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Transfer of phenylpropanoids via the medium between xylem cells in *Zinnia* xylogenic culture

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Abstract

In order to understand cell-cell interactions involved in xylem differentiation, we studied intercellular molecules in an *in vitro Zinnia* xylogenic culture system, where single mesophyll cells transdifferentiate into tracheary elements (TEs) and xylem parenchyma cells. We found that UV-absorbing substances accumulated predominantly in xylogenesis-inducing medium and kept increasing even after the TEs died. This accumulation was inhibited by L-α-aminooxy-β-phenylpropionic acid (AOPP), an inhibitor of phenylalanine ammonia-lyase, and also by brefeldin A, an inhibitor of vesicle transport. These results indicated that living non-TE cells, probably xylem parenchyma cells, secrete some kinds of phenylpropanoids via a vesicle transport system. Further experiment showed that inhibition of brassinosteroid biosynthesis by uniconazole suppressed TE differentiation, but not the secretion of UV-absorbing substances into the medium, implying that differentiation of xylem parenchyma cells might not be strongly affected by the depletion of endogenous brassinosteroids.

Key words: brassinosteroid, coniferyl alcohol, differentiation, secretion, tracheary elements, xylem parenchyma cells, *Zinnia elegans*.

Abbreviations

AOPP, L-α-aminooxy-β-phenylpropionic acid; BA, 6-benzyladenine; BFA, brefeldin A;BR, brassinosteroid; CA, coniferyl alcohol; CA4H, cinnamic acid 4-hydroxylase; CAD, cinnamyl alcohol dehy drogenase; CCoAOMT, *S*-adenosyl-L-methionine: trans-caffeoyl-coenzyme A 3-*O*-methyltransferase; NAA, 1-naphthalenacetic acid; PAL, phenylalanine ammonia-lyase; TE, tracheary element

Introduction

In multicellular organisms, tissue pattern formation requires cell-cell communication based on positional information (Tickle, 1999; Kay, 2002; Teleman *et al.*, 2001). Laser ablation experiments and clonal analysis of plant roots demonstrated that positional cues, rather than cell lineage-information, take precedence for tissue pattern formation during root differentiation (Van Den Berg *et al.*, 1995; Berger *et al.*, 1998; Bouget *et al.*, 1998; Kidner *et al.*, 2000). However, little information is available on the signaling molecules that direct such cell-cell interactions. Vascular tissues in higher plants are composed of several kinds of cells with different tasks. These include tracheary elements (TEs), parenchyma cells, fiber cells, sieve elements and companion cells, all of which are derived from procambial or cambial cells and arranged in an ordered pattern. This organized pattern must be controlled by a complex cell-cell communication among the vascular cells. However, it is difficult to elucidate the characteristics of cell-cell communication in vascular tissues. An *in vitro Zinnia* xylogenic culture system, in which single mesophyll cells transdifferentiate into TEs in liquid medium, has provided important knowledge on TE differentiation (Fukuda, 1997). Furthermore, recent studies indicated that in this system xylem parenchyma-like cells, as well as TEs, are transdifferentiated from mesophyll cells (Shinohara *et al.*, 2000; Endo *et al.*, 2001; Hosokawa *et al.*, 2001; McCann *et al.*, 2001; Ohashi-Ito *et al.*, 2002; Ohashi-Ito and

Fukuda, 2003). These results encouraged us to use the *Zinnia* system for an *in vitro* study of communication between vascular cells. In fact, Motose *et al.* (2001a, b, c) used the *Zinnia* culture and found an arabinogalactan protein, designated xylogen, which was secreted from xylem precursor cells and which promoted TE differentiation. This finding suggests that intercellular signal molecules were secreted into the culture medium before they functioned. Therefore, to understand cell-cell communication that governs vascular cell organization, we aimed to characterize medium substances in relation to xylem cell differentiation in the *in-vitro Zinnia* culture. As a result, we found that UV-absorbing substances (280 and 340 nm) were secreted via a vesicle transport system by living non-TE cells into the medium in a close association with xylem cell differentiation. This secretion was not blocked by the depletion of brassinosteroids (BRs), which inhibited TE differentiation.

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 according to the method of Fukuda and Komanine (1980). The medium for the induction of TE differentiation (D-medium) contained 0.1 mg l^{-1} 1-naphthalenacetic acid (NAA) and 0.2 mg l^{-1} 6-benzyladenine (BA). For control culture, Cp-medium that contained 0.1 mg l^{-1} NAA and 0.001 mg l^{-1} BA was used.

