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Establishment of a new *Zinnia* experimental system including separation of the distinct cell population with flow cytometry and its culture

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Abstract

An *in vitro* culture system of *Zinnia* mesophyll cells has best used for studies on xylogenesis. Our previous studies with *Zinnia* cell culture demonstrated the presence of different cell types in the culture and their cell-cell communication. As the first step to investigate such cell-cell communication, we intended to establish a method to separate different cell populations and to culture a specific one. *Zinnia* mesophyll cells were stained with fluorescein diacetate, and then the fluorescence-positive and negative-cells were separated successfully using flow cytometric cell sorting. The fluorescence-positive cells differentiated into tracheary elements in the presence of an antibiotic, nalidixic acid. These procedures will be useful not only for the analysis of cell-cell interactions in *Zinnia* culture, but also for separating cells with intact cell walls in other plant species.

Key words: Cell-cell communication, Cell culture, Flow cytometric cell sorting, Xylogenesis, *Zinnia elegans*.

Abbreviations

Amt, amphotericin B; Ap, ampicillin; Cf, claforan; Cm, chloramphenicol; FDA, fluorescein diacetate; Gm, gentamycin; Hm, hygromycin B; Nal, nalidixic acid; Sm, streptomycin; Sp, spectinomycin; TE, tracheary element.

Introduction

Vascular tissues function as pathways to transfer water, salt, nutrients, and signal molecules. Vascular tissues are composed of several kinds of cells with different tasks, such as tracheary elements (TEs), parenchyma cells, fiber cells, cambial cells, sieve elements, and companion cells. Regulation mechanism of these variable differentiation processes has been an attractive subject of plant biology.

A *Zinnia* mesophyll cell culture system was established by Fukuda and Komamine (1980) and has been used for the study of cytodifferentiation of TEs, as a typical example of vascular cells, providing basic knowledge on regulation of TE differentiation (Fukuda, 1997; Kuriyama and Fukuda, 2001; McCann *et al.*, 2001; Motose *et al.*, 2001c). However, recent following studies suggested that isolated *Zinnia* mesophyll cells transdifferentiate into not only TEs, but also xylem parenchyma cells. Shinohara *et al.* (2000) isolated a monoclonal antibody recognizing a cell wall component specific for TEs and xylem parenchyma cells, and revealed the presence of xylem parenchyma-like cells as well as TEs in *Zinnia* culture. Hosokawa *et al.* (2001) demonstrated that the monolignols are supplied from non-TE cells to TEs in *Zinnia* culture, leading a presumption that the non-TE cells may be xylem parenchyma cells which supply monolignols to TEs and there is a cell-cell interaction between the different types of cells in *Zinnia* culture. Cell-cell interaction among cultured *Zinnia* cells was also supported by a finding that xylogen, an arabinogaractan protein is secreted from xylem precursor cells and promotes TE differentiation of their surrounding cells (Motose *et al.*, 2001a, b).

The presence of cell-cell interaction via medium in cultured *Zinnia* cells provides an opportunity to analyze cell-cell interaction easily and simply. For such analysis, we need a new technique for sepa-

rating a cell type from the others and for culturing each cell type separately or together. Flow cytometry has been used to isolate different cell types based on their specific characters such as difference of fluorescence levels (Shapiro, 1995). In plants, flow cytometric cell sorting has been applied for relatively limited cells such as maize protoplasts and *Brassica nupus* microspores (Sheen *et al.*, 1995; Schulze and Pauls, 1998), and its application for isolation of plant cells with cell walls has not been reported. With respect to *Zinnia* mesophyll cells, removal of cell walls from mesophyll cells reduces differentiation ability (Kohlenbach and Schöpke, 1981), and therefore, we have to use the isolated mesophyll cells with cell walls as a material for flow cytometry.

In the current study, we tried to establish a procedure for separating fluorescence-stained *Zinnia* cells using flow cyltometer and culturing the sorted cells. The established procedure will enable us to investigate easily the cell-cell interaction during xylem differentiation in an *in vitro* culture system.

