

XYLOGENESIS: INITIATION, PROGRESSION, AND CELL DEATH

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ABSTRACT

Xylem cells develop from procambial or cambial initials in situ, and they can also be induced from parenchyma cells by wound stress and/or a combination of phytohormones in vitro. Recent molecular and biochemical studies have identified some of the genes and proteins involved in xylem differentiation, which have led to an understanding of xylem differentiation based on comparisons of events in situ and in vitro. As a result, differentiation into tracheary elements (TEs) has been divided into two processes. The "early" process involves the origination and development of procambial initials in situ. In vitro, the early process of transdifferentiation involves the dedifferentiation of cells and subsequent differentiation of dedifferentiated cells into TE precursor cells. The "late" process, observed both in situ and in vitro, involves a variety of events specific to TE formation, most of which have been observed in association with secondary wall thickenings and programmed cell death. In this review, I summarize these events, including coordinated expression of genes that are involved in secondary wall formation.

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INTRODUCTION

The formation of xylem, the water-conducting tissue, has been a focus of many studies of differentiation in higher plants, not only because the function of xylem is essential to the existence of vascular plants but also because xylem formation appears to be a good model system for the analysis of differentiation in higher plants (2, 45, 51, 109, 124). Xylem is composed of tracheary elements (TE), parenchyma cells, and fibers. 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. At maturity, TEs lose their nuclei and cell contents and leave a hollow tube that is part of a vessel or tracheid. The ease with which these differentiated cells can be identified by their morphological features, as well as the relatively straightforward induction of TE differentiation *in vitro* and the presence of many biochemical and molecular markers, is very advantageous for studies of cell differentiation (45). At the final stage of TE differentiation, cell death occurs, and therefore this differentiation also focuses our attention on a typical example of programmed cell death in higher plants. Furthermore, xylogenesis is important from an applied and biotechnological perspective, because biomaterials, such as cellulose and lignin in xylem, represent the prominent part of the terrestrial biomass and therefore play an important role in the carbon cycle (17).

Recent progress in studies of xylem differentiation has come from experiments both *in vivo* and *in vitro* and has allowed us to dissect the process of differentiation. Therefore, in this review, I discuss recent findings related to xylem differentiation—in particular, those derived from molecular approaches. The focus is mainly on the formation of the primary xylem, because of space limitation. For further information, I refer the reader to recent reviews on vascular differentiation, including secondary xylem formation and phloem formation (3, 4, 14, 23, 45, 124).

INITIATION

Plant Hormones

IN VIVO Considerable evidence indicates that plant hormones are involved in the initiation of vascular differentiation (2, 4, 45, 99, 123, 129). The continuity

of vascular tissues along the plant axis may be a result of the steady polar flow of auxin from leaves to roots (2, 129). Jacobs (75) showed that indoleacetic acid (IAA), produced by expanding leaves and transported basipetally, was the limiting and controlling factor in the regeneration of xylem strands around a wound. Sachs (129) proposed the "canalization" hypothesis, which suggests that auxin flow that starts initially by diffusion induces the formation of a polar auxin transport cell system, which in turn promotes auxin transport and leads to canalization of auxin flow along a narrow file of cells. This continuous polar transport of auxin through cells finally results in the differentiation of a xylem strand. The differentiation of circular vessels in *Agrobacterium*-inducing crown galls also reflects circular movement of auxin that is produced additionally in the galls (5). The circular vessels also occur normally in healthy tissues such as suppressed buds (7) and branch junctions (94). Aloni & Zimmermann (8) proposed the "six-point" hypothesis, which suggests that high auxin levels near the young leaves induce many TEs that remain small because of their rapid differentiation, while low auxin concentrations further down result in slow differentiation and therefore in fewer and larger TEs. The overproduction of the product of an *Agrobacterium* auxin biosynthetic gene in transgenic *Petunia* plants caused an increase in the number of TEs and a decrease in their size (84). The inactivation of endogenous auxin in tobacco plants, transformed with *Pseudomonas* IAA-lysine synthase gene, decreased the number of TEs and increased their diameter (126). These results supported the hypothesis that the endogenous level of auxin plays a key role in controlling the initiation of TE differentiation and the size of TEs. However, the genes mentioned were expressed universally in various cells of transgenic plants under the control of the cauliflower mosaic virus (CaMV) 35S promoter. More directed modification of IAA levels at specific stages in given cells by use of stage-specific and cell-specific promoters will provide clearer details of the function of IAA in xylem differentiation.

Cytokinin from roots may also be a controlling factor in vascular differentiation (4, 45), although the relatively high levels of endogenous cytokinin in tissues often mask the requirement for cytokinin of xylem differentiation (45). Cytokinin promotes the vessel regeneration in the acropetal direction in the presence of IAA, which suggests that cytokinin increases the sensitivity of cambial initials and derivatives to auxin, which stimulates them to differentiate xylem cells (4, 13). The effect of the overproduction of cytokinin on xylem formation in transgenic plants is still controversial: In some cases, overproduction inhibits xylem formation (101); in other cases, it promotes the formation of a thicker vascular cylinder (97).

IN VITRO Induction of xylem cells has been achieved in callus, in suspension-cultured cells, and in excised tissues and cells (45, 51, 124). In vitro, xylem cells often differentiate as single or clustered TEs or as nested, discrete bands of TEs,

but not as strands of vessels (51). In vitro systems are very useful for studies of TE differentiation at the cellular level because of easy induction, easy external control of differentiation, and in some cases, a high degree of synchrony. Auxin is a prerequisite for the induction of TE differentiation in vitro (4, 45, 123). Auxin is necessary both for the induction of differentiation and for the progression of such differentiation (27, 51).

Cytokinin promotes TE differentiation in a variety of cultured tissues only when present with auxin, but the requirement for cytokinin varies in different cultures, ranging from nonexistent to essential (45, 123). Mizuno & Komamine (105), using cultured phloem slices of carrot, found that the difference among requirements for cytokinin of TE differentiation in different cultivars reflected endogenous levels of cytokinin, which suggests that cytokinin may be essential for induction of differentiation. Cytokinin is necessary for the progression of TE differentiation as well as for its induction, but the requirement for cytokinin may be limited at a much earlier stage of differentiation than that of auxin (27, 51). Molecular approaches to elucidation of the mechanism of induction of differentiation by auxin and cytokinin have just recently been initiated.