Application of chemicals

A stock solution of L-α-aminooxy-β-phenylpropionic acid (AOPP) was prepared at 1 mM in distilled water, sterilized by filtration, and added into the medium to be 10 μM as a final concentration (Ingold *et al.*, 1990). As a control, sterilized distilled water was added instead of AOPP. A stock solution of Brefeldin A (BFA) was prepared at 10 mg ml^{-1} in ethanol and added into the medium to be 8 μg ml^{-1} as a final concentration (Rojas *et al.*, 1999). For the control, ethanol was added instead of BFA. To examine the effects of BRs on the accumulation of phenylpropanoids in the culture medium, 5 μM uniconazole or 5 μM uniconazole plus 10 nM brassinolide were added into the medium at the start of the culture period. In this experiment, all cultures contained a final concentration of 0.5% DMSO, which does not affect xylem differentiation (Fukuda and Komamine, 1981).

Determination of the differentiation rate of TEs

The frequency of TE differentiation was calculated as the proportion of TEs to the total cells, which included TEs and living cells. To identify dying and dead TEs, cells were incubated with Evan's blue (0.05%) for 3 min at room temperature just before counting. Dying TEs still had cytoplasm, which was stained with Evan's blue, while dead TEs had lost their cytoplasm, and thus were not stained. The number of cells from three or four samples was counted under a microscope.

Spectrum analysis of the medium

Cells were removed by centrifugation (3,000 rpm, 5 min) from the medium in which *Zinnia* cells had been cultured. The supernatant was filtered through a 0.22 μm filter (PVDF; Millipore, Bellerica, MA, USA) and its spectrum was obtained in a wavelength region of 250-500 nm with a spectrophotometer.

TLC analysis of UV-absorbing materials in the medium

The medium was concentrated with Sep-Pak C18 cartridges (Waters, Milford, MA, USA) according to the methods of Hosokawa *et al.* (2001). Phenylpropanoids were concentrated in 35 ml of medium with Sep-pak C18 cartridges into 2 ml of a 60% ethanol solution (17.5-fold condensation). Samples corresponding to 1.4 ml of medium were concentrated further by evaporation under a reduced pressure and were dissolved in 10 μl of ethanol. They were then chromatographed on TLC silica gel 60 F_{254} (Merck, Darmstadt, Germany) along with mixed standards for 1 mM *trans*-cinnamic acid, *p*-coumaric acid, ferulic acid, sinapic acid, caffeic acid, sinapaldehyde, sinapyl alcohol, coniferaldehyde, and coniferyl alcohol. The TLC plate was developed first in a mixture of ethyl acetate/formic acid/acetic acid/distilled water (100:11:11:25 v/v/v/v) for 5 cm, and then in benzene/dioxane/acetic acid (70:25:4 v/v/v) for 10 cm. After a brief air-drying, the plate was exposed to UV rays and the UV-absorbing substances were detected as black or fluorescent bands.

Visualization of the lignification of TEs

Autofluorescence of lignin on the secondary walls of TEs was detected using an excitation filter of 330-385 nm and a dichroic mirror of 420 nm with an epifluorescence microscope (Model BX-50, Olympus). All photographs were taken using a CCD camera under the same conditions.

Results

Differentiation-specific accumulation of UV-absorbing substances in the cultured medium

It has been indicated that isolated *Zinnia* mesophyll cells transdifferentiate into different types of vascular cells in culture and that there is a cell-cell interaction among these cells via the medium (Shinohara *et al.*, 2000; Hosokawa *et al.*, 2001; Motose *et al.*, 2001a,b). To understand cell-cell interaction via the medium, which is involved in vascular cell differentiation, we first tried to reveal changes in the factors contained in the medium in association with xylem cell differentiation. For that purpose, we measured the spectra of the culture medium in which *Zinnia* cells had been cultured, the "cultured medium" (**Fig. 1**).

