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An Arabinogalactan Protein(s) is a Key Component of a Fraction that Mediates Local Intercellular Communication Involved in Tracheary Element Differentiation of Zinnia Mesophyll Cells

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Local intercellular communication is involved in tracheary element (TE) differentiation of zinnia (*Zinnia elegans* L.) mesophyll cells and mediated by a proteinous macromolecule, which was designated xylogen. To characterize and isolate xylogen, a bioassay system to monitor the activity of xylogen was developed, in which mesophyll cells were embedded in microbeads of agarose gel at a low (2.0–4.3×10^4 cells ml^{-1}) or high density (8.0–9.0×10^4 cells ml^{-1}) and microbeads of different cell densities were cultured together in a liquid medium to give a total density of 2.1–2.5×10^4 cells ml^{-1}. Without any additives, the frequency of TE differentiation was much smaller in the low-density microbeads than in the high-density microbeads. This low level of TE differentiation in the low-density microbeads was attributable to the shortage of xylogen. When cultures were supplemented with conditioned medium (CM) prepared from zinnia cell suspensions undergoing TE differentiation, the frequency of TE differentiation in the low-density microbeads increased remarkably, indicating the activity of xylogen in the CM. The xylogen activity in CM was sensitive to proteinase treatments. Xylogen was bound to galactose-specific lectins such as *Ricinus communis* agglutinin and peanut agglutinin, and precipitated by β-glucosyl Yariv reagent. These results indicate that xylogen is a kind of arabinogalactan protein.

Key words: Arabinogalactan protein — Local intercellular communication — Tracheary element differentiation — Xylogen — *Zinnia elegans*.

Abbreviations: AGP, arabinogalactan protein; βGlcY, β-glucosyl Yariv reagent; Con A, concanavalin A; CM, conditioned medium; HMWF, high-molecular-weight fraction; LMWF, low-molecular-weight fraction; PNA, peanut agglutinin; PHA, *Phaseolus vulgaris* agglutinin; RCA, *Ricinus communis* agglutinin; TE, tracheary element; UEA, *Ulex europaeus* agglutinin; WGA, wheat germ agglutinin.

Introduction

Recent studies in developmental biology have demonstrated that positional information generated through intercellular communications often plays a key role in controlling cell differentiation and pattern formation. In several multicellular organisms, such intercellular communications have been well characterized and secretory molecules mediating these communications have been identified (Strigini and Cohen 1999, Tickle 1999). Plants also exploit intercellular communications and the resultant positional information to regulate cell differentiation, which have been indicated by laser ablation experiments (Van Den Berg et al. 1995, Berger et al. 1998, Bouget et al. 1999) and clonal analyses (Kidner et al. 2000). However, chemical bases of intercellular communications in plants remain to be elucidated.

Tracheary element (TE) differentiation of isolated mesophyll cells of zinnia have been investigated as a model of plant cell differentiation (Fukuda 1997). The in-vitro culture system of zinnia cells, in which about half of the isolated mesophyll cells are induced to transdifferentiate into TEs in a liquid medium supplemented with adequate concentrations of auxin and cytokinin, offers unique opportunities for characterizing intercellular communications involved in TE differentiation. For the quantitative analysis of such intercellular communications, we previously developed two types of gel-embedding cultures (Motose et al. 2001). One is the thin-sheet culture, in which mesophyll cells were embedded in a thin sheet of agarose gel and cultured on a solid medium, and another is the microbead culture, in which cells were embedded in microbeads of agarose gel and cultured in a liquid medium.

The statistical analysis of two-dimensional distribution of TEs in the thin-sheet culture showed the aggregated distribution of TEs in the field of randomly distributed cells. In the microbead culture, the increase of local cell density (the cell density in each microbead) heightened the frequency of TE differentiation. These results suggest that local cell-cell communication induces or promotes TE differentiation. The factor mediating this intercellular communication was characterized by applying a modified version of the sheet culture,which used two sheets of different cell densities, a low-density sheet and a high-density sheet. TE differentiation in the low-density sheet could be induced only by contacting it with the high-density sheet. Insertion of 25-kDa-cutoff membrane between the low-density sheet and the high-density sheet suppressed such induction of TEs in the low-density sheet. Insertion of agarose sheets

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containing immobilized trypsin also specifically interfered with the induction of TEs in the low-density sheet. Therefore, a proteinous macromolecule of larger than 25 kDa in molecular weight was expected to mediate local intercellular communication required for TE differentiation. This factor was named as "xylogen" with reference to its xylogenic activity (Motose et al. 2001).

In the studies described in this report, a bioassay system based on the microbead culture was developed to monitor the activity of xylogen. This bioassay system allowed us to characterize xylogen and to isolate it from conditioned medium (CM). Results obtained through these studies indicate that xylogen is a kind of arabinogalactan protein (AGP).

Materials and Methods

Plant

Seeds of zinnia (*Zinnia elegans* L. cv. Canary bird) were purchased from Takii Shubyo (Kyoto, Japan). Zinnia seedlings were grown on vermiculite at 25°C under a daily 14-h light period. The first true leaves of 14- to 15-day-old seedlings were used as the source material for isolation of mesophyll cells.