Materials and Methods

Preparation and culture of Zinnia mesophyll cells

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 daily 14-h light period. The first true leaves of 14-day-old seedlings were used to isolate mesophyll cells. Mesophyll cells were isolated mechanically by homogenization of leaves in F-K medium (Fukuda and Komamine, 1980) with a homoblender according to the procedure of Sugiyama and Fukuda (1995). Leaf homogenate was filtered through a 72 μm nylon mesh and centrifuged at 150*g* for 1 min. The precipitated cells were rinsed with the medium, resuspended at a density of $4\text{-}8\times10^4$ cells ml^{-1}, and cultured in F-K medium containing 0.1 mg l^{-1} 1-naphthaleneacetic acid and 0.2 mg l^{-1} 6-benzyladenine in the dark at 27℃ while being rotated at 10 rpm on a revolving drum. Differentiated TEs were counted after 96 h of culture. The frequency of TE differentiation was calculated as the proportions of TEs to the total cells including TEs and living cells.

Preparation of sorting material

After 12 h of culture, *Zinnia* cells were filtered through four nylon meshes of 72, 50, 42, and 32 μm pore sizes. The filtrated cells were concentrated to 10^6 cells ml^{-1} by centrifugation and stained with fluorescein diacetate (FDA) at 1 μg ml^{-1} for 20 min. After washing twice, cells were resuspended in sheath fluid comprised of sterilized 0.3 M phosphate buffer (pH 5.7).

Flow cytometry

Flow cytometry was performed using EPICS Elite EPS flow cytometer (Beckman Coulter) equipped with an argon-ion laser emitting at 488 nm, using a 100 μm flow cell tip. The sheath pressure was 7.5 psi. Forward angle light scatter and fluorescence emission signals corresponding to wavelength of 525±15 nm were collected and the uniparametric histograms of each signal were analyzed using EXPO2 software (Beckman Coulter). Sorting was operated with a crystal drive of about 20 kHz, 38% power. The droplet delay was optimized using Flow-Check Fluorospheres (Beckman Coulter). The positions of the sort gates were determined using uniparameter histograms of log forward angle light scatter and log green fluorescence. Cells from each population were sorted into drops of medium and the efficiency of purification of specific cells was measured. In the case of aseptic cell sorting, the flow cytometer tubing was sterilized by running 70% ethanol according to the user manual of EPICS Elite EPS.

Antibiotics

Nalidixic acid, gentamycin, streptomycin, spectinomycin, claforan, ampicillin, chloramphenicol, hygromycin B, and amphotericin B were used as antibiotics. Bacteria were collected from contam-

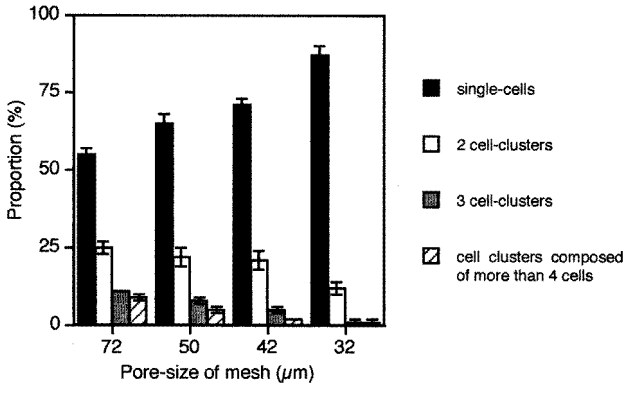

Fig. 1 Effect of sieving with nylon screens on removal of cell clusters.

Zinnia cells were passed through 72, 50, 42, and 32 μm-meshes, and the number of single-cells (solid bars), cell clusters comprised of 2 cells (open bars), cell clusters comprised of 3 cells (shaded bars), and cells clusters comprised of more than 4 cells (hatched bars) were counted under a microscope. The experiments were repeated three times. Data show the mean ± SD of three replicates.

inated *Zinnia* culture. The collected bacteria were diluted with fresh F-K medium and recultured in the presence of each antibiotic. After incubating for 72 h at 27℃ in the dark, OD at 600 nm was measured as an indicator of bacterial growth. The effects of antibiotics on TE differentiation were examined for *Zinnia* cells cultured for 96 h with an antibiotic added at 24th h of culture.

