Tracheary Element Differentiation

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Key Words

xylem, secondary cell wall, programmed cell death, microtubules

Abstract

Tracheary elements (TEs) are cells in the xylem that are highly specialized for transporting water and solutes up the plant. TEs undergo a very well-defined process of differentiation that involves specification, enlargement, patterned cell wall deposition, programmed cell death and cell wall removal. This process is coordinated such that adjacent TEs are joined together to form a continuous network. Expression studies on model systems as diverse as trees and cell cultures have contributed to providing a flood of candidate genes with potential roles in TE differentiation. Analysis of some of these genes has yielded important information on processes such as patterned secondary cell wall deposition. The current challenge is to continue this functional analysis and to use these data and build an integrated model of TE development.

Contents

Tracheids: water-conducting cells that are similar to vessels but with no perforation plate

INTRODUCTION

The formation of xylem cells is one of the most intensely studied aspects of cell differentiation in plants. Part of this interest has been driven by the economic importance of xylem as a major constituent of wood and forage crops. Xylem is composed of a number of cell types, but the highly specialized and easily identifiable water-conducting cells, known as tracheids or vessels, have been most intensively studied. Collectively, these two cell types are frequently referred to as tracheary elements (TEs) and have been used to study many different aspects of plant cell differentiation, including developmentally regulated cell death, cell polarity, patterned secondary cell wall (SCW) deposition, and the role of the cytoskeleton (**Figures 1** and **2**). It is the differentiation of TEs that forms the basis of this review.

Figure 1 shows a summary of TE differentiation illustrating the different stages of vessel development referred to in this review. TEs are only one of several xylem cell types. They are easily identified by their characteristic patterned SCW, which is confined to the lateral sides of the cell and excluded from the ends. This SCW is essential to resist the negative pressures generated in the xylem during transpiration (**Figure 3*a***). The final stages of differentiation are characterized by programmed cell death (PCD), a process that removes the cell contents and leaves the cell empty, resulting in what has been described as a "functional corpse." Mature TEs are connected end-to-end by the perforation plate and form a tube specialized for unimpeded water flow, a process for which coordinated cell-to-cell communication between adjacent cells is clearly vital (**Figures 1** and **2*b***).

The intimate association of vessels with other cells, such as xylem parenchyma and fibers, makes it difficult to study TEs in isolation (**Figures 2** and **3**). However, there is an increasing body of evidence demonstrating that surrounding cells contribute to TE differentiation (see below).

An increasingly large number of *Arabidopsis* mutants have been described with alterations in vascular tissue patterning. These mutants exhibit a variety of phenotypes

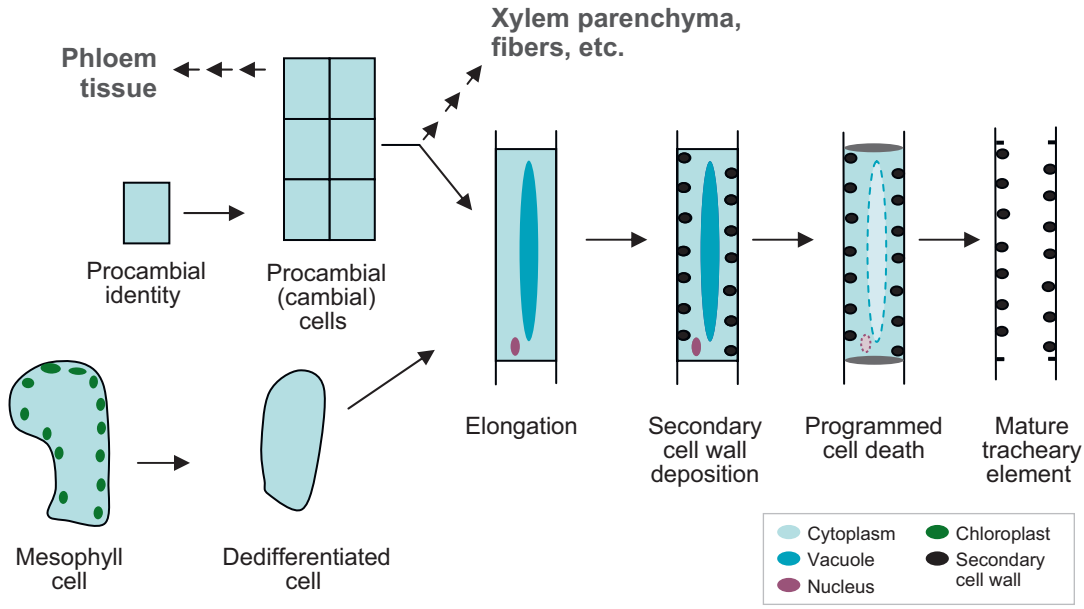

Figure 1

Tracheary element (TE) differentiation. The first sign of primary vascular development is the identification of procambial cells. These cells then divide and differentiate to become both the xylem and phloem. In secondary growth the equivalent cambial cells continue to divide and differentiate over many years. During xylem development procambial/cambial cells form various cell types in addition to TEs. In Zinnia mesophyll cell culture or following wounding, differentiated cells can transdifferentiate to form TEs.

including changes to the spacing and organization of veins of the leaf, or to the organization of xylem and phloem within the vascular bundle. These patterning mutants have been reviewed in this series (129) and recently elsewhere (19, 112) and are not the focus of this review unless they have a clear impact on xylem differentiation.

One distinctive characteristic of recent research on xylem differentiation is the use of widely disparate model systems (**Figure 3**). An ornamental flower, a model weed, and different trees species have all made important contributions to recent progress in the field. Recent research in these systems is characterized by a dramatic increase in data from expression studies and molecular genetic analysis, and these recent developments form the basis of this review.

MODEL SYSTEMS FOR THE STUDY OF XYLEM DIFFERENTIATION

Zinnia Cell Culture

The formation of TEs from isolated leaf mesophyll cells provides an excellent system for studying xylem differentiation (**Figures 1** and **3*b***). No other system offers the ability to regulate differentiation in such a coordinated manner, resulting in the synchronous transdifferentiation of a high percentage (up to 80%) of cells into TEs. Consequently, this enables the study of TE differentiation with minimal interference from other cells types.

Large-scale expression analysis based on cDNA restriction fragment length polymorphisms (RFLPs) identified many genes whose expression alters during Zinnia TE

Vessels: water-conducting cells that are connected together in files via the perforation plate to form a continuous network

TE: tracheary element

SCW: secondary cell wall

Programmed cell death (PCD): a predictable form of cell death. In the xylem, cell contents are removed leaving a hollow conduit for water transport.

