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Tracheary Element Differentiation

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INTRODUCTION

Vascular plants, which are adapted for life on land, first appeared in the late Silurian period, some 400 million years ago. Since then they have evolved to fill a diverse range of habitats all over the earth. The vascular systems of land plants are composed of specialized conducting tissues, the xylem and the phloem, which provide both a pathway for water and nutrient transport and mechanical support for slender plants. The vascular system is also an important conduit for signal-transducing molecules.

Tracheary elements (TEs), which are the distinctive cells of the xylem, are characterized by the formation of a secondary cell wall with annular, spiral, reticulate, or pitted wall thickenings. In the primary xylem, TEs differentiate from procambial cells, whereas in the secondary xylem, they arise from cells produced by the vascular cambium. As they mature, TEs lose their nuclei and cell contents, leaving hollow dead cells that form vessels or tracheids. The final stage of TE differentiation represents a typical example of programmed cell death in higher plants (see Pennell and Lamb, 1997, in this issue).

TEs can also be induced to form in vitro from various types of cells, including cells of the phloem parenchyma and the cortex in roots, the pith parenchyma in shoots, the tuber parenchyma, and the mesophyll and epidermis in leaves (Roberts et al., 1988; Fukuda, 1992). In *Zinnia elegans* cell cultures, single mesophyll cells transdifferentiate directly into TEs without cell division in response to phytohormones (Fukuda and Komamine, 1980). The *Zinnia* system has proven to be particularly useful for studies of the sequence of events during TE differentiation. This is largely because differentiation occurs at a high frequency in *Zinnia* cultures and because the process can be followed in single cells (Chasan, 1994; Fukuda, 1994, 1996). Recently, I presented a general overview of xylogenesis (Fukuda, 1996). In this article, I focus on efforts to elucidate the molecular mechanisms underlying the in vitro differentiation of parenchyma cells into TEs.

INITIATION OF TE DIFFERENTIATION

Phytohormonal Induction

The continuity of xylem tissues along the plant axis has been suggested to result from the steady polar flow of auxin from leaves to roots (Aloni, 1987; Sachs, 1991). Indeed, overproduction of the product of an *Agrobacterium tumefaciens* auxin biosynthetic gene in transgenic petunia plants causes an increase in the number of TEs and a decrease in their size (Klee et al., 1987). By contrast, the inactivation of endogenous auxin in tobacco plants transformed with the *Pseudomonas savastanoi* indoleacetic acid (IAA)–lysine synthetase gene decreases the number of TEs and increases their diameter (Romano et al., 1991). Therefore, the endogenous level of auxin seems to play a key role in controlling the initiation of TE differentiation and the size of TEs (Aloni and Zimmermann, 1983).

Also of relevance are recent data demonstrating that in *Pinus* trees there is a steep gradient of IAA across the vascular cambium and its derivatives, with a peak at the cambium (Uggla et al., 1996). Uggla et al. (1996) suggest that positional information triggering the differentiation of different types of vascular cells may be derived from the gradient of IAA rather than from its concentration. Although this idea is very stimulating, it cannot explain the requirement for auxin in the induction and progression of TE differentiation from isolated single mesophyll cells in vitro (Fukuda, 1992).

Cytokinins also participate in the control of TE differentiation both in vivo, in which case cytokinins may be produced in roots, and in vitro (Fukuda, 1992; Aloni, 1995). However, the relatively high levels of endogenous cytokinin in plant tissues often mask the requirement for cytokinin in TE differentiation (Fukuda, 1992). Cytokinins promote TE formation in the acropetal direction in the presence of IAA, suggesting that cytokinins increase the sensitivity of cambial initials and their derivatives to auxin, which in turn stimulates the initials to differentiate into TEs (Baum et al., 1991). As is the case with auxin, cytokinins are necessary for the progression of TE differentiation as well as for its induction (Church and Galston, 1988).

Inhibitors of ethylene biosynthesis often suppress TE differentiation in vitro. This suggests that ethylene is also involved in the induction and/or progression of TE differentiation, although there is no direct evidence for such a role (Fukuda, 1996).

Wound Induction

Mechanical wounding often induces transdifferentiation of parenchyma cells into TEs, as typically demonstrated by the

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formation of wound vessel members around wound sites (Jacobs, 1952). Wounding interrupts vascular bundles and therefore disturbs hormonal transport. This disturbance may trigger the formation of new vascular tissues around the wound (Aloni, 1995; see Nelson and Dengler, 1997, in this issue). However, Church and Galston (1989) have shown that TE formation from mesophyll cells is substantially promoted in *Zinnia* leaf disks whose upper or lower epidermis is peeled off. Because this effect is not associated with changes in hormonal transport, wounding itself may be involved in the induction of TE differentiation.

