

# Tyloses and Phenolic Deposits in Xylem Vessels Impede Water Transport in Low-Lignin Transgenic Poplars: A Study by Cryo-Fluorescence Microscopy<sup>1[W][OA]</sup>

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Of 14 transgenic poplar genotypes (*Populus tremula* × *Populus alba*) with antisense 4-coumarate:coenzyme A ligase that were grown in the field for 2 years, five that had substantial lignin reductions also had greatly reduced xylem-specific conductivity compared with that of control trees and those transgenic events with small reductions in lignin. For the two events with the lowest xylem lignin contents (greater than 40% reduction), we used light microscopy methods and acid fuchsin dye ascent studies to clarify what caused their reduced transport efficiency. A novel protocol involving dye stabilization and cryo-fluorescence microscopy enabled us to visualize the dye at the cellular level and to identify water-conducting pathways in the xylem. Cryo-fixed branch segments were planed in the frozen state on a sliding cryo-microtome and observed with an epifluorescence microscope equipped with a cryo-stage. We could then distinguish clearly between phenolic-occluded vessels, conductive (stain-filled) vessels, and nonconductive (water- or gas-filled) vessels. Low-lignin trees contained areas of nonconductive, brown xylem with patches of collapsed cells and patches of noncollapsed cells filled with phenolics. In contrast, phenolics and nonconductive vessels were rarely observed in normal colored wood of the low-lignin events. The results of cryo-fluorescence light microscopy were supported by observations with a confocal microscope after freeze drying of cryo-planed samples. Moreover, after extraction of the phenolics, confocal microscopy revealed that many of the vessels in the nonconductive xylem were blocked with tyloses. We conclude that reduced transport efficiency of the transgenic low-lignin xylem was largely caused by blockages from tyloses and phenolic deposits within vessels rather than by xylem collapse.

Secondary xylem in woody plants is a complex vascular tissue that functions in mechanical support, conduction, storage, and protection (Carlquist, 2001; Tyree and Zimmermann, 2002). The xylem must provide a sufficient and safe water supply throughout the entire pathway from roots to leaves for transpiration and photosynthesis. It is well established that enhanced water conductivity of xylem can increase total plant carbon gain (Domec and Gartner, 2003; Santiago et al., 2004; Brodribb and Holbrook, 2005a). According to the Hagen-Poiseuille equation, xylem conductivity

should scale with vessel lumen diameter to the fourth power (Tyree and Zimmermann, 2002). Indeed, xylem conductivity largely depends on anatomical features, including conduit diameters and frequencies (Salleo et al., 1985; McCulloh and Sperry, 2005). However, there are hydraulic limits to maximum vessel diameters, because xylem conduits have to withstand the strong negative pressures of the transpiration stream that could cause cell collapse or embolisms within vessels that are structurally inadequate to withstand these forces (Tyree and Sperry, 1989; Lo Gullo et al., 1995; Hacke et al., 2000). To some extent, stomatal regulation of transpiration limits the negative pressures that the xylem experiences (Tardieu and Davies, 1993; Cochard et al., 2002; Meinzer, 2002; Brodribb and Holbrook, 2004; Buckley, 2005; Franks et al., 2007; Woodruff et al., 2007). Nevertheless, plants rely on an array of structural reinforcements of xylem to ensure the safety of water transport. The size of xylem elements, vessel redundancy, intervessel pit and membrane geometries, and the thickness, microstructure, and chemical composition of cell walls are among the features that regulate tradeoffs between efficiency and safety of xylem water transport (Baas and Schweingruber, 1987; Hacke et al., 2001; Domec et al., 2006; Ewers et al., 2007; Choat et al., 2008).

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The xylem cell wall is made up of cellulose bundles that are hydrogen bonded with hemicelluloses, which are in turn embedded within a lignin matrix (Mansfield, 2009; Salmén and Burgert, 2009). Besides providing this matrix for the cell wall itself, lignin is thought to contribute to many of the mechanical and physical characteristics of wood as well as conferring passive resistance to the spread of pathogens within a plant (Niklas, 1992; Boyce et al., 2004; Davin et al., 2008). Lignin typically represents 20% to 30% of the dry mass of wood and therefore is among the most abundant stores of carbon in the biosphere (Zobel and van Buijtenen, 1989). The complex molecular structure and biosynthetic pathway of various types of lignins have been studied extensively (Boerjan et al., 2003; Ralph et al., 2004, 2007; Higuchi, 2006; Boudet, 2007; Davin et al., 2008). The monomeric composition of lignin varies between different cell types of the same species depending on the functional specialization of the cell (Yoshinaga et al., 1992; Watanabe et al., 2004; Xu et al., 2006). The composition and amount of lignin in wild plants varies in response to climatic conditions (Donaldson, 2002) or gravitational and mechanical demands (Pruyn et al., 2000; Kern et al., 2005; Rüggeberg et al., 2008). It is clear that plants are capable of regulating the lignification pattern in differentiating cells, which provides them with flexibility for responding to environmental stresses (Donaldson, 2002; Koehler and Telewski, 2006; Ralph et al., 2007; for review, see Vanholme et al., 2008).

Whereas some level of lignin is a requisite for all vascular plants, it is often an unwanted product in the pulp and paper industry because it increases the costs of paper production and associated water treatments necessary for environmental protection (Chen et al., 2001; Baucher et al., 2003; Peter et al., 2007). Reducing the lignin content of the raw biomass material may allow more efficient hydrolysis of polysaccharides in biomass and thus facilitate the production of biofuel (Chen and Dixon, 2007). With the ultimate goal of development of wood for more efficient processing, much research has been aimed at the production of genetically modified trees with altered lignin biosynthesis (Boerjan et al., 2003; Boudet et al., 2003; Li et al., 2003; Halpin, 2004; Ralph et al., 2004, 2008; Chiang, 2006; Coleman et al., 2008a, 2008b; Vanholme et al., 2008; Wagner et al., 2009). It is now technically possible to achieve more than 50% reductions of lignin content in xylem of poplar (*Populus* spp.; Leplé et al., 2007; Coleman et al., 2008a, 2008b), but the consequences of such reduction on plant function have received relatively little attention (Koehler and Telewski, 2006). In-depth studies on the xylem structure and functional performance of transgenic plants with low lignin are limited, despite the need to assess their long-term sustainability for large-scale production (Anterola and Lewis, 2002; Hancock et al., 2007; Coleman et al., 2008b; Voelker, 2009; Horvath et al., 2010).

