Monolignol Ferulate Transferase Introduces Chemically Labile Linkages into the Lignin Backbone

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Bioscience Journal club
04-21-14
Ultimate Goal

Redesigning lignin to be amenable to chemical depolymerization can lower the energy required for industrial processing.

Wilkerson C. G.; Ralph J. et al., Science 344, 90-93 (2014)
“Altered trees make digestible wood”

• This research highlighted by Nature:
  “Genetically engineered poplars can make a modified polymer in their wood that breaks more easily than natural forms. Such trees could one day be sources of biofuels.

... J. Ralph and his colleagues inserted a gene into poplar trees (Populus spp.) that produces a molecule that is incorporated in the lignin polymer chains.”

Nature 508, 153 (10 April 2014)
Introduction: importance of this paper

• They had engineered poplar trees designed for deconstruction by introducing ester linkages into the lignin backbone.

• Monolignol ferulate transferase is capable of forming monolignol ferulate conjugates and evidence for the conjugates incorporation into lignin was shown in this paper.

• Improved deconstruction and increased digestibility was pursued based on the labile cleavage.

• Metabolic plasticity of lignification with a range of phenolic monomers was suggested.
Outline

• Background and approach
• Cloning and expression of monolignol ferulate Transferase (AsFMT): enzyme kinetics
• Evidence for generation of FMT protein in vivo and its incorporation in the production of monolignol ferulate conjugates
• Evidence for incorporation of ferulate conjugates into lignin in cell wall in plants
• Evidence for improved cell wall digestibility
Lignin is a complex phenolic polymer that is essential for plant growth and development.

Issue: altering natural lignification process for deconstruction with minimal input for industrial processing.

Previous studies and observations:
- Introduction of exotic conjugation for novel lignin structure in in vitro model.
- The full compatibility of ferulate with lignification.
- Inherent metabolic plasticity of lignification: various non-monolignol phenolic monomers have been shown to actively participate in lignification.
Basic concept

- Concept of introducing readily cleavable ester bonds into the backbone of the lignin polymer.

![Diagram showing normal lignin chain and engineered lignin chain with a 'zip' introduced into the polymer backbone.](image-url)
Approach:

To engineer a plant to synthesize the monolignol ferulate conjugates with the appropriate temporal and spatial control which also has the ability to transport them to the developing wall; the conjugates would ultimately incorporate into the growing lignin polymer.
Chemical structures of lignin monomers

Primary lignin monomers (hydroxycinnamyl alcohol)

- $R_1= H, R_2= H$ : p-coumaryl alcohol
- $R_1=OCH_3, R_2=H$ : coniferyl alcohol (CA)
- $R_1=OCH_3, R_2=OCH_3$ : sinapyl alcohol (SA)

Feruloyl-coenzyme A

- $R_1=OCH_3, R_2=H$ : CA-FA
- $R_1=OCH_3, R_2=OCH_3$ : SA-FA

Monolignol ferulate conjugates

- $R_1=OCH_3, R_2=H$ : CA-FA
- $R_1=OCH_3, R_2=OCH_3$ : SA-FA
Formation of monoferulate conjugates and incorporation into lignin

Predicted that introducing the FA conjugates would make an improved polymer with more digestible biomass.
Overall scheme

Monolignol-ferulate incorporated into Lignin Polymer (with ‘zip’ introduced into the backbone)

Monolignol-Ferulate Conjugate

Continued Lignification (with further monolignols and monolignol-ferulate conjugates)

The final Lignin Polymer (with ‘zips’)

Lignin Oligomers (Highly cleaved Polymer)
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Preparation of AsFMT for enzyme kinetics

- Gene identification and cloning of feruloyl CoA monolignol transferase from *A. senensis* (AsFMT)
- Heterologous expression and purification of AsFMT from *E. coli*.

