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Avoiding transport bottlenecks in an expanding root system: Xylem vessel development in fibrous and pioneer roots under field conditions[1]

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- *Premise of the study:* Root systems develop to effectively absorb water and nutrients and to rapidly transport these materials to the transpiring shoot. In woody plants, roots can be born with different functions: fibrous roots are primarily used for water and nutrient absorption, whereas pioneer roots have a greater role in transport. Because pioneer roots extend rapidly in the soil and typically quickly produce fibrous roots, they need to develop transport capacity rapidly so as to avoid becoming a bottleneck to the absorbed water of the developing fibrous roots and, as we hypothesized, immediately activate a specific type of autophagy at a precise time of their development.
- *Methods:* Using microscopy techniques, we monitored xylem development in *Populus trichocarpa* roots in the first 7 d after emergence under field conditions.
- *Key results:* Newly formed pioneer roots contained more primary xylem poles and had larger diameter tracheary elements than fibrous roots. While xylogenesis started later in pioneer roots than in fibrous, it was completed at the same time, resulting in functional vessels on the third to fourth day following root emergence. Programmed cell death was responsible for creating the water conducting capacity of xylem. Although the early xylogenesis processes were similar in fibrous and pioneer roots, secondary vascular development proceeded much more rapidly in pioneer roots.
- *Conclusions:* Compared to fibrous roots, rapid development of transport capacity in pioneer roots is not primarily caused by accelerated xylogenesis but by larger and more numerous tracheary elements and by rapid initiation of secondary growth.

Key words: black cottonwood; fibrous roots; heterorhizy; pioneer roots; *Populus trichocarpa*; programmed cell death (PCD); root anatomy; Salicaceae; transmission electron microscopy (TEM).

Knowledge of belowground structures and processes is essential for our understanding of factors responsible for tree growth and function. Most studies of root function in relation to root age focus on the role of roots in absorption of water and nutrients or on respiratory activity (Clarkson et al., 1968; Comas et al., 2000; Bouma et al., 2001; Volder et al., 2005; 2009). Yet, in larger plants, rapid transport of water and nutrients to the transpiring leaves may also be a potentially limiting process (Greacen et al., 1976; Passioura, 1988).

Root systems have a complex organization, often with different roots having different functions (Waisel and Eshel, 2002). The ability of plants to produce different types of roots, called heterorhizy (Noëlle, 1910; Hishi, 2007), plays a central role in fine-tuning a root system to satisfy its multiple functions (Barlow, 1993). Using a stream-based ordering system (Pregitzer et al., 2002; Withington et al., 2006), first-order fibrous roots are typically short and fine, with diameters rarely greater than 1 mm. In addition to the ephemeral "fibrous" roots that usually live from several weeks to rarely more than 1–2 yr (Withington et al., 2006; Xia et al., 2010; McCormack et al., 2012), mature root systems usually also produce longer and thicker (1–2 mm diameter) first-order roots, which have been referred to by many names including "pioneer", "long", and "framework" roots (Kolesnikov, 1971; Lyford, 1980; Sutton and Tinus, 1983). Until recently, there have been few detailed studies of the development, structure, and function of these various root types of the same age and order.

It is generally proposed that pioneer roots play a structural function, whereas fibrous roots are responsible for the absorption of water and nutrients (Zadworny and Eissenstat, 2011). The age of roots might also influence their function. As a consequence, anatomical studies of roots of the same age are necessary to assess the functions of different types of root adequately.

Vascular development in roots is a key link between root systems growing enough absorptive surface area for water and nutrient acquisition from the soil and the transport of these materials to meet shoot demands for growth and transpiration. The formation of the vascular system during the primary development of roots depends on root order and distance from the apical meristem (Wilcox, 1962; Hishi and Takeda, 2005; Valenzuela-Estrada et al., 2008; Rost, 2011) as well as the age of the fine lateral root (Zadworny and Eissenstat, 2011). Archic structure might differ among roots from the same branching position (Hishi and Takeda, 2005; Hishi et al., 2006; Zadworny and Eissenstat, 2011). The xylem tracheary elements, which consist of elongated and expanded cells without protoplasts and with thickening of the secondary cell wall, reach maturity between 15 and 40 cm from the tip in maize roots (e.g., McCully, 1999). However, the maize roots analyzed were axial (i.e., framework)

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monocotyledonous roots, not fibrous roots. In other crop species, this distance ranges from 11 to 130 cm (McCully, 1995). As long as vessels remain alive, they are not effective in conducting water. As soon as metaxylem vessels mature, the axial hydraulic resistance within the vessel is no longer rate-limiting (Steudle and Peterson, 1998).

In woody plants, secondary growth is also a key factor to increasing transport capacity. Because the number of xylem vessels increases with root age for roots that exhibit secondary growth, mature roots have a greater capacity to transport water and nutrients along the root axis than young roots (Steudle and Peterson, 1998). However, there is no clearly defined age at which roots initiate secondary growth, particularly because in some species, fibrous first-order roots may never exhibit secondary growth (Eissenstat and Achor, 1999; Xia et al., 2010).

In addition to gross root anatomy, very little is known about the differentiation of tracheary elements and processes of xylogenesis in the roots of woody plants. Xylogenesis is a typical example of developmental programmed cell death (PCD), in which four ontogenic processes can be identified: cell origination, cell elongation and expansion, secondary cell wall deposition, and cell autolysis (Fukuda, 1996; Turner et al., 2007). It has been proposed that autophagy, as a catabolic process involving self-destruction of the cell, is responsible for PCD during xylogenesis in plants. Van Doorn and Woltering, (2005) distinguished three types of this process: microautophagy and macroautophagy (small and large portions of cytoplasm sequestrated in the vacuoles, respectively) as well as mega-autophagy as a rupture of a lysosome or tonoplast that releases hydrolytic enzymes and kills cells (Van Doorn and Woltering, 2005, 2010). Very little is known about autophagy in developmental PCD in relation to the differentiation of tracheary elements, especially in plants growing under field conditions. The stages of PCD have been studied intensively on the cytological and molecular level, for example, in in vitro suspension cell cultures to examine the formation of *Zinnia* tracheary elements from mesophyll cells (Groover et al., 1997; Fukuda, 2000; Kuriyama and Fukuda, 2000; Obara et al., 2001; Fukuda, 2004). Chaffey et al. (1997), however, have queried whether data from in vitro systems are a valid representation of natural field conditions.

