Front. For. China 2009, 4(4): 484–488
DOI 10.1007/s11461-009-0067-6

RESEARCH ARTICLE

Zengfang YIN, Ruwen FAN

Ultrastructural analysis of the differentiation process of secondary xylem vessel element in *Populus deltoides*

Abstract Using electronic microscopy, ultrastructural changes were observed during differentiation in a secondary xylem vessel element (VE) in *Populus deltoides*. Results showed that morphological development of VE differentiation was successively divided into three stages. First was primary cell wall outspread (the initial stage), where the VE was highly vacuolated and the protoplasma distributed along the cell wall. Second was secondary cell wall construction (the pivotal stage), where substances accumulated before the tonoplast broke, and the VE organelle was distinct. Golgi bodies and vesicles, which were associated closely with synthesis and transportation of secondary cell wall substances, were also abundant. After the tonoplast broke, these substances accumulated faster. Simultaneously, the protoplasm was disaggregated and the agglomerated chromatin was distributed over the margin of the nucleus, showing the typical characteristics of programmed cell death (PCD). During secondary cell wall formation, no cell wall substances accumulated between the terminal cell walls of neighboring VEs. In addition, terminal cell wall substances were disaggregated in the post secondary cell wall formation. Later, when the remnant terminal cell wall was broken in the third stage, perforation occurred. Thus, for these successive stages of VE differentiation, the critical stage, when differentiation was not reversible, was at the start of secondary cell wall formation with succeeding VE differentiation similar to a typical PCD process.

Keywords *Populus deltoides*, vessel element, ultrastructure, critical stage, programmed cell death

Translated from *Journal of Zhejiang Forestry College*, 2008, 25(4): 431–436 [译自: 浙江林学院学报]

Zengfang YIN (✉), Ruwen FAN
College of Forest Resources and Environment, Nanjing Forestry University, Nanjing 210037, China
E-mail: zfyin@njfu.com.cn

1 Introduction

The vast commercial value of lumber has promoted research in the differentiation process of secondary xylem elements of trees in recent years (Turner et al., 2007). Poplar, with its small genome, plenty of expressed sequence tags (ESTs), and easy transformation as well as fast growth, is recognized as a model tree of wood formation by the forestry kingdom (Yin et al., 2000; Cui, 2006; Du et al., 2006). The secondary xylem vessel is a very special type of cell that composes hardwood, performing multi-functions such as inorganic salt transportation, signal conduction, and mechanical support. Therefore, it has become one of the most often studied research hotspots in plant developmental biology. There has been great progress in research on the differentiation process and programmed cell death during secondary xylem differentiation. However, in the past, knowledge on the vessel differentiation process was mostly obtained from herbage (Easu, 1982). Research on plant integral systems has proven that the typical position effect exists in the course of plant cell differentiation (Cui, 2006). Therefore, it is very important to observe the development of the vessel systematically. Due to the structural specialty of the secondary xylem, it is very difficult to take samples and sections. So far, there has not been much or systematical research relating to the ultrastructure of vessel differentiation in the secondary xylem. Thus, it is important to observe the dynamic changes of vessel ultrastructure. In this study, the ultrastructural characteristic changes of the secondary xylem vessel in *Populus deltoides* were observed systematically, aiming to enhance fundamental knowledge of plant developmental biology.

2 Materials and methods

The materials and methods for TEM observation were described by Yin et al. (2007).

3 Results

The obvious changes in the protoplast and cell wall occurred during the differentiation of the secondary xylem vessel in *P. deltoides*. There was no protoplast in the mature vessel, and the cell wall of the vessel thickened remarkably. Based on cell wall changes during configuration establishment of the xylem vessel, the course of vessel element differentiation was divided into three stages, i.e., primary cell wall outspread, secondary cell wall construction, and perforation plate formation.

3.1 Primary cell wall outspread

It was observed from the cross section of the stem that the secondary xylem mother cell nearby to the vascular cambium zone expanded radially at the active stage of the vascular cambium. The volume of expanded cells was able to reach 8–15 times the cells derived from the procambium. This indicated that the expanded cell would transform into the vessel element. Meanwhile, the cell was highly vacuolated; the nucleus and cytoplasm were squeezed near the cell wall (Fig. 1(a),(b)). Organelles, such as the mitochondrion, Golgi body and microtubule in the cytoplasm (Fig. 1(b),(c)) were abundant. The outer membrane and cristae of the ellipsoidal mitochondrion were very clear (Fig. 1(b)). Usually, the rough endoplasmic reticulum, instead of a meshwork, was distributed in parallel near the cell wall. The Golgi body surrounded by Golgi vesicles was composed of 4–6 lamellae (Fig. 1(c)). It was also found that some microtubules were distributed stochastically, either parallel or perpendicular to the cell wall.