Genetically modified plants are suitable models for studying fundamental questions of the physiological

role of lignin because of the possibility of controlling lignification without the confounding effects encountered when comparing across plant tissues or stages of development (Koehler and Telewski, 2006; Leplé et al., 2007; Coleman et al., 2008b). Research on *Arabidopsis* (*Arabidopsis thaliana*) and tobacco (*Nicotiana tabacum*) has shown that down-regulation of lignin biosynthesis can have diverse effects on plant metabolism and structure, including changes in the lignin amount and composition (*p*-hydroxyphenyl/guaiacyl/syringyl units ratio) as well as the collapse of xylem vessel elements (Lee et al., 1997; Sewalt et al., 1997; Piquemal et al., 1998; Chabannes et al., 2001; Jones et al., 2001; Franke et al., 2002; Dauwe et al., 2007). Among temperate hardwoods, poplar has been established as a model tree for genetic manipulations because of its ecological and economic importance, fast growth, ease of vegetative propagation, and its widespread use in traditional breeding programs (Bradshaw et al., 2001; Brunner et al., 2004). The question of how manipulation of lignin can affect the anatomy and physiological function of xylem in poplar has been addressed in part by several research groups (Anterola and Lewis, 2002; Boerjan et al., 2003; Leplé et al., 2007; Coleman et al., 2008b). Some studies that involved large lignin reductions reported no significant alterations in the xylem anatomy (Hu et al., 1999; Li et al., 2003). However, in many other experiments, reduced total lignin content was associated with significant growth retardation, alterations in the lignin monomer composition, irregularities in the xylem structure (Anterola and Lewis, 2002; Leplé et al., 2007; Coleman et al., 2008b), and the patchy occurrence of collapsed xylem cells (Coleman et al., 2008b; Voelker, 2009). Furthermore, severely down-regulated lignin biosynthesis has resulted in greatly reduced xylem water-transport efficiency (Coleman et al., 2008b; Lachenbruch et al., 2009; Voelker, 2009). It is generally assumed that the reduced water transport ability of xylem with very low lignin contents is caused by collapsed conduits and/or increased embolism due to the entry of air bubbles into the water-conducting cells (Coleman et al., 2008b; Wagner et al., 2009), but detailed anatomical investigations of the causes of impaired xylem conductivity of low-lignin trees are lacking. Analysis of the anatomical basis for the properties of xylem conduits in plants with genetically manipulated amounts and composition of lignin can provide a deeper understanding of the physiological role of lignin as well as the lower limit of down-regulation of lignin biosynthesis at which trees can still survive within natural environments.

One of the approaches for the suppression of lignin biosynthesis is down-regulation of 4-coumarate:coenzyme A ligase (*4CL*), an enzyme that functions in phenylpropanoid metabolism by producing the monolignol precursor *p*-coumaroyl-CoA (Kajita et al., 1997; Allina et al., 1998; Hu et al., 1998; Harding et al., 2002; Jia et al., 2004; Costa et al., 2005; Friedmann et al., 2007; Wagner et al., 2009). In a 2-year-long field trial on the physiological performance of poplar (*Populus tremula* ×

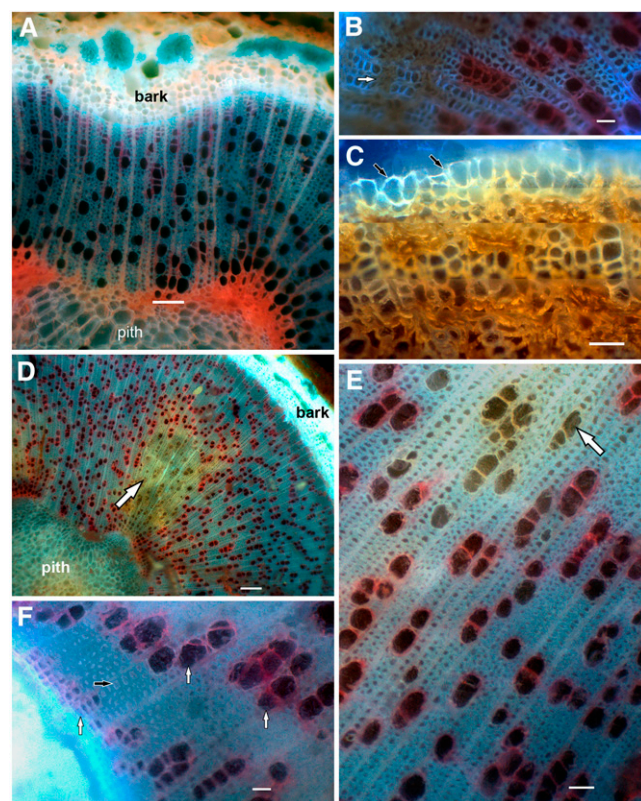
*Populus alba*) transgenic clones, out of 14 genotypes with altered lignin biosynthesis (down-regulated *4CL*), five showed dramatically reduced wood-specific conductivity ( $k_s$ ) compared with that of control trees (Voelker, 2009). Those mutants with the severely reduced  $k_s$  were also characterized by having the lowest wood lignin contents (up to an approximately 40% reduction) in the study. Trees with transgenic events characterized by the formation of abnormally brown wood exhibited regular branch dieback at the end of the growing season, despite having been regularly watered (Voelker, 2009). Our objective was to identify the structural features responsible for reduced transport efficiency in the xylem of transgenic poplars with extremely low lignin contents. We employed fluorescence and laser scanning confocal microscopy for anatomical analyses of xylem structure as well as dye-flow experiments followed by cryo-fluorescence microscopy to visualize the functioning water-conductive pathways in xylem at the cellular level. We report the frequent occurrence of tyloses and phenolic depositions in xylem vessels of strongly down-regulated trees that may be the cause of their reduced xylem conductivity.

