Planta (2001) 213: 121–131

Planta
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Involvement of local intercellular communication in the differentiation of zinnia mesophyll cells into tracheary elements

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Received: 13 July 2000 / Accepted: 4 October 2000

Abstract. The transdifferentiation of isolated mesophyll cells of zinnia (*Zinnia elegans* L.) into tracheary elements (TEs) has been well studied as a model of plant cell differentiation. In order to investigate intercellular communication in this phenomenon, two types of culture method were developed, in which mesophyll cells were embedded in a thin sheet of agarose gel and cultured on solid medium, or embedded in microbeads of agarose gel and cultured in liquid medium. A statistical analysis of the two-dimensional distribution of TEs in the thin-sheet cultures demonstrated their aggregation. In the microbead cultures, the frequency of TE differentiation was shown to depend on the local cell density (the cell density in each microbead): TE differentiation required local cell densities of more than 10^5 cells ml^{-1}. These results suggest that TE differentiation involves cell-cell communication mediated by a locally acting diffusible factor. This presumptive factor 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. Differentiation of TEs in the former could be induced only by bringing it into contact with the latter. Insertion of a 25-kDa-cutoff membrane between the high-density and low-density sheets severely suppressed such induction of TEs in the low-density sheet while a 300-kDa-cutoff membrane suppressed induction only slightly. Insertion of agarose sheets containing immobilized pronase E or trypsin also interfered with the induction of TEs in the low-density sheets. Thus, a proteinaceous macromolecule of 25–300 kDa in molecular weight was assumed to mediate the local intercellular communication required for TE differentiation. This substance was designated "xylogen" with reference to its xylogenic activity. The time of requirement for xylogen during TE differentiation was assessed by experiments in which cells in the low-density sheet were separated from xylogen produced in the high-density sheet at various times by insertion of a 25-kDa-cutoff membrane between the two sheets, and was estimated to be from the 36th hour to the 60th hour of culture (12–36 h before visible thickening of secondary cell walls of TEs).

Key words: Intercellular communication (local) – Tracheary element differentiation – Xylogen – *Zinnia* (tracheary element)

Introduction

The vascular system of plants is composed of specialized conducting tissues, xylem and phloem, which provide transport pathways for water, nutrients, and signaling molecules and support a plant body against mechanical stresses. These functions of the vascular system are realized through fine regulation of the timing and position of vascular differentiation. However, the molecular mechanisms controlling vascular differentiation remain to be elucidated (Nelson and Dengler 1997).

As demonstrated by laser ablation experiments, cell position plays a more important role in determining allocation of fate to a plant cell than cell lineage (Berger et al. 1994, 1998; Van den Berg et al. 1995; Bouget et al. 1998). This is also true for the case of vascular differentiation. Positional information can be assumed to originate from some kind of cell-cell interaction. Thus, it would be expected that the identification of the intercellular communications involved in vascular differentiation might provide a key for understanding the regulatory mechanisms of vascular differentiation.

For direct detection and physiological characterization of such intercellular communications, the in-vitro culture system of zinnia mesophyll cells is eminently suitable. In this system, about half of the isolated mesophyll cells will transdifferentiate into tracheary elements (TEs) in a synthetic medium supplemented

Abbreviation: TE = tracheary element

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with adequate concentrations of auxin and cytokinin (Fukuda and Komamine 1980; reviewed in Fukuda 1994, 1996, 1997). This xylogenic culture has several advantages for investigation of cell-cell interactions. First, intercellular relationships (e.g., distance between cells) can be manipulated experimentally. Second, positional information pre-existing in leaves is canceled by isolation of mesophyll cells and dispersion of isolated cells into the culture medium. Third, in-vitro differentiation of mesophyll cells into TEs mimics in-planta development of the vascular system (Demura and Fukuda 1994).

In the present report, we analyzed the mode of intercellular communications and properties of a putative signaling molecule in xylogenic cultures of zinnia cells. For the quantitative analyses of cell-cell interactions, we developed two types of gel-embedding culture: a thin-sheet culture and a microbead culture. These new culture methods were successfully applied to show the importance in TE differentiation of local cell-cell interactions mediated by a proteinaceous macromolecule.

Materials and methods

Plant material

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 cycle of 14 h of light and 10 h of darkness. The first true leaves of 14-day-old seedlings were used as the source material for isolation of mesophyll cells.

Thin-sheet culture

Mesophyll cells were isolated mechanically by homogenization of surface-sterilized leaves in culture medium according to the procedure of Sugiyama and Fukuda (1995). The liquid culture medium was a slightly modified version of that described by Fukuda and Komamine (1980) and contained 0.1 mg l^{-1} (0.54 μM) 1-naphthaleneacetic acid and 0.2 mg l^{-1} (0.89 μM) benzyladenine as plant growth regulators. In order to increase the percentage of single cells in the population of obtained cells, the leaf homogenate was filtered through a 72-μm nylon mesh and subsequently through a 42-μm mesh (this two-step filtration increased the percentage of single cells up to 80%). Mesophyll cells were precipitated by centrifugation of the filtrate at 150 *g* for 1 min, rinsed with the culture medium, and suspended in the same culture medium at twice the final cell density. The cell suspension was warmed to 30 °C and mixed with an equal volume of medium containing 5.0–5.6% of low-melting-temperature agarose (Low Melt Preparative Grade; Bio-Rad Laboratories, Hercules, Calif., USA), which had been heated to melt agarose and then cooled to 30 °C. The mixture was dropped onto the groove of a glass mold that was specially designed for making gel sheets, overlaid with a coverslip, and cooled down to 18 °C. This produced an even sheet of agarose gel of 9×10 mm^2 in size and 200 μm in thickness. The sheet was transferred onto culture medium gelled with 0.25% gellan gum in a plastic dish and cultured in the dark at 27 °C.

Microbead culture

Cell suspension prepared as described above was mixed with an equal volume of culture medium containing 4.0% of low-melting-temperature agarose at 30 °C. Ten-microliter aliquots of the mixture were dropped onto siliconized glass slides. Each drop was solidified into a lens-shaped microbead of 3 mm in diameter by cooling the slides to 18 °C. The microbeads were transferred into the liquid medium in a test tube and cultured in the dark at 27 °C while being rotated at 10 rpm on a revolving drum.

