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

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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 10⁵ 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...
with adequate concentrations of auxin and cytokinin (Fukuda and Komamine 1980; reviewed in Fukuda 1994, 1996, 1997). This xyleogenic 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 xyleogenic 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℃ 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 Sugiyma 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) benzyladene 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℃ 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℃. 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℃. This produced an even sheet of agarose gel of 9 × 10 mm² in size and 200 μm in thickness. The sheet was transferred onto culture medium gelled with 0.25% gelatin gum in a plastic dish and cultured in the dark at 27℃.

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℃. 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℃. The microbeads were transferred into the liquid medium in a test tube and cultured in the dark at 27℃ 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 (rA) 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 (rE). The parameter R was determined as the ratio of rA to rE. 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 rA is significantly different from rE, the C value, which is the normalized variate of rA, was calculated as C = (R − 1) / 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 rA and rE) < 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 × 10⁴ to 1.0 × 10⁵ cells ml⁻¹ and a high-density sheet where the cell density was from 4.8 × 10³ to 1.4 × 10⁴ 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 (1-1-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℃ for 10 min were coupled to CNBr-activated Sepharose 4B. The enzyme-