STAGE I

A number of cytological, biochemical, and molecular markers for TE differentiation have been identified in the *Zinnia* cell culture system. These markers have facilitated the division of the process of transdifferentiation into three stages: stage I, stage II, and stage III (Figure 1; Fukuda, 1994, 1996).

Stage I, which immediately follows the induction of differentiation, corresponds to the functional dedifferentiation process during which isolated mesophyll cells lose their ability to perform photosynthesis. This dedifferentiation process also involves the expression of wound-induced genes and the acquisition of the cells' ability to grow and differentiate in a new environment. However, this dedifferentiation process is not accompanied by cell division.

A typical example of functional dedifferentiation during stage I is the change in the organization of the reticulate arrays of actin filaments that are thought to anchor chloroplasts to the plasma membrane (Kobayashi et al., 1987). These arrays are reorganized into a three-dimensional network over the entire length of the cell, causing chloroplasts to leave the vicinity of the plasma membrane and the meso-

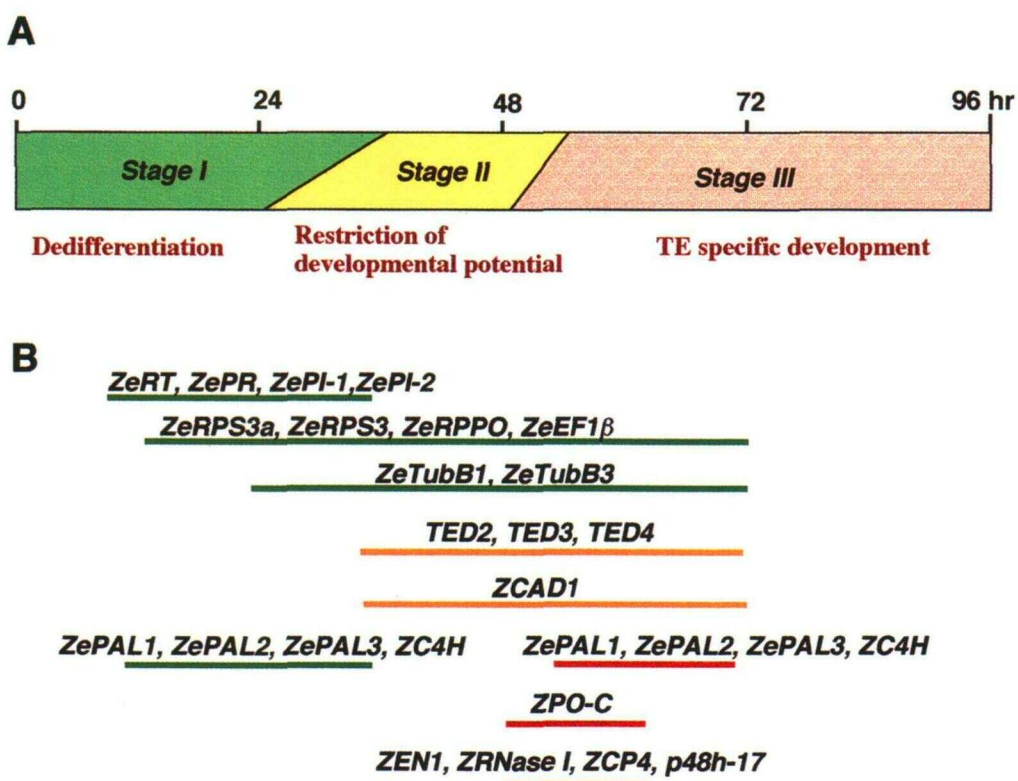

Figure 1. Accumulation of Various Transcripts during the Transdifferentiation of Single *Zinnia* Mesophyll Cells into TEs.

(A) The three stages of transdifferentiation. Stage I corresponds to the functional dedifferentiation process. In stage II, the developmental potential of the dedifferentiated cells becomes restricted from the pluripotent ability to differentiate into immature xylem and/or phloem cells to the single ability to differentiate into TEs. Stage III involves TE-specific events.

(B) Gene expression during transdifferentiation. The accumulation patterns of transcripts for various genes isolated from *Z. elegans* are depicted as bars. Green, yellow, and red bars show transcripts whose accumulation starts during stages I, II, and III, respectively.