Determination of the frequencies of TE differentiation and cell division, and cell viability

For quantitative evaluation of TE differentiation, cell division, and cell viability, TEs, divided cells (cells that divided during culture), and dead cells (non-TE cells that died at cell isolation or during culture), which could be distinguished morphologically under a microscope, were counted for each culture. Here, a cell clump formed through cell division from an initially single cell was scored as one divided cell. A single-cell-derived clump containing TE(s) was scored as one divided cell and also as one TE. The numbers of TEs and divided cells are indicated as percentages of the number of initially living cells, which equals the initial cell number minus the initial number of dead cells. Cell viability is defined as the ratio of initial cell number minus dead cell number to the initial cell number.

Statistical analysis of spatial distribution of cells

The parameter R of Clark and Evans (1954) was applied to the quantitative characterization of the spatial distribution of TEs in the thin-sheet cultures. The distance between the central point of a TE and the central point of its closest TE was measured for 100 TEs. The average of actual distances between TEs (r_A) was calculated from the obtained data. On the other hand, the two-dimensional density of the central points of TEs was measured to estimate the average of distances expected for the same density of TEs distributed randomly (r_E). The parameter R was determined as the ratio of r_A to r_E. This ratio R is less than or greater than 1 according to whether the distribution pattern of TEs is more aggregated or more uniform rather than random, respectively. To assess whether the r_A was significantly different from r_E, the C value, which is the normalized variate of r_A, was calculated as $C = (R - 1) \div 0.052272$. For example, if the absolute value of C is larger than 1.96 or 2.58, then the distribution of TEs is significantly different from random distribution with P (probability of a greater difference between r_A and r_E) $< 5\%$ or $P < 1\%$, respectively. The R parameters and C values were also determined for initially living cells and divided cells in the same way.

Sandwich culture

Sandwich culture was a modified version of the thin-sheet culture, which used two sheets of different cell densities: a low-density sheet where the cell density was from 4.8×10^4 to 1.0×10^5 cells ml^{-1} and a high-density sheet where the cell density was from 4.8×10^5 to 1.4×10^6 cells ml^{-1}. The high-density sheet was placed on the solid medium and the low-density sheet was laid upon the high-density sheet directly or with an insertion such as dialysis membrane (Spectra/Por; Spectrum Laboratories, Huston, Tex., USA) or an agarose-gel sheet containing immobilized enzymes.

The agarose-gel sheets containing immobilized enzymes were prepared as follows. Pronase E (Sigma, St. Louis, Mo., USA) and TPCK (L-1-tosylamido-2-phenylethyl chloromethyl ketone)-treated trypsin (Sigma) were coupled to CNBr (cyanogen bromide)-activated Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. For negative controls, enzymes that had been heat-denatured at 100 °C for 10 min were coupled to CNBr-activated Sepharose 4B. The enzyme-

coupled Sepharose was equilibrated with the culture medium, suspended in the medium, and embedded in agarose gel to form a thin sheet by the same procedure as described for the thin-sheet culture. Each sheet prepared in this way contained either 92 μg pronase E or 157 μg trypsin.

Results

The spatial distribution of tracheary elements

If local intercellular communication is involved in TE differentiation in xylogenic cultures of zinnia cells, TEs would be non-randomly distributed in the population of cultured cells. Inversely, characterization of TE distribution in cultured zinnia cells would be expected to provide evidence for local cellular communication if present. For quantitative analysis of TE distribution, a thin-sheet culture method was developed.

Figure 1 shows the effects of the cell density in a gel sheet. In the range from 2.5×10^4 to 4.0×10^5 cells ml^{-1}, higher cell densities had a more positive influence on TE differentiation, cell division, and viability. At a cell density of 4.0×10^5 cells ml^{-1}, TEs appeared after 72 h in culture and constituted approximately 10% of total cells after 96 h. At this cell density, 50% of cells had divided after 96 h, and viability was almost constant at 60% during 120 h of culture. Reduction of the cell density to 5.0×10^4 cells ml^{-1} severely inhibited TE differentiation and cell division, and decreased viability to 30%. Density reduction to 1.0×10^5 cells ml^{-1} also inhibited TE differentiation strongly while its effects on cell division and viability were only partial.

Inspection of the pattern of TEs that formed in the thin sheets suggested non-random, aggregation of TEs (Fig. 2). To confirm this impression, a statistical analysis of the spatial distributions of cells was conducted for sheets with a cell density of 4.0×10^5 cells ml^{-1} cultured for 96 h. With respect to TEs, the *R* parameter was 0.81, which deviated significantly from the expectation for a random distribution (Table 1). This result indicated that TEs were non-randomly distributed but rather aggregated in the thin-sheets. For divided cells and initially living cells, however, the *R* parameters were not significantly different from those expected for a random distribution.

A population of mesophyll cells used for culture consisted not only of single cells but also contained a small proportion of cell clusters in which a few cells were

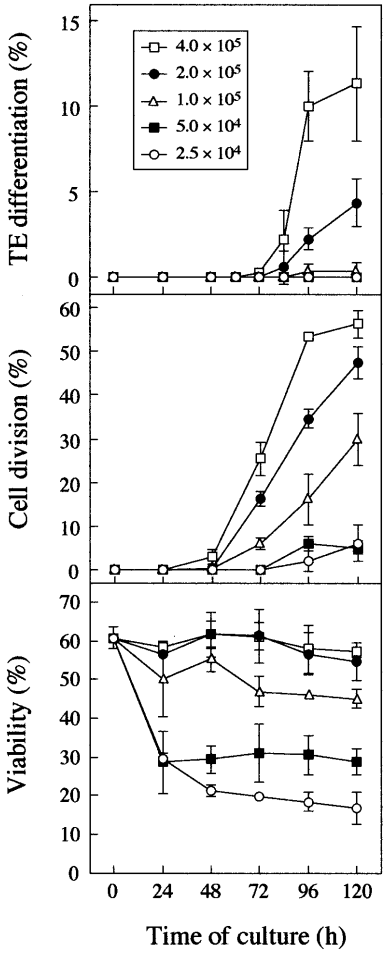

Fig. 1. Effects of cell density on TE differentiation, cell division and viability in thin-sheet cultures. Isolated mesophyll cells of *Zinnia elegans* were embedded in thin sheets of agarose gel at different cell densities as indicated in the uppermost panel (cells ml^{-1}). The frequencies of TE differentiation, the frequencies of cell division, and viability were determined at various times of culture. Data are mean values of three replicates ± SD