Although an exogenous supply of other hormones does not induce xylem differentiation in vitro, endogenous gibberellin and ethylene seem to contribute to xylem differentiation as a hidden promoter or inducer (45). In addition, a role of endogenous brassinosteroid seems likely during the process of TE differentiation in *Zinnia* cells (74). The identification of brassinosteroids from the cambial region of pine trees (83) is consistent with the requirement for brassinosteroids of TE differentiation.

Wounding

Mechanical wounding often induces transdifferentiation of parenchyma cells into TEs, which is typically demonstrated by the formation of wound vessel members around wounding sites (75). Wounding causes the interruption of vascular bundles, which disturbs hormonal transport along the bundles. This disturbed hormonal transport may result in the formation of new vascular tissues around the wound (4). Church & Galston (28) have shown that TE formation from mesophyll cells is substantially promoted in *Zinnia* leaf disks whose upper or lower epidermis is peeled off. This result is consistent with the observation that mechanically isolated mesophyll cells of *Zinnia* differentiate at high frequency (48). In fact, during the first 12 h after their isolation, isolated *Zinnia* mesophyll cells do not seem to react to plant hormones (51), and most of the genes that are expressed preferentially during the first 12 h of culture appear to be induced by the isolation or culture of *Zinnia* cells and not by plant hormones (106). Mechanically damaged cells are known to stimulate rapid induction of wound responses in suspension-cultured cells of *Ly-*

copersicon peruvianum, such as a transient alkalization of the culture medium and ethylene synthesis (41). Plants react to localized mechanical wounding both locally and systemically. Systemin, which is involved in systemic signaling that leads to the induction of protease inhibitors, was able to replace the mechanically damaged cells in the *L. peruvianum* system (41). Similarly, systemic wound signals might be involved in the initiation of transdifferentiation of parenchyma and epidermal cells into TEs.

EARLY PROCESS

The differentiation of TEs is divided into four ontogenetic processes: cell origination, cell elongation, secondary wall deposition and lignification, and wall lysis and cell autolysis (151). In this review, the process of TE differentiation is considered first to be divided into an early process, which includes all the events that occur before TE-specific events such as secondary wall formation and autolysis, and the late process, which includes TE-specific events. These two processes are further dissected below. I first consider the early process of differentiation both in situ and in vitro.

Origin and Development of Procambial Initials

Procambial cells originate in the early embryo. Precursor cells of the procambium of *Nicotiana* and *Trifolium* appear in embryos at the 20-cell stage and the 120-cell stage, respectively (54). In *Arabidopsis*, the procambial primordium, which consists of eight narrow cells, is formed by the late globular stage of the embryo, and it forms the primary xylem tissues in the torpedo-shaped embryo (19). The strong interconnections between cells of the embryo through developed plasmodesmata suggest the presence of diffusible morphogens that are responsible for the organized differentiation of a variety of cells, including vascular cells (76). In plants, the apical meristem serves as a continuous source of the procambial initials (151).

Langdale et al (91) analyzed the cell lineage of vascular tissues in maize leaves using six spontaneous striping mutants. They found that the formation of lateral and intermediate veins was initiated most often by divisions that contributed daughter cells to both the procambium and the ground meristem. They also showed that intermediate veins are multiclonal in both the transverse and the lengthwise directions. Clonal analysis of many plants has shown that cells are not assigned a particular fate early in development (54). The data of Langdale et al (91, 92) also suggest that cells can differentiate according to their position in the leaf, irrespective of their clonal history. Procambial initials elongate and differentiate into different types of vascular cell such as TEs, xylem parenchyma cells, sieve elements, and phloem parenchyma cells. There is little evidence as to when the determination of differen-

tiation into such different types of cell occurs. It is unknown, for example, whether the determination occurs in cambial initials before elongation or in mature procambial cells, and it is not known what signals are involved in such determination.

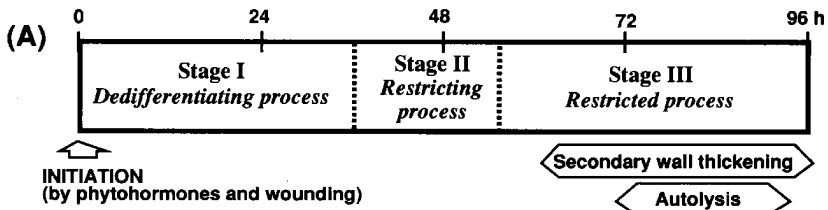
Early ultrastructural observations indicated that the development of intracellular organelles, i.e. the enlargement of the vacuole and nuclei, and the increases in numbers of ribosomes and the extent of the rough endoplasmic reticulum occur during differentiation along the file of cell lineage from procambial initials to xylem cells (51, 90). To date, however, a cytological description is the only information that is related to the origin of procambial precursor cells and even the procambial cells themselves (54). We are anxiously awaiting the identification of molecular and biochemical markers that will allow us to specify particular stages in the development of procambial cells. Some genes that have been isolated from differentiating *Zinnia* cells may provide such markers (31, 32, 158, 159).

Mutants are promising tools for elucidation of details of the initiation and progression of xylem differentiation. However, because a severe defect in vascular tissues is likely to cause the death of the plant, we need a novel strategy for generation of useful mutants. Embryo-lethal mutants of *Arabidopsis*, maize, and rice (71, 102) may help us to analyze the early process of xylem development in the embryo. Jürgens and colleagues (76), who isolated mutants that affect body organization in the *Arabidopsis* embryo, found that in the *gnom* mutant from which the root was absent and in which the cotyledon was strongly reduced in size or eliminated, TEs were present but were not interconnected to form strands. Instead, they were arranged in clusters or as single isolated cells. This mutant may be useful for studies of the involvement of intercellular interactions in vessel formation.

Early Events in Transdifferentiation

The *Zinnia* experimental system in which single mesophyll cells transdifferentiate synchronously into TEs without cell division has proved to be extremely useful for studies of the sequence of events in TE differentiation (26, 45, 46, 124, 144). Detailed analyses of the transdifferentiation have provided a number of cytological, biochemical, and molecular markers that should be useful for the dissection of the process of transdifferentiation (45, 46). The various markers indicate that the process of transdifferentiation can be divided into Stages I, II, and III (Figure 1).

Stage III corresponds to the late process of TE differentiation in situ. At Stage III, various enzymes and structural proteins associated with the secondary wall thickenings and autolysis, as well as the corresponding genes, are expressed.