Absorption maxima at 280 nm was detected in D-cultured medium after 48 h of culture. Thereafter, the absorption at 280 nm increased until 60 h, decreased at 72 h, and increased again after 96 h (**Figs. 1, 2b**). Absorption of the D-cultured medium at 340 nm increased continuously after 48 h of culture (**Figs. 1, 2b**). These increases in A_{280} and A340 were correlated with TE formation, which starts between 48 h and 60 h in xylogenesis-inducing (D) culture (**Fig. 2a**). To examine whether this phenomenon was specific for D culture, absorptions at 280 nm and 340 nm of the Cp-cultured medium were measured (**Fig. 2c**). The Cp medium contains 0.1 mg l^{-1} NAA and 0.001 mg l^{-1} BA and could induce few TEs (**Fig. 2a**). In contrast to the D-cultured medium, the Cp-cultured medium did not exhibit substantial increases in A_{280} and A_{340} (**Fig. 2c**). The measurement of protein contents in the cultured media revealed that the increase in A_{280} was independent of an increase in extracellular protein (data not shown). Analysis by reverse-phase TLC of the D-cultured medium revealed that absorptions in the UV region were derived from many substances (a-n), including a coniferyl alcohol-like substance (b) (**Fig. 2d**). Among them, amounts of b, e, g, h, i and k peaked at 48 h or 60 h of culture, which were similar to a change in A_{280}. Amounts of a, c, d, j and m increased continuously during late culture. However, there was no significant increase in UV-absorbing substances in the Cp-cultured medium (**Fig. 2e**).

Secretion of UV-absorbing substances from living cells other than TEs

In the D culture, there are two types of vascular cells, TEs and xylem parenchyma-like cells (Shinohara *et al.*, 2000; Hosokawa *et al.*, 2001). Because we wanted to determine which type of cells contributes to the secretion of UV-absorbing substances, we studied the timing of accumulation of the UV-absorbing substances and TE cell death in the D culture. Most TEs lost the selective permeability of their cytoplasmic membrane by 84 h of culture (**Fig. 3a**), while the UV absorption levels kept increasing, even at 84 h (**Fig. 3b**). This suggests that cells other than dead TEs provide the UV-absorbing substances. Hosokawa *et al.* (2001) indicated that lignin precursors were secreted into the medium and polymerized on dead TEs. To determine whether the UV-absorbing substances were phenylpropanoids, effects of AOPP, which is an inhibitor of phenylalanine ammonia-lyase (PAL), on the accumulation of the UV-absorbing substances in the D-cultured medium were examined. AOPP severely suppressed the increase in both A_{280} and A_{340} (**Fig. 3b, c**) and reduced almost all the UV-absorbing substances detected on the TLC (**Fig. 3d**). This strongly suggests that the UV-absorbing substances are phenylpropanoids derived from phenylalanine. Furthermore, the lignification suppressed by AOPP was overcome with the addition of the 108 h D-cultured medium (**Fig. 4**). This indicates that most of the UV-absorbing substances elevated in the D-cultured medium are phenylpropanoid derivatives, and at least some can be precursors of lignin. These results strongly suggest that living cells other than dead TEs secrete the lignin-related UV-absorbing substances. To confirm this, effects of BFA, which is an inhibitor of vesicle transport between the endoplasmic reticulum and the Golgi apparatus, on the increase in A_{280} and A_{340} were examined (**Fig. 3b, c**). The BFA treatment from 84 h to 108 h of culture at 8 μg ml^{-1}, which inhibited vesicle transport, but not the viability of

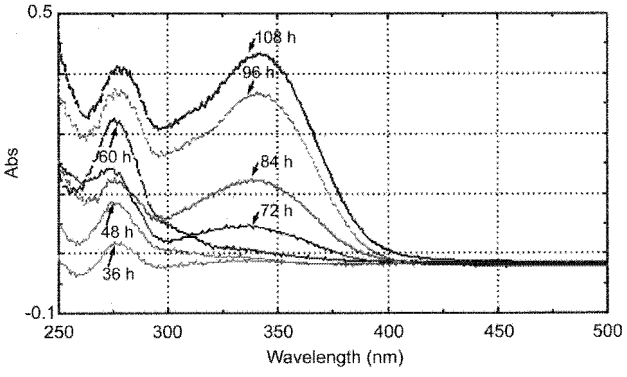

Fig. 1 Changes in the UV-spectrum of the D-cultured medium. The D-medium, in which cells were induced to transdifferentiate into xylem cells, was collected every 12 h from 36 to 108 h of culture and UV-spectra of the samples were recorded.

