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Rapid Report

Brassinosteroids Induce Entry into the Final Stage of Tracheary Element Differentiation in Cultured *Zinnia* Cells

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To elucidate the involvement of brassinosteroids in the progression of tracheary element differentiation in cultured *Zinnia* cells, we analyzed the effects of uniconazole, an inhibitor of brassinosteroid synthesis, and brassinolide, a biologically active brassinosteroid, on the accumulation of mRNAs for various genes that were expressed in different stages of differentiation. Uniconazole specifically suppressed the accumulation of transcripts for genes that were induced in the final stage of differentiation in association with secondary wall formation and cell death. This suppression was recovered with the addition of brassinolide. These results strongly suggest that endogenous brassinosteroids induce entry into the final stage of differentiation.

Key words: Brassinosteroids — Cell death — Gene expression — Secondary wall formation — Tracheary element differentiation — *Zinnia elegans*.

Brassinosteroids are a group of plant steroids that occur in a broad range of plant species. Recent rapid progress in the knowledge about the synthesis and function of brassinosteroids obtained from various biosynthesis and sensitive mutants has furnished conclusive evidence that brassinosteroids are plant growth hormones (Clouse 1996, Hooley 1996, Yokota 1997). Brassinosteroids elicit divergent biological activities, which include functions as developmental regulators of stem elongation, pollen tube growth, leaf bending, root growth, and xylem formation (Yokota 1997). These developmental activities are associated with the promotion of ATPase activity (Cerana et al. 1983), the synthesis of 1-aminocyclopropane-1-carboxylic acid synthase (Arteca 1983), alternation of microtubule orientation (Mayumi and Shibaoka 1995), and the modification of cell walls (Zurek et al. 1994). Recent molecular genetic analysis with *Arabidopsis* mutants has revealed that brassinosteroids function in cell elongation and the suppression of the light-dependent morphogenesis (Li et al. 1996, Szekeres et al. 1996, Kauschmann et al. 1996). Furthermore, brassinosteroids can regulate the expression of specific genes such as *BRU1* (Zurek and Clouse 1994) and *TCH4* (Xu et al. 1995) in elongating tissues. However, the molecular mechanism of brassinosteroid functions in plant development is still open for investigation.

Clouse and Zurek (1991) observed that exogenously supplied brassinolide promoted both tracheary elements (TE) differentiation and cell division in cultured tuber explants of Jerusalem artichoke. Using the *Zinnia* system, in which single mesophyll cells can differentiate directly into TEs, Iwasaki and Shibaoka (1991) also indicated that exogenously supplied uniconazole, (E)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol, prevented uncommitted cells from transdifferentiating into TEs without inhibiting cell division and that brassinolide restored its inhibition. These results have strongly suggested that brassinosteroids are involved in differentiation of parenchyma cells into TEs.

The process of transdifferentiation of *Zinnia* mesophyll cells into TEs is divided into three stages, Stage I, II, and III (Fukuda 1996, 1997). Stage I, which immediately follows the induction of differentiation by phytohormones and wounding, corresponds to a functional dedifferentiation process during which isolated mesophyll cells lose their potential to function as photosynthetic cells. During Stage II, various vascular cell-specific genes such as *TED2, TED3*, and *TED4* begin to be expressed (Demura and Fukuda 1993, 1994). The analysis of Stage II with these genes as markers has suggested that, during Stage II, dedifferentiated cells change into TE precursor cells after becoming procambium-like cells and then immature xylem parenchyma cells, corresponding to the process of differentiation from meristematic cells to TE precursor cells via procambial initials in vivo (Fukuda 1994). Stage III is a final stage that involves secondary wall formation and cell death, which is common both in vitro and in vivo.

This *Zinnia* system facilitates analysis at cellular levels of the mechanisms by which brassinosteroids regulate differentiation. In this report, we show that uniconazole suppresses the accumulation of transcripts for Stage III-spe-

Abbreviations: BA, benzyladenine; NAA, α-naphthaleneacetic acid; PAL, phenylalanine ammonia-lyase;TE, tracheary element.

The nucleotide sequence reported in this paper has been submitted to DDBJ/EMBL/GenBank Nucleotide Sequence Databases under the accession number AB005288.

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cific genes and that exogenously supplied brassinolide releases this suppression, evidence that brassinosteroids may induce entry into Stage III.