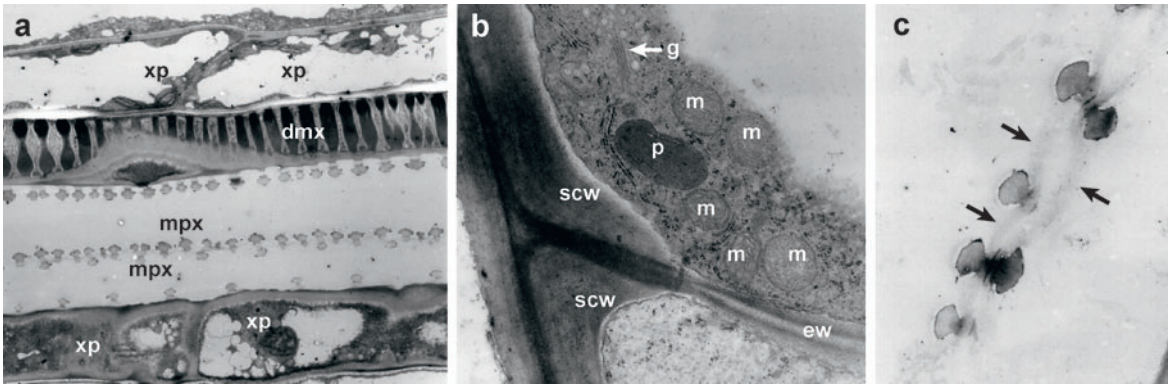

Figure 2

Stages of tracheary element (TE) formation in *Arabidopsis*. (*a*) Stem vascular bundle showing early mature protoxylem (mpx) that is completely differentiated and has lost the cell contents, and developing metaxylem (dmx) that still retains cytoplasm. In xylem tissue, TEs are intimately associated with xylem parenchyma (xp) that retain their contents and form nonpatterned secondary cell walls (SCWs). (*b*) Close-up view of two adjacent TEs. The end wall (ew) will eventually be lost to form the perforation plate that connects the two cells. The lower cell is undergoing cell death and the cytoplasm is degraded. The upper cell shows a very active cytoplasm characteristic of a cell undergoing SCW formation and contains numerous mitochondria (m), plastids (p), golgi (g), and ribosomes as well as extensive endoplasmic reticulum. (*c*) Close-up view of the cell wall between two adjacent mature protoxylem cells. The SCW is clearly visible, and the arrows mark the remnants of the primary cell wall that remains following programmed cell death.

Figure 3

Model systems for studying xylem development. (*a*) Transverse section through a stem vascular bundle from the *Arabidopsis irx3* mutant. Secondary cell walls (SCWs) of the xylem are stained blue and are located opposite the phloem (ph). Metaxylem vessels that have collapsed as a result of a defective secondary cell are marked with arrows. (*b*) Zinnia tracheary elements (TEs) formed as the result of mesophyll cell transdifferentiation. The large cell on the left is fully mature and has a distinct SCW, whereas the adjacent cell on the right just started SCW deposition. The cell above, which contains some chloroplasts and exhibits no signs of TE differentiation, may still contribute to the formation of TEs. (*c*) Wood formation in developing birch showing a transverse section through the birch cambium and the region of wood formation. The cambial initials in the cambial zone (cz) divide to generate new cells that form xylem (dx) and phloem (ph). The distance from the cambial zone is directly related to the stage of xylem differentiation. The wood-forming tissue from the previous year's growth (w) shows the distribution of very large vessels surrounded by numerous fiber cells. The whole tissue is interspersed with ray cells, which are marked by arrows.

differentiation (78). However, the most comprehensive study used microarrays composed of 8000 Zinnia cDNA clones. More than 500 genes were identified whose expression varied more than eightfold during xylem cell differentiation. Hierarchical clustering of these genes identified a number of groupings that exhibit distinctive expression patterns during xylem development (24). Such distinctive patterns can only be easily detected if the differentiation of cells into TEs occurs in a highly synchronized manner and are a testament to the excellence of the Zinnia system.

Although both transient assays and stable transformation may offer some means of carrying out functional analysis of Zinnia genes, rapid progress toward the functional analyis of these genes is being made by analyzing homologous genes from species more amenable to molecular genetic approaches.

Wood Formation in Trees

The study of xylem differentiation in trees is driven by the economic importance of wood. However, other factors, such as the highly organized nature of xylem differentiation during secondary growth, make trees an attractive model. Adjacent files of cells represent a developmental series from undifferentiated cambial initials through to mature xylem (**Figure 3*c***).In Poplar, the ordered arrangement of the xylem and the relatively large size of trees have been exploited to isolate mRNA from sections cut at different stages of xylem formation (45, 110). Recent detailed analysis of the cambial zone has the resolution to distinguish gene expression within the cambial initials from the xylem mother cells and provides some of the most detailed analysis of gene expression during the early stages of xylem formation in any species (110).

The analysis of xylem formation in wood is complicated by the presence of diverse cell types, including vessels, fibers, and ray cells (**Figure 3*c***). However, many of the genes that exhibit alterations in expression during xylem differentiation in trees have clear homologs in *Arabidopsis*, which is likely to facilitate their functional analysis.

Cambial: part of the cambium

Cambium: lateral meristem located between the xylem and phloem that is responsible for secondary growth

Arabidopsis

Arabidopsis has a clear role in the functional analysis of genes identified in both Zinnia and trees. Extensive gene expression studies of xylem development in *Arabidopsis* have already been described (62). In the stem, primary vascular tissue development represents a developmental series that has been exploited to identify genes that may be involved in SCW formation in the xylem (see below) (15, 28). Other investigators have applied weight and auxin to increase secondary growth in the stem and compared tissues undergoing primary growth with those undergoing secondary growth as a means of identifying genes specifically involved in secondary xylem formation (59, 88). The many different cell types in the *Arabidopsis* stem add complexity to the data analysis, a point illustrated by the observation that the expression of more than 5000 genes, around 20% of the genome, is altered during stem development (28). However, detailed bioinformatic analysis has identified candidates for many aspects of xylem differentiation, particularly SCW formation and its regulation (28).

The problems of tissue complexity have been overcome in one study where hypocotyls were separated into xylem, cambium/phloem, and the nonvascular outer tissues, and used to identify genes preferentially upregulated in one of these tissues (132). In one of the few cell type–specific studies in *Arabidopsis*, cell sorting was used to examine gene expression in cell layers of the developing root. This study included the stele that contains both xylem and phloem (9). Cross-referencing expression data from stems with stele-specific expression in the root is an excellent way to screen candidate genes (15, 28). The wealth of publicly accessible expression data available from expression studies in *Arabidopsis* represents a powerful asset that can be used to support custom array data (15). One study (93) relied

Procambial: part of the procambium

IAA: indole-3-acetic acid

solely on 486 essentially randomly selected, publicly available data sets to identify genes likely involved in SCW synthesis.

The use of *Arabidopsis* in genetic analysis is well documented. A focused genetic screen was carried out to identify ectopic expression of a xylem-specific marker gene. Numerous mutants were identified that are likely to define repressors of xylem differentiation (106). An important breakthrough was the development of a TE cell culture system in *Arabidopsis*. Although not as efficient as the Zinnia system (see above), *Arabidopsis* cell suspensions can be induced to form up to 30% TEs within a 96-h culture period in inductive conditions (62, 87).

FACTORS AFFECTING EARLY XYLEM DIFFERENTIATION

Auxin

The role of auxin in the differentiation of vascular tissue both during normal development and wounding is well documented (104). Recent research has greatly increased the understanding of both auxin transport and auxin signaling (see 65 for recent review). Mutants with impaired auxin signaling have clearly identified a role for auxin in the early stages of vascular patterning during the period when procambial cells are formed (reviewed in 19, 112). Studies of secondary growth in pine demonstrate that the highest levels of auxin are found in the cambium, which is consistent with the role of auxin in maintaining cambial cell identity (123).

The role of auxin in promoting procambial cells to differentiate into xylem is unclear. Auxin is an absolute requirement in the Zinnia system, where its addition during a specific 10-min window is sufficient to induce differentiation (77). Several genes involved in auxin signaling are expressed during TE differentiation in Zinnia (24, 78). The complexity of auxin signaling during xylem development is illustrated by studies in Poplar on the Auxin (AUX)/indole-3-acetic acid (IAA) gene family, which is an important component of the auxin signaling pathway. At least eight members of the AUX/IAA gene family are expressed during secondary growth in Poplar (82). The expression of some genes is specifically correlated with high levels of auxin in the dividing cells of the cambium and xylem mother cells, where auxin may be required to maintain a population of dividing cells. In contrast, at least one of the IAA genes was expressed in mature xylem, where it presumably has a role in the later stages of xylem development and must respond to much lower levels of auxin (82). One suggestion is that auxin may act as a morphogen to define the fate of different tissues. In this model auxin concentrations are translated into positional information via the IAA genes, and the expression of individual IAA genes is regulated by different concentrations of auxin. Consequently, expression of IAA genes in the cambial meristem where auxin concentrations are high would contribute to maintaining a population of cambial cells, whereas other IAA genes that are expressed at lower concentrations of auxin facilitate the later stages of xylem development (8).