Markers at each stage

Stage I	Stage II	Stage III
<p>C: Rearrangement of actin filaments (86)</p> <p>B: Repair-type DNA synthesis (142,145)</p> <p>B: Tubulin synthesis (43)</p> <p>M: Expression of <i>ZeTUBB1</i> and <i>ZeTUBB3</i> (52; T Yoshimura, unpublished)</p>	<p>C: Increase in ER, dictyosomes, vesicles, and mitochondria (51)</p> <p>C: Increase in microtubules (43)</p> <p>B: High transcription and translation activities (51)</p> <p>B: Increase in CaM (85)</p> <p>B: Expression of CaM binding proteins (85)</p> <p>M: Expression of <i>TED2</i>, <i>TED3</i>, and <i>TED4</i> (31, 32)</p>	<p>C: Secondary wall thickening (51)</p> <p>C: Reorganization of microtubules (39, 40)</p> <p>C: Reorganization of actin filaments (86)</p> <p>C: Degeneration of organelles (51)</p> <p>B: Xylan synthesis (45)</p> <p>B: Cellulose synthesis (73)</p> <p>B: Lignin synthesis (73)</p> <p>B: Expression of xylan synthase (147), <i>PAL</i> (51), <i>4CL</i> (45), <i>CAD</i> (Y Sato, unpublished), peroxidases (130), nucleases (150), and a cysteine protease (103)</p> <p>M: Expression of <i>PAL</i> (45), <i>C4H</i> (156), <i>CAD</i> (Y Sato, unpublished), <i>ZPO-C</i> (132), and genes for cysteine proteases (p48h-17, 158; <i>ZCP1</i>, A Minami, unpublished)</p>

(B)
In situ



In vitro

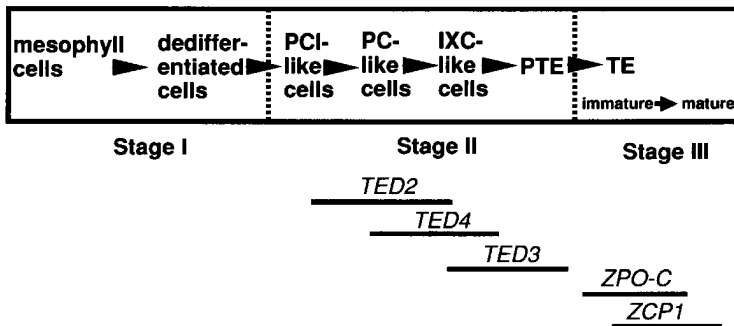


Figure 1 Sequence of events during transdifferentiation of single mesophyll cells of *Zinnia elegans*. (A) Sequence of events during transdifferentiation. C, B, and M represent cytological, biochemical, and molecular markers, respectively. (B) A comparison between the sequences of events in TE differentiation in situ and in vitro. *TED2*, *TED4*, *TED3*, *ZPO-C* (a gene for a peroxidase), and *ZCP1* (a gene for a cysteine protease) are expressed in this order. Bars represent the periods of their expression. MC, meristematic cells; PCI, procambial initials; PC, procambial cells; IXC, immature xylem cells; PTE, precursors of TEs; TE, tracheary elements.

Stage I, which immediately follows the induction of differentiation, corresponds to 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 a new environment. For example, at this stage, the reticulate arrays of actin filaments around chloroplasts, which may anchor chloroplasts to the plasma membrane, turn into a three-dimensional network over the entire length of the cell (86), which causes chloroplasts to leave the vicinity of the plasma membrane and the mesophyll cells to lose their photosynthetic capacity. It should be emphasized that this process of dedifferentiation is not accompanied by cell division. This stage seems not to be specific to TE differentiation but to be necessary for transdifferentiation into TEs (46).

The characterization of molecular markers, isolated as genes (*TED2*, *TED3*, *TED4*) that are expressed prior to the secondary wall thickenings, led to the definition of a new stage of transdifferentiation—Stage II (Figure 1) (31, 32)—between the very early stage (Stage I) and the late stage (Stage III). These genes are expressed preferentially in cells in cultures in which differentiation is induced 12–24 h before the secondary wall thickenings (31, 32). Ye & Varner (158) also isolated a cDNA clone identical to *TED4* from *Zinnia* cells. In situ hybridization demonstrated that the expression of these genes was restricted to cells that were involved in vascular differentiation even during the development of intact plants. *TED3* transcripts were expressed specifically in differentiating TEs or in the cells that were expected soon to differentiate into TEs. The expression of *TED4* transcripts was restricted to vascular cells or future vascular cells and, in particular, to immature xylem cells that did not show any morphological changes. *TED2* transcripts were restricted to procambial regions of root tips as well as to immature phloem and immature xylem cells at the boundary between the hypocotyl and root. Both in situ and in vitro *TED* transcripts were expressed in the same order as follows: *TED2*, then *TED4*, and finally, *TED3*. This experiment suggested that Stage II of transdifferentiation corresponds to the process of differentiation from procambial initials to precursors of TEs in situ (Figure 1) (32, 46). Fukuda (46) proposed the hypothesis that multistep determination occurs during differentiation into TEs from cells that have not been committed, such as meristematic cells and dedifferentiated cells. Their potency to differentiate is restricted from pluripotency to single potency, such that they only form TEs (Figure 1). According to this hypothesis, the final determination occurs just before secondary walls are formed. Therefore, Stages I, II, and III in *Zinnia* can be defined as the dedifferentiating, restricting, and restricted stages, respectively. Gahan & Rana (53, 119), also using a carboxylesterase as a marker of stele tissues, found evidence that multistep programming occurs during the transdifferentiation from parenchyma cells to

TEs in wounded roots of pea, namely, the determination of the differentiation of parenchyma cells into stele cells and the subsequent determination of the differentiation of stele cells into TEs. Further information on the sequence of events during transdifferentiation of *Zinnia* mesophyll cells can be found in earlier reviews (45, 46, 52)

Factors Affecting the Early Process

CELL DIVISION AND DNA SYNTHESIS Cell division is obviously important for the continuous supply of xylem cells. The regeneration of xylem tissues around wound sites involves ordered cell division for generation of well-organized vessels. Therefore, the fine control of the organization of cell division must also be important for the formation of xylem tissues.

