Secondary growth of woody stems involves the coordination of several developmental processes, including the patterning of secondary vascular tissues and differentiation of complex cell types [1]. In the model forest tree genus *Populus*, the cambium is typically believed to contain a single layer of initials, from which xylem mother cells are derived inside the pith of the shoot apical meristem. The cambium is also called the cambial zone, and it consists of meristematic cells that differentiate into xylem or phloem tissue. The cambium is responsible for the production of secondary xylem (wood) and phloem, which are the primary tissues that add thickness to the woody stem. Secondary xylem (wood) or secondary phloem (inner bark) are produced one or more times before differentiating into cell types within the secondary xylem (wood) or secondary phloem (inner bark), respectively. Conspicuous, lignified phloem fibers differentiate in the pith, while xylem and phloem fibers differentiate in the cambium during secondary growth. The cambium is a dynamic tissue that maintains the balance between lignification and cell differentiation.

Although still poorly defined, insights into mechanisms regulating cell differentiation and patterning during the secondary growth of woody tissues are emerging, and have been assisted by the observation that at least some of the key regulatory genes that regulate shoot apical meristems and primary vascular development are also expressed during secondary growth [3,4,5]. For example, Class I KNOX genes are well characterized for their roles in regulating stems and secondary xylem cell differentiation. These genes have been found to be expressed in cambial and cambial daughter cells [6,7]. In contrast, NAC-domain containing transcription factors have been identified that regulate cambial differentiation and cell wall biosynthesis, and these genes are involved in negative regulation of cambial and cambial daughter cells [8]. The role of NAC-domain containing transcription factors is reviewed in [8]. For example, overexpression of VND6 or VND7 results in ectopic differentiation of vessel elements in *Arabidopsis thaliana*, which is accompanied by overexpression of MYB genes (including MYB46, MYB63, MYB83, MYB85, and MYB103) and downstream genes encoding enzymes involved in cell wall biosynthesis and vessel differentiation (including CESA4/IRX5, CESA7/IRX3, CESA8/IRX1, IRX8, IRX10, CCaOMT7, IRX12/LAC4, and...
of meristem size in plants of CNA transcripts was reported not to result in obvious phenotypic changes, but no other dramatic phenotypes [11,27], and RNAi suppression of CNA expression in mutants show slightly increased shoot apical meristem size [11]. Antisense single point mutation abolishing normal miRNA regulation are seen in mutants carrying semidominant alleles of CNA with a moderate dwarfing, upcurling of leaves, and drastic reduction in vascular bundles, but is not expressed in mature leaves [25]. In provascular cells and developing xylem parenchyma of leaf procambial cells in embryos and developing organs, and Class III HD ZIPs are expressed in provascular cells of leaves and roots [25], in A. thaliana Class III HD ZIPs, ATHB8 is expressed in procambial cells in embryos and developing organs, and ATHB8 expression is induced by exogenous auxin [18]. Overexpression of ATHB8 is associated with precocious differentiation of xylem and lignification of cell types that are normally not lignified [19]. All known land plant Class III HD ZIPs contain a binding site for negative regulation posttranscriptionally by miRNA165/166 [20]. Mutations that abolish the miRNA binding sequence without changing amino acid sequence result in dominant phenotypes for CNA [21,12,13,14,15,16,17].

CNA is expressed in provascular cells of leaves and roots [25], in the shoot meristem, in floral meristems, and in ovules [11]. A Z. mays ortholog of CNA, ZnHB-13, is expressed in leaf provascular cells and developing xylem parenchyma of leaf vascular bundles, but is not expressed in mature leaves [25]. In A. thaliana, overexpression of a miRNA-resistant ATHB13 results in moderate dwarfing, upcurling of leaves, and drastic reduction in xylem and lignified interfascicular tissues [21]. Similar phenotypes are seen in mutants carrying semidominant alleles of CNA with a single point mutation abolishing normal miRNA regulation [26,27,28]. Antisense ATHB15 transgenics are severely dwarfed, and display expansion of xylem and interfascicular tissues, and lignification into the pith of stems [21]. In contrast, EMS-induced cna mutants show slightly increased shoot apical meristem size [11] but no other dramatic phenotypes [11,27], and RNAi suppression of CNA transcripts was reported not to result in obvious phenotypic changes [27]. Interestingly, cna is a dramatic enhancer of meristem size in dionaea /2/3 mutants, indicating that CNA may function with CLV genes to promote organ formation [11]. However, neither the genes directly regulated by CNA nor the biological functions influenced by CNA have been identified, and CNA function during secondary growth in plants has not been examined.

Class III HD ZIP transcription factors are evolutionarily ancient and found in all major lineages of land plants [17,20,29]. Importantly, Class III HD ZIPs predate evolution of vascular tissues and adaxial polarity of lateral organs [29], suggesting that many of the functions assigned to Class III HD ZIPs in angiosperms are derived. Ancestral functions of Class III HD ZIPs could include regulation of apical or meristematic growth [20,29], and auxin transport [30]. While Class III HD ZIPs roles during secondary growth have not been characterized, they are known to be expressed during secondary growth from microarray analysis of wood forming tissues in Populus [3,31,32]. Class III HD ZIPs are thus candidates for regulating fundamental developmental processes acquired during the evolution of secondary growth.