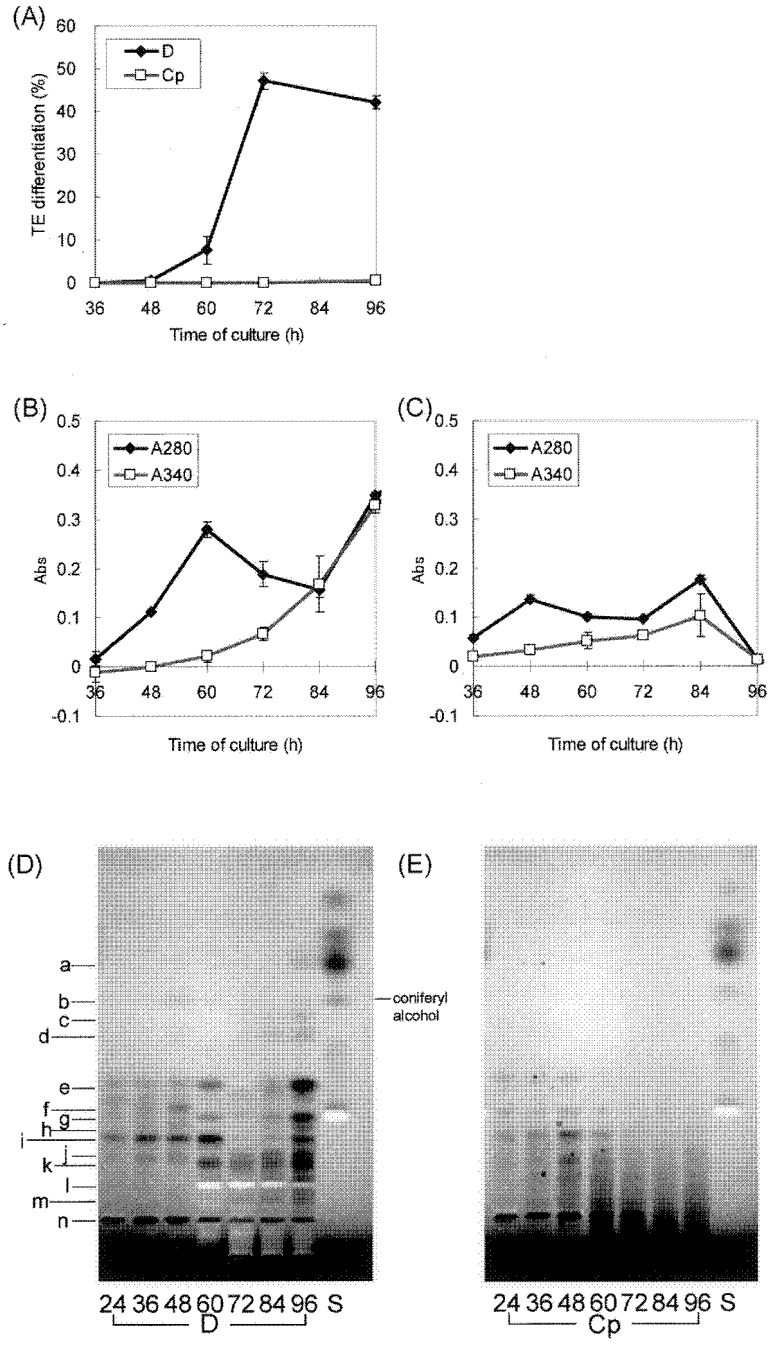

Fig. 2 Accumulation of UV-absorbing substances in the cultured medium during D and Cp culture. (A-C) Time course of tracheary element (TE) differentiation of Zinnia mesophyll cells cultured in the D- or Cp-medium (A), and changes in absorptions at 280 nm and 340 nm of the D-cultured (B), or Cp-cultured medium (C). Data are mean values of three replicates ± SD. (D, E) Reverse-phase TLC analysis of phenolics in the D-cultured (D), or Cp-cultured medium (E). Bands "a"-"n" indicate unknown UV-absorbing substrates in the D-cultured medium. "S" denotes standard phenolics, which include trans-cinnamic acid, *p*-coumaric acid, ferulic acid, sinapic acid, caffeic acid, sinapaldehyde, sinapyl alcohol, coniferaldehyde, and coniferyl alcohol.

cultured *Zinnia* cells (Rojas *et al.*, 1999), strongly suppressed the increase in A_{280} and A_{340} (**Fig. 3c, d**). This finding demonstrates that living non-TE cells secrete the UV-absorbing substances via the vesicle transport system.