3.2 Secondary cell wall construction

Secondary cell wall construction is a crucial step during vessel configuration formation. The development of the secondary cell wall in the vessel element could be divided into two stages, intact tonoplast and tonoplast disaggregation. Cell ultrastructural characteristics at these two stages were significantly different.

3.2.1 Intact tonoplast stage

The third layer of cells near the vascular cambium zone stopped volumetric growth at the active stage of the vascular cambium in *P. deltoides.* In the earlier stage, the vessel element, which expanded to a certain cell volume, still had an intact tonoplast, then the partial cell wall started to thicken, the plasma membrane and tonoplast infolded (Fig. 1(d)), cisternae of ER inflated partially, and the Golgi body produced a mass of vesicles. The substances in the vesicles were similar to those in the cell wall in transparency, which indicated that substance exchanges between the cytoplasm and cell wall were active (Fig. 1(e)).

3.2.2 Tonoplast disaggregation stage

At the fast growing period of *P. deltoides*, the intact tonoplast stage of the vessel element lasted very shortly. Very soon, the tonoplast was broken and cytoplasmic components began to disaggregate. There were two ways of mitochondrion transformation: 1) inner structures of the mitochondrion were mixed up; cristae became unclear, and the inner matrix condensed (Fig. 1(f)); 2) a transparent zone with low electron density within the partial mitochondrion appeared (Fig. 1(g)). The typical change of the ER during vessel cell wall thickening was that cisternae were frothed because of expansion. Later on, the meshwork ER became pieces and the structure was not discernible (Fig. 1(f),(h)). At the earlier stage of vessel wall thickening, the cisternae structure of the Golgi body was

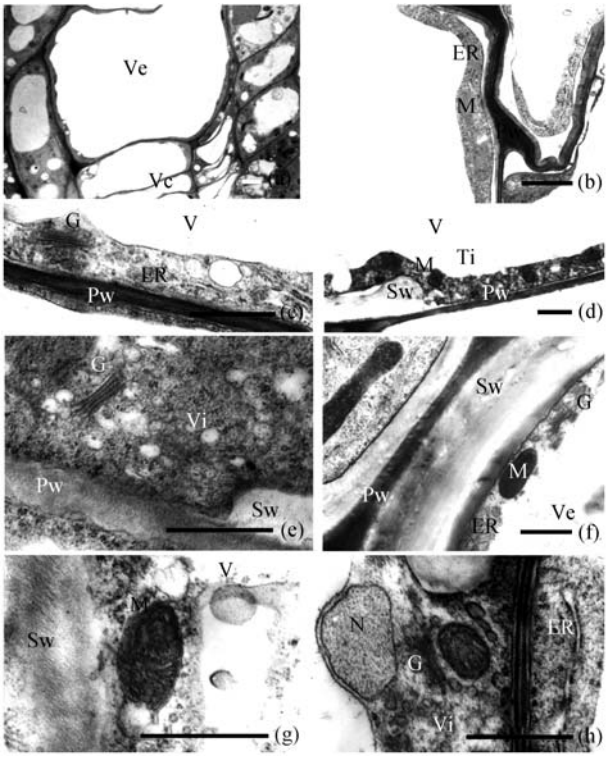

Fig. 1 The outspread of primary cell wall and the establishment of secondary cell wall of vessel element in secondary xylem (bar = 1 μm). (a) The cross section of the stem, showing the vascular cambium and the vessel element (Ve) which was getting on primary cell wall outspread. (b) Another Ve, the plasma distributed along the cell wall. (c) The organelle in earlier development stage of Ve, showing the Golgi body and endoplasmic reticulum. (d) The secondary cell wall substances accumulated partly in Ve with intact tonoplast, showing the tonoplast inflexed. (e) The Golgi body and vesicles during the formation of the secondary wall in Ve. (f) The disaggregated mitochondrion, endoplasmic reticulum and Golgi body in Ve. (g) Another Ve, showing the mitochondrion with transparent area. (h) The later stage of secondary cell wall formation of Ve, showing tonoplast membrane broke and organelle disaggregated.

clear. Later on, the cisternae disaggregated, and ruptured into vesicles. Changes in the nucleus were very complicated during vessel element differentiation. At the earlier stage, the nucleus looked like a spindle. Soon, it became irregular (Fig. 1(h)). As the disaggregation of the tonoplast progressed, the chromatin of the nucleus greatly agglomerated and distributed at the edge of the nucleus (Fig. 2(a)). At the later stage of vessel element differentiation, the nucleus, with an intact nuclear envelope, was still detected while other organelles disaggregated almost completely (Fig. 2(b)). Lastly, the nuclear envelope disappeared, and the nucleus disorganized into debris.