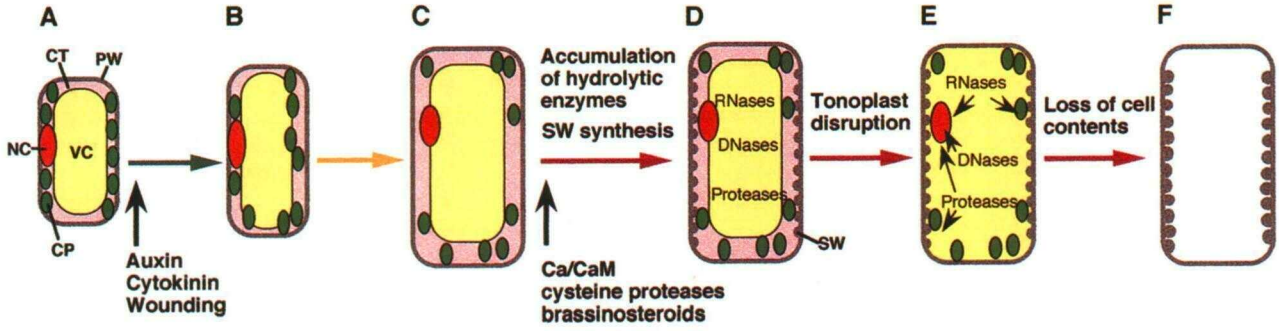

Figure 2. Cellular Events That Occur during Transdifferentiation of *Zinnia* Mesophyll Cells into TEs.

(A) Isolated mesophyll cell.
(B) Dedifferentiated cell.
(C) TE precursor cell.
(D) Immature TE.
(E) Maturing TE.
(F) Mature TE.

Transdifferentiation is induced by wounding and a combination of auxin and cytokinin. Green, yellow, and red arrows show the progress of stages I, II, and III, respectively. The transition from stage II to stage III appears to be regulated by calcium (Ca)/CaM, cysteine proteases, and endogenous brassinosteroids. At the start of stage III, genes that are involved in both secondary wall synthesis and autolysis are expressed. Hydrolytic enzymes, such as DNases, RNases, and proteases, may accumulate in the vacuole. The disruption of the tonoplast causes these enzymes to invade the cytoplasm and attack various organelles, resulting in the formation of a mature TE that has lost its cell contents. CP, chloroplast; CT, cytoplasm; NC, nucleus; PW, primary wall; SW, secondary wall; VC, vacuole.

phyll cells to lose their ability to perform photosynthesis. Calmodulin (CaM) antagonists inhibit TE differentiation when added early in stage I (Roberts and Haigler, 1990), suggesting that progression through dedifferentiation may involve calcium/CaM regulation.

We have isolated cDNA clones corresponding to 12 genes that are induced during stage I (Figure 1; M. Nagata, T. Demura, and H. Fukuda, unpublished results). These genes have been categorized into three groups: group 1 comprises wound-related genes, such as genes encoding protease inhibitors (e.g., *Z. elegans* protease inhibitor 1 and 2 [*ZePI-1* and *ZePI-2*]), and group 2 is made up of genes that encode components of the protein synthesis apparatus, such as ribosomal proteins (e.g., *Z. elegans* ribosomal protein gene S3a, S3, and PO [*ZeRPS3a*, *ZeRPS3*, and *ZeRPPO*]) and an elongation factor (*Z. elegans* elongation factor 1b [*ZeEF1b*]). The remainder of the cDNAs make up group 3. The transcription of all of these genes is induced by wounding but not by phytohormones. However, phytohormones do affect the changes in the level of their transcripts that occur during stage II and stage III of TE differentiation. For example, either naphthaleneacetic acid or benzyladenine must be present for substantial levels of group 2 transcripts to be maintained throughout development. Moreover, the accumulation of transcripts of group 1 genes is reduced only in cells that have been cultured in differentiation-inducing media that contain specific ratios of naphthaleneacetic acid and benzyladenine. These results suggest that the dedifferentiation process involves wound-induced events and the activation of protein synthesis, both of which are regulated by phytohormones at a later stage in the transdifferentiation process.