→ The *E. coli* expressed FMT enzyme was used to generate kinetic data by using different substrates (e.g. acetyl-CoA, *p*-coumaroyl-CoA, coniferyl alcohol, sinapyl alcohol, etc.) to compare enzyme kinetics.
Enzyme activity assay and kinetic analysis

Table 1. Kinetic data for AsFMT purified from *E. coli*. Michaelis constant \( (K_m) \) and specific activity \( (V_{max}) \) data are calculated from the mean of at least three replicates ± SE. There was essentially no detectable activity with p-coumaroyl-CoA, so data are not given. \( K_{cat} \) is the catalysis rate; 1 nkat = 1 nMol product per second.

<table>
<thead>
<tr>
<th>Varying substrate</th>
<th>Saturating substrate</th>
<th>( K_m \pm SE ) (μM)</th>
<th>( V_{max} \pm SE ) (nkat mg(^{-1}))</th>
<th>( K_{cat} ) (s(^{-1}))</th>
<th>( K_{cat}/K_m ) (μM(^{-1}) s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feruloyl-CoA</td>
<td>Coniferyl alcohol</td>
<td>0.97 ± 0.14</td>
<td>8547 ± 144</td>
<td>426</td>
<td>438.81</td>
</tr>
<tr>
<td>Coniferyl alcohol</td>
<td>Feruloyl-CoA</td>
<td>182 ± 20</td>
<td>8060 ± 298</td>
<td>401</td>
<td>2.21</td>
</tr>
<tr>
<td>Sinapyl alcohol</td>
<td>Feruloyl-CoA</td>
<td>204 ± 31</td>
<td>2212 ± 118</td>
<td>110</td>
<td>0.54</td>
</tr>
<tr>
<td>p-Coumaryl alcohol</td>
<td>Feruloyl-CoA</td>
<td>373 ± 43</td>
<td>14540 ± 721</td>
<td>724</td>
<td>1.94</td>
</tr>
</tbody>
</table>

- Kinetic analysis shows the desired transferase activity: high activity with feruloyl-CoA and low activity with p-coumaroyl–CoA.
- Selectivity to use feruloyl-CoA.
- Three monolignols can be a target substrate.
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Planta expression of FMT

• Preparation of hybrid poplar (populus alba x gradidentata)
• Expression of FMT gene in the tissues of the hybrid plant was driven by universal 3S promoter and CesA8 xylem-specific promoter.
• Tagging the FMT with YFP to show its generation and vascular tissue-specific expression with the CesA8 xylem-specific promoter.
Evidence for generation of the FMT in planta

FMT protein was being produced and facilitated localization for each promoter used.
Evidence for a need for FMT protein

- In vitro synthesis of CA-FA was performed with isolated FMT protein from these transgenic plants.

The target conjugates were produced only with FMT.
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Are ferulate conjugates incorporated into lignin?

- **DFRC:** Derivatization Followed by Reductive Cleavage

- Breaking down the lignin polymer for analysis

Released coniferyl/sinapyl dihydroferulate diacetates (CA-FA and SA-FA) are quantifiable by GC-MS.
Evidence for the incorporation of monolignol ferulate conjugates: GC-MS

- Compounds 8 are diagnostic markers indicating the ferulate conjugates incorporation into lignins.
- Monitoring the released monolignol ferulate conjugates (8) by GC-MS.
- Their transgenic poplars are performing all of the biochemistry and chemistry desired.
Evidence for the incorporation of monolignol ferulate conjugates

- Transgenic CesA8::FMT-6 vs. WT
- All the data for the transgenic poplar-released conjugates match (e.g. r.t, MS, fragmentation) and the diagnostic conjugates were above those of WT at levels.
Estimation of the monolignol ferulate incorporation level

• Isotopically-labeled reagent(*) modification to the DFRC method.

• The expected differentially deuterated compounds 8 (CA-FA)
Determination of etherification profile of CA-FA

- The expected deuterated compounds were confirmed by MS MRM analysis.

- The measured ion ratio determined by GC-MRM/SIM-MS and the MS fragmentation ions from labeled compounds 8G.

- Thus, how well the conjugate incorporation was revealed: 53% of ferulate and coniferyl alcohol in the releasable conjugates.
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Improvement in saccharification

- Utility of these altered lignins is determined by industrial amenability to cell wall deconstruction.
- Substantial improvement in saccharification after mild alkaline pretreatment (6.25 mM NaOH, 90°C, 3 hrs) was shown.