The main aim of this study was to determine how and when vascular tissues develop in roots of fundamentally different functions. Because of the importance of transport functions in pioneer roots, we hypothesized that both primary and secondary xylogenesis proceeds more quickly in pioneer than fibrous roots. Our approach was to examine the differentiation and maturation (including all PCD stages) of primary xylem vessels in fibrous and pioneer roots of the same age and branching order in black cottonwood (*Populus trichocarpa*). We purposely chose to work with roots formed from well-established trees under field conditions, as roots from seedlings and adults may differ in development (Eissenstat and Achor, 1999) and function (Espeleta and Eissenstat, 1998). This is the first comparative study of daily rates of xylem tracheary elements formation within fibrous and pioneer first-order roots under any experimental condition.

MATERIALS AND METHODS

***Plant material*—**All experiments were performed on five mature trees of *Populus trichocarpa* (Torr. & Gray) (Salicaceae) growing at the experimental field site of the Institute of Dendrology, Polish Academy of Sciences in Kórnik (52°14′40″N, 17°06′27″E). The daily rate of root growth was examined under field conditions, using root boxes (rhizotrons) with a transparent window, which allow individual roots to be observed from early stages without removal and then sampled at a known age (Comas et al., 2000; Resendes et al., 2008; Zadworny and Eissenstat, 2011). In autumn 2008, five root boxes ($0.6 \times 0.5 \times 0.4$ m deep) were installed in pits that had been dug to the same size as the box and were ca. 1 m from the trunk of each tree and at least 2 m from neighboring boxes. The transparent window of each root box faced the tree. Soil from that location (for soil parameters see site 2 in Karliński et al., 2010) was used to provide good contact between the window and the surrounding soil. The observation windows (0.55×0.3 m) were made of acetate film and were insulated with a foamed polystyrene board to avoid temperature fluctuations. Windows and boxes were covered when not being used to avoid light intrusion. Observations began at least 6 mo after the boxes were installed, which allowed the soil to settle and undisturbed new roots to grow against the window. Observations were performed during two seasons (June and July 2009 and 2010). New roots that appeared in the window were traced with a black permanent marker. New root growth visible on the acetate was traced daily with different color extrafine markers each day (see Resendes et al., 2008). Individual roots were cut directly through the acetate at soil depths ranging from 5 to 30 cm. Each harvested root was immediately divided with thin double-edged razors into sections that corresponded to each day's increment of growth and analyzed separately. On the basis of root length, the roots were pooled across root boxes for statistical analyses because the root box from which the root was collected was not a significant source of variation at $P > 0.05$ (e.g., for fibrous roots total length: 1st day $F_{4,12} = 0.09$; 2nd day $F_{4,16} = 1.48$; 3rd–4th day $F_{4,43} = 1.56$; 6th–7th day $F_{4,7} = 0.51$; and for pioneer roots: 1st day $F_{4,17} = 2.06$; 2nd day $F_{4,17} = 0.05$; 3rd–4th day $F_{4,37} = 1.36$; 6th–7th day $F_{4,14} = 0.56$). The number of roots analyzed from each age category is presented in Table 1. Fibrous and pioneer roots were identified in the field based on their morphology (larger diameter of pioneer roots). Root growth observations and harvests were done when daily average air temperature was 17.9 ± 0.5°C, relative humidity was 80.4 ± 1.4%, and dew point temperature was 14 ± 0.4°C.

***Root anatomical and cytological studies*—**For histological analysis, harvested samples of fibrous and pioneer roots were fixed immediately in 2% glutaraldehyde and 2% formaldehyde in 0.05 mol/L cacodylate buffer (v/v) (pH 6.8; overnight with one change of solution; Polysciences, Warrington, USA). Samples were rinsed three times with the and dehydrated in a graded ethanol series (10–100%). They were then rinsed with butanol and finally infiltrated and embedded in Paraplast Plus (Sigma, St. Louis, Missouri, USA). Sections that were 12 μm thick were prepared with a HM 340E rotary microtome (Microm, Walldorf, Germany). The sections were double-stained with safranin O and fast green. For observations of the extent of xylem vessel formation and the degree of cell wall lignification, sections were examined with light and fluorescence microscopy (excitation = 470 nm; emission = 510 nm; Axioscope A1, Carl Zeiss, Jena, Germany). All details in relation to size and anatomical structures were studied in cross and longitudinal (radial and tangential) sections.

Samples for TEM were preserved as close as possible to their native state by fixing them (2% glutaraldehyde and 2% formaldehyde (pH 6.8) for 2 h at room temperature, then overnight at 4°C) immediately after they were harvested and the roots dissected. Segments were then rinsed three times with cacodylate buffer (0.05 mol/L, pH 6.8; Polysciences, Warrington, USA), and postfixed with 1% osmium tetraoxide (v/v in water) for 2 h at room temperature. This fixation method has been used successfully for assessing autophagic cell death in human cancer cell lines (Shao et al., 2004) and for visualizing autophagic bodies in *Arabidopsis* roots (Yoshimoto et al., 2004) and xylogenesis in poplar (Arend and Fromm, 2003).

Fixed material for TEM was counterstained with 2% uranyl acetate (w/v) and embedded in low viscosity resin using the method described by Zenkteler and Bagniewska-Zadworna (2005). Ultrathin sections (0.1 μm) were cut with a diamond knife on an ultramicrotome EM UC6 (Leica-Reichert, Bensheim, Germany) and collected on Formvar-coated copper grids. The sections were stained with uranyl acetate and lead citrate (Reynolds, 1963), then examined with a JEM 1200 EX II transmission electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 80 keV.

For cytological studies, at least five root segments from each age category were harvested. An average of three copper grids per sample was examined under electron microscopy.

***Statistical analysis*—**Data were evaluated using the Statistica PL 9.0 software (StatSoft Polska, Cracow, Poland). Values in percentages were transformed by Bliss' formula. For each root age, the measurements were averaged for each root segment. The number of roots harvested for anatomical analyses is in Table 1. The significance of differences between variables was assessed using one-way ANOVA at $P = 0.05$.

RESULTS

***Root growth rate and anatomical analysis*—**Rate of root growth varied significantly between root types. Mean daily growth of pioneer roots was 10.3 ± 0.4 mm·day^{-1}, whereas fibrous roots grew 2.7 ± 0.3 mm·day^{-1}. Roots differed also in diameters with pioneer roots having about 4-fold greater diameter than fibrous roots at a particular root age (1st day $F_{1,22}$ = 186.28, $P < 0.001$; 2nd day $F_{1,22}$ = 329.92, $P < 0.001$; 3rd–4th day $F_{1,55}$ = 769.18, $P < 0.01$; 6th–7th day $F_{1,29}$ = 296.4, $P < 0.01$) (Table 1).