We report here the cloning and characterization of a Populus CNA ortholog, POPCORONA. We defined roles for PCN during secondary growth by examining PCN expression and mutant phenotypes in transgenic Populus expressing either an artificial miRNA targeting PCN transcripts or overexpressing a miRNA-resistant form of PCN. Together, our results suggest that PCN regulates development of secondary vascular tissues, potentially through regulation of transcriptional modules associated with cell differentiation and/or hormone-mediated processes.

Results

POPCORONA encodes a Class III HD ZIP transcription factor

The POPCORONA (PCN) gene (also called Pt-ATHB.12; Joint Genome Institute Populus v.1.1 gene modeligenesh4_pm.C. LG_I000560; Phytoseiulus Populus v2.0 gene model POPTR_0001s18930: GenBank XM_002299699) was amplified from cDNA of the sequenced Populus trichocarpa individual [33] based on similarity to the Arabidopsis thaliana ATHB15/CORONA (Materials and Methods). To determine the relationship of PCN to other Class III HD ZIPs, a phylogenetic analysis of Class III HD ZIP sequences from whole-genome sequencing projects was undertaken (Materials and Methods). Maximum parsimony analysis from the nucleotide sequence alignment of the coding sequences of Class III HD ZIPs found in 17 plant species and deduced from amino acid sequence alignments yielded a single tree, with clade support from Bayesian and bootstrap analysis (Fig. 1). Sequences from Physcomitrella patens, a moss, were used as outgroups to root the tree. Sequences from the lycophyte representative, Selaginella moellendorffii, formed a clade (53% bootstrap, 1.00 Bayesian) sister to sequences from the angiosperm taxa, which formed a strongly supported (100% bootstrap, 1.00 Bayesian) clade. These results all reflect previously reported Class III HD ZIP gene family relationships [17,20]. Among the angiosperm sequences, three strongly supported clades were formed, REV, PHB/PHV, and C8, which is in agreement with previous reports [17]. REV and PHB/PHV clades are sister to each other forming a larger clade that is sister to C8. Relationships within each of the three clades are generally consistent with currently accepted ideas about angiosperm phylogeny [34]. Our analysis includes four representative species from the grass lineage; O. sativa, Z. mays, B. distachyon, and S. bicolor, allowing further details in the divergence of homologues within this monocot lineage. Sequences from these four species form monophyletic clades separate from the eudicot species. Within these clades two duplication events appear to have occurred after the monocot-eudicot split. This duplication likely represents the grass whole genome duplication event [35,36].

The major duplication events previously reported are mostly in agreement with our phylogenetic tree, with a few exceptions. Our phylogeny does not include representatives of species within the ferns, basal angiosperms, or gymnosperms, and therefore our analysis cannot dispute or support duplication events that were suggested previously to have occurred in these lineages. In our parsimony tree (Fig. 1), bootstrap supports ATHB8 and ATHB8 (100% bootstrap), and MgHB7 (80% bootstrap) as sister to the rest of the CNA/HB8 eudicot clade, while Bayesian analysis supports them within the HB8 clade (1.00 Bayesian). Supporting ATHB8, ATHB8, and MgHB7 within the HB8 clade is evidence for a duplication event resulting in the HB8 and CNA clades, as previously reported (Prigge and Clark, 2006). Therefore, our parsimony and Bayesian analysis are not in agreement with their placement, and only partially support previous suggestions of a
duplication event in C8 clade. This inconsistency may be an artifact of limited and uneven sampling, as discussed above.

Within *Populus*, each Class III HD ZIP is represented by two paralogs (Fig. 1), reflecting the genome duplication event whose signature was found in analysis of the *P. trichocarpa* genome [33]. Independent duplication events are present in the PHV/PHB clade in the *A. thaliana* and *Populus* lineages (Fig. 1). Two paralogs of *A. thaliana* ATTHB15/CNA are present, including PCN and Pt-ATHB.11 (Phytozome *Populus* v2.0 gene model POPTR_0003s04860; Joint Genome Institute *Populus* v1.1 gene model estExt_fgenesh4_pg_C_433).

**PCN** is expressed during secondary growth

*PCN* is expressed in shoot apices, leaves, stems, and roots of young *Populus* plants. Transcript levels were determined for organs of tissue culture-grown *Populus alba* x *tremlula* using quantitative real time PCR (Materials and Methods). Primers amplifying *PCN* transcripts revealed highest expression in stems and apices, with lower but significant expression in leaves and roots (Fig. 2a). Primers amplifying a *PCN* paralog, Pt-ATHB.11, showed similar expression patterns for this gene, although expression was slightly higher in apices relative to stem tissues (Fig. 2b).

*PCN* is expressed broadly in the cambial zone and xylem of *Populus* shoots. Whole mount *in situ* hybridization was used to visualize *PCN* expression in tangential sections from different developmental stages of *Populus* stems (Materials and Methods). During primary growth and the transition to secondary growth, *PCN* is expressed broadly in the cambial zone and in developing xylem (Fig. 3a,b). Later in development, phloem fiber differentiation becomes evident (Fig. 3c,f) and weak signal is seen outside of the cambial zone at this stage in the developing phloem, including the phloem fibers (Fig. 3c,f). Moving further down the stem into developmentally older tissues, *PCN* expression is maintained within developing xylem, and is most pronounced in rays (Fig. 3 i,j). At the base of the stem, strongest expression is found in the cambial zone, with reduced expression in the secondary xylem (Fig. 3m,n). However, expression is still pronounced within the rays traversing the secondary xylem (Fig. 3n). In comparison to sense-probe negative controls (Fig. 3c,g,k,o), the in situ staining of experimental sections with the *PCN* antisense probe is specific and has relatively low background. However, based on comparison of *PCN* antisense and sense negative control sections we cannot exclude the possibility of low *PCN* expression, cross hybridization to related genes, or diffusion of the stain in epidermis, pith cells, or other tissues/cell types. Differential staining caused by differences in cytoplasmic density of cell types is revealed by an antisense probe for a presumably ubiquitously expressed gene encoding a 50S ribosomal protein (Fig. 3d,h,l,p).