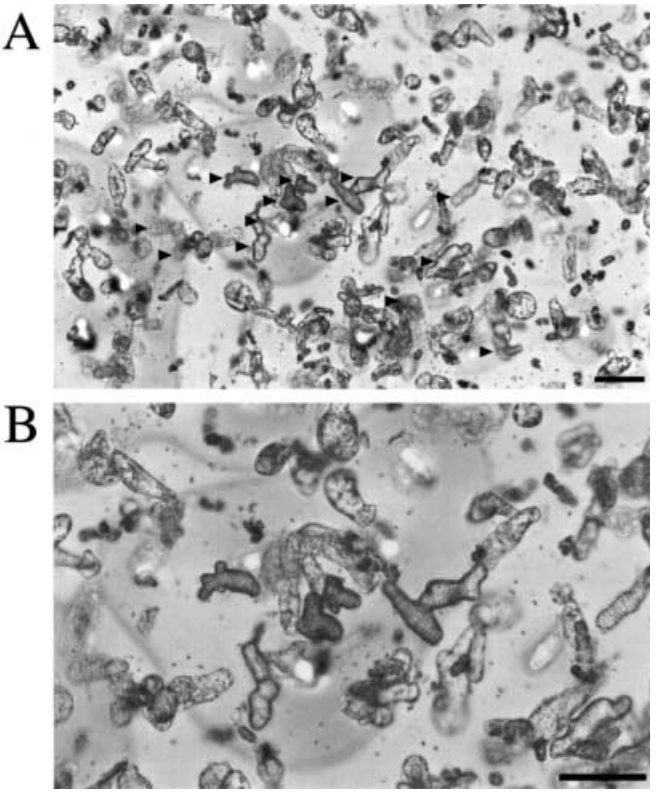

Fig. 2A,B. Tracheary element distribution in thin-sheet cultures. **A** A thin sheet (4.0×10^5 cells ml^{-1}) observed at the 96th hour of culture. *Arrowheads* indicate TEs. **B** Higher magnification of **A**. Scale bars = 100 μm

Table 1. Statistical analysis of the distribution of initially living cells, TEs, and divided cells in the thin-sheet culture. r_A = the average of actual distances between cells. r_E = the average of distances expected for the same density of cells distributed randomly. R = the ratio of r_A to r_E. C = the normalized variate of r_A. P = probability of a greater difference between r_A and r_E

	Individual cells					Inclusive field of single cells and cell clusters				
	r_A (μm)	r_E (μm)	R	C	P%	r_A (μm)	r_E (μm)	R	C	P%
Initially living cells	46.8	44.1	1.06	−1.15	25.29	57.2	53.9	1.06	−1.13	26.12
TEs	117.4	144.5	0.81	3.59	0.05	125.5	147.7	0.85	2.87	0.50
Divided cells	53.7	58.9	0.91	1.73	8.67	69.1	63.8	1.08	−1.14	11.73

attached to each other as they were in the mesophyll tissue. If cells in the same cluster tended to be channelled into the same cell fate according to the position or the clonal origin in the mesophyll, it might induce the aggregation of TEs independently of cell-cell communication in culture. To exclude from the statistical analysis such a possible effect caused by cell clusters, the R parameter and the C value were determined for the inclusive field of both single cells and cell clusters, each of which was dealt with as one point in the measurement of distance (Table 1). This treatment caused a small increase in the R parameters for TEs up to 0.85, which was still significantly different from the expectation for a random distribution. Therefore, aggregation of TEs can be attributed to local intercellular communication inducing or promoting TE differentiation, instead of the above-mentioned effect of cell clusters.

Effects of local and total cell densities on TE differentiation

To reinforce involvement of local intercellular communication in TE differentiation, a microbead culture method was developed, by which a local cell density (cell density in a bead) and a total cell density (average of cell density in a culture container) could be manipulated separately. Out of these, the local cell density was considered to influence local intercellular communication more specifically.

First, the effects of total cell density on TE differentiation, cell division, and viability were investigated. In this experiment, total cell densities were controlled by changing the number of beads and the volume of liquid medium in a culture container, whereas the local cell density in each microbead was fixed at 7.5×10^5 cells ml^{-1}. At a total density of 1.0×10^2 cells ml^{-1}, the frequency of TE differentiation was nearly zero (Fig. 3). When the total cell density was increased to 3.0×10^2 cells ml^{-1}, which was much lower than the cell density (from 5.0×10^4 to 1.0×10^5 cells ml^{-1}) adequate for the induction of TE differentiation in the standard procedure of zinnia xylogenic culture (Matsubayashi et al. 1999b), about 10% of cells could differentiate into TEs. As long as the total cell density did not exceed 3.0×10^4 cells ml^{-1}, an increase in the cell density always gave more favorable condition for TE differentiation. However, higher cell densities than 3.0×10^4 cells ml^{-1} were rather inhibitory for TE differentiation. Thus, the optimum total cell density was 3.0×10^4 cells ml^{-1} in the microbead culture with a local cell density of 7.5×10^5 cells ml^{-1}, and at this total density TE differentiation frequency reached approximately 40%. In the range of the total cell densities tested, the frequency of cell division increased as the total density increased. Although the dependency of viability on the total cell density was not so clear, it seemed to be related

