Overexpression of a developing xylem cDNA library in transgenic poplar generates high mutation rate specific to wood formation

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Summary

We investigated feasibility of the Full-length complementary DNA OvereXpression (FOX) system as a mutagenesis approach in poplar, using developing xylem tissue. The main goal was to assess the overall mutation rate and if the system will increase instances of mutants affected in traits linked to the xylem tissue. Indeed, we found a high mutation rate of 17.7%, whereas 80% of all mutants were significantly affected in cellulose, lignin and/or hemicellulose. Cell wall biosynthesis is a major process occurring during xylem development. Enrichment of mutants affected in cell wall composition suggests that the tissue source for the FOX library influenced the occurrence of mutants affected in a trait linked to this tissue. Additionally, we found that FlcDNAs from mutants affected in cell wall composition were homologous to genes known to be involved in cell wall biosynthesis and most recovered FlcDNAs corresponded to genes whose native expression was highest in xylem. We characterized in detail a mutant line with increased diameter. The phenotype was caused by a poplar homolog of LONELY GUY 1 (LOG1), which encodes an enzyme in cytokinin biosynthesis and significantly increased xylem proliferation. The causative role of LOG1 in the observed phenotype was further reaffirmed by elevated cytokinin concentration in the mutant and recapitulation overexpression experiment wherein multiple independent lines phenocopied the original FOX mutant. Our experiments show that the FOX approach can be efficiently used for gene discovery and molecular interrogation of traits specific to woody perennial growth and development.

Keywords: poplar, genetics, fox hunting system.

Introduction

T-DNA insertional mutagenesis approaches have been instrumental in the functional dissection of many plant traits but have been largely limited to the Arabidopsis model (Alonso et al., 2003). Most of these approaches generate recessive loss-of-function mutations requiring rounds of selfing to expose the phenotype. Thus, these approaches are impractical in trees, which have long nonflowering juvenile periods lasting from years to decades.

Nevertheless, dominant insertional mutagenesis approaches, like activation tagging, have been successfully applied to poplar (Busov et al., 2011) and other nonmodel plants (Aulakh et al., 2015; Lu et al., 2014; Mathews et al., 2003; Zubko et al., 2002). Thus far, in poplar, generation and screening of activation tagging population of few hundreds to few thousand lines have been undertaken (Busov et al., 2011; Dash et al., 2017; Harrison et al., 2007; Mathews et al., 2003). The screens of these populations have yielded extremely important discoveries with fundamental and applied value (Dash et al., 2017; Mathews et al., 2003; Yordanov et al., 2010, 2014). However, when populations of restricted sizes are used, the rate of mutant discovery is a major parameter that determines the efficiency of the overall effort. In poplar, we have reported much higher mutation rates of activation tagging compared to Arabidopsis, which makes the application of the method feasible (Busov et al., 2011). However, when a specific trait is of interest, the rate of mutants affecting this specific trait diminishes because of the random nature of the insertion process, and thus, the mutants affected in this trait are only a fraction of all mutants. Therefore, the overall mutation rate and the mutation recovery within a specific process are of major importance.

The Full-length complementary DNA OvereXpression (FOX) system was first developed in Arabidopsis (Ichikawa et al., 2006; Kondou et al., 2010). The system uses en block transformation of a normalized full-length complementary DNA (FlcDNA) library, directionally cloned between the strong 35S promoter and terminator into transgenic plants (Carninci et al., 1996; Seki et al., 1998). The resulting independent transgenic lines are then screened for changes in traits of interest. Like activation tagging, FOX generates dominant gain-of-function mutations (Kondou et al., 2010). One of the most appealing features of the FOX system is the very high mutation rates: in Arabidopsis studies ranging between ~10% and ~17% (Ichikawa et al., 2006; Nakamura et al., 2007). To date, the FOX system has been implemented in Arabidopsis (Dubouzet et al., 2011; Fujita et al., 2007; Ichikawa et al., 2006; Kondou et al., 2009, Nakamura et al., 2007), Lotus corniculatus (bird’s-eye trefoil; Himuro et al., 2011) and rice (Hakata et al., 2010). This method has aided the discovery of genes with fundamental and applied value (Dubouzet et al., 2011; Fujita et al., 2007; Kondou et al., 2009).
FOX FLcDNA libraries could be generated from RNA derived from specific source (tissue, developmental stage, response to environmental cue etc.), which is closely linked to a trait of interest and thus enriched in genes that are associated with this specific trait (Seki et al., 2002; Seki and Shinzaki, 2009). Therefore, the FOX system could potentially increase the proportion of mutations affecting this specific trait. This potential utility remains to be tested. In this study, we implemented the FOX system in poplar using a library derived from developing xylem. We selected this tissue because it is important for wood formation, a process of significant environmental and economic significance. Our main goal was to implement the system in poplar and assess the overall mutation rate and if the system will increase instances of mutants affected in traits linked to the tissue from which the RNA was derived.