Both genetic and inhibitor studies have identified a clear role for polar auxin transport in developing vascular networks and this work was recently reviewed elsewhere (19, 112). In the Zinnia system, xylogenesis may be blocked by auxin efflux inhibitors, which appear to activate metabolism of intracellular auxin, resulting in it degrading more rapidly. Consequently, the concentration of intracellular auxin is lower in these treated cells and this is presumed to be the cause of the low rates of differentiation (130).

A 10-min exposure to both auxin and cytokinin can be sufficient to induce TE differentiation (77); however, exposure to either auxin or cytokinin alone has no effect. This suggests that high levels of both exogenous auxin and cytokinin are required for a commitment step, after which point they are no longer required for TE formation. At least 68 genes are upregulated within 30 min of

induction by auxin and cytokinin, and some of these genes were previously implicated in auxin signaling. Of the genes upregulated, a substantial proportion are upregulated by auxin or cytokinin alone (24, 78). However, many genes are upregulated by these hormones, whether or not they are involved in xylem development. Therefore functional analysis of the upregulated genes is essential to establish their roles, if any, in xylem differentiation. One method of narrowing the list of candidates is to cross-reference the Zinnia expression data with that from trees where no application of hormones is required (24).

Cytokinin

The *Arabidopsis woodenleg* (*wol*) mutants contain fewer cell files in the vascular cylinder of the primary root and all the cells differentiate as xylem (108). *wol* is allelic with *cre1*, which is a cytokinin receptor (50, 72). Cytokinin acts via a signaling pathway similar to the bacterial two-component relay systems, with members of the *Arabidopsis* Response Regulator (ARR) family acting downstream of the receptor. Overexpression of one *ARR* gene (*ARR22*) results in the vascular cylinder of the primary root being composed exclusively of xylem, phencopying the *wol* mutants (57). A recent study overexpressed a cytokinin oxidase gene to deplete cytokinin specifically in the procambium (71). The resulting plants also phenocopy the *wol* mutation and provide very good evidence for an essential role for cytokinin in maintaining procambial cells and preventing their differentiation into xylem. In Zinnia cells, cytokinin is essential for differentiation (see above), which seems at odds with its role in maintaining procambial cell identity. However, it is possible that in the Zinnia system, cytokinin promotes mesophyll cell de-differentiation prior to transdifferentiation into TEs.

Brassinosteroids

Although a great deal of attention has been focused on the role of brassinosteroids (BRs) in regulating cell expansion (23), several independent experiments suggest BRs are also involved in the regulation of xylem development. BRs have been detected in developing pine cambium, and more direct evidence has come from the Zinnia system. The addition of Uniconazole, a known inhibitor of BR and gibberellin biosynthesis, to the Zinnia systems blocks the transdifferentiation of TEs in a manner that can be overcome by the addition of exogenous BRs but not gibberellin (54). Uniconazole appears to block the later stages of TE differentiation and prevents the expression of genes associated with PCD and SCW deposition (127). Consistent with the idea that BRs are required for the later stages of TE differentiation is the identification of five different BRs that accumulate both within the cells and in the TE culture medium when TEs are differentiating (128).

Independent confirmation of a role for BRs in xylem development came from the study of the known *Arabidopsis* BR biosynthesis mutants. *CPD*, *DWF7*, and *DET2* encode a hydroxylase, sterol desaturase, and reductase, respectively, and are all essential for BR biosynthesis (22, 84, 114). *cpd* and *dwarf7* have fewer vascular bundles, with each vascular bundle having comparable amounts of phloem to the wild type, but reduced xylem (22, 114), whereas *det2* has more phloem cells per vascular bundle at the expense of the xylem (18). *bri1* encodes a membrane-bound kinase believed to be part of the plasma membrane BR receptor (66). *bri1* mutants exhibit a similar phenotype to the biosynthetic mutants with an increase in phloem relative to xylem, whereas plants overexpressing *BRI1* have increased amounts of xylem (18). Three other proteins (BRL1, 2, and 3) have been identified based on their similarity to BRI1. Both BRL1 and BRL3, but not BRL2, bind to BRs and may also function as BR receptors (18). *brl1* mutants exhibit a similar phenotype to *bri1*, and the double mutant exhibits an enhanced vascular defect. A triple mutant of *bri1*, *brl1*, and *brl3* has greatly reduced vascular tissue, with both fewer xylem and phloem cells.

Procambium: meristematic cells that generate both the xylem and phloem of the primary vascular tissues

These results suggest that in addition to promoting xylem development at the expense of phloem, BRs also promote cell divisions in the procambial cells to provide the precursors for vascular cells (18).

Both auxin (see above) and BRs control a number of similar processes, such as cell elongation and vascular development. The induction of several auxin-inducible IAA genes by BRs (83) suggests a common link in the signaling of these two hormones; however, how these two hormones act together to regulate vascular development remains unclear. One important outcome of the discovery of the role of BRs in xylem differentiation is the development of an *Arabidopsis* cell culture system for TE differentiation in which the addition of BRs to the medium is essential (62, 87).

Xylogen

It is well established that one of most important factors in obtaining a high frequency of TE differentiation in the Zinnia cell culture system is a high cell density in the starting culture. Analysis of Zinnia cells immobilized in a thin sheet of agarose demonstrates that TEs typically form in clusters of cells, suggesting that differentiation of TEs tends to promote differentiation of neighboring cells (80). Furthermore, media isolated from cultures that have already undergone TE differentiation (called conditioned media) can be used to induce TE formation in cultures with a low starting density (80). Both an oligosaccharide (97) and a sulphated pentapeptide (Phytosulfokine) (75) have been suggested as compounds that accumulate and promote TE differentiation. Recently, a compound called xylogen was biochemically purified from differentiating Zinnia TEs (81). The purified protein (ZeXYP1) contains an N-glycosylation site, a signal peptide, and a putative glycosylphosphatidylinositol **(**GPI) anchor, which are all characteristics of Arabinogalactan proteins (AGPs) (81). Xylogen synthesized heterologously in tobacco cells can promote TE differentiation in the Zinnia cell system. Furthermore, a double mutant combination of two *Arabidopsis* homologs (*AtXYP1* and *AtXYP2*) resulted in leaf veins with a simpler, poorly coordinated pattern and xylem vessels that develop unconnected to the network. Interestingly, xylogen preferentially accumulates at only one end of the cell. Xylogen does not appear to be an essential determinate for xylem development because TEs still differentiate in the *AtXYP1/AtXYP2* double mutant. Taken together, these data suggests that xylogen coordinates the deposition of vascular tissue and that its polar secretion contributes to the continuity of the vascular network by promoting differentiation of the adjacent cells (81).

CLE Peptides

Clavata3-like/ESR (CLE) genes encode small peptides that appear to act as extracellular signaling molecules. The most widely studied member is CLV3, which functions together with the CLV1/CLV2 receptor complex as part of a pathway essential for regulating the organization of the *Arabidopsis* shoot apical meristem. There are at least 25 members of the CLE family in *Arabidopsis*, including several expressed in the vascular tissue. Recently, Ito and colleagues (53) used a bioassay for Zinnia TE differentiation to isolate a 12–amino acid peptide that inhibited TE differentiation. Comparison with the cDNA sequence revealed that the peptide was part of a larger precursor that exhibited homology to CLE genes. Only CLE peptides containing the specific 12–amino acid sequence could prevent TE differentiation and they had no effect on the root apical meristem. The study defines two distinct signaling pathways for CLE genes products: the regulation of apical meristem activity and the control of TE differentiation (53).