Anatomical analyses were done for the first 9 d of root growth until cambial tissue was formed. The two classes of roots, pioneer and fibrous, differed distinctly in their anatomical structure with respect to the variables analyzed. Total root length (1st day $F_{1,37}$ = 32.81, $P < 0.001$; 2nd day $F_{1,41}$ = 45.63, $P < 0.001$; 3rd–4th day $F_{1,88}$ = 131.14, $P < 0.01$; 6th–7th day $F_{1,29}$ = 54.04, $P < 0.001$), stele diameter (1st day $F_{1,22}$ = 220.49, $P < 0.001$; 2nd day $F_{1,22}$ = 437.84, $P<0.001$; 3rd–4th day $F_{1,43}$ = 581.06, $P < 0.01$; 6th–7th day $F_{1,29}$ = 1763.6, $P < 0.01$), number of tracheary elements per xylem poles (3rd–4th day $F_{1,41}$ = 41.28, $P < 0.001$; 6th–7th day $F_{1,46}$ = 258.17, $P < 0.01$), and protoxylem (3rd–4th day $F_{1,33}$ = 88.36, $P < 0.001$; 6th–7th day $F_{1,45}$ = 95.82, $P < 0.01$) and metaxylem (3rd–4th day $F_{1,60}$ = 180.53, $P < 0.01$; 6th–7th day $F_{1,42}$ = 107.99, $P<0.001$) vessel diameters were much higher in pioneer roots than in fibrous roots, independently of the root age (Table 1). In addition on cytological level, protoxylem vessels of pioneer roots had thicker cell walls than those of fibrous roots at 6th–7th day (3rd–4th day $F_{1,58}$ = 1.23, $P > 0.27$; 6th–7th day $F_{1,31}$ = 12.11, $P < 0.001$); however, there was no significant differences between metaxylem cell wall thickness between roots analyzed (3rd–4th day $F_{1,66}$ = 0.68, $P > 0.43$; 6th–7th day $F_{1,42}$ = 3.15, $P > 0.08$) (Table 1).

The histological arrangement of the structure of the stele and the configuration of the vascular tissue differed distinctly in the two root types. All pioneer roots had more primary xylem poles (tetrarchic) than did fibrous roots (all diarchic) (Fig. 1). Consistent with the faster extension rate in pioneer roots, cell differentiation took place farther from the root tip than did cell differentiation in fibrous roots.

Fibrous roots—On the first day of growth, fibrous root cells started to differentiate to form vascular tissues (except meristematic tissues close to the root tip). In transverse section, the primordial tracheary elements were noticeable, mainly due to their enlarged diameter (Fig. 1a). Both protoxylem and metaxylem precursors expanded laterally. The diarchic structure was visible at this stage; however, cell walls remained thin and primary, without secondary cell wall deposition (Fig. 1a, b). On the second day of root development, the cell walls of developing tracheary elements were visibly thicker (Fig. 1c); however, secondary cell wall deposition was not accompanied by lignification. Viewed with fluorescence microscopy, sections had only a few early-differentiated vessels, which were mostly developing metaxylem, with lignin autofluorescence (Fig. 1d). During the next stages (3–7 d of growth), expansion of xylem vessels was detectable (Fig. 1e–h). Secondary xylem formation was not observed in the 11 fibrous roots examined during 9 d of growth.

Pioneer roots—On the first day of growth, only the meristematic procambial tissue was observed, regardless of distance from the root tip (Fig. 1i, j). On the second day of pioneer root growth, primordial tracheary elements were detected in a tetrarchic arrangement (Fig. 1k, l). Each of the xylem vessel precursors underwent dramatic changes in size during the second day, but cell walls were not lignified (Fig. 1l). Later in development (3–4 d of growth), four xylem poles formed (Fig. 1m). In each xylem pole, 9–11 vessels lignified cell walls were observed (Fig. 1n). On days 6–7, the xylem poles elongated and expanded laterally (Fig. 1o), and the cell walls of the vessels underwent intensive lignification (Fig. 1p). At this stage of development, the initiation of cambium formation and divisions were detected ~70 mm from the root tip in 100% of the roots examined (N = 11) (Fig. 2a), and on 9-d-old pioneer roots, secondary xylem was already visible in all of the roots examined (Fig. 2b).

TABLE 1. Mean values (±SE) for anatomical and cytological characteristics of fibrous and pioneer roots of *Populus trichocarpa*.

	1st day		2nd day		3rd–4th day		6th–7th day	
Characteristic	Fibrous	Pioneer	Fibrous	Pioneer	Fibrous	Pioneer	Fibrous	Pioneer
Root feature								
Mean root length (mm)	4.1 ± 0.5^a	10.8 ± 0.9^b	6 ± 0.8^a	19.9 ± 1.9^b	8.8 ± 0.7^a	33.9 ± 2.2^b	9.7 ± 1.4^a	53.1 ± 4.6^b
Root diameter (μm)	299.7 ± 20.3^a	1119.9 ± 56.2^b	371.8 ± 32.7^a	1667.0 ± 63.4^b	435.9 ± 22.8^a	1956.9 ± 54.8^b	519.7 ± 12.9^a	2035.3 ± 55.0^b
Archic structure	Diarchic	Tetrarchic	Diarchic	Tetrarchic	Diarchic	Tetrarchic	Diarchic	Tetrarchic
Stele diameter (μm)	84.7 ± 6.2^a	277.8 ± 11.3^b	119.1 ± 4.4^a	466.4 ± 16.0^b	129.7 ± 4.6^a	504.9 ± 13.4^b	148.2 ± 4.0^b	553.7 ± 6.7^b
Stele % in root diameter	30.0 ± 3.1^a	25.3 ± 1.4^a	31.8 ± 2.2^a	27.5 ± 0.7^a	31.2 ± 1.9^a	29.5 ± 0.4^b	29.3 ± 0.8^a	28.2 ± 0.6^a
No. of tracheary elements *	—	—	3.5 ± 0.3	—	5.8 ± 0.2^a	9.8 ± 0.6^b	12.3 ± 0.9^a	47.7 ± 2.0^b
No. of roots	17	22	21	22	48	42	12	19
Protoxylem								
Vessel diameter (μm)	—	—	—	—	4.1 ± 0.2^a	8.4 ± 0.3^b	4.1 ± 0.1^a	8.3 ± 0.4^b
Mean cell wall thickness	—	—	—	—	0.2 ± 0.0^a	0.3 ± 0.0^a	0.2 ± 0.0^a	0.4 ± 0.0^b
Cell wall thickness % in vessel diameter (μm)	—	—	—	—	4.4 ± 0.2^a	4.0 ± 0.2^a	5.8 ± 0.5^a	9.4 ± 1.1^b
Metaxylem								
Vessel diameter (μm)	—	—	—	—	8.3 ± 0.3^a	18.2 ± 0.7^b	10 ± 0.3^a	20.3 ± 1.0^b
Mean cell wall thickness	—	—	—	—	1.1 ± 0.1^a	1.1 ± 0.0^a	1.4 ± 0.1^a	1.6 ± 0.1^a
Cell wall thickness % in vessel diameter (μm)	—	—	—	—	13.1 ± 0.7^a	6.1 ± 0.2^b	13.8 ± 0.8^a	7.5 ± 0.3^b

Notes: * with secondary cell wall thickening counted per xylem pole; —: no measurements taken. Superscripts a and b indicate significant differences at P = 0.05 within fibrous and pioneer roots segments of the same age.