Misregulation of *PCN* results in whole plant phenotypes

To examine the function of *PCN* during plant development, the *Populus* clone INRA 717-IB4 (*Populus alba* x *P. tremula*) was transformed with recombinant DNA constructs to either up- or down-regulate *PCN* transcript levels. To upregulate *PCN* transcript...
levels, a *PCN* cDNA was modified to change the miRNA165/166 recognition sequence without changing the amino acid sequence when translated into protein, thus making the transcript transparent to negative regulation by miRNA165/166 (Materials and Methods). This cDNA was cloned behind a 35S cauliflower mosaic virus promoter in a T-DNA vector to create construct 35S::PCN-miRNAd, and transformed into *Populus* using an *Agrobacterium*-based method (Materials and Methods). To down-regulate *PCN* transcripts levels, an artificial miRNA [37,38] was designed to target both *PCN* and its paralog, Pt-ATHB.11, and cloned behind the 35S promoter in a T-DNA vector to create construct 35S::miRNA-PCN, which was then transformed into *Populus* (Materials and Methods). Two plants independently transformed with 35S::PCN-miRNAd, and two plants independently transformed with 35S::miRNA-PCN were recovered and used in the analyses here. Quantitative Reverse Transcription Polymerase Chain Reaction (Q-RT-PCR) analysis of the transformants and wild-type controls showed that for plants transformed with 35S::PCN-miRNAd, one line (35S::PCN-miRNAd-4-1) had approximately two-fold increase of *PCN* transcript abundance relative to controls, while line 35S::PCN-miRNAd-4-3 had over twelve-fold increase in *PCN* transcript abundance.

Figure 3. Expression of *PCN* during *Populus* stem development revealed by whole mount in situ hybridization. Antisense *PCN* (first and second columns), sense negative control (third column), and positive control (fourth column) probes were hybridized to stem sections from two month old tissue culture grown trees. (a) Section from first elongating internode hybridized with antisense *PCN* probe. *PCN* is expressed broadly during primary growth, with strongest expression associated with procambium. (b) Higher magnification of first elongating internode hybridized with antisense *PCN* probe. (c) Section from first elongating internode hybridized with sense *PCN* probe (negative control), showing minimal background hybridization. (d) Section from first elongating internode hybridized with antisense pop50S probe (positive control). (e) Section from the fourth internode, hybridized with antisense *PCN* probe. *PCN* is expressed broadly in the cambial zone, and strongly in differentiating xylem. (f) Higher magnification of (e). (g) Section from the fourth internode hybridized with negative control sense *PCN* probe. (h) Section from fourth internode hybridized with positive control antisense pop50S probe. (i) Section from seventh internode hybridized with antisense *PCN* probe. *PCN* expression is mostly associated with differentiating xylem cells and lightly in cambial zone. (j) Higher magnification of (i). (k) Section from seventh internode hybridized with sense *PCN* probe (negative control). (l) Section from seventh internode hybridized with positive control antisense pop50S probe. (m) Section from the base internode hybridized with antisense *PCN* probe. *PCN* expression is largely limited to the differentiating xylem cells and cambial zone. (n) Higher magnification of (m). (o) Section from the base internode hybridized with sense *PCN* probe (negative control). (p) Section from the base internode hybridized with positive control antisense pop50S probe. Cambial zone (Ca), Phloem fiber (Pf), Procambium (Pc), Ray (r), Xylem (Xy), Bar = 100 μm. doi:10.1371/journal.pone.0017458.g003
Comparison of PCN transcript abundance in all transgenics compared to the wild-types. (a) Comparison of quantitative real-time PCR (qRT-PCR) reactions of independent RNA samples for each transgenic and the wild-type prepared from different batches of two month-old plants. T test (P<0.05) comparison showed significant differences of expression levels (mean ± SE) were calculated from triplicate qRT-PCR reactions in wild-type and transgenic plants. 

Figure 4. PCN expression levels in PCN 35S::miRNA-PCN gain of function and 35S::miRNA-PCN knockdown transgenic plants relative to wild-type controls. PCN expression levels were detected by quantitative real-time PCR (Materials and Methods). Relative expression levels (mean ± SE) were calculated from triplicate qRT-PCR reactions of independent RNA samples for each transgenic and the wild-type prepared from different batches of two month-old plants. T test (P<0.05) comparison showed significant differences of expression levels in wild-type and transgenic plants. (a) Comparison PCN transscripts in wild-type and 35S::miRNA-PCN gain of function plants. (b) Comparison PCN transcripts in wild-type and 35S::miRNA-PCN plants. 