HD-Zip Genes and MicroRNAs

Analysis of the homeodomain leucine zipper (HD-Zip) proteins illustrates the

Table 1 Multiple functions of HD-ZIP genes[1]

Arabidopsis gene no.	Gene name	*Arabidopsis* Mutant	Loss of function Phenotype	Zinnia homolog	Overexpression Phenotype
AT4G32880	*ATHB8*	—	Not apparent	ZeHB-10	Overexpression produces weakly radialized vascular bundles
AT2G34710	*ATHB14*	*Phabulosa* (*phb*)	Radialized leaves and vascular tissue	—	—
AT1G30490	*ATHB9*	*Phavoluta* (*phv*)	Radialized leaves and vascular tissue	—	—
AT5G60690		*Revoluta/ interfascicular fiberless* (*rev/ifl*)	Loss of interfascicular fibers, alterations in auxin transport, meristem initiation	ZeHB-11 ZeHB-12	Overexpression produces weakly radialized vascular bundles
AT1G52150	*ATHB15*	*Corona* (*cna*)	Decreased vascular tissue development, increased apical meristem cell numbers	ZeHB-13	

[1]Further information on genes and phenotypes can be found in References 37, 91, 94, 111, and references therein.

complexities involved in relating gene expression patterns in developing xylem to gene function. This family of putative transcription factors has been divided into four classes (I-IV) (111). In *Arabidopsis*, class III is composed of five members (**Table 1**). Mainly due to expression studies in *Arabidopsis* and Zinnia, the role of class III HD-ZIP genes during vascular development has been the focus of much attention (3, 56, 89, 90, 111).

Analysis of the role of the HD-Zip III genes in vascular development is complicated by the complex interactions between family members, and the pleiotropic nature of the mutant phenotypes, which include alteration in the size of the apical meristem and changes in organ polarity (**Table 1**). Promoter-GUS studies using *ATHB8* (an HD-Zip III) show that it is an early marker for procambial development (3). Overexpression of *ATHB8* causes a proliferation of xylem and precocious initiation of secondary growth; however, loss of function mutations in *ATHB8* cause no obvious vascular phenotype (4). The absence of a vascular phenotype in the *ATHB8* knockout cannot be explained by redundancy among the HD-ZIP III group of genes (4) because comprehensive genetic analysis of insertional mutants in all five members of the HD-ZIP group does not support this idea (94). The proliferation of xylem caused by overexpression of *ATHB8* may result from the activation of genes normally regulated by members of the HD-ZIP II class. The rice HD-Zip III, *OsHOX1*, is expressed early in vascular development and appears to enhance the rate at which procambial cells differentiate into xylem (107).

In contrast to results with *ATHB8*, down-regulation of *ATHB15* either by mutation (94) or by antisense (58) gives a clear phenotype and results in plants with increased vascular tissue. Overexpression of this gene leads to smaller vascular bundles, consistent with the role of *ATHB15* as a negative regulator of procambial cell specification or proliferation. Recently, three Zinnia class III HD-Zip genes expresed during TE formation were overexpressed in *Arabidopsis* (91). This study included mutant forms that should be resistant to microRNA (miRNA)-mediated cleavage (see below). Overexpression of the mutated *ZeHB10* and *ZeHB12* resulted in much

MicroRNA (miRNA): 20–25 nucleotide RNA derived from a stem-loop region of a longer transcript complementary to a gene sequence

higher mRNA levels, but the principle phenotype was weakly radialized vascular bundles. This study highlights the problem of trying to dissect the role of genes that are specific to vascular development when large changes in plant morphology occur simultaneously. Whether alterations in vascular development reflect an alteration in organ polarity and to what extent expression of genes within the vascular tissue influences organ polarity or other changes in plant morphology need further attention (13, 19, 112).

HD-Zip III genes were among the first plant genes identified as targets of miRNA regulation (55). Two miRNAs (mi165 and mi166) appear to regulate expression of HD-Zip class III genes. This is illustrated by the characterization of the *men1* mutants. The phenotype of *men1* plants resembles different loss-of-function mutant combinations of the HD-Zip III gene family that includes *ATHB15* (94), but appears to result from the overexpression of mi166 (58). *ATHB15* has a clear role in vascular development (see above), and mRNA for *ATHB15* is barely detectable in the *men1* mutant. Although mi166 can mediate the cleavage of *ATHB15* mRNA, resulting in a proliferation of vascular tissue, the role of mi166 in regulating the expression of *ATHB15* during normal development is still unclear. By analogy with other miRNAs, it is likely that mi166 restricts the spatial expression pattern of *ATHB15*.

More indirect evidence for a role for miRNAs in regulating xylem differentiation has come from work that identified 21 miRNA gene families from Poplar, many of which exhibited vascular-specific expression (70). The 200 *Arabidopsis* genes that exhibit homology to miRNAs include several that are regulated by auxin (40, 73). Normal regulation of these genes by miRNAs appears to be an essential part of auxin-regulated developmental processes, such as root cap formation (125). miRNAs may play a role in the regulation of vascular development by auxin, although there is currently no direct evidence supporting this.

Other Genes Involved in Regulating the Early Stages of Xylem Development

Transcription factors are the focus of considerable work with the aim of identifying genes regulating the early stages of xylem differentiation. A NAC transcription factor is upregulated within 30 min of transfering Zinnia cells to inductive conditions (78). Similarly, a family of seven NAC genes in *Arabidopsis* is upregulated in *Arabidopsis* cell cultures during TE differentiation, but with varying expression patterns. The corresponding proteins have been termed vascular-related NAC-domain (VND) proteins. VND6 and VND7 localize to the nucleus and overexpression of either of these genes results in ectopic TE development, which supports their role in regulating TE differentiation (62). It is not clear exactly which steps VND6 and 7 regulate during TE differentiation, and their primary function may be the regulation of SCW deposition (see below).

In a comprehensive study of gene expression during stem development, Elthing and colleagues (28) identified 271 transcription factors whose expressions were altered during stem development. More than 190 of these genes were upregulated in a manner consistent with a role in xylem development. Cross-referencing with other expression data identified 19 genes that were upregulated in at least two independent studies and would therefore be good candidates for regulating some aspect of xylem development. Further analysis is needed to determine whether this is correct and, if so, what aspect of xylem development the genes regulate.

It has been noted that transcription factors are particularly abundant among the target genes for plant miRNAs (95). In addition to the HD/ZIP genes described above, both the MYB and NAC family of transcription factors have been identified as potential targets for miRNA (95). Ehlting and coworkers (28) identified 15 MYB and NAC family genes upregulated during stem development in a

manner consistent with a role in xylem development. In addition, Kubo and colleagues (62) identified six more NAC family genes upregulated during TE differentiation in *Arabidopsis* cell culture; however, only one of these genes, *ATMYB33* (At5g06100), is a potential target for a miRNA (mi159). Other members of these gene families have binding sites for sRNAs either upstream (At4g13480) or downstream (At1g35515, At5g22380) of their coding sequence (**http://asrp.cgrb.oregonstate.edu/db/**); however, the significance of these observations has not been examined experimentally.

Identifying the downstream targets for these putative transcription factors is essential to understanding the transcriptional network that controls TE differentiation. This process has already begun for NAC6 and NAC7, and this represents the first step in unraveling the complex transcriptional processes that control all aspects of TE differentiation.

