Phenylcoumaran Benzylic Ether Reductase Prevents Accumulation of Compounds Formed under Oxidative Conditions in Poplar Xylem

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Phenylcoumaran benzylic ether reductase (PCBER) is one of the most abundant proteins in poplar (Populus spp) xylem, but its biological role has remained obscure. In this work, metabolite profiling of transgenic poplar trees downregulated in PCBER revealed both the in vivo substrate and product of PCBER. Based on mass spectrometry and NMR data, the substrate was identified as a hexosylated 8–5-coupling product between sinapyl alcohol and guaiacylglycerol, and the product was identified as its benzyl-reduced form. This activity was confirmed in vitro using a purified recombinant PCBER expressed in Escherichia coli. Assays performed on 20 synthetic substrate analogs revealed the enzyme specificity. In addition, the xylem of PCBER-downregulated trees accumulated over 2000-fold higher levels of cysteine adducts of monolignol dimers. These compounds could be generated in vitro by simple oxidative coupling assays involving monolignols and cysteine. Altogether, our data suggest that the function of PCBER is to reduce phenylpropanoid dimers in planta to form antioxidants that protect the plant against oxidative damage. In addition to describing the catalytic activity of one of the most abundant enzymes in wood, we provide experimental evidence for the antioxidant role of a phenylpropanoid coupling product in planta.

INTRODUCTION

Wood is the most abundant renewable natural material on earth and the primary source for timber and pulp and paper. In addition to these traditional uses, wood is increasingly considered as a promising alternative for the production of second generation biofuels. Considering the worldwide importance of wood, it is surprising that the biological role of one of the most abundant proteins in poplar (Populus spp) wood, phenylcoumaran benzylic ether reductase (PCBER) (Vander Mijnsbrugge et al., 2000a), remains to be elucidated. PCBER occurs mostly in young differentiating xylem, xylem parenchyma, and young differentiating phloem fibers (Vander Mijnsbrugge et al., 2000b). It belongs to the pinoresinol-lariciresinol, isoﬂavone, phenylcoumaran benzylic ether reductase family (Min et al., 2003; Davin et al., 2008). Members of this plant-specific family have been shown to catalyze reductive reactions in isoflavonoid, lignan, and neolignan biosynthetic pathways. Isoﬂavonoids are a class of ﬂavonoids primarily present in legumes (Lapcík, 2007), where they are mainly involved in plant defense against pathogens or herbivores. Lignans and neolignans are derived from monolignol coupling, with the term lignan being used for the 8–8- or 5–5-linked monomers, whereas the term neolignan refers to the 8–5– or 5–5–linked monomers (Figure 1).

Whereas lignans and neolignans are a subject of intense research due to their health-promoting activities (Davin et al., 2008; Kim et al., 2010a), their exact function in plant metabolism remains unclear. Lignans have been proposed to have a role in defense against pathogens (Davin et al., 2008; Naoumkina et al., 2010), but varying activities have been reported in different experimental systems. For example, the lignan pinoresinol has been described to have antioxidant activity (Kim et al., 2010a; Leu et al., 2011), to act as an antifungal agent (Hwang et al., 2010), to exert cytotoxicity (Menendez et al., 2008), or to delay molting and induce mortality in insects (Cabral et al., 1999). Most of the evidence for an involvement of lignans in plant defense

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against insects arises from the testing of how compounds isolated from plant species that are resistant to particular insect pests deter feeding (Harmatha and Dinan, 2003). It is likely that the reported differences between the effects of lignans and neolignans are due not only to their structural similarities but also to their redox state. For example, reduced forms of phenolic compounds typically exhibit antioxidant activities, whereas their oxidized counterparts are cytotoxic (Lattanzio et al., 2006). However, despite the wealth of in vitro data that support the antioxidant properties of PCBER, there is no direct evidence to date for such a function in planta.

Poplar PCBER reduces in vitro the 8-5 linkage-associated phenylcoumaran unit of the coniferyl alcohol-derived neolignan dehydrodiconiferyl alcohol (1 DDC) to form the corresponding isohydroxyhydrogenated alcohol (2 IDDC; Figure 1) (Gang et al., 1999). DDC has been shown to accumulate in the culture medium of lignifying zinnia (Zinnia elegans) cell cultures and in developing poplar xylem and has been proposed to be an intermediate in the lignification process (Morreel et al., 2004; Tokunaga et al., 2005). According to other studies, DDC as well as other lignans and neolignans exhibit phytotoxic activities by inhibiting seed germination (Cutillo et al., 2003) or plant growth (Nishiwaki et al., 2005). According to these studies, DDC and its redox products are cytotoxic and inhibiting seed germination (Cutillo et al., 2003) or plant growth (Nishiwaki et al., 2005). On the other hand, the glycosylated form of DDC as well as other lignans and neolignans exhibit phytotoxic activities by inhibiting seed germination (Cutillo et al., 2003) or plant growth (Nishiwaki et al., 2005). According to other studies, DDC as well as other lignans and neolignans exhibit phytotoxic activities by inhibiting seed germination (Cutillo et al., 2003) or plant growth (Nishiwaki et al., 2005). It is likely that the reported differences between the effects of lignans and neolignans are due not only to their structural similarities but also to their redox state. For example, reduced forms of phenolic compounds typically exhibit antioxidant activities, whereas their oxidized counterparts are cytotoxic (Lattanzio et al., 2006). However, despite the wealth of in vitro data that support the antioxidant properties of PCBER, there is no direct evidence to date for such a function in planta.

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Here, we downregulated PCBER in poplar via RNA interference (RNAi) and phenotyped the transgenic lines at the plant, tissue, and molecular levels to uncover the biological role of the protein and unequivocally identify its natural substrate. As we show here, metabolite profiling coupled with a combination of mass spectrometry (MS), NMR, and biochemical techniques revealed the structure of the in vivo substrate and product. Furthermore, metabolite profiling and mass spectrometry also identified the structures of compounds that accumulated over 2000-fold in the PCBER-downregulated poplars and showed that they could be produced in vitro from monolignols in the presence of cysteine under oxidative conditions. Altogether, we propose that PCBER protects xylem cells from the oxidative environment provoked by the lignification process, by reducing products derived from oxidative coupling of monolignols. In addition, we provide experimental evidence for an antioxidant role of a phenylpropanoid coupling product in planta.