First leaves of 14-d-old seedlings of *Zinnia elegans* L. cv. Canary bird were macerated with a homoblender in a culture medium (Fukuda and Komamine 1980). Isolated mesophyll cells were cultured in a differentiation-inducing medium that contained 1.0 mg liter^{-1} benzyladenine (BA) and 0.1 mg liter^{-1} α-naphthaleneacetic acid (NAA). Uniconazole and/or brassinolide were added into the culture medium at the start of culture. Uniconazole was kindly supplied by Sumitomo Chemical Co., Ltd. (Hyogo, Japan). A mixture of S-and R-optical isoforms (8 : 2) of uniconazole was used for experiments, because Iwasaki and Shibaoka (1991) demonstrated that both the isoforms have similar inhibitory effects on TE differentiation.

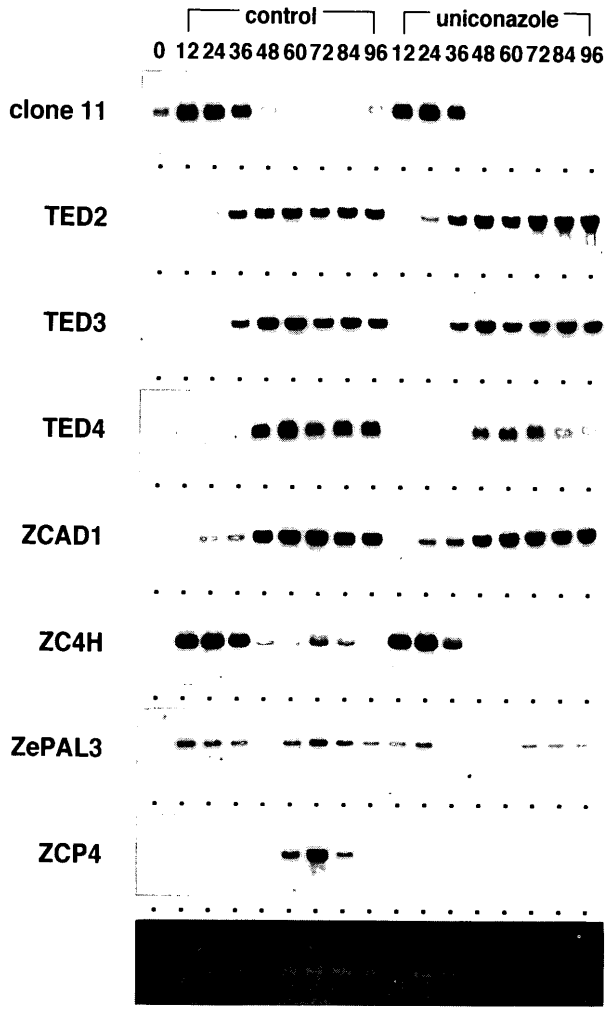

Fig. 1 Changes in the accumulation of mRNAs for various genes during TE differentiation in the presence (-uniconazole-) or absence of 5 μM uniconazole (control). Total RNA was isolated from *Zinnia* mesophyll cells that had been cultured for indicated periods in a TE differentiation-inducing medium. RNA gel blot hybridization was performed with digoxigenin-labeled antisense RNA probes of clone11 (a cDNA clone whose corresponding transcripts are expressed during Stage I), *TED2, TED3, TED4* (TE-differntiation related genes), *ZCAD1* (a gene encoding cinnamyl alcohol dehydrogenase), *ZePAL3* (a gene encoding PAL), *ZC4H* (a gene encoding cinnamate 4 hydroxylase), *ZCP4* (a gene encoding cysteine protease). Ethidium bromide-stained bands of rRNA is shown to demonstrate the equal loading (10 μg) of RNA.

As a marker of Stage I, we used a cDNA (clone 11) whose mRNAs are strongly expressed 12 h after the start of culture (Fig. 1, Miyo Nagata, Taku Demura and Hiroo Fukuda, unpublished). As markers of Stage II, we used *TED2, TED3*, and *TED4* (Demura and Fukuda 1993, 1994) and *ZCAD1* encoding cinnamyl alcohol dehydrogenase (Sato et al. 1997). The expression of these mRNAs is known to start during Stage II. Stage III includes two key events, secondary wall formation and cell death. As a marker of secondary wall formation, we isolated a cDNA (*ZePAL3*) for phenylalanine ammonia-lyase (PAL) from differentiating *Zinnia* cells by PCR and subsequent screenings of a cDNA library of differentiating *Zinnia* cells with the PCR fragment as a probe. A cDNA for cinnamic acid 4-hydroxylase (*ZC4H*) was also used as a maker of secondary wall formation (Ye et al. 1994). *ZCP4* (Atsushi Minami, Taku Demura and Hiroo Fukuda, unpublished), which encodes cysteine protease, involved in autolysis was used as a cell death marker.