ALTERATIONS IN CELL MORPHOLOGY

Cell Expansion

Although cell elongation in vessels has not been a topic of intense study, expression patterns of genes likely involved in cell wall loosening, such as expansins and xyloglucan endotransglucosylase/hydrolase (XTH) have been examined. Three expansin genes were cloned from differentiating xylem cells, two of which appeared to correlate with SCW deposition. In situ mRNA hybridization localized the transcripts from these two genes (*ZeEXP1* and *ZeEXP2*) to xylem parenchyma cells adjacent to the vessels. Surprisingly, the transcripts were localized at either the basal (ZeExp2) or apical (ZeExp1) end of the cell (49). The authors concluded that they may have a role in tip growth, which is consistent with work demonstrating that differentiating Zinnia TEs enlarge by tip growth (99). Expansins are also abundantly expressed in developing xylem of Poplar, where expansion of xylem fibers by intrusive tip growth has been well documented. mRNA for at least one Poplar expansin gene localizes to the ends of xylem fibers, consistent with a role for this gene in tip growth (36). An XTH protein that may participate in cell wall remodeling during expansion has also been localized to developing xylem cells in Poplar (12).

Recent analyses of gibberellin (GA) levels and expression of GA biosynthesis genes, and known GA signaling molecules in developing wood are all consistent, with GA acting early in the process of xylem differentiation (51). Given the known functions of GA, it is likely involved in the expansion of developing xylem cells.

THE ROLE OF THE CYTOSKELETON

Microtubules

Xylem vessels exhibit a very characteristic patterned secondary cell wall deposition. However, attention to this subject has been further stimulated by the observation that in whole plants and cultured cells bands of microtubules (MTs) underlie areas of SCW deposition (**Figure 4**) (86). These bands of MTs may be visualized prior to any visible signs of SCW formation and thus appear to predict the sites of SCW deposition. The relationship between MTs and cell wall deposition has been reviewed in detail by Baskin (7). Although most reports support a role for MTs in determining the sites of SCW deposition, one recent study suggests that the situation may be more complicated. Adding dyes, such as Congo Red or Evans Blue, to differentiating Zinnia TEs disrupts cellulose deposition during SCW formation and results in a more irregular pattern of SCW deposition at localized sites that do not correspond to bands of cortical MTs (98). Evans Blue interferes with cellulose microfibril crystallization, suggesting that cellulose synthesis and crystallization are required for normal SCW deposition (98). Careful observation suggests that MTs are lost

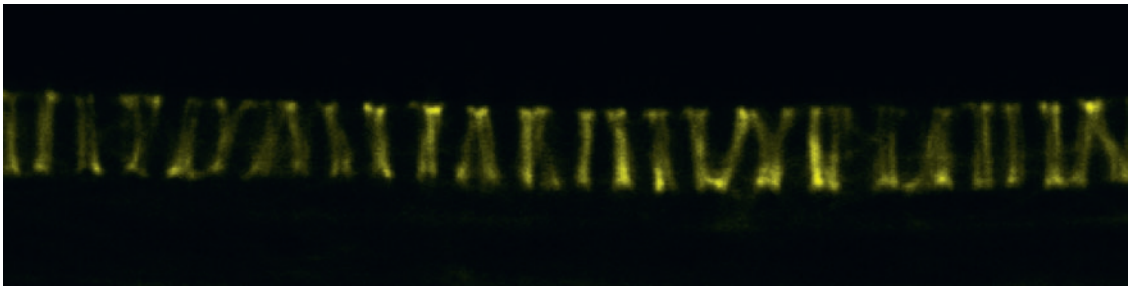

Figure 4

Microtubules (MTs) in developing xylem. MTs were visualized using yellow fluorescent protein (YFP) attached to an MT-binding protein and exhibit the characteristic banding pattern that marks sites of secondary cell wall deposition. (Photograph courtesy of Raymond Wightman.)

MT: microtubule

DCB: 2,6-dichlorobenzonitrile

before SCW deposition is complete and that MTs may only be required to pattern the early stages of SCW deposition (98).

The debate on the role of MTs in SCW deposition often focuses on the relationship between MTs and cellulose deposition (7). It is clear from numerous studies, however, that bands of cortical MTs in developing TEs mark not only the site of cellulose deposition in SCW, but also the site of deposition of other cell wall components such as lignin, hemicellulose, and proteins (46). Both the cellulose synthase complex and hemicelluloses are likely transported in Golgi vesicles, which must be targeted to specific regions of the cell membrane. Furthermore, other cell wall components, such as lignin, are incorporated into the wall in a localized manner (**Figure 5**). Consequently, bands of cortical MTs in TEs mark the sites for vesicle transport for a number of components, making it a unique example of MT-targeted vesicle fusion.

Inhibition of cellulose deposition by 2,6-dichlorobenzonitrile (DCB) is reported to give wider SCW bands, which do not protrude as far into the cell. These cells are also reported to have a dispersed pattern of lignin deposition (117). These observations support a mechanism in which the patterned deposition of cellulose directs the assembly of lignin to the same regions. Although this is an attractive idea, it is hard to reconcile with genetic studies. Mutants that appear to lack cellulose in the SCW, such as *irx3*, exhibit more uneven but distinctly narrower SCW thickening in contrast to the broader banding seen in DCB-treated cells (41, 122). An alternative hypothesis was presented by Hogetsu (46), who observed the pitted xylem vessels of pea. In these cells MTs and microfibrils are parallel to one another. However, under the pit, where no SCW deposition occurs, the MTs tended to be randomly orientated, with the exception of a distinct accumulation of MTs around the border of the pits. Based on these observations, Hogetsu (46) suggested that the plasma membrane is partitioned into two distinct domains. Domains under SCW thickenings are preferential sites for inserting vesicles containing hemicellulose and cellulose synthase rosettes. Bundles of MTs are postulated to determine and maintain the boundaries between plasma membrane domains (46) (**Figure 5**). If colchicine is used to depolymerize the MTs, the SCW thickenings become rough and poorly defined (87), consistent with the idea that MTs might maintain two different domains in the plasma membrane (46) (**Figure 5**).

Adding MT-stabilizing drugs, such as taxol, to differentiating TEs does not prevent bands of MTs from forming (30). Furthermore, whether the bands are orientated in the longitudinal or transverse plane appears to reflect the organization of MTs prior to TE differentiation. These observations

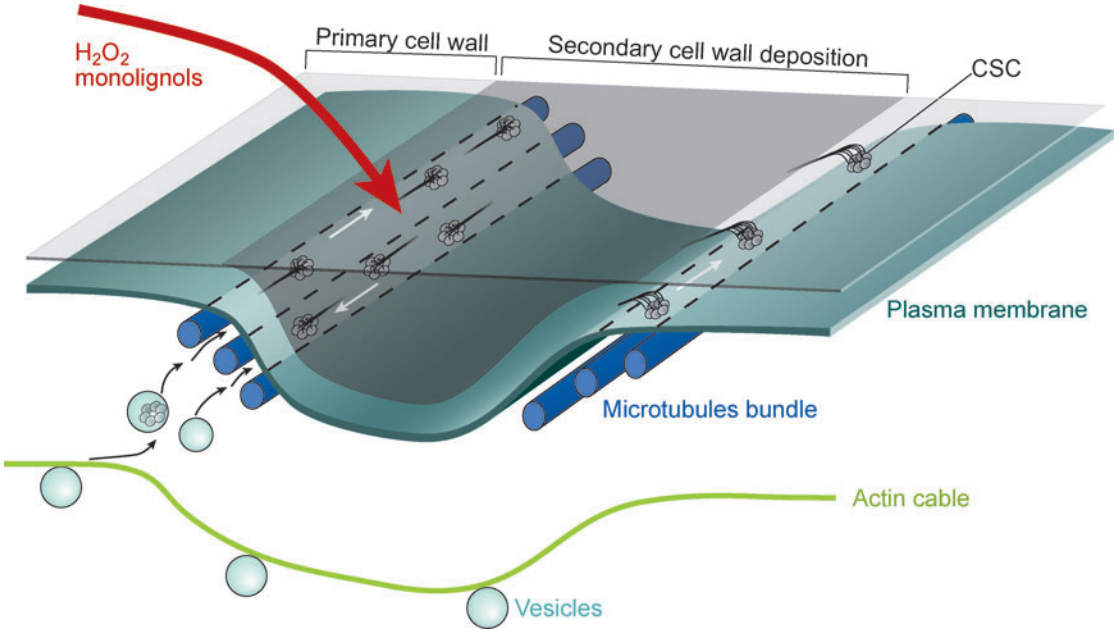

Figure 5

A model for secondary cell wall deposition. The actin cytoskeleton directs the delivery of vesicles containing hemicellulose and the cellulose synthase complex to the plasma membrane. Microtubule (MT) bundles mark the sites of vesicle insertion and maintain plasma membrane partitions, ensuring that the cellulose synthase complex moves parallel to the MTs, coaligning the MTs and the cellulose microfibrils. Adjacent cells deliver components that contribute to the synthesis of lignin in the secondary cell wall thickenings.