RESULTS

Overall Growth and Cell Wall Phenotyping of Transgenic Poplars Downregulated in PCBER

In order to study the in vivo function of the PCBER enzyme, four transgenic poplar lines downregulated in PCBER were produced using RNAi. The relative amount of PCBER produced in the transgenic lines was assessed by immunoblot analysis using polyclonal antibodies raised against the poplar PCBER and was compared with that of the wild type. All four transgenic lines showed severely reduced levels of PCBER protein in xylem (Figure 2). Given that PCBER is a particularly abundant protein in wood, we first investigated whether downregulation had any effect on the visible phenotype. To this end, the stem length of plants grown in the greenhouse was measured on a weekly basis for 15 consecutive weeks and the total fresh weight was recorded at the end of the experiment. No significant differences in growth or biomass production were observed between the wild-type and any of the transgenic lines (Supplemental Figure 1A). No significant difference could be observed either in a similar experiment conducted outdoor in a caged area in Göttingen, Germany (Supplemental Figure 1B).

The downregulation of PCBER was expected to result in a cell wall phenotype because wood consists essentially of cell walls. However, no histological differences in cell wall structure were...
PCBER-RNAi-1, PCBER-RNAi-2, PCBER-RNAi-3, PCBER-RNAi-4: RNAi lines downregulated for PCBER. Each sample is taken from a different individual. Molecular masses (MM) are indicated in kilodaltons. PCBER has a molecular mass of ~36 kD.

observed when stem sections of PCBER-downregulated and wild-type poplar lines were analyzed by transmission electron microscopy (Supplemental Figure 2). Chemical analyses to determine the cellulose (sulfuric acid-phenol method) and lignin (acetylbromide method) contents as well as lignin structure analysis by NMR also did not reveal any significant differences between the wild-type and transgenic trees (Supplemental Figures 3 and 4). Altogether, these data show that downregulation of PCBER does not affect cell wall structure and composition under standard growth conditions.

Molecular Phenotyping

Because PCBER has been described to reduce a phenylcoumaran [1, DDC, alternatively referred to as \(G(\beta-5)G\) in vitro, metabolite profiling of xylem extracts was performed by reversed phase liquid chromatography-Fourier transform-ion cyclotron resonance-mass spectrometry in the negative ionization mode to reveal possible molecular consequences of PCBER downregulation on phenolic metabolism. In total, 3179 peaks were integrated and aligned and were estimated to correspond to at least 290 compounds (Supplemental Methods). Based on both univariate (nested ANOVA; \(\alpha = 0.05\) and multivariate (orthogonal partial least squares discriminant analysis, minimum loading factor = 0.016) statistics, 24% of all peaks, corresponding to ~70 compounds, were significantly different in abundance between the wild-type and transgenic poplars, indicating a role of PCBER in secondary metabolism. The levels of 19 compounds were increased in the transgenic lines whereas 51 others were decreased.

When a subset of these samples (four samples for each of the wild-type and RNAi lines) was analyzed in the positive ionization mode, 2118 peaks that were estimated to correspond to ~180 compounds could be integrated and aligned. From these, seven compounds accumulated in the transgenic lines, whereas five others were strongly depleted with differences greater than 100-fold. Supplemental Table 1 shows a combined list of the most significantly quantitatively affected compounds as judged from conservative thresholds in univariate and multivariate statistics. The corresponding accurate masses, putative chemical formulae, and MS° fragmentation patterns are also provided when available. Interestingly, most of the compounds accumulating in PCBER-downregulated plants contain nitrogen. Several of these also contain sulfur and were putatively identified as cysteine adducts of dilignols. These compounds were barely detectable in the wild-type poplars, but they clearly accumulated in the transgenic trees with differences in abundance in excess of 2000-fold. Two nitrogen-containing compounds with a dilignol backbone were among the most strongly depleted metabolites in the transgenic poplars, with decreased levels 1000- and 500-fold lower than in the wild type.

The previously reported in vitro substrate (1, DDC) and product (2, IDDDC) of PCBER were not present among the affected compounds and were not even detectable in the xylem extracts, suggesting that PCBER has either a different substrate/product or additional ones in vivo. Because our data indicated that downregulating PCBER has many, probably indirect, effects on plant metabolism, and because data in the literature had suggested that the PCBER enzyme is an NADPH-dependent reductase (Gang et al., 1999), we screened for compounds that might be directly derived from such an activity based on their differential abundance in silenced and wild-type plants (Supplemental Methods; Morreel et al., 2014). Only one putative substrate-product pair was identified (Supplemental Table 1), suggesting PCBER activity in planta. The putative substrate was more than 4 times more abundant in the PCBER-downregulated poplars than in the wild type, whereas the product was more than 7 times less abundant in the transgenic trees. Both the putative substrate and product were purified and used for enzymatic activity assays and structural elucidation by NMR.

Elucidation of the PCBER Substrate and Product

The PCBER substrate was annotated as the anion eluting at 9.52 min with \(m/z\) 583.20252 and corresponded to a compound having the formula \(C_{27}H_{36}O_{14}\). This compound is predicted to contain 10 double-bond equivalents (double bonds and/or rings), which indicated a highly conjugated and/or aromatic structure. The accurate masses of the three most abundant first product ions were recorded in the ion cyclotron resonance cell upon MS° fragmentation in the preceding linear ion trap (Figure 3). These three ions were associated with losses of water (\(m/z\) 565.19243), formaldehyde (\(m/z\) 553.19192), and combined water-formaldehyde (\(m/z\) 535.18259), indicative of the presence of a 1,3- or 1,4-diol function in which one of the hydroxyl groups is a primary alcohol (Eklund et al., 2008; Morreel et al., 2010a). Supplemental Figure 5 provides an illustration of the fragmentation pathways, including the structures of the resulting ions.

The MS° spectrum of the first product ion at \(m/z\) 535, which had already lost one hydroxyl via water elimination, also showed a formaldehyde loss (30 D), as is characteristic of the presence of a second primary alcohol (Ye et al., 2005; Eklund et al., 2008; Morreel et al., 2010a). A hexose loss (162 D) was evident from most of the MS^n spectra presented in Figure 3. As no losses of 30, 60, or 90 D were observed, this hexose is likely to be linked at its anomeric position (Carroll et al., 1995). Furthermore, the MS^4
The spectrum of the dehydrated aglycone at m/z 403 showed a major methyl radical loss, yielding the base peak at m/z 388. This is typical for a methyl allyl or methyl aryl ether (Bowie, 1990). The abundance of this peak suggested at least two such groups. The third product ions at m/z 385, 373, and 355 indicated that this dehydrated aglycone had lost the diol moiety. A third product ion at m/z 237, which was reminiscent of the 1,2B⁻ ion resulting from an interunit cleavage mechanism typical of phenylcoumaran-type neolignans, was also clearly present (Morreel et al., 2010a). The latter ion indicated that the diol function was present on the aliphatic end group of the neolignan. Taking the accurate mass into account, the aglycone structure was resolved as S(8–5)G in which the side chain of the guaiacyl end group was altered to a glycerol, for convenience denoted as S(8–5)Gglycerol, i.e., compound 32 (Supplemental Figure 6). As PCBER was not able to reduce phenylcoumarans that carry a hexose on the phenolic end-group (see below) and because both primary alcohol functions were shown to cleave off before the hexose loss in the gas phase, the hexose is likely attached to the 7–O- or 8–O-position of the glycerol end group. The 7–O-hexoside, assumed to be the most likely candidate, corresponds to compound 34 in Supplemental Figure 6.