Results
Generating and screening the FOX poplar population

A normalized FLcDNA library was generated using RNA extracted from the developing xylem of an aspen tree and was transformed into WT (see “Materials and methods” for details). A total of 113 transgenic lines were regenerates, validated for the presence of the transgene and screened under greenhouse conditions for a battery of traits (Table 1). At least one of the measured traits had to be significantly different from WT at \( P < 0.01 \) for the line to be considered mutant (also referred in the text as FOX mutant, mutant line or FOX mutant line). Using this criterion, 20 of the 113 FOX poplar lines had significant phenotypic changes in at least one measured trait: a mutation rate of 17.7% (Table 1). All other traits differing from WT at \( P < 0.05 \) were also recorded as phenotypic attributes of these mutants (Table 1). On average, a mutant line was affected in 4–7 traits.

Significant enrichment in mutant affected in cell wall chemistry

Affected traits of FOX mutants were analysed to determine for over- and underrepresentation (Figure 1). The only significantly enriched trait among the mutants was cell wall composition (i.e. lignin, cellulose and/or hemicellulose); in fact, 80% of mutants (16/20) exhibited significant changes in cell wall composition. Stem density, stem diameter and leaf dry weight were significantly underrepresented among the recovered mutants.

Molecular characterization of inserted FLcDNAs

The FLcDNA from the 20 mutant FOX lines were PCR-amplified using promoter and terminator specific primers. This analysis showed that 13 lines had single and seven lines had double insertions (Figure S1). In total, the 20 lines had 27 different insertions. On average, a mutant line had 1.35 insertions, with the large majority (13) having only one insertion.

Of the 27 amplified FLcDNAs, 24 were successfully isolated and sequenced (Table 2). In addition, 32 FLcDNA from nonphenotypic FOX lines were additionally recovered and sequenced (Table S2). The closest Arabidopsis and Populus trichocarpa homologs were identified through homology searches for all recovered sequences (Tables 2 and S2).

FLcDNAs are highly overexpressed in poplar FOX lines

The majority of the FOX poplar lines showed highly increased expression for their corresponding inserted FLcDNA: approximately half showed \( \geq 100 \)-fold expression increases, while the other subset showed huge up-regulation levels, some showing levels of over 1000-fold (Figure 2). On average, FLcDNA overexpression was 1423-fold higher than WT.

| Table 1 Summary of mutant FOX phenotypes |
|-----------------|-----------------|
| FOX ID          | P-values |
| 1F1-2 C6        | <0.01  |
| 1F1-3 C5, height, MC% | C5, diameter base, Den-D, DW leaves, DW stem base, DW whole stem, internode, diameter 20th |
| 1F1-5 C6        | Den-D, DW stem base, DW whole stem, height, internodes, lignin, MC% |
| 1F2-4 C6, lignin | C5, DW stem base, DW whole stem, height, internodes, lignin, MC% |
| 1F3-2 Lignin     | C5     |
| 1F3-4 C6, diameter 20th | Den-D, DW stem base, DW whole stem, height, lignin, MC% |
| 1F3-9 MC%       | DW whole stem, height, internodes, lignin |
| 1F43-3 MC%      | C5, DW whole stem, lignin |
| 1F47-4 C5, MC%  | Height, lignin |
| 2F1-1 DW leaves, height, internodes | Den-D |
| 2F6-5 C5        | DW leaves |
| 2F5B-1 Lignin    | MC%    |
| 3F10-6 Den-G     | C5     |
| 3F10-7 Den-G     | MC%    |
| 3F10-8 Lignin    | MC%    |
| 3F12-7 Height    | DW leaves, internodes |
| 3F16-5 C5        | DW leaves |
| 3F16-6 DW leaves, DW whole stem, height, internodes, diameter 20th | C5, height, internodes, DW whole stem, lignin, MC%—moisture content percentage, 20th—20th internode. See Table S2 for detailed trait descriptions. |

\( P \)-values determined using a Student t test (unpaired, unequal variance, \( n \geq 3 \)).

A line was considered mutant as long as one trait displayed significant difference at \( P < 0.01 \). All traits displaying difference at \( P < 0.05 \) are considered attributes of a mutant phenotype and are also listed. An average mutant phenotype was affected in \( \leq 4 \) measured traits. Growth conditions and traits’ measurements described in “Materials and methods”.

3FOX16-6 line is affected in xylem proliferation

Using published RNA-seq expression data (Shi et al., 2017), we studied native gene expression in different tissues for the homologs to the recovered FLcDNAs (Figure 3). Most of these genes showed highest expression in xylem congruent to the tissue source, from which the FLcDNA library was derived.