suggest that the bands of MTs seen in differentiating TEs result from the bundling of existing cortical MTs (29). MT dynamics has been studied extensively in expanding cells (26). However, few studies address how and where bands of MTs form in developing TEs, although they represent a well-defined system from which to study cytoskeleton organization and dynamics. This point is illustrated by the *wilted-dwarf* mutant of tomato. During normal vessel development, SCW deposition must be excluded from the ends of the cell where the perforation plate forms. This reflects the polarity of the cytoskeleton and consequently restricts the banding of MTs to the lateral wall and excludes them from the wall ends. In mutant plants, the thickening, and presumably the MTs, cover the ends of the cell and partially occlude the perforation plate, thus causing the wilted phenotype (1). Understanding this mutant or the identification of other mutants with altered patterns of MT banding will likely provide insight into the regulation of cell polarity and how this polarity helps determine MT dynamics in developing xylem and other plant cells.

MT bundling proteins, such as MAP65, have been shown to cross-link MTs by forming cross-bridges between them (21). A MAP65 recently isolated from Zinnia (ZeMAP65-1) is upregulated during Zinnia TE differentiation and is expressed in xylem cells of Zinnia vascular bundles. ZeMAP65-1 is associated with bundles of MTs in differentiating Zinnia TEs. When overexpressed in *Arabidopsis* suspension cells it causes bundling of cortical MTs in a manner reminiscent of developing TEs (74). However, expression of ZeMAP65-1 cannot be the complete explanation because in situ experiments

Metaxylem: later-forming xylem; distinguished by larger pitted cells with reticulate SCW patterning

Protoxylem: early-forming xylem; distinguished by spiral (helical) or annular SCW patterning

35S: highly expressed cauliflower mosaic virus RNA

suggest that the gene is strongly expressed in developing xylem parenchyma, which do not exhibit MT banding or patterned SCW deposition (74).

Live cell imaging of MTs has confirmed previous observations that initial transverse bands of MTs branch to form the more complex patterns seen in reticulate pitted metaxylem vessels (87). Although MT behavior and dynamics may have partially explained MT patterning, recent work suggests that transcriptional changes are also important in MT patterning in developing TEs. Kubo and coworkers (62) used xylogenic *Arabidopsis* cultures to identify a family of seven NAC transcription factors, including VND6 and VND7, expressed during TE differentiation. Overexpression of *VND6* and *VND7* resulted in ectopic vessels (see above). Most surprisingly, in Cauliflower mosaic virus strong promoter *35S:VND6* plants both ectopic and root TEs exhibited reticulate or pitted patterns of cell wall thickening characteristic of metaxylem. In contrast, the TEs of *35S:VND7* plants exhibited spiral or annular patterns of SCW thickenings characteristic of protoxylem. Overexpression of the VND6 protein fused to the SRDX strong repression domain driven by the 35S promoter resulted in the repression of metaxylem development in the root, although protoxylem was normal. The converse was found when VND7 was used. These results demonstrate that these two transcription factors independently regulate aspects of protoxylem or metaxylem development (62). It will be intriguing to compare the downstream targets of these two transcription factors and determine how they differ.

Actin

Early reports suggested that actin filaments exhibited a similar pattern to MTs in developing Zinnia TEs. Furthermore, during TE differentiation in Zinnia, disrupting actin with cytochalasin B prevents the reorientation of MTs from a predominantly longitudinal to a transverse orientation that occurs, suggesting a role for actin in reorientating MTs (60, 61). However, studies with intact *Arabidopsis* roots and in trees suggest that actin cables run mostly longitudinally during TE differentiation, which suggests a more probable role in vesicle transport of cell wall components (20, 34).

An MT-independent role for actin microfibrils in the SCW is suggested by the *Arabidopsis* mutants *fra3*, *fra4*, and *fra7*, which are caused by mutation in a GTPase, inositol polyphosphate 5-phosphotase, and phosphoinositide phosphostase, respectively. All these mutants have thinner SCWs and altered patterns of actin cables, but normal patterns of MTs (48, 134, 135). The relationship between the actin defect and decreased SCW deposition is unclear, but it is likely that phoshoinositides regulate the actin cytoskeleton and the resulting cell wall defect may be due to reduced vesicle trafficking of cell wall components (**Figure 5**).

SECONDARY CELL WALL BIOSYNTHESIS

Cellulose

Studies using *Arabidopsis* mutants have demonstrated that three different cellulose synthase (CESA) proteins [IRREGULAR XYLEM (IRX)1, IRX3, and IRX5] are required for cellulose synthesis in the SCW (118). This organization appears conserved between *Arabidopsis* rice and various tree species. IRX1, IRX3, and IRX5 initially localize within the cell and bands of cortical MTs precede their localization to the plasma membrane. At later stages of TE development, all three proteins colocalize at the plasma membrane with bands of cortical MTs (34), which is consistent with much earlier work that used electron microscopy to localize the cellulose synthase complexes to regions of SCW thickenings (109). The absence of any one of IRX1, IRX3, or IRX5 results in the remaining subunits being retained within the cell, presumably as a result of the inability to form an

intact cellulose synthase complex. However, the organization of cortical MTs appears unaffected (34), suggesting that the cellulose synthase complex does not have a role in stabilizing MTs during SCW deposition.

The endoglucanase KORRIGAN is also required for cellulose synthesis in the SCW. It does not appear to be part of the cellulose synthase complex and its role in cellulose synthesis remains unclear (115). Other candidates for genes involved in SCW synthesis have come from microarray studies. Using microarray data, a number of genes were identified that exhibited good coexpression with *IRX1*, *IRX3*, and *IRX5* (15, 93). Mutations in several of these genes gave an *irx* phenotype characteristic of a SCW mutant. However, cell wall analysis revealed that only one mutation in the *COBRA-LIKE4* (*CBL4*) gene exhibited a specific reduction in cellulose (15). Mutation in the rice homolog of *CBL4* had previously been shown to cause a cellulose-deficient phenotype (67). *COBRA* was previously described as a gene required for cellulose microfibril orientation during anisotrophic growth (101, 102), but the analysis described above suggests a wider role for this gene family in cellulose deposition.

Two additional *fra* mutants have provided some insight into how MTs might control the orientation of cellulose deposition. *fra2* is caused by a mutation in a katenin-like gene that encodes a protein with MT severing properties. *fra2* plants exhibit both altered cell shape and MT organization in a wide variety of cell types. However, in the SCW of fibers, cellulose microfibril orientation is clearly altered in a way that reflects altered MT organization, providing one of the best pieces of evidence to support a role of MTs in controlling the orientation of cellulose microfibrils (16, 17). In contrast, *fra1* mutants exhibit normal patterns of MTs, but cellulose microfibril orientation is altered, which results in plants with much weaker cell walls. The *fra1* phenotype is caused by a mutation in a kinesin-like protein and it remains unclear how this might alter cellulose deposition (133). One possibility is that the kinesin is involved in the transport of cell wall components that contribute to cellulose orientation.