The peak representing the PCBER product had a retention time of 6.68 min and an m/z of 585.21814, corresponding to

Figure 3. Elucidation of the Structure of the PCBER in Vivo Substrate by MS^n.

Accurate mass data of the substrate, fragmentation patterns of the first and second product ions, and a proposed structure of the substrate are presented.
a compound having a chemical formula of C₂₃H₂₄O₁₄. The MS² spectra showed essentially the same neutral losses as observed for the PCBER substrate (Supplemental Figure 7), resulting in product ions with an m/z value increased by 2 D. The presence of the 1,2-B₂– third product ion at m/z 237 in the MS⁴ spectrum of m/z 405 indicated that the aliphatic end group was unchanged and that the reduction was in the phenylcoumaran ring. Therefore, the structure of the aglycone was elucidated as the reduced form of S(8–5)G in which the side chain of the guaiacyl end group is altered to a glycerol, i.e., the reduced form of S(8–5) Gglycerol (compound 33 in Supplemental Figure 6). By analogy with the PCBER substrate, the hexose is likely connected to the 7–O- or 8–O-position of the glycerol end group. The 7–O-hexoside is, again, assumed to be the most likely candidate, corresponding to compound 35 in Supplemental Figure 6.

**NMR Analysis of the PCBER Product and Its Validation**

The PCBER product isolated from poplar xylem was also subjected to an independent structural elucidation by NMR using a sensitive 600-MHz instrument fitted with a microcryoprobe. The proton spectrum was difficult to analyze even with water suppression, but complementary 2D-NMR experiments (correlation spectroscopy, heteronuclear single quantum coherence [HSQC], and heteronuclear single quantum coherence-total correlation spectroscopy [HSQC-TOCSY]) strongly suggested that the product was a benzyl-reduced 8–5-dimer, formally from sinapyl alcohol and guaiacylglycerol (see structures in Figure 4).

The aromatic ring substitutions were determined by the single cross-polarization spectroscopy [HSQC-TOCSY] spectrum (Figure 4C). The pair of Cα correlations indicates their belonging to a benzylic CH₂ group. The nature of the D-ring moiety of the side chain had the characteristic signature of a glycerol, as observed previously (DellaGreca et al., 1998; Matsunaga et al., 2004; Gan et al., 2008), and as could be seen by comparison to the glycerol model compounds we synthesized (Ralph et al., 2006; Moreel et al., 2010b; Yue et al., 2012). Chemical synthesis of the assumed substrate and product proved to be particularly difficult. Indeed, very low yields are typically obtained for products of syringyl coupling between a syringyl unit and a guaiacyl unit. In addition, the removal of protecting groups following the production of the glycerol side chain was particularly difficult. Nevertheless, a mixture of the two compounds 32 and 33 (and other minor products) was synthesized and their structures were confirmed by detailed NMR experiments and by liquid chromatography-mass spectrometry. The data from the synthesized benzyl-reduced model (containing rings C and D; Figure 4B) perfectly matched those from the isolated PCBER product in Figure 4A, proving that the major aromatic component of that isolate was the benzyl-reduced 8–5-crossed dimer from sinapyl alcohol and guaiacylglycerol, i.e., compound 33 in Supplemental Figure 6.

It is noteworthy that structural determination by NMR was conducted independently of the MS analysis and resulted in the same structure. However, the product identified by NMR is the aglycone 33 instead of compound 35 suggested by ultra-performance liquid chromatography-mass spectrometry studies. This is probably because the fraction analyzed by NMR contained a very low amount of the glycosylated product, whereas the aglycone was present in sufficient quantity for NMR analysis (see also product purification in Methods).

**Enzymatic Activity Assays**

PCBER was heterologously produced in *Escherichia coli* to complement the results obtained by metabolite profiling with data from in vitro assays. First, we investigated whether the previously described in vitro substrate, DDC 1, which was below the detection limit in the poplar samples, could be used as a substrate by the recombinant PCBER. To this end, chemically synthesized DDC was added to the enzymatic assay. After incubation, the reduced form (IDDDC 2) was indeed produced (Table 1). Control assays in which NADPH was omitted did not result in DDC reduction, confirming that PCBER requires NADPH or an equivalent cofactor.

In an effort to confirm the in vivo substrate-product pair identified by molecular phenotyping, we first incubated methanol extracts from wild-type and PCBER-downregulated poplars with the recombinant PCBER enzyme. The amount of putative product increased in the reaction mixture in the presence of NADPH, whereas the levels of putative substrate decreased proportionally (Figure 5A). These data support the hypothesis that the putative substrate-product pair identified by molecular phenotyping corresponded to those involved in PCBER enzymatic activity. Importantly, none of the other compounds accumulating in the PCBER-downregulated poplars were reduced by the recombinant enzyme. Subsequently, enzymatic assays were performed with the purified substrate (m/z 583). As shown in Figure 5B, this compound was fully converted into the expected product (m/z 585) upon incubation with the heterologously produced PCBER and NADPH, thereby confirming the identity of the substrate and product pair.

A number of structurally related compounds synthesized chemically were tested as alternative substrates of PCBER to shed light on the enzyme specificity (Table 1; Supplemental Figure 6 for structures). Compound S(8–5)G 5 was reduced by PCBER, further supporting our observation that PCBER is able to reduce the phenylcoumaran in coupling products containing both S and G units. Compound 9, H(8–5)H, does not contain any methoxyl group on either of the aromatic rings, but was also reduced by PCBER. This, together with the previous results, indicates that the enzyme is able to recognize the phenylcoumaran unit, but that it is not specific for any particular ring substitution (or at least the ones likely to be found in planta [H, G, and S]). Furthermore, the enzyme was able to reduce 8–5-linked tetramers, such as compounds G(8–5)G(8–5)G(8–5)G 17 and G(8–5)G(4–O–)G(5–5)G(8–5)G 19, indicating that further 5-substitution on the aromatic A-ring, due to the radical coupling reactions, is also accommodated.