A unique phenotype was observed with mutant line 3FOX16-6 (3F16-6) (Table 1; Figure 4). We observed a highly significant...
Table 2 Functional annotation of mutant FOX FLcDNAs

| FOX line | E-value | Pt Gene ID | At gene ID | Gene name/Description |
|----------|---------|------------|------------|-----------------------|
| 1F1-2    | 0       | Po02G104400| AT2G22500  | DICARBOXYLATE CARRIER 1 (DIC1) |
| 1F1-3    | 1.9E-127| Po01G242300| AT5G59480  | HALOACID DEHALOGENASE LIKE HYDROLASE (HAD) |
| 1F1-5.1  | 8.2E-145| Po02G070200| AT1G21320  | Nucleic acid/nucleotide binding protein |
| 1F2-4.1  | 8.9E-132| Po03G173300| AT1G27920  | MICROTUBULE ASSOCIATED PROTEIN 65-B (MAP65-B) |
| 1F2-4.2  | 6.8E-67 | Po01G036000| AT3G17880  | HSP70 INTERACTING PROTEIN 2 (HIP2) |
| 1F3-2    | 0       | Po00G033200| AT3G05330  | TANGLED1 (TAN1) |
| 1F3-4    | 0       | Po01G029600| AT5G13530  | KEEP ON GOING (KEG) |
| 1F3-9    | 0       | Po16G086400| AT2G37090  | IRREGULAR XYLEM 9 (IRX9) |
| 1F4-3    | 0       | Po07G061600| AT4G36210  | DUF726 |
| 1F4-7.1  | 1E-133  | Po13G013000| AT1G56230  | DUF1399 |
| 1F4-7.2  | 2.4E-102| Po05G101400| AT1G20693  | HIGH MOBILITY GROUP B2 (HMGB2) |
| 2F1-1    | 0       | Po14G045100| AT4G16780  | HOMEobox LEUCINE ZIPPER PROTEIN 4 (HAT4) |
| 2F6-5.1  | 1.3E-113| Po03G132000| AT1G63910  | MYB DOMAIN PROTEIN 103 (MYB103) |
| 2F6-5.2  | 2.4E-35 | Po02G160200| AT2G45910  | U-box domain-containing protein kinase family protein |
| 2F5-1    | 5.4E-115| Po02G216000| AT1G54790  | GDSL-motif esterase |
| 3F10-6.1 | 0       | Po02G051400| AT5G36740  | Acyl-CoA N-acyltransferase with RING/FYVE/PHD-type zinc finger protein |
| 3F10-6.2 | 2.4E-164| Po02G067600| AT1G21090  | Cupredoxin superfamily protein |
| 3F10-7.1 | 0       | Po06G279100| AT4G31270  | HARBINGER TRANSPON DERIVED PROTEIN 2 (HDP2) |
| 3F10-8.2 | 4.5E-86 | Po13G086700| AT5G36290  | Uncharacterized protein family (UPF0016) |
| 3F12-7   | 0       | Po18G047700| AT2G26280  | CTC-INTERACTING DOMAIN 7 (CID7) |
| 3F16-5   | 2.1E-176| Po04G210800| AT2G28430  | C3HC4 type family protein |
| 3F16-6   | 3.5E-89 | Po09G010800| AT2G28305  | LONELY GUY 1 (LOG1) |
| 3F17-7   | 8.9E-176| Po08G020900| AT3G54260  | TRICHOME BIREFRINGENCE-LIKE 36 (TBL36) |
| 3F90-4   | 0       | Po08G012400| AT5G06390  | FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 17 (FLA17) |

Lines with more than 1 insertion are indicated with the same line number but with the ’1’ and ’2’ extensions. E-values based on Basis Local Alignment Sequencing Tool (BLAST) analysis against P. trichocarpa genome. At—Arabidopsis thaliana, Pt—P. trichocarpa.
Majority of poplar LOGs are expressed in wood forming tissues in a specific manner

Using poplar cell level resolution RNA-seq expression profiles (Sundell et al., 2017), we surveyed gene expression spanning wood forming tissues for all 14 poplar LOG genes (Figure 5a). The majority (11/14) were expressed in the wood forming tissues with distinct expression profiles in different developmental zones. Two clusters, each comprising four LOG genes, could be distinguished: both groups showed expression within the cambial zone but the cluster LOG5b/6/7/a8d showed expression further into the xylem, likely in cell layers undergoing cell differentiation and lignification. Outside of these clusters are three separate genes: one exclusively expressed in phloem and two exclusively in differentiating and expanding xylem.

