Overexpression of a Prefoldin β subunit gene reduces biomass recalcitrance in the bioenergy crop Populus

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Summary
Prefoldin (PFD) is a group II chaperonin that is ubiquitous in the eukaryotic kingdom. Six subunits (PFD1-6) form a jellyfish-like heterohexameric PFD complex and function in protein folding and cytoskeleton organization. However, little is known about its function in plant cell wall-related processes. Here, we report the functional characterization of a PFD gene from Populus deltoides, designated as PdPFD2.2. There are two copies of PFD2 in Populus, and PdPFD2.2 was ubiquitously expressed with high transcript abundance in the cambial region. PdPFD2.2 can physically interact with DELLA protein RGA1_8g, and its subcellular localization is affected by the interaction. In P. deltoides transgenic plants overexpressing PdPFD2.2, the lignin syringyl/guaiacyl ratio was increased, but cellulose content and crystallinity index were unchanged. In addition, the total released sugar (glucose and xylose) amounts were increased by 7.6% and 6.1%, respectively, in two transgenic lines. Transcriptomic and metabolomic analyses revealed that secondary metabolic pathways, including lignin and flavonoid biosynthesis, were affected by overexpression of PdPFD2.2. A total of eight hub transcription factors (TFs) were identified based on TF binding sites of differentially expressed genes in P. deltoides transgenic plants overexpressing PdPFD2.2. In addition, several known cell wall-related TFs, such as MYB3, MYB4, MYB7, TT8 and XND1, were affected by overexpression of PdPFD2.2. These results suggest that overexpression of PdPFD2.2 can reduce biomass recalcitrance and PdPFD2.2 is a promising target for genetic engineering to improve feedstock characteristics to enhance biofuel conversion and reduce the cost of lignocellulosic biofuel production.

Introduction
A protein’s function is partially controlled by its three-dimensional structure and is dependent on a complex network of molecular chaperones (Kim et al., 2013). Molecular chaperones are crucial for cellular development and play important roles in protein assembly, folding, trafficking and degradation (Hartl et al., 2011). Prefoldin (PFD) was first described as a co-chaperone protein and is ubiquitously present across the eukaryotic kingdom (Vainberg et al., 1998). PFD is now classified as a group II chaperonin, which is found in archaea and eukaryotes and appears to be central to a complex network of co-chaperones. Eukaryotic PFD is polymeric and consists of six subunits: two α subunits (PFD3 and PFD5) and four β subunits (PFD1, PFD2, PFD4 and PFD6) (Siebett et al., 2000). These six subunits form a ‘jellyfish-like’ heterohexameric complex, which can deliver newly synthesized unfolded protein to cytosolic chaperonins containing TCP-1 for protein folding and protecting unfolded proteins in this process (Siebett et al., 2000).

Prefoldin plays a central role in cellular development and its function has been well studied from yeast to mammalian species. In yeast, PFD can bind to tubulin and actin to facilitate productive folding and binding inside the chaperonin cavity, and deletion of a single or multiple PFD subunits results in cytoskeleton disruption (Geissler et al., 1998). Reduction of PFDs causes Caenorhabditis elegans embryonic lethality, and silencing of PFD1, PFD2, PFD3 and PFD6 subunits reduces microtubule growth (Lundin et al., 2003). Prefoldin is involved in various cellular functions, such as cellular development, differentiation, stress response, cell secretion and cell cycle progression. In mammals, PFDs are involved in protein folding in the endoplasmic reticulum and induce the formation of ER tubules (Kim et al., 2020). In Arabidopsis thaliana, several PFDs are involved in abiotic stress responses, cell wall metabolism and hormone signaling (Zhang et al., 2020). In rice, PFDs are involved in the regulation of cell wall structure and cell expansion (Jiang et al., 2020). In poplar, PFDs are involved in secondary cell wall metabolism, lignin biosynthesis and cell expansion (Zhang et al., 2020). In this study, we report the functional characterization of a PFD gene from Populus deltoides, designated as PdPFD2.2.
Human PFD inhibits amyloid-β fibrillation and is involved in the nontoxic amyloid-β aggregation, which is consistent with its role in Alzheimer’s disease (Broer et al., 2011; Sörgjerd et al., 2013). Eukaryotic PFD is highly conserved, as evidenced by the fact that plant and human subunits can functionally complement yeast pfd mutants (Geissler et al., 1998; Rodríguez-Milla and Salinas, 2009). In Arabidopsis, only one copy of PFD is present for each of the six subunits (Hill and Hemmingsen, 2001). Among these, PFD3, PFD5 and PFD6 are required for normal microtubule dynamics and organization (Gu et al., 2008; Rodríguez-Milla and Salinas, 2009). The Arabidopsis pfd6-1 mutant exhibits a range of microtubule defects, including hypersensitivity to oryzalin, and defects in cell division, cortical array organization and microtubule dynamics (Gu et al., 2008). In addition, Arabidopsis pfd3 and pfd5 mutants showed reduction of α- and γ-tubulin, alteration in developmental patterns and microtubule organization and hypersensitivity to salt stress (Rodríguez-Milla and Salinas, 2009). As a fundamental component of plant cell wall, cellulose is synthesized by cellulose synthase (CESA) and is associated with the dynamics of the cytoskeleton via actin filaments and microtubules (Cai et al., 2011). During secondary cell wall formation, the cellulose synthase complex (CSC) has been observed to form bands beneath sites of secondary cell wall synthesis, which is dependent upon underlying bundles of microtubules and the thick actin cables (Paredes et al., 2006; Wightman and Turner, 2008). Therefore, PFD subunits may have a role in cell wall-related processes, but this has been unstudied.