Compounds G(8–5)Gglycerol 3, G(8–5)FA 7, and dihydrodehydrodiconiferyl alcohol (DDDC) 11, which have modifications on the B-ring side chain, were also reduced by the enzyme,
Figure 4. Elucidating the Structure of the PCBER in Vivo Product by NMR.
2D $^1$H-$^1$C correlation (HSQC and HSQC-TOCSY) NMR experiments in D$_2$O were performed using 600- and 500-MHz cryoprobe-equipped NMRs.
showing that PCBER can accommodate modifications to the side chains of the 5-coupled moiety in 8–5-coupled compounds. Compounds S(8–O–4)G(8–5)G 27 and G(8–5)G glucose 28, on the other hand, were not reduced by the enzyme. In these compounds, the phenol function of the G(8–5)G is unavailable to the enzyme, indicating more conclusively that a free phenolic function is necessary for the enzyme to operate on the substrate. PCBER was also unable to reduce compound Me-pCA (8–5)Me-pCA 29, which has modifications in the phenylcoumaran side chain, i.e., the 8-linked moiety in the 8–5-structure, and also in the 5-linked moiety. Compounds S(8–5)FA-Et 13 and Et-FA(8–5)G 15, which only have one of the two above-mentioned moieties modified, were reduced by PCBER, indicating that the enzyme can tolerate modifications on one of the moieties. However, modifying both moieties together (as in compound 29) perhaps changes the shape of the resulting compound to an extent that prevents its optimal binding to the active site of the enzyme.

Compounds S(8–S) 21, G(8–8)G 23, and H(8–8)H 24 were tested to see whether the enzyme was able to accept 8–8-coupled products as substrates. Only S(8–S) 21 was reduced, but not G(8–8)G 23 or H(8–8)H 24, indicating that the enzyme was not totally specific for phenylcoumaran structures. Compounds G(8–O–4)G 25, G(8–O–4)G hexose 26, C(8–O–4)C 30, and G(8–O–4)CA-Me 31 were used to test whether the enzyme was able to accept 8–O–4-coupled products as substrates (whether they are normal aliphatic β-aryl ethers or cyclic benzoxazoxines). The β-aryl ethers and benzoxazoxines were not reduced, indicating at least some level of enzyme specificity. From these data, it can be concluded that PCBER primarily, but not completely specifically, reduces 8–5-coupled products with a free phenolic function and that it can accommodate various degrees of aromatic ring methoxylation and other 5-substitution (resulting from radical coupling) as well as modifications in the A- and B-ring side chains. PCBER is therefore quite flexible in its substrate tolerance, and these findings or requirements are all

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**Table 1. PCBER Enzyme Assays with Substrate Analogs**

<table>
<thead>
<tr>
<th>No.</th>
<th>Shorthand Name [Trivial Name]</th>
<th>m/z</th>
<th>Activity Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>G(8–5)G [DDC]</td>
<td>357</td>
<td>++</td>
</tr>
<tr>
<td>3</td>
<td>G(8–5)Gglycerol</td>
<td>391</td>
<td>++</td>
</tr>
<tr>
<td>5</td>
<td>S(8–5)G</td>
<td>387</td>
<td>++</td>
</tr>
<tr>
<td>7</td>
<td>G(8–5)FA</td>
<td>371</td>
<td>++</td>
</tr>
<tr>
<td>9</td>
<td>H(8–5)H</td>
<td>297</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>G(8–5)DHCA [DDDC]</td>
<td>359</td>
<td>++</td>
</tr>
<tr>
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<td>S(8–5)FA-Et</td>
<td>429</td>
<td>++</td>
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<td>Et-FA(8–5)G</td>
<td>399</td>
<td>+</td>
</tr>
<tr>
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<td>713</td>
<td>+</td>
</tr>
<tr>
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See Supplemental Figure 6 for the structures corresponding with the compound number (No.). The m/z value for the precursor ion is based on negative ion MS. The shorthand name is based on Moreel et al. (2004). Units derived from p-coumaryl alcohol, caffeoyl alcohol, coniferyl alcohol, sinapyl alcohol, dihydroconiferyl alcohol, p-coumaric acid, caffeic acid, and ferulic acid are indicated as H, G, C, S, DHCA, pCA, CA, and FA, respectively. Hexosylation, methylation, or ethylation of the units is indicated as Hex, Et, or Me, respectively. A side chain representing a glycerol moeity is indicated in superscript. +, PCBER reduces the compound and the product peak is <10% of the substrate peak; ++, PCBER reduces the compound and the product peak is >10% of the substrate peak; –, PCBER does not reduce the compound; DDC, dehydrodiconiferyl alcohol; DDDC, dihydrodehydrodiconiferyl alcohol.
compatible with the proposed structures of the in vivo substrate and product.

**MS Spectral Evidence for Cysteine-Dilignol Adducts**

As stated above, the PCBER-downregulated poplars accumulated putative cysteine adducts of dilignols, which were identified by MS in the positive ionization mode (three and two peaks with 510.17920 and 540.18990 as m/z values corresponding with the chemical formulae C_{24}H_{32}O_{9}NS and C_{26}H_{34}O_{10}NS, respectively). Ionization in the negative mode was performed to elucidate the structure of the adducts. This was necessary because the positive-mode ionization of phenylpropanoid coupling products is not well described in the literature. Two anions that elute at 10.2 and 11.3 min were observed, with m/z values of 508 and 538, respectively. These parent ions yielded isotopic peaks at m/z 510 and 540 with relative abundances of —4.0 to 4.5% of the parent peak, confirming the presence of sulfur in both anion structures. Because the nominal m/z values were even, this also indicated the presence of an odd number of nitrogen atoms in their chemical formulas. Additional information was obtained from their MS² spectra, which were similar for the two anions. In the case of the precursor ion with m/z 508, collision-induced dissociation lead to four major product ions at m/z 220, 250, 328, and 387 (Supplemental Figure 6). The latter product ion arose from a neutral loss of 121 D, indicating a cysteine residue. The fragmentation pattern is also typical of β-aryl ethers (Moreel et al., 2010a); therefore, this compound can be annotated as S²⁷⁸(8-O–4)G. Analogously, analysis of the MS² spectra of the precursor ion with m/z 538 identified it as S²⁷⁸(8-O–4)S. Notably, no peak indicative of a compound with a fragmentation pattern corresponding to cysteine coupled to either 8–5- or 8–8-linked dilignols could be found, suggesting that the 8–O–4-linked quinone methide intermediate (following radical coupling) is more prone to cysteine addition. This situation is expected as cysteine addition competes with water addition in the 8–O–4-linked intermediate, but must compete with the faster internal trapping of the quinone methide in the 8–5- or 8–8-linked intermediates. Figure 5 shows how the cysteine adducts of dilignols might be formed.