Results and Discussion

Preparation of single mesophyll cells for flow cytometry

In order to perform flow cytometry, homogeneous single cells are needed as a staining material. However, a mesophyll cell population prepared with a usual method includes cell clusters in which cells join each other with their cell walls. We tried to prepare single-cell suspensions by using nylon screens with finer pore sizes. When cells were passed through the 72 μm-pore screen, which is commonly used, single cells accounted for about 50%. A series of filtration with 60, 42, and 32 μm-pore nylon screens removed cell clusters and eventually proportion of single cells rose to 87% (**Fig. 1**). The recovery of cells was about 20% with this procedure.

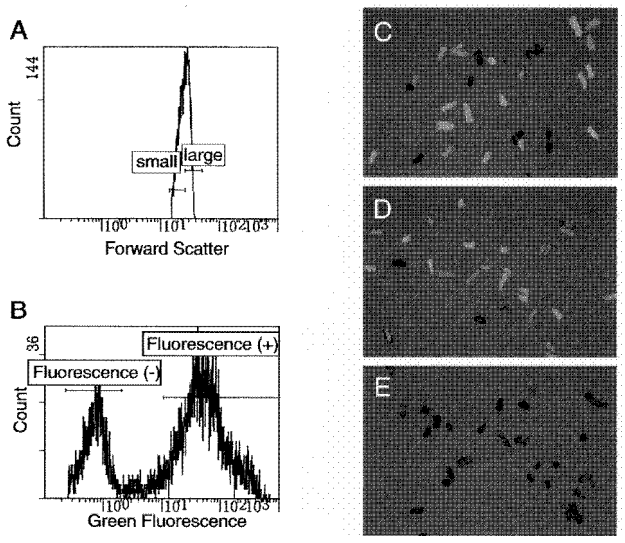

Fig. 2 Flow cytometric analysis of *Zinnia* mesophyll cells.

FDA-stained mesophyll cells were subjected to flow cytometry. Uniparametric analysis based on forward scatter (A) and green fluorescence (B) was performed to determine the sorting gates indicated by horizontal bars. Fluorescent images for FDA-stained cells, cells sorted from "fluorescence (+)" and "small" gates, and cells sorted from "fluorescence (-)" gate are shown in (C), (D) and (E), respectively.

Flow sorting of FDA-stained mesophyll cells

Using the single cell population, we attempted to separate specific cell types of *Zinnia* cells with fluorescence-activated cell sorting. For this purpose, we used FDA as a marker showing cell viability. Mechanically isolated mesophyll cells were stained with FDA, which visualized fluorescence-positive living cells (fluorescence (+)-cells) and fluorescence-negative dead cells (fluorescence (-)-cells) (**Fig. 2C**). Fluorescence (+)-cells occupied 68% of total mesophyll cells before sorting. These cells were sorted into two populations based on fluorescence (**Fig. 2B**). The fluorescence (-)-population was composed of approx. 100% fluorescence (-)-cells (**Fig. 2E, Table 1**). On the other hand, the fluorescence (+)-population contained 90% single fluorescence (+)-cells, 5% cell clusters with fluorescence (+)-cells, and 5% fluorescence (-)-cells (**Fig. 2D, Table 1**). Because the contamination of fluorescence (-)-cells in the fluorescence (+)-population seemed to be due to temporal adhesion of fluorescence (-)-cells and fluorescence (+)-cells, the forward scatter "small" gate was adopted to remove cell clusters (**Fig. 2A**). The sorting with combination of the "fluorescence (+)" gate and the "small" gate resulted in an increase in single fluorescence (+)-cells to 94% (**Table 1**), but the fluorescence (-)-

Table 1 Efficiency of purification of viable cells.