Lignin

Lignin is a complex phenolic polymer that is essential for SCW structure and is formed from the oxidative cross-linking of monolignols. Lignin biosynthesis has been revised many times in recent years and was recently reviewed in this series (10); therefore, it is not covered in detail here. In cases where the genes encoding individual steps in lignin biosynthesis are unknown, candidate genes have been identified from microarray data (28). Lignification has identified an intriguing relationship between vessels and the surrounding xylem parenchyma. Evidence from Zinnia TEs suggests that lignification proceeds after cell death. Studies suggest that TEs can use monolignols or dilignols supplied by other cells or added to the media, and these can be incorporated into the cell walls of TEs that have already undergone cell death (47, 120). Similarly, Barcelo (6) looked for the site of H_20_2 production that is presumed to be required for lignin polymerization in developing Zinnia TEs. H_20_2 was generated at the plasma membrane of both vessels and thin-walled cells that are not undergoing lignification. Barcelo concluded that the latter cells provide the H_20_2 necessary for lignification in developing TEs (6). These results also imply that the restriction of lignin to sites of SCW thickening in vessels is determined by the localization of enzymes that polymerize lignin cross-linking. This is consistent with a recent report of a peroxidase from Zinnia specifically localized to the SCW (105).

Hemicellulose

Xylan is the predominant hemicellulose of SCWs (reviewed in 27) and consequently one of the most abundant naturally occurring polymers. Surprisingly, no gene for xylan

synthesis has been identified, although the characterization of three xylan-deficient mutants generated using reverse genetics has identified three glycosyltransferases that are good candidates for xylan biosynthetic enzymes (15).

Glucomannans are also found in *Arabidopsis* SCWs (42), and the enzyme that synthesizes the backbone of this polymer was recently identified. The enzyme has broad substrate specificity and is a member of the Cellulose synthase-like A (*CslA*) gene family (25, 68).

It is assumed that hemicellulose is synthesized in the Golgi and transported to the cell wall in Golgi-derived vesicles. Identifying more of the enzymes involved in hemicellulose synthesis should clarify the relationship between the sites of hemicellulose synthesis and its localized deposition in the SCW of xylem vessels.

Cell Wall Modification During Cell Death

During cell death, the end walls connecting adjacent cells are removed to form the perforation plate while areas of the lateral primary cell wall not covered by the SCW are extensively modified (**Figures 1** and **2**). This is particularly apparent in protoxylem, in which the regions of SCW deposition are well spaced. In protoxylem, vessels face a particular problem in maintaining the integrity of the vessel for water transpor, while being passively stretched by the elongation of the growing plant. During cell death many of the components of the primary cell wall are digested, with only the cellulose microfibrils and an electron-dense material remaining (**Figure 2*c***). Immunological data suggest that glycine-rich proteins (GRPs) are abundant components of this electron-dense material and link together regions of SCW thickening as well as the SCW of adjacent protoxylem (103). It is suggested that these GRPs are highly specialized to act as load-bearing components that stabilize the hydrolyzed primary cell wall. In doing so they maintain the integrity of the protoxylem vessels for transporting water even as the vessels become stretched during plant growth (96).

Regulation of Secondary Cell Wall Deposition

The regulation of cell wall deposition and particularly lignin deposition by transcription factors has received considerable attention. MYB genes in particular have been implicated in the regulation of lignin biosynthesis. MYB genes from *Arabidopsis* and *Antirrhinum* have been shown to control phenylpropanoid biosynthesis (11, 116). Similarly, a MYB from pine is sufficient to direct lignification in tobacco (92). More recently, a Eucalyptus MYB (*EgMYB2*) that alters lignin deposition in tobacco was identified. However, *EgMYB2*-overexpressing plants generally exhibit much thicker SCWs, suggesting that *EgMYB2* regulates many aspects of SCW synthesis, not just lignification (35). In addition to the two NAC transcription factors described above (*VND6* and 7) (62), another NAC transcription factor appears to have a role in regulating SCW deposition. *NST1* falls within a different NAC subfamily to the VND genes, but results in ectopic SCW when overexpressed (79). In the epidermis, *NST1* overexpression results in cell walls that are patterned and are reminiscent of TEs, whereas ectopic SCWs in mesophyll and other cell types is not patterned, suggesting that *NST1* regulates SCW deposition independently of cell wall patterning. Interestingly, repressing these genes results in the anthers failing to dehisce as a result of cells in the endothecium which surround the pollen grains failing to undergo secondary thickening (79). A similar phenotype was also described for mutations in a MYB gene (113). This highlights an important point: Many features of TE differentiation, such as patterned cell wall deposition, may be shared by other cell types and may be regulated by common mechanisms.

Programmed Cell Death

PCD is an active area of research in plants. Several molecular components have been identified over the past few years, but there is as yet no coherent picture. It appears that plants have evolved PCD mechanisms, but it is not clear what these mechanisms have in common with animal PCD, and, consequently, it is inappropriate to refer to plant PCD as plant apoptosis. Cell death of the TE has long been recognized as a prime example of developmental PCD in plants. It occurs among healthy cells in a predictable pattern that indicates regulation by a developmental program. It is an active process whereby a TE cell up-regulates genes that trigger its destruction. New protein synthesis is required, because inhibiting translation using cycloheximide blocks cell death of TEs in the Zinnia system (63).

The most striking feature of xylem cell death is the collapse of the vacuole that coincides with the digestion of the nucleus (31, 38). Vacuolar collapse activates or releases hydrolytic enzymes, including proteases (32), DNAses (52) and RNAses (64), into the cell, some of which may cause its destruction. Vacuolar collapse, without accumulation of these enzymes is not sufficient for PCD as it does not induce digestion of the nucleus in non-TE cells in Zinnia cultures (85). In addition, the clearest proof for the essential nature of the hydrolytic enzymes during PCD is demonstrated by the introduction of an antisense for the DNase *ZEN1* gene that suppresses nucleus digestion (52). After the accumulation step, the enzymatic activity of these hydrolytic enzymes dramatically increases at the time of vacuolar collapse, possibly because of their release from the vacuole. In support of this, a vacuolar localization was demonstrated for the protease XCP1 (32). By contrast, there is at least one RNase that accumulates in the ER of TEs and not in the vacuole (64). In fact, some of the hydrolytic enzymes detected may be cytosolic and activated by the acidification of the cytosol that results from vacuolar collapse. Hydrolytic enzymes associated with TE PCD have also been detected in the proteome of maize xylem sap (2) and this may support the idea that some of them are involved in defense and not PCD, as has been suggested for the xylem protease XCP1 (32). Of the several xylem-specific proteases detected, none have yet been shown to be required for the cell death process.