**Oxidative Coupling Assays**

We hypothesized that the in planta synthesis of S²⁷⁸(8-O–4)G and S²⁷⁸(8-O–4)S is the result of a nucleophilic attack of cysteine on the quinone methide intermediate resulting from the radical coupling reaction during dilignol formation. In order to test this hypothesis, oxidative coupling assays were performed in vitro. Horseradish peroxidase was used because it is known to mediate oxidative coupling of monolignols (Freudenberg et al., 1958; Freudenberg and Neish, 1968). When monolignols and cysteine were incubated with horseradish peroxidase in the presence of H₂O₂, compounds were formed that had the same masses and fragmentation patterns in the positive-mode ionization as the putative cysteine-dilignol adducts that accumulated in the PCBER-downregulated poplars (Figure 7). When any of the components (i.e., cysteine, horseradish peroxidase, or H₂O₂) were omitted, these products were not formed. In agreement with the molecular phenotyping data, we could not find compounds with fragmentation patterns corresponding to cysteine coupled to either 8–5- or 8–8-linked dilignols, even though these dilignols are known to be well represented in the mixtures resulting from such in vitro coupling reactions (Katayama and Fukuzumi, 1978).

**Amino Acid Analysis**

To investigate whether the elevated cysteine dilignol adducts could have been caused by higher cysteine levels in the cell, we performed amino acid quantification by gas chromatography-mass spectrometry and liquid chromatography-ultraviolet spectroscopy (Supplemental Tables 2 and 3). However, no significant
differences in amino acid levels could be observed between wild-type and PCBER-downregulated poplars.

**DISCUSSION**

**PCBER Affects Secondary Metabolism**

One of the most abundant proteins in poplar wood, PCBER, has previously been proposed to reduce the phenylcoumaran G(8-5)G (1, DDC), based on in vitro enzymatic activity assays (Gang et al., 1999). Knowing that coniferyl alcohol is one of the main monomers of lignin, that lignification is one of the most important processes in wood development, and that PCBER is among the most abundant proteins in poplar xylem (Vander Mijnsbrugge et al., 2000b), and is localized mainly in cells that are about to undergo lignification (Vander Mijnsbrugge et al., 2000a), strongly suggested a role for PCBER in lignification. However, it has been difficult to determine exactly what role it might play, particularly given that benzyl-reduced compounds are unknown in lignins.

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Figure 6. Cysteine-Dilignol Adduct Formation.
Proposed mechanism for the formation of cysteine adducts of dilignols by cysteine trapping of the quinone-methide intermediary (right) as opposed to water trapping (left).
Reversed phase liquid chromatography-positive ion-mass spectrometry chromatograms representing the m/z ranges 509.5 to 510.5 and 539.5 to 540.5. Chromatograms were obtained from the phenolic profiling of xylem extracts from PCBER-downregulated poplars are shown. The insert MS² spectra are derived from the phenolic profiling of the transgenic poplars. The two MS² spectra at the bottom were obtained from the liquid chromatography-mass spectrometry analysis of the oxidative coupling assays. Peaks that were also observed in the latter chromatogram are shown in dark gray.

Figure 7. Dilignol Cysteine Adducts.
from dicots. Our phenotypic analysis of PCBER-downregulated plants showed that PCBER was not essential for plant growth and development under normal growth conditions, nor for cell wall biosynthesis and lignification, as evidenced by chemical analyses of the cell wall NMR and electron microscopy experiments. On the other hand, phenolic profiling of xylem tissues of PCBER-downregulated trees by reversed phase liquid chromatography-Fourier transform-ion cyclotron resonance mass spectrometry revealed that ~24% of all the compounds detected were differentially abundant compared with the wild type, indicating a strong effect on secondary metabolism.

**S(8–5)Gglycerol Glycoside, and/or Its Aglycone, Is a Natural Substrate for PCBER**

The first evidence for the in vivo activity of this enzyme was the presence of a possible substrate-product pair revealed by comparative metabolic phenotyping of wild-type and PCBER-downregulated poplars. The existence of this substrate-product pair was confirmed by enzymatic activity assays using a recombinant PCBER. A combination of MS and NMR analysis enabled us to propose structures for both substrate and product. The in planta PCBER substrate was annotated as a S(8–5)Gglycerol glycoside, with the glycosyl moiety attached to the glycerol side chain at the 7–O- or 8–O-position (Figure 3). The aglycone structure of the product was authenticated by comparison with NMR data from a synthesized standard. We assume that either the glycoside or its aglycone (or both) is (are) in the in planta substrate(s) and that both the phenoxybenzyl unit and/or the reduced aglycone can be glycosylated in planta. This clearly demonstrates that such a compound exists in plants and also that an in vivo activity is shown for PCBER.

Furthermore, enzymatic activity assays with synthetic substrates showed that PCBER is able to reduce several hydroxycinnamyl alcohol or hydroxycinnamate coupling products containing H, G, or S units (Table 1; Supplemental Figure 6). Our data demonstrate that poplar PCBER can reduce coupling products with different degrees of methoxylation and other 5-substitutions and also accommodates modifications of the side chains such as hydroxylation in the case of G(8–5)Gglycerol (and hydroxylation and glycosylation in the case of the in planta substrate) and even oxidized side chains, e.g., in G(8–5)FA in the phenylicoumaran unit. The fact that the enzyme was neither able to reduce the S(8–O–4)G(8–5)G trimer nor the G(8–5)G phenolic glucoside strongly implies that a free phenolic function is necessary for the PCBER-catalyzed reduction to occur. This supports the proposed reaction mechanism for PCBER involving a conjugated-enone intermediate (Gang et al., 1999). Furthermore, the proposed structure of the in vivo substrate, which also has a free phenolic function and modifications in one of the side chains, is in agreement with our new in vitro data. The degree of promiscuity shown in vitro by PCBER suggests that it has a general function in reducing various products that arise from the oxidative coupling of monolignols. It is possible that, depending on the particular cellular conditions, different oxidative coupling products are generated and subsequently reduced by PCBER. Because PCBER is a cytoplasmic enzyme (Vander Mijnsbrugge et al., 2000a), our observations further imply that 8–5-coupled dimers are present in the cytoplasm.

**The Possible Origin of the PCBER Substrate S(8–5)Gglycerol Glycoside**

The structure of the S(8–5)Gglycerol aglycone indicates that it is made from 8–5 oxidative coupling of a sinapyl alcohol radical with the radical from a coniferyl alcohol-derived phenolic unit, suggesting that the cells in which it is formed have the ability to oxidize monolignols and other phenolics to their radicals. It has been proposed that during xylem lignification the majority of monomers are supplied by the adjacent parenchyma cells (Hosokawa et al., 2001). This was supported by Pesquet et al. (2013) who showed that, in zinnia, the lignification of tracheary elements occurs post mortem. The parenchyma cells express the lignin monomer biosynthetic genes and have the capacity to produce and export lignin monomers into the apoplast (Pesquet et al., 2013). Another study showed that lignin deposition is not exclusively a post mortem event, but also occurs prior to cell death, and that parenchyma cells contribute to lignin deposition in the xylem, whereas lignification of fibers is cell autonomous (Smith et al., 2013). The lignification process itself requires H2O2 as a cofactor for peroxidases, and parenchyma-like cells are the main sites of H2O2 production in zinnia cell cultures (Gómez Ros et al., 2006).