FDA-stained cells were sorted from the gates shown in **Fig. 2** into a drop of medium on the slide glass. The sorted cells were counted under a microscope and divided into 4 classes; single fluorescence (+)-cells, cell clusters with fluorescence (+)-cells, single fluorescence (-)-cells, fluorescence (-)-cell clusters. Data are mean values ± SD of three replicates.

sorting gate	proporsion of each category (%)			
	single fluorescence (+)- cells	cell clusters with fluorescence (+)- cells	single fluorescence (-)- cells	fluorescence (-)- cell clusters
Fluorescence (+)	90 ± 8	5 ± 5	5 ± 3	0 ± 1
Fluorescence (-)	0 ± 0	0 ± 0	97 ± 2	3 ± 2
Fluorescence (+) + small	94 ± 6	2 ± 2	3 ± 4	0 ± 0
Fluorescence (+) + large	75 ± 8	12 ± 2	13 ± 7	0 ± 0

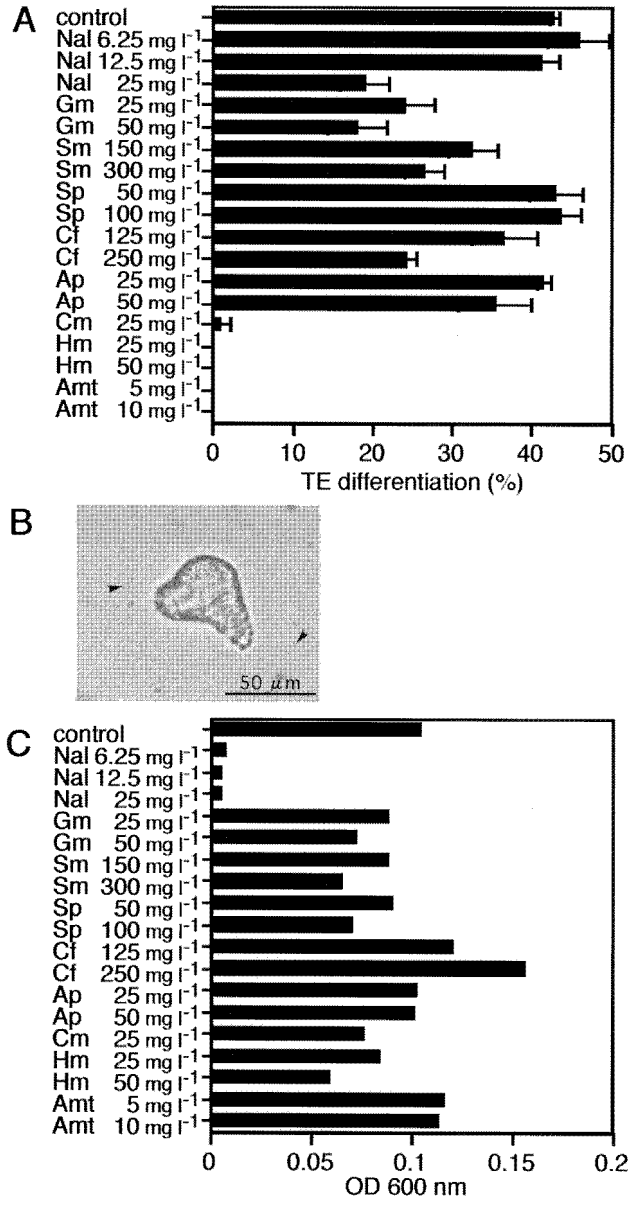

Fig. 3 Effects of antibiotics on TE differentiation and growth of bacteria.

(A) Effects of antibiotics on TE differentiation. Each antibiotic was added into cell suspension at 24th h of culture. The frequency of TE differentiation was determined after 96 h of culture. (B) Bacteria and a differentiating TEs in culture. Arrow heads indicate bacteria observed in culture following cell sorting. (C) Bacterial growth in the presence of different antibiotics was monitored. Each antibiotic was added to bacteria cultured in F-K medium. OD 600 nm as an indicator of bacterial growth was measured after 72 h of culture. Nal: nalidixic acid, Gm: gentamycin, Sm; streptomycin, Sp: spectinomycin, Cf: cla foran, Ap: ampicillin, Cm: chloramphenicol, Hm: hygromycin B, Amt: amphotericin B.

cells were still remained. Repeated sorting did not improve frequency of the fluorescence (+)-cells. Therefore small part of fluorescence (+)-cells may be injured to die and changed to fluorescence (-)-cells during or after sorting. We concluded from the experiment described above that a pure single population can be sorted perfectly by negative-signal sorting and almost completely by positive-signal sorting.