Expression of LOGs coincides with the expression of their putative upstream regulators

In Arabidopsis root meristem, LOG3 and LOG4 are targets of a regulatory module, the interconnect incoherent feed-forward loop (IFFL), involving bHLH transcription factors LONESOME HIGHWAY (LHW) and TARGET OF MONOPTEROS 5 (TMOS; Figure 5c). AtLOG3/4 gene regulation largely controls root xylem proliferation. This gene module is further upstream regulated through MONOPTEROS 1 (MP1) also called AUXIN RESPONSE FACTOR 5 (ARF5; De Rybel et al., 2014; Fischer et al., 2019; Hardtke et al., 2004; Ohashi-Itô et al., 2014). Since poplar LOG1 shows high similarity to the AtLOG3/4 (Figure S2), we hypothesized that poplar secondary growth could be regulated by a similar mechanism. We assayed expression of poplar homologs for LHW, TMOS, MP1 and their ‘LIKE’ genes (poplar is an ancient polyploidy and has many duplications) (Figure 5b); many of these genes showed very similar expression to poplar LOG1. This could suggest that similar regulatory interactions may govern poplar LOG1 expression and influence secondary growth in trees.

Recapitulation of poplar LOG1 overexpression phenotype

To recapitulate the effect of the poplar LOG1 in the 3F16-6 mutant line, we constructed an overexpression construct (OE) using the poplar LOG1 FLcDNA under the same 3S5 promoter. We regenerated multiple (15) transgenic lines. We further characterized four independent transgenic lines that showed high up-regulation of the poplar LOG1 (Figure S3a). We observed nearly complete phenocopying of the original 3F16-6 phenotype in the four lines (Figure S4a–d).

Discussion

We generated a FOX poplar population using a developing xylem FLcDNA library. We selected this tissue for its importance in wood formation a trait of substantial environmental and economic value. Our study developed the tools needed for the implementation of the method in poplar including the vector needed for the generation of the FOX library and the pipeline linking the phenotype to genotype. We also established important parameters of how this method works in poplar. Specifically, we found that the majority of lines harboured only single insertions. The rest of the analysed lines had two insertions. This significantly simplifies the isolation of the FLcDNA causative to the phenotype. We further demonstrated, through recapitulation experiments for two mutant lines, the causative effect of the overexpressed FLcDNAs to the observed phenotypes (Figures S3 and S4).

A high mutation rate of 17.7% was also found using a stringent statistical cut-off value of \( P < 0.01 \) for identification of the mutant plants (Table 1). This mutation rate is similar to mutation rates seen in the studies employing the same method in Arabidopsis (Ichikawa et al., 2006; Nakamura et al., 2007). The high mutation rates suggest the FOX system could significantly improve feasibility and reduce cost of gene discovery obtained through mutagenesis in poplar.
The feasibility and cost-effectiveness of a mutagenesis method is determined by the overall mutation rate but also the proportion of mutants affected in a specific trait/process of interest. Because the FOX method is based on overexpression of FLcDNAs derived from cDNA library linked to a trait or process of interest (Seki et al., 2002; Seki and Shinozaki, 2009), it could influence the frequency of mutations affected in this process/trait. Having used developing xylem FLcDNAs, we expected increased frequency of FOX lines affected in wood formation.

Indeed, we found a disproportionate overrepresentation of mutant lines affected in cell wall composition: 80% of all the discovered mutants were significantly affected in at least one of the three main cell wall constituents (cellulose, hemicellulose or lignin; Table 1; Figure 1). Significance of the differences determined using a Student t test (unpaired, unequal variance, \( n \geq 4 \)); one, two or three asterisks indicate significances at \( P < 0.05, 0.01 \) and 0.001, respectively (g–m).

Figure 4 The 3F16-6 lines show dramatic phenotypic changes. Whole plant photograph with three ramets of WT (left) and 3F16-6 (right) (a). Fully developed leaves from 15th internode of WT (b) and 3F16-6 (c). Transverse stem sections taken from 15th internodes of WT (d) and 3F16-6 (e). Scale bars, 20 cm (a); 5 cm (b, c); 50 µm (d, e). The biosynthesis and activation pathway for the iP type of CK (f): (1) ISOPENTYL TRANSFERASE (IPT) modifies an adenine base of AMP generating iPRMP; (2) first step in the two-step pathway: nucleosidase removal of inorganic phosphate from iPRMP generates iPR; (3) second step in the two-step pathway: nucleosidase removal of ribose group from iPR generates iP. LOG genes encode CK nucleoside 5-monophosphate phosphoribohydrolase which directly convert iPRMP to iP in a single step (Kurakawa et al., 2007; Sakakibara, 2006). Measured traits from WT (light grey) and 3F16-6 (dark grey), including: height (g); leaf area (h); diameter at 20th internode (i); xylem width (j); inactive and active cytokinin concentrations in both leaf and stem (k, l); and cell wall composition (m). Significance of the differences determined using a Student t test (unpaired, unequal variance, \( n \geq 4 \)); one, two or three asterisks indicate significances at \( P < 0.05, 0.01 \) and 0.001, respectively (g–m). AMP—adenosine monophosphate, C5—cellulose, C6—hemicellulose, DMAPP—dimethylallyl diphosphate, iP—isopentenyl adenine, iPR—isopentenyl riboside, iPRMP—isopentenyl adenosine monophosphate, LOG—LONELY GUY, m/z—mass to charge ratio, P—Pith, Ph—phloem, X—xylem.