Effects of brassinosteroids on the accumulation of lignin precursors and xylem cell differentiation

We have revealed that biosynthesized BRs initiate the final differentiation of TEs (Yamamoto *et al.*, 1997, 2001). Uniconazole, an inhibitor of cytochrome P450 enzymes involved in BR and gibberellin synthesis, suppresses the differentiation of TEs.

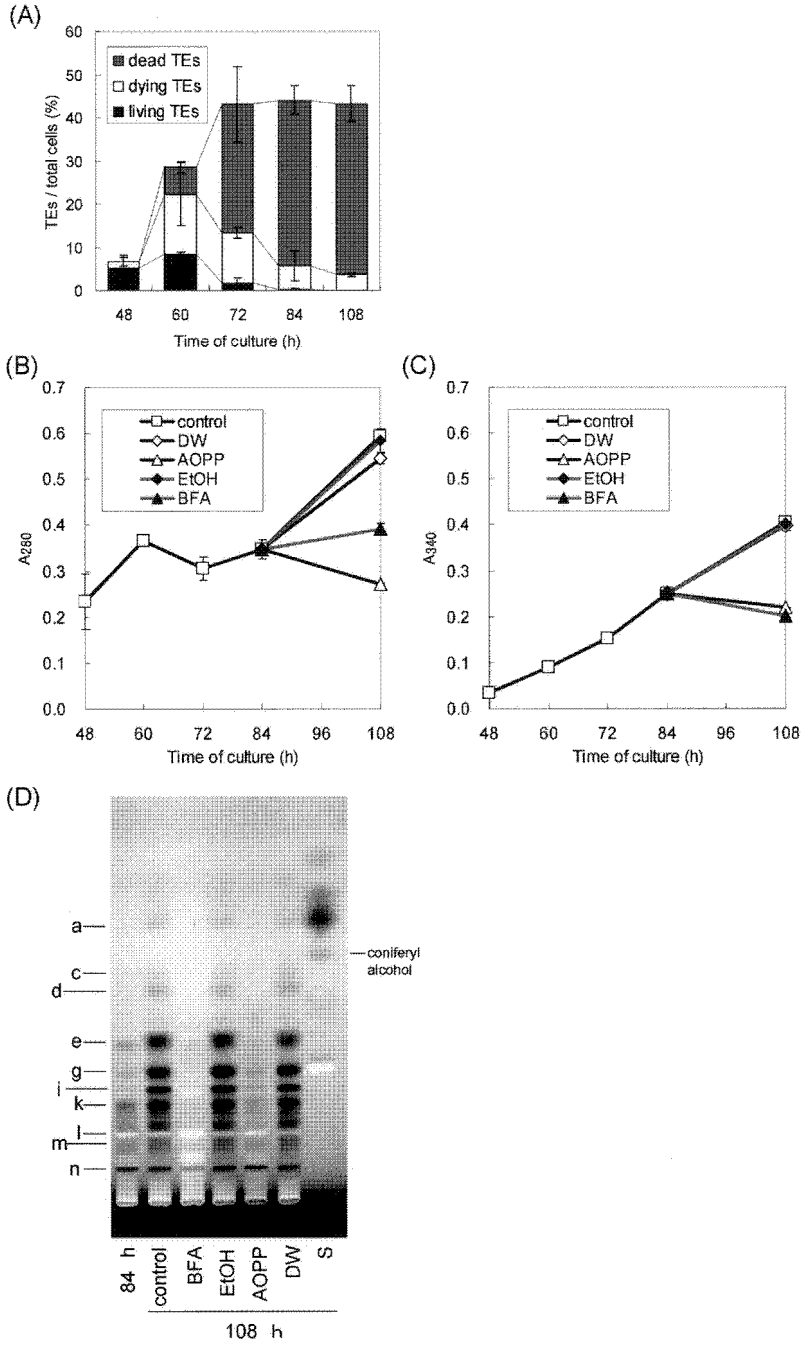

Fig. 3 Effects of brefeldin A (BFA) and *L*-α-aminooxy-β-phenylpropionic acid (AOPP) on the accumulation of UV-absorbing substances. (A) Changes in percentages of living tracheary elements (TEs), dying TEs, and dead TEs. Living TEs, dying TEs, and dead TEs were detected by Evan's blue staining. Data are mean values of three replicates ± SD. (B-D) At 84 h of culture, 8 μg ml^{-1} BFA or 10 μM AOPP was added to the cell cultures. Cells were cultured for another 24 h and the UV-absorbing substances in the medium analyzed. As a control, ethanol or distilled water rather than BFA or AOPP, respectively was added to the cell cultures. Changes in UV absorbances at 280 nm (B) and 340 nm (C). Data are mean values of three replicates ± SD. (D) Reverse-phase TLC analysis of UV absorbing materials. "S" indicates the standard substrates described in **Fig. 2.**