Besides the cytoskeletal complexes, PFD subunits can also assemble non-cytoskeletal complexes in the cytoplasm. For instance, a PFD-like complex participates in the cytoplasmic assembly of RNA polymerase II (Boulon et al., 2010) and in the stabilization and assembly of phosphatidylinositol 3-kinase-related kinases in cooperation with the R2TP complex (Horejsi et al., 2010). In addition, PFD is involved in hormonal signalling pathways. The PFD complex directly interacts with DELLAs proteins, and its subcellular localization has been shown to be gibberellin (GA)-dependent (Locascio et al., 2013). PFD also shuttles between the cytoplasm and the nucleus and acts on DNA-binding proteins. In yeast, all of the PFD subunits can be detected in the nucleus (Millan-Zambrano et al., 2013). An α-class PFD-like subunit, UXT, is also located in human centrosomes, associated with γ-tubulin, and its overexpression disrupts the centrosome structure (Zhao et al., 2005). In plants, PFD5 and PFD6 are also found in the nucleus of tobacco and Arabidopsis leaf cells. Depending on the physical interaction with the nuclear DELLAs proteins, the PFD complex can stay in the nucleus to affect microtubule orientation when GA is absent or can be localized in the cytoplasm when GA is present (Locascio et al., 2013). Localization of PFD in the nucleus may be the result of the regulation of its cytoplasmic functions by means of a cytoplasm-exclusion mechanism, as has been demonstrated in Arabidopsis (Locascio et al., 2013). PFD has also been shown to play transcriptional roles in mammalian cells. For example, human PFD5 can interact with c-Myc, EGR1 and p73 (Satou et al., 2001; Watanabe et al., 2002); PFD3 can interact with HIV integrase, HMM54 and NF-xB (Kim et al., 2008; Mousnier et al., 2007); another PFD-like subunit UXT can bind to EVIL, NF-xB, ALS2, LRP16, TAF130, Sp1, etc., to play regulatory roles (Millan-Zambrano and Chavez, 2014). In plants, except for its interaction with DELLA in Arabidopsis (Locascio et al., 2013), little is known about the transcriptional or regulatory roles of PFD. Presently, plant cell walls are considered to be a renewable resource for the conversion and production of biofuels (Pauly and Keegstra, 2010). The cell wall composition, that is, lignin, cellulose and hemicellulose content, and cell wall properties, for example, lignin monomer ratios and hemicellulose types, impact sugar release efficiency during biofuel conversion (Himmel et al., 2007; Zhang et al., 2019). However, the effect of monolignol composition on biomass digestibility varies greatly in different species or under different treatment conditions. For example, S/G ratio is negatively related to the enzymatic hydrolysis of untreated biomass of maize, poplar and eucalyptus. On the other hand, high S/G ratio can enhance saccharification of poplar and Arabidopsis biomass with hot water pretreatment (Li et al., 2010; Studer et al., 2011) and poplar biomass with steam explosion pretreatment (Mansfield et al., 2012). In this study, we describe the characterization of a PFD β subunit gene in Populus deltoids, PtPFD2.2, encoded by the locus Potri.008G153900. We overexpressed this gene in P. deltoids ‘WV94’ and found that it altered the lignin composition and sugar release characteristics in transgenic poplar plants. We also performed transcriptomic and metabolomic analyses to explore the molecular mechanism of changes in cell wall precursors. Our results suggest that PFD2.2 is likely involved in reactions leading to structural changes in cell wall composition. This study provides insights into the role of a prefoldin protein in the genetic control of the dynamic changes in cell wall composition, and provides a foundation for reducing cell wall recalcitrance in the bioenergy crop Populus.

Results

PTD2 genes in Populus

To identify PFD2 proteins in Populus, we performed a Blastp search against the P. trichocarpa genome (V3.0) using Arabidopsis PFD2 (AT3G22840) as a query. Two copies of PFD2 (Potri.001G240200 and Potri.008G153900) were identified and named PtPFD2.1 and PtPFD2.2, respectively (Figure 1a), with both containing the Prefoldin_2 motif. To explore the evolutionary relationship of PFD2 in the plant kingdom, we compared PFD2 from 12 plant species, including four woody dicots: Populus (P. trichocarpa), Salix (S. purpurea), Eucalyptus (E. grandis) and grape (Vitis vinifera); three herbaceous dicots: Arabidopsis (A. thaliana), Medicago (M. truncatula) and soybean (Glycin max); four monocots: rice (Oryza sativa), maize (Zea mays), Brachypodium distachyon and Sorghum (S. bicolor); and one ancient nonvascular moss: Physcomitrella patens (Figure 1a). One to three PFD2 copies were identified from each of these 12 species. Salix and soybean contain three copies of PFD2, whereas Arabidopsis, Medicago, Eucalyptus, grape and Brachypodium contain only one copy in each genome. To examine the evolutionary relationships of PFD2 proteins across different plant species, we performed a maximum likelihood (ML) phylogenetic analysis using the full-length amino acid sequences of PFD2 proteins. Except for Arabidopsis PFD2, the PFD2 genes from woody dicots, herbaceous dicots, monocots and moss were grouped into different clades (Figure 1a), which implies that their functions might have been diverged during evolution.

