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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. 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) programmed cell death (PCD) to empty the cell content, (ii) lateral secondary cell wall formation to strengthen the cell sides and (iii) thinning/perforation of the cell terminal end to provide access to the cell lumen. Altogether these processes lead to the formation of a hollow cell, terminally perforated and with a reinforced lateral cell wall. 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. 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 (hydroxy), G (guaiacyl) and S (syringyl), respectively. 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. 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. The resulting in vitro TEs present all the morphological, genetic and biochemical characteristics associated to TEs in whole plants, making the Zinnia elegans system an ideal tool to study TE formation.

Keywords: lignin, non-cell autonomous process, post-mortem lignification, secondary cell wall, tracheary elements, xylem/wood vessels

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.
Lignin synthesis is suggested to control the spatial deposition in plants. One of the mechanisms suggests a tight spatial regulation of lignin therefore dependent on the cell type and abundance of lignin between the apoplast/primary cell wall and the secondary cell wall (SCW) is restricted to the SCW and absent from the primary cell wall that is visible between the lateral SCW thickenings (Fig. 1B).

The differential localization 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.

**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 = 50 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.

Lignin deposition in TE secondary thickenings is the specific localization of oxidizing enzymes. The H2O2-dependent peroxidase ZPO-C (AB023959) is co-regulated with SCW polysaccharide synthesis genes and localized to TE secondary cell walls. Similarly, O2-dependent laccases 4 and 17 are also specifically localized in the secondary cell walls of Arabidopsis TEs. Lignification is therefore defined to specific areas of the cell wall at the time when the polysaccharide SCWs are synthesized but before deposition of the bulk lignin. During TE formation in Zinnia cell cultures, the timing of lignification is regulated as its deposition has been shown to occur after the deposition of the SCW polysaccharides. Lignin synthesis in Zinnia TE SCWs directly depends on 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 (Ca2+) 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 continues for several hours. Accordingly, blocking cell death in TE cell cultures by silver thiosulfate also blocks lignification, while inhibiting lignification with piperonylic acid (PA) does not affect TE cell death. In order to demonstrate that TE corpses are able to fully lignify post-mortem, TEs were inhibited to lignify using PA until all TEs died. These TE corpses were then supplied with 60 μM coniferyl alcohol (G-OH) and/or sinapyl alcohol (S-OH). TE lignification was estimated at the single cell level using FT-IR microspectroscopy, which showed that dead TEs were able to lignify only when supplied with external monomers. Multivariate analysis confirmed that
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 -C=C- vibrations, characteristic of lignin, Fig. 2A and B). 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 Zinnia plants and similarly confirmed an increase in lignin with increasing age of the TEs. TE post-mortem lignification was also shown to occur in Arabidopsis root TEs 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 Zinnia TE differentiating cell cultures, about 30–40% of the cells become TEs and die while the rest of the cells remain alive. 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. During TE differentiation in Zinnia cell cultures, the gene expression of the lignin monomer synthesis genesPAL (phenylalanine ammonia-lyase), C4H (cinnamate-4-hydroxylase), CCR (cinnamoyl-CoA reductase) and CAD (cinnamyl-alcohol dehydrogenase) was monitored.}

**Figure 2.** Post-mortem lignification of Zinnia elegans TEs. (A) Principle component analysis (PCA) of FT-IR spectra of differently treated TEs from Zinnia elegans cell cultures. Samples include TEs without 50 μM piperonylic acid (PA) (white squares), TEs with 50 μM PA and 60 μM coniferyl alcohol (G-OH) (gray), TEs with 50 μM 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 PA treated samples with G-OH compared to PA treated samples only. (C) Hierarchical clustering of average FT-IR spectra of Zinnia non-TEs with or without 50 μM PA, TEs with 50 μM PA and 60 μM G-OH or sinapyl alcohol (S-OH), TEs with or without 50 μM PA and TEs with PA 60 μM G-OH and 60 μM S-OH. (D) Average peak area of the 1505 cm\(^{-1}\) FT-IR band (aromatic –C=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 –C=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 ± SD.
dehydrogenase) continues long after the TEs have committed cell death. \(^{12,17,31,32}\) Similarly, the enzymatic activities of C4H and CAD can also be detected several days after TEs have died. \(^{31,32}\) 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, \(^{12,13}\) the extracellular medium accumulates in TE differentiating conditions known lignin monomers such as coniferyl and sinapyl alcohols beyond the TE lifespan. \(^{13,17,18,27}\) 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. \(^{17}\) TE non-cell autonomous lignification also concerns the production of \(H_2O_2\) (necessary for peroxidase activity) which is produced by both differentiated parenchyma cells in \(Zinnia\) cell cultures and xylem parenchyma in \(Zinnia\) plants. \(^{33,34,35}\) Interestingly, pharmacological inhibition of \(H_2O_2\) 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 \(H_2O_2,\) also for lignin polymerization. \(^{17}\)

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. \(^{17}\) These included the lignin monomer biosynthesis gene C4H (AT2G30490), the RADICAL-INDUCED CELL DEATH 1 RCD1 (AT1G32230) and the transcription factor MYB13 (AT1G06180). \(^{17}\) Cell specific expression was analyzed by using the promoter driven \(\beta\)-glucuronidase (GUS) reporter system in 7-d old \(Arabidopsis\) thaliana seedlings (representative picture of \(n = 12\) independent seedlings), stem sections (upper image) and hypocotyl sections (lower image) of C4H (A), RCD1 (B) and MYB13 (C); TE, tracheary element; arrows indicate expression in xylem parenchyma; bars (seedling) = 200 \(\mu\)m; bars (stem/hypocotyl) = 50 \(\mu\)m. (D) Pyrolysis-GC/MS analysis of 2-month old \(Arabidopsis\) hypocotyls of Columbia-0 (Col-0) and Landsberg erecta (Ler) wild-type plants and \(c4h-3,\) \(myb13,\) rcd1-1 and rcd1-2 mutant plants \((n = 3\) replicated experiments). All mutants are in Col-0 background except for \(myb13,\) which is in Ler background. Pyrolysis-GC/MS profiles of the different mutants and wild-type controls (Col-0 and Ler) were compared using heat-map hierarchical clusterization of 54 cell wall-related pyrolysis product peaks according to. \(^{36}\) Heatmap color scale indicates fold changes in specific peak accumulation compared to wild-type (WT).
proteins, such as RCD1 and MYB13, in this process as well.

Conclusion

The differential distribution of lignin in the cell wall and the apoplasm 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 \( \text{H}_2\text{O}_2 \), 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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