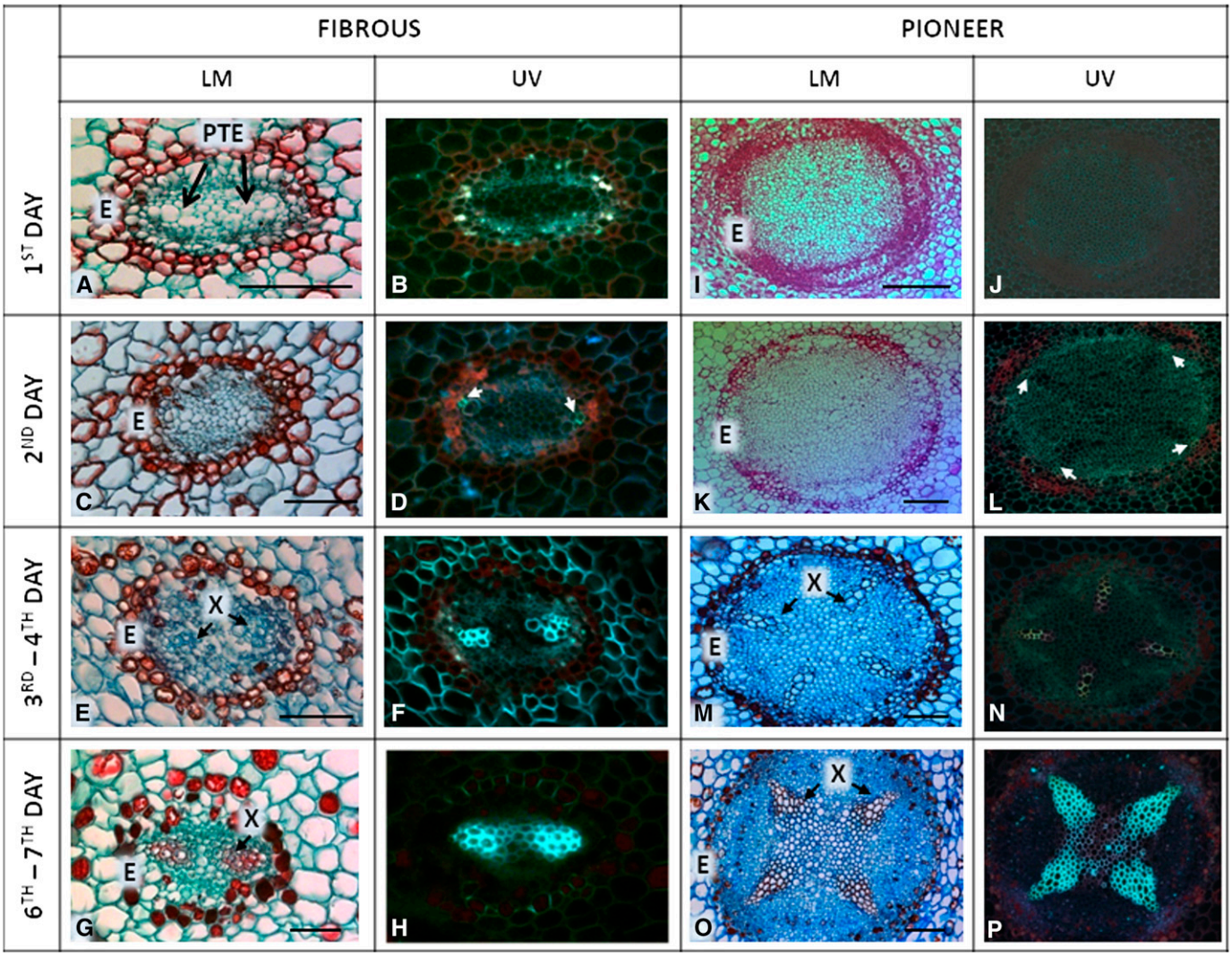

Fig. 1. Comparative anatomy of *Populus trichocarpa* fibrous and pioneer roots of different ages viewed with light (LM) and fluorescence (UV) microscopy. Note the lack of xylem differentiation in pioneer roots on first day of their growth (J). The first lignified vessels, visible under UV, are marked by white arrows in (D) and (L). Scale bars (A–H) = 50 μm; (I–P) = 100 μm. *Abbreviations:* E, endodermis; PTE, primordial tracheary elements; X, xylem.

***Cytological analysis of xylogenesis*—**For transmission electron microscopy, both fibrous and pioneer roots segments were sampled during the first 7 d of their development. Primordial tracheary elements were firstly detected starting 1–2 mm from the tip of fibrous roots and were clearly visible at 15–20 mm from the tip of pioneer roots, which corresponded to the first and second day of development, respectively (Fig. 3a, b). These cell types were identified easily on the basis of their elongation and expansion compared with neighboring cells. Moreover, the primordial tracheary elements were highly vacuolated. In the fibrous roots on the first day of growth, autophagic-like structures inside vacuoles were detected (Fig. 3a). Only living xylem vessels (Fig. 3c, d), with protoplasts still remaining inside, were detected in fibrous roots ~5 mm from the root tip, and at ~20 mm in pioneer roots. At this stage, apposition of the secondary cell wall was visible as cell wall thickening was evident mainly in fibrous roots. Nonliving xylem vessels, usually without end walls, which would be associated with much higher axial conductivity (Fig. 3e; minimum three per xylem pole), were observed on days 3–4 in both fibrous (>8 mm from the root tip) and pioneer roots (>30 mm). The summary of the early xylogenesis events is presented on Fig. 4.