The same study showed that both lignifying and nonlignifying cells live in a high H2O2 environment during the transdifferentiation process. Furthermore, xylem parenchyma cells were shown to be the main source of H2O2 necessary for lignification in planta (Ros Barceló, 2005). H2O2 is relatively stable and is able to pass through cell membranes either by diffusion or via aquaporins. It is therefore likely that it is present in substantial amounts in cells adjacent to lignification sites. By itself, toxicity of H2O2 is low, but in the presence of transition metals, a Fenton-type reaction occurs that produces hydroxyl radicals (OH•), the most reactive oxygen species (Sakihama et al., 2002). Altogether, these observations strongly suggest that xylem parenchyma cells, but also vessels and fibers that have not completed their programmed cell death process, have an oxidative intracellular environment during the time the biosynthesis of monolignols takes place. Together with the presence of intracellular peroxidases (class I peroxidases), this results in the oxidation of monolignols to monolignol radicals and their subsequent combinatorial coupling to dilignols, among which is S(8–5)G, in the cytosol. Under oxidative conditions, the cinnamyl alcohol end of these dilignols can be oxidized to form arylglycerols, as we have demonstrated in vitro (Ralph et al., 2006; Supplemental Figure 9); for example, the cinnamyl alcohol side chain in DDC becomes oxidized to the glycerol, in low yield, simply by H2O2 in vitro. Attack by a hexose on the epoxide that is the likely intermediate in the H2O2 oxidation of the cinnamyl alcohol side chain, may give rise to the in planta substrate of PCBER (Figure 8). Alternatively, the hexosylated substrate of PCBER could be formed by glycosylation of the arylglycerol of S(8–5)G. Together, these studies support our contention that the PCBER substrate, S(8–5)Gglycerol glycoside, is formed spontaneously as a consequence of the oxidative environment in lignifying cells.
PCBER Protects Xylem Cells against Lignification-Induced Oxidative Damage

*PCBER*-downregulated poplars accumulated cysteine-dilignol adducts at more than 2000-fold their wild-type control levels. Notably, cysteine adducts were only observed for the 8-O-4-dilignols, but not for 8-5- and 8-8-coupled dilignols. The enzymatic assays did not give any indication that the cysteine-dilignol adducts could be reduced or modified in other ways by the PCBER enzyme (data not shown). They are therefore secondary consequences of the PCBER downregulation. How then can we explain their synthesis and accumulation? As we have demonstrated, the cysteine-dilignol adducts can be formed by peroxidase-mediated oxidative coupling in the presence of cysteine in vitro (again providing compelling evidence that xylem cells of *PCBER*-downregulated poplars are in an oxidative environment); monolignol radicals couple by one of three coupling modes to produce quinone methides, of which only the 8-O-4-bonded quinone methide is rearomatized by trapping with an external nucleophile such as water or, in the case of *PCBER*-downregulated plants, by cysteine.

Why then do these cysteine adducts accumulate in *PCBER*-downregulated poplars? It is possible that reduced phenylpropanoid coupling products are generally better radical scavengers than nonreduced ones, as was shown for reduced 8-8-coupling products (Masuda et al., 2010). Therefore, by reducing phenylpropanoid coupling products, PCBER might increase the radical scavenging capacity of the cytosol, hence preventing other oxidative coupling products, such as the cysteine adducts, from forming (Figure 9). In addition to trapping of the quinone methide by cysteine from the cytosol, the dilignol quinone methides may also trap cysteine units that are part of peptides and proteins, potentially disturbing their function. Indeed, it was recently demonstrated that amino acids and proteins couple to monolignol dimers and lignin under oxidative conditions in vitro (Cong et al., 2013; Diehl et al., 2014). Further research might determine whether dilignol adducts with peptides and proteins are present in the *PCBER*-downregulated poplars.

If the reduced dilignols are radical scavengers, they must be oxidized back to the PCBER substrate, forming an antioxidant cycle such as the well known glutathione cycle, as shown in Figure 9. Zanarotti (1982) originally showed that phenolics with an benzylic-CH$_2$ group could form the quinone methides, via radical disproportionation as shown in Supplemental Figure 10 (Naphl et al., 2009). In the case of 8-5-coupled products, the quinone methide formed is exactly the same intermediate moiety as produced by oxidative coupling of a monolignol (at its 8-position) with a phenolic guaiacyl unit, i.e., the same as that produced during lignification. Such quinone methides are rearomatized by internal-phenol trapping to form the five-membered-ring phenylcoumaran...
Figure 9. Proposed Function for PCBER in Protecting Plant Cells against Lignification-Induced Oxidative Stress.

Monolignol biosynthesis produces monolignols inside the cytoplasm of living cells (1). Most monolignols are exported to the apoplast (2) where they are oxidized by peroxidases (POX) and then polymerized into lignin (3). Some monolignols couple inside the cytosol (4). In the presence of PCBER, the monolignol coupling products are reduced and in turn neutralize cytosolic H₂O₂ (5). In the absence of PCBER (6), H₂O₂ that is not consumed by the oxidation of the reduced dilignols is consumed by the oxidation of monolignols instead, promoting the formation of cysteine adducts (7). R = hexose or hydroxyl group. [●], H₂O₂ not consumed by oxidation of reduced (8–5) dilignol, but of monolignols instead.
structure. We therefore hypothesize that the PCBER-generated reduced product could and would be cycled back to the original phenylcoumaran under radical-generating oxidative conditions, i.e., POX-H₂O₂. We demonstrated here (Supplemental Figure 11) that this indeed occurs using an analog of compounds 6 and 33 in which the upper-ring side chain is simply a propanol group for simplicity (and improved stability). Thus, compound 12 (Supplemental Figure 6), an analog of 6 and 33, forms the parent phenylcoumaran 11, an analog of 5 and 32, in vitro using POX-H₂O₂. To our knowledge, this is the first demonstration of such a reaction and is particularly relevant here as it provides a pathway to effectively scavenge H₂O₂.

