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Direct Evidence for Cytodifferentiation to Tracheary Elements without Intervening Mitosis in a Culture of Single Cells Isolated from the Mesophyll of *Zinnia elegans*

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ABSTRACT

A serial observation of the process of tracheary element differentiation from single cells isolated from the mesophyll of *Zinnia elegans* L. cv. Canary bird provided the first direct evidence for the cytodifferentiation without intervening mitosis. Percentage of the tracheary elements formed without cell division was about 60% of total tracheary elements formed on the 4th day of culture. The number of tracheary elements formed without intervening mitosis was not reduced in the presence of colchicine at the concentrations blocking cell division. These facts clearly indicate that cell division is not a prerequisite for tracheary element differentiation in this system.

It is an important but controversial problem of xylogenesis whether or not cell division is a prerequisite for tracheary element formation.

Fosket (4) observed that inhibitors of mitosis and of DNA synthesis almost completely blocked the differentiation of wound vessel members in explanted *Coleus* stem segments and suggested that cells must divide in order to initiate cytodifferentiation. The view was supported by other work based on histological examination and cell size comparisons (1). On the other hand, several workers have reported that certain parenchyma cells differentiated to tracheary elements without an immediately preceding cell division. These reports were also based on cell size comparisons (2, 8) or inhibitor experiments (17). The work of Torrey (15) and Kohlenbach and Schmidt (10) suggested that cells in suspension could differentiate to tracheary elements without intervening mitosis. However, no direct evidence for tracheary element formation without intervening mitosis has been provided and it still remains unresolved whether or not the precursor cells must divide before cytodifferentiation.

One of the best approaches to solve this question is to follow the sequence of cytodifferentiation to tracheary elements in individual cells. In the previous paper (7) we established a system for the study of cytodifferentiation in which tracheary element formation occurs from single cells isolated from the mesophyll of *Zinnia elegans*. Using that system, we present direct evidence that single cells can differentiate to tracheary elements without intervening mitosis. Experiments using colchicine were also performed to confirm the evidence.

MATERIALS AND METHODS

Single cells were isolated from the first leaves of 2-week-old seedlings of *Z. elegans* L. cv. Canary bird and cultured in the liquid medium in the dark at 27 C with rotation in a rotating drum at 10 rpm, as described previously (7).

Suspension cells in a microchamber fixed on the stage of an inverted microscope, Olympus Type CK (Olympus Optical Co., Ltd., Tokyo), equipped with a camera were cultured for 4 days in the dark at 27 C and photographs were taken each 6 h or 12 h throughout the culture period.

Various concentrations of colchicine were added to the medium immediately after isolation of cells and given continuously throughout the culture, in experiments to test the effect of colchicine on tracheary element differentiation. Number of tracheary elements detected with phloroglucinol test (14) were counted 4 days after culture.

The number of cells and tracheary elements was counted by using a hemocytometer, Tatai type (Kayagaki Irikakogyo Co., Ltd., Tokyo) without enzymic maceration, as previously described (7).

RESULTS

Serial Observation. A serial observation of the process of tracheary element differentiation from single cells isolated from the mesophyll of *Z. elegans* is shown in Figure 1. The cell indicated by arrow, which was a palisade parenchyma cell, showed little change in the first 48 h of culture and at 71 h culture showed some enlargement but no sculptured pattern which is characteristic for tracheary elements. However, the pattern appeared suddenly at 77-h culture, indicating that secondary wall thickenings occurred in those 6 h. The formed tracheary element was mature at 96 h of culture. Spongy parenchyma cells also differentiated to tracheary elements without cell division.

Types of Formed Tracheary Elements. On the 4th day of culture, three types of tracheary elements were observed and referred to as types 1, 2 and 3 in Figure 2, respectively. Tracheary elements of type 1 which were formed without intervening mitosis were a major type, but tracheary elements with cell plates shown as type 2 in Figure 2 were also observed, indicating that some cells isolated from the mesophyll could differentiate to tracheary elements with cell division. The proportion of tracheary elements of type 1 was approximately 60% of total tracheary elements and that of type 2 was approximately 30%. Tracheary elements of type 3, which had partial secondary wall thickenings, occupied approximately 10%.

