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NOTE

Tracheary elements that resemble secondary xylem in calli derived from the conifers, *Torreya nucifera* and *Cryptomeria japonica*

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Abstract Differentiated cells were recognized in calli derived from needles of *Torreya nucifera* and in calli derived from immature zygotic embryos of *Cryptomeria japonica*. Some differentiated cells resembled tracheary elements of primary xylem with spiral or reticulate thickening of cell walls. Other cells resembled tracheary elements with thick cell walls and bordered pits, which are features of secondary xylem. These tracheary elements were formed in cell clusters. Tracheary elements in calli of *T. nucifera* formed more highly developed structures, such as bordered pits and spiral thickening, than those of *C. japonica*. Cultured cells derived from conifers might provide a good model for studies of the differentiation of secondary xylem in vitro.

Keywords Bordered pits · Conifers · Secondary xylem · Secondary wall · Tracheary elements

Introduction

Tracheary elements, such as tracheids and vessel elements, play an important role in water transport in plants and, thus, the formation of tracheary elements is an important aspect of xylem differentiation. Since wood consists of secondary xylem cells derived from the cambium of trees, a full understanding of the mechanism of differentiation of secondary xylem, and, in particular, of the deposition of secondary walls is needed for improvement of wood quality.

An in vitro model of tracheary element differentiation would be useful for investigations of the differentiation of secondary xylem. A model system, using isolated mesophyll cells of *Zinnia elegans*, was developed by Fukuda and Komamine [1] for studies of xylem differentiation and has provided extensive information at the cellular level [2]. In addition, a system of tracheary element differentiation in vitro, using *Arabidopsis thaliana* cells in suspension, has been used for analyses of the cytoskeleton and related proteins during the formation of the cell walls [3, 4]. In such differentiation systems in vitro, the main products are tracheary elements that resemble primary xylem, with spiral or reticulate thickening of secondary walls. They do not yield tracheary elements that resemble secondary xylem, with broad areas of secondary wall thickenings and bordered pits. By contrast, an in vitro system for tracheary element differentiation using the conifer, *Pinus radiata*, developed by Möller et al. [5–7], yielded tracheary elements with helical, scalariform, reticulate and pitted types of secondary wall thickening. Therefore, we postulated that it might be possible to induce the formation of more highly developed types of tracheary element from cultured cells of conifers.

In this report, we describe the presence of tracheary elements in cultured cells of the conifers *Torreya nucifera* and *Cryptomeria japonica*. Some of these tracheary elements resembled secondary xylem, with broad areas of secondary wall thickenings and bordered pits.

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Materials and methods

Cell culture in vitro

Calli were induced from young needles of *Torreya nucifera* trees that were growing in the campus of the Tokyo University of Agriculture and Technology, Fuchu, Tokyo. For induction of calli, young needles were placed on a modified version of MS medium [8], with the concentration of KNO_3 changed to 3.4 g/l and without NH_4NO_3 but including 3 % sucrose and 100 mg/l *myo*-inositol, with the pH adjusted to 5.7, and solidified with 0.3 % gellan gum. As a plant regulator, 2,4-dichlorophenoxy acetic acid (2,4-D) were added to the medium at 1.99 mg/l. The needles and resultant calli were incubated at 25 °C in darkness, with subculture in the same medium at 4-week intervals.

Calli of *Cryptomeria japonica* were induced from immature zygotic embryos in a modified version of CD medium [9] in which the concentration of NH_4NO_3 was reduced to 800 mg/l, and to which 2,400 mg/l L-glutamine, 3 % sucrose, and 1 g/l *myo*-inositol were added. The pH was adjusted to 5.7, the medium was solidified with 0.2 % gellan gum [10], and 2,4-D was added at 0.66 mg/l as a plant growth regulator. The embryos and resultant calli were incubated at 25 °C in darkness, with subculture in the same medium at 4-week intervals.