Cell suspension culture

Mesophyll cells of zinnia were isolated mechanically by homogenization of surface-sterilized leaves in a culture medium with a homoblender according to the procedure of Sugiyama and Fukuda (1995). Leaf homogenate was filtered through a 72-μm nylon mesh and the filtrate was centrifuged at 150×*g* for 1 min. The precipitated cells were rinsed with the medium and resuspended at a density of 5.0–8.0×10^4 cells ml^{-1}. This suspension was cultured in the dark at 27°C while being rotated at 10 rpm on a revolving drum. The culture medium was a slightly modified version of that described by Fukuda and Komamine (1980). The culture medium for the induction of TE differentiation (D medium) contained 0.1 mg $liter^{-1}$ 1-naphthaleneacetic acid and 0.2 mg $liter^{-1}$ 6-benzyladenine. For control cultures, in which few tracheary elements were differentiated, Cp medium that contained 0.1 mg $liter^{-1}$ 1-naphthaleneacetic acid and 0.001 mg $liter^{-1}$ 6-benzyladenine was used instead of the D medium.

Preparation of CM

The supernatant of cell suspension culture was separated from cultured cells by centrifugation at 1,000×*g* for 3 min or by filtration through a 15-μm nylon mesh. The supernatant was further filtered though a PVDF membrane with a pore size of 0.22 μm (Millipore, Bedford, MA, U.S.A.) and this filtrate was used as CM.

Ultrafiltration of CM

Ultrafiltration of CM was conducted by centrifugal concentrators with molecular weight cutoff of 5 kDa, such as Centricon, Centricon Plus, or Ultrafree (Millipore). To the solution retained on the ultrafiltration membrane, 10 volumes of a fresh medium was added and ultrafiltration was performed again. After this procedure was repeated three times, the solution finally retained on the membrane was used as a high-molecular-weight fraction (HMWF). The first filtrate was referred to as a low-molecular-weight fraction (LMWF). HMWF and LMWF were filter-sterilized and stored under –80°C.

Bioassay

Mesophyll cells were isolated as described above but the filtration procedure of leaf homogenate was slightly modified. In order to exclude cell clusters in which cells were attached to each other as they were in the mesophyll tissue, leaf homogenate was filtered through 72-μm nylon mesh and subsequently through a 42-μm mesh so that the percentage of single cells increased up to 80%. Isolated mesophyll cells were suspended in the D medium either at the high density of 1.60–1.80×10^6 cells ml^{-1} or at the low density of 4.0–8.6×10^4 cells ml^{-1} which were twice the final cell density. Each of two kinds of cell suspensions was mixed with an equal volume of the D medium containing 4.0% low-melting-temperature agarose (Bio-Rad Laboratories, Hercules, CA, U.S.A.) at 30°C. Ten-microliter aliquots of each mixture were dropped on siliconized glass slides and each drop was solidified into a lens-shaped microbead by cooling at 20°C. A set of the high-density microbeads and the low-density microbeads were transferred into the liquid D medium at the total cell density (the average cell density in a culture container) of 2.1–2.5×10^4 cells ml^{-1}, and cultured in the dark at 27°C while being rotated at 10 rpm on a revolving drum. CM or various fractions of CM to be tested for the activity of xylogen were supplied in the liquid medium.

Determination of the frequencies of TE differentiation and cell division

TEs, divided cells, and dead cells, which are morphologically distinguishable, were counted under a light microscope. The frequencies of TE differentiation and cell division were calculated as the proportions of TEs and divided cells to the number of initially living cells, which equals the initial cell number minus the initial number of dead cells, respectively.

Heat treatmen of HMWF

HMWF was prepared from CM of cell suspension cultured for 72 h in the D medium. HMWF was boiled for 10 min and centrifuged at 10,000×*g* for 20 min. The supernatant was filter-sterilized and subjected to the bioassay for the activity of xylogen.

Proteinase digestion of HMWF

HMWF was prepared from CM of cell suspension cultured for 72 h in the D medium and applied on a PD-10 column (Amersham Pharmacia Biotech, Uppsala, Sweden). HMWF was eluted from the PD-10 with 20 mM KH_2PO_4-KOH buffer (pH 7.5) and digested with 50 μg ml^{-1} pronase E (Sigma, St. Louis, MI, U.S.A.) at 30°C for 3 h. Alternatively, HMWF was eluted from the PD-10 with 20 mM Tris-HCl buffer containing 10 mM $CaCl_2$ (pH 8.0) and digested with 50 μg ml^{-1} TPCK-treated trypsin (Sigma) at 30°C for 3 h. For control experiments, pronase E and trypsin that had been denatured by boiling for 10 min were used. HMWFs digested with proteinases were boiled for 10 min to inactivate proteinases and centrifuged at 10,000×*g* for 20 min. The supernatant was applied on a PD-10 column, eluted with the D medium, filter-sterilized, and then subjected to the bioassay for the activity of xylogen.

Lectin column chromatography

CM was separated from cell suspension cultured for 72 h in the D medium, and subjected to ultrafiltration through a 5-kDa-cutoff membrane. By repeating addition of buffer A (10 mM KH_2PO_4-KOH containing 150 mM NaCl, pH 7.2) to HMWF retained on the membrane and ultrafiltration three times, HMWF was prepared in buffer A. This HMWF was applied onto lectin-agarose columns (Seikagaku Kogyo, Tokyo, Japan) that had been equilibrated with buffer A. Lectins used for column chromatography were *Phaseolus vulgaris* agglutinin (PHA), concanavalin A (Con A), *Ricinus communis* agglutinin (RCA), peanut agglutinin (PNA), *Ulex europaeus* agglutinin (UEA), and wheat germ agglutinin (WGA). After unbound materials were washed out from the column with buffer A, bound materials were eluted with

buffer A containing 0.2 M hapten sugars: methyl-α-D-glucoside for Con A, *N*-acetyl-D-glucosamine for WGA, fucose for UEA, and lactose for RCA and PNA. In the case of PHA-agarose column chromatography, buffer A containing 0.2 M potassium tetraborate was used as the elution buffer. The buffer of the HMWFs fractionated by lectin column chromatography was exchanged to the culture medium using an ultrafiltration unit before bioassay for xylogen activity.