The role of cell division as an initiator of transdifferentiation into TEs has been discussed (34, 51). A requirement of transdifferentiation for cell division or DNA replication during the S phase of the cell cycle has been proposed mainly on the basis of results with inhibitors of cell division or DNA synthesis (137). However, transdifferentiation can occur without the progression of cell division or the S phase in *Zinnia* and Jerusalem artichoke, which indicates that cell cycling is not essential for the initiation of transdifferentiation (49, 116). On the basis of a detailed analysis of the time course of cell division and differentiation in cultured *Zinnia* cells, Fukuda & Komamine (49) suggested that the early process of transdifferentiation (Stages I and II) may be compatible with the progression of the cell cycle. Nevertheless, it was also evident early on in their study that a variety of inhibitors of DNA synthesis prevented transdifferentiation (50), even in the *Zinnia* system in which replication of DNA in the S phase is not required (49). Thus, there might be a requirement for some minor DNA synthesis of transdifferentiation (50). Sugiyama and colleagues (141, 143, 145) suggested that this minor DNA synthesis may be repair-type DNA synthesis, a suggestion that is consistent with the fact that inhibitors of poly(ADP-ribose) polymerase, which plays an important role in DNA excision repair, prevent TE differentiation in *Zinnia* (142), pea, and Jerusalem artichoke (117). This repair-type DNA synthesis may be involved in the early process of transdifferentiation from parenchyma cells to TEs.

CALCIUM IONS AND CALMODULIN The essential role of calcium ions and calmodulin (CaM) in certain types of signaling has been established in a variety of organisms including plants (20, 122). It has also been suggested that TE differentiation may be regulated by the calcium/CaM system, because differentiation can be inhibited specifically by a decrease in the intracellular concentration of calcium ions, a calcium channel blocker, and CaM antagonists (85, 121). CaM antagonists were only effective early in Stage I and late in Stage II of the transdifferentiation of *Zinnia* mesophyll cells into TEs (85, 121). At Stage II,

CaM levels increase transiently and, subsequently, a few CaM-binding proteins start to be expressed in a differentiation-specific manner (85), which suggests that the calcium/CaM system is involved in the progression of Stage II, i.e. in the differentiation from procambial initials to TE precursor cells. The possibility that calcium/CaM is involved in the progression of Stage II is also supported by higher levels of membrane-associated calcium ions in TE precursor cells than in control *Zinnia* cells (120). The formation of the secondary walls might itself also be under the control of calcium/CaM (47, 121).

LATE PROCESS

The most striking features of TE formation are the development of patterned secondary walls and the autolysis of cell contents and walls, which occur in the late process of differentiation. The secondary cell wall is composed of cellulose microfibrils arranged parallel to one another and to the bands of the secondary wall and of cementing substances that contain lignin, hemicellulose, pectin, and protein, which add strength and rigidity to the wall. These substances are synthesized and deposited cooperatively during secondary wall formation. The autolysis is a typical example of programmed cell death and involves activation of the expression of degradative enzymes such as nucleases and proteases.

Pattern Formation of Secondary Walls

Microtubules in differentiating TEs are located as bands over the thickenings of secondary walls in various plants (59). Disturbance of microtubule organization by colchicine and taxol caused the formation of unusual secondary wall thickenings (40). Such cytological studies led to the suggestion that microtubules determine the wall pattern by defining the position and orientation of secondary walls, probably by guiding the movement of the cellulose-synthesizing complex in the plasma membrane (59). Microtubule arrays change dramatically from a longitudinal to a transverse orientation prior to secondary wall formation in *Zinnia* (39, 40). This rearrangement of microtubule arrays is under the control of actin filaments (47).

The number of cortical microtubules increases prior to the reorganization of microtubules during TE differentiation in cultured *Zinnia* cells (43) and *Azolla* (63). The increase is caused by an increase of tubulin levels, which results from tubulin synthesis *de novo*. This synthesis starts as early as 4 to 8 h after the induction of differentiation (43). The degradation of tubulin is also associated with the reorganization of microtubules (44). The small genome of *Arabidopsis* contains at least six genes for α -tubulin and at least nine genes for β -tubulin (55). Recent studies have provided evidence of the differential expression of various isotypes of plant tubulin genes in specific organs and

tissues during development, in addition to the regulation of their expression by environmental factors such as temperature and phytohormones (55). Each tubulin gene seems to have a unique spatial and temporal pattern of expression. Three different cDNAs for β -tubulin—*ZeTUBB1*, *ZeTUBB2*, *ZeTUBB3*—were isolated from differentiating cells of *Zinnia* (52). RNA gel blot analysis indicated that the levels of expression of *ZeTUBB1* and *ZeTUBB3* transcripts increased rapidly late in Stage I. The very recent results of in situ hybridization suggest the preferential expression of *ZeTUBB1* transcripts in differentiating xylem cells, as well as in actively dividing cells (T Yoshimura, T Demura & H Fukuda, unpublished data).

Polysaccharide Synthesis

During the formation of secondary walls, levels of cellulose and hemicellulose increase, and the deposition of pectin ceases (109). Putative complexes of cellulose-synthesizing enzymes, visible as rosettes, are localized in the plasma membrane over regions of secondary wall thickenings in TEs (61). The rosettes are also present in the Golgi apparatus throughout the enlargement of secondary wall thickening, which suggests that new rosettes are continuously inserted into the plasma membrane via Golgi vesicles during secondary wall formation (61). Northcote et al (110), using an antiserum against β 1-4 oligoxylosides, demonstrated the presence of xylose not only in the secondary thickening but also in the Golgi vesicles of TEs. Hogetsu (69), in experiments with fluorescein-conjugated wheat germ agglutinin (WGA), also found that a hemicellulose(s), probably xylan, was localized in the secondary walls of TEs in many angiosperm plants.

Polysaccharide synthesis is regulated in the level of the availability of nucleoside phosphate sugars as precursors by their location, transport, and feedback controls during secondary wall formation (108). In addition, control of the activity of the membrane-bound enzymes involved in polysaccharide synthesis is critical to secondary wall formation during xylem differentiation (109). Increased activity of xylosyltransferase (16, 147) and decreased activities of polygalacturonic acid synthase (15) and arabinosyltransferase (16) have been noted in conjunction with secondary wall formation during TE differentiation. Rodgers & Bolwell (125) succeeded in the partial purification of Golgi-bound arabinosyltransferase and xylosyltransferase from French bean. The xylosyltransferase with a molecular mass of 40 kDa, with increased activity during secondary wall formation, was suggested to be a xylan synthase. Particulate membrane preparations isolated from differentiating xylem cells of *Pinus sylvestris* included an enzyme complex that was active in the synthesis of hemicellulose (30). This enzyme complex, resembling that for cellulose synthesis, is constructed in part on the endoplasmic reticulum and is then transferred to the Golgi apparatus, where it is modified and sorted.