Next, we analysed expression patterns of the Populus PFD2 genes across various tissues and organs in the Populus Gene Atlas (https://phytozome.jgi.doe.gov/phymotome/beginn). Both PtPFD2.1 and PtPFD2.2 were highly expressed in stem (internode and node) and immature leaf (Figure 1b). Based on the AspWood gene expression database, which contains high-resolution RNA-Seq data from nanometre-scale tissues of the
P. tremula stem development, PtPFD2.1 and PtPFD2.2 were highly expressed in the cambium with decreased expression during xylem expansion, but increased again during xylem maturation (Figure 1c). Notably, PtPFD2.2 has higher transcript abundance than PtPFD2.1 in both the stem and cambium (Figure 1b, c). We then constructed a co-expression network of PtPFD2.2 based on global gene expression patterns across different tissues and under various stresses. Based on the functional classification, genes related to proteolysis (54 genes), cell wall (26 genes), transport (14 genes), growth (13 genes) and cytoskeleton (4 genes) were co-expressed with PtPFD2.2 (Figure 1d). GO enrichment analysis showed that ‘protein modification’-related GO terms were significantly enriched in the network (Figure S1). To explore the potential regulatory mechanism of PtPFD2.2 expression, we analysed the cis-acting elements of the promoter region (3000 bp upstream of the translation initiation site) of PtPFD2.2. The identified cis-acting elements were classified into four groups (development, hormone, stress and other) according to their potential functions. Notably, a total of six MYB binding sites, including three MBS (MYB binding site involved in drought inducibility), two MRE (MYB binding site involved in light responsiveness) and one CCAAT-box (MYBHv1 binding site), were found in the promoter of PtPFD2.2 (Figure S2). For hormone-related cis-elements, five gibberellin-responsive elements (four GARE-motifs and one P-box) and four salicylic acid-responsive elements (TCA-elements) were identified (Figure S2).

Protein structure of Populus PFD2.2

In order to understand the functional mechanism of PtPFD2.2, we analysed its protein structure. PtPFD2.2 is 145 aa in length. It has two predicted α-helices connected by two β-sheets, and the majority of the whole protein is constructed as the Prefoldin_2 motif (13-118 aa; Figure S3). Based on the predicted posttranslational modification, a total of 20 phosphorylation, one sumoylation and four ubiquitination sites were identified. The phosphorylation sites were enriched in the first α-helix and the C-terminal tail. For ubiquitination sites, one is located in the first

**Figure 1** Phylogenetic analysis and expression patterns of the *Populus* PFD2 genes. (a) Phylogenetic tree constructed using full-length amino acid sequences of PFD2 from 12 plant species by the neighbour-joining (NJ) method with 1000 bootstrap replicates. (b) Expression patterns of the PtPFD2 genes across various tissues. (c) Expression patterns of the PtPFD2 genes during wood formation. The data were retrieved from the AspWood database (http://aspwood.popgenie.org/aspwood-v3.0), where relative expression is shown for aspen stem samples, which consist primarily of phloem, cambium, expanding xylem and maturing xylem. (d) Co-expression network of PtPFD2.2.

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α-helix and three in the C-terminal tail (Figure S3). Three-dimensional structure prediction by I-TASSER analysis revealed that two alpha-helices are in parallel arrangement.

**PFD2.2 physically interacts with DELLA protein in Populus**

To explore whether the interaction between PFD and DELLA proteins is conserved in *Populus* as observed in *Arabidopsis*, we examined the protein-protein interaction between *PDPFD2.2* and PIDEELA proteins. First, we sought to identify the *Populus* DELLA homolog that may potentially interact with *PDPFD2.2* by comparing the expression correlation with *PDPFD2.2* among four DELLA genes in the *Populus* genome. Within the DELLA gene family, two genes (*RGA1_8g* and *RGA1_10g*) showed high expression correlation with *PDPFD2.2* in both tissue samples and wood formation process (Figure 2a). RGA1_8g and RGA1_10g are paralogous, and both proteins contain a nuclear localization signal (NLS) peptide (Figure 2b). Although the two proteins showed high sequence similarity, their protein 3D structures were different due to the N-terminal sequence divergence (Figure 2c).

To determine the subcellular localization of *PFD2.2*, RGA1_8g and RGA1_10g, each of these three proteins was fused with Yellow Fluorescent Protein (YFP) at their N-terminus and were transiently expressed in the *Populus* leaf mesophyll protoplasts (Figure 2d). Signals of YFP-RGA1_8g and YFP-RGA1_10g (green colour) overlapped with the red signal of the nuclear marker mCherry-VirD2NLS (Lee et al., 2008), indicative of the nuclear localization of RGA1_8g and RGA1_10g. In contrast, PFD2.2 appeared to be localized outside of the nucleus, because the signal of YFP-PFD2.2 had no overlap with the signal of mCherry-VirD2NLS.

To investigate the *in vivo* association of *PFD2.2* with RGA1_8g or RGA1_10g, we performed the bimolecular fluorescence complementation (BiFC) assay. In the BiFC assay, the *PFD2.2* protein was fused with the C-terminal fragment of cyan fluorescent protein (cCFP). RGA1_8g and RGA1_10g proteins were fused with the N-terminal fragment of Venus (vVenus). We then introduced paired proteins into the *Populus* leaf mesophyll protoplasts by PEG-calcium-mediated transfection. The interaction of cCFP-tagged protein with vVenus-tagged protein is indicated by yellow fluorescence due to the formation of an intact YFP. As shown in Figure 2e, BiFC signals (green colour) resulted from PFD2.2 and RGA1_8g interaction were present as distinct nuclear speckles (shown as yellow colour). Such interaction was not detected in either the PFD2.2-RGA1_10g pair or the negative controls. Collectively, these results indicate that RGA1_8g may associate with PFD2.2 and possibly guide PFD2.2 into the nucleus.

**Growth and cell wall components in *Populus* transgenic plants overexpressing *Pdpfd2.2***

To characterize the function of *PFD2.2* in *Populus*, the full-length CDS of *PFD2.2* was cloned from *P. deltoides* ‘VV94’ (*Pdpfd2.2*) and overexpressed in ‘VV94’. From five independent transgenic lines, two lines that had relatively high *Pdpfd2.2* expression levels were selected for further study and labelled as #1 and #2. The transcript level of *Pdpfd2.2* was examined by qRT-PCR and both *Pdpfd2.2* transgenic lines were confirmed to overexpress *Pdpfd2.2* (Figure S4).