TEs with visible secondary walls appeared around 60 h of culture, and the percentage of differentiated cells reached a maximum (40%) at 72 h. Uniconazole, when added into the culture medium at a concentration of 5 μM at the start of the culture, prevented TE formation almost completely (see Fig. 2), but did not affect cell division (data not shown). RNA was isolated at 12-h intervals from *Zinnia* cells cultured in the presence or absence of uniconazole, and RNA gel blot analysis was carried out using digoxigenin-labeled antisense RNA probes for the cDNAs

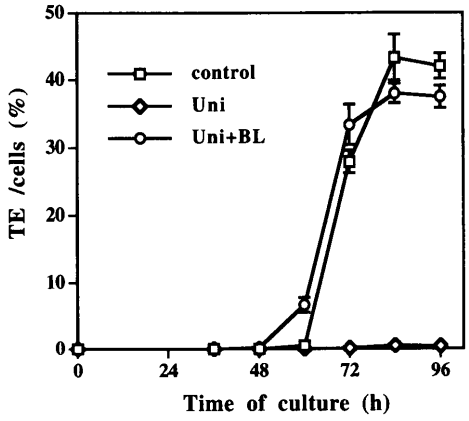

Fig. 2 Effects of uniconazole and brassinolide on the formation of TEs. Isolated mesophyll cells were fed with 5 μM uniconazole (◇), 5 μM uniconazole and 10 nM brassinolide (○), or 0.5% (v/v) DMSO only (□) at the start of culture and cultured for various periods.

(Fig. 1). mRNAs for clone 11 accumulated greatly between 12 and 36 h of culture and slightly between 84 and 96 h. This accumulation was not affected by uniconazole that was added at the start of culture. The accumulation of mRNAs for two other genes that begin to be expressed in Stage I was not suppressed by uniconazole (data not shown). The accumulation of *TED2* and *ZCAD1* mRNAs started at 24 h of culture, then increased rapidly, and decreased after 60 or 72 h. *TED3* and *TED4* mRNAs increased after 36 h and continued to accumulate throughout the culture period with a slight decrease after 72 h. The accumulation of these mRNAs was not prevented significantly by uniconazole, although the accumulation of *TED4* mRNAs was slightly inhibited by uniconazole. In contrast, *ZCP4* mRNAs, which were transiently expressed between 60 and 84 h, did not appear at all in the presence of uniconazole. Lin and Northcote (1990) have reported that there are two peaks of the accumulation of PAL mRNAs during TE differentiation in cultured *Zinnia* cells. It is known that the first accumulation that occurs during Stage I is induced by wounding and that the second that occurs during Stage III is induced in relation to lignin synthesis (Fukuda 1996). Like the changes in total PAL mRNAs, *ZePAL3* mRNAs appeared 12 h of culture, decreased after 24 h, then increased again after 60 h, and decreased after 72 h. Uniconazole did not affect the first accumulation that occurs during Stage I but severely reduced the accumulation during Stage III. Because low but substantial levels of PAL mRNAs were accumulated after 48 h of culture even in non-differentiating cells (Lin and Northcote 1990), the low accumulation of *ZePAL3* mRNAs during Stage III in the presence of uniconazole may be due to non-differentiating cells. The accumulation pattern of *ZC4H* mRNAs was very similar to that of *ZePAL3* mRNAs, indicating that uniconazole suppressed the increase in *ZC4H* mRNAs during Stage III of differentiation. Such specific inhibition of mRNA accumulation by uniconazole was also observed for a gene encoding lignin-related peroxidase that is expressed specifically and transiently during Stage III (data not shown). These results indicate that uniconazole suppresses the accumulation of mRNAs for genes that start to be expressed during Stage III in association with secondary wall formation and cell death, although it does not inhibit the accumulation of mRNAs for genes that start to be expressed during Stage I and Stage II.