Exogenously supplied brassinolide, an active BR, releases this suppression (Iwasaki and Shibaoka. 1991). To determine whether endogenous BRs affect the accumulation of the UV-absorbing substances, we treated *Zinnia* cells with uniconazole and brassinolide. Although uniconazole prevented TE differentiation almost completely, it did not suppress the accumulation of the UV-absorptions (**Fig. 5**). Interestingly, A_{280} kept increasing, even in the presence of uniconazole, whereas it increased

until 48 h and then increased again after 96 h in the absence of uniconazole (control). Brassinolide altered the continuously increasing pattern in the uniconazole-treated culture to the control pattern, although A_{280} in uniconazole-brassinolide treated culture was slightly lower than that in the non-treated control culture. Treatment with uniconazole or uniconazole plus brassinolide did not alter significantly the accumulation pattern of phenylpropanoid-related substances which were detected by reverse-phase TLC (**Fig. 5d**).

Discussion

Xylem parenchyma cells secrete phenylpropanoids via a vesicle transport system

Many UV-absorbing substances were detected in the D-cultured medium by TLC. Accumulation of the UV-absorbing substances was inhibited by AOPP, which indicates that the UV-absorbing substances were phenylpropanoids. The finding that cultured medium containing the UV-absorbing substances restored the inhibition of lignification of AOPP treated TEs indicates that the UV-absorbing substances contained phenylpropanoids that were lignin precursors. Because the absorption maxima of already-known lignin precursors, CA, sinapyl alcohol, coniferaldehyde, and sinapaldehyde are about 270, 275, 340, and 350, respectively (data not shown), the substances with 280 nm and 340 nm maxima may be derivatives of such aromatic alcohols and aldehydes, respectively. Indeed HPLC and NMR analyses of the D-cultured medium revealed that all of the major 4 peak substances are dimer of coniferyl alcohol and could restore the inhibition of TE lignification by AOPP (N.Tokunaga *et al*, unpublished). This result supports that the UV-absorbing substances are derived from these lignin precursors.

The UV-absorbing substances accumulated even after almost all TEs had died. In *Zinnia* culture, transcripts of *ZePAL3, ZC4H*, and *ZCAD1* continue to accumulate after the TEs die (Sato *et al.*, 1997; Yamamoto *et al.*, 1997; Demura *et al.*, 2002). Cultured cells also retain high activities of PAL, cinnamic acid 4-hydroxylase (CA4H), *S*-adenosyl-L-methionine:*trans*-caffeoyl-coenzyme A 3-*O*-methyltransferase (CCoAOMT), cinnamyl alcohol dehydrogenase (CAD) after the TEs die (Fukuda and Komamine, 1982; Lin and Northcote, 1990; Ye *et al.*, 1994; Ye, 1996; Sato *et al.*, 1997). These facts suggest that living non-TE cells may synthesize lignin precursors. Our finding that the accumulation of UV-absorbing substances was inhibited by BFA demonstrated that living non-TE cells secrete phenylpropanoids related to lignin via a vesicle transport system. The secretion of lignin precursors via a vesicle transport system has also been suggested by autoradiographic studies with an electron microscope showing that Golgi vesicles as well as cell walls were labeled in the developing wheat coleoptile primary xylem exposed to tritiated cinnamic acid, a lignin precursor (Pickett-Heaps, 1968). Because this secretion occurs only in TE-inductive culture, in which TEs and xylem parenchyma cells differentiate, non-TE cells that secrete lignin precursors may be xylem parenchyma cells.