Isolation of AGPs from HMWF by Yariv reagent

β-Glucosyl Yariv reagent (1,3,5-tris[4-β-D-glucopyranosyloxyphenylazo]-2,4,6-trihydroxybenzene, βGlcY) was purchased from Bio Supplies Australia (Australia) or prepared according to the method of Yariv et al. (1962). HMWF was prepared from CM of cell suspension cultured for 72 h in the D medium. To the HMWF, βGlcY was added in an approximate ratio of 1 mg βGlcY to 2 mg AGPs. NaCl was also added to give a final concentration of 170 mM. The concentration of AGPs was determined as described by Van Holst and Clarke (1985). After overnight incubation at 4°C, the HMWF supplemented with βGlcY and NaCl was centrifuged at 5,000×*g* for 20 min at 4°C to pellet the AGP-βGlcY complex. The supernatant was referred to as a non-AGP fraction, which was tested for xylogen activity after the free molecules of βGlcY were removed from the fraction. The precipitate was washed with 170 mM NaCl, suspended in pure water, and centrifuged at 5,000×*g* for 20 min at 4°C to remove undissolved residues. The AGP-βGlcY complex was then reprecipitated at 4°C by addition of NaCl to the supernatant at a final concentration of 170 mM. This procedure consisting of precipitation with 170 mM NaCl and extraction with water was repeated until no undissolved residues were observed. Finally, the complex was dissolved in pure water and dissociated with 10% (w/v) sodium dithionite at room temperature. After the solution became colorless, it was applied on a PD-10 column that had been equilibrated with pure water. AGPs were eluted from the PD-10 column with pure water.

Electrophoresis

Proteins were separated by the tricine-SDS-PAGE acoording to the method described by Schaegger and Von Jagow (1987). Gels were silver-stained using Silver Stain Kanto II (Kanto Chemicals, Tokyo, Japan) or Silver Stain Kit Wako (Wako Pure Chemical Industries, Osaka, Japan). For visualizing sugars in the gel, it was stained with silver following oxidation with periodic acid as described by Dubray and Bezard (1982). In some cases, after SDS-PAGE, proteins were electroblotted onto a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech). Total proteins on the membrane were stained with SYPRO Ruby (Molecular Probes, Eugene, OR, U.S.A.) and detected using FLA-2000 (Fuji Film, Tokyo, Japan). AGPs on the membrane were stained with 50 μg ml^{-1} βGlcY in 170 mM NaCl.

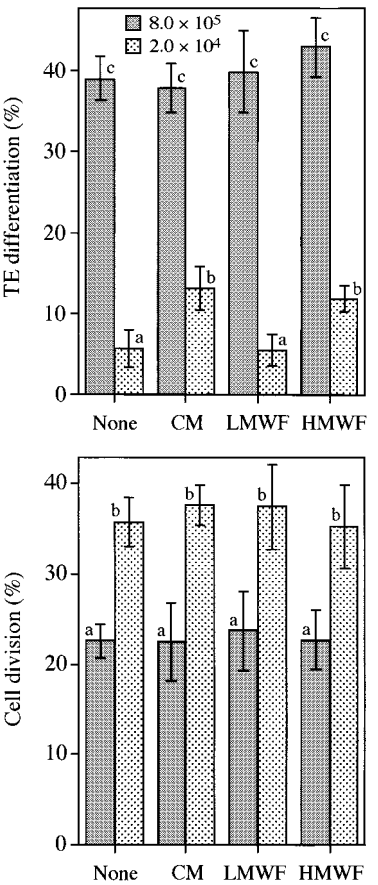

Fig. 1 Effects of CM supplement on TE differentiation and cell division in the high-density and low-density microbeads. Mesophyll cells of zinnia were embedded in microbeads of agarose gel at the local cell density of 8.0×10^5 cells ml^{-1} (high-density, shaded bars) or 2.0×10^4 cells ml^{-1} (low-density, stippled bars). A set of the high-density and low-density microbeads were cultured together to give the total cell density of 2.1×10^4 cells ml^{-1} in the absence (None) or the presence of CM, LMWF, or HMWF. CM was prepared from cell suspensions undergoing TE differentiation after 72 h in culture in the D medium, and HMWF and LMWF were prepared from this CM. CM, HMWF, and LMWF were added to the microbead cultures at the final concentrations equivalent to 15% of the original concentrations in cell suspensions. The frequencies of TE differentiation and cell division in each microbead were determined after 72 h in culture. Data are mean values of three replicates ± SD. Values designated by different letters are significantly different at the 0.05 level in Student's *t*-test.

Results

Detection of the activity of xylogen in HMWF of CM

In the microbead cultures, the cell density in each microbead (local cell density) makes a critical effect on TE differentiation. TE differentiation is suppressed significantly in the low-density microbeads of which local cell density is less than 10^5 cells ml^{-1}. Our previous studies attributed such suppression of TE differentiation in the low-density microbeads to the shortage of xylogen, a proteinous macromolecule implicated in local intercellular communication specifically required for TE differentiation, in these microbeads (Motose et al. 2001). Accordingly, the activity of xyogen should be detected as the ability of compensating for the shortage of xylogen to restore TE differentiation in the low-density microbeads. Indeed addition of CM prepared from xylogenic suspension cultures, where xylogen secreted from cells was expected to accumulate, increased the frequency of TE differentiation in the low-density microbeads (Fig. 1). When CM was fractionated into HMWF and LMWF and each fraction was added to the microbead cultures separately, only HMWF showed a positive effect on TE differentiation in the low-density microbeads.

