Cooperative lignification of xylem tracheary elements

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The development of xylem tracheary elements (TEs) – the hydro-mineral sap conducting cells - has been an evolutionary breakthrough to enable long distance nutrition and upright growth of vascular land plants. To allow sap conduction, TEs form hollow laterally reinforced cylinders by combining programmed cell death and secondary cell wall formation. To ensure their structural resistance for sap conduction, TE cell walls are reinforced with the phenolic polymer lignin, which is deposited after TE cell death by the cooperative supply of monomers and other substrates from the surrounding living cells.

Xylem is the vascular tissue in land plants which transports water and minerals from the roots to the leaves.1 To do so, specialized cells named tracheary elements (TEs) form conductive cellular structures which assemble end-to-end and laterally to establish a complex vascular network throughout the plant body. To fulfill their conductive role, TEs undergo 3 main morphological modifications including (i) thinning/perforation of the cell wall formation to strengthen the cell sides and (iii) thinning/perforation of the cell terminal end to provide access to the cell lumen.2 Altogether these processes lead to the formation of a hollow cell, terminally perforated and with a reinforced lateral cell wall.3 This lateral reinforcement corresponds to the deposition of a secondary cell wall which is composed of cellulose (40–50%), hemicellulose (25–30%) and the phenolic polymer lignin (20–30%). Addition of lignin to TE cell walls provides an increased mechanical resistance (extra 25–75 MPa in tensile strength and 2.5–3.7 GPa in Young’s modulus) and impermeability.4,5 The formation of lignin results from the oxidative polymerization of at least 3 different 4-hydroxyphenylpropene monomers - p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol - which form the 3 main lignin subunits: H (hydroxyl), G (guaiacyl) and S (syringyl), respectively.6 Oxidizing enzymes, such as H2O2-dependent peroxidases and O2-dependent laccases, generate monomer radicals which directly polymerize into lignin in the cell wall. Genetic or pharmacological disturbances of TE formation in plants result in dramatic defects including collapsed TEs, reduced growth, greater sensitivity to environmental stress and pathogen infections.6 To study TE formation without hindering plant development, simplified cell culture systems have been established such as the Zinnia elegans TE differentiating cell cultures. In this system, TE differentiation can be triggered hormonally from freshly isolated mesophyll cells: the differentiation is synchronous, morphological changes progress chronologically within 4 days, with 30–40% of the cells being fully differentiated into dead lignified TEs.7 The resulting in vitro TEs present all the morphological, genetic and biochemical characteristics associated to TEs in whole plants,7,8 making the Zinnia elegans system an ideal tool to study TE formation.

Lignin deposition is controlled spatio-temporally

Lignin is differentially distributed between the different parts of the cell wall, which can be easily visualized by UV-autofluorescence coupled with confocal microscopy (Fig. 1A). Arabidopsis thaliana hypocotyl cross-sections exhibit the highest level of lignin autofluorescence in the cell corners (CC) and the middle
The proper synthesis of secondary polysaccharides as pharmacological disturbance of cellulose synthesis leads to dispersed and lower lignification of TEs. In contrast, treatments with inhibitors of lignin synthesis do not affect the spatial organization of TE SCWs. Thus a chronological sequence occurs during TE SCW formation, starting with polysaccharide synthesis and ending with lignin deposition. This sequence of cell wall polymer deposition is confirmed when disassembling TE cell walls as degradation of *Zinnia* TE secondary cellulose and xylan is enhanced when first removing lignin.

### TEs are lignified post-mortem

TE differentiation is completed by cell death and secondary cell wall lignification. In differentiating TE cell cultures, cell death is triggered once xylan and cellulose have been deposited in the secondary cell wall. This process includes an influx of calcium (Ca\(^{2+}\)) and a change of the tonoplast permeability, which leads to the inflation of the vacuole and finally bursting of the tonoplast and release of the vacuolar hydrolytic contents (proteases and nucleases) into the cytoplasm. Once cell death is accomplished, the gradual autolysis of the protoplast remnants by the released enzymes occurs rapidly; it takes less than 10 minutes to completely remove the nucleus and several hours for the chloroplasts. Remarkably, lignification of TEs occurs mainly after cell death and secondary cell wall lignification.

In differentiating TE cell cultures, cell death and secondary cell wall lignification.

