Programmed Cell Death During Secondary Xylem Differentiation in *Eucommia ulmoides*

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**Abstract:** Programmed cell death (PCD) during secondary xylem differentiation in *Eucommia ulmoides* Oliv. was examined using electron microscopy and by investigation of DNA fragmentation and degradation of caspase-like proteases (CLPs). DNA ladders were detected in developing secondary xylem by gel electrophoresis. DNA fragmentation was further confirmed by using the TdT-mediated dUTP nick-end labeling (TUNEL) method. Western blotting analysis showed that CLPs (caspase-8- and caspase-3-like proteases) and PARP (poly (ADP-ribose) polymerase) were degraded during secondary xylem differentiation. The results thus indicated that secondary xylem differentiation in *E. ulmoides* was a typical process of PCD and the degradation of CLPs might be a constitutive PCD event during secondary xylem differentiation.

**Key words:** caspase-like protease: DNA fragmentation: *Eucommia ulmoides*: poly (ADP-ribose) polymerase: programmed cell death: secondary xylem differentiation

It is now generally accepted that many plant developmental processes are achieved through programmed cell death (PCD) (Gan and Amasino, 1997). For example, cell death during xylogenesis has been extensively studied (Mittler and Lam, 1995; Ye and Varner, 1996; Groover et al., 1997; Wang and Cui, 1998; Fukuda, 2000; McCann et al., 2000), mostly by use of the *Zinnia* (*Zinnia elegans*) culture system (Fukuda and Komamine, 1980a). In this system, isolated mesophyll cells from young *Zinnia* leaves can be induced to transdifferentiate into "xylem" elements (TEs) by culture medium manipulation (Fukuda and Komamine, 1980b). The uniformity, accessibility and reduced complexity of the *Zinnia* system make it an effective tool for studying regulation of TE PCD (McCabe and Leaver, 2000): a number of genes associated with TE formation have been isolated and characterized (Fukuda, 1997; Milioni et al., 2001), and induction of hydrolytic enzymes, including proteases, serine proteases, and nucleases has been demonstrated (Thelen and Northcote, 1989; Ye and Droste, 1996; Ye and Varner, 1996; Beers and Freeman, 1997; Aoyagi et al., 1998; Groover and Jones, 1999; Obara et al., 2001). However, although the conversion of mesophyll cells into lignified elements has been termed xylogenesis (Fukuda, 1996), the relevance of the *Zinnia* system to the formation of tree secondary vascular systems (SVS) can be disputed. Some of the shortcomings of the *Zinnia* system have been discussed by Chaffey (1999), for example, it is single cell system, these "xylem" elements as being either protoxylem- or metaxylem-like, and the products remain single-cell.

With the introduction of the annual herbaceous plant *Arabidopsis* as a genetic system for studying plant growth and development, *Arabidopsis* has been adopted as a powerful system for genetic dissection of vascular differentiation and pattern formation (Ye, 2002). Unfortunately, this has meant that the natural system (the tree) has tended to be overlooked in favour of systems that are more amenable to manipulation and investigation (Chaffey, 1999). However, it is also unlikely that *Arabidopsis* will be a suitable model for the SVS of hardwood trees, especially in view of its restricted (Dolan et al., 1993) or negligible (Dharmawardhana et al., 1992) secondary growth, and lack of perenniality.

Of course, *Zinnia* and *Arabidopsis* have been proven to be invaluable testing ground for the development of techniques and ideas which can later be tested in the natural system, but the bulk of future work on the tree SVS should, and must, be performed with the natural system (Chaffey, 1999). Earlier studies on whole organisms identified the cytological and biochemical changes associated with secondary xylem differentiation. Ultrastructural observations of secondary xylem cell death demonstrated the rapid and progressive degeneration of organelles, and finally the removal of proplasts and parts of uninified primary walls (O'Brien and Thimann, 1967; Srivastava and Singh, 1972; Wodzicki and Humphreys, 1972, 1973; Esau and Charvat, 1978; Savidge and Barnett, 1993). Few investigations considered the...
mechanism of PCD during this process, and the hallmarks of PCD events such as DNA ladder formation and caspase (Cysteinyl-aspartate-specific proteinases) degradation have not been reported during secondary xylem differentiation in intact plants.