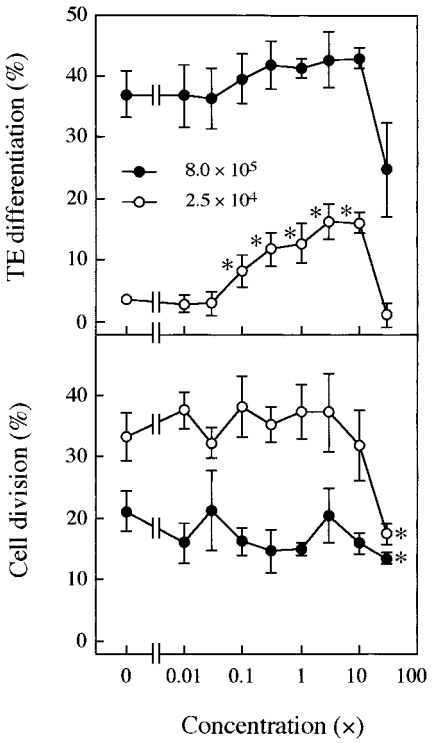

Fig. 2 Dose-dependency of the effects of HMWF on TE differentiation and cell division in the high-density and low-density microbeads. A set of the high-density (8.0×10^5 cells ml^{-1}) and low-density microbeads (2.5×10^4 cells ml^{-1}) were cultured together to give a total cell density of 2.2×10^4 cells ml^{-1} in the presence of various concentrations of HMWF. HMWF was prepared from cell suspensions undergoing TE differentiation after 72 h in culture in the D medium and added to the microbead cultures. Numbers along the horizontal axis indicate the final concentrations of HMWF relative to its original concentration in the source suspension culture. The frequencies of TE differentiation and cell division were determined after 72 h in culture for the high-density microbeads (closed circles) and the low-density microbeads (open circles). Data are mean values of three replicates ± SD. Values specified by asterisks were significantly different from the control values in the absence of HMWF at the 0.05 level in Student's *t*-test.

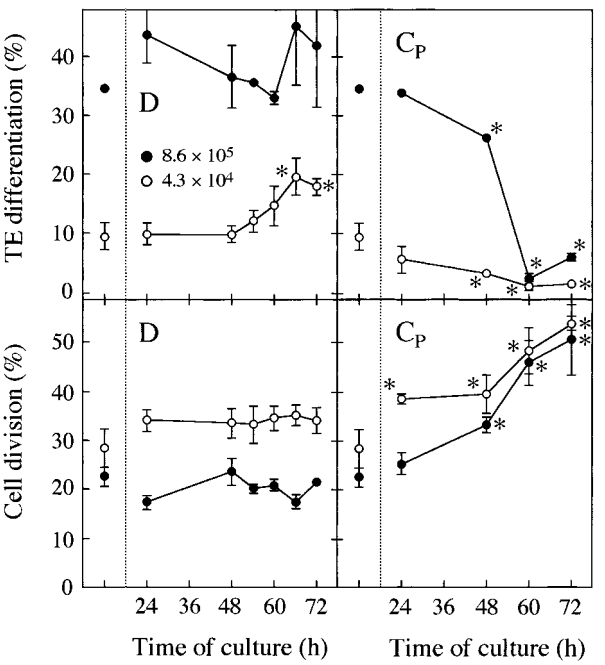

Fig. 3 Changes in the activity of xylogen during suspension culture of zinnia cells. HMWFs were prepared from xylogenic suspension culture (culture in the D medium) or control suspension culture (culture in the Cp medium) at different times of culture indicated along the horizontal axis. These HMWFs were added to the microbead culture, which contained a set of the high-density (8.6×10^5 cells ml^{-1}) and low-density microbeads (4.3×10^4 cells ml^{-1}) at the total cell density of 2.4×10^4 cells ml^{-1}, at the final concentrations equivalent to threefold of the original concentrations in the source cell suspensions. The frequencies of TE differentiation and cell division were determined after 72 h in culture for the high-density (closed circles) and low-density microbeads (open circles). The leftmost values of each panel indicate the values obtained from the control microbead culture without addition of HMWF. Data are mean values of three replicates ± SD. Values specified by asterisks were significantly different from the control values in the absence of HMWF at the 0.05 level in Student's *t*-test.

This effect was dose-dependent (Fig. 2). The highest concentration of HMWF tested here impeded both TE differentiation and cell division, indicating non-specific inhibitory substance(s) in HMWF. The presence of such substance(s) besides xylogen in HMWF might account for incomplete restoration of TE differentiation in the low-density microbeads by addition of the optimal concentration of HMWF. In the range of concentrations effective for increasing the frequency of TE differentiation, HMWF did not influence cell division. Thus the activity of xylogen in HMWF of CM could be detected successfully in the microbead culture, which was used as a bioassay system in later experiments.