*Fig. 4. Digestibility data on various mild alkaline-pretreated transgenic poplar lines compared to WT. Error bars indicate SD from the mean of triplicate determinations; *P < 0.01; **P < 0.005.*
• Inducing plants to use monolignol ferulate conjugates during lignification turned out to be feasible for deconstruction.

• This supports the prediction that the inherent metabolic plasticity of lignification to alter lignins can be utilized for societal benefit by inducing plants to synthesize novel phenolic monomers.

• Introduction of ester bonds into the lignin polymer backbone portends the production of crop plants with reduced energy and/or chemical inputs.
Complexity of combinatorial radical coupling of coniferyl ferulate (CA-FA)
Needs for FMT enzyme
• Mass spectra of synthetic and authentic compounds 8Ga-d, ions monitored by SIM are colored and the MRM parent ion in bold.

• Chromatograms from GC-MRM-MS of the DFRC products from transgenic Poplar CesA8::FMT-5
• GC-MRM-MS chromatogram of the product ions from DFRC analysis of transgenic Poplar CesA8::FMT-5, using labeled reagents.
Crude estimating CA-FA levels with model system

- To estimate the amount of CA-FA incorporated into the lignin polymer, extrapolation based on the ectopic CA-FA lignigation of corn lignin model system.

CesA8::FMT poplar trees are incorporation ~7 to 23% of ferulate conjugates into their lignin. It is crude estimation; however currently no method exists for better quantification.
**Conjugate incorporation estimation**

**Table S4.** Levels of released DFRC conjugates 8, estimation of %CA-FA incorporated in Poplar lignification, and ratios of the two released conjugates 8.

<table>
<thead>
<tr>
<th>Sample</th>
<th>%CA-FA of KL</th>
<th>%CA-FA monomer (calculated)</th>
<th>CA-FA : SA-FA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn lignin 0%CA-FA</td>
<td>0.00 ± 0.00</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Corn lignin 20%CA-FA</td>
<td>0.59 ± 0.04</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Corn lignin 40%CA-FA</td>
<td>0.84 ± 0.05</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Corn lignin 60%CA-FA</td>
<td>1.35 ± 0.28</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Poplar Wild-type (WT)</td>
<td>0.04</td>
<td>&lt;2</td>
<td>70 : 30</td>
</tr>
<tr>
<td>Poplar CesA8::FMT-5*</td>
<td>0.45</td>
<td>20</td>
<td>70 : 30</td>
</tr>
<tr>
<td>Poplar CesA8::FMT-6</td>
<td>0.41</td>
<td>18</td>
<td>50 : 50</td>
</tr>
<tr>
<td>Poplar CesA8::FMT-7</td>
<td>0.52</td>
<td>23</td>
<td>40 : 60</td>
</tr>
<tr>
<td>Poplar CesA8::FMT-8</td>
<td>0.15</td>
<td>7</td>
<td>70 : 30</td>
</tr>
</tbody>
</table>

*Poor internal standard recovery, SA-FA signal too weak to quantify.

“%CA-FA of KL” is the DFRC-released CA-FA (+SA-FA) level on a Klason Lignin basis.

“%CA-FA monomer” is the estimated for the poplars graphically determined from the corn data.

“CA-FA : SA-FA” is the ion ratio detected by MS from the released DFRC conjugates (CA-FA = 8G, SA-FA = 8S).

Note: CA-FA in columns 1 and 3 indicates the monomer conjugate that went into lignification, i.e., compound 2G; CA-FA in columns 2 and 4 refers to the conjugate released by DFRC, i.e., compound 8G; the two have only two mass units difference.

Note: Now that the sensitivity of our detection method has dramatically improved, there appears to be a (consistent) trace of CA-FA, but not SA-FA, in the WT controls; in the past we were not able to see any evidence for the conjugates in the controls. If it is real, as it appears to be, it is possibly a result of activity from the related (but currently unknown) native p-hydroxybenzoyl-CoA:monolignol transferase in Poplar.