Figure 1. Lignin distribution in xylem cell walls of *Arabidopsis thaliana* hypocotyls and in isolated TEs. (A) UV confocal microscopy of transverse sections of the hypocotyl; lignification in the secondary xylem is visualized by artificial color 8-bits intensity scale (from 0 to 256). SCW, vessel secondary cell wall; fSCW, fiber secondary cell wall; CML, compound middle lamella; CC, cell corner. Bar = 30 μm. (B) UV confocal microscopy of a TE from differentiating cell cultures; lignification is visualized by artificial color 8-bits intensity scale. SCW, secondary cell wall; PCW, primary cell wall. Bar = 8 μm. (C) Quantification of the lignin UV autofluorescence intensity of hypocotyl transverse sections of *Arabidopsis thaliana* ecotypes Columbia-0 (Col-0) and Landsberg erecta (Ler). Error bars indicate ± SD.
the changes observed when adding external lignin monomers to PA-treated dead TEs were due to aromatic compounds built into the cell wall, based on bands at 1505 cm\(^{-1}\) and 1595 cm\(^{-1}\) (aromatic \(-\text{C} -=\text{C}-\) vibrations, characteristic of lignin, Fig. 2A and B).\(^{28}\) Hierarchical clustering of average FT-IR spectra revealed that dead PA-treated TEs achieved normal post-mortem lignification when supplied with a mixture of both lignin monomers (Fig. 2C). Moreover, estimation of the 1505 cm\(^{-1}\) and 1595 cm\(^{-1}\) band area (integrals) confirmed full post-mortem lignification of dead PA-treated TEs (Fig. 2D-E). To evaluate if post-mortem TE lignification also occurs in intact plants, FT-IR microspectroscopy was performed on proto- and metaxylem TEs at different internodes of 5-weeks old \textit{Zinnia} plants and similarly confirmed an increase in lignin with increasing age of the TEs.\(^{17}\) TE post-mortem lignification was also shown to occur in \textit{Arabidopsis} root TEs\(^{29}\) suggesting that TE post-mortem lignification is a general event in angiosperms. However, the mechanisms controlling the triggering of lignin formation after TE cell death are still unknown.

Lignification of TEs is non-cell autonomous

TE post-mortem lignification implies that the substrates necessary for lignin formation (monomers and/or H\(_2\)O\(_2\)) are either released in the extracellular medium when TEs die and/or secreted by the surrounding living parenchyma cells. In \textit{Zinnia} TE differentiating cell cultures, about 30–40\% of the cells become TEs and die while the rest of the cells remain alive.\(^{7}\) Although these parenchyma cells do not exhibit distinct morphological features, they are differentiated cells which express specific genes that are also expressed in xylem parenchyma of whole plants.\(^{30}\) During TE differentiation in \textit{Zinnia} cell cultures, the gene expression of the lignin monomer synthesis genes PAL (phenylalanine ammonia-lyase), C4H (cinnamate-4-hydroxylase), CCR (cinnamoyl-CoA reductase) and CAD (cinnamyl-alcohol dehydrogenase) was also confirmed.