## RESULTS

### General Xylem Anatomy and Active Water Transport Pathways

#### *Method of Detection of the Active Water Conduction Pathways*

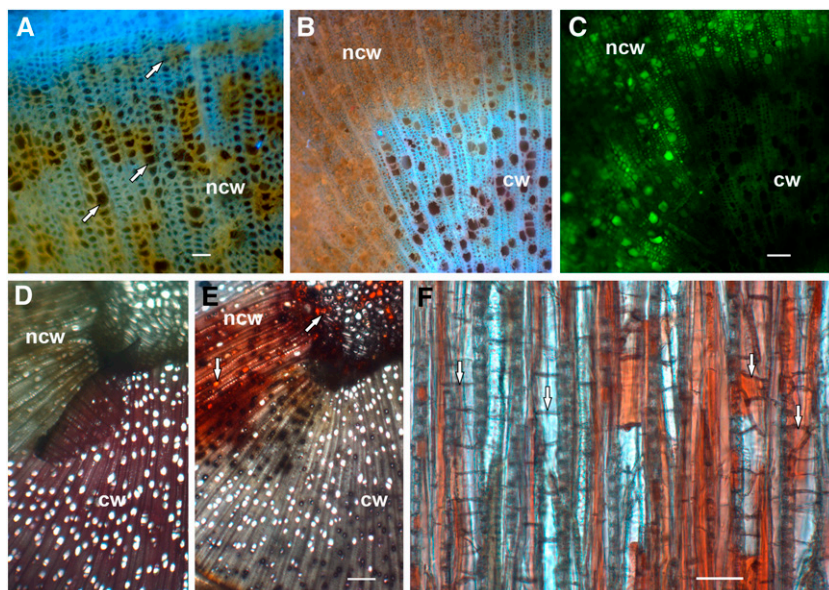
The introduction of acid fuchsin solution in the transpiration stream followed by cryo-fixation clearly revealed conductive and nonconductive portions of xylem. By cryo-fluorescence microscopy, the cell walls of conductive vessels were stained red by the acid fuchsin solution, whereas fluorescence was not detected in the frozen solution of this dye within the vessel lumens (Fig. 1). The lumens of nonconductive vessels were filled with a frozen substance that appeared with a faint yellow color because of a weak fluorescence or reflectance (Figs. 1E and 2A). Therefore, in addition to the absence of acid fuchsin-stained walls, nonconductive wood could be distinguished by the yellowish fluorescence of cell walls and cell contents (Fig. 1, D and E). These yellowish or brown fluorescence patches corresponded to the abnormal brown wood areas seen on cross sections with the naked eye, whose presence and extent had been characterized earlier in the same transgenic clones (Voelker et al., 2010). However, small numbers of nonconducting vessels were also detected outside of the brown wood areas (Supplemental Movie S1). By cryo-fluorescence microscopy, ice in fibers had no fluorescence but appeared whitish because of light reflectance (Fig. 1F). In contrast, a lumen-filling substance, which was later determined to be phenolics, remained within the fibers and vessels of the nonconductive wood after thawing or freeze drying of the samples (Fig. 2, B and



**Figure 1.** Xylem anatomy of branch cross sections of a control and low-lignin hybrid poplar trees. Samples were first perfused with acid fuchsin, frozen in liquid nitrogen, cryo-planed, and then visualized by cryo-fluorescence. Cell walls of conducting vessels are stained in red. A, Control, general view. B, Sample 712-2 showing an outer part of the xylem growth ring. Nonconductive vessels are brown and are partly or entirely collapsed (arrow). C, Magnified area of sample 712-2 showing collapsed vessel elements in the outer region of the growth ring. Stitched images were taken at different focal planes. The cambium side is at the upper side of the image. The outermost layers of xylem cells have thinner secondary walls and are incompletely differentiated (arrows). D, Sample 712-5. The arrow points to nonconductive wood. E, An enlarged view of sample 712-5 showing conductive vessels (red) and nonconductive vessels (yellowish-brown; arrow). All vessels are filled with frozen dye solution or phenolics. F, An enlarged area from D showing vessels (white arrows) at the outer region of the xylem ring filled with frozen dye solution. All fibers are filled with ice (black arrow). Bars = 100  $\mu\text{m}$  (A), 25  $\mu\text{m}$  (B, C, and F), 200  $\mu\text{m}$  (D), and 50  $\mu\text{m}$  (E).

C). In addition to cryo-fluorescence microscopy, freeze drying of cryo-planed samples in which the dye had been fixed within vessels also showed a clear distinction between conductive and nonconductive portions of the wood (Figs. 2, B and C, and 3). There was no difference between the frozen and freeze-dried samples with regard to the fixation of the dye tracer; both of the methods could be used for the detection of active conduction pathways in xylem (Figs. 1 and 3). Furthermore, in freeze-dried samples, the phenolic substance was observed in cell lumens of nonconductive xylem, clearly distinguishing it from conductive xylem (Figs. 2, B and C, and 3, C–E; see also three-dimensional [3D] movies in Supplemental Movies S1–S4).





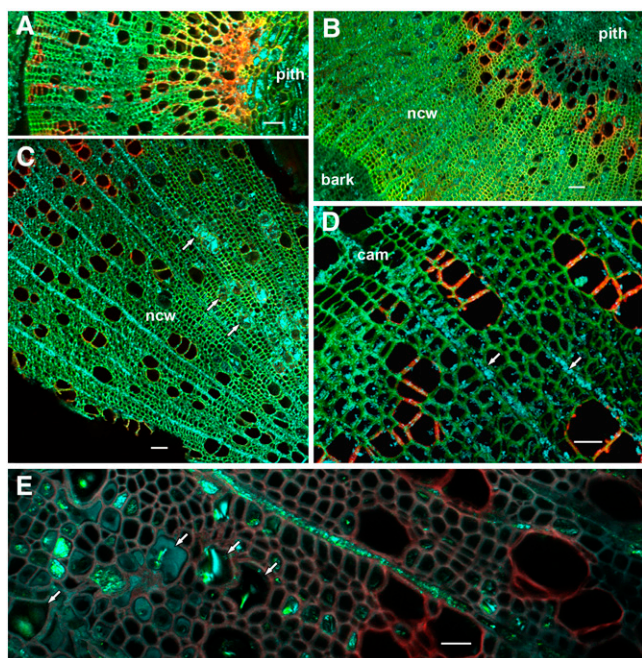
**Figure 2.** Xylem anatomy of branches of low-lignin hybrid poplar (sample 671-10) by various light microscopy methods. A to C, Cryo-planed cross sections showing conductive wood (cw) and nonconductive wood (ncw). D to F, Detection of phenolics by the vanillin-HCl test and bright-field microscopy. A, Frozen sample visualized by cryo-fluorescence (LP 420) showing wood at the outer part of the xylem ring and adjacent to the cambium. The direction of cambium is toward the top of the image. The vessels (arrows) are filled with ice or phenolics and have brown walls (weak fluorescence in the green and red spectra), whereas the walls of fibers have stronger fluorescence in the blue spectrum and appear blue or yellowish. The vessels are not collapsed compared with those in the outer areas of wood in sample 712-2 (Fig. 1, B and C) but also contain no dye and hence are not actively conducting. B, Nonconductive (brown) and conductive (blue fibers and red vessels) wood visualized by wide-field fluorescence after freeze drying (LP 420). C, The same area as in B but visualized with BP 510 to 530. The condensed phenolics strongly fluoresce in the green spectrum. D, A sample perfused with acid fuchsin solution showing conductive wood (red) and nonconductive wood (gray or brown). E, The same area as in D but after vanillin-HCl test. The condensed phenolics in the nonconductive wood and in the pith are stained in red (arrows), and at the same time the acid fuchsin has been removed from the conductive wood by the vanillin and HCl solutions. F, Longitudinal tangential section of nonconductive wood showing the distribution of phenolics (red) and tyloses walls (arrows). Part of the phenolics is possibly removed during the sectioning and the vanillin-HCl test. Bars = 25  $\mu\text{m}$  (A), 100  $\mu\text{m}$  (C), 200  $\mu\text{m}$  (E), and 50  $\mu\text{m}$  (F).