Synthesis of Cell Wall Proteins

Recent progress in molecular approaches has permitted us to identify structural proteins, many of which are insoluble and therefore difficult to study directly, on the cell walls of vascular tissues (22, 77, 109). They belong to four classes: hydroxyproline-rich glycoproteins (HRGPs), proline-rich proteins (PRPs), glycine-rich proteins (GRPs), and arabinogalactan proteins (AGPs) (22).

One family of HRGPs contains a characteristic Ser-(Hyp)₄ pentapeptide sequence. Extensins are the best-characterized proteins among the HRGPs. Extensins contribute to the mechanical strength of cell walls (22) and also to the control of intracellular organization, e.g. the control of microtubule organization (1). Extensins have been located in the cell wall of specific types of cell, including vascular cells (21). The expression of mRNAs for HRGPs is regulated developmentally, as well as by environmental cues such as wounding and infection. Some *HRGP* genes are expressed strongly in vascular cells, including cambial and phloem cells, in soybean tissues (157), and in regions where vascular cells and sclerenchyma are initiated in embryos, leaves, and roots of maize (139). However, such expression is not specific to vascular tissues. Recently, Bao et al (9) isolated an extensin-like protein from the xylem of loblolly pine, and they indicated that the protein was present in secondary cell walls of xylem cells during lignification and remained as a structural component of the cell walls in the wood.

GRPs are a class of proteins with a very repetitive primary structure. About 60% of their residues are glycine residues that are arranged predominantly in (Gly-X)_n repeats (77). Some GRPs such as the *Petunia* GRP1 and French bean GRP1.8 are present on cell walls of vascular cells (77). GRP1.8 is localized on the cell walls of the primary xylem elements and primary phloem of many plant species (80, 157). Detailed immunoelectron microscopic studies suggested that GRP1.8 is produced by xylem parenchyma cells that export the protein to the walls of protoxylem vessels (127).

AGPs are characterized by high levels of arabinose and galactose in the carbohydrate moiety. The protein part, which contributes only 2–10% of the molecular mass, is commonly characterized by high levels of hydroxyproline, serine, and alanine (22). Two antibodies against AGPs—JIM13 and JIM14—bind to developing xylem cells and sclerenchyma cells in maize coleoptiles (134). In carrot, JIM13e binds to the future xylem as well as the epidermis of the root apex, whereas JIM14e binds to all cells of the root.

WGA, which has a strong affinity for a sequence of three β -(1-4)-linked *N*-acetyl-D-glucosamine residues, binds specifically to the secondary walls of TEs (69, 155). Wojtaszek & Bolwell (155) isolated three novel glycoproteins that bind to WGA. An antibody against one of the glycoproteins, SWGP90, revealed that this glycoprotein was localized in the secondary walls of TEs,

xylem fibers, and phloem fibers. Another novel type of wall protein, Tyr- and Lys-rich protein from tomato, has also been shown to be localized in the secondary walls (35). Thus, evidence is accumulating that some specific proteins are localized in secondary walls and function in the formation of the networks of the secondary walls.

Lignin Synthesis

Lignin is one of the most characteristic components of secondary walls and has been more intensively studied than any of the other macromolecules whose synthesis is closely associated with secondary wall formation. However, the synthesis of lignin is also induced by wounding or infection. I focus here on the lignification that occurs in association with xylem differentiation. The details of lignification can be found in several excellent reviews (17, 68, 95).

Lignins are cross-linked polymers composed of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units (68). Gymnosperm lignins consist essentially of G units. Angiosperm lignins are mainly mixtures of G and S units. Grass lignins are composed of H, G, and S units. The distribution of the G and S units is modified in the xylem cells and phloem fibers of *Coleus* by auxin and gibberellic acid (6). Terashima et al (149) indicated that H and S units are deposited, respectively, at the initial stage of lignification within middle lamella and, later, in the secondary walls of xylem cells. Thus, the composition of lignins also involves developmental and spatial events. An inhibitor of phenylalanine ammonia-lyase (PAL) activity, α -aminooxy- β -phenylpropionic acid not only blocked lignin synthesis but also caused the release of polysaccharides containing xylose into the culture medium of *Zinnia* cells (73). Inhibitors of cellulose synthesis such as 2,6-dichlorobenzonitrile dispersed deposits of lignin and reduced the level of xylan in the thickenings of differentiating TEs of *Zinnia* (146, 148). These results suggest that there is a mechanism whereby some components of secondary walls mediate patterning of deposition of other components. Indeed, lignin can make cross-links with many cell wall polymers such as polysaccharides and proteins through ester, ether, and glycosidic linkages (72). The cross-links, together with the hydrophobicity and chemical stability of lignins, reinforce and waterproof the walls, providing mechanical support, a pathway for solute conductance, and protection against pathogens.

THE GENERAL PHENYLPROPANOID PATHWAY The biosynthesis of lignin involves three pathways, known as the shikimate, the general phenylpropanoid, and the specific lignin pathways (95). The general phenylpropanoid pathway involves PAL, cinnamate hydroxylase (C4H), *O*-methyltransferase (OMT), and 4-coumarate:CoA ligase (4CL), all of which seem to be expressed in association

with xylogenesis and have been suggested as markers of lignification during xylem differentiation (45). Ye et al (156) indicated that the activity and transcripts of caffeoyl-CoA-3-*O*-methyltransferase (CCoAOMT) were expressed specifically in differentiating TEs both in the in vitro culture system and in intact plants of *Zinnia*, whereas the expression of caffeic acid-OMT transcripts was not correlated with lignification during TE differentiation in vitro or in the plant (160). These results strongly suggest that CCoAOMT is involved in an alternative methylation pathway to lignin that is predominant in differentiating *Zinnia* cells, although the question remains as to whether this pathway is predominant in differentiating TEs in many plant species. The end products of this general pathway, the hydroxycinnamoyl CoAs, however, are precursors not only of lignins but also of other phenolic compounds such as flavonoids and stilbenes (60), a fact that complicates the analysis of lignin synthesis.