Secondary xylem differentiation involves thickening of the secondary cell wall (Maushet, 1988). Therefore, the formation of secondary cell wall and PCD seems to be coupled with each other during secondary xylem differentiation. Mittler and Lam (1995) reported that fragmentation of nDNA was detected in nuclei of vessel elements containing mature, lignified secondary cell wall structures in pea root. In the Zinnia system, Groover et al. (1997) reported that although 5% of the cells with pronounced secondary cell walls were labeled by the TUNEL assay, most of the developing TEs with pronounced secondary cell wall formation could not be labeled, indicating that nuclear degradation did not occur until the secondary cell wall formation was at or near completion. This process has not been satisfactorily elucidated by studies of plant development.

The present study was conducted on a living woody plant, Eucnemis ulmoides Oliv., and its aim was to investigate the occurrence of PCD in vivo during secondary xylem differentiation. We demonstrated that DNA ladder formation and degradation of CLPs (caspase-8- and caspase-3-like proteases) and PARP could be detected during the process. These results provide a basis for further studies of secondary xylem PCD in natural system.

1 Materials and Methods

1.1 Plant materials

Five vigorously growing Eucnemis ulmoides Oliv. trees were selected in a natural stand located in the Peking University campus, Beijing, China. The trees were about 20 years old, 10 m tall, and had a diameter of about 20 cm measured 1.3 m above the ground level. Samples containing phloem, cambium, differentiating xylem and mature xylem were taken from the main stem at the height of 1.0 m above the ground in June 2002. They were cut into 1 mm³ blocks for electron microscopy or 2 mm³ blocks for light microscopy.

For DNA and protein extraction, samples were isolated from the main stem. Trees were girdled as previously described (Li et al., 1982). The exposed surface of the tree was scraped with a sterilized scalpel to obtain a fraction. As revealed by light microscopy, samples contained several layers of cambial region and enriched in expanding xylem elements. Samples were placed in liquid N₂ immediately after being collected.

1.2 Light microscopy (LM) and TUNEL assay

The 2 mm³ blocks were fixed in 2% glutaraldehyde in 0.02 mol/L sodium cacodylate buffer, pH 7.0 for 4 h at room temperature, dehydrated in a graded ethanol series, and embedded in Technovit 7100 resin. Continued sample sections (5 µm in thickness) mounted on glass slides were first examined and photographed under an Olympus BH-2 polarizing microscope, then treated with the in situ Cell Death Detection Kit AP according to the manufacturer’s instructions (Roche, Germany). For positive control, DNase I (grade I, 0.5 mg/mL in 50 mmol/L Tris-HCl, pH 7.5, 1 mg/mL BSA) was added before TUNEL reaction mixture for 10 min at room temperature to induce DNA strand breaks. The negative control was conducted by similar incubations in the absence of TdT. Samples were counter-stained with 0.5% methyl green for 30 s.

1.3 Electron microscopy

Tissue blocks (1 mm³) were fixed in 2.5% glutaraldehyde in 0.05 mol/L sodium cacodylate buffer, pH 7.2 for 6 h at room temperature, post-fixed overnight at 4 °C in 1% OsO₄ in the same buffer, and then dehydrated and embedded in Epon 812 resin (Mollenhauer, 1964). Ultrathin sections stained with uranyl acetate and lead citrate (Reynolds, 1963) were examined with a JEM 100 CX transmission electron microscope.

1.4 DNA analysis

Total DNA of xylem was extracted by standard procedures (Dellaporta et al., 1983). Samples were ground in liquid N₂. The frozen powder was suspended in extraction buffer (2% (W/V) cetyltrimethylammonium bromide, 1.4 mol/L NaCl, 0.2% (V/V) 2-mercaptoethanol, 20 mmol/L EDTA, 100 mmol/L Tris-HCl, pH 8.0). The suspension was incubated at 65 °C for 1 h and extracted twice with chloroform: isomyl alcohol (24:1). Subsequently, DNA was precipitated with cold isopropanol. After RNAse treatment, DNA samples were analyzed by electrophoresis on a 1.0% agarose gel loaded with 4 µg DNA per lane.