STAGE II

The accumulation of transcripts of the *TED2*, *TED3*, and *TED4* (tracheary element differentiation–related 2, 3, and 4) genes, which begins 12 to 24 hr before secondary wall thickening, defines the second stage of transdifferentiation in cultured *Zinnia* cells (Figure 1; Demura and Fukuda, 1993, 1994). In situ hybridization experiments have also demonstrated that the expression of these genes is restricted to cells that are involved in vascular differentiation in intact plants. For example, *TED3* transcripts are expressed specifically in TE precursor cells. Similarly, *TED4* transcripts are restricted to vascular cells or future vascular cells and in particular to immature xylem cells that do not show any morphological changes. *TED2* transcripts are restricted to procambial regions, immature phloem, and immature xylem cells. These data suggest that stage II of transdifferentiation in vitro corresponds to the process of differentiation from procambial initials to precursors of TEs in vivo (Figure 2).

Although transdifferentiation of *Zinnia* and Jerusalem artichoke cells does not require cell division or progression through the S phase of the cell cycle (Fukuda and

Komamine, 1981a; Phillips, 1981), a variety of inhibitors of DNA synthesis prevent transdifferentiation (Fukuda and Komamine, 1981b). Sugiyama et al. (1995) have suggested that repair-type DNA synthesis is involved in TE differentiation. This hypothesis is consistent with the fact that inhibitors of poly(ADP-ribose) polymerase, which plays an important role in DNA excision repair, prevent TE differentiation without any significant decrease in the frequency of cell division in both *Zinnia* (Sugiyama and Komamine, 1987; Shoji et al., 1997) and pea (Phillips and Hawkins, 1985). These inhibitors also prevent the accumulation of *TED2*, *TED3*, and *TED4* transcripts (Demura and Fukuda, 1993), suggesting that repair-type DNA synthesis may be necessary for entry into stage II.

Stage II also includes a marked increase in the transcription of *ZeRPS3a*, *ZeRPS3*, *ZeRPPO*, and *ZeEF1β*, which encode components of the protein synthesis apparatus (Figure 1; M. Nagata, T. Demura, and H. Fukuda, unpublished results). The increase in the transcription of these genes correlates with the dramatic elevation of protein and RNA synthesis that has been demonstrated in experiments with radioactive tracer molecules (Fukuda and Komamine, 1983).

The cytoskeleton also undergoes dynamic changes during stages I and II. Molecular evidence for this comes from analyses of the expression of tubulin genes. For example, the expression of *ZeTubB1* and *ZeTubB3*, which encode *Z. elegans* β-tubulins, also starts during stage I (Figure 1; Yoshimura et al., 1996). Tubulin synthesis continues during stage II and brings about an increase in the number of microtubules. These microtubules facilitate the regulation of secondary wall formation in stage III (see below; Fukuda, 1987). In situ hybridization analyses suggest that *ZeTubB1* is preferentially expressed in both differentiating xylem cells and actively dividing cells (Yoshimura et al., 1996).

There are also changes in actin organization during stage II, when three-dimensional networks of actin filaments form thick cables, mainly in the longitudinal direction. These cables function in cytoplasmic streaming (Kobayashi et al., 1987).

TRANSITION FROM STAGE II TO STAGE III

The transition from stage II to stage III seems to be an irreversible check point in TE differentiation. During stage II, CaM levels increase transiently. Subsequently, the expression of several CaM binding proteins is induced (Kobayashi and Fukuda, 1994). Moreover, CaM antagonists specifically prevent cultured *Zinnia* cells from entering stage III (Kobayashi and Fukuda, 1994). These data suggest that the calcium/CaM system may be involved in progression through stage II and/or entry into stage III (Figure 2). This possibility is also supported by the detection of higher levels of membrane-associated calcium ions in TE precursor cells than in control cells of *Zinnia* (Roberts and Haigler, 1989).

Entry into stage III is also regulated by other factors (Figure 2). Iwasaki and Shibaoka (1991) have shown that the inhibition of brassinosteroid biosynthesis by uniconazole prevents TE differentiation from *Zinnia* mesophyll cells. This conclusion has been substantiated by recent investigations of the inhibitory effect of uniconazole on the expression of genes involved in TE differentiation. Uniconazole does not inhibit the expression of genes induced during stage I or stage II, such as *ZePI-2*, *ZePR*, and the *TED*s, but does prevent the expression of stage III–specific genes, including those encoding enzymes involved in secondary wall thickening (e.g., a peroxidase; ZPO-C) and enzymes involved in cell death (e.g., a cysteine protease; ZCP4) (R. Yamamoto, T. Demura, and H. Fukuda, unpublished results).