Colchicine Treatment. The relationship between cell division and tracheary element formation was investigated furthermore by using colchicine, an inhibitor of cell division.

Colchicine has been reported to affect secondary wall thickenings (9, 12, 13). In our work, tracheary elements with abnormal secondary wall thickenings were observed in the presence of colchicine at concentration above 10^{-5} M (Fig. 3); more than 90%

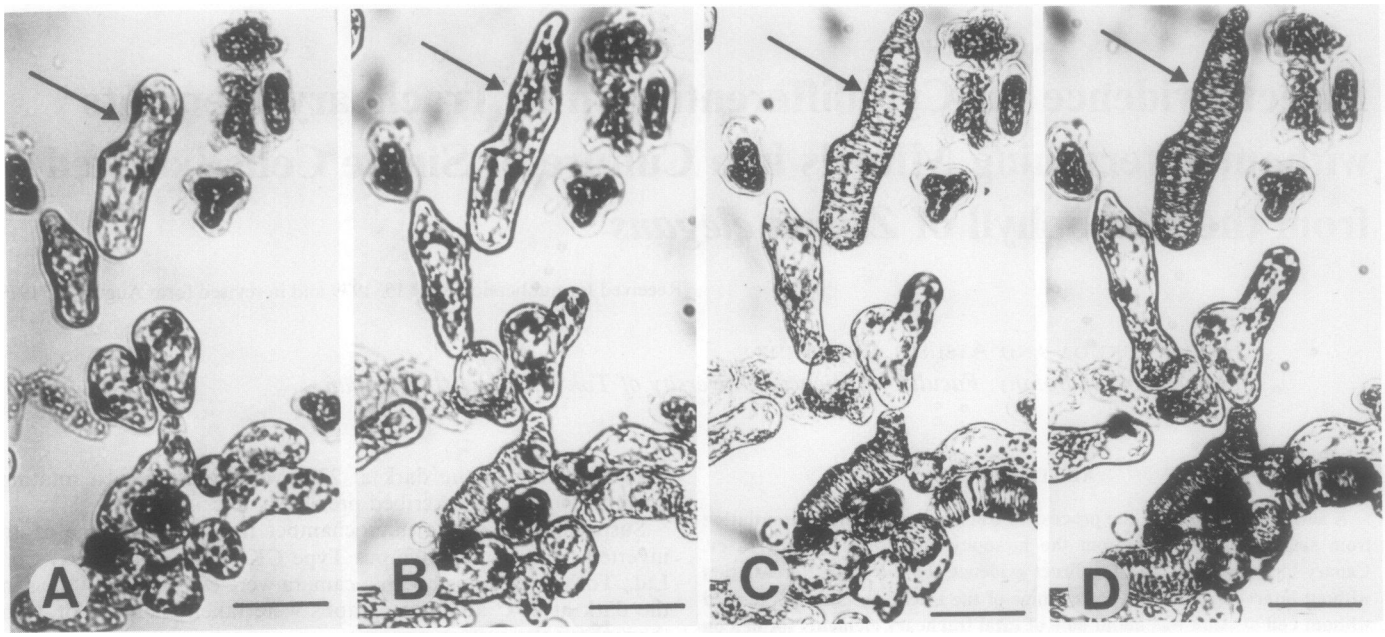

FIG. 1. A serial observation of the process of tracheary element differentiation from single cells isolated from the mesophyll of *Z. elegans*. Photographs were taken at 48 h (A), 71 h (B), 77 h (C), and 96 h (D) of culture. Bar represents 50 μm in each photograph.