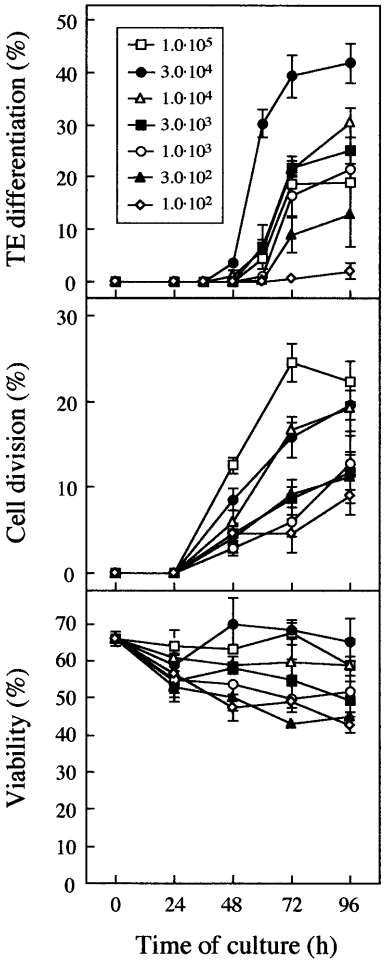

Fig. 3. Effects of total cell density on TE differentiation, cell division and viability in microbead cultures. Agarose gel microbeads containing zinnia mesophyll cells at the same local cell density (7.5×10^5 cells ml^{-1}) were incubated in liquid medium at different total cell densities as indicated in the uppermost panel (cells ml^{-1}). The frequencies of TE differentiation, the frequencies of cell division, and viability were determined at various times of culture. Data are mean values of three replicates ± SD

somewhat positively to the total cell density. These results suggested that long-range intercellular communication pervading the culture medium is necessary for both TE differentiation and cell division, and is possibly involved in survival of cells.

Next, the dependency of TE differentiation, cell division, and viability on the local cell density was assessed. For this purpose, a set of microbeads of different cell densities was cultured in a given volume of the liquid medium in a test tube to adjust the total cell density to 2.7×10^4 cells ml^{-1}. The frequency of TE differentiation after 96 h in culture varied from 5% to 40%, depending on the local cell density (Fig. 4). The maximum level of TE differentiation required local cell densities of 3.2×10^5 to 8.6×10^5 cells ml^{-1}, which was much higher than the optimum of the total cell density. By contrast, the frequency of cell division was reduced by increasing the local cell density. Cell viability was almost unaffected by the local cell density in the density range examined. These results illustrated the presence of local intercellular communication specifically acting on TE differentiation.

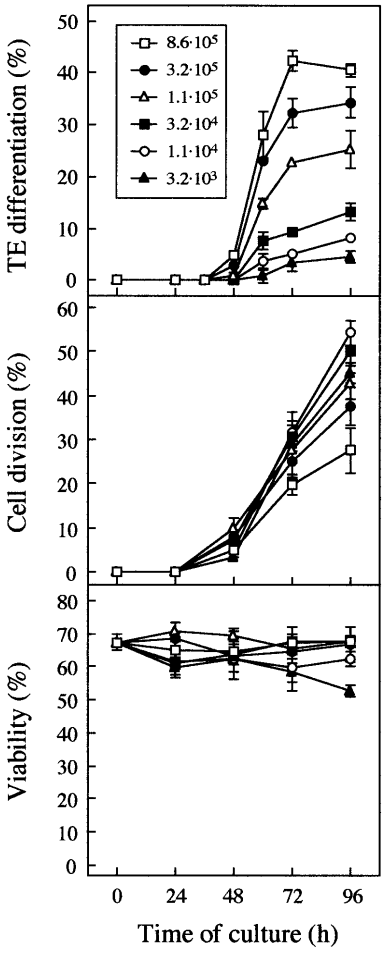

Fig. 4. Effects of local cell density on TE differentiation, cell division and viability. Isolated zinnia mesophyll cells were embedded in microbeads of agarose gel at different local cell densities as indicated in the uppermost panel (cells ml^{-1}). A set of these microbeads was cultured in the same test tube containing 1.5 ml of the liquid medium at the total cell density of 2.7×10^4 cells ml^{-1}. The frequencies of TE differentiation, the frequencies of cell division, and viability were determined at various times of culture. Data are mean values of three replicates ± SD

Characterization of local intercellular communication by sandwich cultures

Tracheary element differentiation scarcely took place at cell densities of less than 1.0×10^5 cells ml^{-1} in the thin-sheet culture (Fig. 2). However, TE differentiation in these low-density sheets was induced when they were cultured in contact with high-density sheets that contained cells at higher densities than 4.0×10^5 cells ml^{-1} (e.g., Fig. 5, PC). Contact between the low-density sheet and the high-density sheet also increased the frequency of cell division in the low-density sheet. Accordingly, the high-density sheet was considered to supply some secretory factors involved in TE differentiation and cell division to the low-density sheet, which could not produce sufficient amounts of such factors by itself.

To estimate the molecular masses of these factors, sandwich cultures in which dialysis membranes of various cutoff sizes were placed between the low-density sheet and the high-density sheet were carried out. When a 1-, 15-, or 25-kDa-cutoff membrane was inserted, the induction of TE differentiation in the low-density sheet by contact with the high-density sheet was significantly suppressed (Fig. 5). In contrast, substantial induction of TE differentiation was observed in the low-density sheet when a 300-kDa-cuttoff membrane was inserted. Insertion of a 50-kDa-cutoff membrane had a weak inhibitory effect on TE induction in the low-density sheet. An effect of membrane insertion on the high-density-sheet-dependent stimulation of cell division in the low-density sheet was not detected even when using a 1-kDa-cutoff-membrane. The effects of inserting dialysis membranes demonstrated the presence of a secretory factor of 25–300 kDa specifically inducing or promoting TE differentiation and a secretory factor smaller than 1 kDa stimulating cell division.