PCD processes during xylogenesis—Xylogenesis progressed in a similar manner in fibrous (Fig. 5) and pioneer (Fig. 6) roots. However, the timing and distance of xylogenesis from the root tip varied between the two root types. During the first stage of xylogenesis, cells were elongated and expanded. Vacuoles were usually small and electron-lucent, and nuclei and other organelles did not display any changes in their structure. Shortly afterward, uniformly distributed cisternae of rough endoplasmic reticulum (Figs. 5a, 6a) and fully formed mitochondria were also visible. In many cells, the reticulum cisternae were swollen slightly (Fig. 6a) and sometimes formed elongated or irregularly shaped vesicles (Fig. 6b). Golgi bodies that contained many dictyosomes, each of which comprised several cisternae (Figs. 5b, 5c, 6b), and Golgi-derived vesicles were conspicuous in the protoplast, as well as close to the cell walls. This pattern was observed mostly during the first stage of secondary wall deposition. The Golgi often formed vesicles that were larger

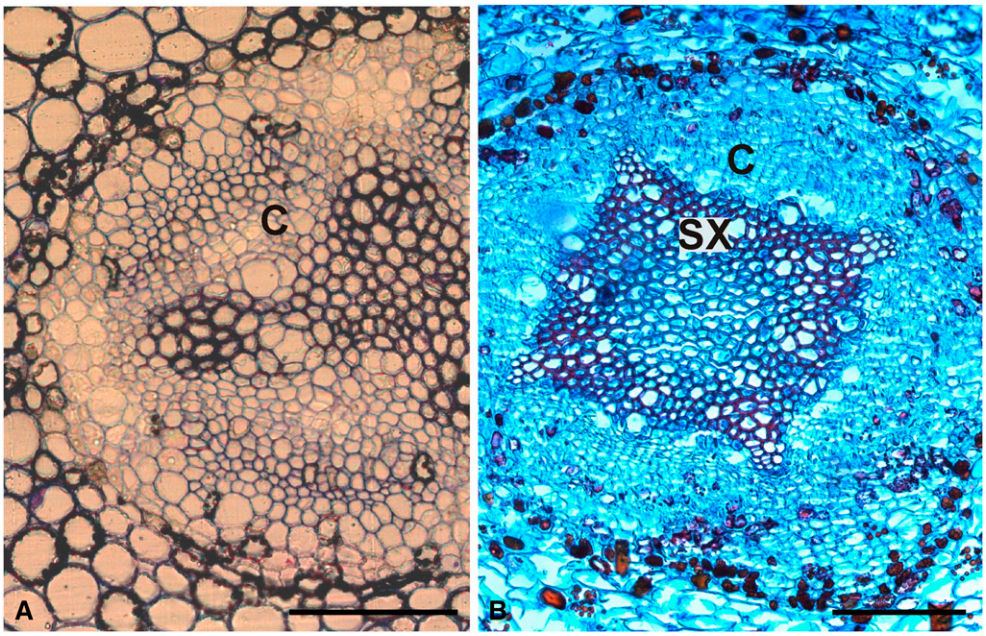

Fig. 2. Secondary xylem formation of pioneer roots of *Populus trichocarpa*. Note cambium activation in 7-d-old roots (A) and secondary xylem development in 9-d-old roots (B). Scale bars = 100 μm. *Abbreviations:* C, cambium; SX, secondary xylem.

and varied in shape and size, and these larger vesicles were observed to have transported matrix substances (Fig. 5c, d) to the cell wall. Frequently, fusion of the vesicles with the plasma membrane could also be detected and resulted in the release of matrix substances toward the outer walls and their incorporation into the cell wall (Fig. 6c, g). Subsequently, several alterations were noted in other organelles. In many cells, large portions of cytoplasm including small organelles, formed autophagosomes with double membranes derived from endoplasmic reticulum (Figs. 5e, 5f, 6d), and consequently, after fusion of their extended membrane with tonoplast, they were sequestered in the vacuole. Frequently vacuoles that contained several autophagic-like bodies were detected (Figs. 5g, 5h, 6f). Interestingly, larger vesicles were also transported and merged with vacuoles (Fig. 6g, h). At this stage, vacuoles were usually enlarged, located centrally in the cell, and occupied almost the entire cell (Fig. 5i, 6i). In longitudinal sections (not shown), a few large vacuoles were noted. The remaining organelles were positioned along the cell wall. The characteristic features that were observed at this point were complete disintegration of the organelles and the presence of vesicles, which were sometimes cracked and varied in size, shape, and number (Fig. 5j). The last step in xylogenesis was the occurrence of fully mature, dead xylem vessels in both fibrous (Fig. 5k) and pioneer roots (Fig. 6j) by day 3 or 4.

DISCUSSION

Root systems are necessary not only for water and nutrient acquisition, but also for the rapid transport of these materials to meet shoot functions. The development of primary root tissues in the first days of a root's life is poorly understood, especially under field conditions. By examining daily development of tracheary elements in fibrous and pioneer first-order roots, we were able to uncover all stages of early vessels formation, indicating that even such distinctly different roots as fibrous and pioneer performed primary xylogenesis in a similar way and have functionality at the same age. The key difference in these roots seems to be in secondary vascular development, which occurs in little over a week in pioneer roots and was not observed in any of the fibrous roots. The lack of secondary development in fibrous roots in consistent with studies of a much longer duration (Xia et al., 2010; Zadworny and Eissenstat, 2011). Our work underscores the importance of secondary growth in pioneer roots to meet the transport demands of the developing root system.

On the first day of development, we found that the anatomy of the roots already varied, which indicated differences in the rate of xylem vessels differentiation. The beginning of the primordial tracheary elements formation was detected earlier and much closer to the root tip (first day of growth) in fibrous than in pioneer roots. Tracheary elements of pioneer roots started to differentiate on the second day of growth and at greater distances from the root tip than in fibrous roots. However, in pioneer roots, the tracheary elements matured quickly, which suggested that total length of an individual root cannot be treated as the sole criterion for the stage of root development. Contrasting root development in different root types, we found that even if xylogenesis occurred at different rates (slowly for fibrous and quickly for pioneer roots), both types developed functional xylem vessels at 3 to 4 d after their emergence.

Indeed, to properly characterize xylogenesis in two types of roots in black cottonwood, which was the main object of our study, we needed to specify the PCD events during xylogenesis. Programmed cell death is an active process that occurs at the end of tissue differentiation in some cell types, e.g., mature xylem vessels, and is programmed genetically. The best recognized study system is the transdifferentiation of the *Zinnia elegans* (Jacq.) suspension cell cultures, in which mesophyll cells are induced to differentiate into tracheary elements (Fukuda, 2000, 2004; Kuriyama and Fukuda, 2000). For this type of