Analysis of transgenic lines showed no significant difference in basal stem diameter or plant height compared to empty vector control plants (Figure 3a, b). When both longitudinal and lateral growth rates were combined, line #1 showed a significant increase in above-ground biomass as measured by diameter² × height (D²H) (Figure 3c). Based on molecular beam mass spectrometry (MBMS) measurements, lignin content in the *Populus* transgenic lines overexpressing *Pdpfd2.2* was not significantly different from that in the control plants (Figure 3d). However, the lignin S/G ratio was increased by 7.9% and 7.3% in the two *Pdpfd2.2* overexpression lines (Figure 3e). In addition, we analysed the cellulose and hemicellulose contents in *Pdpfd2.2* overexpression lines. There were no significant differences in cellulose and hemicellulose contents between the control and two overexpression lines (Figure S5a, b). To explore if *Pdpfd2.2* overexpression affected cellulose crystallinity, we analysed the cellulose crystallinity index (CrI), the number-average degree of polymerization (DPn) of cellulose, the weight-average degree of polymerization (DPw) of cellulose and the polydispersity index (PDI) of cellulose. No significant differences in each of these cellulose parameters were found between control and *Pdpfd2.2* overexpression lines (Figure S6), indicating that *Pdpfd2.2* may not be associated with cellulose biosynthesis or cellulose crystallinity in *Populus*.

**Saccharification efficiency of *Populus* transgenic plants overexpressing *Pdpfd2.2***

To assess the sugar release performance of the *Pdpfd2.2* overexpression lines, glucose and xylose release during the enzymatic hydrolysis was compared between transgenic and control plants. As shown in Figure 3f–h, both glucose and xylose release from the two *Pdpfd2.2* overexpression lines was greater than the control plants. The total released sugar from the two lines was increased by 7.6% and 6.1%. In order to rule out the possible effects caused by changes in cellulose and hemicellulose contents, we determined the glucose and xylose release relative to cellulose and hemicellulose contents, respectively, and notably, the *Pdpfd2.2* transgenic lines still showed high glucose and xylose release (Figure S5c, d). It should be noted that although there is a relatively large difference (~three-fold) in the *Pdpfd2.2* transcript level in the two overexpression lines (Figure S4), these two transgenic lines displayed similar phenotypes (Figure 3), implying higher *Pdpfd2.2* expression may not necessarily result in larger phenotypic effects; presumably, it may have reached the dosage threshold for affecting phenotypes.

**Transcriptome analysis of *Populus* transgenic plants overexpressing *Pdpfd2.2***

To explore how overexpressing *Pdpfd2.2* may affect cell wall-related phenotypes in poplar, we conducted transcriptomic analysis using RNA-Seq. For each transgenic line and control plants, library construction and RNA-Seq analyses were performed using two biological replicates. The correlation (R²) between replicates were 0.902, 0.936 and 0.885 in control, line #1 and line #2, respectively (Figure S7), suggesting high repeat accuracy between biological replicates. In the two transgenic lines, a total of 752 (514 up and 238 down) and 4109 (2063 up and 2046 down) differentially expressed genes (DEGs) were identified in line #1 and #2, respectively. Among them, 332 up- and 180 down-DEGs overlapped in the two lines and were defined as core-DEGs (Figure 4a). The hierarchical clustering of core-DEGs indicates that the expression patterns of core-DEGs were consistent among transgenic lines and replicates, and the fold changes of DEGs were higher in #2 than #1 (Figure 4b), which is consistent with the *Pdpfd2.2* expression level in the two transgenic lines (Figure S4). We then used the MapMan functional classification system to classify these core-DEGs. For cell
wall-related MapMan bins, a total of 23 up- and 17 down-DEGs belong to cell wall, secondary metabolism, glycolysis or major/ minor carbohydrates. In addition, 43 (27 up and 16 down), 32 (25 up and 7 down) and 31 (18 up and 13 down) DEGs were classified as related to protein, stress and development, respectively (Figure 4c and Table S1). We examined the gene expression patterns in metabolism and found that genes related to cell wall precursor synthesis, including pectin esterase, pectate lyases and polygalacturonases, were up-regulated in\textit{\textit{PdPFD2.2}} overexpression lines. In secondary metabolism, genes associated with terpenes and phenylpropanoids biosynthesis were up-regulated, whereas genes associated with flavonoids were down-regulated (Figure 4d).

To seek potential hub regulatory transcription factors (TFs) that play key roles in \textit{PdPFD2.2} transgenic poplars, we analysed the TF binding sites (TFBS) in the promoter regions of the 322 up and 180 down core-DEGs (Table S2). After being ranked, eight TFs with potential to bind to the promoter of most core-DEGs were identified as hub TFs, which include one \textit{HB1}, one \textit{CIB}, two \textit{WRKYs} (\textit{WRKY6} and \textit{WRKY35}), one \textit{MYB-like} (\textit{MYBD}) and three \textit{NACs} (\textit{NAC029b}, \textit{NAC047} and \textit{NAC056}) (Figure 4e and Table S3). Furthermore, we selected 14 TFs, including eight hub TFs and six known TFs related with cell wall biogenesis (\textit{XND1}, \textit{GT2a}, \textit{MYB4}, \textit{TT8}, \textit{MYB3} and \textit{MYB7}), to validate their up- or down-expression using qRT-PCR. As shown in Figure 4f, six TFs were up-regulated and eight TFs were down-regulated in the two transgenic lines, which are consistent with the results from RNA-Seq. It is plausible that these TFs may act downstream of \textit{PdPFD2.2} to regulate those cell wall-related phenotypical changes observed in the \textit{Populus} transgenic plants overexpressing \textit{PdPFD2.2}.