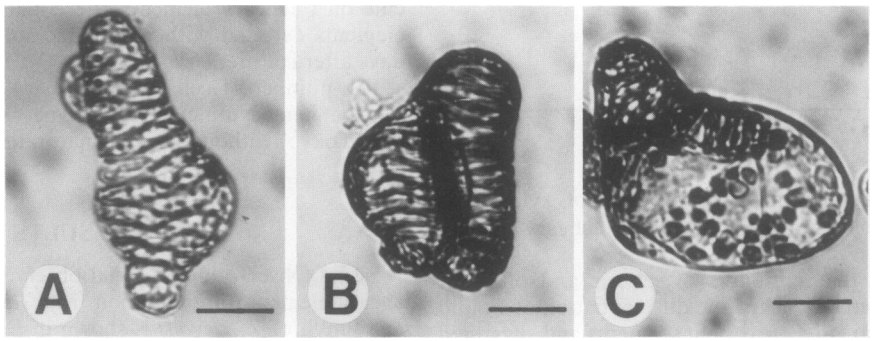

FIG. 2. Three different types of tracheary elements formed 4 days after culture. A: tracheary element formed without intervening mitosis (type 1); B: tracheary element with a cell plate (type 2); C: tracheary element which has partial secondary wall thickenings (type 3). Bar represents 20 μm in each photograph.

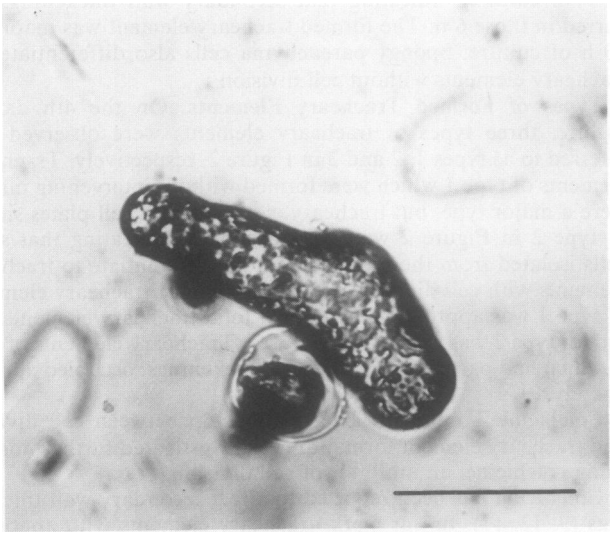

FIG. 3. An abnormal tracheary element formed 4 days after culture in the medium containing 10^{-4} M colchicine. The bar represents 50 μm.

of tracheary elements formed were normal in the presence of colchicine of 10^{-6} M, but more than 90% of them were abnormal at concentrations above 10^{-5} M (Table I).

Cell division was almost completely blocked in the presence of colchicine at concentrations above 10^{-5} M and almost all cells were composed of single cells (Fig. 4). Colchicine in this range of concentrations did not reduce the number of tracheary elements of type 1, but the formation of tracheary elements of types 2 and 3 was almost completely inhibited (Fig. 5).

DISCUSSION

Fosket and Torrey (4–6, 16) have proposed that cell division is a prerequisite for cytodifferentiation to tracheary elements on the basis of experiments using inhibitors. Several workers (2, 8, 17) have reported that tracheary elements can be formed without an immediately preceding cell division. However all the work was based on indirect evidence using multicellular systems.

Our work consisted of a serial observation of the process of cytodifferentiation in a single cell isolated from the mesophyll of *Z. elegans* (Fig. 1) provided the first direct evidence for tracheary element formation of a single cell without intervening mitosis,

Table I. *Formation of Tracheary Elements in the Presence of Colchicine*

Isolated mesophyll cells of *Z. elegans* were cultured in the medium containing various concentrations of colchicine for 4 days.

Colchicine	Tracheary Elements		
	Normal	Abnormal[a]	Normal/ total
M	× 10^{-4} cells/ml		%
0	2.19 ± 0.21[b]	0	100
10^{-6}	1.91 ± 0.12	0.04 ± 0.03	98
10^{-5}	0.14 ± 0.10	1.27 ± 0.36	10
5×10^{-5}	0	1.25 ± 0.10	0
10^{-4}	0	0.99 ± 0.13	0

[a] Tracheary elements, in which the ridges of secondary walls staining with phloroglucinol had a wavy, undulating appearance, were regarded as abnormal.

[b] Mean ± standard deviation (N = 3).