Changes in the activity of xylogen during culture

To assess changes in the extracellular activity of xylogen during culture, HMWFs were prepared from xylogenic (D) and control (Cp) suspension cultures at various times and subjected to the bioassay of xylogen (Fig. 3). The activity of xylogen (the increase in the frequency of TE differentiation in the low-density microbeads) in the D suspension culture increased after 48 h of culture and reached the maximum level within the subsequent 24 h. This time course of the xylogen activity appeared similar to that of TE formation in the D suspension culture. In the Cp suspension culture, no activity of xylogen was detected throughout the culture period. Instead HMWFs prepared from the Cp suspension culture inhibited TE differentiation strongly, indicating the accumulation of inhibitory macromolecule(s) in the medium of the Cp suspension culture. With respect to cell division, HMWF from the D suspension

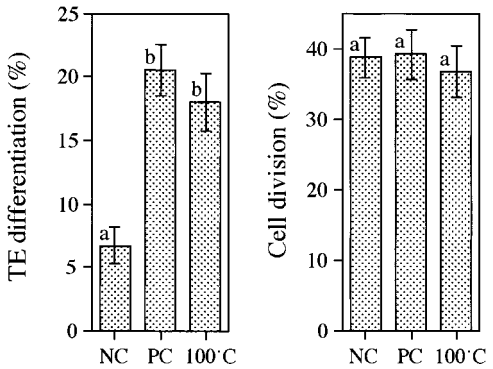

Fig. 4 Effect of heat treatment on the activity of xylogen in the low-density microbeads. HMWF that had been prepared from the 72-h cell suspensions undergoing TE differentiation was incubated at 100°C for 10 min and added to the microbead culture, in which a set of the high-density (8.3×10^5 cells ml^{-1}) and the low-density microbeads (2.3×10^4 cells ml^{-1}) were cultured to give a total cell density of 2.3×10^4 cells ml^{-1}. The final concentration of HMWF in the microbead culture was threefold higher than its original concentration in the source suspension culture. NC (negative control) and PC (positive control) represent no addition of HMWF and addition of non-boiled HMWF, respectively. The frequencies of TE differentiation and cell division in each microbead were determined after 72 h in culture. Only data for the low-density microbeads are shown in this figure. All of the treatments had no effect on TE differentiation and cell division in the high-density microbeads. Data are mean values of three replicates ± SD. Values designated by different letters are significantly different at the 0.05 level in Student's *t*-test.

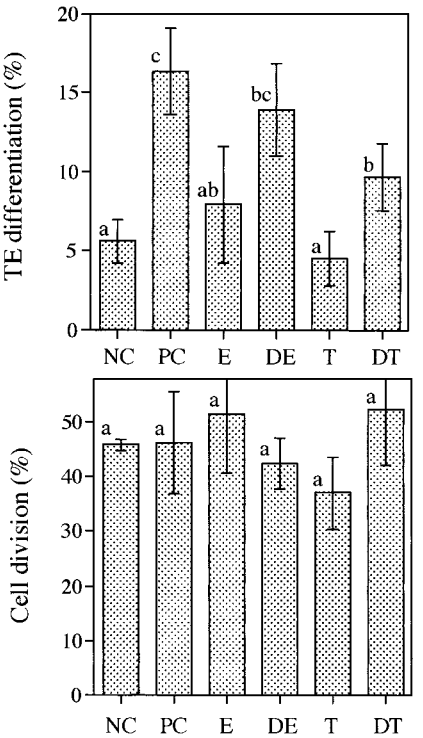

Fig. 5 Effect of proteinase digestion on the activity of xylogen in the low-density microbeads. HMWF that had been prepared from the 72-h cell suspensions undergoing TE differentiation was incubated with pronase E (indicated as E), denatured pronase E (DE), trypsin (T), or denaured trypsin (DT) at 30°C for 3 h and added to the microbead culture, in which a set of the high-density (8.0×10^5 cells ml^{-1}) and the low-density microbeads (2.5×10^4 cells ml^{-1}) were cultured to give a total cell density of 2.2×10^4 cells ml^{-1}. The final concentration of HMWF in the microbead culture was threefold higher than its original concentration in the source suspension culture. NC (negative control) and PC (positive control) represent no addition of HMWF and addition of crude HMWF before proteinase digestion, respectively. The frequencies of TE differentiation and cell division in each microbead were determined after 72 h in culture. Only data for the low-density microbeads are shown in this figure. All of the treatments had no effect on TE differentiation and cell division in the high-density microbeads. Data are mean values of three replicates ± SD. Values designated by the same letter are not significantly different at the 0.05 level in Student's *t*-test.

culture had little effect while HMWF from the Cp suspension culture showed a promotive effect. This promotion might be a secondary effect caused by suppression of TE differentiation and the resultant increase of the population of non-TE cells capable of dividing.

Heat tolerance of xylogen

Heat tolerance of xylogen was examined by measuring the xylogen activity of HMWF before and after boiling it for 10 min. This boiling treatment did not reduce the activity of xylogen significantly (Fig. 4), and thus xylogen was shown to be very stable at high temperatures.

Sensitivity of xylogen to proteinase digestion

HMWF was incubated with pronase E or trypsin and tested for the residual activity of xylogen. Both proteinases eliminated most of the activity of xylogen (Fig. 5). Denatured proteinases did not have such effect. This result confirms that xylogen is a proteinous factor.

Fractionation of HMWF by lectin-affinity chromatography

HMWF was fractionated by chromatography with several kinds of lectin-agarose columns. The activity of xylogen was detected in the fractions adsorbed by RCA, PNA, and Con A but not in the fractions adsorbed by WGA, PHA, and UEA (Fig. 6). In view of sugar-specificity of these lectins described by Osawa and Tsuji (1987) and Cummings (1994), xylogen was inferred to bear glycosyl side-chain(s) containing galactose and mannose. The fractions bound to lectin-agarose columns were further separated by SDS-PAGE (Fig. 7). A broad band with apparent molecular weights of larger than 75 kDa was detected in all of the xylogen-positive fractions and absent in any of the xylogen-negative fractions. As this band resembled migration pattern reported for AGPs, suggesting that xylogen is a kind of AGP.