Involvement of brassinosteroids in secretion of the UV-absorbing substances and xylem cell differentiation

BRs are generally involved in plant development (Clouse, 2002). In particular, a tight coupling of xylem differentiation and BRs is well known (Iwasaki and Shibaoka, 1991; Yamamoto *et al.*, 1997; Nagata *et al.*, 2001; Yamamoto *et al.*, 2001; Clay and Nelson, 2002; Ohashi-Ito *et al.*, 2002; Ohashi-Ito and Fukuda, 2003). Iwasaki and Shibaoka (1991) indicated that uniconazole suppressed TE differentiation by preventing the endogenous synthesis of BRs. Our data confirm their result (**Fig. 5a**). However, the UV-absorbing substances continued to accumulate in the presence of uniconazole. The composition of the UV-absorbing substances were not affected either by uniconazole. These results imply that the secretion of lignin precursors is not substantially suppressed by uniconazole. As discussed in the previous section, xylem parenchyma cells secrete lignin precursors in *Zinnia* xylogenic culture. Therefore, these results strongly suggest that the depletion of endogenous BRs does not suppress the differentiation of xylem parenchyma cells, but rather suppresses TE differentiation. Yamamoto *et al.* (1997) revealed that uniconazole repressed the accumulation of transcripts of lignin-related genes when TE differentiation occurred. However, there were some differences in the suppression of gene expression by uniconazole among genes. Uniconazole blocked completely the accumulation of transcripts of a TE-specific peroxidase involved in lignification, as well as a TE-specific cysteine protease involved in programmed cell death. However, it did not completely suppress that of PAL and CA4H, which are involved in lignin precursor biosynthesis. This result can be explained by the idea that the depletion of BRs does not strongly affect the differentiation of xylem parenchyma cells, but rather of TE differentiation.

In summary, our results strongly suggest that 1) at

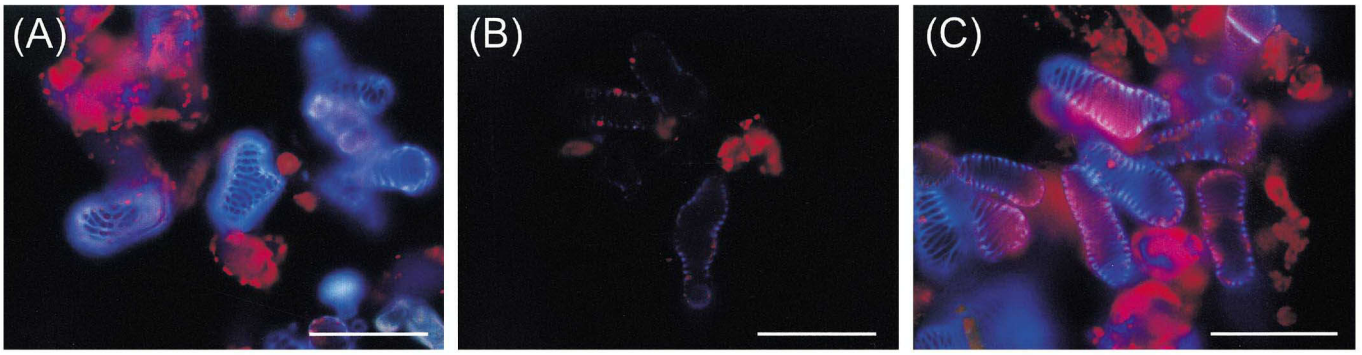

Fig. 4 Restoration of lignification in L-α-aminooxy-β-phenylpropionic acid (AOPP)-treated TEs by the D-cultured medium. (A) A fluorescence image of TEs cultured for 96 h. Lignified secondary wall of TEs exhibits auto-fluorescence upon exposure to UV. (B) A fluorescence image of AOPP-treated TEs. TEs have only faint autofluorescence derived from lignin. 10 μM AOPP was added into medium at the start of the culture period. (C) A fluorescence image of TEs formed when cells were cultured for 96 h with AOPP, and the D-cultured medium in which cells had been cultured for 108 h. The same volume of the D-cultured medium was added to the cell culture at 48 h of culture. Note that the addition of the D-cultured medium restores the lignification of AOPP-treated TEs. Bar = 100 μm.