Further evidence that brassinosteroids specifically affect the transition from stage II to stage III comes from analyses of the expression of genes encoding phenylalanine ammonia-lyase (PAL; *ZePAL1*, *ZePAL2*, and *ZePAL3*) and cinnamic acid-4-hydroxylase (*ZC4H*). Expression of these genes is usually induced during both stages I and III (Figure 1); however, uniconazole does not inhibit their induction during stage I, only during stage III. By contrast, brassinolide and several intermediates of brassinosteroid biosynthesis can circumvent the uniconazole-induced inhibition of both TE differentiation and gene expression. These data imply that endogenous brassinosteroids may induce entry into stage III.

STAGE III

Stage III involves secondary wall formation and autolysis, which occur both in vitro and in vivo. During stage III, various enzymes and structural proteins associated with the formation of secondary walls and with cellular autolysis are expressed (Fukuda, 1996).

Secondary Wall Formation

The Cytoskeleton

Microtubules determine wall pattern by defining the position and orientation of secondary walls, probably by guiding the movement of cellulose-synthesizing complexes in the plasma membrane (Gunning and Hardham, 1982). Microtubule arrays in cultured *Zinnia* cells change dramatically from a sparse scattered distribution in stage I to the several thick transverse bundles that appear before secondary wall formation via a longitudinal orientation of dense microtubules (Falconer and Seagull, 1985; Fukuda and Kobayashi, 1989).

Actin filaments show more dynamic changes than do microtubules during TE differentiation. Longitudinal actin cables change to transverse bands of actin filaments at the beginning of stage III (Kobayashi et al., 1987). Double staining of microtubules and actin filaments and treatment with agents

that depolymerize cytoskeletal components indicate that there is 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).

Secondary Wall–Specific Proteins

Several proteins are specifically associated with secondary walls of TEs. These proteins include an extensin-like protein, which was isolated from the xylem of loblolly pine (Bao et al., 1992). This protein is present in secondary cell walls of xylem cells during lignification and remains as a structural component of cell walls in wood. Arabinogalactan proteins are also present in secondary walls of TEs, and several antibodies directed against arabinogalactan proteins bind to immature xylem cells in some species of plant (Schindler et al., 1995). For instance, the JIM13 monoclonal antibody binds to immature and mature TEs in cultured *Zinnia* cells (Stacey et al., 1995).

Wheat germ agglutinin, which has a strong affinity for a sequence of three β-(1-4)-linked *N*-acetyl-D-glucosamine residues, also binds specifically to secondary walls of TEs (Hogetsu, 1990; Wojtaszek and Bolwell, 1995). Wojtaszek and Bolwell (1995) have isolated three novel glycoproteins that bind to wheat germ agglutinin. By using an antibody against one of these glycoproteins, SWGP90, they show that the protein is localized in secondary walls of TEs, xylem fibers, and phloem fibers. Another novel type of wall protein, a Tyr- and Lys-rich protein from tomato, has also been localized to secondary walls (Domingo et al., 1994). Finally, *GRP1.8* was isolated as a cDNA clone encoding a protein that is localized in the cell walls of the primary xylem elements and the primary phloem of many plant species (Keller et al., 1988; Ye et al., 1991). Detailed immunoelectron microscopy studies indicate that GRP1.8 is produced by xylem parenchyma cells and exported to the walls of protoxylem vessels (Ryser and Keller, 1992).

The bean *GRP1.8* promoter has been analyzed in detail. The promoter possesses both a negative element that represses promoter activity in nonvascular cells and a positive element that directs vascular tissue–specific expression (Keller and Baumgartner, 1991; Keller and Heierli, 1994). Recently, Keller's group indicated that a novel basic leucine zipper transcription factor binds to a 28-bp element, termed *vs-1*, that partially overlaps with the negative element in the *GRP1.8* promoter (Schumann et al., 1996).

Expression of Lignin Biosynthetic Enzymes

Lignin is one of the most characteristic components of secondary walls of TEs; its biosynthesis involves the shikimate, general phenylpropanoid, and specific lignin pathways (Boudet et al., 1995). In cultured *Zinnia* cells, activities of PAL and caffeoyl-CoA-3-*O*-methyltransferase are induced preferentially during stages I and III, mirroring the increased accumulation of the corresponding transcripts (Lin and Northcote, 1990; Ye et al., 1994). Detailed analysis of *ZePAL1*, *ZePAL2*, *ZePAL3*, and *ZC4H* transcript accumulation suggests that the expression of these phenylpropanoid pathway–related genes is coordinately induced during stages I and III (Figure 1; R. Yamamoto, T. Demura, and H. Fukuda, unpublished results). During stage I, the expression of these genes is induced by wounding, whereas during stage III, it is associated with lignin synthesis (Fukuda, 1996).