Misregulation of PCN alters secondary growth

Wild-type Populus make a gradual transition from primary to secondary growth. Under our culture conditions, wild-type Populus clone INRA 717-IB4 (Populus alba × P. tremula) is already transitioning to secondary growth by the fourth elongating internode from the apex, as seen in stem cross sections stained with toluidine blue (Fig. 6). By the seventh internode, secondary xylem characterized by cell files derived from the cambial initials is apparent (Fig. 6a). Nascent phloem fibers are apparent by the seventh node but are not yet highly lignified. At the base of the stem, cell files of lignified secondary xylem can be seen emanating from the cambial zone, and fully differentiated and lignified phloem fibers are seen at the periphery of the phloem (Fig. 6c). At lower magnification (Fig. 6d), the radial organization of the stem is seen, with successive layers of pith, secondary xylem, cambial zone, secondary phloem and phloem fibers, cortex, and epidermis.

In 35S::PCN-miRNAd-4-3 plants, cell files within the cambial zone are more readily apparent than in wild-type control plants at the fourth internode (Fig. 6e). The premature appearance of differentiating phloem fibers also distinguishes these stems from the wild-type, although the fibers are not lignified (Fig. 6e). Internode seven of 35S::PCN-miRNAd-4-3 plants is characterized by a distinct cambial zone, modest secondary xylem, and un lignified cells presumed to be differentiating phloem fibers (Fig. 6f). A section from the base of the stem shows relatively well-developed secondary xylem, but is largely lacking the lignified phloem fibers that conspicuously occur in the periphery of the phloem of wild-type plants. Thus, although phloem fiber differentiation appears to initiate prematurely in 35S::PCN-miRNAd plants, they do not complete normal differentiation and lignification.

As mentioned, 35S::miRNA-PCN plants had only a modest reduction in PCN transcript abundance (Fig. 4), and no whole plant phenotype was apparent (Fig. 5). However, cross sections revealed subtle defects in stem development. At the fourth internode from the apex, lignified phloem fibers are already apparent in 35S::miRNA-PCN plants, while they are lacking at this stage of development in the wild-type (Fig. 6f). Also at this position, cambium activity is evident as cell files within the cambial zone, and modest amounts of secondary xylem have formed. By internode seven, 35S::miRNA-PCN plants have more abundant secondary and noticeably more highly lignified phloem fibers than the wild-type (Fig. 6g). Sections through the bottom of the stem are similar to the wild-type, except that there is abnormal lignification of some cells within the pith (Fig. 6h). These abnormally lignified cells are primarily found adjacent to the position of primary vascular bundles (Fig. 6i). In summary, misexpression or down-regulation of PCN results in differences in the number of phloem fibers, number of xylem cell layers, and number of lignified pith cells, as quantified in Fig. 7.

Misregulation of PCN changes expression of genes associated with gene regulation, hormones and cell differentiation

To begin to understand the biological processes and genes regulated directly or indirectly by PCN during secondary growth, global transcript abundance was assayed using microarrays for
stems from wild-type control and 35S::PCN-miRNAd-4-3 plants (Materials and Methods). RNA was isolated from stems of four plants each of 35S::PCN-miRNAd-4-3 and matched wild-type controls (Materials and Methods). The RNA quality and quantity was assayed with Agilent Bioanalyzer before being labeled and hybridized to Affymetrix Populus whole transcriptomes gene chips (Materials and Methods). The resulting data were normalized and analyzed using dChip software (http://biosun1.harvard.edu/complab/dchip/) to identify genes with statistically differential expression between the wild-type and 35S::PCN-miRNAd stems (Materials and Methods). Data are available as accession GSE19467 through NCBI GEO (http://www.ncbi.nlm.nih.gov/geo/).

Of the approximately 45,000 genes in the Populus genome [33], 237 genes showed statistically significant two-fold or greater differential transcript abundance between the wild-type and 35S::PCN-miRNAd stems (see Supplemental Table S3 for analysis of all probes with statistically different transcript levels). Of these genes, those belonging to three biological categories (transcription, hormone-related, and cell wall-related) are discussed here in more detail with the idea that they are potentially associated with the function of PCN and the phenotype of PCN transgenics. It should be noted that presumably only a subset of these genes may be direct targets of PCN.

Sixteen genes encoding transcription factors from ten different families are misregulated in 35S::PCN-miRNAd stems (Table 1). Interestingly, this group includes Populus orthologs of A. thaliana transcription factors that have been implicated in a transcriptional network controlling vascular cell differentiation and lignification. SECONDARY CELL WALL-ASSOCIATED NAC DOMAIN (SND) and VASCULAR RELATED NAC DOMAIN (VND) proteins have been shown to positively regulate expression of specific MYB transcription factors [9,39,40,41]. In 35S::PCN-miRNAd stems, transcript levels of Populus orthologs of NAC7/VND4 (gw1.III.864.1) and SND2 (eugene3.01240095) are upregulated, as are orthologs of previously proposed VND/SND targets MYB83 (gw1.IX.3293.1), MYB85 (gw1.IX.3293.1), MYB32 (eugene3.00120054) [9,39]. Additionally, a Populus ortholog (fgenesh4_pm.C_scaffold_66000095) of XYLEM ENDOPEPIDASE 1 (XCP1) as well as categories (cellulose synthesis-related, lignin-related) and specific orthologs of cell wall-related genes (including IRREGULAR XYLEM3 and IRX8 orthologs) that are targets of these MYB transcription factors [39] are misregulated in 35S::PCN-miRNAd stems (Table 2).