#### *Location and Morphology of the Nonconductive Xylem*

Nearly all vessels of the control trees were perfused with acid fuchsin solution, suggesting that they were conductive (Figs. 1A and 3, A and D; Supplemental Movie S2). In contrast, low-lignin trees contained nonconductive patches of xylem where the vessels tended to be occluded or collapsed. Although poplar xylem is diffuse-porous (Richter and Dallwitz, 2000), the first-formed vessels around the pith (metaxylem and first-formed secondary xylem) have larger lumen diameters than the last-formed vessels of the same growth ring (Figs. 1A and 3A). These first-formed vessels were rounded in cross section, and they were almost always perfused with dye in the low-lignin trees (Fig. 3B). Near the distal parts of the branches of the low-lignin trees, many vessels in the outer layers of the growth rings (formed later in the growing season and thus smaller in diameter) were collapsed and not stained, showing that they were not able to transport water (Fig. 1, B and C). At the basal and middle parts of branches, both conductive and nonconductive vessels were found in the outer xylem (Figs. 1, D and F, and 3D), and nonconductive wood also occurred as iso-

lated patches in the inner portions of the growth rings (Figs. 1, D and E, and 3C). No dye ascent was observed in the fibers of control or transgenic events, and the dye tracer was strictly localized within conductive vessels.

Collapsed vessels were seen in both frozen and freeze-dried samples of the low-lignin events, demonstrating that cell collapse did not result from sample preparation (Figs. 1, B and C, and 3E; Supplemental Movie S4). The walls of the collapsed cells, as well as those that were not collapsed but nonconductive, had very weak blue fluorescence, suggesting lower lignin concentrations (Figs. 1B and 2A). Reduced lignin content of cell walls of nonconductive xylem was confirmed with phloroglucinol-HCl staining (Supplemental Fig. S1). Collapsed cells were seen in all samples of the event with larger areas of brown wood (712) and were rarely seen in the event with more restricted brown wood (671-10). In both 712 and 671-10 events, the majority of nonconductive wood vessels were not collapsed. Collapsed cells occurred only in the outer portion of the growth rings, where vessels had smaller diameters, and collapsed cells were more frequent toward the branch tips (Fig. 1, B and C).



**Figure 3.** Confocal microscopy showing conductive vessels (red walls) and nonconductive wood (ncw) with phenolics. Samples were first prepared as those in Figure 1 but freeze dried and observed at room temperature. A, Control plant where all vessels contained the dye and no collapsed vessels were seen. Phenolics were present in pith parenchyma cells but not in xylem vessels. A maximum intensity projection image is shown (for details and a 3D movie, see Supplemental Movie S2). B, Sample 712-2. Only vessels around the pith are conductive. The nonconductive wood is occluded with phenolics (green or blue), and some vessels are partly or entirely collapsed. A maximum projection image is shown (for details and a 3D movie, see Supplemental Movie S3). C, Sample 712-5, inner region of the xylem ring. Some of the nonconductive vessels are filled with phenolics (green or blue; arrows). A maximum projection is shown of four optical sections at 1- $\mu\text{m}$  intervals with band-pass filters (BP 420–480, BP 505–530, BP 625–700). D, Sample 712-5, outer region of the xylem ring. Inactive cambium (cam) is at the top left corner. Acid fuchsin is seen in the walls of conductive vessels adjacent to the cambium. A maximum projection is shown of four optical sections at 1- $\mu\text{m}$  intervals with the same band-pass filters as in C. E, An enlarged detail of sample 712-2 showing the boundary between conductive and nonconductive wood (for a 3D movie, see Supplemental Movie S4). Most of the nonconductive wood is filled with phenolics (green-blue), and some vessels are partially collapsed (arrows). A single optical section is shown with a band-pass filter combination (BP 420–480, BP 530–600, BP 604–625) that shows a better differentiation between phenolics and cell walls. Note that unstained lignified cell walls have a very broad autofluorescence spectrum, including red fluorescence, but with a very low intensity compared with walls stained with acid fuchsin. Bars = 50  $\mu\text{m}$  (A–C) and 20  $\mu\text{m}$  (D and E).

Some peculiarities were noticed with regard to the stage of differentiation of the last-formed xylem elements in the mutant events. Cells adjacent to nonconductive wood in the low-lignin trees appeared to have thinner cell walls and incomplete differentiation (Fig. 1C). In contrast, the last-formed elements of the conductive wood of both low-lignin and control trees

appeared fully differentiated (Figs. 1F and 3D). Fibers within the conductive wood did not contain the dye tracer but were filled with ice, which shows that they contained water, although they still were not actively conducting after 60 to 90 min of dye introduction (Figs. 1F and 2A). Gelatinous (tension wood) fibers frequently occurred in the low-lignin trees but were rarely observed in the controls, in accordance with previous observations (Voelker, 2009). The gelatinous fiber wall layer has weak natural fluorescence and was not clearly visible in unstained sections with the fluorescence microscope. However, the gelatinous layer of the tension wood fibers was clearly visible after extraction of the nonstructural phenolics and staining with calcofluor (Fig. 4, A and C).