The promoters of genes encoding PAL and 4-coumaric acid:CoA ligase (4CL) share conserved sequences that may mediate their coordinate regulation. For example, the AC-rich sequence ACII, which has been identified in PAL- and 4CL-encoding genes of a number of plants, functions as a negative element that suppresses gene expression in the phloem (Hauffe et al., 1993; Hatton et al., 1995). ACII is thought to be a Myb binding site, which implies that Myb proteins may be involved in the coordinated expression of genes encoding phenylpropanoid pathway–related enzymes.

By contrast, the activity of cinnamyl alcohol dehydrogenase, which is involved in the specific lignin pathway, is highest at stage III, but its transcript (*ZCAD1*) begins to accumulate during stage II (Figure 1; Sato et al., 1997). Interestingly, uniconazole does not inhibit the expression of *ZCAD1*, which is consistent with the above-mentioned observation that uniconazole does not inhibit the expression of genes induced during stages I and II.

A second enzyme in the specific lignin pathway, P5, which is a cationic peroxidase isozyme that is ionically bound to the cell walls, is known to be involved in the final step of lignin synthesis in differentiating *Zinnia* cells—the polymerization of cinnamyl alcohols into lignin (Sato et al., 1993, 1995). Transcripts of the corresponding gene, *ZPO-C*, accumulate specifically and transiently during stage III (Figure 1; Y. Sato and H. Fukuda, unpublished results). The accumulation of *ZPO-C* transcripts precedes that of the *ZePALs* and *ZC4H*, and *ZPO-C* transcript levels are elevated for a shorter period of time. Therefore, genes encoding lignin biosynthetic enzymes appear to be regulated in a complex manner during TE differentiation in cultured *Zinnia* cells.

Programmed Cell Death

Differentiation into TEs is a typical example of developmentally programmed cell death in higher plants (see Pennell and Lamb, 1997, in this issue). TEs reach maturity after the loss of cell contents, including the nucleus, and the partial digestion of primary walls. Pores open at the ends of individual vessel elements, which are longitudinally arranged to form a long vessel tube. The cell death process that takes place during stage III of TE differentiation is coupled tightly to the formation of secondary walls. Indeed, it has not been

possible to separate experimentally cell death from secondary wall formation during TE differentiation.

Induction of Cell Death

In animals, a variety of factors, such as Fas ligands, tumor necrosis factors, and hormones, are involved in the induction of apoptosis. Death signals induced by these factors activate the interleukin-1β–converting enzyme (ICE)–like protease cascade, which triggers cell death (White, 1996). Genetic analysis of the nematode *Caenorhabditis elegans* has provided the most direct evidence that the initiation of cell death during development is controlled by specific genes, such as *ced-3*, *ced-4*, and *ced-9* (Yuan and Horvitz, 1990; Hengartner et al., 1992). The nematode *ced-3* and *ced-9* genes have mammalian counterparts that are responsible for initiating apoptosis in mammals (White, 1996).

In plants, toxins, high concentrations of salts, and some chemicals can induce the apoptosis-like death of particular cells (Katsuhara and Kawasaki, 1996; Wang et al., 1996). However, inducers of developmental cell death have not been identified in higher plants. During TE differentiation, inhibitors of CaM action (Kobayashi and Fukuda, 1994), cysteine protease activity (Y. Watanabe and H. Fukuda, unpublished results), and brassinosteroid synthesis (R. Yamamoto, T. Demura, and H. Fukuda, unpublished results) prevent TE precursor cells from entering stage III (Figure 2).

The transcripts for both cell death–related enzymes and enzymes involved in secondary wall formation begin to accumulate at the same time (Figure 1). These observations imply that a common signal(s) may induce both cell death and secondary wall formation. Furthermore, the inhibition of the entry into stage III by cysteine protease inhibitors may suggest the presence of an unknown protease cascade similar to the ICE cascade, although inhibitors of ICE do not prevent cell death during TE differentiation (H. Kuriyama and H. Fukuda, unpublished results; see also Pennell and Lamb, 1997, in this issue).