Nineteen cell wall-related genes have altered transcript levels in 35S::PCN-miRNAd (Table 2). Four of the eight cell wall-related genes with increased transcript levels are involved in cellulose biosynthesis, including the previously mentioned orthologs of IRX3 and IRX8 as well as orthologs of CESAl/RSW1 (grail3.0052005901) and CSLA9/RAT4 (estExt_fgenesh4_pm.C_LG_VIII0087). The remaining eleven wall-related genes have lower transcript levels in 35S::PCN-miRNAd and participate in functions including lignification (putative laccase, cinnamoyl CoA reductase, chalcone synthase), and pectin biosynthesis and modification (putative pectin methylesterases and pectinesterase).

Six hormone-related genes are misregulated in 35S::PCN-miRNAd (Table 3). Two putative auxin-responsive genes (grail3.0050017401, grail3.0061005101) show elevated transcript levels in 35S::PCN-miRNAd. While two gibberellic-related genes have higher transcript levels in 35S::PCN-miRNAd, one (GA 20-
oxidase, fgenesh4_pm.C.LG_II000702) is putatively involved in GA biosynthesis [42] while the other (GA 2-oxidase, estExt_fgenesh4_pg.C.LG_XI0670) is putatively involved in GA catabolism [43]. Transcript abundance for a gene encoding a putative cytokinin oxidase (gw1.VI.2253.1) involved in inactivation of cytokinin [44] and a gene encoding a cytokinin response factor (Table 1, gw1.I.8677.1) are lower in 35S::PCN-miRNAd. Auxin, cytokinin, ethylene, and gibberellins have been implicated in various studies as regulators of vascular development and secondary growth, although their precise roles remain uncertain [1].

**Discussion**

Class III HD ZIP transcription factors have been implicated in regulating diverse developmental processes, but their function during secondary vascular development has not been addressed. In addition, the genes and cellular processes regulated by Class III HD ZIPS are poorly understood. We examined here the expression and function of a Populus Class III HD ZIP, POPCORONA (PCN), during secondary growth and correlated PCN expression with changes in anatomy and gene expression that are consistent with a role in influencing cell differentiation.

Class III HD ZIPS are best characterized in *Arabidopsis thaliana*, where they have been shown to be involved in meristem initiation and function, polarity of lateral organs, and vascular development. Interestingly, among the Class III HD ZIPS in *A. thaliana*, only REVOLUTA and CORONA (CNA) present loss of function phenotypes [10,11,45,46,47,48]. The cna loss of function phenotype is subtle, and largely limited to a slightly increased meristem size [11]. In *A. thaliana*, CNA is among the earliest expressed markers of vascular development [11,25], and is expressed in procambial cells in leaves, shoot apical meristems, floral meristems, stamens, and carpels [11,25]. A zinnia ortholog of CNA, ZeHB-13, is also expressed in procambial cells of developing leaves, but is not expressed at detectable levels in mature leaves [25]. In addition, ZeHB-13 is expressed in tracheary elements differentiating in vitro, but is not directly induced by cytokinin or auxin in that system [25]. We found that PCN is not restricted to provascular cells or primary xylem in *Populus* stems during primary growth. In *Populus*, PCN is also expressed during secondary growth, with strongest expression in phloem fibers, the cambial zone,
PCN expression is maintained in older secondary xylem, and potentially pith (Figure 3). Interestingly, PCN expression is maintained in secondary vascular tissues but not primary vasculature of leaves could reflect differences among species, but more likely reflects differences among these tissue types. Specifically, cambial and ray tissues are not present in A. thaliana as has been described for A. thaliana Class III HD ZIPs [10]. While the A. thaliana cna loss of function phenotype is subtle [11], A. thaliana plants expressing an antisense CRF2 transgene present a strong phenotype that includes dwarfing, expansion of xylem and interfascicular tissues, and lignification into the pith (Kim et al., 2005). Possible explanations for the discrepancy between these phenotypes include that cna alleles characterized may not be complete loss of CRF2 function, or that the sense transgene affected expression of multiple Class III HD ZIP family members.

Populus overexpressing a miRNA-resistant (non-cleavable) PCN (35S::PCN-miRNAAd) had stronger stem phenotypic changes, with delayed differentiation of secondary xylem and severe reduction of phloem fiber differentiation (Figure 6). Cells consistent with nascent phloem fibers form in appropriate positions in 35S::PCN-miRNAAd plants, but fail to complete differentiation or fully lignify. Phloem fibers could be considered abaxial in their position within the stem, as their position is analogous to and continuous with the abaxial phloem of primary vascular tissues in leaves. Although one possible interpretation of this phenotype is that overexpression of PCN promoted adaxialization of the stem affecting phloem fiber development, we do not favor this interpretation, in part because phloem fibers initiate in an appropriate position, and simply fail to properly complete differentiation. Additional evidence supporting primary affects of PCN on cell differentiation vs. patterning was provided by gene expression profiling of PCN mutant stems. Genes associated with the biosynthesis, response, or catabolism of auxin, gibberellins, and cytokinin were misregulated in 35S::PCN-miRNAAd stems (Table 3). In addition, an AP2-like transcription factor orthologous to Cytokinin Response Factor 2 (CRF2) is downregulated in 35S::PCN-miRNAAd stems. CRF2 is upregulated by cytokinin, and acts with other CRFs to regulate a large fraction of genes involved in cytokinin response [49]. Interestingly, these same hormones have all been implicated in fiber differentiation, with auxin and gibberellins flowing basipitally through the stem from leaves [50,51], and cytokinin from roots [52]. Indeed, mutations in the related A. thaliana Class III HD ZIP REVOLUTA/INTERFASCICULAR FIBERLESS 1 can result in defects in interfascicular fiber differentiation [47], which are associated with polar auxin transport defects in stems [48]. These results are also consistent with the implication of Class III HD ZIP genes in affecting expression of PIN1, an auxin efflux transporter, during embryogenesis in A. thaliana [30]. Thus, we favor the interpretation that PCN phenotypes do not reflect patterning or polarity defects, but more likely hormone-related defects and changes in genes involved in cell differentiation and cell wall biosynthesis, as discussed below.