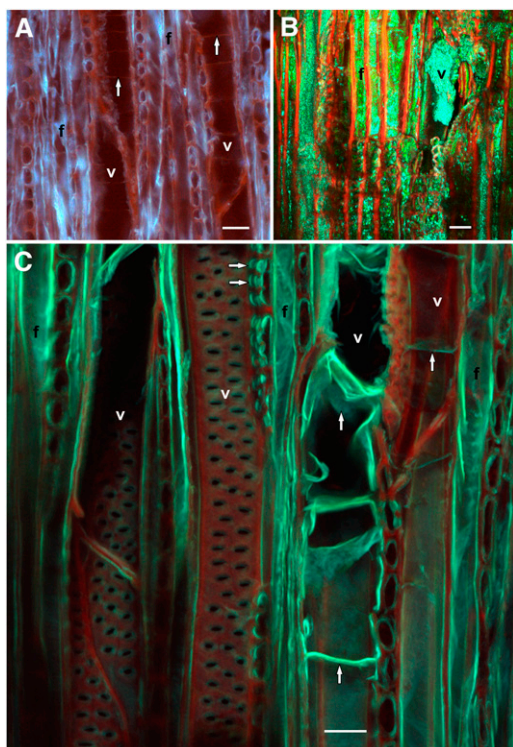
### Occlusions with Phenolics

Whereas dye was transported through almost all vessels in the control plants, groups of occluded nonconductive vessels were common in the low-lignin plants. As noted earlier, these nonconducting vessels were occluded with a substance that appeared white or colorless by dissecting microscopy. In frozen samples viewed with the cryo-fluorescence microscope, the occluding substance was distinguishable from acid fuchsin solution or water by its weak yellow fluorescence (Fig. 1E). Moreover, in freeze-dried samples, the occluding substance remained deposited within the xylem cells and strongly fluoresced green (Figs. 2C, 3, C–E, and 4B). The occlusions were phenolics (condensed tannin), as shown by the dark red staining with the vanillin histochemical test, and these tannins were also present in the rays and pith parenchyma (Fig. 2, D–F). Cryo-fixation and cutting with a cryo-microtome helped retain the phenolic occlusions within cells. With the confocal microscope, the occlusions were clearly distinguishable from the cell walls with their stronger blue-green natural fluorescence (Fig. 3, C and E). The 3D reconstructions revealed that all xylem cell types in the nonconductive wood were entirely filled with phenolics (Fig. 4B; Supplemental Movies S3 and S4). In contrast, the phenolic occlusions were only occasionally found in vessels and fibers outside of the brown wood, but they were present in the rays (Fig. 3E; Supplemental Movie S1). Both cryo-fluorescence and confocal microscopy showed that vessels filled with phenolics were not collapsed or were only partially collapsed (Figs. 1E, 2C, and 3, C and E), whereas those vessels that were entirely collapsed (both in frozen and in freeze-dried samples) were apparently devoid of phenolic contents (Fig. 1; Supplemental Movie S4).

### Occlusions with Tyloses

Tyloses were not observed in the controls, but they were observed in almost all vessels of nonconducting wood in the low-lignin events. The thin transverse walls of tyloses were seen in longitudinal sections of wood (Fig. 2F), but their presence in the frozen and freeze-dried samples appeared to have been masked





**Figure 4.** Tyloses in vessels, phenolic deposits, and gelatinous fibers in nonconductive wood of mutants. A and C show 60- $\mu\text{m}$ -thick tangential sections of sample 712-5 stained with calcofluor after extraction of phenolics, and B shows a cryo-planed and freeze-dried radial-longitudinal plane of sample 671-10. A, The cellulosic walls of gelatinous fibers are seen in blue. Thin walls of tyloses are also visible (arrows). Image by wide-field fluorescence with LP 420. B, Phenolics are blue or green; lignified walls are red. Image by confocal laser scanning microscopy, maximum projection of 11 optical sections at 1- $\mu\text{m}$  intervals with band-pass filters (BP 420–480, BP 505–530, BP 590–650). C, Tyloses (vertical arrows) and lignified walls (red). Horizontal arrows point to vessel-ray pits of contact ray cells. Image by confocal laser scanning microscopy (for a 3D movie, see Supplemental Movie S5), maximum projection of 31 optical sections at 0.95- $\mu\text{m}$  intervals with the same band-pass filters as in B. f, Fiber; v, vessel element. Bars = 25  $\mu\text{m}$  (A) and 20  $\mu\text{m}$  (B and C).

by the abundant phenolic extractives deposited in the same cells. The tyloses' thin walls were evident after extraction of the phenolics (Fig. 4, A and C; Supplemental Movie S5). Tyloses as well as phenolics were seen in some vessels outside of the brown patches of nonconducting wood, which could explain the observation of nonconducting vessels by the dye-flow experiment outside of the brown wood boundaries (Supplemental Movie S1).

## DISCUSSION

### Determination of the Active Conductive Paths in the Xylem

An earlier study on the appropriateness of different dyes for the analysis of flow paths in woody tissue showed that acid fuchsin solution is useful for tracing

plant water transport because it is conducted in xylem at the same rate as water (Umebayashi et al., 2007). Our study showed that acid fuchsin dye flow can be examined by fluorescence and confocal microscopy, which allows observation of thick sections or planed surfaces. Previously, the cryo-fixation of dyes was followed by freeze drying, then resin embedding, and bright-field conventional microscopy of thin sections for tracing the dye within individual cells (Sano et al., 2005; Umebayashi et al., 2007). Here, we employed a novel protocol of dye stabilization and cryo-fluorescence microscopy that enabled us to visualize the dye at the cellular level and to identify actively conducting paths within the xylem without the need of resin embedding and thin sectioning of the samples. Cryo-fluorescence was previously used for the observation of cell collapse in leaves (Cochard et al., 2004; Brodribb and Holbrook, 2005b). We used a different cryo-fluorescence technique where cryo-fixed branch segments were planed in the frozen state on a sliding cryo-microtome and observed with an epifluorescence microscope equipped with a cryo-stage. Cryo-planed samples could also be freeze dried and observed at room temperature with an epifluorescence or a confocal microscope. After freeze drying, the dye was localized within conductive vessels, and an occluding substance within nonconductive cells, which was later determined to be phenolic, was clearly visible by its strong blue and green fluorescence. The cryo-fluorescence microscope provided lower resolution compared with what can be achieved by cryo-scanning electron microscopy (Utsumi et al., 2003; Cobb et al., 2007) but enabled us to clearly distinguish between phenolic-occluded xylem, actively conducting (stained), and not actively conducting (unstained) water-filled xylem cells. It was previously discussed by Umebayashi et al. (2007) that prolonged immersion of plant stems in dye solution may cause the dye tracer to diffuse from vessels to adjacent xylem elements. In our experiments, the branches were cryo-fixed when the dye ascent reached leaf petioles and no dye diffusion from vessels was noticed. Also, no dye was observed in the xylem fibers of the controls and the low-lignin events.

The nonconductive wood in severely *4CL*-down-regulated plants tended to occur predominantly toward the later-formed, outer xylem cell layers in the distal parts of branches but had various locations in the lower parts of branches. In the same poplar clones, brown wood was found frequently at stem-branch junctions and in distal portions of branches, but there was no discernible pattern or arrangement of the brown xylem in the main stem (Voelker et al., 2010). In another study, Leplé et al. (2007) found that distribution of colored wood in cinnamoyl-CoA reductase-down-regulated poplar was more pronounced toward branch-stem junctions and opposite to the distribution of tension wood that typically forms at the upper side of branches. Irregular distribution of lignin-deficient patches of wood was previously reported in transgenic poplar xylem (Leplé et al., 2007; Coleman et al., 2008b).