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Indeed, we found a disproportionate overrepresentation of mutant lines affected in cell wall composition: 80% of all the discovered mutants were significantly affected in at least one of the three main cell wall constituents (cellulose, hemicellulose or lignin; Table 1; Figure 1). This is unsurprising since wood formation requires massive synthetic effort of secondary cell wall, and consequently, our FLcDNA library reflected this and ultimately affected mutant phenotypes. Second, several mutant lines with affected cell wall composition harboured FLcDNAs homologous to Arabidopsis genes already known to regulate cell wall biosynthesis: IRREGULAR XYLEM 9 (IRX9), MYB103, MICROtUBULE ASSOCIATED PROTEIN 65-8 (MAP65-8) and FASCICLIN-LIKE ARABINOGALACTAN 17 (FLA17). IRX9 is a non-CLS (cellulose-synthase-like gene) involved in xylan biosynthesis (York and O’Neill, 2008); MYB transcription factors have been found to regulate lignin biosynthesis (Öhman et al., 2013); MAP65 genes organize the microtubule scaffolds which associated to cellulose assembly (Chan et al., 2003; Lloyd and Chan, 2004); and FLA gene knockdown has been shown to decrease cellulose and lignin xylem cell wall composition (Wang et al., 2014). Third, the majority of recovered FLcDNAs corresponded to poplar genes with high native expression in xylem (Figure 3). Collectively, these results demonstrate that the FOX system can be used as a mutagenesis approach to identify genes important for a specific trait of interest.

Mutant lines also uncovered a sizable number (15% of all FOX mutant lines) of uncharacterized genes that impacted cell wall composition (Table 2). Cell wall synthesis is a commercially important trait to agriculture, forestry and bioenergy industry and has been intensively studied for decades (Nieminen, Robischon, Immanen, and Helariutta, 2012). Thus, this study demonstrates
that using a small FOX population can discover novel genes in an intensively studied process. This finding demonstrates the potential high value of the FOX method as a gene discovery tool.

We expected an enrichment for mutant FOX poplars with altered diameter growth because developing xylem is this trait’s main driver for expansion. Contrasting this, we observed an underrepresentation of mutant lines affected in stem diameter (Table 1; Figure 1). There are multiple reasons why this may have occurred. First, diurnal and seasonal context could have affected the mRNA abundance of specific genes involved in xylem diameter expansion (Guerriero et al., 2013). Second, the cambium zone on the xylem side is only 4–5 cell layers thick (Du and Groover, 2010): its contribution to FLCdNA would be disproportionately small and among all sampled cells, thus lowering the abundance of genes involved in expansion and overrepresenting gene involved in cell wall synthesis. Third, genes involved in regulation of cambium are often strong developmental and hormonal regulators, which are expressed at very low levels and in a manner highly specific to cell/tissue types (Du and Groover, 2010; Shi et al., 2017): this compounds with the dilution effect associated with the small proportion of cambial cells and further reduces the proportion of stem girth related genes. In summary, we believe underrepresentation of FOX mutants lines affected in diameter growth is linked to the underrepresentation of genes expressed in cambial cells. This underscores a need for precision when selecting tissues for FLCdNA source material.

Despite this, we still identified some FOX plants with altered diameter traits: one of these was the 3F16-6 line. The increased diameter (Figure 4d,e,i,j) in this line suggested the single FLCdNA insert (Table 2) positively affects diameter growth. Sequencing the insert (Table 2), demonstrating its up-regulation (Figure 2), validating increased bioactive CK (Figure 4k,l) and most importantly recapitulation of the phenotype in multiple transgenic lines via retransformation of the gene under the same promoter (Figures S3a and S4a–d) suggests up-regulation of poplar LOG1 and increased bioactive CK are causal for the observed phenotype.

Stems of transgenic poplars have greatly advanced under-standings of CK role in secondary growth (Immanen et al., 2013; Ursache et al., 2013): poplar lines overexpressing Arabidopsis ADENOSINE PHOSPHATE-ISOPENTYLTRANSFERASE 7 (AtIPT7) (Immanen et al., 2016) and CYTOKININ OXIDASE 2 (AtCKX2) (Nieminen et al., 2008), respectively, increased and decreased CK concentrations and subsequently increased and decreased diameter. Despite these advances, the mechanisms outlining regulatory genes controlling CK levels and signalling in poplar stem remain unclear.