In addition to hormone-related genes discussed above, other transcription factors as well as cell wall biosynthetic genes are misexpressed in 35S::PCN-miRNAAd stems (Table 1, 2). Interestingly, these genes include homologs of SECONDARY CELL WALL-ASSOCIATED NAC DOMAIN (SND), VASCULAR RELATED NAC DOMAIN (VND), and MYBs that have been implicated in a hierarchical pathway regulating genes involved with tracheary element development and cell wall synthesis and lignification [9,39,40,41]. Cell differentiation and cell wall-related genes associated with these transcriptional regulators also show misregulation in 35S::PCN-miRNAAd (see Results). The changes in cell...
wall–related genes, including downregulation of lignin-related genes in 35S::PCN-miRNAd stems are consistent with anatomical phenotypes, which include inhibition of phloem fiber lignification in 35S::PCN-miRNAd and abnormal lignification of cells in the pith of 35S::miRNA-PCN. Importantly, it should be noted that the two SND/VND genes misexpressed in 35S::PCN-miRNAd are orthologous to SND2 and NAC7/VND4, not the better characterized SND1 and VND6&7 [8], and the expression relationships of MYBs to putative targets is altered. Thus, in addition to differences expected between species and tissue types, the relationships among the transcriptional networks underlying secondary growth in Populus and primary growth in A. thaliana will require further study to comprehensively compare.

Given that Class III HD ZIPs are evolutionarily ancient and predate evolution of vascular tissues [29], the function of PCN during secondary growth must be derived. PCN and its orthologs in A. thaliana and Zinnia [11,17,25] are not uniquely expressed during secondary vascular development, and the proteins’ biochemical functions are presumably the same in the different tissues where it is expressed. However, it is possible that target genes of PCN vary between tissues, because of differences in available partners for dimerization, dominant negative LITTLE-ZIPPERs [53,54], different co-factors, or differences in regulation by miRNA165/166 in different tissues. Although beyond the scope of the work presented here, it will ultimately be very informative to examine changes in gene expression for PCN or related mutants in the various tissues in which these genes are normally expressed. Unfortunately there are no published reports of gene expression profiling for A. thaliana CNA mutants to date. An example of a potentially important difference for PCN/CNA

| JGI Gene Accessiona | Fold Changeb | Arabidopsisc | Definition Linea |
|---------------------|-------------|-------------|-----------------|
| gw1.IX.329.1        | 3.82        | AT2G26800   | MYB domain protein 85 (MYB85) |
| gw1.III.861.1       | 3.47        | AT1G12260   | NAM protein, NAC7/VND4 |
| eugene3.00120054    | 3.36        | AT1G17950   | MYB domain protein 52 (MYB52) |
| *grail3.0001068002  | 3.24        | AT1G10200   | transcription factor LIM |
| eugene3.0001068002  | 3.21        | AT1G10200   | transcription factor LIM |
| gw1.IV.991.1        | 2.96        | AT3G28310   | heat shock transcription factor A68 (HSFA68) |
| eugene3.001310019   | 2.73        | AT1G17950   | MYB domain protein 52 (MYB52) |
| eugene3.001240095   | 2.65        | AT4G28500   | NAM protein, SND2 |
| gw1.II.2517.1       | –2.83       | AT3G60530   | GATA transcription factor 4 (GATA4) |
| gw1.XI.648.1        | –3.26       | AT1G01030   | DNA-binding protein |
| fgenesh4_pg.C_570199| 2.99        | AT4G36740   | A. thaliana homeobox protein 40 (ATHB40) |
| gw1.V.991.1         | 2.96        | AT3G28310   | heat shock transcription factor A68 (HSFA68) |
| eugene3.001310019   | 2.73        | AT1G17950   | MYB domain protein 52 (MYB52) |
| eugene3.001240095   | 2.65        | AT4G28500   | NAM protein, SND2 |
| gw1.II.2517.1       | –2.83       | AT3G60530   | GATA transcription factor 4 (GATA4) |
| gw1.XI.648.1        | –3.26       | AT1G01030   | DNA-binding protein |
| fgenesh4_pg.C_570199| 2.99        | AT4G36740   | A. thaliana homeobox protein 40 (ATHB40) |

* Accession number assigned by the Joint Genome Institute (http://genome.jgi-psf.org).
* Fold Change is expressed as the ratio of gene expression in PCN gain of function transgenics to wild type control.
* Accession number of the best Arabidopsis BLAST return using the JGI gene model as query.
* Definition line is from the Arabidopsis accession at TAIR (http://www.arabidopsis.org).
* Transcript level differences confirmed by qRT-PCR.

