Molecular Mechanism of Transdifferentiation of Isolated Mesophyll Cells into Xylem Cells

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INTRODUCTION

Differentiation in plant cells is plastic. Differentiated cells can transdifferentiate *in vitro* to other types of cells. Xylem cell differentiation is an excellent example of transdifferentiation occurring at the cellular level in higher plants. *In situ,* vessels and tracheids are formed from cells referred to as tracheary elements, which are differentiated from cells of the procambium of the root and shoot in primary xylem or from cells produced by the vascular cambium in secondary xylem. Tracheary elements are characterized by the formation of a secondary cell wall with annular, spiral, reticulate or pitted wall thickenings. At maturity, differentiating tracheary elements lose their nuclei and cell contents, forming a hollow tubular system.

We have established an *in vitro* experimental system as a useful model for the study of tracheary element differentiation: Single mesophyll cells isolated from *Zinnia* leaves can transdifferentiate directly into tracheary elements without cell division, synchronously and at high frequency by the initiation of wounding and a combination of auxin and cytokinin (Fukuda and Komamine, 1980, Fukuda 1989, 1992). We report here our recent work on the differentiation of *Zinnia* mesophyll cells into tracheary elements.

MATERIALS AND METHODS

Single mesophyll cells were isolated mechanically from the first true leaves of 14-day-old seedlings of *Zinnia elegans*. Isolated cells were cultured in a liquid medium containing 0.1 mg/l 1-naphthaleneacetic acid (NAA) and 1 mg/l benzyladenine (BA), resulting in 30-60% of differentiation into tracheary elements synchronously between 60 and 80 h of culture (Fukuda and Komamine, 1980, Fukuda, 1989). As controls, cells which were cultured in the presence of 0.1 mg/l NAA and 0.001 mg/l BA or in the absence of hormones were used.

RESULTS

1. INITIATION

Both auxin and cytokinin were prerequisites for the induction of differentiation of *Zinnia* mesophyll cells into tracheary elements. Wounding was also essential for the initiation of differentiation. Efforts are underway to isolate genes induced rapidly by these signals.

2. EVENTS OCCURRING DURING DIFFERENTIATION

A. cytoskeleton

Microtubules in differentiating tracheary elements are localized as bands over the thickenings of secondary walls. Disruption of microtubules with an agent such as colchicine causes the formation of unlocalized secondary wall thickenings. These observations have led people to the hypothesis that microtubules determine the wall pattern by predicting the position and orientation of secondary walls. Therefore, we examined changes in organization of the cytoskeleton including microtubules and actin filaments during tracheary element differentiation (Kobayashi et al., 1988). We found that both microtubules and actin filaments are involved in the regulation of localized thickening of secondary walls which is a specific feature of tracheary elements. We indicated a coordinated mechanism in which actin filaments are involved in the reorganization of microtubules which, in turn, regulate the spatial disposition of secondary walls (Fukuda and Kobayashi, 1989).

In association with the dynamic organization of microtubules, their number increased, and the increase was dependent on new synthesis of tubulin containing both α and β subunits (Fukuda, 1987). Detailed analysis of the expression of β-tubulin genes indicated that three β-tubulin genes are expressed differentially during tracheary element differentiation in *Zinnia* cells.

B. Lignin synthesis

Lignification occurs specifically on secondary wall thickenings and is one of the most characteristic biochemical markers of tracheary elements. Lignin biosynthesis is catalyzed by many enzymes including phenylalanine ammonia-lyase (PAL) and peroxidase. We have shown that PAL and wall bound peroxidases, which are composed of ionically bound and tightly bound peroxidase, increased in a differentiation-specific manner (Fukuda and Komamine, 1982).

The ionically bound peroxidase fraction contained at least 5 cationic isozymes (P1-5; Sato et al., 1993). Although all cationic isozymes increased with the age of culture, only P4 and P5 increased in a differentiation-specific manner. The increase in P5 activity precedes that in P4 activity. Cells cultured for 72 h were separated into 4 Percoll density gradient fractions with mature tracheary elements concentrated in the >20% Percoll fraction. P5, a differentiation-specific isozyme, was a major component of the ionically bound peroxidases extracted from the mature tracheary element-rich fraction, and P1 and P2 were also found to be present in the fraction. This suggests that although P5 is essential for lignification, other peroxidases may also share different functions in this process.

In differentiating *Zinnia* cells, at least 4 different PAL genes were found to be expressed. We constructed a binary vector containing a fragment of PAL DNA in an antisense orientation, and introduced it into *Zinnia* leaf segments from which roots were induced. In these transformed roots, the antisense PAL DNA suppressed not only the endogenous PAL activity but also normal development of xylem.