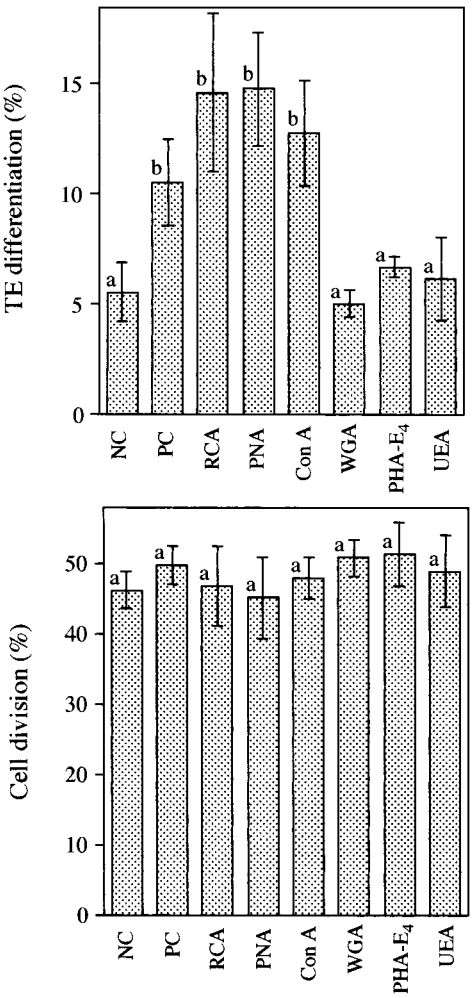

Fig. 6 Effects of lectin-bound fractions on TE differentiation and cell division in the low-density microbeads. HMWF was prepared from 72-h cell suspensions undergoing TE differentiation in the D medium and separated into lectin-bound and lectin-unbound fractions with various lectin-agarose columns. Lectin-bound fractions were added to the microbead culture, in which a set of the high-density (9.0×10^5 cells ml^{-1}) and low-density microbeads (3.0×10^4 cells ml^{-1}) was cultured at the total cell density of 2.5×10^4 cells ml^{-1}. The final concentration of each fraction in the microbead culture was threefold higher than its original concentration in the source suspension culture. NC (negative control) and PC (positive control) represent no addition of HMWF and addition of total HMWF, respectively. The frequencies of TE differentiation and cell division in each microbead were determined after 72 h in culture. Only data for the low-density microbeads are shown in this figure. All of the treatments had no effect on TE differentiation and cell division in the high-density microbeads. Data are mean values of three replicates ± SD. Values designated by different letters are significantly different at the 0.05 level in Student's *t*-test.

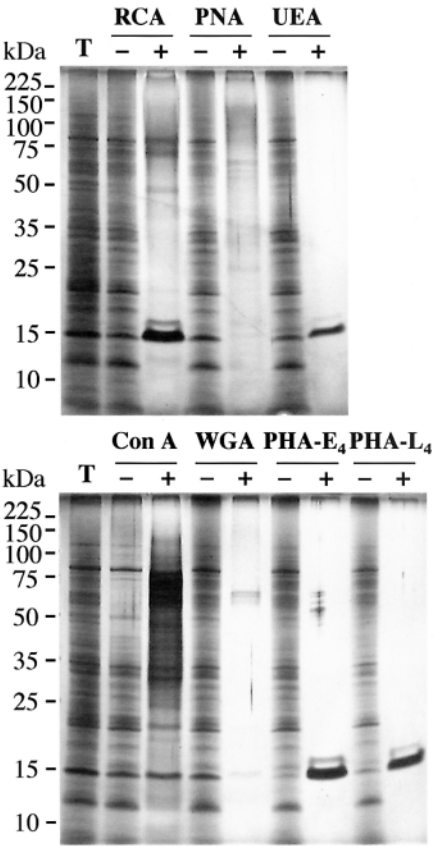

Fig. 7 SDS-PAGE analysis of fractions bound and unbound to lectins. HMWF was prepared from 72-h cell suspensions undergoing TE differentiation in the D medium and separated into lectin-bound and lectin-unbound fractions with various lectin-agarose columns. These fractions were subjected to SDS-PAGE and gels after electrophoresis were silver-stained. T indicates total HMWF before lectin-affinity chromatography. Minus (–) and plus (+) mean unbound and bound fractions, respectively. Lane T contained 16 μg proteins and each of the minus lanes contained 11 μg proteins. On each of the plus lanes, an aliquot equivalent to 21.4 ml of the source suspension culture was loaded.

Xylogen activity of AGP

To answer the question of whether xylogen is an AGP, AGPs were isolated from HMWF using βGlcY, which interacts specifically with AGPs to form a complex, and assayed for xylogen activity (Fig. 8). Addition of the AGP fraction increased the frequency of TE differentiation in the low-density microbeads in a dose-dependent manner but had little effect on cell division. Such positive effect on TE differentiation could not be detected in the non-AGP fraction (data not shown). These results indicate that some species of AGP harbor the activity of xylogen. Proteins and sugars in the AGP fraction migrated as one broad band with apparent molecular weights of larger than 100 kDa on SDS-PAGE (Fig. 9).