Effects of antibiotics on TE differentiation and bacterial growth

Because it was difficult to perform cell sorting under complete aseptic condition, culture of sorted cells was often subjected to bacterial contamination (**Fig. 3B**). Therefore, we tried to use appropriate antibiotics to prevent the growth of bacteria during the culture. Nine antibiotics, nalidixic acid, gentamycin, streptomycin, spectinomycin, claforan, ampicillin, chloramphenicol, hygromycin B, and amphotericin B, were tested for their effects on TE differentiation in *Zinnia* cell culture (**Fig. 3A**) and bacterial growth (**Fig. 3C**). No antibiotic at the indicated concentration reduced viability of *Zinnia* cells (data not shown). Less than 12.5 mg l^{-1} of nalidixic acids, 150 mg l^{-1} of streptomycin, less than 100 mg l^{-1} spectinomycin, 125 mg l^{-1} claforan, and less than 50 mg l^{-1} ampicillin did not prevent mesophyll cells from TE differentiation (**Fig. 3A**). When the influence of the nine antibiotics on bacterial growth was examined within the range of concentrations that did not substantially affect cell viability or TE differentiation in *Zinnia* culture, only nalidixic acid suppressed bacterial growth. Nalidixic acid belongs to the family of the quinolones and has been shown to inhibit DNA gyrase, one of type II topoisomerases that catalyze the breakage and rejoining of DNA in *E. coli* (Levine *et al.*, 1998). Heinhorst et al. (1985) showed that nalidixic acid at low concentrations (5 and 20 mg l^{-1}) preferentially inhibits plastid DNA synthesis but has only limited effects on nuclear DNA formation in suspension cells of *Nicotiana tabacum*. This limited effect on nuclear DNA synthesis in plant cells may be the reason why nalidixic acid suppressed bacterial growth without severely affecting severely TE differentiation.

Culture of the sorted cells

Finally, in the presence of 6.25 mg l^{-1} nalidixic acid, we cultured *Zinnia* cells that had been sorted using the established sorting procedures (**Fig. 4**). Although cells sorted as fluorescence (+)-cells showed 12-h delay of TE formation, the final percentage of TE formation in the sorted cells was rather higher than in non-treated control. Interestingly, time course and final percentage of TE for-

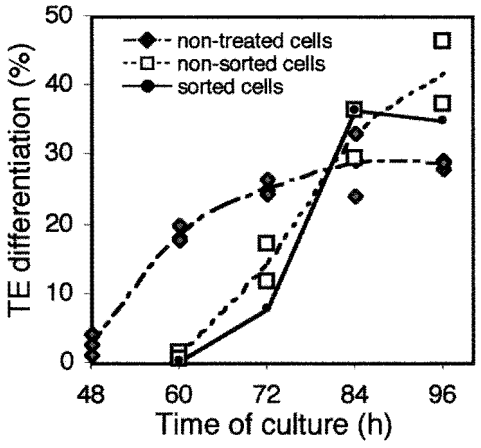

Fig. 4 Time course of TE formation in culture of sorted cells in the presence of 6.25 mg l^{-1} nalidixic acid.

Cells were sorted from the "fluorescence (+)" and "small" gates as described in **Fig. 2**, and designated "sorted cells". Cells subjected to the cell sorter but collected without sorting from any gates were designated "non-sorted cells". The sorted cells (closed circle) and non-sorted cells (open square) as well as freshly isolated mesophyll cells (designated "non-treated cell", closed diamond) were cultured with 6.25 mg l^{-1} nalidixic acid in TE-induction medium.

mation did not differ between the fluorescence-positive cell population and non-sorted cell population, suggesting that dead cells do not affect much TE differentiation.

In conclusion, here we succeeded in providing the first protocol for separation and culture of isolated *Zinnia* cells. To the best of our knowledge, flow sorting of plant cells with cell wall has not been reported yet. This procedure will enable us to separate specific cell populations that are labeled positively or negatively with fluorescent dyes and to analyze cell differentiation processes with homogeneous cell populations or with artificially mixed populations. In addition, this procedure will also be applicable to other many cell culture systems including *Asparagous* mesophyll cell culture.

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