An important and recent development in our understanding of the role of proteases in plant PCD has been the demonstration of the importance of caspase-like proteases in this process (reviewed in 100). There are two main classes of plant caspase-like protease: metacaspases and proteases with caspase-like activity. The plant proteases most related to caspases are the metacaspases, which do not have caspase activity. One antisense study showed a metacaspase to be required for the cell death of the suspensor in embryogenic cell cultures of Norway spruce (14). It is interesting to note that only one of the nine *Arabidopsis* metacaspases is upregulated during the late stages of TE differentiation and this member of the gene family could therefore be one mediator of the PCD process (**Figure 6**). Few plant proteases with caspase-like activity have been identified thus far (100). One of them, the protease vacuolar processing enzyme (VPE), which has caspase 1 activity, is relevant to TE differentiation. Silencing of VPEs suppresses vacuole collapse in TMV-infected leaves. VPE may therefore be required to activate various vacuolar proteins involved in the disintegration of vacuoles that occurs during plant PCD (44). One member of the VPE family in *Arabidopsis* is upregulated during TE formation and could therefore be involved in vacuole collapse (**Figure 6**). However, the absence of any effect following the addition of caspase inhibitors (ICE inhibitors, general inhibitors) in the Zinnia system has been reported (31, 76), but the details have not been published. This suggests that the TE PCD may differ from many plant PCD systems that are clearly sensitive to caspase inhibitors (100). In this context, a study

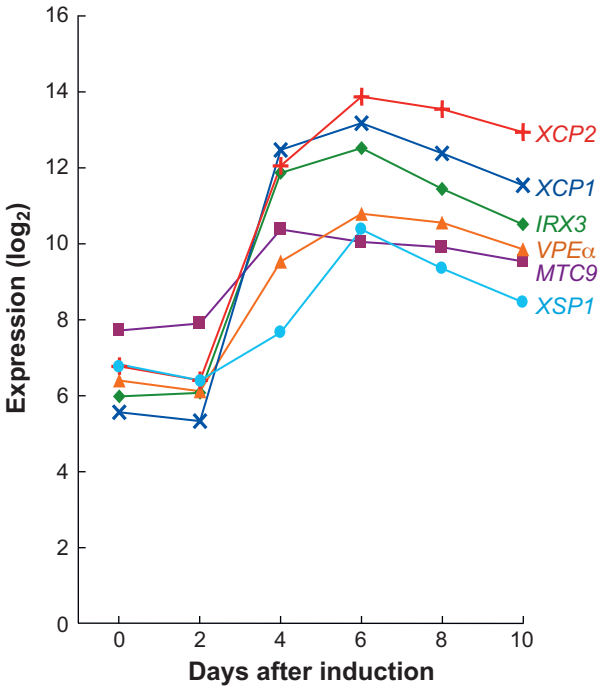

Figure 6

The expression patterns of programmed cell death (PCD)-associated genes during TE differentiation. Expression data from *Arabidopsis* cell suspension cultures (62) were used to examine transcript abundance of numerous putative PCD-related genes. The *IRX3* gene is included as a marker for the later stages of TE differentiation. The genes analyzed include those believed to encode vacuolar processing enzymes (VPE), metacaspases, xylem endopeptidases gene families, and genes involved in autophagy (32, 43, 119, 124). Only genes showing upregulation consistent with a role in PCD are shown. *MTC9*, metacaspase 9; *VPEα*, vacuolar processing enzyme; *XCP*, xylem cysteine proteases.

using the full range of caspase inhibitors available would prove very informative, as would knockout and overexpression studies of metacaspases and caspase-like proteases.

It has been suggested that TE cell death is a form of autophagy (126). There is little evidence to support this. On the contrary, autophagy may limit PCD, as shown in TMV-induced cell death (69). In addition, the upregulation of a suite of genes is a marker for autophagy, but in our microarray analysis, none of the 13 autophagy genes from *Arabidopsis* are upregulated at any time in xylem differentiation (**Figure 6**). This suggests that autophagy is not a component of PCD during TE differentiation.

Another important development in plant PCD has been the realization of a role for mitochondria. In particular, the release of cytochrome C from mitochondria is an early marker of cell death despite the fact that this does not appear to activate PCD, as described in animal models (5). One study showed that TE mitochondria are involved in the PCD process and cytochrome C is released before the vacuole ruptures (131). This suggests that vacuole collapse is not a trigger of TE PCD but more likely a final execution stage. Vacuole collapse is such a dramatic and visible event that it has attracted a lot of attention, possibly to the detriment of earlier PCD events that remain to be characterized.

Despite a wealth of information on the demise of the TE, it is striking that virtually nothing is known of the induction pathway for TE PCD at the biochemical or molecular level. Some studies have suggested that calcium (39) and nitric oxide (33), two known PCD regulators in plants, may be involved. However, little is known about which genes are regulating the process with such contained tissue localization. Is the trigger for PCD cell autonomous? Is PCD an integral part of the TE differentiation program or is it a general cell death module that is activated at the end of TE differentiation? The mutant *gpx* shows that PCD can occur without the completion of SCW synthesis and it is conceivable that in other mutants, secondary cell wall deposition could occur in the absence of PCD (79, 121). It is striking that so far there are no TE differentiation mutants in which only the PCD step is absent or has been delayed. Such mutants would represent an important breakthrough and their identification is likely to require specific genetic screens.

CONCLUSIONS

The variety of experimentally amenable models and the economic importance of wood have led to intensive study of TE differentiation. Consequently, a lot of data have recently

been generated on this subject. This is particularly true of transcriptional data. To maximize the utility of these data it will be necessary to integrate microarray data from diverse species such as Poplar, Zinnia, and *Arabidopsis*. It is clearly not possible to assign a function to a gene simply based on expression analysis. Consequently, one of the biggest challenges is to analyze these data and select target genes for further functional analysis. More proteomic data and metabolomics data will likely be available shortly; a future goal will be to integrate these data to develop a real systems model for TE differentiation. Although such a model is still some way off, TE differentiation is well ahead of the study of many plant cell types and appears on course to pioneer this approach.

SUMMARY POINTS

1. A large amount of transcriptomic analysis, including recent comprehensive microarray data from *Arabidopsis*, has led to the identification of many genes whose expression is altered during TE differentiation.
2. Functional analysis of many genes expressed during TE differentiation is still needed.
3. Initiation of TE differentiation is regulated by complex interactions of the plant growth regulators auxin, cytokinin, and BR.
4. Xylogen, an AGP that promotes TE differentiation and that exhibits a polar secretion, appears to contribute to the continuity of the xylem network.
5. Transcriptional regulation plays a role in various aspects of TE differentiation. In some cases, such as the HD-ZIP III genes where the corresponding mutants have pleiotropic phenotypes, it is difficult to separate their effects on TE differentiation from those of other aspects of plant development.
6. The cytoskeleton and MTs in particular appear to determine the site of SCW synthesis and are confined to the lateral walls and excluded from the end wall in a way that reflects the highly polarized organization of the cell.
7. The analysis of two transcription factors VND6 and VND7 uncovered a role for transcription in determining patterns of cell wall deposition.
8. Although large increases in hydrolytic enzymes, vacuole collapse, and cell wall digestion characterize the later stages of PCD during TE differentiation, factors triggering early events during PCD have not been identified.

FUTURE ISSUES

1. Several plant growth regulators such as auxin, BRs, and cytokinin influence TE differentiation, and understanding the network that integrates these signals is essential.
2. The identification of an AGP (xylogen) and a CLE peptide, which regulate TE differentiation, appears to define new signaling pathways. Which other components are involved in these pathways and how these components function remain to be determined.

3. Many putative transcriptional regulators are expressed during TE differentiation. Identification of their downstream targets and how they integrate to form a transcriptional network is a major challenge.
4. A proper understanding of TE differentiation will not be complete without more comprehensive proteomic and metabolomic data, which will need to be integrated with the transcriptomic data.
5. Recent work suggests that different patterns of transcription lead to altered patterns of SCW deposition, and a better understanding of how these transcriptional changes translate to alteration in the cytoskeleton is needed.
6. Identifying mutants that better separate the different stages of TE development, such as mutants that fail to undergo PCD, will greatly help with functional analysis of genes expressed during TE differentiation.
7. Mutation in several genes that are upregulated in TE differentiation results in very pleiotropic phenotypes. Dissecting the role of these genes in vascular development from their role in other aspects of plant development requires further analysis.

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15. Both custom and publicly available data were used to identify genes upregulated during secondary cell wall formation and to identify novel components required for cellulose and xylan biosynthesis.

24. Utilized a large cDNA array to examine transcriptional changes during TE differentiation in Zinnia.

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Annual Review of Plant Biology
Volume 58, 2007

Contents

Indexes

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