Induction of the differentiation of tracheary elements

Calli of *T. nucifera* were transferred to suspension culture in liquid medium of the same composition as the solid medium but without gellan gum. Then cells were agitated on a rotary shaker at 110 rpm at 25 °C in darkness. Suspension-cultured cells were washed five times on nylon mesh with hormone-free subculture medium. Approximately 0.8 g (fresh weight) of cells was transferred to 20 ml aliquots of induction medium. The composition of the induction medium was the same as that of the hormone-free subculture medium but with the addition of activated charcoal at 5 g/l.

In the case of *C. japonica*, small pieces of calli, 4 weeks after subculture, were transferred to induction medium, which had the same composition as the hormone-free subculture medium, with the addition of activated charcoal at 5 g/l. All cultures were maintained at 25 °C in darkness.

Microscopic observations of tracheary elements

Cells on the upper side of calli that were harvested suspended in distilled water and observed by bright-field or polarized-light microscopy or differential interference contrast microscopy (Axioskop; Carl Zeiss, Oberkochen, Germany). We defined cells with strong birefringence as tracheary elements because of the presence of secondary walls with well-ordered cellulose microfibrils. The area percentage of tracheary elements was determined by calculating the ratio of total area of tracheary elements per total area of cells with image-analysis software (Image-J; National Institutes of Health, MD, USA).

Calli were stained for 30 min with a 0.01 % aqueous solution of safranin for observations of secondary cell walls and examined by confocal laser scanning microscopy (LSM310; Carl Zeiss) with a 590-nm long-pass filter and excitation by an argon ion laser (488 nm) [11, 12].

Results

Tracheary elements in calli of *Torreya nucifera*

Soft white calli were induced from young needles of *T. nucifera* trees 1 week after the start of culture. After 4 weeks, calli were no longer soft and white but were brown and hard. Polarized-light micrograph revealed tracheary elements in calli with strong birefringence (Fig. 1). Tracheary elements were frequently observed in cell clusters. Almost all of them had a thick secondary cell wall around the entire cell (Fig. 2a). Some tracheary elements developed bordered pits, which are a typical feature of tracheids and vessel elements of secondary xylem (Fig. 2b, d). Bordered pits' pairs were apparent between neighboring cells (Fig. 2c). Other tracheary elements developed spiral thickenings, which are characteristic of the tracheids of *T. nucifera*, on the most inner surface of the secondary walls (Fig. 2d).

Tracheary elements in calli of *Cryptomeria japonica*

Calli of *C. japonica* contained tracheary elements (Fig. 3a) with strong birefringence under polarized light (Fig. 3b). Numbers of tracheary elements differed among calli derived from different zygotic embryos (Fig. 3). By contrast to those in calli of *T. nucifera*, the secondary wall thickenings of tracheary elements in calli of *C. japonica* mainly resembled those of primary xylem (Fig. 4a), being either helical (Fig. 4b) or reticulate (Fig. 4c). In some tracheary elements, the thickening of secondary walls was complex, being a mixture of the helical and reticulate types (Fig. 4d).

Induction of the differentiation of tracheary elements

Four weeks after the start of culture, we transferred calli of *T. nucifera* to liquid medium and maintained the cells in suspension culture. Tracheary elements were apparent in