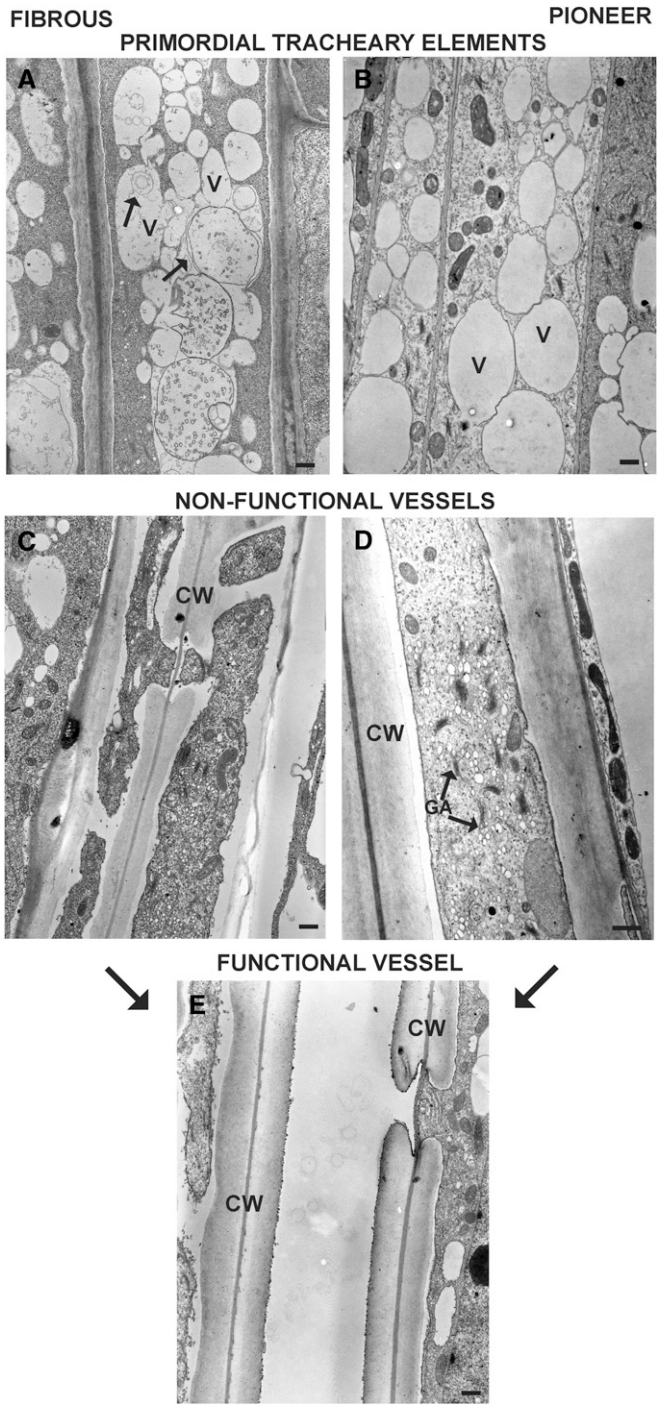

Fig. 3. Xylogenesis in fibrous and pioneer roots. (A, B) Primordial tracheary elements. (C, D) Non-functional xylem vessels. (E) Functional vessels, identical for both root types. Note autophagic-like bodies inside vacuoles of the primordial tracheary elements in fibrous roots (arrows). Scale bars = 1 µm. *Abbreviations:* GA, Golgi apparatus dictyosomes; V, vacuole; CW, cell wall.

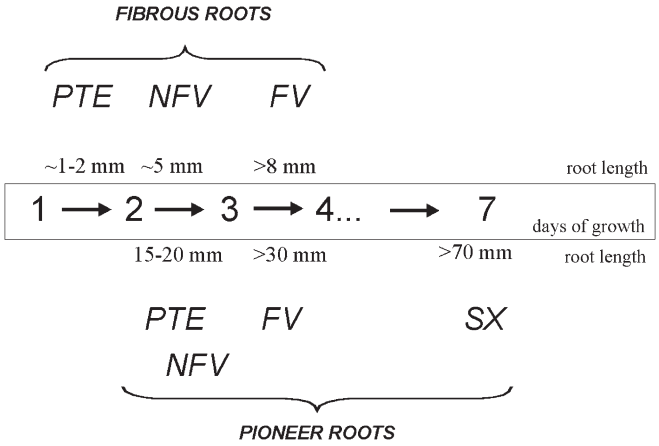

Fig. 4. The early events of xylogenesis in fibrous and pioneer roots of *Populus trichocarpa*. *Abbreviations:* FV, conductive (functional vessels: minimum 3 per xylem pole); NFV, nonfunctional vessels; PTE, primordial tracheary elements; SX, secondary xylem.

PCD, several stages were noted on the structural level: secondary cell wall formation, vacuole tonoplast rupture, lack of cytoplasmic circulation, changes in endoplasmic reticulum structure, and finally complete disintegration of the protoplast (Groover et al., 1997; Fukuda, 2000; Obara et al., 2001). According to the latest nomenclature, suggested by Van Doorn and Woltering (2005, 2010), a typical example of mega-autophagy with collapse of the central vacuole was noted in *Zinnia*; however, it occurred several hours after formation of the secondary cell wall (Fukuda, 1996). While detailed studies of PCD during aboveground tissue differentiation, mainly during secondary wood development of the poplar shoot, has been reported by Arend and Fromm (2003), Moreau et al. (2005) and Courtois-Moreau et al. (2009), very little is known about xylogenesis in the tree roots, and to our knowledge, nothing is known about this process under field conditions. It is of special importance, since root developmental processes under field conditions may differ dramatically from those formed in artificial laboratory cultures (McCully, 1999).

In the field study described here, the xylogenesis was gradual: first stage involved cell elongation and expansion, followed by formation of the secondary cell wall. During these first stages of xylem vessel development, lignification did not occur. Lignified cell walls were observed later throughout the next days of growth. This observation corroborated the results of other investigations of developing primary xylem tissue, in which cell walls remained unlignified (Wardrop, 1981). At this stage, the role of both the Golgi apparatus and the endoplasmic reticulum seem to be crucial. In our study, numerous Golgi-derived vesicles were observed along cell walls and were probably involved in the synthesis and delivery of matrix substances to the developing secondary cell walls. Cisternae of rough endoplasmic reticulum with swollen ends were also visible, and shortly afterward, the ends detached and formed larger deposits, which likely fused subsequently with vacuoles. Similarly, an increase in vesicles was reported by Arend and Fromm (2003) in poplar stem tissues during secondary xylem formation, and the extension of the endoplasmic reticulum was also observed in the *Zinnia* in vitro transdifferentiation system (Groover et al., 1997; Obara et al., 2001). There is also strong evidence that endoplasmic reticulum could be crucial both in signaling and autophagy (Cacas, 2010; Klionsky and Emr, 2000; Van Doorn and Woltering, 2005). It is probably involved in the formation of preautophagic structures, which form autophagosomes after the end membranes close. The other crucial role of the endoplasmic reticulum in PCD is the transportation of hydrolytic enzymes to