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**Figure 2** \textit{PFD2.2} physically interacts with a DELLA protein in \textit{Populus}. (a) Expression correlation between \textit{PFD2.2} and two DELLA genes (\textit{RGA1\_8g} and \textit{RGA1\_10g}) in tissue samples (24 samples including different developmental stages of bud, leaf, stem, root and female and male catkins) and wood formation samples (25 samples from phloem, cambium and expanding and maturing xylem). (b) Sequence alignment of \textit{RGA1\_8g} and \textit{RGA1\_10g} proteins and nuclear localization signal (NLS). (c) Protein structures of \textit{RGA1\_8g} and \textit{RGA1\_10g}. (d) Subcellular localization of \textit{PFD2.2}, \textit{RGA1\_8g} and \textit{RGA1\_10g} in \textit{Populus} protoplast (green). The nuclear marker mCherry-VirD2-NLS is shown in red. Bar = 5 \textmu m. (e) Bimolecular fluorescence complementation (BiFC) assay of \textit{PFD2.2} and two DELLA proteins (\textit{RGA1\_8g} and \textit{RGA1\_10g}). The nuclear marker mCherry-VirD2-NLS is shown in red. Bar = 5 \textmu m.
Metabolite profiles of Populus transgenic plants overexpressing PdPFD2.2

To further examine the function of PdPFD2.2 at the metabolomic level, we analysed the metabolite profiles of PdPFD2.2 transgenic lines. Overexpression PdPFD2.2 had greatly altered the leaf metabolite profiles relative to control plants (Figure 5a). Among a total of 165 identified metabolites by GC-MS, 91 metabolites were changed, including 28 up- and 63 down-regulated metabolites in PdPFD2.2 transgenic lines (Figure 5a). Based on the metabolic pathway analysis, the most enriched pathway from the 91 metabolites was ‘flavone and flavonol biosynthesis’, followed by ‘phenylpropanoid biosynthesis’, ‘alanine, aspartate and glutamate metabolism’, ‘galactose metabolism’ and ‘flavonoid biosynthesis’ (Figure 5b). The flavone and flavonol biosynthesis pathway include the synthesis of numerous flavonoid metabolites, and it was this class of metabolites that was most down-regulated in the two transgenic lines (Figure 5c), including rutin [Figure 5c; a glycoside of the flavonol quercetin], luteolin, isorhamnetin, dihydroquercetin (taxifolin), kaempferol, catechin, epicatechin, quercetin and gallo catechin (Figure 5a). In contrast, one flavonoid that was up-regulated was genistin (GNT) (Figure 5a). Examples of phenylpropanoid metabolites that were significantly reduced included the upstream hydroxycinnamate precursors of lignin biosynthesis, p-coumaric acid (pCA), caffeic acid and ferulic acid (FA), and the downstream products, coniferyl alcohol (G-monolignol), coniferin and its glucoside storage product (Figure 5a, c). Notably, sinapyl alcohol (S-monolignol) was up-regulated (Figure 5a), which is consistent with increased S/G ratios observed in the PdPFD2.2 transgenic plants (Figure 3e).

Whereas most flavonoids declined, secondary metabolites of the higher-order salicylate (salicylic acid (SA), its esters and glucoside conjugates) pathway were both up and down-regulated. For example, grandidentatin and purpurein were accumulated in transgenic lines, as were several simple salicylate-related metabolites and simple conjugates, including salicylic alcohol, gentisic acid 5-O-glucoside, salirepin, isosalicin and salicyl-saliclylate (Figure 5a). Notably, several of the larger, more complex higher-order salicylate metabolites were down-regulated in transgenic lines, including salicortin and salicyl-saliclylol-2-O-glucoside, as well as SA (Figure 5a). Taken together, these results further validate the role of PFD2.2 in metabolite responses and are consistent with changes in cell wall properties and the transcriptome of Populus transgenic plants overexpressing PdPFD2.2.

Discussion

As a group II chaperonin, PFD plays important roles in newly synthesized protein folding and protection (Cao, 2016). Several studies in different species have also indicated that PFD is involved in cytoskeleton organization (Geissler et al., 1998; Gu et al., 2008; Lundin et al., 2008) and transcriptional regulation (Locascio et al., 2013; Millan-Zambra and Chavez, 2014; Millan-Zambra et al., 2013). However, its function in plants is largely unknown. In this study, we provide evidence that a Prefoldin β subunit gene in Populus, Pfd2.2, has a role in modifying cell wall properties and that Pfd2.2 can be used to reduce biomass recalcitrance in the bioenergy crop Populus.

The co-expression network and posttranslation modification indicate that many proteolysis-related proteins are co-expressed with Pfd2.2 (Figure 5f). One of the common ubiquitination and sumoylation sites (Figure S3) is consistent with Pfd2.2 functioning as a chaperonin. Cell wall-related genes were co-expressed with Pfd2.2 (Figure 1), and Pfd2.2 was highly expressed in cambium (Figure 1). These imply that Pfd2.2 may have a role in cell wall-related processes. In Arabidopsis, PFD is involved in cytoskeleton-related processes, including microtubule dynamic and organization. The pfd4-1 mutant has reduced tubulin proteins abundance and exhibits defects in cell division.
and microtubule dynamicity (Gu et al., 2008). The components of cytoskeleton are tightly connected with cambium cell division and secondary vascular differentiation in woody perennial plants. For example, in dividing cambial cells, myosin and callose are localized at the cell plate, while actin microfilaments (MFs) are alongside the cell plate; both tubulin and actin are associated with the phragmoplast (Chaffey and Barlow, 2002). MFs were rare in ray cambial cells; but were abundant and axially arranged in their derivatives once cell elongation had begun. Microtubules were randomly oriented in ray and fusiform cells of the cambial zone (Chaffey et al., 2002). Thus, our findings suggest that the altered cell wall properties in the PdPFD2.2 transgenic poplars might be due to its action in cytoskeleton-related processes.