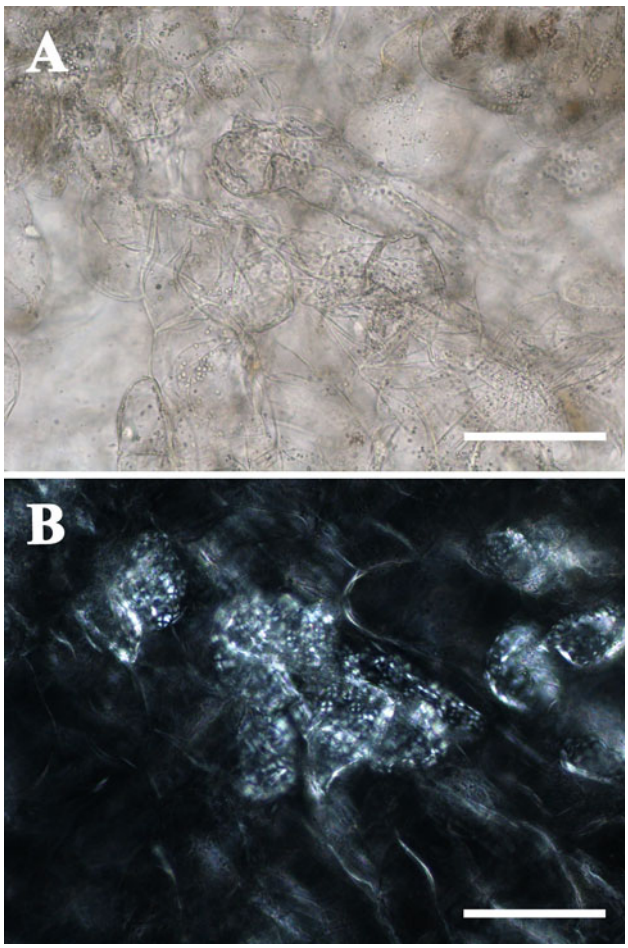

Fig. 1 Tracheary elements in calli of *Torreya nucifera*. **a** Bright-field micrograph of callus tissue. **b** Polarized-light micrograph of the same field of view as in **a**. *Bars* 100 μm

suspension-cultured cells (Fig. 5a, b). The number of tracheary elements in suspension-cultured cells increased when calli were transferred to hormone-free medium that contained activated charcoal (Fig. 5c, d). Image analysis showed that the area percentage of tracheary elements was 1.6 % in Fig. 5c and 47.2 % in Fig. 5d. Tracheary elements were frequently found in cell clusters. However, almost all the induced tracheary elements resembled primary xylem, with the reticulate type of secondary wall thickening (Fig. 6).

In the case of *C. japonica*, when the calli themselves had included small numbers of tracheary elements (Fig. 3c), incubation in hormone-free medium plus activated charcoal increased the numbers of tracheary elements in calli (Fig. 3d). Image analysis showed that the area percentage of tracheary elements was 1.2 % in Fig. 3c and 31.0 % in Fig. 3d. By contrast, when calli already included many tracheary elements, activated charcoal had no obvious effect on the number of tracheary elements. The induced tracheary elements tended to be located in cell clusters (Fig. 3b, d) and almost all of them resemble primary xylem cells, having reticulate thickenings of their secondary walls.

Discussion

We found tracheary elements in calli derived both from needles of *Torreya nucifera* and from immature zygotic embryos of *Cryptomeria japonica*. Some of the tracheary elements in calli of *T. nucifera* formed bordered pits that are characteristic of secondary xylem. Möller et al. [5, 7] also observed the development of pitted tracheary elements in calli of *Pinus radiata*. In our study, we found more highly developed pits and, even, bordered pits pairs (Fig. 2b, c). In addition, the tracheary elements in calli of *T. nucifera* had the spiral thickening of secondary walls that is a typical feature of tracheids of *T. nucifera*. Therefore, cultured cells of *T. nucifera* appear to provide a good model for the induction of the secondary xylem type of tracheary elements in vitro. By contrast, calli of *C. japonica* contained only tracheary elements with secondary wall thickenings typical of primary xylem, such as the helical or reticulate type. Thus, the nature of types of tracheary elements in calli appears to differ among species of conifers.

The numbers of tracheary elements in calli of *T. nucifera* and *C. japonica* increased after calli were transferred to hormone-free medium supplemented with activated charcoal. Möller et al. [5, 7] also observed such an increase in tracheary elements in calli of *P. radiata* in the presence of activated charcoal. Activated charcoal might absorb phenolic compounds and plant hormones from the incubation medium [13]. A putative decrease in levels of plant hormones, such as auxin, in the medium might induce differentiation into tracheary elements. However, the induced tracheary elements had only the spiral and reticulate types of secondary wall thickenings (Fig. 6). Therefore, activated charcoal might be an effective enhancer of the number of tracheary elements in calli of the conifers *T. nucifera* and *C. japonica* but is not associated with induction of the development of tracheary elements.