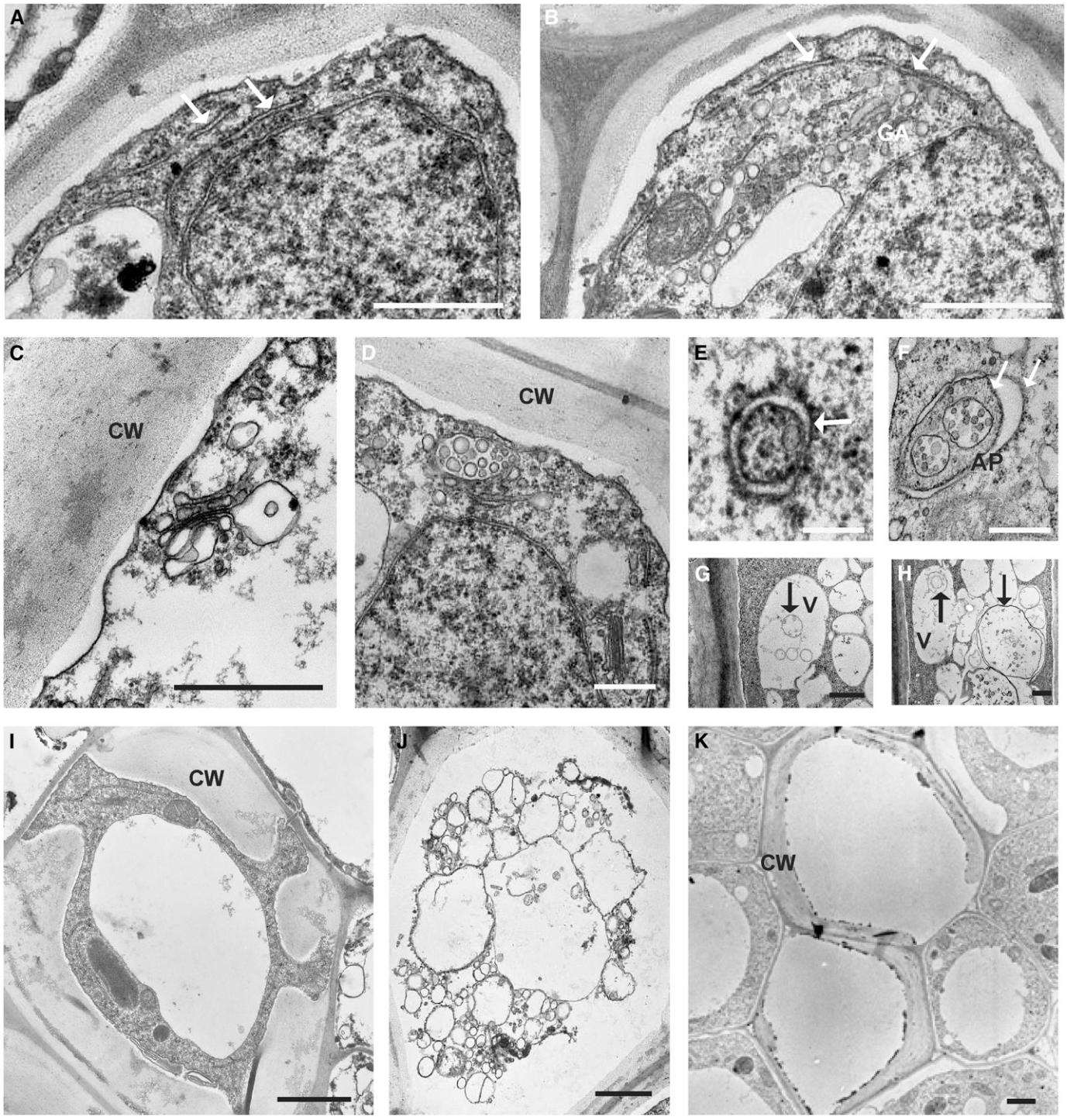

Fig. 5. Stages of programmed cell death during xylogenesis of fibrous roots of *Populus trichocarpa*. (A, B) Endoplasmic reticulum (white arrows) and accumulation of Golgi apparatus close to the cell walls. (C, D) Note the formation of Golgi-derived vesicles, which contain matrix substances for secondary cell wall formation. (E, F) Formation of (E) preautophagic structures derived from endoplasmic reticulum (white arrow) and autophagosomes. Note double membrane of the autophagosome structure (F, white arrows). (G, H) Autophagic bodies (black arrows) inside vacuoles. (I) Enlargement of the central vacuole followed by tonoplast rupture. (J) Organellar disintegration. (K) Functional mature xylem vessels in cross section. Scale bars: (A-C, E) = 200 nm, (D, F) = 500 nm, G-K = 1 μm. *Abbreviations:* AP, autophagosome; CW, cell wall; GA, Golgi apparatus dictyosomes; N, nucleus; V, vacuole.

the vacuole and their subsequent accumulation there (Müntz, 2007; Piszczek and Gutman, 2007).

Using images from transmission electron microscopy, we confirmed that in black cottonwood roots, both micro- and macroautophagy occur at the beginning of tracheary elements formation. Mega-autophagy was detected as the last, probably very quick, step of cell death after the formation of the secondary cell wall was completed, and it was an irreversible, definitive