Changes in the amount of AGP during culture

HMWFs derived from xylogenic (D) and control (Cp) suspension cultures at different times were subjected to SDS-PAGE followed by electroblotting onto a nitrocellulose membrane. The membrane was stained with βGlcY to visualize

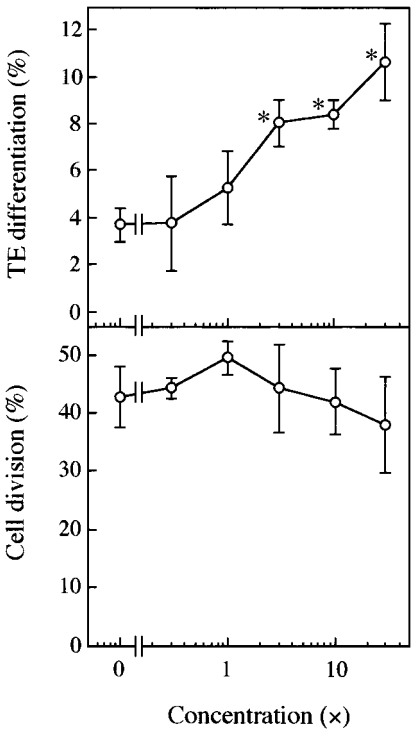

Fig. 8 Dose-dependency of the effects of AGPs on TE differentiation and cell division in the low-density microbeads. A set of the high-density (9.0×10^5 cells ml^{-1}) and low-density microbeads (3.0×10^4 cells ml^{-1}) were cultured together to give a total cell denisty of 2.5×10^4 cells ml^{-1} in the presence of various concentrations of AGPs. HMWF was prepared from 72-h cell suspensions undergoing TE differentiation in the D medium. AGPs in this HMWF were precipitated with βGlcY, dissolved in the culture medium, and added to the microbead culture. The final concentrations of AGPs in the microbead culture were 0.3-, 1-, 3-, 10-, and 30-fold higher than its original concentration (0.87 μg ml^{-1}) in the source suspension culture. The frequencies of TE differentiation and cell division in each microbead were determined after 72 h in culture. Only data for the low-density microbeads are shown in this figure. All of the treatments had no effect on TE differentiation and cell division in the high-density microbeads. Data are mean values of three replicates ± SD. Values specified by asterisks were significantly different from the control values in the absence of AGP at the 0.05 level in Student's *t*-test.

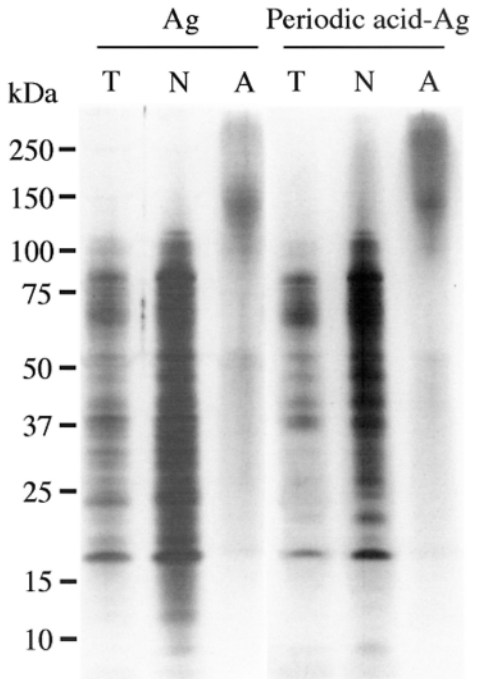

Fig. 9 SDS-PAGE analysis of the AGP fraction. HMWF was prepared from 72-h cell suspensions undergoing TE differentiation in the D medium and separated into non-AGP (N) and AGP (A) fractions using βGlcY. Total HMWF (T) and these fractions were subjected to SDS-PAGE. Gels were stained with silver for visualizing proteins (Ag) or with silver following treatment with periodic acid for visualizing sugars (Periodic acid-Ag). Lanes T and N contained 10 μg and 15 μg of proteins, respectively. Lane A contained 27.4 μg of AGPs.

AGPs (Fig. 10). In the D suspension culture, AGPs began to accumulate after 36 h in culture and increased gradually during the subsequent 36 h. In the Cp suspension culture, accumulation of AGPs was undetectable for the first 60 h of culture and only a trace amount was detected at the 72nd h.

Discussion

TE differentiation of zinnia mesophyll cells requires local intercellular communication mediated by a proteinous macromolecule, which we designated "xylogen" (Motose et al. 2001). The purpose of the present study was to identify xylogen biochemically. A bioassay system based on the microbead culture method was developed to this end and successfully applied to isolate and characterize xylogen. Finally we have reached the conclusion that xylogen is a type of AGP. This conclusion was supported mainly by the experiments using βGlcY, which detected the activity of xylogen in the AGP fraction isolated through specific interaction between AGP and βGlcY (Fig. 8). Heat tolerance (Fig. 4) and binding to galactose-specific lectins (Fig. 6) of the xylogen activity together with the electrophoretogram of the xylogen-positive fractions (Fig. 7, 9) also supports this conclusion, in the light of our knowledge about the properties of AGPs (Fincher et al. 1983, Nothnagel 1997, Serpe and Nothnagel 1999). To verify strictly that xylogen is an AGP, however, structural analysis of glycosyl chains of xylogen would be necessary.