A gene for *S*-adenosylmethionine synthase (sam-1), which serves as a donor of methyl groups in numerous transmethylation reactions, was reported to be expressed preferentially in vascular tissues of *Arabidopsis* (115). This finding suggests that during the differentiation of xylem cells, genes involved in the synthesis of compounds that are not specific to but are necessary for differentiation are also expressed preferentially.

THE SPECIFIC LIGNIN PATHWAY Hydroxycinnamoyl-CoA esters are converted to their corresponding alcohols by cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD), which are specific to the lignin pathway. CCR, isolated from differentiating xylem of *Eucalyptus gunnii*, had approximately equal affinity for *p*-coumaroyl CoA, feruloyl CoA, and sinapoyl CoA, which suggests that CCR may not contribute to the regulation of monomer composition of lignins in plants (56).

CADs and their corresponding cDNA clones have been isolated from various angiosperms and gymnosperms (17). Two different isoforms with different molecular masses and different substrate-specificities exist in angiosperms such as tobacco (57), while gymnosperms have a single CAD isoform (112). This difference between angiosperms and gymnosperms has been discussed in terms of the progressive evolution of lignins from G lignin to S lignin (17). The expression of β -glucuronidase (GUS), driven by the *Eucalyptus* CAD promoter, indicated that CAD genes are expressed preferentially in the primary xylem vessels in young regions of the stem and in the xylem rays in more mature regions of the stem (42).

In differentiating TEs, the cinnamyl alcohols are delivered to the cell walls by vesicles that are derived from the Golgi apparatus or the endoplasmic reticulum. At the cell walls, they are polymerized into lignin by wall-bound peroxidase isoenzymes via free-radical formation in the presence of H₂O₂ (95). In differentiating *Zinnia* cells, a cationic isozyme of peroxidase (P5), bound ionically to the cell walls, was shown to be involved in lignin

synthesis (130, 131). It was suggested that the enzyme was fixed tightly into the secondary walls with the progression of lignin deposition (130). A histochemical test for H_2O_2 (114) and observations by electron microscopy (29) indicated that H_2O_2 is localized preferentially in cells that are undergoing lignification such as TEs, although Schopfer (135) also showed the presence of H_2O_2 in the phloem and sclerenchyma, but not in the xylem, by tissue blots of soybean. Many genes for peroxidases have been isolated from a variety of plant species (89). However, there is no direct evidence for the isolation of a peroxidase gene that is specifically responsible for lignin synthesis during the differentiation of TEs (45). A cDNA clone that may correspond to P5 was recently isolated from a cDNA library of *Zinnia* differentiating cells (132). The characterization of this clone may provide interesting insights.

Laccase, which is a ubiquitous polyphenol oxidase and oxidizes polyphenols in the absence of H_2O_2 , is another candidate for the enzyme that catalyzes the oxidation of cinnamyl alcohols. Laccases or laccase-like oxidase are colocalized with lignin in the secondary walls of the xylem in various plant species, and they can catalyze the polymerization of lignin precursors in vitro (10, 37). However, there is also a report that xylem cells are only capable of oxidizing diaminobenzidine on the secondary walls in the presence of H_2O_2 (11). In addition, a serious problem is related to the difficulties encountered in attempts to separate laccases from other oxidases (17). The laccases and laccase-like oxidases that have been isolated from different plant species have different molecular masses, e.g. 38 kDa for the protein from mung bean (24), 90 kDa for that from *Pinus* (10), and 56 or 110 kDa for that from sycamore (37). O'Malley et al (113) speculated that the heterogeneity of laccases might result in the formation of heterologous lignins. The isolation of laccases specific for lignin synthesis and their genes are now of considerable importance.

Lignification proceeds even after TEs have lost their cell contents (138). The expression of GUS under the control of promoters of *CAD*, *PAL*, and *4CL* suggested that genes for these lignin-related enzymes are expressed in cells adjacent to differentiating TEs, as well as in differentiating TEs (42, 66). Immunolocalization studies have also confirmed that *PAL* and *4CL* are accumulated in developing metaxylem tissues and in cells adjacent to the metaxylem (138). It appears, therefore, that lignin precursors can be supplied by parenchyma cells adjacent to differentiating TEs. During lignin deposition in conifers, the monolignol precursors may be transported to the cell walls of TEs as glucosides such as coniferin (133). A β -glucosidase specific for coniferin has been isolated from lodgepole pine, and its activity seems to be localized to the area of differentiating xylem (33). Thus, in conifers, lignin precursors may be supplied as β -glucosides from the adjacent parenchyma cells to the secondary walls of differentiating TEs. However, lignification in ma-

ture TEs occurs even in isolated single *Zinnia* cells in the absence of direct cell-to-cell interactions (H Fukuda, unpublished observation). It is very important now to identify the cells in which lignin precursors are synthesized, how these precursors are transported, and whether the site of their synthesis changes with developmental stage or from tissue to tissue.

MUTANTS IN LIGNIN BIOSYNTHESIS Mutants that have a defect in the pathway to lignin are useful not only for the analysis of the complex regulation of lignin synthesis but also for the improvement of biomass via modifications of lignin content and composition (17). The *brown midrib* mutants of maize have reddish-brown pigmentation in the leaf midrib, which is associated with lower levels and a change in quality of lignins (88). Vignols et al (152) demonstrated that the *brown midrib3* mutation of maize was located in the gene for OMT. Pilonel et al (118) also found that the *brown midrib6* mutant of *Sorghum* resulted from a genetic lesion at a single locus, which resulted in decreased activities of both CAD and OMT. This result suggests that the mutated gene may be a regulatory gene that is responsible for control of the expression of CAD and OMT genes. The *Arabidopsis* mutant *fah1*, which produces abnormal lignins that lack syringyl units, seems to have a genetic lesion that results in a malfunctioning feruloyl-5-hydroxylase (25).

Genetic manipulation of lignin metabolism by antisense and sense suppression of gene expression has been attempted for several enzymes including PAL (12), OMT (38), and CAD (62). The analysis of transgenic plants in which PAL expression was suppressed indicated that the reaction catalyzed by PAL became the dominant rate-limiting step in lignin biosynthesis when PAL levels were below 20–25% of wild-type levels (12). Antisense expression of a homologous CAD gene caused strong suppression of CAD activity in tobacco plants, with the resultant production of modified lignin in which less cinnamyl alcohol monomers and more cinnamyl aldehyde monomers were incorporated into lignin than in control plants, with little quantitative change in lignin (62). The “antisense plants” had red-brown xylem tissues, resembling the *brown midrib* mutants.