Tracheary elements were frequently observed in cell cluster, and it seems plausible that the cells that differentiated first into tracheary elements might induce neighboring cells to develop into tracheary elements. Motose et al. [14, 15] identified the arabinogalactan protein "xylogen" as an inducer or promoter of the differentiation of tracheary elements in *Zinnia* system. Xylogen or some similar

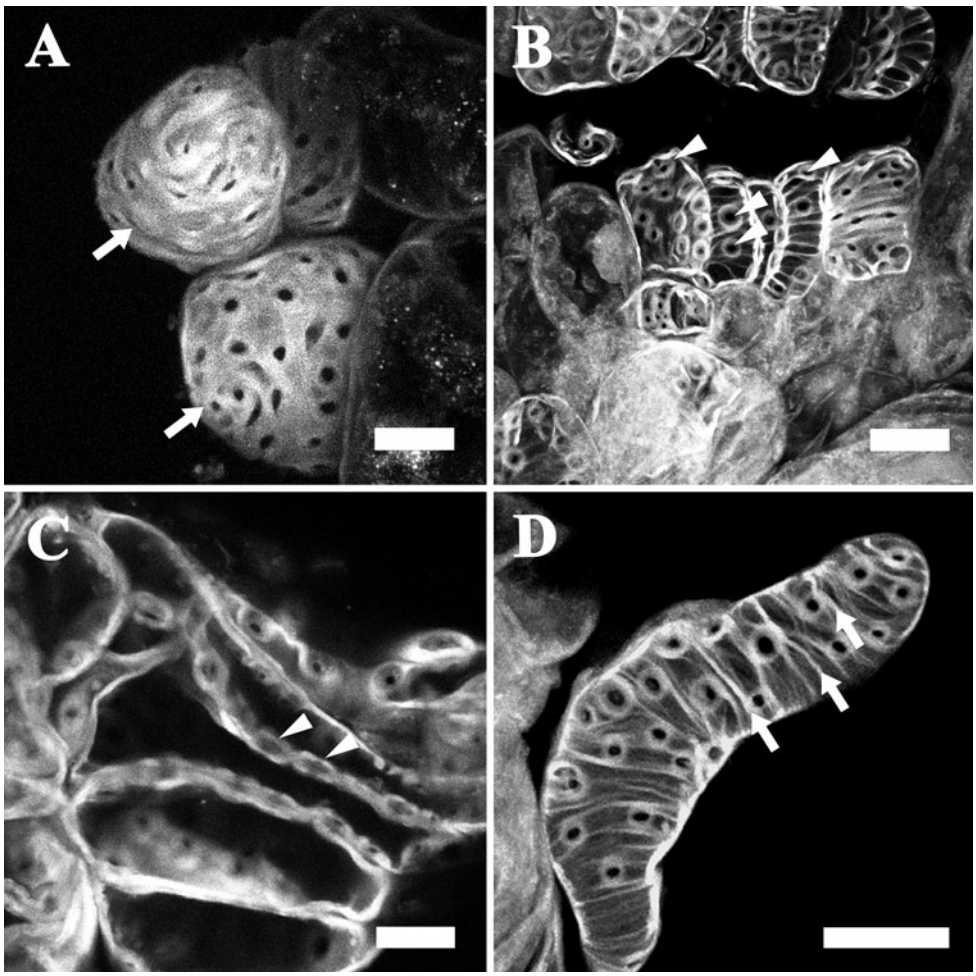

Fig. 2 Confocal laser scanning micrographs showing tracheary elements in calli derived from needles of *Torreya nucifera*. **a** Tracheary elements formed broad areas of secondary cell wall thickening (*arrows*). **b** Tracheary elements formed bordered pit (*arrowheads*). **c** Bordered pits' pairs between neighboring tracheary elements (*arrowheads*). **d** Spiral thickening of inner surfaces of secondary walls (*arrows*). *Bars* 25 μm

