE initials initiate secondary-cell-wall deposition in response to high auxin concentration or increased sensitivity to auxin. (c) As long as a TE initial continues to expand, secondary-cell-wall deposition will not commence. Cessation of expansion and stimulation of secondary-cell-wall development may be regulated by factors such as decreased availability of or sensitivity to EIF and increased availability of or sensitivity to auxin. (d) Cell dimensions at the time of TE differentiation influence the pattern of secondary-cell-wall deposition.

Many aspects of this model remain to be clarified. For example, we have proposed an inhibitory effect of cell expansion on TE differentiation (Roberts and Haigler, 1994), but it is not clear whether secondary-cell-wall deposition is antagonized by longitudinal cell expansion, radial cell expansion, or both. It is also unknown whether the pattern of secondary-cell-wall deposition is influenced by the expansion history of the TE initial or its shape at the time of differentiation.

Partial characterization of the EIF from *Z. elegans* mesophyll suspension cultures has shown that it is heat stable to 121°C, passes through a 1000-kD molecular mass cutoff ultrafiltration membrane, and is resistant to hydrolysis by trypsin. These characteristics indicate that the factor is not a protein. The EIF activity was not adsorbed by activated charcoal, which has strong affinity for uncharged aromatic compounds (Hassler, 1974), especially those that are hydrophobic (Walters and Luthy, 1984) and of low molecular mass (Kilduff et al., 1996). This indicates that the factor is unlikely to be a phenolic. Although phenolic-binding PVPP did adsorb EIF, PVPP has affinity for other compounds, such a carboxylic acids, that can form hydrogen bonds by donating a proton (Olsson and Samuelson, 1974). Finally, adsorption of EIF by Con A indicates that the factor contains α-glucosyl or α-mannosyl residues. The activity was eluted with α-methylmannopyranoside, indicating that the adsorption is reversible. Adsorption by Con A was not complete, as indicated by (a) occasional incomplete reversal of the EIF activity following adsorption and (b) the need to reduce the volume of the eluent relative to the volume of CM adsorbed to demonstrate activity. CM contains large amounts of mannitol, Suc, and probably other secreted oligosaccharides. These substances may have interfered with the complete adsorption of EIF.

Biologically active oligosaccharides previously identified in conditioned plant cell culture media include xyloglucan oligosaccharides isolated from suspension cultures of spinach (Fry, 1986), unconjugated *N*-glycans isolated from CM of *Silene alba* (Priem et al., 1990, 1994), and a pectic oligosaccharide from tobacco suspension cultures (Schroder and Knoop, 1995). Unlike the pectic oligosaccharide described by Schroder and Knoop (1995), EIF is completely adsorbed by PVPP. Experiments aimed at further characterizing EIF by testing its sensitivity to glycosidases were difficult to interpret. Whole-CM as well as high- and low-molecular-mass fractions (<3-kD molecular mass cutoff) that had been hydrolyzed with pectolyase inhibited differentiation and decreased cell viability when added to cultures of freshly isolated cells. Pectins are commonly found in conditioned suspension culture media (Fry, 1980; Kikuchi et al., 1995), including that of *Z. elegans* (Stacey et al., 1995), and some fragments are known to be involved in the hypersensitive response (Hahn et al., 1989). It is possible that large pectic fragments, cleaved into biologically active fragments by the enzyme, were responsible for the inhibitory and toxic effects of the pectolyase hydrolysis. Long incubations of CM with mannosidase also produced toxic products. As a result, it was not possible to draw conclusions about the structure of EIF based on its sensitivity to pectolyase and mannosidase.

Other possibilities for the nature of the EIF activity that are excluded by our data include: (a) an increase in the buffering capacity of the medium, (b) changes in the auxin concentration of the medium, and (c) depletion of one or more components of the medium. We have shown by titration that the buffering capacity of CM is reduced compared with fresh medium and that pH fluctuations in CM-supplemented cultures are similar to those in control cultures. Addition of IAA, NAA, or 2,4-D does not mimic the effect of CM, and when CM was tested for the presence of total secreted IAA, only physiologically insignificant levels were detected. The highest detected concentration was 5.75 ng/mL or 32.8 nM; in comparison, the auxin-binding protein ABP1 has a K_D IAA of 3.9 μM (Jones, 1994). Depletion of hormones or any other medium component can be ruled out, since activity was present when EIF was eluted from Con A into full-strength, fresh xylogenic medium.

It is clear that the expansion-inducing potency of individual batches of CM can vary substantially. In some cases this variation appears to be related to the density of dividing and expanding cells in the culture, as explained above. However, we have not ruled out the possibility that the other culture variables, such as subtle changes in pH, may influence EIF secretion or stability. An alternative explanation for variable EIF potency is variation in the expansion-inhibiting activity of different batches of CM. The observation that the potency of CM can sometimes be increased by heating or removal of high-molecular-mass components is consistent with the presence of a heat-labile, high-molecular-mass, expansion-inhibiting factor in some cultures. Alternatively, a heat-labile, high-molecular-mass factor that destroys or reduces the activity of EIF may be present. Either way, these observations suggest the possibility that cell expansion and differentiation may be regulated by more than one secreted factor.