1.5 Protein extraction

To extract soluble proteins from xylem, samples were thoroughly ground in liquid N₂ in a prechilled mortar. The powder was suspended in extraction buffer (2% (W/V) SDS, 0.5 mol/L Tris-HCl, pH 6.8, 5% 2-mercaptoethanol, 10% glycerol). After centrifugation at 15 000g for 15 min at 4 °C, the supernatant was collected and the pellet was washed three times with the same buffer. Supernatants were combined for use. The protein concentration of the extracts was determined by using Bradford's method, with bovine serum albumin as standard (Darbre, 1986).
1.6 Western blotting analysis

Twenty to forty microgram aliquots of protein were separated on 15% (caspase-8 and caspase-3) or 8% (PARP) SDS-PAGE and transferred onto nitrocellulose membranes. Nonspecific binding was blocked for 1 h at room temperature with 5% non-fat milk in PBS (pH 7.4) containing 0.1% Tween-20. Anti-caspase-8 polyclonal antibody (H-134, Santa Cruz Biotechnology) was diluted 1:200 with 1% non-fat milk in PBS containing 0.1% Tween-20. Anti-caspase-3 polyclonal antibody (H-277, Santa Cruz Biotechnology) was diluted 1:200, and anti-PARP monoclonal antibody (Sigma) 1:1 000. After 1 h incubation at 37 °C, membranes were washed three times in PBS and then once in TBS (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl). Second antibodies were alkaline phosphatase anti-rabbit IgG (caspase-8 and caspase-3) or anti-mouse IgG (PARP) at a dilution of 1:1 000. After 1 h incubation at 37 °C, membranes were washed three times in TBS and the protein bands were visualized with BCIP/NBT color substrate (Promega).

2 Results

2.1 Light microscopy and TUNEL analysis

Light microscopy gave an anatomical overview of secondary xylem differentiation (Figs 1, 2). The cambial region (Ca), differentiating xylem (Dx) and mature xylem (Mx) were easily distinguished. The locations of secondary cell wall are visible under the polarizing microscope: secondary cell wall is strongly anisotropic and shows substantial
double refraction and appears bright in polarized light (Esau, 1977). As shown in Figs. 1 and 2, secondary cell wall was visible in the differentiating xylem region at approximately 15–18 layers inward from the cambial region because of the abrupt increase in wall brightness. Cells in the cambial region were not labeled by the TUNEL assay, but positive signals (indicated by purple staining) could be detected in immature secondary xylem (approximately 7–9 cell layers inward from the cambial region) without or with unpronounced secondary cell wall as well as the developing secondary xylem with pronounced secondary cell wall (Figs. 1, 2). In positive control sections, all cell nuclei in cambium cells and immature xylem cells were positive labeled (Fig. 3), while in negative control sections, no

Figs.5–8. Electron micrographs of differentiating xylem cells, showing the autolytic degeneration of protoplasts. 5. Cells in the cambial region were lightly vacuolated, numerous organelles could be observed in cytoplasm, and a single nucleolus was seen at the midpoint of the cell. 6. Many small autolytic bodies were observed in the cytoplasm and the large vacuole. 7. The still visible nucleus contained condensed chromatin (arrowhead). 8. Autolized debris connected with one of the adjacent cells through the pits (arrowhead). Bar = 2 μm. Abbreviations: CW, cell wall; ER, endoplasmic reticulum; G, dictyosome; M, mitochondria; N, nucleus; V, vacuole.
TUNEL-positive cells were detected in the nuclei (Fig. 4).

2.2 Electron microscopic analysis

Transmission electron microscopy showed that secondary xylem differentiation was a typical process of PCD, which consisted of two synchronous processes of secondary cell wall deposition and proplastid degeneration ubiquitously existing during wood formation.

Cells in the cambial region were shown to be lightly vacuolated with the cytoplasm confined to a thin parietal layer (Fig. 5). Numerous cytoplasmic organelles such as dictyosomes, mitochondria, and endoplasmic reticulum could also be observed. A single nucleus was seen at the midpoint of the cell.