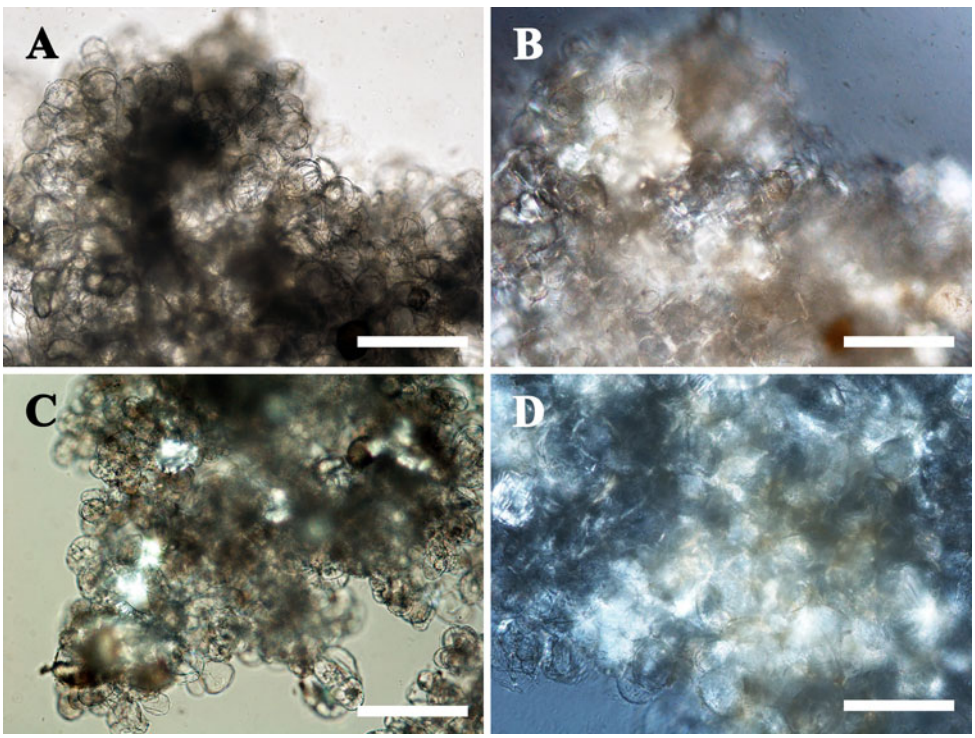

Fig. 3 Tracheary elements in calli derived from *Cryptomeria japonica*. **a** Bright-field micrograph of calli. This cell line yielded large numbers of tracheary elements. **b** Differential interference contrast micrograph of the same field of view as in **a**. **c** Differential interference contrast micrograph of calli. This cell line yielded low numbers of tracheary elements. **d** Differential interference contrast micrograph of calli of the same cell line as in **c** after 4 weeks of incubation in hormone-free medium supplemented with activated charcoal. The number of tracheary element was considerably greater than in **c**. *Bars* 200 μm