This nonuniformity in the distribution of low-lignin and nonconductive brown patches of xylem could be what is referred to as “phenotypic inconsistency” or “patchiness” at the cellular level in gene silencing (Baucher et al., 1996; Tsai et al., 1998; Meyermans et al., 2000; Pilate et al., 2002; Wagner et al., 2009). Our observations showed that even in the most severely modified xylem structure, the first-formed vessels surrounding the pith were always conductive. The inner vessels that surround the pith have larger lumen diameters than the rest of the vessels in 1-year-old branches (ring-porous-like structure); consequently, the inner vessels of the first growth ring should make an important contribution to the water flow not only in the mutant plants but in the control plants as well.

The normally colored xylem of the low-lignin plants was conductive and identical in structure to the controls (Figs. 1, A and F, and 3, A and D). Some wood anatomical and mechanical characteristics of the same plant material were previously studied by Voelker (2009), who found no significant difference in vessel diameter and cell wall thickness between the controls and the low-lignin events. However, the fraction of brown wood in stem cross sections was negatively correlated with xylem transport efficiency and positively correlated with shoot dieback of low-lignin transgenic events (Voelker, 2009). Furthermore, the main stems of the “brown wood” events had lower stiffness but a similar safety factor to buckling due to having more stem taper than controls, whereas “normal wood” transgenic events were transitional in taper (Voelker, 2009). Our cryo-fluorescence microscopy analysis showed that conductive and nonconductive wood occurred as normal and brown wood, respectively, which could explain the positive relationship between colored wood formation and the impaired hydraulic function of wood.

#### Occlusion of Vessels with Phenolics

In contrast to traditional methods of histological preparations for light microscopy, cryo-fixation and cryo-microtomy retained the phenolic substances, and they were apparently more abundant in planed surfaces than in thin sections of the same specimen. Condensed tannins (leucoanthocyanins and catechins; Gardner, 1975) were detected by the vanillin-HCl test in all cells within the brown wood regions of the xylem rings. In transgenic poplar event 712, the wood extractives were determined to be naringenin, dihydrokaempferol, and their 7-*O*-glucosides (Voelker et al., 2010). These substances were nearly undetectable in the control event. Similar flavonoids can be heavily deposited in the knots of *Populus* wood (Pietarinen et al., 2006). However, naturally occurring heartwood in *Populus* is little different or the same in color as the sapwood (Richter and Dallwitz, 2000). Therefore, it is unknown whether the same signals responsible for heartwood formation caused the deposition of the abnormal brown wood extractives we observed.

The autofluorescence of wood in the control plants was similar to that of the normally colored conductive wood in the mutants and was apparently different from that of the nonconductive patches of xylem. The intensity of UV and blue light-induced fluorescence of secondary walls is indicative for their total lignin contents (Donaldson, 2002). However, in our freeze-dried samples of 4CL-down-regulated trees, the lignin autofluorescence was obscured by the occurrence of phenolics. Furthermore, blue light autofluorescence in colored zones of xylem may indicate the presence of secondary metabolites or cell wall structures that are undetectable in noncolored zones (Leplé et al., 2007). It was clear that the distinct fluorescence of nonconductive wood was mainly due to the abundance of phenolics that fluoresced strongly green under blue light excitation. In sections that were cleared of phenolic extractives, the autofluorescence of lignified cell walls in nonconductive xylem was apparently less intense than that in conductive xylem, suggesting lower lignin contents of the nonconductive xylem. The phloroglucinol-HCl test also indicated lower lignin contents in the cell walls of the nonconductive wood in the mutant trees (Supplemental Fig. S1).

Abnormal coloration of wood usually occurs in genetically modified poplar and other species with reduced cell wall lignin (Baucher et al., 1996; Kajita et al., 1997; Piquemal et al., 1998; Leplé et al., 2007; Voelker, 2009). Similarly, the transgenic events with very low xylem  $k_s$  used in this study formed patches of brown wood that were associated with increased extractives contents (Voelker et al., 2010). In a different study, a real-time PCR revealed that orange-brown wood was more down-regulated in cinnamoyl-CoA reductase than normally colored wood (Leplé et al., 2007). The same authors attributed the discolored wood to the incorporation of ferulic acid into the lignin. Similarly, in tobacco plants with down-regulated lignin biosynthesis, the brown coloration of xylem was associated with an increase of the ferulic acid bound to cell walls (Piquemal et al., 1998). Our microscopic observations showed that brown wood patches were entirely impregnated with phenolics that filled vessels, fibers, and rays. The occlusions had a faint yellowish color in frozen samples and may have contributed to the abnormal discoloration of the associated wood. However, after extraction of the soluble phenolics through the ethanol series, the red-brown patches remained in the transgenic wood samples. Therefore, the discoloration may also be due to changes in the cell wall chemistry, as indicated in other lignin-deficient plants (Piquemal et al., 1998; Leplé et al., 2007). Moreover, the distribution of phenolics, as well as nonconductive wood, was not restricted to the brown-stained wood but occurred sporadically in normal-colored wood adjacent to brown wood. Other studies also found increased amounts of phenolic extractives in transgenic poplar stems, and it was suggested they formed as a result of a shift in carbon flux away from the lignin biosynthetic pathway and into the production of secondary metabolites

(Meyermans et al., 2000; Voelker et al., 2010). Increased amounts of nonstructural phenolics were also found in a *4CL*-down-regulated gymnosperm (Wagner et al., 2009). It is known that the down-regulation of *4CL* affects the production of *p*-coumaroyl-CoA, a precursor for the production of lignins as well as secondary plant metabolites such as flavonoids (Boudet, 2007). After the production of *p*-coumaroyl-CoA, the phenylpropanoid pathway is divided to different metabolic routes, one of them leading to the synthesis of flavonoids and the other leading to the synthesis of lignins (Tsai et al., 2006). A recent study demonstrated that silencing of hydroxycinnamoyl-CoA shikimate/quinic hydroxycinnamoyl transferase (HCT), which catalyzes the formation of guaiacyl and syringyl lignin precursors, rerouted the metabolic pathway to increased formation of flavonoids (Besseau et al., 2007). The degree of flexibility in biochemical rerouting of carbon earlier in the phenylpropanoid pathway, such as in plants with down-regulated *4CL*, is not well understood. However, the presence of naringenin and other flavonoids in the brown wood of low-lignin *4CL* poplar (Voelker et al., 2010) and the ability of naringenin to inhibit *4CL* activity in lignifying xylem (Voo et al., 1995) argue that perturbations to upstream phenylpropanoid enzymes can induce complex dynamics or even positive feedbacks between lignin and secondary metabolite production.