C. Autolysis

The first visible signal of autolysis during tracheary element differentiation is the disruption of the tonoplast. In differentiating *Zinnia* cells, the tonoplast was disrupted several hours after the formation of visible secondary walls, and cell contents were lost the remaining several hours later. We examined the activities of various hydrolytic enzymes in differentiating *Zinnia* cells. Among the enzymes, protease activity was found to increase more conspicuously in the differentiation-inductive culture than in a control culture, in the late process of differentiation. Partial purification of the protease that was expressed preferentially in differentiation-induced cultures showed it to be a thiol-protease.

D. Others-Expression of newly isolated genes in relation to differentiation

Around 12 h before secondary wall deposition starts, minor but differentiation-specific changes were observed on two-dimensional polypeptide maps of *Zinnia* cells (Fukuda and Komamine, 1983). Two newly synthesized polypeptides appear in cells cultured in medium that induces formation of tracheary elements, but not in cells in a control medium, and their synthesis continues at least until the time at which a secondary wall begins to form.

We isolated, through differential screening of a cDNA library, four clones that contained cDNA inserts whose corresponding mRNAs were expressed preferentially in cells in the culture in which differentiation was being induced (Table 1; T. Demura and H. Fukuda, submitted). The cDNAs were designated TEDs (for Tracheary Element Differentiation-related genes) 1 to 4, respectively. TED 1 cDNA corresponded to the mt atpA gene (mitochondrial gene for F1-ATPase a-subunit) in *Zinnia*. A homology search revealed significant similarity between TED 2 and the gene for zeta-crystallin, which was recently demonstrated to function as an NADPH:quinone oxidoreductase, from the guinea pig lens. The polypeptide sequence deduced from TED 3 cDNA showed that TED 3 encodes a hydrophilic protein with the Asp-Gly-Tyr motif which is repeated fifteen times. The polypeptides encoded by TED 3 were suggested to be a protein that is located in the secondary walls of tracheary elements. TED 4 cDNA encodes a polypeptide of 10kDa with eleven hydrophobic amino acids which may be a transit peptide, at the N-terminus. A homology search with the nucleotide and deduced amino-acid sequences of TED

Table 1. Characteristics of TED cDNAs

cDNA	NA (bp)	AA	Characteristics	Homology
TED1	(1700)			mitochondria F1-ATPase α–subunit
TED2	1183	325	hydrophobic regions	ζ-crystallin of guinea pig ADH
TED3	1435	319	(NGY) motifs repeated amino acid sequences	GRP
TED4	535	95	transit peptide metal-binding "finger"	aleurone-specific-protein (B11E) of barley

4 revealed significant similarity to those of the barley aleurone-specific clone, B11E. Each gene is expressed at a detectable level after 36 h of culture of *Zinnia* cells and is expressed preferentially in cells in the differentiation-induced culture. This timing is considered to correspond to a shift from the early to the late process of differentiation. *In situ* hybridization using probes from these cDNAs revealed that these genes were expressed specifically in differentiating xylem cells in cotyledons of *Zinnia* seedlings. Thus, these genes may play roles in the initiation or the progression of the late process of xylem differentiation in both *in vitro* and *in situ*.