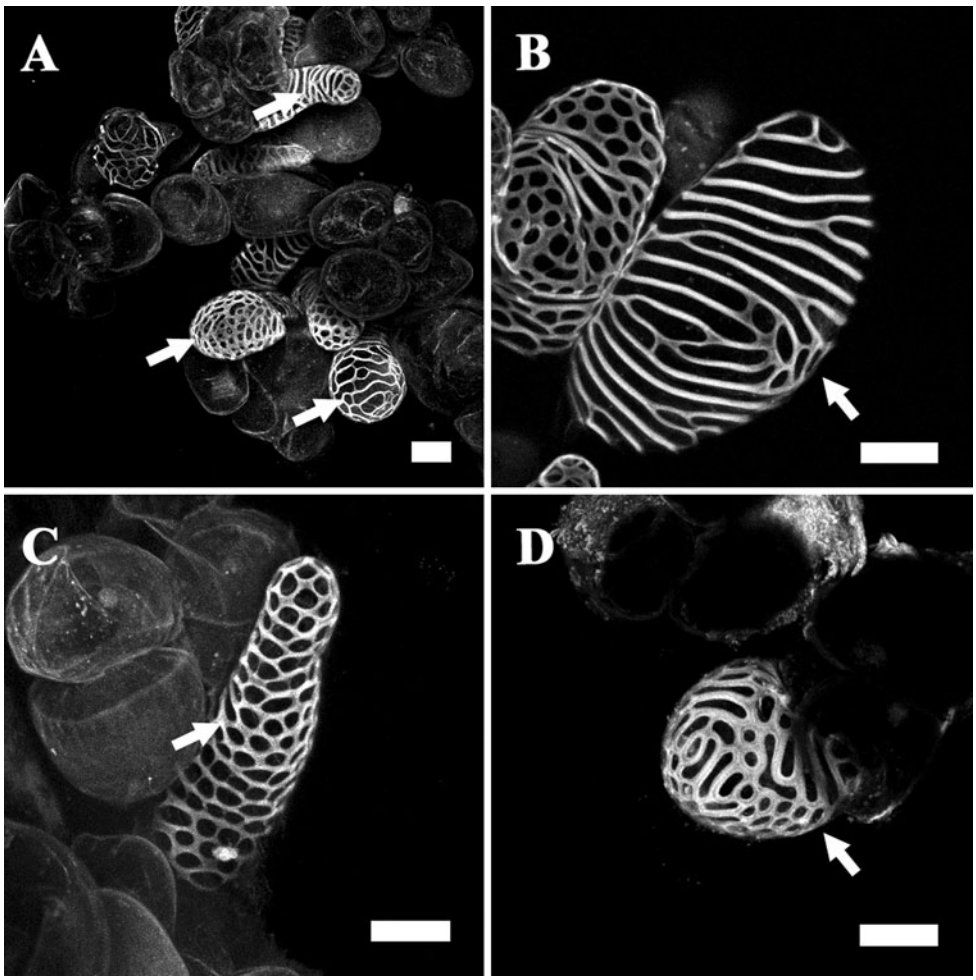

Fig. 4 Confocal laser scanning micrographs of tracheary elements in calli of *Cryptomeria japonica*. **a** Tracheary elements (*arrows*) with different types of secondary wall. **b** Tracheary element with helical thickening of the secondary wall (*arrow*). **c** Tracheary element with reticulate thickening of the secondary wall (*arrow*). **d** Tracheary element with a mixture of helical and reticulate thickening of the secondary wall (*arrow*). *Bars* 25 μm