In Arabidopsis, PFD regulation of cortical microtubule organization depends on its physical interaction with DELLAs, which are nuclear proteins that mediate transcriptional regulation of cell expansion and other developmental processes by GAs (Cheng et al., 2004; de Lucas et al., 2008; Ubeda-Tomas et al., 2008). In rice, a DELLA protein SLR1 can interact with a NAC TF in the top layer of secondary wall formation to regulate cellulose biosynthesis (Huang et al., 2015). In the present study, we found that PFD2.2 physically interacts with a DELLA protein RGA1_8g in Populus, but not with the other tested DELLAs (Figure 2). The differences in protein–protein interaction of the two DELLAs with PFD2.2 might be caused by the protein structure difference of RGA1_8g and RGA1_10g (Figure 2). The nuclear
localization of PFD2.2 is likely guided by RGA1_8g. However, the cellulose content was not significantly affected in the Populus transgenic plants overexpressing PdPFD2.2, suggesting that the interaction of PFD2.2-DELLA is not directly involved in cellulose biosynthesis in Populus.

As a key player in phytohormone cross-talk, DELLA is also involved in other hormone signalling pathways. For example, DELLA-like1 is co-localized with endogenous biologically active GAs in expanding xylem cells in poplar (Israelsson et al., 2005) and in a feedback response to increased GA and IAA levels (Björkland et al., 2007). DELLA can also alter the relative strength of defense hormones SA and JA. In Arabidopsis, DELLA has different effects on SA and JA. Navarro et al. (2008) reported DELLA promoted JA perception and/or signalling and antagonized SA to modulate immunity. Unlike in Arabidopsis, a rice DELLA protein, Slender Rice 1 (SLR1), acts as a positive regulator of hemibiotrophic resistance by integrating and amplifying SA- and JA-dependent defense signalling (De Vleesschauwer et al., 2016).

In the present study, overexpression of PdPFD2.2 altered the SA-related metabolites (e.g. salicyl-salicyloyl-2-O-glucoside, salicortin, salicyl alcohol and salicylate) in transgenic poplar (Figure 5a), which provides clues for the connection between PdPFD2.2 and the SA signalling pathway. Furthermore, WRKYs belong to a large family of TFs and are known as key regulators responding to

Figure 5 Metabolomic changes of Populus transgenic plants overexpressing PdPFD2.2. (a) Metabolite profile in mature leaf of two PdPFD2.2 overexpression lines (#1 and #2) compared to control plants. (b) Pathway analysis of differential metabolites between control (Ctrl) and Populus transgenic plants overexpressing PdPFD2.2. Global metabolic alterations of the most relevant pathways induced by PdPFD2.2 were revealed using the MetaboAnalyst. Small P-value and large pathway impact factor indicate that the pathway is greatly influenced. (c) Abundance of rutin, p-coumaric acid (pCA), caffeic acid and ferulic acid (FA) in flavone and flavonol biosynthesis pathway in Ctrl and PdPFD2.2 overexpression lines. The graphs depict the abundance and chemical structure of these metabolites.
pathogen attack and to the endogenous SA (Eulgem and Somssich, 2007). In addition, WRKY TFs are involved in the activation of SA biosynthesis. For example, WRKY28 and WRKY46 directly activate the expression of SA biosynthesis genes IC51 and PBS3 (van Verk et al., 2011). In the present study, two WRKYs (WRKY6 and WRKY35) were identified as potential downstream regulators of PdPFD2.2 in RNA-Seq analysis (Figure 4e), implying that PdPFD2.2 may also potentially be involved in defense-related processes, but this has not been experimentally tested.

Among the eight hub TFs, three NAC (NAC029b, NAC047 and NAC056) and one MYB (MYB40) were down-regulated (Figure 4e). In contrast, other known TFs involved in cell wall-related processes, such as XND1, MYB3, MYB4, MYB7 and TT8, were also affected in *Populus* transgenic plants overexpressing PdPFD2.2 (Figure 4f). XND1 is a negative regulator of lignocellulose synthesis and programmed cell death in xylem (Zhao et al., 2008), and it was up-regulated by PdPFD2.2 overexpression (Figure 4f). Among the four down-regulated TFs (MYB3, MYB4, MYB7 and TT8), MYB3 is a repressor of phenylpropanoid biosynthesis, MYB4 is a repressor of lignin biosynthesis, and MYB7 and TT8 work as repressor and activator in flavonol biosynthesis, respectively (Zhang et al., 2018). The changes of these key secondary metabolic TFs by PdPFD2.2 is likely the primary mechanism driving the flavonoid responses and the hydroxycinnamate and monolignol responses of the lignin pathway.

Generally, cell wall recalcitrance is gauged by biomass enzymatic hydrolyzability (McCann and Carpita, 2015). Among various components in cell walls, lignin is one of the most significant recalcitrance contributors (Pu et al., 2011). In biomass, cell wall recalcitrance was related to lignin content, structure, subunit composition and linkages with polysaccharides. The relative abundance of H-, S- and G-units of lignin have been proved to be associated with biomass recalcitrance. Biomass recalcitrance is a multi-scale, multi-factor property that is influenced by a variety of factors such as lignin content, lignin structural features, cellulose degree of polymerization and crystallinity, hemicellulose content/structures and accessibility (Meng et al., 2017). In this study, although overexpression of PdPFD2.2 slightly increased the lignin content in the transgenic lines, the increase was not statistically significant when compared to the control plant. On the other hand, S/G ratio was significantly increased in the PdPFD2.2 transgenic plants, compared to the control plant. Because among all the examined cell wall-related parameters (Figures 3, 55, 56), including the content of lignin, cellulose or hemicellulose, cellulose crystallinity index and degree of cellulose polymerization, lignin S/G ratio was the only changed parameter in the PdPFD2.2 transgenic plants, we deduced that the increased sugar release observed in the PdPFD2.2 transgenic plants (Figure 3) is likely due to the increased lignin S/G ratio.