In addition to the cysteine-dilignol adducts, other nitrogen-containing compounds accumulated in PCBER-downregulated poplar (Supplemental Table 1). These compounds, however, did not contain sulfur and their fragmentation was also inconsistent with the presence of a cysteine or any other amino acid residue, so they remain unidentified to date. Given their high carbon-to-hydrogen ratios and neutral losses corresponding to a p-hydroxybenzoic acid (−120 D and −138 D) (Supplemental Table 1), these compounds probably contain phenolic groups. A way by which nitrogen-containing secondary metabolites can be formed under oxidative conditions is through nitration. Peroxidase-mediated nitration of simple phenolics (such as p-coumaric acid) has been demonstrated in vitro (Sakihama et al., 2003) and has been proposed to be involved in reactive oxygen species and reactive nitrogen species detoxification in vivo. It is therefore possible that higher molecular mass phenol-containing secondary metabolites perform a similar function and that they accumulate in PCBER-downregulated plants as a consequence of oxidative stress.

Taken together, our data reveal a protective function for PCBER against oxidative damage. Figure 9 provides a conceptual scheme of the proposed pathways involving monolignol coupling products under oxidative conditions and the role PCBER might play. Due to the intracellular oxidative environment during lignification, a fraction of the monolignols is oxidized, coupling to dilignols. These dilignols are reduced by PCBER to generate products that can scavenge excess H₂O₂, in this way preventing oxidative damage, as demonstrated by the accumulation of cysteine adducts of dilignols when PCBER is downregulated. The oxidized dilignols can in their turn be reduced by the abundantly present PCBER, making an effective radical scavenging cycle. Thus, PCBER makes a virtue of necessity; dilignols that are themselves products from the damaging oxidative environment are turned into radical scavenging molecules by the action of PCBER. This provides evidence supporting a role for reduced phenylpropanoid coupling products acting against oxidative damage in planta. It is possible that related proteins, such as pinoresinol reductases, perform similar functions to PCBER.

METHODS

Plant Material

An RNAi construct to downregulate PCBER expression in poplar (Populus tremula × tremuloides) was generated as follows: the primers 5′-GGG-GACCACTTTTGTACAAAGAAGCTGGTCTCCGAGATGGCAATGCCAAG-3′ and 5′-GGGGACAGTTTTGTACAAAAAAGCAGGCTTTGAGAGCAGGAAGACGAGCAGG-GATGCGCAG-3′ were used to amplify a 415-bp fragment of the PCBER coding sequence from P. tremula × tremuloides cDNA. The resulting fragment was cloned into the Gateway destination vector pK7GWG2(I) used for RNAi downregulation (Karimi et al., 2002). A BLAST search of the PCBER-RNAi construct sequence against the T98 raw genome sequence (UPSC draft genome release; http://popgenie.org) revealed four sequences with significant similarity to the construct (scaffold_3197, scaffold_5833, scaffold_548, and scaffold_469). The corresponding gene models all belong to the PCBER family (PCBER1, PCBER3, PCBER5, and PCBER7). PCBER1 (the target) and PCBER7 are similar enough that they may be both downregulated by the construct. There were no significant hits outside the PCBER gene family; therefore, it is unlikely that the construct will downregulate unintended targets. P. tremula × tremuloides clone T98 was transformed by Agrobacterium tumefaciens-mediated transformation as previously described (Nilsson et al., 1992). Four independent transgenic lines downregulated for PCBER were obtained (PCBER-RNAi-1, PCBER-RNAi-2, PCBER-RNAi-3, and PCBER-RNAi-4).

Transgenic poplars downregulated for PCBER and the wild type were micropropagated in vitro and then transferred to the greenhouse (21°C, 60% humidity, 16-h-light/8-h-dark regime, 40 to 60 μmol m⁻² s⁻¹ photosynthetic photon flux) as previously described (Moree et al., 2010b). At least 10 ramets (vegetatively propagated plants) of each of the four RNAi-downregulated PCBER lines and 31 wild-type poplars were grown for 3 months, after which the stems were cut at 10 cm above soil level and harvested. At that time, the trees were ~2 m tall. From each tree, a 40-cm-long piece from the basal part of the stem was debarked, immediately flash frozen in liquid nitrogen, and stored at −70°C until used for all analysis except compound isolation. After harvesting, the tree stumps were watered to allow the development of new shoots. Only the largest shoot for each plant was allowed to grow, whereas the other shoots were removed 3 weeks after harvesting. The poplars were grown for another 4 months, and the stems harvested in the same way as described above. This material was used for compound isolation and purification.

Xylem Tissue Preparation for Protein and Metabolite Extraction

Young developing xylem was scraped (~0.5 cm deep) with a scalpel while frozen, from each of the 40-cm-long debarked stems harvested from the greenhouse grown poplars. The material was then ground to a fine powder in a mortar cooled with liquid nitrogen and immediately processed or stored at −70°C.

Immunoblot Analysis

Proteins were extracted from 500 μL of the ground poplar xylem with 300 μL extraction buffer containing 50 mM Tris-HCl, pH 7.4, 80 mM NaCl, 10% glycerol, 0.1% Tween 20, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 50 μM MG132 (Sigma-Aldrich), and complete protease inhibitor (Roche). After centrifugation at 4°C (1400 rpm in an Eppendorf centrifuge 5417R) for 15 min, the supernatant was collected. Total proteins (20 μg) were separated on SDS-PAGE gel according to standard procedures (Laemmli, 1970). The separated proteins were transferred by wet blotting onto a polyvinylidene fluoride membrane (Millipore). Immunodetection was according to standard procedures using a polyclonal antibody raised against recombinant poplar PCBER (Vander Mijnsbrugge et al., 2000a).