Bioactivation of CK can be accomplished through enzymatic one- or two-step pathways (Iio et al., 2008; Kuroha et al., 2009; Figure 4f). The single step catalysing enzyme LONELY GUY (LOG) was named after the phenotype of a mutant rice plant that identified the gene: LOG knockout severely suppressed stamen development (Kurakawa et al., 2007). LOG is a CK riboside 5’-monophosphate phosphoribohydrolase and performs the two hydrolase activities leading to CK activation simultaneously. This separation of CK biosynthesis from activation allows for precise temporal and spatial control of CK activity. Poplar LOG1 has the highest expression in xylem tissues and thus provides tissue-specific control of bioactive CK levels. Poplar LOG1 shows the highest sequence homology to AtLOG1/3/4 (Figure S2). These three Arabidopsis LOGs show distinct substrate specificities from the other LOGs and identical pH optimum (Kuroha et al., 2009). In Arabidopsis root, AtLOG3/4 are targets of LONESOME HIGHWAY (LHW) and TARGET OF MONOPTEROS 5 (TMOS; De Rybel et al., 2013; Ohashi-Ito et al., 2014). Both LHW and TMOS encode bHLH proteins that heterodimerize and regulate vascular tissue organization (De Rybel et al., 2013; Ohashi-Ito et al., 2014). Moreover, the TMOS-LHW complex transcriptional activation of LOG3/4 promotes xylem cell fate specification and proliferation (De Rybel et al., 2014). Our data suggest that a similar mechanism may operate in stem xylem differentiation and proliferation during secondary woody growth of poplar. First, poplar LOG1 has the highest native expression in xylem tissues (Figure S5). Second, overexpression of poplar LOG1 resulted in increased secondary growth observed through a near doubling (1.9-fold) in xylem proliferation (Figure 4). Third, poplar LOG1 shows close homology to AtLOG3/4, genes implicated in xylem cell specification in the Arabidopsis (De Rybel et al., 2014). Forth, RNA-seq data derived from high-resolution sampling through the wood forming tissues of aspen stems further support the proposed mechanism (Sundell et al., 2017): various aspen homologs for MP, LHW, TMOS and respective ‘LIKE’ genes had similar cambial zone expression to multiple LOG genes including poplar LOG1. Finally, we observed multiple GfBox sites within the 3 kbp upstream regions for all poplar LOG genes with stem tissue expression profiles (Table S3). These cis-element sequences often serve as binding sites for bHLH transcription factors (like TMO5 and LHW; Liu et al., 2015; Toledo-Ortiz et al., 2003). Further investigation may uncover the gene module like the TMO-LHW HIGHWAY (LHW) and TARGET OF MONOPTEROS 5 (TMOS; De Rybel et al., 2013; Ohashi-Ito et al., 2014). Both LHW and TMOS encode bHLH proteins that heterodimerize and regulate vascular tissue organization (De Rybel et al., 2013; Ohashi-Ito et al., 2014). Moreover, the TMOS-LHW complex transcriptional activation of LOG3/4 promotes xylem cell fate specification and proliferation (De Rybel et al., 2014). Our data suggest that a similar mechanism may operate in stem xylem differentiation and proliferation during secondary woody growth of poplar. First, poplar LOG1 has the highest native expression in xylem tissues (Figure S5). Second, overexpression of poplar LOG1 resulted in increased secondary growth observed through a near doubling (1.9-fold) in xylem proliferation (Figure 4). Third, poplar LOG1 shows close homology to AtLOG3/4, genes implicated in xylem cell specification in the Arabidopsis (De Rybel et al., 2014). Forth, RNA-seq data derived from high-resolution sampling through the wood forming tissues of aspen stems further support the proposed mechanism (Sundell et al., 2017): various aspen homologs for MP, LHW, TMOS and respective ‘LIKE’ genes had similar cambial zone expression to multiple LOG genes including poplar LOG1. Finally, we observed multiple GfBox sites within the 3 kbp upstream regions for all poplar LOG genes with stem tissue expression profiles (Table S3). These cis-element sequences often serve as binding sites for bHLH transcription factors (like TMO5 and LHW; Liu et al., 2015; Toledo-Ortiz et al., 2003). Further investigation may uncover the gene module like the TMO-LHW HIGHWAY (LHW) and TARGET OF MONOPTEROS 5 (TMOS; De Rybel et al., 2013; Ohashi-Ito et al., 2014). Both LHW and TMOS encode bHLH proteins that heterodimerize and regulate vascular tissue organization (De Rybel et al., 2013; Ohashi-Ito et al., 2014). Moreover, the TMOS-LHW complex transcriptional activation of LOG3/4 promotes xylem cell fate specification and proliferation (De Rybel et al., 2014). Our data suggest that a similar mechanism may operate in stem xylem differentiation and proliferation during secondary woody growth of poplar. First, poplar LOG1 has the highest native expression in xylem tissues (Figure S5). Second, overexpression of poplar LOG1 resulted in increased secondary growth observed through a near doubling (1.9-fold) in xylem proliferation (Figure 4).
About 7 μg of total RNA was used for FLcDNA synthesis with a modified SMART™ method using random and oligo dT primers (Zhu et al., 2001). Double-stranded cDNAs were obtained by primer extension using Pfu polymerase (Fermentas) and purified. The FLcDNAs were normalized, re-amplified and purified. Re-amplified cDNAs were SfiI-digested and size-fractioned on a 1% agarose gel. Fragments greater than 1kb cDNA were purified for directional cloning. The purified digested cDNA fragments were ligated into the new modified FOX binary vector (see above). The ligation products were transformed into DH10B-T1 cells (Invitrogen, Waltham, MA, USA). Following transformation, cells were stored in 15% glycerol at −80 °C. About 1.0 × 10^5 clones were grown per large Petri dish (about 10 dishes for each library), then washed and collected into 10 tubes for each library. A mixture from 10 tubes from each original library was used for a DNA Maxi-Prep (Qiagen). About 1.9 × 10^5 clones were grown per dish (20 dishes in total), washed, collected and resuspended in 50% glycerol. The 0.5 mL aliquots of the glycerol stock were stored at −80 °C. The 0.5 mL aliquots were used to inoculate 100 mL of Lennox liquid culture supplemented with the appropriate antibiotics. The culture was grown overnight at 28 °C and gently shaken and used for transformation (described below).