Coordinated Regulation of Gene Expression

Lignin-related enzymes are coordinately expressed (87). Recent progress in promoter analyses of their genes is helping to elucidate the mechanism of such coordination. PAL is encoded by a single-copy gene in gymnosperms (140) but by a small family of genes in angiosperms (100). The isotypes of *PAL* genes in angiosperms are expressed differently in different organs and in response to different environment stimuli, such as wounding, infection, and light (98). 4CL is encoded in parsley by two highly homologous genes, which show no evidence of differential regulation (36), and it is encoded in

Arabidopsis by a single gene (93). Histochemical analysis of transgenic tobacco plants with a bean *PAL2* promoter-GUS chimeric gene and a parsley *4CL-1* promoter-GUS chimeric gene revealed that the promoters were active in differentiating xylem cells but were also active in other types of cell. Both promoters appear to transduce a complex set of developmental and environmental stimuli into an integrated spatial and temporal program of gene expression (65, 96).

Analysis of the regulatory properties of promoters of the bean *PAL2* and parsley *4CL-1* in transgenic tobacco plants showed that functionally redundant *cis* elements, located in regions proximal to the transcription start site, are essential for expression in xylem (64, 65, 96). A sequence in the *PAL* promoter, (T)CCACCAACCCC (C) (ACII), contained a negative element that suppresses the activity of a cryptic *cis* element required for expression in phloem (64, 96). This sequence corresponds to a conserved motif in a number of *PAL* promoters from bean, parsley, and *Arabidopsis*, which have a consensus sequence of CCA(A/C)C(A/T)AAC(C/T)CC (100). A similar element was also found in the negative regulatory sequence of *4CL-1* (65). Thus, the two enzymes in the general phenylpropanoid pathway seem to be regulated coordinately by a negative *cis* element. However, this motif has been found in a number of other stress-inducible promoters (60, 64), and moreover, the motif is also involved in gene expression specific to petals (64). Thus, other factors may be necessary for gene expression specific to xylem cells. The ACI motif in *PAL2* promoter has also been suggested to suppress gene expression in phloem (64). These negative elements also promote the gene expression in xylem tissues. The maize Myb protein P binds to overlapping hexameric repeats of CC(T/A)ACC and CCACC (58). The ACI and ACII elements are both composed of the overlapping hexamers, suggesting that tissue-specific *trans*-acting elements that bind to AC elements may be Myb proteins (64, 128).

The expression of the bean *GRP1.8* promoter has also been analyzed in detail (77). Transgenic tobacco plants containing a chimeric gene of a fragment starting at nucleotide -427 of the *GRP1.8* promoter fused to the GUS reporter gene expressed GUS activity in the vascular tissue of roots, stems, leaves, and flowers (81). The promoter contained at least five different regulatory elements that are involved in tissue-specific expression: SE1, SE2, RSE, VSE, and NRE (78, 79). RSE was essential for the promoter activity in roots and induced vascular tissue-specific expression. Keller & Heierli (79) also indicated that five tandem aligned RSE elements could confer vascular tissue-specific expression on the CaMV 35S minimal promoter. However, it is unknown which cells in vascular tissues express this gene at present. VSE was also essential for vascular tissue-specific expression. NRE seems to be a unique negative regulatory sequence that represses promoter activity in non-

vascular cells of leaves and stems. Thus, negative control of tissue-specific expression of genes could be a general mechanism in vascular differentiation. The *GRP1.8* promoter is regulated by a combinatorial mechanism that can integrate the actions of different regulatory elements to yield a vascular tissue-specific pattern of expression (79). Keller's group isolated a cDNA clone for a bZIP-type transcription factor that binds to the NRE element (77). It has also been reported that a nuclear factor that binds to the promoter of an extensin gene, EGBF-1, is present in the phloem and xylem of carrot roots (70).

Cell Death

Genetically programmed cell death plays an important role in the development of multicellular organisms. Differentiation into TEs is a typical example of programmed cell death in higher plants, and mature TEs are completed by the loss of cell contents including the nucleus, plastids, mitochondria, Golgi apparatus, and the endoplasmic reticulum, and by the partial digestion of the primary walls (51, 111, 124). In animals, programmed cell death is often apoptotic (18), and the death of TEs has been discussed in terms of apoptosis (104, 134). However, many observations by electron microscopy have failed to reveal any substantial similarity between apoptosis and the autolysis that occurs during TE differentiation (90, 111). There is no direct evidence of the occurrence of apoptosis-specific events in the autolytic process, such as DNA laddering, nuclear shrinkage, cellular shrinkage, and the formation of apoptotic bodies. [For details of apoptosis-specific events, see Kerr & Harmon (82).] Recently, Mittler & Lam (104) reported the presence of fragmented nuclear DNA in differentiating TEs. However, they did not provide direct evidence of the DNA laddering. Because of increases in levels of several different nucleases, as shown by Thelen & Northcote (150), which may be a common feature of differentiating TEs, the results of Mittler & Lam might also be explainable in terms of DNA fragmentation by such nucleases, rather than by apoptosis-specific endonucleases. The autolytic process seems to be a type of programmed cell death that is unique to higher plants. Figure 2 summarizes the process of degradation for each type of organelle during the autolysis of differentiating *Zinnia* TEs (153). This figure indicates that no signs of autolysis are apparent before the disruption of the tonoplast and that the degeneration of all organelles starts after its disruption. The disruption of the tonoplast occurs several hours after secondary wall thickenings become visible in *Zinnia* cells. After the disruption of the tonoplast, organelles with a single membrane such as Golgi bodies and the endoplasmic reticulum become swollen and then disrupted. Subsequently, organelles with double membranes are degraded (Figure 2). Therefore, the disruption of the tonoplast may be an irreversible step toward cell death.