#### Occlusion of Vessels with Tyloses

Tyloses with thin walls can be formed in poplar stems (Richter and Dallwitz, 2000), but to our knowledge, the presence of tyloses in poplar has been very rarely reported and has not been investigated in genetically modified poplars. This investigation showed that severe down-regulation of lignin biosynthesis in poplar resulted in the formation of tyloses. Phenolic extraction followed by calcofluor staining allowed for a clearer observation of the tyloses' thin walls as well as for the observation of gelatinous fiber walls using fluorescence microscopy. The occurrence of tyloses-occluded vessels not only within the discolored wood was in agreement with the dye-flow experiments that detected nonconductive vessels within the normally colored wood.

#### Vessel Occlusion or Vessel Collapse

Drought-tolerant plants can overcome occasional or minor hydraulic dysfunction in their xylem through various mechanisms. These include redundancy of xylem vessels (Ewers et al., 2007), the co-occurrence of vessels and tracheids (Carlquist, 1985), the ability to refill embolized conduits (Holbrook and Zwieniecki, 1999), or cell walls and pit membranes that are particularly mechanically resistant to the hoop stresses exerted on vessels at very negative hydraulic pressures (Hacke et al., 2001; Hacke and Sperry, 2003). However, poplar is a mesophytic, diffuse-porous species with comparatively low wood density and has

only limited safety features to protect the plant against xylem embolism. Poplars rely on water transport through a number of active xylem rings, whereas ring-porous species are able to supply the crown with water through one or a few growth rings. Furthermore, the xylem of the low-lignin poplar events was significantly less resistant to embolism than the controls (Voelker, 2009). When the strength and integrity of vessel walls is compromised by reduced lignification, the conduits are more vulnerable to dysfunction through collapse or air-seeding and embolism.

Collapsed vessels occurred in the outer (latest-formed) parts of the xylem rings in our samples of transgenic plants. Similarly, collapsed vessels were found in other studies of transgenic low-lignin poplar (Coleman et al., 2008b; Voelker, 2009). Despite fundamental structural and physiological differences between angiosperm and coniferous gymnosperm xylem, a tendency toward collapse was also noticed in lignin-deficient tracheids in *Pinus radiata* (Donaldson, 2002; Wagner et al., 2009). The reasons for the frequently documented collapse of cells in low-lignin xylem have not been studied, but it is generally assumed that without sufficient lignin the vessel or tracheid walls are not strong enough to withstand the xylem water tensions produced by transpiration. In studies of lignin-deficient plants, the capacity of the xylem to function properly is often inferred by the occurrence of normal or collapsed xylem conduits (Donaldson, 2002; Coleman et al., 2008b). Unlike in the transgenics, collapse of xylem conduits in wild plants as a direct result of water stress has been rarely reported, and only as a reversible collapse of certain tracheids in conifer leaves (Cochard et al., 2004; Brodribb and Holbrook, 2005b). Cell collapse should not occur in normal xylem because strong negative xylem pressures would induce the air seeding of embolism prior to cell collapse (Domec et al., 2006).

Cases of reduced or variable lignification of woody cells have been reported in naturally growing dicotyledonous or conifer trees (Daniel et al., 1991; Donaldson, 1991; Downes et al., 1991; Gindl and Grabner, 2000). The reduced lignification was attributed to a virus disease (Beakbane and Thompson, 1945), nutrient or water deficiency (Barnett, 1976; Downes et al., 1991; Donaldson, 2002), or decreased autumn temperature (Gindl and Grabner, 2000). Barnett (1976) suggested that poor lignification of tracheid walls might be due to ceased tracheid differentiation resulting from drought, and Donaldson (1991) found evidence that latewood tracheids of the last-formed growth ring in *P. radiata* were not completely lignified until the next growing season. Moreover, on microscope slides, conifer tracheids with severely compromised lignification were often observed to be collapsed (Barnett, 1976; Donaldson, 2002). The reason for the xylem cell collapse, in the current study as well as in previous studies (Barnett, 1976; Donaldson, 2002; Coleman et al., 2008b), may be a compromised rigidity of cell walls due to reduced lignin. We are uncertain whether



xylem collapse occurs after cells are fully formed and integrated into the functional xylem network without the support of turgor pressure (when xylem cells lose their protoplasts at maturation) or whether collapse occurs during cell differentiation because the biosynthetic mechanisms are so altered. Mechanical pressures exerted by growth, in which developing cells are confined between the cambium, phloem, and bark on one side and the mature tissues on the other side, could have contributed to the collapse in structurally compromised cells near the cambium. The cells that may be particularly vulnerable to collapse are the vessel elements that were formed late in the growing season and that appeared to have had incomplete cell wall differentiation (Fig. 1C). One unexpected observation was the inverse relationship between the occurrence of collapsed vessels in the low-lignin wood and the occurrence of phenolics and tyloses. Collapsed vessels still had a small lumen area but were devoid of phenolics, while intact or partially collapsed vessels were filled with phenolics. This pattern suggests, if cell collapse is caused by hydraulic tension, that phenolics and tyloses may have functioned to isolate certain vessels from the actively conducting xylem, such that these cells never reached the tensions that caused collapse in nonisolated vessels that had low-lignin cell walls. In wild plants, tyloses and phenolics are formed as defense reactions against pathogens and for physical and chemical isolation of wounds. In aspen species, the amounts of flavonoids were considerably higher in the knots relative to the stem wood (Pietarinen et al., 2006). In addition, some flavonoids are considered as endogenous auxin transport regulators (Peer and Murphy, 2007) and can apparently inhibit 4CL activity (Voo et al., 1995). Increased accumulation of flavonoids was associated with reduced auxin transport and reduced growth of HCT-deficient *Arabidopsis* plants (Besseau et al., 2007). However, more recent work suggests that lignin reductions rather than the accumulation of flavonoids was the primary cause of the stunted growth in HCT-deficient plants (Li et al., 2010). In a different system, involving field-grown low-lignin poplar, we have shown that the accumulation of nonstructural phenolics was associated with reduced xylem water-transport function. Because phenolics occurred in parenchymatic tissues beyond the nonconductive portions of wood, other processes, including auxin transport, may have been affected as well. However, the physical blockages with phenolics and tyloses of large portions of the vessel networks constitute a direct cause of the occurrence of shoot dieback in the extremely low-lignin events.

The same transgenic poplar events that we used in this study were previously investigated for their hydraulic properties, and it was shown that the xylem with severely reduced lignin contents was significantly more vulnerable to embolism compared with the controls (Voelker, 2009). However, similar to the study by Coleman et al. (2008b), the trees in our experiment were never subjected to water stress; there-

fore, greater vulnerability to embolism was not likely the cause of the measured decreases in xylem  $k_s$  in low-lignin trees. Furthermore, to assess the full potential of the stems to conduct water, Voelker (2009) removed embolisms before measuring the initial  $k_s$ . The majority of nonconducting vessels were not collapsed, and collapsed cells occurred only at the periphery of the xylem rings. Because of the small vessel size and restricted occurrence, these entirely collapsed vessels would have little impact on the total hydraulic conductivity. Our results show that tyloses and phenolic depositions, but not cell collapse, were the main cause of decreased xylem water conductivity of the mutant plants.