3.3 Perforation plate formation

The perforation can form between the end walls of the vessel elements. The perforation plate in *P. deltoides* was usually oblique, with an angle of 60 degrees to the long axes of the vessel element (Fig. 2(c)). At the early stage of vessel differentiation, secondary cell wall thickening did not occur at the end wall of neighboring vessel elements (Fig. 2(d)). Also, as the vessel's secondary cell wall thickened and the abundant plasmalemma infolded; this seems to relate to substance disaggregation of the primary cell wall (Fig. 2(e)). Eventually, the whole end cell wall started to bend, and the bent end cell wall broke up in some vessels (Fig. 2(f)). Therefore, it is inferred that the disappearance of the end cell wall of the vessel in *P. deltoides* might be accomplished with the aid of transpiration flow (Easu, 1982).

4 Discussion

4.1 Dynamic changes of organelles during secondary xylem vessel differentiation

The dynamic changes of inner cell organelles ran through the whole process of secondary xylem vessel differentiation in *P. deltoides* and played a crucial role in vessel differentiation. As the secondary cell wall substance accumulated, the cisternae of the ER expanded to a great extent so that the ER collapsed into short pieces nearby the cell wall, and the membrane structure disorganized gradually until it disappeared completely. The Golgi body plays an important role in the formation of the vessel's secondary cell wall. Plenty of vesicles, which carry substances similar to those of the cell wall in electron density, were distributed along the thickening cell wall. Some cytological research has proven that the Golgi body is closely related to the formation of the cell wall. For example, it was proved that the noncellulose amylose of cell walls is synthesized and transported by the Golgi body. The Golgi vesicles enwrapping the precursor substance of the cell wall move around to the cell plate along the phragmoplast microtubule during plant cell cytokinesis (Easu, 1982). Xu et al. (2006) observed that Golgi vesicles obviously increased in the formation of the helical vessel secondary cell wall in *Cucurbita moschata*, and thus, the Golgi body was closely related to the secondary cell wall. The mitochondrion was one of the organelles which disaggregated slowly during secondary xylem vessel differentiation in *P. deltoides*, and its representation in the vessel differentiation was very similar to that in *Eucommia ulmoides* Oliv (Wang et al., 1998). Two types of mitochondrial structural changes occurred during the development of the vessel in *P. deltoides*. One occurred often at the later stage of vessel differentiation, at which the condensed inner matrix formed a sort of substance with great electronic density. Another one was seen mostly at the early-middle stage, at which the partial mitochondrion became transparent. Wang et al. (1998) thought of this as one way of mitochondrion disaggregation. We believe that is a kind of unstable status during the active metabolism rather than mitochondrion disaggregation.

The vacuole underwent great changes during vessel formation. At the early stage of secondary cell wall substance aggregation in the vessel, the tonoplast remained intact and the protoplast structure was clear. Later on, the tonoplast was broken, and organelles in the cell began to disorganize. By observing the tracheary elements during differentiation in *Zinnia elegans*, Fukuda (1996) found that the tonoplast was broken in several hours after secondary cell wall thickening, and subsequently, the organelle started to disaggregate. The cytoplasma disappeared completely after the tonoplast broke. This indicated that the broken tonoplast is the key step in tracheary element death. Wang et al. (1998) found that the vacuole functioned as a lysosome which phagocytoses various organelle remnants during secondary xylem vessel element differentiation in *E. ulmoides*. The vacuole broke up at the later stage of vessel differentiation. It might have released a hydrolytic enzyme which induced a more disordered cell structure, then the resulting hydrolysate could have been involved in formation of the secondary cell wall.

Like in *E. ulmoides* and other trees (Wang et al., 1998), the nucleus was the last organelle to disaggregate during secondary xylem vessel differentiation in *P. deltoides*. The nucleus had no signs of disaggregation after the secondary cell wall thickened. At the later stage of vessel differentiation, as the tonoplast was broken, the chromatin condensed and distributed at the margin of the nucleus. Using in situ hybridization techniques, Wang et al. (1998) detected fragmentation of DNA in the vessel nucleus in *E. ulmoides*, and thought there were apoptotic bodies during the differentiation of the secondary xylem vessel, just like those in animal cells. This also illuminated the programmed cell death of the vessel nucleus in *E. ulmoides* (Wang et al., 1998). We found that after almost all other components disaggregated in the secondary xylem vessel of *P. deltoids*, the nuclear envelope still remained intact; the nucleus was broken up into debris after secondary wall