The sandwich culture method was also used to assess the susceptibility of these secretory factors to proteinases. Insertion of an agarose gel sheet containing immobilized pronase E interfered with both TE differentiation and cell division in the low-density sheet (Fig. 6) whereas insertion of a gel sheet containing immobilized trypsin only suppressed TE differentiation (Fig. 7). Inactivation of pronase E before immobilizing it abolished its inhibitory effects on TE differentiation and cell division. Inactivation of trypsin abolished its inhibitory effects on TE differentiation. The inhibitory effect of treatment with pronase E on TE differentiation and cell division showed that both TE differentiation and cell division in the low-density sheet were dependent on proteinaceous factors secreted from the high-density sheet. In addition, differential effects of trypsin on TE differentiation and cell division indicated that TE differentiation requires a trypsin-sensitive proteinaceous factor that is distinct from a trypsin-insensitive protei-

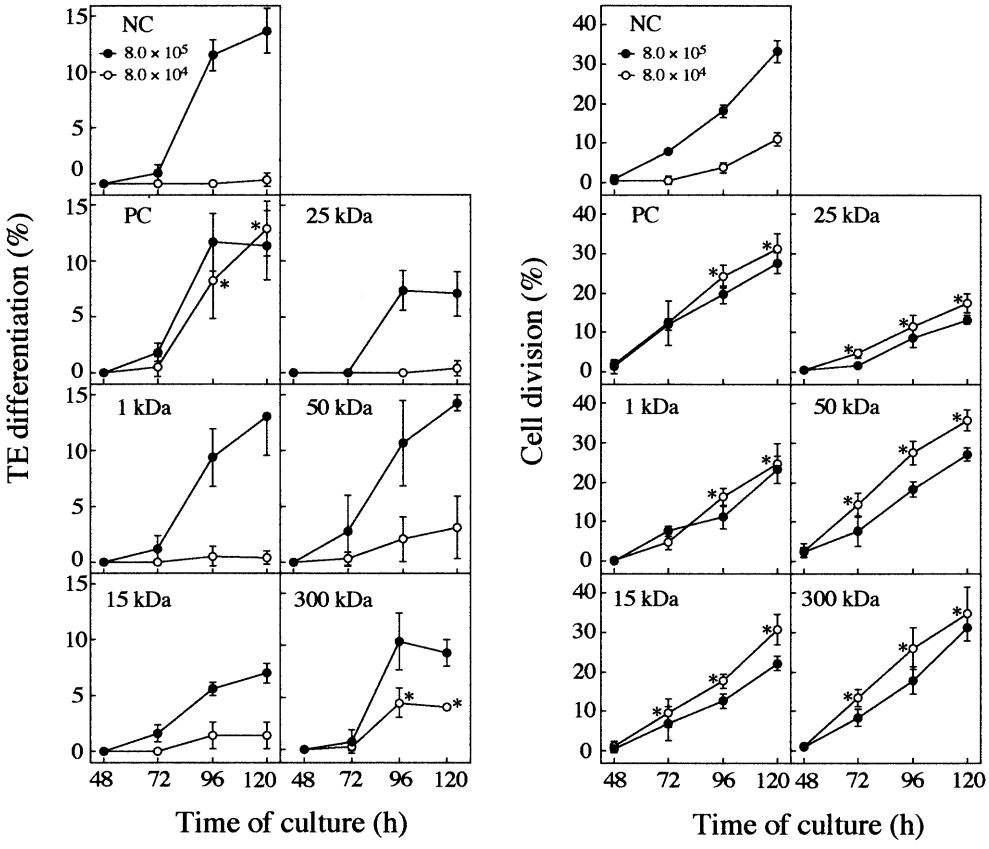

Fig. 5. Effects of the insertion of a dialysis membrane on TE differentiation and cell division in sandwich cultures. Low-density sheets of agarose gel containing zinnia mesophyll cells at a density of 8.0×10^4 cells ml^{-1} were laid on high-density sheets containing cells at 8.0×10^5 cells ml^{-1} with insertion of dialysis membranes of the specified pore size (indicated as a molecular-weight-cutoff value in each panel). *NC* represents the negative control, in which the low-density sheets were cultured separately from the high-density sheets. *PC* represents the positive control, in which the low-density sheets were laid directly on the high-density sheets without membrane insertion. The frequencies of TE differentiation and cell division were determined at various times of culture for the low-density (*open circles*) and high-density (*closed circles*) sheets. Data are mean values of three replicates ± SD. Values specified by asterisks were significantly different from the values in the low-density sheet of *NC* at the $P = 0.02$ level in the Student's *t*-test

naceous factor stimulating cell division. Taken together with the results of membrane-insertion experiments, it seemed quite reasonable to assume the presence of a secretory and proteinaceous macromolecule of 25–300 kDa with the specific function of inducing or promoting TE differentiation. Hereafter, this unidentified substance is referred to as "xylogen" in consideration of its xylogenic activity.

Time at which xylogen is required for TE differentiation

As indicated above, insertion of a 25-kDa-cutoff membrane hampered the supply of xylogen from the high-density sheet to the low-density sheet while insertion of a 300-kDa-cutoff membrane did not. This property of xylogen was exploited to define the period when xylogen is necessary for TE differentiation.

A couple of experiments were conducted. In the first experiment, the low-density sheet was initially cultured on the 25-kDa-cutoff membrane laid on the high-density sheet and transferred onto the 300-kDa-cutoff membrane on the high-density sheet at various times during culture (Fig. 8). This treatment effectively separated cells in the low-density sheet from xylogen produced in the high-density sheet until its transfer. In the low-density sheets transferred up to the 30th hour, TE differentiation was not suppressed. Tracheary element differentiation was slightly suppressed in the low-density sheet transferred at the 36th hour. Transfer after the 48th hour strongly suppressed TE differentiation.

In the second experiment, the low-density sheet was initially cultured on the 300-kDa-cutoff membrane laid on the high-density sheet and transferred onto the 25-kDa-cutoff membrane on the high-density sheet at various times (Fig. 9). This treatment transiently exposed cells in the low-density sheet to xylogen produced in the high-density sheet until its transfer. In the low-density sheet transferred up to the 60th hour, TE differentiation was suppressed strongly. However, TE differentiation was not suppressed in the low-density sheets transferred after the 72nd hour. In neither experiment was cell division or viability influenced by the transfer of the low-density sheets at any time.

Data obtained from these two experiments were combined to estimate that zinnia mesophyll cells required xylogen for TE differentiation between the 36th hour and 60th hour of culture, which corresponded to 12–36 h before visible thickening of secondary cell walls of TEs.