**Transformation**

FOX library was transformed into the *Populus tremula × Populus alba* (genotype INRA 717-IB4) wild type (WT) using method described in Han et al. (2000). A total of 113 independent transgenic events were regenerated. Transgene presence was PCR-verified using primers specific to the NptII kanamycin selection marker (Table S1). Transformation of the recapitulation constructs was performed using the same method and Agrobacterium strain carrying the respective binary vector construct (described below).

**Generation of recapitulation constructs**

The 2F1-1 and 3F16-6 FLcDNA were PCR-amplified using B1 Pro35S and reverse B2 ocsCZ primers with B1 and B2 tails for GATEWAY cloning, respectively (Table S1) and Pfu polymerase (Fermentas). Amplified fragments were inserted into the pDonr221 vector using BP Clonase II (Thermo Fisher Scientific Inc., Waltham, MA, USA). The reactions were transformed into DH10B T1R cells. Successful transformants were selected for using 50 mg/L kanamycin LB growth media. Plasmids were extracted and sequence validated.

A sequence-validated clone was used to transfer poplar *LOG1* cDNA into the pK7WG2 binary vector between CamV35S promoter and T35S terminator using LR Clonase II (Thermo Fisher Scientific Inc., Waltham, MA, USA). The reactions were transformed into DH10B T1R cells. Successful transformants were selected for using 50 mg/L kanamycin LB growth media. Plasmids were extracted and sequence validated.

**Figure 5** Poplar homologs of *Arabidopsis* genes involved in the LHW-TMOS interconnected incoherent feed-forward loop (IFFL) show tissue-specific expression during wood formation. All poplar LOG genes with distinctive expression profiles in woody tissues (a). Various IFFL homologs with high gene expression in cambial cells (Fischer et al., 2019; b). Heat map columns represent different sampled sites of aspen stem: phloem range 01–04, cambial zone range 05–09 (marked in blue), expanding xylem range 10–19 and lignified xylem range 20–25 (a, b). Expression scaled per gene by z-scoring: red and blue z-scores, respectively, indicate z-scores higher and lower than average gene expression; z-score range indicated by z-score vs. density plot; figures generated using Aspwood interface (Sundell et al., 2017; a, b). Brief outline of IFFL gene module (c): MP transcription factors induce expression of TMOS transcription factor; TMOS heterodimerizes with LHW; TMOS-LHW heterodimer induces LOG gene expression; LOG genes catalyse single step activation of CK precursors (De Rybel et al., 2014; Ohashi-tito et al., 2014). LOG—OLONELY GUY, LHW—LONESOME HIGHWAY, MP—MONOPTEROS, TMOS—TARGET OF MONOPTEROS 5. Gene accession IDs listed in Table S4.
Scientific Inc.). Successful transformants were selected on 50 mg/L spectinomycin LB media.

The construct was transformed into AGL-1 Agrobacterium using the freeze and thaw method. Transformants were selected on Lennox growth media containing 50 mg/L spectinomycin, 50 mg/L rifampicin and 50 mg/L kanamycin. The presence of the binary vector was PCR-verified with HindIII/BSFII120I restriction digestion. Individual colonies validated for the presence of the binary vector were used for transformation as previously described (Han et al., 2000).