It has been reported that the influence of S/G ratio on the hydrolyzability of biomass varies on a number of factors, such as plant species, lignin content, pretreatment method, enzyme types, as well as natural and transgenic lines (Li et al., 2016b). For example, Chen reported that S/G ratio alone did not correlate well with the sugar release in transgenic alfalfa lines (Chen and Dixon, 2007). Lignin S/G ratio was also reported to have negative effect on biomass saccharification in *Miscanthus* after alkaline pretreatment (Li et al., 2014; Xu et al., 2012), which was partially attributed to the effective co-extraction of lignin–hemicellulose complexes in G-rich lignin samples under the alkaline pretreatment conditions. For poplar wood, saccharification was positively correlated with S/G ratio in poplar biomass after hot water pretreatment (Studer et al., 2011) and steam explosion pretreatment (Mansfield et al., 2012). The positive effect of high S/G ratio on saccharification has been related to the S-lignin structural features: (i) higher level of labile β-O-4 linkages in S-lignin which are readily cleavable during pretreatment and (ii) relatively higher occurrence of β-β bonds in S-lignin leading to lower molecular weight which could facilitate lignin migration and removal during pretreatment. It was also reported that the G unit of lignin could form a more cross-linked lignin structure generating a larger physical barrier against the substrate accessibility (Yu et al., 2014). These results further suggest the complexity of the influence of S/G ratio on saccharification of biomass and the difficulty in comparison between various biomass species and pretreatments.

In this study, the sugar release performance of poplar was measured after hot water pretreatment by using a method similar to the one used by Studer et al. (2011), further supporting that higher lignin S/G ratio improves sugar release under this condition. Furthermore, in a recent study (Alam et al., 2019), biomass porosity and cellulose accessibility were proposed as the finalized determinant on biomass enzymatic hydrolysis. It would be worthwhile to investigate whether the increased S/G ratio in the PdPFD2.2 transgenic plants affects the biomass porosity and the cellulose surface accessibility after chemical pretreatments for co-extraction of lignin and hemicellulose in future studies.

Recalcitrance is also associated with the hydroxycinnamates in lignin, which include pCA, FA, caffeic acid, etc. (Li et al., 2016b). pCAs are usually present as esters pendently linked at the γ-position of S-lignin and acylated to polysaccharides (Petrik et al., 2014). In maize, the cell wall enzymatic digestibility is negatively correlated with the esterified pCA and lignin content (Zhang et al., 2011). In contrast, FA mediates the cross-link reaction of polysaccharides-lignin and polysaccharides-poly saccharides (Azapira et al., 2011) and reduced FA-mediated cross-linking improved digestibility (Jung and Phillips, 2010). In *Populus* transgenic plants overexpressing PdPFD2.2, the contents of pCA, FA and caffeic acid were significantly decreased (Figure 5), which is consistent with its reduced recalcitrance.

In summary, overexpression of PdPFD2.2 enhances lignin S/G ratio and sugar release in transgenic poplar. Therefore, PdPFD2.2 plays a role in overcoming plant cell wall recalcitrance, which provides a path to reduce recalcitrance via modifying cell wall composition. As such, PdPFD2.2 can be a potential target for genetic engineering to improve biofuel conversion and reduce the cost of lignocellulosic biofuel production.

**Experimental procedures**

**Plant materials**

The full-length open-reading frame of PdPFD2.2 was amplified from *Populus deltoides* ‘WV94’. The complementary DNA (cDNA) was cloned into the pAGW560 binary vector in which the expression of PdPFD2.2 was driven by the *Ubiquitin* 3 promoter. Agrobacterium-mediated transformation into *P. deltoides* ’WV94’ was conducted at ArborGen Inc. (Ridgeway, SC) (Biswal et al., 2015). Then transgenic plants were transferred and grown in the greenhouse at Oak Ridge National Laboratory (Oak Ridge, TN) at 25 °C and 16 h/8 h photoperiod. To estimate stem cylinder volume, plant height and stem base diameter were measured in ~6-month-old plants. We measured primary stem length from stem base to shoot tip for plant height and measured the diameter of stem base.
Bioinformatics analysis

To identify PFD2 protein in Populus, the full-length amino acid sequence of Arabidopsis PFD2 (AT3G222840) was subjected to Blastp search integrated in the Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html). Each identified PpFD2 homolog was then used as a new query to search for the PFD2 protein in Salix purpurea, Eucalyptus grandis, Vitis vinifera, Medicago truncatula, Glycin max, Oryza sativa, Zea mays, Brachypodium distachyon, Sorghum bicolor and moss (Physcomitrella patens) genomes. The full-length amino acid sequences with E-value < 1E-10 were selected and subjected to the Pfam database (http://pfam.xfam.org/) to validate the presence of the Prefoldin_2 domain PF01920.

To conduct a phylogenetic analysis, a maximum likelihood (ML) tree was constructed with full-length amino acid sequences of collected PFD2 proteins. Multiple sequence alignment of PFD2 proteins were performed using the Clustal X2.1 (Larkin et al., 2007). A phylogenetic tree was constructed using maximum likelihood (ML) method by MEGA7 with 1000 bootstrap replicates (Kumar et al., 2016).

Normalized FPKM values of the PpFD2 genes from various tissues and organs were obtained from the Populus Atlas database integrated in Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html), which includes 24 samples from bud (predormant bud I, predormant bud II, early dormant bud, late dormant bud and fully opened bud), leaf (young, immature and first fully expanded leaf), stem (inode, node, stem_ammonia, stem_nitrate and stem_urea), root (root, root tip, root_ammonia, root_nitrate and root_urea), female catkin (BESC423.ZL 7 female early, BESC842.ZL 22 female late and BESC443.ZG 43 female receptive) and male catkin gene expression during wood formation, a nm-scale high-resolution gene expression database AspWood (http://aspwood.popgenie.org/aspwood-v3.0/) was used, which includes 25 samples from phloem, cambium and expanding and maturing xylem. A co-expression network of PpFD2.2 was created according to the method reported by Li et al. (2016a) using data obtained from the co-expressed biological processes database for P. trichocarpa (http://webs2.kazusa.or.jp/kagiana/cop0911/). Cytoscape (Smoot et al., 2011) was used to visualize the resulting network.