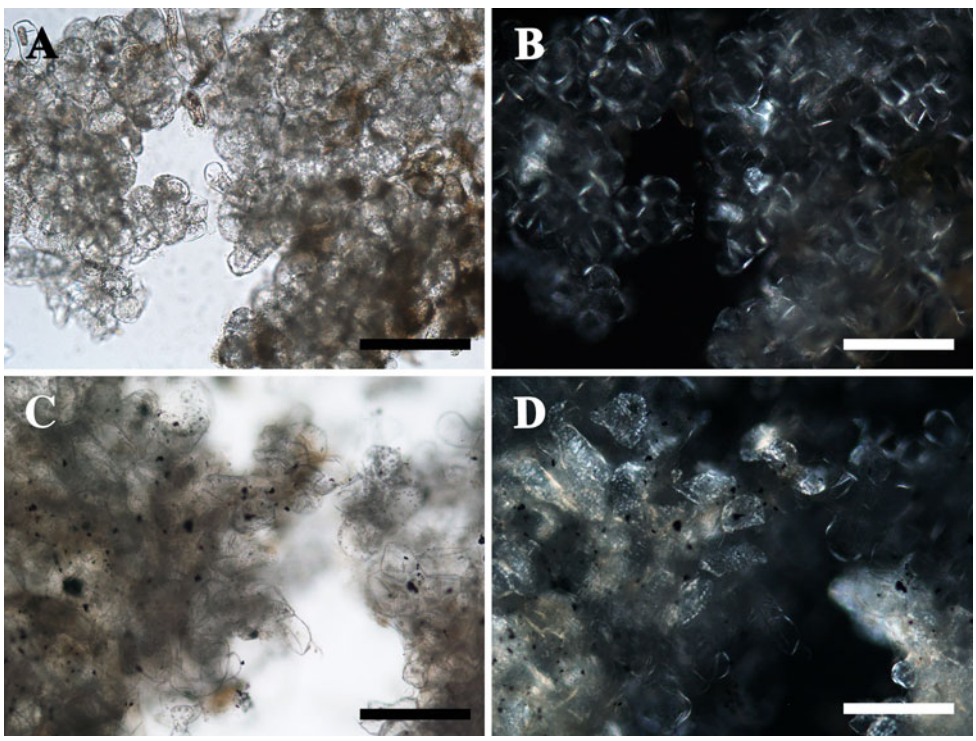

Fig. 5 Tracheary elements in calli derived from *Torreya nucifera*. **a** Bright-field micrograph of callus tissue. **b** Polarized-light micrograph of the same field of view as in **a**. **c** Bright-field micrograph of callus tissue after 4 weeks of incubation in hormone-free medium supplemented with activated charcoal. **d** Polarized-light micrograph of the same field of view as in **c**. The number of tracheary elements clearly increased during the incubation. *Bars* 200 μm