Greenhouse growth and measurements

Plants used in the experiments were in vitro propagated and adapted to greenhouse conditions. Post-adaptation, plants were grown for approximately 3 months in the greenhouse in a completely randomized design with three replications. The following traits were measured at the end of the trial: height (cm), number of internodes, diameter at base (mm), diameter at 20 internode (mm), dry weight stem base (dw stem base) (g), green stem density (den-g) (g/cm³), dry stem density (den-d) (g/cm³), moisture content percentage (MC%), dry weight whole stem (dw whole stem) (g) and dry weight leaves (dw leaves) (g). Density (den-g and den-d) measurements were performed using the volume displacement. The MC% was determined by comparing fresh and kiln-dried weight of tissues. Kiln-drying was performed at 105 °C. Cell wall content was determined by pyrolysis molecular-beam mass spectrometry analysis (PyMBMS) as described in Sykes et al. (2009). 3FOX16-6 was characterized with nine replications. Statistical analysis was performed using Microsoft Excel and R Statistical Software.

Molecular characterization of FOX lines

The FlcDNAs from transgenic lines were recovered by a standard set of primers designed for annealing to promoter and terminator regions (Table S1). The expression levels of the various FlcDNAs in the FOX lines were determined using quantitative real-time PCR (qRT-PCR) using primer sets specific to the respective FlcDNAs (Table S1). qRT-PCR was performed using a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific Inc.), Maxima SYBR Green qPCR master mix (Thermo Fisher Scientific Inc.). 0.2 μM of each primer and 2 μL 10× diluted cDNA in a 20 μL final volume. Default StepOnePlus cycling parameters were used. Three biological replicates were analysed for each sample, and relative transcript abundance was calculated using ubiquitin as an internal standard (Schmittgen and Livak, 2003; Tsai et al., 2006).

Cytokinin analysis

A 20 mg of freeze-dried and homogenized material was extracted from the leaves and stems of four independent 3FOX16-6 and WT lines. The determination of CKs included extraction and purification (Dobrev and Kaminok, 2002) followed by quantitation with LC-MS/MS (Dobrev and Vankova, 2012). Samples were extracted with extraction solvent (methanol/H2O/ formic acid, 15 : 4 : 1, v : v : v) supplemented with stable isotope-labelled CK internal standards (10 pmol), to check the recovery during the purification and to validate the quantification. After incubation for 30 min at −20 °C, the extract was centrifuged at 20 000 g and pellet re-extracted. Pooled super-natants were evaporated in vacuum concentrator (Alpha RVC, Christ), and then, a sample residue was dissolved into 0.1 M formic acid and applied to mixed-mode reversed-phase—cation exchange SPE column ( Oasis-MCX, Waters). CKs were eluted with 0.35 M NH4OH in methanol. Eluate was evaporated to dryness in a vacuum concentrator and dissolved into 30 μL of 15% acetonitrile. An aliquot was analysed on HPLC (Ultimate 3000, Dionex) coupled to a hybrid triple quadrupole-linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems, Waltham, MA, USA) set in selected reaction monitoring mode. Quantification of hormones was done using an isotope dilution method. Data processing was carried out with Analyst 1.5 software (Applied Biosystems).

Sequence and phylogenetic analyses

Sequence homology searches and analyses were performed using the Phytozome (http://www.phytozome.net/Poplar.php) and the National Center for Biotechnology Information BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/). Protein sequences were downloaded and aligned using ClustalW (Thompson et al., 1994) and analysed using MEGA7 (Kumar et al., 2016). The phyloge-netic tree was constructed using neighbour-joining. Confidence at each branch was estimated using bootstrap analyses of 1000 replications.

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Conflict of interest

The authors declare no conflicts of interest.

Author contributions

V.B., Y.Y. and J.R. designed the experiment. J.R., Y.Y. and V.B. performed the experiments. J.R. and V.B. analysed the data and wrote the manuscript. P.D. and R.V. performed cytokinin experiments. R.S. performed cell wall composition experiments. C.K. revised the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 Summary of primers used in experimentation.
Table S2 Functional annotation of the FLcDNAs identified in the transgenic lines.
Table S3 Summary cis-elements found 3kbp upstream of LOG gene sequences.
Table S4 Accession numbers for genes used in Fig. 8 and 9.
Figure S1 Mutant FOX lines contain 1.35 inserted FLcDNAs on average.
Figure S2 PtaLOG1 was most homologous and nearly identical to PtLOG1: both genes are listed on the same branch and are referred to by poplar LOG1.
Figure S3 Poplar LOG1 and HAT4 are overexpressed within lines phenocopying 3F16-6 and 2F1-1 (respectively).
Figure S4 Poplar LOG1 and HAT4 overexpressing lines phenocopy 2F16-6 and 2F1-1, respectively: four overexpression lines completely (L1, L2, L4) and partially (L3) phenocopied the 3F16-6 line; three overexpression lines completely (H2) and partially (H1, H3) phenocopied the 2F1-1 line.
Figure S5 Poplar LOG1 gene expression highest in xylem.
Figure S6 Trait changes of mutant poplar lines.