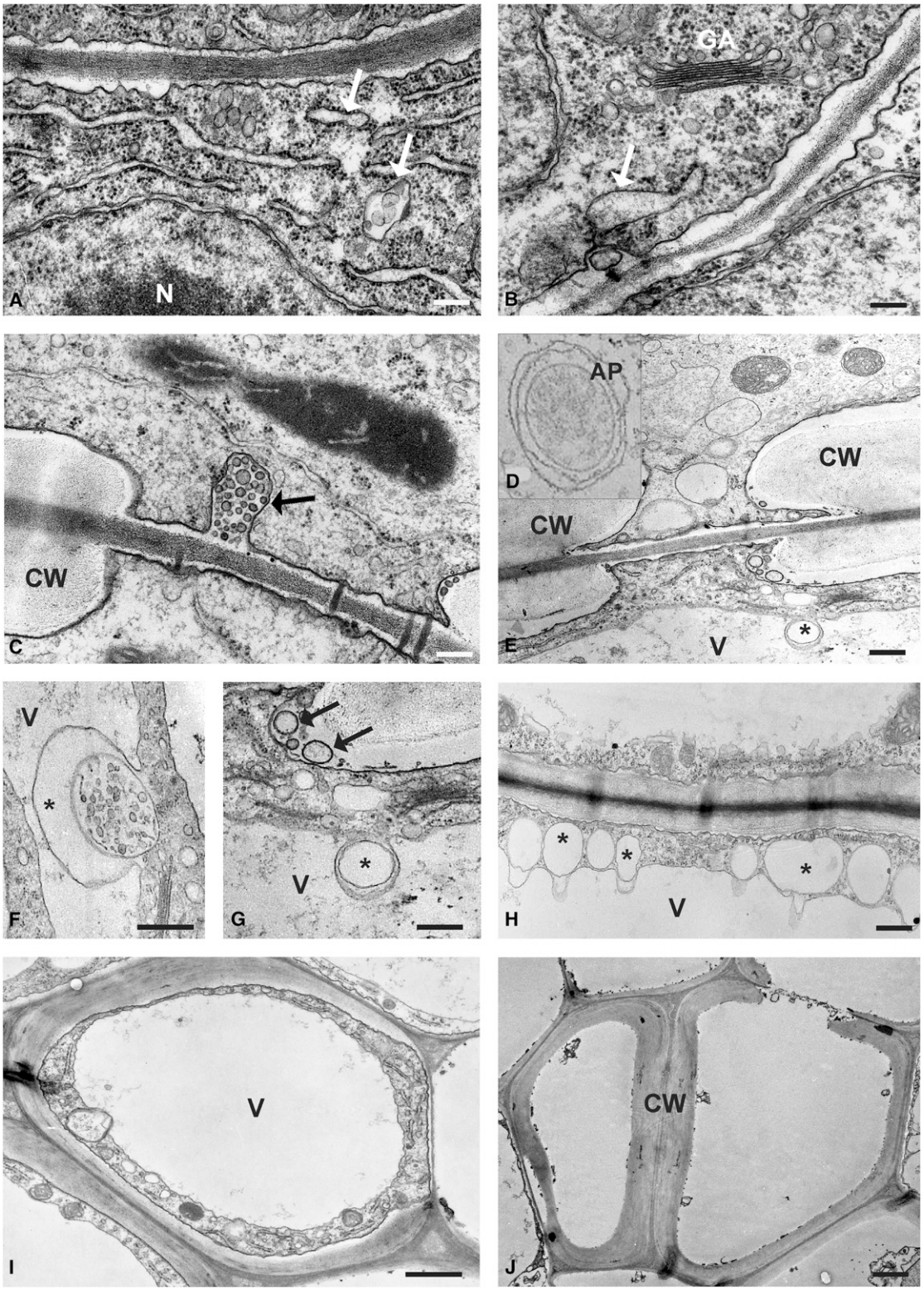

Fig. 6. Programmed cell death events during xylogenesis of pioneer roots. (A, B) Swollen cisternae of endoplasmic reticulum forming irregular elongated vesicles (white arrows). (C) Golgi-derived vesicles, which contain cell wall matrix substances (black arrow), and fusion with the plasma membrane. (D) Autophagosome formation in the cytoplasm (note the double membrane, derived from ER cisternae). (E-H) Enlargement of the central vacuole, with tonoplast invagination and formation of autophagic bodies (asterisks). (G) Note the cell wall matrix substances embedded in the formatting cell wall thickening (black arrows). (G, H) Vesicles with electron-lucent content merged with central vacuole. (I) Large, central vacuole, which occupied most of the lumen of the developing xylem vessel. (J) Functional, mature xylem vessels in cross section. Scale bars: (A-G) = 500 nm, (H-J) = 1 μm. *Abbreviations:* AP, autophagosome; CW, cell wall; N, nucleus; GA, Golgi apparatus dictyosomes; V, vacuole.

step toward cell death. Van Doorn and Woltering (2005) suggested that the three types of autophagy cannot be mutually exclusive. However, in published reports, these types of PCD are usually separated. Courtois-Moreau et al. (2009) has indicated that, in poplar stems, PCD of xylem fibers is gradual and results in complete loss of the cytoplasmic contents well before the loss of vacuolar integrity, which is considered to be the moment of death. In this case, mega-autophagy plays a role in cell death and the recycling of cell material, suggesting that other types of autophagy could occur in developmental PCD. Cytological observations of degenerating intergeneric embryos (Bagniewska-Zadworna et al., 2010) revealed that different types of autophagy occurred at the same time, although two different types of vacuolization of embryo cells were noticed, resulting in different modes of PCD.

For the poplar roots examined in this study, we postulate that the mega-autophagy as the last step in PCD, quickly followed by disappearance of vessel element end walls, enabled xylem cells to effectively conduct water. Given that plants are equipped with such an irreversible mechanism, cells can activate this mechanism if necessary. Although this mechanism appeared to happen more quickly in pioneer roots, it also started at a later age, causing vessels to become functional at a similar age in pioneer and fibrous roots.

In contrast to pioneer roots, where cambium formation and initiation of secondary development was observed after 7–9 d, fibrous roots were unable to form secondary growth in this time frame. Our observation of 21-d-old fibrous roots also did not show any symptoms of secondary growth (data not shown). Others have also failed to observe secondary growth of 1st-order fibrous roots in woody plants (Eissenstat and Achor, 1999; Valenzuela-Estrada et al., 2008; Xia et al., 2010; Zadworny and Eissenstat, 2011). This lack could be explained by the ephemeral character of lower order fibrous roots, which, to have a living cortex for nutrient absorption and support of mycorrhizal fungi, must restrict secondary vascular development.

The anatomy and cytology of xylem elements can strongly influence the function of individual roots. Compared to the case in fibrous roots, the larger vessel diameters in pioneer roots both in proto- and metaxylem should lead to higher vessel conductance (Steudle and Peterson, 1998), and the thicker cell walls in protoxylem elements should allow for greater resistance to collapse from high tensions. A pioneer root (6–7-d-old) had almost 4-fold more vessels per xylem pole and twice the number of poles on average than a fibrous root of the same age. Calculating the average of each radius of the vessel raised to the fourth power (Steudle and Peterson, 1998; Valenzuela-Estrada et al., 2009) and taking the average, pioneer roots had a 2-fold greater vessel radius for conduction per one xylem pole. Thus, the overall root hydraulic conductance of the primary xylem of pioneer roots was ~135-fold greater than that of fibrous roots. The magnitude of differences in conductivity of the primary xylem suggests that the transport bottleneck of developing pioneer roots is avoided by both the greater investment in the number and diameter of vessels in the primary xylem and the rapid development of the secondary vascular tissue. Thus, by the time fibrous roots begin emerging from a pioneer root, the vascular system is already in place to enable transport from the increased influx of water and nutrients.

In summary, the anatomical and cytological data obtained in this study advance our understanding of how trees increase their exploration of soil resources through heterorhizy of first-order roots. Differences in histogenesis are consistent with the view that the main functions of pioneer roots are soil exploration and assurance of long-distance, rapid transport of water and nutrients as well as provision of a framework for the whole root system. In contrast, the primary function of fibrous roots appears to be the absorption of nutrients and water. Specialization of root development enables plants to respond to their environment and modify absorption and transport functions through selective formation of pioneer and fibrous roots.

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