Concomitantly with the differentiation of secondary xylem, cell wall thickening was initiated and increased drastically. With the further wall thickening of xylem cells, many small autolytic bodies could be observed in the cytoplasm and the large vacuole (Fig. 6). Organelles were clearly disorganized and swollen. The nucleus was still visible and contained condensed chromatin (Fig. 7). In some cells the condensed proplast containing all autolyzed debris might be connected through the pits with one of the adjacent cells (Fig. 8). The mature xylem vessel members were dead cells with only thick cell walls; the cellular contents were completely degraded.

2.3 Detection of DNA ladders

Another hallmark of PCD is DNA laddering. To test whether secondary xylem differentiation is correlated to PCD, DNA extracted from this tissue was analyzed in 1.0% agarose gels. Results indicated that although obvious smearings of DNA appeared in differentiating secondary xylem (Fig. 9, lane 2), low molecular DNA signals with the sizes of approximately 200 bp and 530 bp could be detected in this process. As a control, DNA extracted from young *E. ulmoides* leaves (just developed) were examined at the same time and showed high molecular weight without DNA laddering (Fig. 9, lane 1). The result supported the occurrence of DNA degradation during secondary xylem differentiation.

2.4 Western blotting analysis

It was found that CLPs (caspase-8- and caspase-3-like proteases) and PARP were degraded during secondary xylem differentiation. As clearly shown in Fig. 10, differentiating secondary xylem contained the characteristic 20-kD caspase-8 subunit (Fig. 10a, lane 2), 27-kD caspase-3 subunit (Fig. 10b, lane 2) and 85-kD PARP subunit (Fig. 10c, lane 2). However, these degradations were not found in young *E. ulmoides* leaves (Fig. 10a, lane 1, Fig. 10b, lane 1, Fig. 10c, lane 1).

3 Discussion

3.1 Secondary xylem differentiation entails typical PCD

In this study, we examined the manner of cell death during secondary xylem differentiation in *E. ulmoides* trees. Ultrastructural studies showed that secondary xylem underwent a type of autolysis of proplast in the process of differentiation. Concomitant DNA degradation and cleavage of CLPs and PARP were observed. These results indicated that secondary xylem differentiation in *E. ulmoides* involved PCD.

The DNA ladder has not previously been demonstrated.

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**Fig. 9.** Photograph of electrophoresis of total DNA isolated from secondary xylem cells (lane 2). Note the ladder-like low molecular fragments (530- and 200-bp) as indicated by arrows. DNA extracted from young *E. ulmoides* leaves showed high molecular weight without DNA laddering (lane 1). M, a DNA molecular marker of 100-bp.

**Fig. 10.** Photographs of western blotting analysis, showing the degradation of CLPs and PARP during secondary xylem differentiation. a. caspase-8-like protease (lane 2). b. caspase-3-like protease (lane 2). c. PARP (lane 2). Degradations were not found in young leaves (a, lane 1; b, lane 1; c, lane 1).
during secondary xylem differentiation in vitro or in vivo. In this paper, DNA ladders were detected in developing secondary xylem (Fig. 9, lane 2) in comparison with high molecular weight DNA detected in young leaves which were proved not undergoing PCD by our previous study (Fig. 9, lane 1) (Cao et al., 2003). We expected to detect DNA ladders resembling those shown during induced synchronous PCD. The result, however, exhibited much DNA smearing and less laddered fragments. We could presume that this indistinct laddering related to the asynchrony of PCD during secondary xylem differentiation in vivo.

In animal cells, the activation of initiator caspases, such as caspase-8 and caspase-9, is known to transduce apoptotic signals and activate executioner caspases such as caspase-3 or caspase-7, to initiate a caspase cascade leading to PCD. Another feature of animal PCD is the cleavage of key proteins by caspases. PARP is among the first target proteins specifically cleaved by caspases (Lazebnik et al., 1994), and degradation of PARP is an important event in apoptosis. PARP is involved in DNA repair and chromatin structure formation.