Expression of PCBER Recombinant Protein in Escherichia coli

An available clone containing the poplar (Populus trichocarpa) PCBER cDNA in a Bluescript II SK—vector (Gang et al., 1999) was used as template for PCR-based cloning. Recombinant PCBER was expressed in the E. coli strain BL21 codon + plC2a that was transformed with the pLH36 PCBER in which expression is induced by isopropyl β-D-thiogalactopyranoside under control of a pL-promotor developed by the Protein Service Facility of VIB (WO 98/48025, WO 04/074488). The pLH36 plasmid is provided with
a His$_6$-tag followed by a murine caspase-3 site. The murine caspase-3 site can be used for the removal of the His$_6$-tag attached at the N terminus of the protein of interest during purification. The transformed bacteria were grown in Luria Bertani medium supplemented with ampicillin (100 µg/mL) and kanamycin (50 µg/mL) overnight at 28°C before 1/100 inoculation in a 20 liters of fermenter provided with Luria Bertani medium supplemented with ampicillin (100 µg/mL) and 1% glycerol. The initial stirring and airflow was 200 rpm and 1.5 L/min, respectively. Further, this was automatically adapted to keep the pO$_2$ at 30%. The temperature was kept at 28°C. The cells were grown to an optical density of $A_{600nm} = 1.0$, transferred to 20°C, and expression was induced by addition of 1 mM isopropyl β-D-thiogalactopyranoside overnight. Cells were then harvested and frozen at −20°C. After thawing, the cells were resuspended at 3 mL/g in 20 mM Na$_2$HPO$_4$, pH 7.4, 500 mM NaCl, 20 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, and 0.1% emigten (Merck). The cytoplasmatic fraction was prepared by sonication of the cells followed by centrifugation at 18,000 rpm for 30 min. All steps were conducted at 4°C. The clear supernatant was applied to a 60 mL Ni-Sepharose 6 FF column (GE Healthcare), equilibrated with 20 mM NaH$_2$PO$_4$, pH 7.4, 500 mM NaCl, 20 mM imidazole, and 0.1% emigten. The column was eluted with 20 mM Na$_2$HPO$_4$, pH 7.4, 20 mM NaCl, 400 mM imidazole, and 0.1% emigten after an extra wash step with 50 mM imidazole in the same buffer. The elution fraction was diluted 1/20 with 20 mM Tris, pH 8.5, and 0.1% emigten and loaded on a 20-mL Source 15Q column (GE Healthcare) to remove contaminants. After equilibration, the protein of interest was eluted by a linear gradient over 20 column volumes of NaCl from 0 to 1 M in 20 mM Tris, pH 8.5, and 0.1% emigten. To the PCBER-containing fractions, activated murine caspase-3 (1/100/10% murine caspase-3/PCBER) with 10 mM DTT was added to remove the His$_6$-tag. After 1 h incubation at 37°C, the reaction solution was reloaded on a Ni-Sepharose 6 FF column to capture the His$_6$-tag that was attached to PCBER and the murine caspase-3 that was also provided with a His$_6$-tag. PCBER without fusion tag stayed in the flow-through of the column. Finally, the recombinant protein was injected on a HiLoad 26/60 Superdex prep grade with PBS as running solution for formulation and to remove minor contaminants. The obtained fractions were analyzed by SDS-PAGE and the concentration was determined using the Micro-BCA assay (Pierce).

**Enzymatic Activity Assays**

Enzymatic activity assays with G8-(S)G (1, DDC; Figure 1) and plant extracts were performed according to Gang et al. (1999). Each assay used 100 µg of PCBER recombinant protein, the substrate to be tested (either 2.5 mM DDC, lyophilized methanol extract, or purified fractions) and 10 mM DTT in 150 µL PBS final volume. The reactions were initiated by adding NADPH (from Merck) to a final concentration of 5 mM, and then the assays were incubated for 20 h at 23°C with shaking. After incubation, the tubes were centrifuged briefly (1400 rpm in an Eppendorf centrifuge 5417R) and 15 µL of the supernatant directly subjected to RPLC-IT-MS. Each assay was performed in duplicate. Control assays were performed in which recombinant PCBER was omitted.

**Oxidative Coupling Assays**

For the oxidative coupling assays, 1 mM coniferyl alcohol (dissolved in 5 µL aceton; Sigma-Aldrich), 1 mM sinapyl alcohol (dissolved in 5 µL aceton; Sigma-Aldrich), 1 mM L-cysteine (Sigma-Aldrich), and 0.1 units of horseradish peroxidase (type VI; Sigma-Aldrich) were dissolved in 90 µL Bis-Tris propane buffer (20 mM, pH 7). The assays were initiated by adding 2 mM H$_2$O$_2$ (Sigma-Aldrich) after which the mixtures were incubated for 20 h at 23°C with shaking. After incubation, the samples were subjected to RPLC-IT-MS as described earlier. Control assays were performed in which the cysteine, the horseradish peroxidase, or the H$_2$O$_2$ were omitted.

**NMR Analysis of Fractions Containing the Purified PCBER Product**

The PCBER product was purified from poplar xylem as described above. Approximately 1 µg of material was isolated in this manner and consisted of the glycosylated compound and its aglycon in a 1:3 ratio, as judged from MS analysis. NMR spectra of the sample in D$_2$O were acquired on a Bruker Biospin Avance III 600 MHz spectrometer equipped with a cryogenically cooled 1.7-mm 1H/13C/15N cryogenic gradient micro-probe with inverse geometry (proton coils closest to the sample). Spectra run with typical parameters were: 1H with “water suppression using excitation sculpting with gradients” (Hwang and Shaka, 1995), Bruker pulse program “zgssgp”; HSQC Bruker pulse program “hsqcdgpsisp2.2” (as above) (Palmer et al., 1991; Kay et al., 1992; Schleucher et al., 1994); HSQC-TOCSY, Bruker pulse program “hsqcdgpsisp2.2”; correlation spectroscopy, Bruker pulse program “cosyqgpffq” (using a double-quartum filter) (Shaw et al., 1996; Ancian et al., 1997). Spectra of the model compound mixture in Figure 4B, again in D$_2$O, were run on the Bruker Avance 500 spectrometer described above using the same pulse programs. The central residual water peak at 4.63 ppm (1H only) was used as internal reference.

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL database under accession numbers AJ162452 and AJ005803. The RNAi construct to downregulate PCBER expression in poplar (P. tremula × tremuloides) was generated based on the ESTA017P67U, GenBank reference AJ162452 (Sterky et al., 1998). The GenBank accession number AJ005803 was used for heterologous expression.

**Supplemental Data**

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

**Supplemental Figure 1.** Growth Rate Plots.

**Supplemental Figure 2.** Electron Microscopy.

**Supplemental Figure 3.** Cellulose Quantification.

**Supplemental Figure 4.** Lignin Quantification.

**Supplemental Figure 5.** MS$^2$ Fragmentation Pathways of the PCBER in Vivo Substrate.

**Supplemental Figure 6.** Structures of Compounds Used in This Study.

**Supplemental Figure 7.** MS Data for the PCBER Product.
Supplemental Figure 8. MS² Data of S⁰ν(8–O–4)G.
Supplemental Figure 9. 2D NMR Spectra of Dilignol Glycerols.
Supplemental Figure 10. IDDDC Conversion to DDC.
Supplemental Figure 11. 2D HSQC NMR Spectra of Ag₂O Oxidation of TDDC.
Supplemental Table 1. Differential Compounds Between PCBER-Downregulated and Wild-Type Plants.
Supplemental Table 2. Compounds Detected by GC-MS.
Supplemental Table 3. Amino Acid Profiling.
Supplemental Methods.
Supplemental References.

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Phenylcoumaran Benzylic Ether Reductase Prevents Accumulation of Compounds Formed under Oxidative Conditions in Poplar Xylem

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