Discussion

Local intercellular communication specifically involved in TE differentiation

Positional information resulting from various kinds of intercellular communication seems to play a critical role in tissue differentiation in plants. With respect to intercellular communications involved in vascular differentiation, however, there have been few investigations

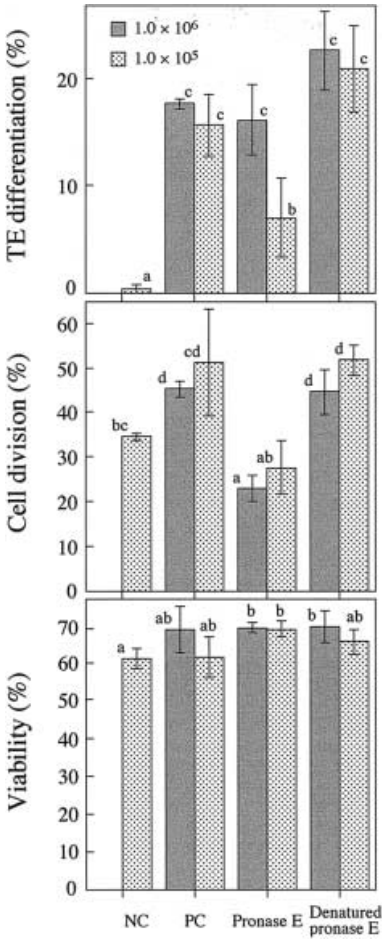

Fig. 6. Effects of insertion of an agarose gel sheet containing immobilized pronase E on TE differentiation, cell division, and viability in sandwich cultures. Low-density sheets of agarose gel containing zinnia mesophyll cells at a density of 1.0×10^5 cells ml^{-1} were laid on high-density sheets containing cells at 1.0×10^6 cells ml^{-1} with insertion of agarose sheets containing native or denatured pronase E. *NC* represents the negative control, in which the low-density sheets were cultured separately from the high-density sheets. *PC* represents the positive control, in which the low-density sheets were laid on the high-density sheets with insertion of cell-free, enzyme-free sheets of agarose. The frequencies of TE differentiation, the frequencies of cell division, and viability were determined after 96 h in culture for the low-density (*stippled bars*) and high-density (*shaded bars*) sheets. Data are mean values of three replicates ± SD. Values designated by the same letter are not significantly different at the $P = 0.05$ level in the Student's *t*-test

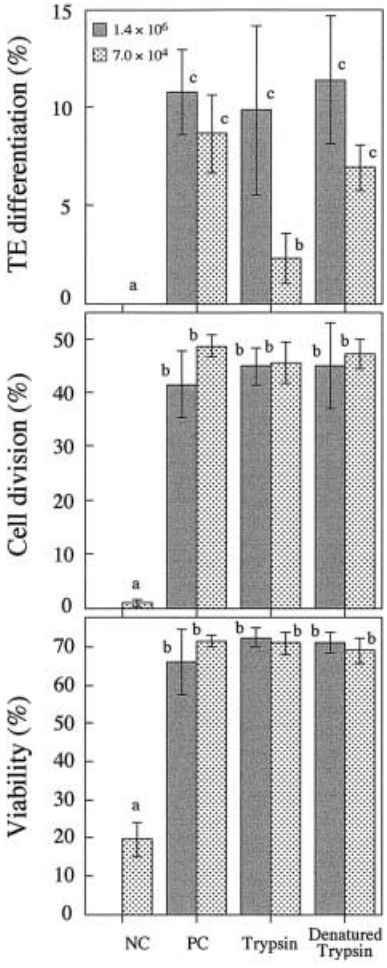

Fig. 7. Effects of insertion of an agarose gel sheet containing immobilized trypsin on TE differentiation, cell division, and viability in sandwich cultures. Low-density sheets of agarose gel containing zinnia mesophyll cells at a density of 7.0×10^4 cells ml^{-1} were laid on high-density sheets containing cells at 1.4×10^6 cells ml^{-1} with insertion of agarose sheets containing native or denatured trypsin. *NC* represents the negative control, in which the low-density sheets were cultured separately from the high-density sheets. *PC* represents the positive control, in which the low-density sheets were laid on the high-density sheets with insertion of cell-free, enzyme-free sheets of agarose. The frequencies of TE differentiation, the frequencies of cell division, and viability were determined after 96 h in culture for the low-density (*stippled bars*) and high-density (*shaded bars*) sheets. Data are mean values of three replicates ± SD. Values designated by the same letter are not significantly different at the $P = 0.05$ level in the Student's *t*-test

reported. In the present research, we developed two types of gel-embedding xylogenic culture system, thin-sheet culture and microbead culture, which enabled us to detect and characterize the intercellular communication involved in TE differentiation.

In the thin-sheet culture, mesophyll cells were two-dimensionally and randomly dispersed in an agarose gel sheet. After 4 days of culture with auxin and cytokinin, 10% of cells in the sheet differentiated into TEs. Statistical analysis indicated that the distribution of these TEs was not random but rather aggregated. The distribution of cells that had divided during culture was shown not to deviate significantly from a random distribution. These results imply that local intercellular communication specifically induces or promotes TE differentiation.

Further characterization of intercellular communication was conducted with the microbead culture in which the total cell density and the local cell density could be manipulated separately. Changes in the total and local cell densities are expected to affect inter-microbead (long-range) and intra-microbead (local) communications, respectively. A decrease in total cell density reduced the frequency of TE differentiation, the frequency of cell division, and also viability. This pleio-