## CONCLUSION

Cryo-fluorescence microscopy allowed us to look at structural features, such as cell wall integrity, tyloses formation, and the patterns of phenolic deposition, in relation to xylem water conductivity. Low-lignin poplar trees in our study had compromised integrity of xylem vessels and abundant tyloses and phenolic deposits. None of these was observed in the control plants. Our analyses revealed that reduced transport efficiency of genetically modified xylem with very low-lignin contents was caused by the formation of tyloses and the deposition of phenolics within vessels. To provide knowledge that may help in the development of biotechnologies for sustainable poplar crops, further research should address the mechanisms of tyloses formation, phenolic deposition, and their biological role in transgenic poplars with down-regulated lignin biosynthesis.

## MATERIALS AND METHODS

### Plant Material

Hybrid white poplars (*Populus tremula* × *Populus alba* [INRA-France 717-1B4]; all female plants) were used for all controls and transformants. Forty- to 50-d-old in vitro-grown plantlets were produced from microcuttings and served as explant sources. To ensure that transformation events were independent, a single clone per individual explant was selected for further propagation after confirmation of transgene presence by PCR. The antisense *Pt4CL1* construct for repression of *4CL* was generated by fusing the *Pt4CL1* cDNA coding sequence in an antisense orientation with respect to a duplicated-enhancer cauliflower mosaic virus 35S promoter (the gene construct was provided by Dr. Vincent Chiang at North Carolina State University). PCR-positive events plus controls derived from the same explants without genetic transformation were propagated for 4 months in the greenhouse. A total of 14 transgenic events and numerous copies of the control were produced and planted in the field in the fall of 2005, after 3 months of acclimatization in a cold frame. The field trial was north of Corvallis, Oregon. Details of the plant transformation methods and growing conditions are available elsewhere (Voelker, 2009).

Samples for this study were collected in September 2008 (when leaves were still green and intact). Three individual trees from the two transgenic events that had the greatest reduction of lignin contents were selected for study. Two of the trees (712-2 and 712-5) represented an event with various percentages of brown wood in the branches and that experienced substantial shoot dieback, and one tree (671-10) represented an event with a smaller degree of brown wood and less shoot dieback (Voelker, 2009). Samples from two control trees were also collected. Five current-year shoots per tree were cut at dawn, under

water, and quickly transported to the laboratory with the cut ends under water.

## Dye-Flow Experiments and Microscopy

The branches were transferred into a beaker containing a 0.2% acid fuchsin solution, which is brilliant red and can be seen in small concentrations. While transferring the branches, their cut ends were always kept immersed in small plastic bottles of water to prevent xylem embolism. Branches were placed in sunlight under a fan to increase leaf transpiration. As soon as acid fuchsin was observed to reach the petioles, the branches were snap frozen in liquid nitrogen (while the cut ends remained immersed in dye). The cryo-fixed branches were cut into 2- to 3-cm-long segments and stored at  $-12^{\circ}\text{C}$  in a cold room (walk-in freezer). Then, branch segments were planed in the frozen state at  $-10^{\circ}\text{C}$  to  $-30^{\circ}\text{C}$  on a sliding cryo-microtome. At least two segments from the basal, middle, and distal parts of branches from each tree were observed at  $-30^{\circ}\text{C}$  with an epifluorescence microscope (Nikon E400) equipped with a cryo-stage (Kitin et al., 2008). Images were recorded with a digital CCD camera (Q Imaging; Micropublisher 5.0 RTV). Other replicates of the cryo-planed samples were freeze dried and observed at room temperature with the epifluorescence microscope (emission filters LP 420 and LP 520) or a confocal microscope (LSM-510; Carl Zeiss) using single-track, triple-channel imaging with 405-, 488-, and 543-nm laser lines.

## Histological Observation for Tyloses

Cryo-fixed branch segments were gradually thawed by transferring them through  $-8^{\circ}\text{C}$  and  $4^{\circ}\text{C}$  in a cold room and then room temperature. Transverse or longitudinal sections ( $40\text{--}60\ \mu\text{m}$  in thickness) were then cut the same day using a sliding microtome. The sections were passed through a 30%, 50%, and 95% ethanol series to remove extractives and stored in 50% ethanol. For fluorescence microscopy, we placed a drop of 0.5% calcofluor (Calcofluor White M2R; Sigma Chemical) on each section and then protected it from light for 30 min. Sections were then mounted in 50% glycerin on microscope slides and observed with the fluorescence or confocal microscope as described above.

## Histochemical Tests for Phenolics

Thick transverse and tangential longitudinal sections were cut on a sliding microtome as described above or with a razor blade. In order to visualize nonstructural phenolics, the sections were treated with a drop of saturated alcoholic vanillin followed by the addition of concentrated HCl to form vanillin-tannin condensates that can be easily distinguished by their bright-red color (Gardner, 1975). A phloroglucinol-HCl test (Wiesner reaction) was performed on some sections for visualization of lignified cell walls by first pouring a drop of 1% phloroglucinol ethanol solution on a 10- to 20- $\mu\text{m}$ -thick cross section of wood (after the extractives have been removed as described above), next by adding a drop of 35% HCl, and then covering the section with a coverslip. The intensity of red coloration (absorption peak at 570 nm) is proportional to the quantity of coniferyl and sinapyl aldehyde units in lignins (Yoshizawa et al., 2000; Pomar et al., 2002). However, because the intensity of coloration is not stable over time, the phloroglucinol-HCl test is poorly quantitative; nevertheless, it is still widely used for making a distinction between strongly and weakly lignified cell walls. The sections were observed with a conventional transmitted light microscope and bright-field illumination.

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** A phloroglucinol-HCl test (Wiesner reaction) for visualization of lignified cell walls in low-lignin poplar wood (specimen 712-2).

**Supplemental Movie S1.** 3D movie showing functional and nonfunctional vessels within normal-colored wood of specimen 712-5 visualized by confocal laser scanning microscopy.

**Supplemental Movie S2.** Control plant perfused with acid fuchsin solution

**Supplemental Movie S3.** Poplar with severely down-regulated lignin biosynthesis, specimen 712-2.

**Supplemental Movie S4.** Nonconductive wood (sample 712-2).

**Supplemental Movie S5.** Tyloses in vessels in nonconductive wood of specimen 671-10.

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