Morphological Features of the Cell Death Process

Apoptosis in animals involves nuclear shrinkage, cellular shrinkage, membrane bubbling, the formation of apoptotic bodies, and digestion by macrophages (Kerr and Harmon, 1991; see also Pennell and Lamb, 1997, in this issue). The process of cell death during TE differentiation, which has been studied in detail by electron microscopy, does not exhibit any substantial similarity to these typical morphological features of animal apoptosis (reviewed in Fukuda, 1996). For example, in differentiating *Zinnia* cells, the visible degeneration of all organelles, including the nucleus, starts only after the tonoplast ruptures, which occurs several hours after the secondary wall thickenings become visible. After the disruption of the tonoplast, organelles with a single membrane, such as Golgi bodies and the endoplasmic reticulum, become swollen and then rupture. Subsequently, organelles with double membranes are degraded. The nucleus is also degraded, but this degradation is not preceded by nuclear shrinkage and fragmentation. TEs lose most of their organelles within a few hours after disruption of the tonoplast, and the entire contents of the cell disappear within ~6 hr after the first visible evidence of secondary wall thickening. These data imply that disruption of the tonoplast may be a critical event in TE cell death.

Concomitant with autolysis, cell walls of TEs are modified, although lignification of secondary walls begins before tonoplast disruption. Around the time of tonoplast rupture, lignin is deposited on the outer layer of primary walls of TEs, after which the regions of primary walls that have not been lignified are digested. Perforation of the ends of elongated TEs, which presumably are not thickened, also follows tonoplast disruption.

These cell wall modifications must be controlled strictly. Indeed, we recently identified a cell wall degrading activity that is specific to differentiating TEs in cultured *Zinnia* cells and that is initiated before tonoplast disruption (Y. Ohdaira, M. Sugiyama, and H. Fukuda, unpublished results). Moreover, Burgess and Linstead (1984) have reported that the middle lamella between a cell that is differentiating into TEs and one that is not is resistant to degradation, whereas the middle lamella between two neighboring cells that are both differentiating is digested completely. These observations imply that both cell wall modification and expression of hydrolytic enzymes may be necessary for the characteristic degradation of TE walls.

Molecular Features of the Cell Death Process

The rapid degeneration of organelles during autolysis must be the result of a variety of highly active hydrolytic enzymes. Minami and Fukuda (1995) have found that the activity of a 30-kD cysteine protease increases transiently just before the start of autolysis and is specific to differentiating *Zinnia* TEs; this protease is most active at pH 5.5. Moreover, the specific inhibition of intracellular cysteine protease activity in differentiating TEs suppresses nuclear degeneration, suggesting that a cysteine protease(s) plays a key role in this process (Y. Watanabe and H. Fukuda, unpublished results).

Two cDNAs, p48h-17 (Ye and Varner, 1996) and *ZCP4* (A. Minami, T. Demura, and H. Fukuda, unpublished results), that may encode cysteine proteases have been isolated from *Zinnia*. The deduced amino acid sequences of these cysteine proteases suggest that their precursors, which resemble papine, are probably transported into the vacuole, where they are processed into activated forms. The p48h-17 and *ZCP4* transcripts also accumulate transiently just before autolysis and are found specifically in differentiating TEs in vitro and in vivo (Ye and Varner, 1996; A. Minami, T. Demura, and H. Fukuda, unpublished results).

In addition to the cysteine proteases, a 145-kD serine protease has been detected specifically in differentiating TEs of cultured *Zinnia* cells on substrate-impregnated gels (Beers and Freeman, 1997). Moreover, an ~60-kD serine protease has been reported to appear preferentially in differentiating TEs (Ye and Varner, 1996; Beers and Freeman, 1997). These results indicate that a complex set of proteases is involved in the autolytic process. Nonetheless, the substrates, cellular location, and mode of induction of each of these proteases are unknown.

During apoptosis, DNA laddering is caused by the digestion of DNA into nucleosome-sized units by endonucleases. Among the candidates for these nucleases in animal cells are NUC 18, DNase I, DNase II, and DNase (Peitsch et al., 1993; Tanuma and Shiokawa, 1994). DNase is present in the nucleus, activated by Ca^{2+} and Mg^{2+}, and inhibited by Zn^{2+} (Tanuma and Shiokawa, 1994). Similarly, Mittler and Lam (1995a) have detected a 36-kD DNase, NUC III, that is expressed in association with cell death during the hypersensitive reaction (see Pennell and Lamb, 1997, in this issue). This nuclease has characteristics similar to apoptosis-related DNases in that it is present in the nucleus and shows Ca^{2+}-mediated activation and Zn^{2+}-mediated inhibition. However, this DNase is not expressed during cell death in senescence.