A large amount of AGPs accumulated in the medium of the xylogenic suspension culture whereas few AGPs were detected in the control culture medium (Fig. 10). AGP accumulation in the xylogenic suspension culture started slightly ahead of visible TE formation and increased in association with the rise of the percentage of TEs. Similar pattern of AGP accumulation was reported by Stacey et al. (1995), who used a mono-

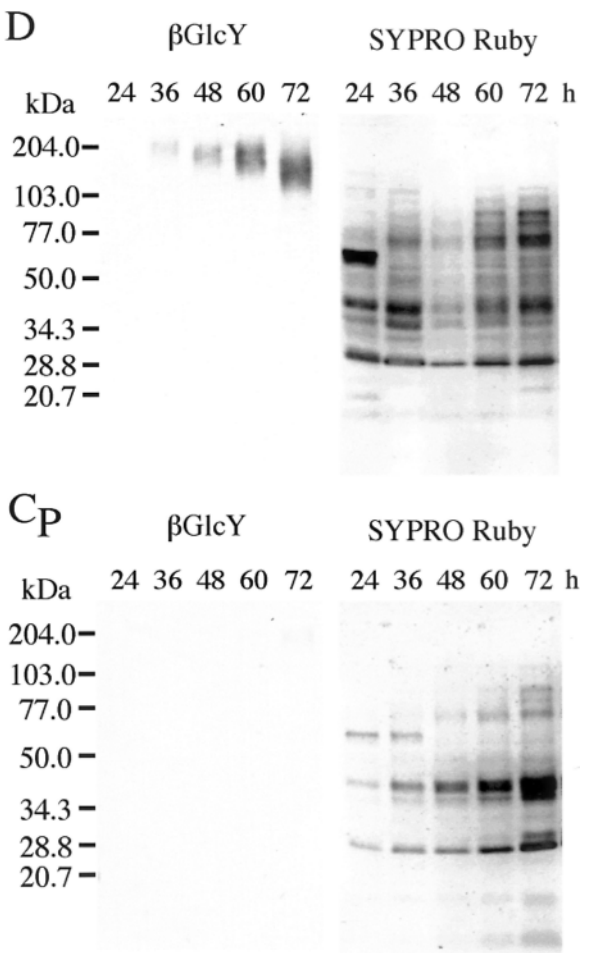

Fig. 10 Changes in the electrophoresis profiles of AGPs and proteins in the medium during suspension culture of zinnia cells. HMWFs were prepared from xylogenic suspension culture (culture in the D medium) or control suspension culture (culture in the Cp medium) at various times of culture, which were indicated above each lane. An aliquot of HMWF equivalent to 6 ml of the source suspension culture was loaded on each lane and subjected to SDS-PAGE followed by electroblotting onto nitrocellulose membranes. The membranes were stained with SYPRO Ruby for visualizing proteins and then stained with βGlcY for visualizing AGPs.

clonal antibody JIM13 to recognize some kinds of AGPs. Changes in the xylogen activity in the medium of cell suspension cultures were also closely correlated with TE formation (Fig. 3). Taking the function of xylogen to induce or promote TE differentiation together with these results, we can assume a positive feedback loop in which cells are drawn into the pathway towards TE differentiation dependently on the concentration of xylogen and such cells come to produce more xylogen. This is a fascinating possibility to be tested in the context of spatial regulation of TE differentiation *in planta*.

The AGP fraction isolated by use of βGlcY migrated as a broad band on SDS-PAGE (Fig. 9). It can be hardly distinguished which this broad band represents heterogeneity of glycosyl side chains of a single AGP species, xylogen, or presence of multiple species of AGPs besides xylogen. A parallelism between accumulation patterns of AGPs (Fig. 10) and xylogen (Fig. 3) during culture suggests that the major component of the AGP fraction is xylogen. However, our preliminary result of crossed electrophoresis indicates that the AGP fraction contains at least two types of AGPs. Further separation of the AGP fraction to purify xylogen is under investigation.

AGP is a group of extracellular proteoglycans structurally characterized by extensive O-glycosylation with type II arabinogalactan consisting of a main chain of (1→3)β-D-galactan and side chains of (1→6)β-D-galactan to which arabinose residues are bound at the remaining O-3 or O-6 positions (Nothnagel 1997, Serpe and Nothnagel 1999, Majewska-Sawka and Nothnagel 1997). Hitherto a number of AGPs have been discovered from various tissues of diverse plant species. These AGPs are often categorized into classical and non-classical types on the basis of their protein backbone structures (Du et al. 1996). Sensitivity of xylogen to proteinase treatments suggests that xylogen belongs to the non-classical type since protein backbones of classical AGPs are protected by glycosylation totally covering them against proteinases while protein backbones of non-classical AGPs are not. Some of non-classical AGPs were reported to possess N-linked glycans containing mannose residues in addition to O-linked arabinogalactan chains (Wang et al. 1993, Sommer-Knudsen et al. 1996). The presence of mannose residues in xylogen suggested by its binding to Con A implies that xylogen might be an N-glycosylated, non-classical AGP like these AGPs.

In spite of increasing researches on plant AGPs, physiological functions have been specified for only a few AGPs: carrot AGPs, which promote or inhibit somatic embryogenesis (Kreuger and Van Holst 1993, Kreuger and Van Holst 1995, McCabe et al. 1997, Toonen et al. 1997), and transmitting tissue specific (TTS) proteins of *Nicotiana tabacum* and *N. alata*, which attract pollen tubes and stimulate their growth (Cheung et al. 1995, Wu et al. 2000). Although there are several papers reporting vascular-specific or -preferential localization of AGPs (Knox et al. 1989, Stacey et al. 1990, Dolan et al. 1995, Loopstra and Sederoff 1995, Schindler et al. 1995, Stacey et al. 1995, Casero et al. 1998, Gao et al. 1999, Loopstra et al. 2000), functional relationships between these AGPs and vascular differentiation have never been assessed. The present research on xylogen, an AGP mediating local intercellular communication involved in TE diffrentiation, shed light on a novel aspect of AGP physiology.

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