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Table 2. Genes up or down-regulated in PCN transgenics involved in hormone related processes.

| JGI Gene Accessiona | Fold Changeb | Arabidopsisc | Definition Linea |
|---------------------|-------------|-------------|-----------------|
| *fgenesh4_pg.C_LI0000702 | 4.67  | AT4G25420 | gibberellin 20-oxidase |
| *grail3.0005017401  | 3.78  | AT3G62100  | auxin-responsive protein |
| *estExt_fgenesh4_pg.C_LI0000702 | 3.16 | AT1G78440 | gibberellin 2-oxidase |
| *grail3.00061005101 | 3.08  | AT3G62100  | auxin-responsive protein |
| gw1.VI.2253.1       | –2.41 | AT5G21482  | putative cytokinin oxidase |
| estExt_Genewise1_v1.C_LG_XI03374 | –7.1  | AT4G22070 | WRKY DNA-binding protein 31 (WRKY31) |

* JGI gene accession refers to the accession number assigned by the Joint Genome Institute (http://genome.jgi-psf.org).
* Fold Change is expressed as the ratio of gene expression in PCN gain of function transgenics to wild type control.
* Arabidopsis refers to the accession number of the best Arabidopsis BLAST return using the JGI gene model as query.
* Definition line is from the Arabidopsis accession at TAIR (http://www.arabidopsis.org).
* Transcript level differences confirmed by qRT-PCR.

Table 2. Genes up or down-regulated in PCN transgenics involved in hormone related processes.
Table 3. Genes up or down-regulated in PCN transgenics involved in cell wall synthesis related processes.

| JGI Gene Accession* | Fold Changeb | Arabidopsis* | Definition Lineb |
|---------------------|--------------|--------------|------------------|
| estExt.Geneview1_v1.C_LG.V02188 | 3.76 | AT5G17420 | cellulose synthase, CESA7/IRREGULAR XYLEM 3 (IRX3) |
| estExt.fgenesh4.pm.C_LG.V080087 | 3.43 | AT5G03760 | cellulose synthase like, CSLA9/RTA4 |
| grail3.0052005901 | 3.35 | AT4G32410 | cellulose synthase, CESA1/RADIIALLY SWOLLEN1 (RSW1) |
| eugene3.00111083 | 3.10 | AT5G54690 | galacturonosyltransferase, IRX8 |
| gw1.XV.2531.1 | 2.89 | AT5G61750 | merin-like protein-like |
| fgenesh8.pg.C_LG._003312 | 2.82 | AT1G23460 | polygalacturonase |
| gw1.VII.534.1 | 2.72 | AT4G28380 | extensin-like protein |
| gw1.VI.781.1 | 2.44 | AT4G30420 | nodulin-like protein; MNI21 gene product |
| gw1.XIII.2619.1 | −2.52 | AT2G23910 | cinnamoyl CoA reductase-like |
| gw1.131.165.1 | −2.55 | AT1G56710 | polygalacturonase |
| grail3.0048017501 | −2.61 | AT1G03870 | fasciclin-like arabinoagalactan 9 (FLA9) |
| gw1.856.4.1 | −2.83 | AT3G22142 | proline-rich cell wall protein |
| estExt.Geneview1_v1.C_LG.V11401 | −2.97 | AT5G09760 | pectin methyltransferase-like protein |
| gw1.VIII.2100.1 | −3.37 | AT5G05390 | LACCASE 12 (LAC12) |
| eugene3.00140920 | −3.55 | AT5G13930 | chalcone synthase, TRANSPARENT TESTA 4 |
| estExt.fgenesh4.pm.C._1480004 | −3.78 | AT2G44480 | glycosyl hydrolase family 1 |
| fgenesh8.pg.C.LG.V000014 | −3.95 | AT5G09760 | pectin methyltransferase-like protein |
| gw1.X3259.1 | −4.32 | AT3G01720 | pectinesterase |
| gw1.III.2731.1 | −6.6 | AT1G62500 | putative proline-rich cell wall protein |

*Accession number assigned to the assayed gene model by the Joint Genome Institute (http://genome.jgi-psf.org).

bFold Change is expressed as the ratio of gene expression in PCN gain of function transgenics to wild type control.

*Arabidopsis accession at TAIR (http://www.arabidopsis.org).

*Definition line is from the Arabidopsis accession at TAIR (http://www.arabidopsis.org).

Nucleotide sequences were translated into amino acid sequences using DAMBE [55]. Amino acid alignment was achieved using ClustalX (version 1.8) [56] with all default settings. The alignment was modified manually using MacClade 3.08 [57] and exported in NEXUS format for further analysis with the final alignment containing 3261 sites. A nucleotide alignment was created based on the amino acid alignment using DAMBE [55].

Maximum parsimony analysis of the nucleotide alignment was performed using PAUP* version 4.0b10 for Macintosh [58], with heuristic searches using the TBR branch-swapping algorithm and 1000 random taxon addition replicates; the maxtrees setting was allowed to increase automatically as necessary. Gaps within the alignment were treated as missing data. Relative support for clades was assessed using 1000 bootstrap replicates with 10 random taxon addition replicates per bootstrap replicate and maxtrees capped at 100. Bayesian analyses, using the GTR+G model of sequence evolution as selected by MrAIC [59], were implemented in MrBayes 3.1.1 [60]. Double analyses were run with four chains for 1,000,000 generations, sampling every 100 generations. Trees from the first 250,000 generations (2,500 trees) from each run were discarded as burn-in, following the authors’ recommendations and consistent with the observation that the likelihood scores from both runs had stabilized. The sampled trees from both analyses were pooled and majority-rule consensus trees were constructed from the resulting 15,000 trees in order to estimate Bayesian clade credibility values.