ACKNOWLEDGMENTS

For the IAA analyses, we thank Drs. Janet Slovin and David M. Ribnicky for supervision and contributed expertise and Dr. Jerry Cohen (U.S. Department of Agriculture-Agricultural Research Service, Beltsville, MD) for the use of his laboratory. We thank Ralph Vaughn and Sue Pujari for technical assistance.

Received March 17, 1997; accepted June 20, 1997.

LITERATURE CITED

Aldington S, McDougall GJ, Fry SC (1991) Structure-activity relationships of biologically active oligosaccharides. Plant Cell Environ **14:** 625–636

Aloni R (1992) The control of vascular differentiation. Int J Plant Sci Suppl **153:** S90–S92

Brower DL, Hepler PK (1976) Microtubules and secondary wall deposition in xylem: the effects of isopropyl *N*-phenylcarbamate. Protoplasma **87:** 91–111

Chen K-H, Miller AN, Patterson GW, Cohen JD (1988) A rapid procedure for the purification of indole-3-acetic acid prior to GC-MS-SIM analysis. Plant Physiol **86:** 822–825

Cohen JD, Slovin JP, Bialek K, Sutter EG, Baldi BF, Michalczuk L, Ribjicky DM, Tam YY, Cooke TJ (1992) Stable isotope techniques for the analysis of indole auxin metabolism in normal and mutant plants. *In* CM Karssen, LC van Loon, D Vreugdenhil, eds, Progress in Plant Growth Regulation. Kluwer Academic, Dordrecht, The Netherlands, pp 859–873

Cordewener J, Booij H, van der Zandt H, van Engelen F, van Kammen A, de Vries S (1991) Tunicamycin-inhibited carrot somatic embryogenesis can be restored by secreted cationic peroxidase isoenzymes. Planta **184:** 478–486

Cote F, Hahn MG (1994) Oligosaccharins: structures and signal transduction. Plant Mol Biol **26:** 1379–1411

Cousson A, Toubart P, Tran Thanh Van K (1989) Control of morphogenetic pathways in thin cell layers of tobacco by pH. Can J Bot **67:** 650–654

Cousson A, Tran Thanh Van K, Trinh TH (1992) Changes with time in the H+ concentration of the culture medium influences morphogenesis in tobacco thin cell layers. Physiol Plant **85:** 102–110

Darvill A, Augur C, Bergmann C, Carlson RW, Cheong J-J, Eberhard S, Hahn MG, Lo V-M, Marfa V, Meyer B, and others (1992) Oligosaccharins–oligosaccharides that regulate growth, development and defence responses in plants. Glycobiology **2:** 181–198

de Jong AJ, Cordewener J, Lo Schiavo F, Terzi M, Vandekerckhove J, van Kammen A, de Vries SC (1992) A carrot somatic embryo mutant is rescued by chitinase. Plant Cell **4:** 425–433

Eberhard S, Doubrava N, Marfa V, Mohnen D, Southwick A, Darvill A, Albersheim P (1989) Pectic cell wall fragments regulate tobacco thin-cell-layer explant morphogenesis. Plant Cell **1:** 747–755

Falconer MM, Seagull RW (1985) Immunofluorescent and calcofluor white staining of developing tracheary elements in *Zinnia elegans* L. suspension cultures. Protoplasma **125:** 190–198

Filippini F, Lo Schiavo F, Terzi M, Branca C, Bellincampi D, Salvi G, Desiderio A, de Lorenzo G, Cervone F (1992) Phytoalexin elicitor-active α-1,4-D-oligogalacturonides reduce auxin perception by plant cells and tissues. *In* CM Karrsen, LC van Loon, D Vreugdenhil, eds, Progress in Plant Growth Regulation, Vol 13. Kluwer Academic, Dordrecht, The Netherlands, pp 517–521

Fry SC (1980) Gibberellin-controlled pectinic acid and protein secretion in growing cells. Phytochemistry **19:** 735–740

Fry SC (1986) In-vivo formation of xyloglucan nonasaccharide: a possible biologically active cell-wall fragment. Planta **169:** 443–453

Fry SC, Aldington S, Hetherington PR, Aitken J (1993) Oligosaccharides as signals and substrates in the plant cell wall. Plant Physiol **103:** 1–5

Fukuda H (1992) Tracheary element formation as a model system of cell differentiation. Int Rev Cytol **136:** 289–332