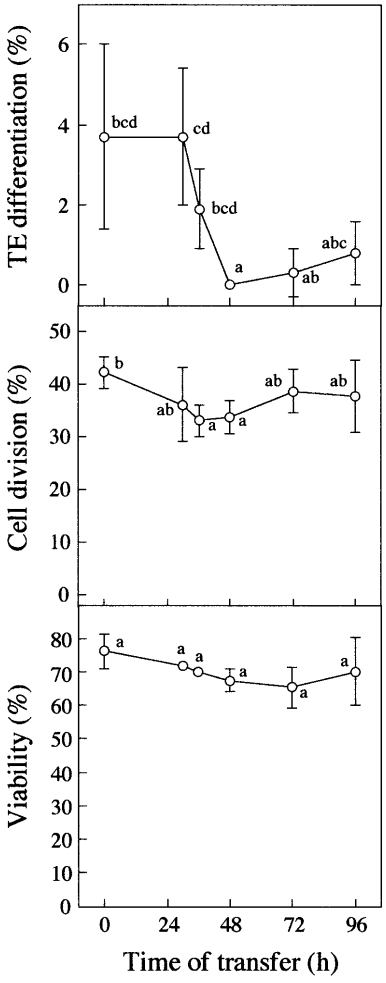

Fig. 8. Effects of sheet transfer from the top of a 25-kDa-cutoff dialysis membrane to the top of a 300-kDa-cutoff membrane on TE differentiation, cell division, and viability of zinnia cells in sandwich cultures. All dialysis membranes were laid on high-density sheets containing cells at a density of 5.5×10^5 cells ml^{-1}. Low-density sheets that contained cells at 5.5×10^4 cells ml^{-1} were initially cultured on the 25-kDa-cutoff membrane, and transferred onto the 300-kDa-cutoff membranes at various times of culture. The frequencies of TE differentiation, the frequencies of cell division, and viability were determined at the 96th hour for the low-density sheets. Data are mean values of three replicates ± SD. The frequencies of TE differentiation, the frequencies of cell division, and viability in the high-density sheets after 96 h of culture were on average 8.7%, 33%, and 66%, respectively, and were almost unaffected by sheet-transfer manipulations. Values designated by the same letter are not significantly different at the $P = 0.05$ level in the Student's t-test

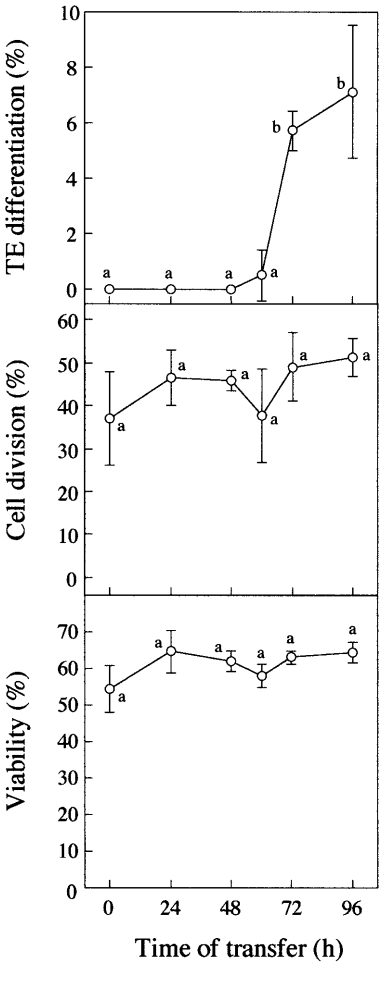

Fig. 9. Effects of sheet transfer from the top of a 300-kDa-cutoff dialysis membrane to the top of a 25-kDa-cutoff membrane on TE differentiation, cell division, and viability of zinnia cells in sandwich cultures. All dialysis membranes were laid on high-density sheets containing cells at a density of 4.8×10^5 cells ml^{-1}. Low-density sheets that contained cells at 4.8×10^4 cells ml^{-1} were initially cultured on the 300-kDa-cutoff membrane, and transferred onto the 25-kDa-cutoff membranes at various times of culture. The frequencies of TE differentiation, the frequencies of cell division, and viability were determined at the 96th hour for the low-density sheets. Data are mean values of three replicates ± SD. The frequencies of TE differentiation, the frequencies of cell division, and viability in the high-density sheets after 96 h of culture were on average 9.6%, 41%, and 60%, respectively, and were almost unaffected by sheet-transfer manipulations. Values designated by the same letter are not significantly different at the $P = 0.05$ level in the Student's t-test

tropic effect of total cell density can be explained by the assumption of long-range intercellular communication commonly necessary for TE differentiation, cell division and the survival of cultured cells. In contrast, a decrease in local cell density suppressed TE differentiation strongly without reducing the frequency of cell division and viability. The specific effect of local cell density on TE differentiation suggests that local intercellular communication specifically facilitates TE differentiation. This local intercellular communication would result in the aggregation of TEs in the thin-sheet culture. Thus, the local intercellular communication detected in the microbead culture might correspond to that demonstrated in the thin-sheet culture.

Xylogen as a mediator of local intercellular communication

Local intercellular communication specifically involved in TE differentiation was speculated to be mediated by a secretory factor that diffuses slowly from the source and acts locally. To identify and characterize such factor, sandwich cultures were conducted. When an agarose gel

sheet (low-density sheet) containing cells at a density of less than 1.0×10^5 cells ml^{-1}, which was usually insufficient for TE differentiation, was cultured in contact with an agarose gel sheet (high-density sheet) containing cells at a density higher than 4.0×10^5 cells ml^{-1}, TE differentiation was induced in the low-density sheet as well as in the high-density sheet. This high-density-sheet-dependent TE differentiation in the low-density sheet was suppressed severely by insertion of a 25-kDa-cutoff membrane between these two sheets but only slightly by insertion of a 300-kDa-cutoff membrane. Insertion of a gel sheet containing immobilized pronase E or trypsin interfered with high-density-sheet-dependent TE differentiation in the low-density sheet. These results demonstrate that TE differentiation in the low-density sheet requires a proteinaceous factor of 25–300 kDa in molecular weight supplied by cells in the high-density sheet, which we have referred to as xylogen because of its xylogenic activity. Diffusion of xylogen was not completely free from the influence of insertion of a dialysis membrane even when its cutoff size was increased up to 300 kDa. It can be inferred from this fact that xylogen may be too large, although smaller than 300 kDa, to move rapidly across a 300-kDa-cutoff membrane.

Xylogen was estimated to have a large molecular mass and accordingly a relatively small diffusion rate. Since insertion of the 25-kDa-cutoff membrane and trypsin-immobilized sheet between the low-density and high-density sheets specifically affected TE differentiation without influencing cell division and viability, the function of xylogen seemed to be restricted to the induction or promotion of TE differentiation. These properties of xylogen are completely in agreement with those presumed for a mediator of local intercellular communication involved in TE differentiation. Therefore, it is reasonable to conclude that xylogen mediates local intercellular communication to facilitate TE differentiation.