Three-dimensional structure prediction was performed by means of the I-TASSER (iterative threading assembly refinement) suite (Yang et al., 2015).

Subcellular localization

For the analysis of protein subcellular localization, PFD2.2, RGA1_8g and RGA1_10g were cloned into the YFP fusion vector and expressed in the P. trichocarpa root, root tip, root_ammonia, root_nitrate and root_urea, female catkin (BESC423.ZL 7 female early, BESC842.ZL 22 female late and BESC443.ZG 43 female receptive) and male catkin gene expression during wood formation, a nm-scale high-resolution gene expression database AspWood (http://aspwood.popgenie.org/aspwood-v3.0/) was used, which includes 25 samples from phloem, cambium and expanding and maturing xylem. A co-expression network of PpFD2.2 was created according to the method reported by Li et al. (2016a) using data obtained from the co-expressed biological processes database for P. trichocarpa (http://webs2.kazusa.or.jp/kagiana/cop0911/). Cytoscape (Smoot et al., 2011) was used to visualize the resulting network.

Three-dimensional structure prediction was performed by means of the I-TASSER (iterative threading assembly refinement) suite (Yang et al., 2015).

Bimolecular fluorescence complementation (BiFC) assay

For BiFC, CDS sequence of PpPD2.2 was cloned into the pSAT1-cCFP-C vector (ABRC# CD3-1068). RGA1_8g or RGA1_10g was cloned into the pSAT1-nVenus-C vector (ABRC# CD3-1076). Then, each of the expression cassettes of these three constructs were cloned into the transient expression vector pUC119-RCS (ABRC# CD3-1747) by Ascl. To perform BiFC, 8 µg of cCFP construct, 8 µg of nVenus construct and 4 µg of nuclear marker VirD2LS-mCherry construct were co-transfected into 200 µL of protoplasts. After a 12-h incubation under weak light, YFP and mCherry fluorescence were examined and photographed. Images were collected on a Zeiss LSM 710 confocal microscope and were processed using the Zeiss ZEN software package.

Chemical composition analysis

Four milligrams of dried, ground [40 mesh] stem biomass was placed into a pyrolysis molecular beam mass spectrometry chamber, and then, using 17 eV electron impact ionization, mass spectral data were acquired on a Merlin Automation data system version 3.0 from 30 to 450 m/z (Sykes et al., 2009). Lignin estimates were determined as described previously (Sykes et al., 2009). S/G ratios were determined by summing the area under the peaks attributed to syringyl moieties (i.e. m/z 154, 167, 168, 182, 194, 208 and 210), and dividing this area by the area under the peaks attributed to guaiacyl moieties (i.e. m/z 124, 137, 138, 150, 164 and 178). Cellulose and hemicellulose were analysed according to Methods S1.

Gel permeation chromatographic (GPC) analysis

The weight-average molecular weight (Mw) and number-average molecular weight (Mn) of cellulose were measured by GPC after tricarbanilation (Li et al., 2017). Briefly, the α-cellulose was derivatized with phenyl isocyanate in an anhydrous pyridine system prior to GPC analysis. Size-exclusion separation was performed on an Agilent 1200 HPLC system (Agilent Technologies, Inc., Santa Clara, CA) equipped with Waters Styragel columns (HR1, HR2 and HR6; Waters Corporation, Milford, MA). Number-average degree of polymerization (DPn) and weight-average degree of polymerization (DPw) of cellulose were obtained by dividing Mn and Mw, respectively, by 519 g/mol, the molecular weight of the tricarbanilated cellulose repeating unit. The results were reported as the average value of duplicate measurements.

Cellulose crystallinity analysis by solid-state NMR

Cellulose crystallinity was measured with cross-polarization magic angle spinning (CP/MAS) solid-state NMR according to published procedure (Li et al., 2017). Cellulose for solid-sate NMR analysis was isolated from the holocellulose (150 mg) by hydrolysis at 100 °C with HCl (8 mL of 2.5%) for 2 h. The isolated cellulose was retained at 30%–50% moisture. The NMR samples were packed into 4-mm cylindrical Zirconia MAS rotors. CP/MAS NMR analysis of cellulose was carried out on a Bruker Avance-400 spectrometer operating at frequencies of 100.59 MHz for 13C in a Bruker double-resonance MAS probe head at spinning speeds of 8 kHz. CP/MAS experiments utilized a 5 ms (90°) proton pulse, 1.5 ms contact pulse, 4 s recycle delay and 2048 scans. The cellulose crystallinity index (CrI) was determined from the areas of the crystalline and amorphous C-4 signals using the following formula:

\[
\text{CrI} = \frac{A_{86.5-92 ppm}}{A_{86.5-92 ppm} + A_{87.8-88.6 ppm}}
\]

Saccharification assay

Dried and Wiley-milled (40 mesh) stems of the Populus control and transgenic plants were used for saccharification assays.
Overexpressing PFD2.2 reduces recalcitrance in poplar

Conflict of interest
The authors declare no conflict of interest.

Author contributions
JZ, WM and JGC designed this study. JZ performed experiments, conducted data analysis and wrote the manuscript. MX performed subcellular localization analysis. ML and JD performed chemical compositional analysis. WR, KAW and CMC generated Populus transgenic lines. VS, EAL, KB and JS generated RNA-Seq data. ACB, SJJ and LEG measured biomass production. NLE and TJJ generated and analysed metabolomics data. XY designed the construct for Populus transformation. GAT, WM and JGC conceived the study, coordinated research and contributed to experimental design and data interpretation. JZ, YP, TJJ, GAT and JGC revised the manuscript. All authors read and approved the final manuscript.

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