Hahn MG, Bucheli P, Cerone F, Doares SH, O'Neill RA, Darvill A, Albersheim P (1989) Roles of cell wall constituents in plant-pathogen interations. *In* T Kosuge, EW Nester, eds, Plant-Microbe Interactions. Molecular and Genetic Perspectives, Vol 3. McGraw-Hill, New York, pp 131–238

Hasegawa K, Mizutani J, Kosemura S, Yamamura S (1992) Isolation and identification of lepidimoide, a new allelopathic substance from mucilage of germinated cress seeds. Plant Physiol **100:** 1059–1061

Hassler JW (1974) Purification with Activated Carbon: Industrial, Commercial, Environmental. Chemical Publishing, New York, p 390

Jones AM (1994) Auxin-binding proteins. Annu Rev Plant Physiol Plant Mol Biol **45:** 393–420

Kikuchi A, Satoh S, Nakamura N, Fujii T (1995) Differences in pectic polysaccharides between carrot embryogenic and non-embryogenic calli. Plant Cell Rep **14:** 279–284

Kilduff JE, Karanfil T, Weber WJ Jr (1996) Competitive interactions among components of humic acids in granular activated carbon adsorption systems: effects of solution chemistry. Environ Sci Technol **30:** 1344–1351

Lo Schiavo F, Filippini F, Cozzani F, Vallone D, Terzi M (1991) Modulation of auxin-binding proteins in cell suspensions. I. Differential responses of carrot embryo cultures. Plant Physiol **97:** 60–64

McDougall GJ, Fry SC (1989) Anti-auxin activity of xyloglucan oligosaccharides: the role of groups other than the terminal α-1-fucose residue. J Exp Bot **40:** 233–238

McDougall GJ, Fry SC (1990) Xyloglucan oligosaccharides promote growth and activate cellulase: evidence for a role of cellulase in cell expansion. Plant Physiol **93:** 1042–1048

O'Brien TP (1981) The primary xylem. *In* JR Barnett, ed, Xylem Cell Development, Castle Publishing, Turnbridge Well, UK, pp 14–46

Olsson L, Samuelson O (1974) Chromatography of aromatic acids and aldehydes and phenols on cross-linked polyvinylpyrrolidone. J. Chromatogr **93:** 189–199

Priem B, Morvan H, Gross KC (1994) Unconjugated N-glycans as a new class of plant oligosaccharins. Biochem Soc Trans **22:** 398–402

Priem B, Morvan H, Hafez AMA, Morvan C (1990) Influence d'un xylomannoside d'origine vegetal sur l'elongation de l'hypocotyle de lin. CR Acad Sci **311(III):** 411–416

Roberts AW, Haigler CH (1990) Tracheary-element differentiation in suspension-cultured cells of *Zinnia* requires uptake of extracellular Ca^{2+}. Experiments with Ca-channel blockers and calmodulin inhibitors. Planta **180:** 502–509

Roberts AW, Haigler CH (1994) Cell expansion and tracheary element differentiation are regulated by extracellular pH in mesophyll cultures of *Zinnia elegans* L. Plant Physiol **105:** 699–706

Schroder R, Knoop B (1995) An oligosaccharide growth factor in plant suspension cultures: a proposed structure. J Plant Physiol **146:** 139–147

Smith DL, Krikorian AD (1992) Low external pH prevents cell elongation but not multiplication of embryogenic carrot cells. Physiol Plant **84:** 495–501

Stacey NJ, Roberts K, Carpita NC, Wells B, McCann MC (1995) Dynamic changes in cell surface molecules are very early events in the differentiation of mesophyll cells from *Zinnia elegans* into tracheary elements. Plant J **6:** 891–906

Torrey JG, Fosket DE, Hepler PK (1971) Xylem formation: a paradigm of cytodifferentiation in higher plants. Am Sci **59:** 338–352

Tran Thanh Van K, Mutaftschiev S (1990) Signals influencing cell elongation, cell enlargement, cell division and morphogenesis. *In* HJJ Nijkamp, LHW Van der Plas, J Van Aartrijk, eds, Progress in Plant Cellular and Molecular Biology. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 514–519

Tran Thanh Van K, Toubart P, Cousson A, Darvill AG, Gollin DJ, Chelf P, Albersheim P (1985) Manipulation of the morphogenetic pathways of tobacco explants by oligosaccharins. Nature **314:** 615–617

van Engelen FA, de Vries SC (1992) Extracellular proteins in plant embryogenesis. Trends Genet **8:** 66–70

Walters RW, Luthy RG (1984) Equilibrium adsorption of polycyclic aromatic hydrocarbons from water onto activated carbon. Environ Sci Technol **18:** 395–403

York WS, Darvill AG, Albersheim P (1984) Inhibition of 2,4-dichlorophenoxyacetic acid stimulated elongation of pea stem segments by a xyloglucan oligosaccharide. Plant Physiol **75:** 295–297