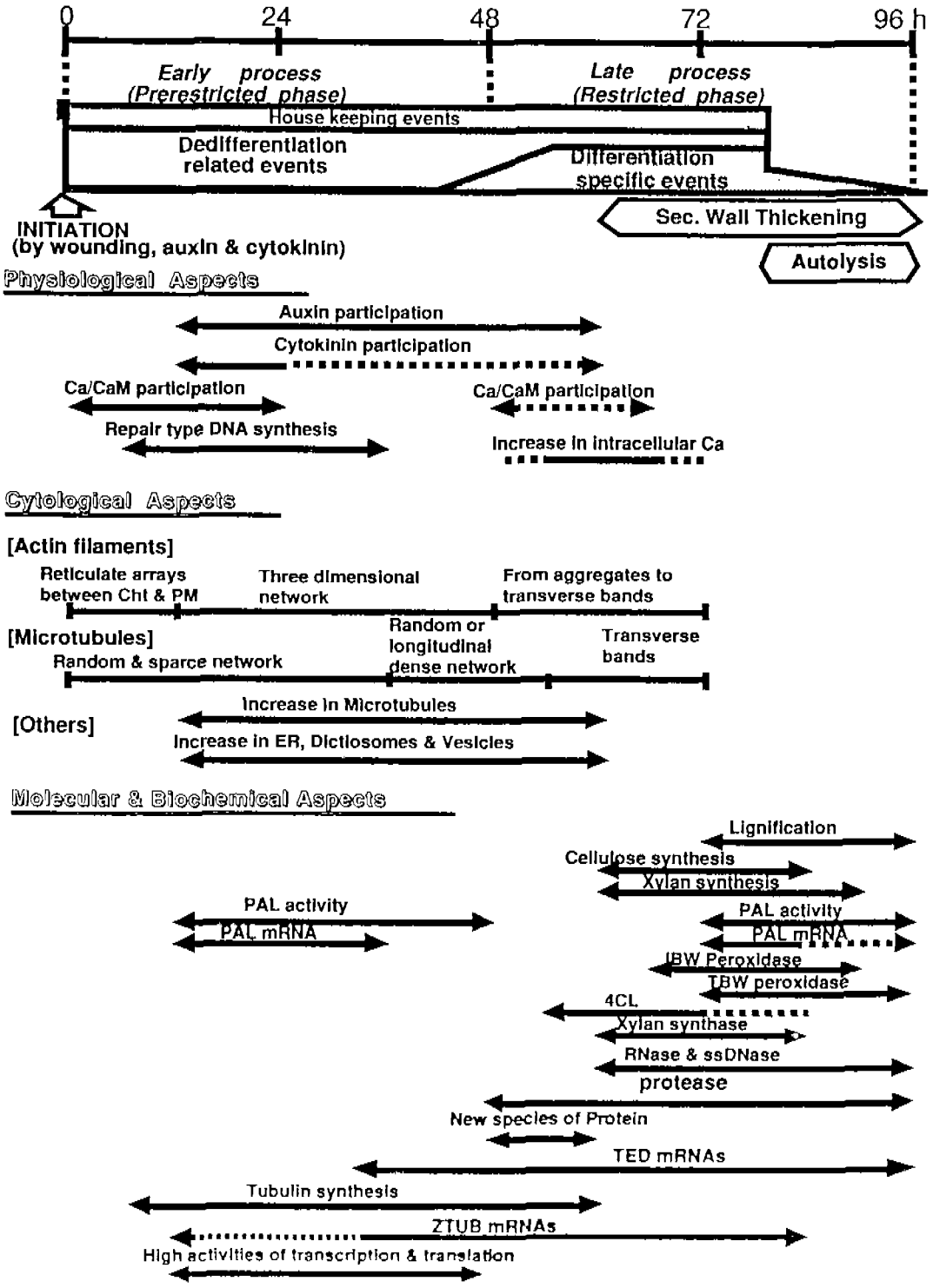

Fig. 1. Sequential events in the process of tracheary element differentiation from single mesophyll cells of *Zinnia elegans*.

DISCUSSION

Figure 1 is a summary of various events that occur during transdifferentiation of isolated mesophyll cells of *Zinnia elegans* into tracheary elements (Fukuda, 1992). The initiation of differentiation occurs by wounding and phytohormones, leading isolated mesophyll cells to differentiate into tracheary elements. The process of differentiation is divided into early and late processes, which may be defined as prerestricted and restricted processes, respectively.

The early process is thought to be complex, involving a variety of events which can be classified into three groups, that is, housekeeping events, dedifferentiation-related events and differentiation-specific events. Most of the events in this early process occur in almost all cultured cells, that is, in both differentiating and non-differentiating cells and, therefore, they are not specific for differentiation. However, they are necessary for the progression of differentiation. For instance, the expression of tubulin genes in the early process is observed both in differentiation-induced and control cultures. However, tubulin synthesis is essential for the construction of new arrays of microtubules in the late process of differentiation, which controls the patterned thickenings of secondary walls. Such events may be involved in the dedifferentiation process during which isolated mesophyll cells lose their potential to function as photosynthetic cells and acquire the ability to grow and differentiate in the new environment. Unfortunately, no differentiation-specific events have been defined at the early stage.

The late process involves a variety of differentiation-specific events that are common to systems *in vitro* or *in vivo*, and to all of plant species examined. As shown in Figure 1, however, all genes involved in the late process are not expressed simultaneously. This observation implies that such genes may be induced by different components of a putative signal cascade that controls the late process of differentiation.

Studies of the molecular events during the shift from the early to the late process have just begun. The analysis of the function of TED products, and *cis* and *trans*-acting factors that control expression of TED genes, may provide new insights into the control mechanism of tracheary element differentiation.

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