Plant Cultivation and Transformation

Hybrid aspen clone INRA 717-IB4 (Populus alba × P. tremula) was used for all experiments. Plants were propagated and
transformed using previously published methods [61]. Two independently transformed lines were used for PCN gain of function analysis (35S::PCN-miRNAad 2-1 and 35S::PCN-miRNAad 2-3). Two independently transformed lines were used for PCN knock down analysis (35S::miRNA-PCN 6-1, and 35S::miRNA-PCN 6-4). All experiments were repeated at least twice using each of the above transformed lines and matched wild-type controls with similar results, unless otherwise stated.

Whole Mount in situ Hybridization
Whole mount in situ hybridization was performed as previously described [7]. A 220- bp fragment from the 5' end of the PCN coding region and a 292-bp fragment from the 5'9 end of the gene were selected to design primers to generate the template of probes using the gene-specific primers: PtCN-F: 5'CACCCTCGAGGAGAATGATGAAGGTGTGTGATGATATG-3' and PtCN-R: 5'CTCCCACCCCTTTGATGTTGCTT-3'. T7 promoter sequence 5'-TAA-TACGACTCACTATAGGG-3' was added to the 5' end of the PCN-R primer and Pop50S-R primer sequence to generate templates of antisense probes for PCN and pop50S. T7 promoter sequence was added to the 5' end of PCN-F primer sequence and Pop50S-F primer sequence to generate templates of sense probes for PCN and pop50S.

Recombinant DNA Constructs
The overexpression of Class III HD Zip genes is not expected to yield strong phenotypes, as these genes contain a microRNA binding site which negatively regulates transcript levels. To prevent this mechanism masking the effects of overexpression of the gene of interest, the microRNA binding site of PCN was changed to abolish microRNA recognition while leaving the protein sequence unchanged, as previously described [13]. The coding sequence of PCN was amplified with PCR primers which replaced base pairs in the microRNA binding site PCN-F 5'-CTGCCA- GAACGCTTGAACCAGTTCCCAG-3' and PCN-R 5'-GCACCAGTTCCTAATCCGTTAGTG-3'. T7 promoter sequence 5'-TAA-TACGACTCACTATAGGG-3' was added to the 5' end of the PCN-R primer and Pop50S-R primer sequence to generate templates of antisense probes for PCN and pop50S. T7 promoter sequence was added to the 5' end of PCN-F primer sequence and Pop50S-F primer sequence to generate templates of sense probes for PCN and pop50S.

Microarray Analysis and Q-RT-PCR
For each genotype, three bulks of three plants each were combined for RNA isolation from shoot apices, leaves, stem, and root tissue of two-month-old tissue culture trees for microarray analysis and Q-RT-PCR. Affymetrix GeneChip® Poplar Genome Array oligonucleotide microarrays were used for all microarray hybridizations. For microarray hybridizations, total RNA was isolated from entire, defoliated stems of two months old tissue culture grown plants. Three independent biological replicate RNAs were isolated for each of three overexpression and miRNA lines, and four independent biological replicates of matched wild-type controls. Total RNA quantity and quality was determined using an Agilent Bioanalyzer (Agilent Technologies, USA). Biotin labeling of target RNAs was performed with one-cycle target kit (One-Cycle Target Labeling and Control Reagents, Affymetrix, P/N 900493), and hybridized according to the manufacturer’s protocol.

Analysis of microarray data was performed with dChip (http://biosun1.harvard.edu/complab/dchip/). Data were normalized across arrays using median probe intensity of the baseline array. Preliminary analysis established filtering and statistical cutoff thresholds, using as criteria the false discovery rate, the identification of biologically meaningful genes, and the inclusion of genes confirmed as being misexpressed by Q-RT-PCR as primary criteria. In the final analysis, model-based expression data were filtered by removing genes whose representative probes did not exceed >= 40% (presence call %) on a given array and >= 50% among arrays. Filtered genes were then compared based on fold expression difference and t-test (p-value of 0.05). False discovery rate was determined by 50 permutations to be 2.4% in the final analysis. MIAME-compliant information about samples, array platform, microarray data and further details of the samples are available through NCBI GEO (GSE19467). Output from microarray analysis is shown at the probe-level in Table S2 (and includes probes designed against Populus ESTs), and for P. trichocarpa gene models in Table S3.

Gene expression differences estimated by microarray analysis were confirmed using qRT-PCR for the indicated genes in Tables 1, 2 and 3. Gene-specific PCR primers were designed to target genes showing differential expression in the microarray comparison of 35S::PCN-miRNAad, 35S::miRNA-PCN and wild-type trees. Primers with Tm of >59°C were designed to produce a product 200-300bp. A tubulin-encoding gene [JGI accession estExt_fgenesh1_pm.C_LG_HII0736] was used as reference gene for Q-RT-PCR. Q-RT-PCR was performed with an MJ Mini Opticon (BioRad) following the manufacturer’s protocols.

Supporting Information
Table S1 Gene sequences from the Class III HD Zip genes family recovered from species that have complete genome sequence using the BLAST function in phytozome.net. (TXT)
Table S2 Output from microarray analysis at the probe-level, including probes designed against Populus spp. ESTs. (XLS)
Table S3 Output from microarray analysis at the level of *P. trichocarpa* gene models, excluding probes designed against *Populus* spp. ESTs.

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