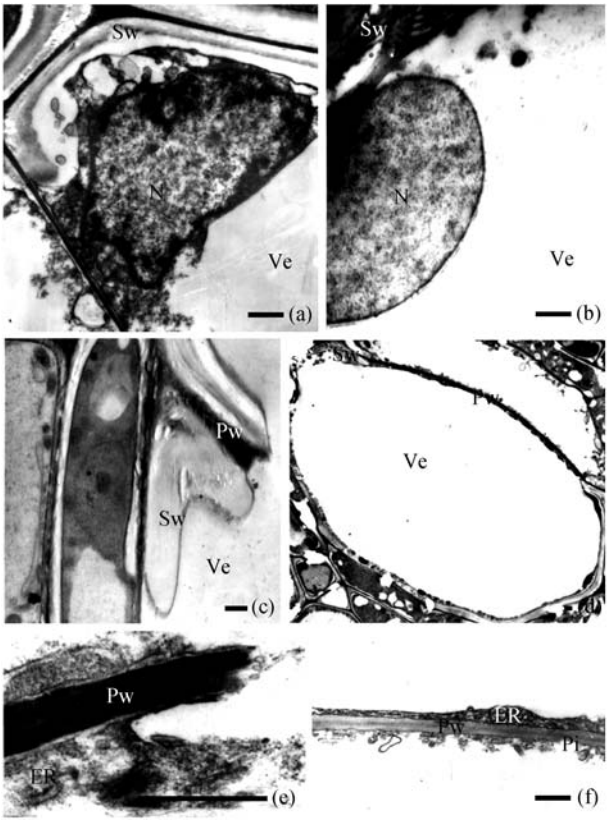

Fig. 2 The disaggregation of nuclear and formation of perforation plate of secondary xylem vessel element (bar = 1 μm). (a) The disaggregation of vessel element (Ve) nuclear, showing agglomerated chromatin distributed in the margin of nuclear. (b) The later differentiation stage of Ve, the plasma disaggregated mostly, but the nuclear structure is clear. (c) The longitudinal section of Ve, showing the perforation plate development. (d) The skew section of Ve, showing the developing perforation plate. (e), (d) enlarged partly, showing organelle, and plasma membrane inflexed near the end cell wall of Ve. (f) The end cell wall between the neighboring Ves was broken.

formation and vanished eventually. Therefore, we presumed that cytoplasm disaggregation is an orderly progress controlled by some signals from the nucleus.

4.2 Crucial time limit of irreversible vessel differentiation in secondary xylem

The process of plant cell differentiation could be divided into sequential phases one after the other, and programmed cell death might be the last phase (Fukuda, 1996; Fan et al., 1999). Cui (1997) pointed out that a crucial time limit could exist in the process of tracheary element differentiation. The cell differentiation was reversible before the crucial time limit, that is, the cell could dedifferentiate. Once the crucial time limit was over, differentiation was irreversible. Despite the application of inhibitor caspase-3 or Dnase to *Populus tomentosa* Carr., its vascular cambium still maintained cell division, and the derivative cells expanded; however, the secondary cell wall could not form. This indicated that the formation of the secondary cell wall happened later to when the nuclear DNA was broken (Cui, 2006). An investigation on the *Z. elegans* experimental system *in vitro* showed that lignin accumulation starts after the tracheary element dies. Moreover, the monolignol and dilignol could be provided by the surrounding cells (Hosokawa et al., 2001; Tokunaga et al., 2005; Turner et al., 2007). However, the tonoplast of the vessel did not rupture when the secondary cell wall substance accumulated during the vessel differentiation of *P. deltoides*. Here, the organelle structure of the vessel was clear, and the vesicles derived from the Golgi body were very abundant. It indicated that the synthesis of the secondary cell wall substance was provided by the vessel self at the beginning. We thought that secondary cell wall formation was the essential period in vessel differentiation, and lignin accumulation occurred already before the vessel tonoplast broke. Furthermore, the irreversible crucial time limit was triggered by the accumulation of lignin. The formation of the secondary cell wall represented the starting point of the irreversible process of vessel differentiation and the programmed cell death of the vessel. After the tonoplast was broken, the PCD of the vessel was still discernible. Complete disaggregation of the nucleus was the sign of the end of vessel programmed cell death.

Acknowledgements This work was supported by the National Natural Science Foundation of China (Grant Nos. 39730350, 30671657) and the Natural Science Foundation of Jiangsu Province, China (No. BK2005132). We thank Dr. Li Guiying (Auburn University, USA) for helpful suggestions and a critical reading of the English manuscript.

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