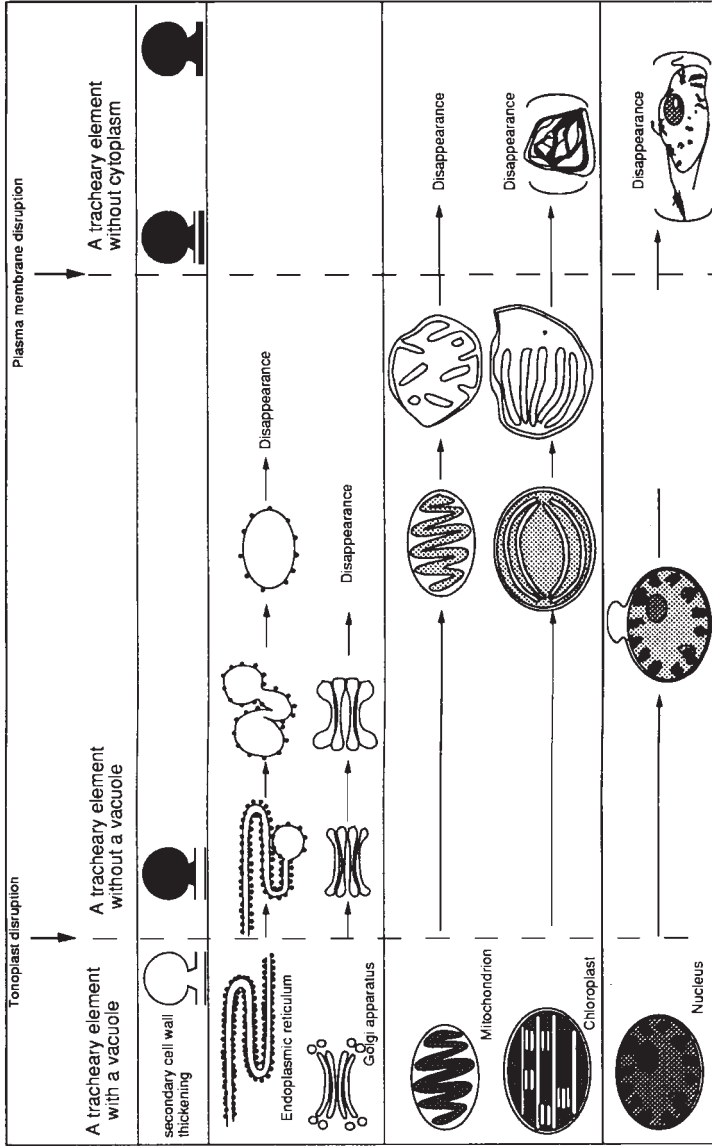


Figure 2 Structural changes in organelles during autolysis of differentiating *Zinnia* TEs. [Courtesy of Y. Watanabe.]

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* (67, 161). The nematode *ced-3* and *ced-9* genes have mammalian counterparts, the gene for interleukin-1 β -converting enzyme/CPRE-32 and *bcl2*, respectively, which are also responsible for the initiation of mammalian apoptosis (154). The molecular and biochemical analysis of the autolytic events in higher plants is just beginning, and no genes or proteins responsible for the initiation of the autolysis during TE differentiation have been identified. Is the initiation of autolysis coupled with the initiation or the progression of secondary wall formation? This is an important question because nobody has yet succeeded in separating the two processes.

The degeneration of organelles during autolysis may involve an increase in the activity of various hydrolytic enzymes that degrade large intracellular molecules. Thelen & Northcote (150) found that DNase and RNase activities were closely associated with TE differentiation in *Zinnia* cells. Nucleases—43, 22, and 25 kDa—appeared 12 h prior to the visible formation of TEs, and their levels increased conspicuously during the maturation phase of differentiation. An RNase of 17 kDa was detected transiently during the initial induction of other nucleolytic enzymes. One of the nucleases appeared before the onset of secondary wall thickenings. Minami & Fukuda (103) also found that the activity of a cysteine protease increased transiently at the start of autolysis and was specific to differentiating TEs in *Zinnia*. The mRNA corresponding to the cysteine protease was shown to be expressed specifically in differentiating TEs in *Zinnia* hypocotyls by in situ hybridization (A Minami, T Demura & H Fukuda, unpublished observation). Ye & Varner (158) also reported that the deduced amino acid sequence of a cDNA whose corresponding mRNA was expressed specifically in differentiating cells was homologous to papain, a typical cysteine protease. The inhibition of the activity of intracellular cysteine proteases by a specific inhibitor suppressed the loss of the nucleus, which suggests that a cysteine protease(s) plays a key role in nuclear degeneration (153). The addition of the inhibitor just before the start of secondary wall thickenings prevented TE differentiation itself, and therefore it seems possible that some cysteine protease(s) might be involved in the final determination of TE differentiation. CED3 protein, which is responsible for the initiation of cell death in animals, is a cysteine protease responsible for the cleavage of poly(ADP-ribose) polymerase (107). Our speculations are not contradicted by this result.

Sheldrake & Northcote (136) suggested that auxin and cytokinin, produced as a consequence of cellular autolysis, may induce the formation of new TEs. Other substances released from autolyzing TEs might also promote new cytodifferentiation. Thus, autolysis may be not only the final step in dif-

ferentiation, but it might also be the first step toward differentiation of an adjacent cell.

CONCLUDING REMARKS

TE differentiation is induced by a combination of auxin and cytokinin and, in some cases, by wounding. The differentiation can be divided into early and late processes. Each process has been dissected by use of genes and proteins that have been identified as markers associated with TE differentiation. The early process is complex and seems to involve several different stages. The early stage of transdifferentiation into TEs *in vitro* appears to involve functional dedifferentiation and the conversion of dedifferentiated cells into TE precursors via procambium-like cells. The latter process may be common to the process of meristematic cells, which may correspond to dedifferentiated cells, into TE precursor cells during differentiation of the primary xylem *in situ*.

The late process is common to systems *in vitro* and *in situ* and involves a variety of TE-specific events that are associated with secondary wall formation and cell death. Molecular analysis of genes that are expressed in the late process suggested the presence of putative *cis*-elements that direct the xylem-specific expression of a certain set of genes. Cell death begins with the disruption of the tonoplast, and the process differs from apoptotic cell death in animals.

Many problems remain to be solved. In particular, the following questions require our urgent attention. What are the different stages into which the early process can be subdivided by use of new cytological, biochemical, and molecular markers, and what is the signal(s) responsible for the transition from one stage to the next? At what point are the thickening of cell walls and autolysis separated in the putative signal cascade? What is the mechanism of coordinated gene expression? How is the coordinated differentiation of different types of xylem cell regulated?

To answer these questions, we need to develop: (a) a method by which many differentiation stage-specific marker genes can easily be isolated, (b) a cultured cell system in which gene transfer is easy and in which the effects of an introduced gene on TE differentiation can easily be analyzed, and (c) a mutation system in which mutants that have a defect in xylem differentiation can easily be isolated.

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