Thelen and Northcote (1989) have found that several DNase and RNase activities are closely associated with TE differentiation in *Zinnia* cells. These 43-, 22-, and 25-kD nucleases appeared 12 hr before the visible formation of TEs, and their levels increased conspicuously during the maturation phase of differentiation. The 43-kD nuclease is the only one that has DNase activity in differentiating TEs. This nuclease requires Zn^{2+} for activation and can hydrolyze RNA, single-stranded DNA, and double-stranded DNA. Recently, Mittler and Lam (1995b) detected fragmented nuclear DNA in differentiating TEs. However, their results could be explained by invoking the activity of a general nuclease such as this rather than that of apoptosis-specific endonucleases.

The characteristics of the 43-kD nuclease resemble those of a nuclease that is expressed in barley aleurone cells during germination (Brown and Ho, 1986). Moreover, a Zn^{2+}-activated DNase has also been found in senescing leaves (Blank and McKeon, 1989). We have isolated two cDNA clones, *BEN1* (barley endonuclease 1) and *ZEN1* (*Zinnia* endonuclease 1), that correspond to the Zn^{2+}-activated nucleases from the barley aleurone and from differentiating *Zinnia* TEs, respectively. The nucleotide and deduced amino acid sequences show that BEN1 and ZEN1 are similar to each other and to the S1 nuclease of *Aspergillus* (S. Aoyagi, M. Sugiyama, and H. Fukuda, unpublished results). These data suggest that Zn^{2+}-activated nucleases similar to S1 are commonly involved in programmed cell death processes that take place throughout plant development.

Recently, *ZRNase1*, a cDNA encoding the 22-kD (or 25-kD) RNase detected by Thelen and Northcote (1989), has been isolated from *Zinnia* (Ye and Droste, 1996). The mRNA for *ZRNase1* also accumulates specifically in differentiating TEs in vitro and in vivo. Like those for *ZEN1*, *ZCP4*, and *p48h-17*, *ZRNase1* transcripts also accumulate transiently just before autolysis starts (Figure 1). Therefore, these genes may be regulated via a common mechanism that relies on the same *cis*- and *trans*-activation factors. Interestingly, *ZPO-C*, a gene encoding lignin-related peroxidase, is also expressed in a pattern very similar to that of the autolysis-related genes, suggesting that gene expression, in association with both secondary wall formation and autolysis, may be regulated at least in part by a common mechanism.

Their deduced amino acid sequences show that ZEN1, ZRNase1, ZCP4, and p48h-17 have signal peptides at their N termini. This implies that these proteins may be transported to the vacuole (Figure 2). Disruption of the tonoplast during stage III of TE differentiation causes hydrolytic enzymes to invade the cytoplasm and attack various organelles. It is interesting that the optimal pH of both the DNase and cysteine protease is 5.5, which corresponds to the pH in the vacuole and probably in the cytoplasm after the tonoplast ruptures. Even so, one of the most pressing issues regarding the cell death process during TE differentiation—the mechanism controlling tonoplast disruption—is still open for investigation.

CONCLUSIONS AND FUTURE PERSPECTIVES

Transdifferentiation into TEs in vitro is induced by a combination of auxin and cytokinin and, in some cases, by wounding. The transdifferentiation process can be divided into three stages: stage I, stage II, and stage III (Figures 1 and 2). Stage I corresponds to the functional dedifferentiation process. In stage II, the developmental potential of the dedifferentiated cells becomes restricted from the pluripotent ability to differentiate into immature xylem and/or phloem cells to the single ability to differentiate into TEs. This process seems to correspond to the in vivo process during which meristematic cells change into procambial cells and then into TE precursor cells. Stage III involves TE-specific events, such as secondary wall formation and autolysis, and is shared both in vitro and in vivo. A two-step process underlying the differentiation of parenchyma cells into stele cells and then of stele cells into TEs has also been suggested for transdifferentiation in wounded pea roots (Rana and Gahan, 1983).

One of the most promising approaches to the elucidation of the mechanism of TE differentiation is the analysis of mutants that have a defect in the process of TE differentiation. However, because plants that have a severe defect in TE differentiation are likely to be inviable, they may be hard to identify. Moreover, transgenic plants in which a TE-essential gene is overexpressed or suppressed may also face problems with lethality during regeneration.

The use of transgenic organs may overcome this problem. Indeed, we have recently established a transgenic *Zinnia*

root system into which we plan to introduce TE-specific genes so that we may analyze their function in TE differentiation during the development of vascular bundles in roots (Y. Tateishi, H. Choi, and H. Fukuda, unpublished results). We are using this system to investigate the roles of the *TED*s and of several genes involved in lignin biosynthesis, and initial indications are that this system may be useful for the study of the role of various gene products in TE differentiation. Such new approaches should provide marked increases in our understanding of TE differentiation in plants in the near future.

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