![Figure 2. Post-mortem lignification of \textit{Zinnia} elegans TEs. (A) Principle component analysis (PCA) of FT-IR spectra of differently treated TEs from \textit{Zinnia} elegans cell cultures. Samples include TEs without 50 \(\mu\)M piperonylic acid (PA) (white squares), TEs with 50 \(\mu\)M PA and 60 \(\mu\)M coniferyl alcohol (G-OH) (gray), TEs with 50 \(\mu\)M PA (black). (B) Correlation scaled loadings of the first principal component, showing lignin characteristic bands (1505 cm\(^{-1}\) and 1595 cm\(^{-1}\)), accumulating in PA untreated samples as well as in PA treated samples with G-OH compared to PA treated samples only. (C) Hierarchical clustering of average FT-IR spectra of \textit{Zinnia} non-TEs with or without 50 \(\mu\)M PA, TEs with 50 \(\mu\)M PA and 60 \(\mu\)M G-OH or sinapyl alcohol (S-OH), TEs with or without 50 \(\mu\)M PA and TEs with PA and 60 \(\mu\)M G-OH and 60 \(\mu\)M S-OH. (D) Average peak area of the 1505 cm\(^{-1}\) FT-IR band (aromatic \(-\text{C} -=\text{C}-\) vibration associated to G-type lignin in au, arbitrary units) of non-TE and TE samples mentioned in (C). (E) Average peak area of the 1595 cm\(^{-1}\) FT-IR band (aromatic \(-\text{C} -=\text{C}-\) vibration associated to S-type lignin in au, arbitrary units) of non-TE and TE samples mentioned in (C). The asterisks indicate statistically significant difference from TEs treated with PA by t-test with Welch correction (*\(P < 0.05\), **\(P < 0.01\), and ***\(P < 0.001\)). Error bars indicate \(\pm\) SD.](https://www.tandfonline.com/e1003753-3)
dehydrogenase) continues long after the TEs have committed cell death. Similarly, the enzymatic activities of C4H and CAD can also be detected several days after TEs have died. The extended gene expression and protein activity beyond the TE lifespan suggests that the remaining parenchyma cells are involved in the non-cell autonomous supply of lignin monomers. Although no significant increase of intra- and extracellular total phenolics is visible during TE formation in Zinnia cell cultures, the extracellular medium accumulates in TE differentiating conditions known lignin monomers such as coniferyl and sinapyl alcohols beyond the TE lifespan. The fact that dead, non-lignified TEs were able to lignify after washing away the lignin-biosynthesis inhibitor PA demonstrates that the living parenchyma cells present enable the post-mortem lignification of TEs by directly exporting lignin monomers into the extracellular medium. TE non-cell autonomous lignification also concerns the production of H2O2 (necessary for peroxidase activity) which is produced by both differentiated parenchyma cells in Zinnia cell cultures and xylem parenchyma in Zinnia plants. Interestingly, pharmacological inhibition of H2O2 production in differentiated parenchyma cells effectively reduced post-mortem lignification of TEs in cell cultures, suggesting that the living parenchyma cells provide other substrates, such as H2O2, also for lignin polymerization.

Genetic evidence for the TE non-cell autonomous post-mortem lignification was presented by the identification of Arabidopsis genes that were specifically expressed in xylem parenchyma and that affected xylem lignification in a reverse genetic analysis. These included the lignin monomer biosynthesis gene C4H (AT2G30490), the RADICAL-INDUCED CELL DEATH 1 RCD1 (AT1G32230) and the transcription factor MYB13 (AT1G06180). Cell specific expression was analyzed using the promoter driven β-glucuronidase (GUS) reporter system in 7-d old Arabidopsis thaliana seedlings. C4H was expressed along the entire root except for the root apical meristem as well as in the vascular system of the leaves. RCD1 was highly expressed in the root apical meristem and in the youngest leaf primordia and to a lower extent in the entire root and in the vasculature of the leaves. MYB13 was expressed in the hypocotyl, the shoot apical meristem and to a lower extent in the vasculature of the leaves and the root apical meristem. GUS analysis in stem and hypocotyl cross sections confirmed the expression of C4H, RCD1 and MYB13 in the xylem parenchyma cells surrounding TEs. Hypocotyl lignin analysis of the corresponding loss-of-function mutants using pyrolysis-GC/MS showed significant changes in both lignin quantity and composition. The c4h-3 and myb13 mutants exhibited a significant reduction in lignin, essentially due to a decrease in lignin G units whereas the rcd1-1 mutant showed an increase in lignin due to a higher amount of G and H units while S units were not affected. Taken together, these analyses elucidate the action of the monomer biosynthetic machinery, including C4H, in cooperative lignin biosynthesis and reveal novel

![Figure 3. Xylem parenchyma expression of C4H, RCD1 and MYB13 and mutational impact on lignin quantity and composition.](image-url)
proteins, such as RCD1 and MYB13, in this process as well.

**Conclusion**

The differential distribution of lignin in the cell wall and the apoplast of specific cell types is a tightly controlled process. Among the lignifying cells in plants, TEs undergo *post-mortem* secondary cell wall lignification enabled by a non-cell autonomous supply of lignin monomers and H_{2}O_{2}, provided by the surrounding living parenchyma cells. The quantity and composition of lignin in TEs depend on a tight coordination and cooperation between TEs and the surrounding parenchyma cells, perhaps to enable optimal sap conduction as the plant grows.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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