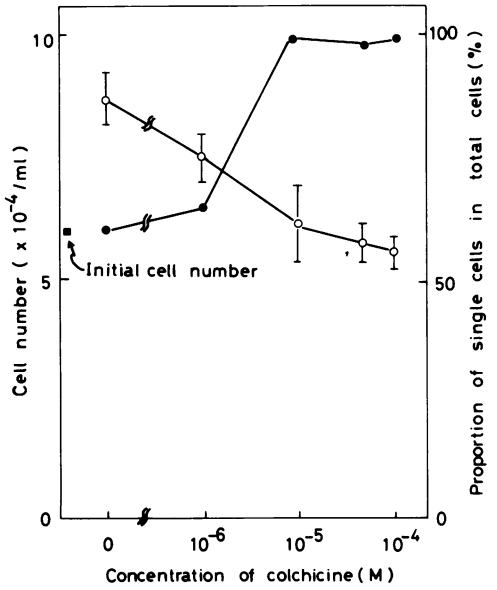

FIG. 4. Effect of colchicine on cell division; cell number (○) and proportion of single cells in total cells (●). Isolated mesophyll cells of *Z. elegans* were cultured in the medium containing various concentrations of colchicine for 4 days.

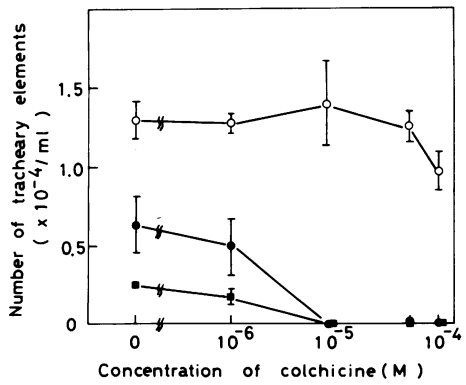

FIG. 5. Effect of colchicine on the formation of three different types of tracheary elements: type 1 (○), type 2 (●), and type 3 (■). Isolated mesophyll cells of *Z. elegans* were cultured in the medium containing various concentrations of colchicine for 4 days. Number of tracheary elements formed at concentrations above 10^{-5} M of colchicine includes both normal and abnormal types of tracheary elements.

indicating clearly that cell division is not a prerequisite for tracheary element formation.

The observation that secondary wall thickenings on cells occurred within 6 h indicated that cytodifferentiation was expressed visibly in a short time. Torrey (15) stated on the basis of indirect evidence in a suspension culture of *Centaurea* that a mature parenchyma cell could differentiate to a tracheary element without intervening cell division but the differentiation occurred at much higher frequency after parenchyma cells had undergone recent cell division. Contrary to the results of his work, the proportion of tracheary elements formed without intervening cell division occupied approximately 60% of total tracheary elements formed in our system, indicating that isolated mesophyll cells of *Z. elegans* can differentiate without intervening mitosis at high frequency.

Roberts and Baba (13) reported that colchicine at 2.5×10^{-4} M permitted wound vessel number formation with abnormal wall sculpturing but blocked at 9×10^{-3} M. Fosket (4) and Comer (1) also reported that colchicine at 10^{-3} M reduced wound vessel member formation indicating that cell division was a prerequisite for cytodifferentiation. However, it is possible that such high concentrations of colchicine as used in these two reports would block not only cell division but also have certain side effects, *e.g.* on DNA synthesis (3), on ethylene biosynthesis (11), and on membrance structure (18), which may lead to inhibition of tracheary element formation. Therefore, colchicine should be used at as low concentration as possible. In the suspension culture of isolated mesophyll cells of *Z. elegans*, 10^{-5} M colchicine, which is much lower concentration than those used in the works mentioned above, almost completely blocked cell division. But the number of single tracheary elements (type 1) formed in the presence of this concentration of colchicine was the same as in the absence of it, indicating that this concentration of colchicine did not affect the initiation of cytodifferentiation to single tracheary elements, even if it had any side effects. The fact that colchicine did not reduce the number of tracheary elements formed without intervening mitosis also supports the view that cell division is not a prerequisite for the cytodifferentiation.

We reported here that cell division is not a prerequisite for tracheary element formation in this system. However, another important question, whether or not the precursor cell of tracheary element becomes committed to the new pathway of cytodifferentiation at some particular phase of the cell cycle, still remains unresolved. Approaches to the question are in progress using the single cell culture of the mesophyll of *Z. elegans*.

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