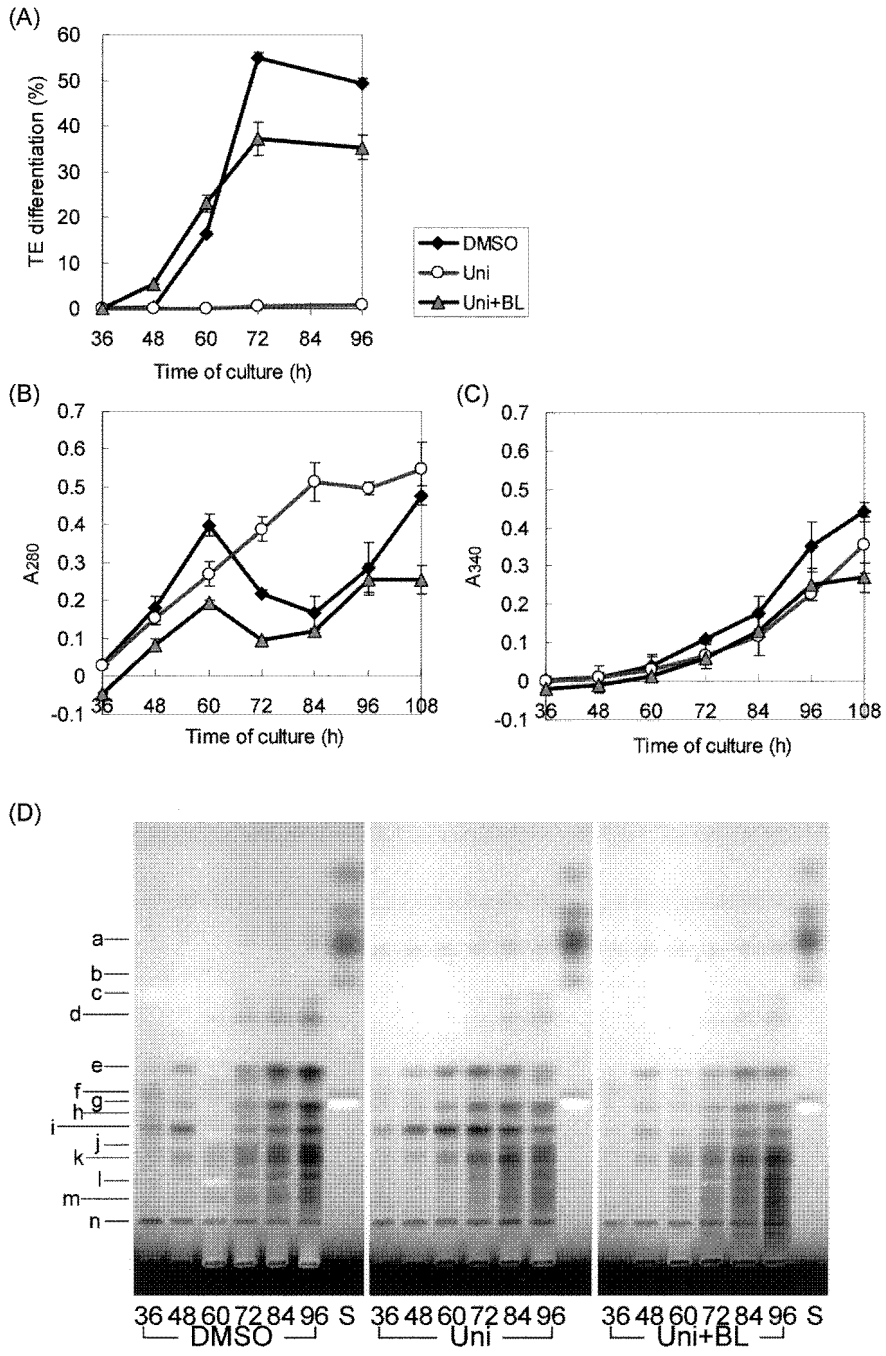

Fig. 5 Effects of uniconazole and brassinolide on the accumulation of A_{280}- and A_{340}-absorbing substances in the cultured medium. *Zinnia* mesophyll cells were cultured in the D-medium with 5 μM uniconazole (Uni), 5 μM uniconazole plus 10 nM brassinolide (Uni + BL), or 0.5% (v/v) DMSO only as a control (DMSO). (A-C) Time course of tracheary element (TE) differentiation (A) and change in absorptions at 280 nm (B) and 340 nm (C). Data are mean values of three replicates ± SD. (D) Reverse-phase TLC analysis of phenolics. "S" indicates the standard substrates described in **Fig. 2.**

least two types of xylem cells, TEs and xylem parenchyma cells, are differentiated in *Zinnia* xylogenic culture, 2) the two types of cells communicate via lignin precursors in medium, and 3) BRs promote differentiation only one type of xylem cells, TEs.

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