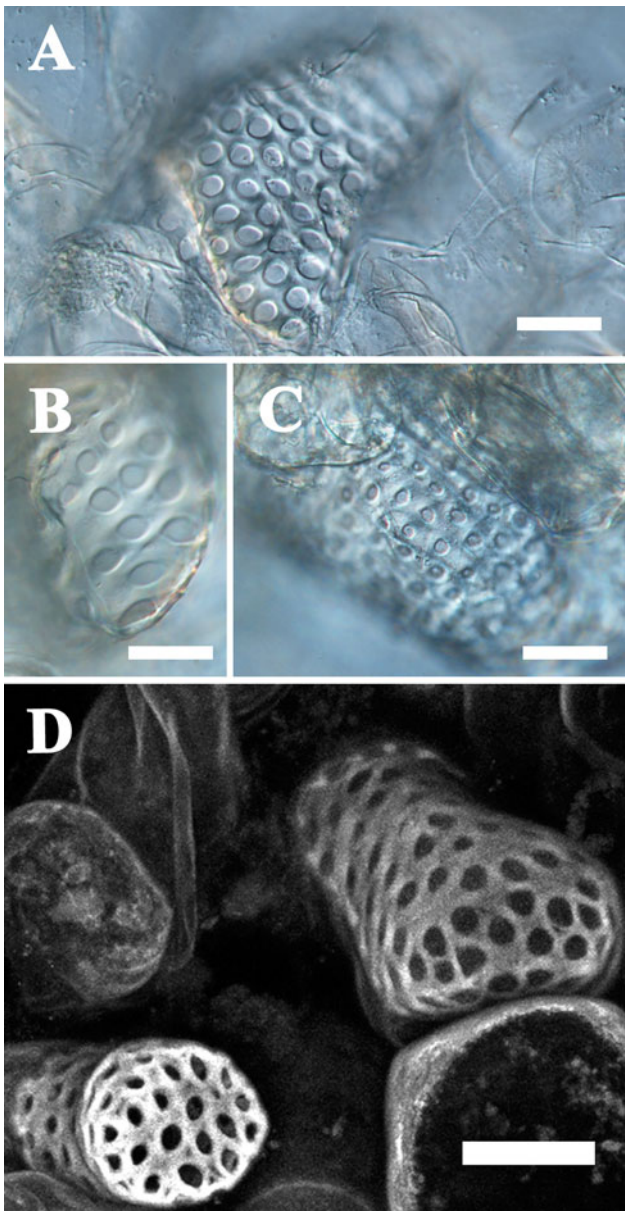

Fig. 6 Tracheary elements in calli derived from *Torreya nucifera* after 4 weeks of incubation in hormone-free medium supplemented with activated charcoal. **a–c** Differential interference contrast micrographs showing tracheary elements with reticulate secondary wall thickening. **d** A confocal laser scanning micrograph showing tracheary elements with a reticulate pattern of secondary wall thickening. *Bars* 25 μm

proteins might be involved in intercellular communication during the differentiation of tracheary elements in calli of *T. nucifera* and *C. japonica*.

In conclusion, we found that calli of *T. nucifera* developed tracheary elements that resembled secondary xylem with broad areas of secondary wall thickening and bordered pits. Such a system might be useful for studies of the mechanism of differentiation of secondary xylem cells in vitro.

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References

1. Fukuda H, Komamine A (1980) Establishment of an experimental system for the study of tracheary element differentiation from single cells isolated from the mesophyll of *Zinnia elegans*. Plant Physiol 65:57–60
2. Fukuda H (2004) Signals that control plant vascular cell differentiation. Nat Rev Mol Cell Biol 5:379–391
3. Oda Y, Mimura T, Hasezawa S (2005) Regulation of secondary cell wall development by cortical microtubules during tracheary element differentiation in *Arabidopsis* cell suspensions. Plant Physiol 137:1027–1036
4. Pesquet E, Korolev AV, Calder G, Lloyd CW (2010) The microtubule-associated protein AtMAP70-5 regulates secondary wall patterning in *Arabidopsis* wood cells. Curr Biol 20:744–749
5. Möller R, McDonald AG, Walter C, Harris PJ (2003) Cell differentiation, secondary cell-wall formation and transformation of callus tissue of *Pinus radiata* D. Don. Planta 217:736–747
6. Möller R, Koch G, Nanayakkara B, Schmitt U (2005) Lignification in cell cultures of *Pinus radiata*: activities of enzymes and lignin topochemistry. Tree Physiol 26:201–210
7. Möller R, Ball R, Henderson A, Modzel G, Find J (2006) Effect of light and activated charcoal on tracheary element differentiation in callus cultures of *Pinus radiata* D. Don. Plant Cell Tissue Organ Cult 85:161–171
8. Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497
9. Campbell RA, Durzan DJ (1975) Induction of multiple buds and needles in tissue cultures of *Picea glauca*. Can J Bot 53: 1652–1657
10. Nakagawa R, Ogita S, Kubo T, Funada R (2006) Effect of polyamines and L-ornithine on the development of proembryogenic masses of *Cryptomeria japonica*. Plant Cell Tissue Organ Cult 85:229–234
11. Begum S, Nakaba S, Oribe Y, Kubo T, Funada R (2010) Changes in the localization and levels of starch and lipids in cambium and phloem during cambial reactivation by artificial heating of main stems of *Cryptomeria japonica* trees. Ann Bot 106:885–895
12. Nakaba S, Sano Y, Kubo T, Funada R (2006) The positional distribution of cell death of ray parenchyma in a conifer, *Abies sachalinensis*. Plant Cell Rep 25:143–1148
13. Thomas TD (2008) The role of activated charcoal in plant tissue culture. Biotechnol Adv 26:618–631
14. Motose H, Fukuda H, Sugiyama M (2001) Involvement of local intercellular communication in the differentiation of *Zinnia* mesophyll cells into tracheary elements. Planta 213:21–131
15. Motose H, Sugiyama M, Fukuda H (2004) A proteoglycan mediates inductive interaction during plant vascular development. Nature 429:873–878