Although to date no functional homologs of animal caspases have been identified in plants, a vast amount of indirect evidence suggesting the existence in plants of true caspase-like activity and its functional involvement in plant cell death has accumulated (del Pozo and Lam, 1998; Sun et al., 1999; Korthout et al., 2000; Tian et al., 2000; Mejnek and Prochůzka, 2002). In Zinnia system, increases in TE-specific cysteine peptidase activities have been observed (Minami and Fukuda, 1995; Ye and Varner, 1996; Beers and Freeman, 1997), and a cDNA encoding a papain-type cysteine endopeptidase has been cloned from differentiating Zinnia TE cells (Ye and Varner, 1996). In our study, we provided evidence that CLPs (caspase-8- and caspase-3-like proteases) and PARP degraded during secondary xylem differentiation in E. ulmoides and that this might play an identical role in the PCD pathway in plant cells (Fig. 10). To our knowledge, this was the first report implicating degradation of CLPs and PARP in secondary xylem PCD in woody plants. Further studies on the identification and characterization of these endogenous proteases and their protein targets are necessary for elucidating the PCD pathway in plant cells.

3.2 Relationship between PCD and secondary cell wall formation

TUNEL assay was performed with the aim of understanding specific degradation of nucleus (Gavrieli et al., 1992). It was reported that although the nuclei of TE cells close to their maturation were labeled with the TUNEL assay (Mittler and Lam, 1995; Groover et al., 1997), the nuclei of most of the developing TE cells with pronounced secondary cell wall were not labeled (Groover et al., 1997), indicating that DNA degradation does not precede cellular changes. In addition, Groover and Jones (1999) proposed a model in which secondary cell wall synthesis and cell death were coordinated by the concomitant secretion of the 40-kD protease and secondary cell wall precursors; subsequent cell death was triggered by a critical level of protease activity or the arrival of substrate signal precursor corresponding to the completion of a functional secondary cell wall. However, in our study, positive TUNEL signals could be detected in immature secondary xylem without or with unpronounced secondary cell wall as well as the developing secondary xylem with pronounced secondary cell wall (Figs. 1, 2). This result revealed that DNA degradation appeared earlier in secondary xylem differentiation and lasted for a long time, and the nuclease for DNA degradation were released prior to vacuole collapse. The nucleus was one of the most durable organelles, implying that the initiation of nuclear DNA breaking as visualized by TUNEL labeling might not greatly limit cell function. The exact function of earlier DNA degradation and its possible relationship with secondary wall formation require further study.

Schulz and Jensen (1981) and You (1985) reported that vacuole and dilated rough endoplasmic reticulum (RER) function as lysosomes. In the present study, electron microscopic analysis revealed that the vacuole autophagized debris from other organelles during PCD. The results suggested that materials in degenerated cells throughout PCD could be used to build up the secondary cell wall. This fate might explain why secondary cell wall formation is concomitant with degeneration.

Within the SVS of the tree, cell-cell interactions and the positional information related thereto are important to the proper development of the plants (Hake and Char, 1997). The probability of transfer of information between adjacent cells, such as via plasmodesmata (Barnett, 1995; McLean et al., 1997), coordinating cell division and cell differentiation is high in vivo. Our results indicated that in some cells the condensed protoplast containing all the autolyzed debris might be connected with adjacent cells through the pits. Further studies on this connection are necessary for elucidating the mechanism of PCD during secondary xylem differentiation. The degree of complexity, with the likelihood of cell-cell communication, is absent from the Zinnia system where an individual cell may actively and autonomously degrade its contents without the assistance of neighboring cells, denying us
the opportunity of gaining and insight into this fundamental feature of the SVS (Chaffey, 1999).

3.3 About the mechanism of secondary xylem PCD

In view of the multiplicity of cell types and the various organizations of xylem tissues, one could imagine that the molecular mechanisms controlling secondary xylem differentiation were complicated, involving many genes (Ye, 2002). Most studies on initiation of xylem differentiation have indicated that auxins, especially IAA, act as a pattern-forming agents (Sawidge and Waring, 1981; Sawidge, 1983; Aloni, 1987; Cui et al., 1992; Cui and Little, 1993). However, little is known about the signal transduction pathways of auxin that lead to xylem cell formation. Many auxin response factors have been identified (Guilfoyle, 1998), and studies of their functions will further elucidate xylem cell differentiation.