Effects on TE differentiation in the low-density sheet of transient insertion of a 25-kDa-cutoff membrane between the low-density and high-density sheets showed that xylogen plays an essential role in TE differentiation from the 36th hour to 60th hour of culture (12–36 h before secondary wall thickening became visible). The process of transdifferentiation of zinnia mesophyll cells into TEs is divided into three stages (Fukuda 1994, 1996, 1997): stage 1 (time zero to the 30th hour in the standard culture), during which mesophyll cells dedifferentiate to become pluricompetent cells similar to procambial cells; stage 2 (the 30th to 50th hour), during which dedifferentiated cells restrict their competence of differentiation to become precursors of TEs; and stage 3 (the 50th hour and thereafter), during which TE precursors complete secondary wall formation, execute programmed cell death, and mature into TEs. According to this stage definition, the period during which xylogen is required for TE differentiation corresponds to the stage 2. Therefore, xylogen appeared to control the fate of dedifferentiated cells towards vascular cell differentiation and finally towards TE differentiation. The above discussion, which is based on the temporal requirement of TE differentiation in the low-density sheet for xylogen, says nothing about the time course of xylogen production in the high-density culture. Our preliminary experiments designed to address this aspect of xylogen have suggested that xylogen production is not constitutive but dependent on the stage of transdifferentiation (data not shown). This problem is of great interest and should be investigated more precisely.

Several lines of data obtained in the present study suggest the existence of a secretory factor influencing cell division as well as TE differentiation. The positive effects of total cell density on TE differentiation and cell division in the microbead culture indicate that long-range cell-cell interaction is involved in both TE differentiation and cell division. This long-range communication is probably mediated by a small molecule whose molecular mass is less than 1 kDa since the increase in the frequency of cell division in the low-density sheet as a result of contact with the high-density sheet was not suppressed by insertion of a 1-kDa-cutoff membrane in the sandwich culture. Insertion of a gel sheet that contained immobilized pronase E suppressed both TE differentiation and cell division in the low-density sheet while insertion of a trypsin-immobilized sheet suppressed only TE differentiation. This means that a secretory factor involved in cell division is sensitive to pronase E but not to trypsin. The most likely candidate for this factor is phytosulfokine-alpha as mentioned below.

It is typical that plant cells dispersed in a liquid medium cannot divide at initial cell densities lower than a critical value. Supplying conditioned medium, which is the supernatant from a cell-suspension culture, to cells cultured at less than the critical cell density is sometimes able to induce cell proliferation. This phenomenon has indicated that the conditioned medium contains a secretory factor(s) necessary for cell division, designated the ''conditioning factor'' or ''conditioned medium factor''. Recently, using a highly sensitive bioassay, phytosulfokine-alpha was discovered as a conditioned medium factor in asparagus cell-suspension cultures (Matsubayashi and Sakagami 1996). This compound is a sulfated pentapeptide of 846 Da in molecular weight, sensitive to pronase E, and resistant to trypsin. Later, phytosulfokine-alpha was identified from suspension cultures of maize, rice, carrot, and zinnia as well as asparagus, and shown to restore cell division, somatic embryogenesis, and TE differentiation in the low-density cultures of these plant cells (Matsubayashi et al. 1997, 1999a,b; Matsubayashi and Sakagami 1999; Kobayashi et al. 1999; Yang et al. 1999; Hanai et al. 2000). Taking all these properties of phytosulfokine-alpha into consideration, this molecule is very likely to mediate the long-range intercellular communication discussed in the previous paragraph. Here it should be noted that xylogen is presumed to mediate local intercellular communication specifically involved in TE differentiation and is absolutely distinct from phytosulfokine in this respect. Both phytosulfokine and xylogen, globally

acting and locally acting secretory factors, respectively, might be necessary for TE differentiation.

Possible function of xylogen in planta

As a fundamental mechanism underlying vascular pattern formation, two hypotheses have been proposed so far, the auxin signal flow canalization hypothesis and the diffusion-reaction prepattern hypothesis. The auxin canalization hypothesis assumes that a promotive effect of auxin flux on the capacity for polar auxin transport, forming a positive feedback loop, leads to canalization of the flow of auxin, which induces vascular differentiation (Sachs 1991, 2000). The diffusion-reaction prepattern hypothesis is based on diffusion-reaction wave theory, derived from the pioneering model of Turing (Turing 1952), which postulates interaction among at least two diffusible substances with different diffusion rates, resulting in autonomous formation of patterns (Meinhardt 1982, 1996; Koch and Meinhardt 1994). In the simplest case, an autocatalytic, local activator and a long-range inhibitor are necessary and sufficient for pattern formation.

The auxin canalization hypothesis accounts excellently for experimental induction of vascular strands by auxin application. Moreover, recent studies of vascular development with a predominant focus on auxin polar transport appear to be very supportive of this hypothesis (Przemeck et al. 1996; Mattsson et al. 1999). As pointed out by Koizumi et al. (2000), however, the results obtained through these studies have never verified the essential points of the auxin canalization hypothesis and are not contradictory to the diffusion-reaction prepattern hypothesis at all. Some aspects of vascular pattern formation, such as simultaneous formation of minor-vein networks and genetic fragility of continuous network formation, are in favor of the diffusion-reaction prepattern hypothesis rather than the auxin canalization hypothesis (Nelson and Dengler 1997; Koizumi et al. 2000).

In the light of the above argument, one of the fascinating possibilities for the in-planta function of xylogen is that xylogen may participate as an autocatalytic, local activator in the generation of a diffusion-reaction wave, which directs the position of vascular differentiation. Isolation and detailed characterization of xylogen at the molecular level would enable us to test this possibility.

We thank Dr. Y. Matsubayashi and Prof. Y. Sakagami, Nagoya University, for their helpful discussions and personal communications. This study was supported by Grants-in-Aid from the Ministry of Education, Science and Culture, Japan (Nos. 10304063, 10219201, and 10182101) and from the Japan Society for the Promotion of Science (JSPS-RFTF96L00605).

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