The inhibition of TE differentiation by uniconazole was recovered by the addition of 10 nM brassinolide at the start of culture (Fig. 2) or 24 h after culture began (data not shown). The presence of exogenously supplied brassinolide throughout culture hastened TE formation. The addition of brassinolide also restored the suppression of accumulation of mRNAs for *ZePAL3, ZC4H* and *ZCP4* by uniconazole (Fig. 3). The accumulation of mRNAs was also sped up by brassinolide in accordance with the time course

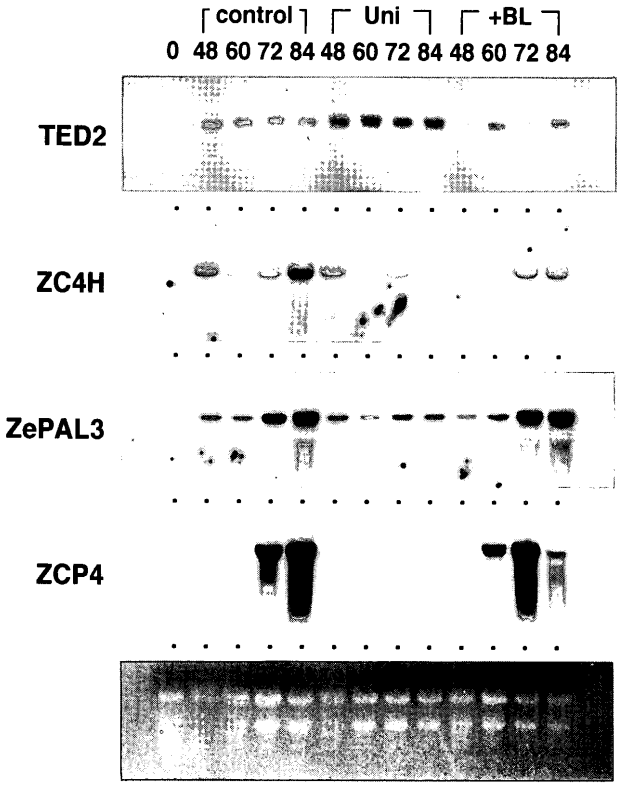

Fig. 3 Recovery from uniconazole-inducing suppression of transcript levels by brassinolide. Total RNA was isolated from *Zinnia* mesophyll cells that had been cultured for indicated periods with 0.5% (v/v) DMSO (control), 5 μM uniconazole (Uni), and 5 μM uniconazole plus 10 nM brassinolide (+BL). RNA gel blot hybridization was performed with digoxigenin-labeled antisense probes of *TED2, ZePAL3, ZC4H* and *ZCP4*. Ethidium bromide-stained bands of rRNA are also shown.

of TE formation.

A detailed analysis by Iwasaki and Shibaoka (1991) has indicated that the inhibitory effect of uniconazole on TE differentiation is due to the suppression of brassinosteroid synthesis in cultured *Zinnia* cells. In our experiments, uniconazole did not affect the accumulation of mRNAs for genes that start to be expressed during Stage I. This result implies that enodogenous brassinosteroids are not involved in the expression of genes in Stage I, and probably not in the progression of Stage I. This conclusion is supported by the fact that the addition of brassinolide at 24th h of culture releases, without any delay of differentiation, the inhibitory effects of uniconazole on TE differentiation supplied at the start of culture. In situ hybridization strongly suggested that the expression of *TED* genes is restricted in vascular cells; *TED2* is expressed in procambium, immature phloem, and immature xylem, *TED4* is mainly in immature xylem and *TED3* is specifically in TE precursor cells (Demura and Fukuda 1994). TE precursor cell-specific expression of *TED3* was demonstrated by the fact that

TED3 promoter fused to the GUS gene directed the specific expression of GUS activity in TE precursor cells of all organs in plants and even of callus (Megumi Igarashi, Taku Demura and Hiroo Fukuda, unpublished). Based on these results, we have suggested that Stage II involves the changes of dedifferentiated cells into TE precursor cells after becoming procambial-like cells and then immature xylem cells (Demura and Fukuda 1994, Fukuda 1994). Uniconazole did not suppress the accumulation of *TED* mRNAs. This result strongly suggests that enodogenous brassinosteroids are not involved in the progress of Stage II. However, uniconazole suppressed the accumulation of mRNAs for genes that are involved both in cell death and in secondary wall formation and that are induced during Stage III. Moreover, the inhibition was completely recovered by bassinolide. Thus, our results imply that endogenous brassinosteroids may induce the transition from Stage II into Stage III, which is the final step of TE differentiation. This is the first report suggesting that endogenous brassinosteroids are involved in the induction of a specific step of differentiation in plants through gene expression.

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