It was mentioned earlier that animal PCD is due to an orchestration of mechanisms involving effectors, adaptors, regulators and signals (Krishnamurthy, 2000). The key effector molecules of animal PCD are the caspases. Once caspases are activated, they cleave a large number of proteins within the cell, resulting in the morphological changes observed during apoptosis (Nicholson and Thornberry, 1997). In particular, the protein inhibitor (ICAD) of the caspase-activated DNase (CAD) is cleaved and CAD activation results in the fragmentation of DNA (Enari et al., 1998). In plants, protease activity has also been correlated with developmental events culminating in TE PCD (Minami and Fukuda, 1995; Ye and Varner, 1996; Beers and Freeman, 1997). In our study, CLPs and PARP degraded during secondary xylem differentiation and the DNA ladder was detected. Even if the role of these proteases in secondary xylem differentiation is not established, it has been proposed that they might play a role in regulating or executing cell death (del Pozo and Lam, 1998; Sun et al., 1999, Korthout et al., 2000; Tian et al., 2000; Miejscek and Prochózka, 2002).

Some morphological and biochemical features, such as cytoplasmic condensation and shrinkage, chromatin condensation, and nucleosomal DNA fragmentation, appear to be common between plants and animals irrespective of the type of cell undergoing PCD (Ellis et al., 1991; Kerr and Harmon, 1991; Havel and Durzan, 1996; Ryerson and Heath, 1996; Wang et al., 1996; Sun et al., 1999; Wei et al., 2002; Li et al., 2003; Cao et al., 2003). At the very end of animal apoptosis, apoptotic bodies are formed and phagocytosed. Since phagocytes are absent in plants, the degenerated cytoplasm and the nucleus have to be eliminated by a different process of vacuolar autophagy and plastolysis. In addition, in plants a number of cell types start functioning only after the cell death. In secondary xylem differentiation, the formation of secondary cell wall continues while the protoplast is degraded by an autolytic process (Groover et al., 1997; Wang and Cui, 1998).

The emerging picture was that, despite differences in initial and final events, there was considerable resemblance between plants and animals in some middle pathway of the PCD process. Figure 11 shows a hypothetical pathway for secondary xylem differentiation in woody plants. After reception of signals coming from the auxin, cambial region cells were induced for xylem differentiation. Initiator CLPs (caspase-8-like protease, etc.) could closely couple to pro-PCD signals. Once activated, these CLPs cleaved downstream effector CLPs (caspase-3-like protease, etc.), which in turn cleaved nuclear proteins such as PARP and ICAD and induced secondary xylem PCD. Subsequently, DNase was activated and DNA degradation could be detected in developing secondary xylem. Secondary cell wall thickening was initiated and increased drastically. In the final stage, organelles were clearly disorganized and swollen, and in

![Fig.11. Diagram of a hypothetical pathway during secondary xylem differentiation in woody plants. After reception of signals coming from the auxin, cambial region cells were induced for xylem differentiation. A series of CLPs were degraded and these enzymes in turn cleaved a discrete subset of key polypeptides, such as PARP and ICAD and induced secondary xylem PCD. Subsequently, DNase was activated, and DNA degradation could be detected in developing secondary xylem. In this action, secondary cell wall thickening was initiated and increased drastically. In the final stage of secondary xylem differentiation, organelles were clearly disorganized and swollen. The mature xylem vessel members are dead cells with only thick cell walls; the cellular contents are completely degraded.](image-url)
some cells, condensed protoplasts containing all autolyzed debris might be connected with one of the adjacent cells through the pits. The mature xylem vessel members are dead cells with only thick cell walls; the cellular contents are completely degraded.

In conclusion, we have examined secondary xylem differentiation in *E. ulmoides* and described the associated PCD. Although we have found no morphological evidence that secondary xylem PCD proceeded by an apoptotic mechanism, this PCD process have shown a subset of apoptotic features: activation of a mechanism of DNA degradation, cleavage of CLPs and PARP. This would provide a basis for the better understanding of the PCD process during secondary xylem differentiation in higher plants.

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(Managing editor: WANG Wei)