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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.

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 Shinozaki, 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 regenerated, 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 traits.

Table 1 Summary of mutant FOX phenotypes

	P-values	
FOX ID	<0.01	<0.05
1F1-2	C6	DW whole stem, height, internodes
1F1-3	C5, height, MC%	C6, 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, 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%	C6, DW whole stem, lignin
1F47-4	C5, MC%	Height, lignin
2F1-1	DW leaves, height, internodes	Den-D
2F6-5	C5	
2F58-1	Lignin	
3F10-6	Den-G	MC%
3F10-7	Den-G	C6
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	
3F17-7	Lignin	
3F90-4	C5	

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 ~4 measured traits. Growth conditions and traits' measurements described in "Materials and methods". C5—cellulose, C6—hemicellulose, Den-D—density dry weight, Den-G—Density green weight, DW—dry weight, MC%—moisture content percentage, 20th—20th internode. See Table S2 for detailed trait descriptions.

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 ≤100-fold expression increases, while the other subset showed huge up-regulation levels, some measuring above 1000-fold (Figure 2). On average, FLcDNA overexpression was 1423-fold higher than WT.

Genes corresponding to the FLcDNAs are predominantly expressed in xylem tissue

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.

3FOX16-6 line is affected in xylem proliferation

A unique phenotype was observed with mutant line *3FOX16-6* (*3F16-6*) (Table 1; Figure 4). We observed a highly significant

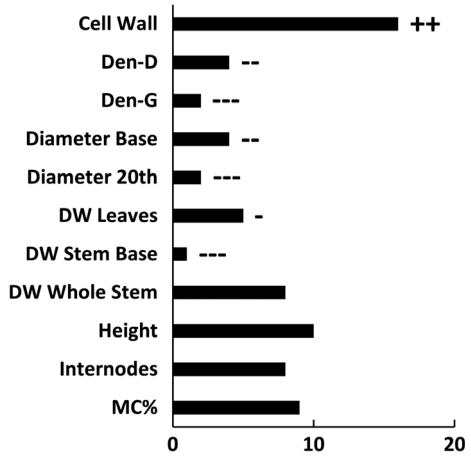

Figure 1 Affected traits are significantly over- and underrepresented among the mutant FOX lines. Along the *x*-axis are measured traits. Along the *y*-axis is the number of mutant lines demonstrating significant difference from WT. *P*-values determined using chi-square test (unpaired, unequal variance, $P = 0.5$, $n = 20$, E = 10, DF = 1). The '+' indicates an overrepresented trait. The '−' indicates an underrepresented trait. One, two or three of the '+/−' indicates significance levels: $P < 0.05$, 0.01 and 0.001, respectively. Den-D—density dry weight, Den-G—density green weight, DW—dry weight, MC%—moisture content, 20th—20th internode.

increase in stem diameter due to a near doubling (1.9×-fold) in xylem proliferation with no impact on cell wall composition (Figure 4e,d,i,j,m). This line also was also significantly impacted in several other traits (Table 1).

3FOX16-6 FLcDNA encodes LONELY GUY 1 homolog

The single overexpressed FLcDNA of *3F16-6* (Figure S1) showed highest homology to LONELY GUY 1 (LOG1) of *Arabidopsis* and *P. trichocarpa* (Table 2; Figure S2). We named the gene *PtaLOG1* (*Populus tremula* × *alba* = Pta). Since the *PtaLOG1* sequence had highest homology to *PtLOG1*, we refer to both collectively as poplar *LOG1* (Figure S2). LOG genes constitute families of nine and 14 members in *Arabidopsis* and poplar, respectively (Immanen *et al.*, 2013; Tokunaga *et al.*, 2012). These genes encode enzymes that directly convert inactive cytokinins (CK) precursors to their biologically active forms (Kurakawa *et al.*, 2007; Figure 4f). We therefore compared levels of CK precursor (isopentenyl adenosine monophosphate = iPRMP) and its respective active form (iP = isopentenyl adenine) in *3F16-6* and WT (Figure 4k,l). Consistent with the biochemical function of LOG, we observed significantly higher concentrations of the active CK (iP) in both stem and leaf tissues of the mutant line. Interestingly, we found significantly decrease of the precursor (isopentenyl adenosine monophosphate = iPRMP) in leaves but not in stems.

Table 2 Functional annotation of mutant FOX FLcDNAs

FOX line	*E*-value	*Pt* Gene ID	*At* gene ID	Gene name/Description
1F1-2	0	Potri.002G104400	AT2G22500	DICARBOXYLATE CARRIER 1 (DIC1)
1F1-3	1.9E−127	Potri.001G242300	AT5G59480	HALOACID DEHALOGENASE LIKE HYDROLASE (HAD)
1F1-5.1	8.2E−145	Potri.002G070200	AT1G21320	Nucleic acid/nucleotide binding protein
1F2-4.1	8.9E−132	Potri.003G173300	AT1G27920	MICROTUBULE ASSOCIATED PROTEIN 65-8 (MAP65-8)
1F2-4.2	6.8E−67	Potri.015G036000	AT3G17880	HSP70 INTERACTING PROTEIN 2(HIP2)
1F3-2	0	Potri.005G033200	AT3G05330	TANGLED1 (TAN1)
1F3-4	0	Potri.001G029600	AT5G13530	KEEP ON GOING (KEG)
1F3-9	0	Potri.016G086400	AT2G37090	IRREGULAR XYLEM 9 (IRX9)
1F43-3	0	Potri.007G016100	AT4G36210	DUF726
1F47-4.1	1E−133	Potri.013G013000	AT1G56230	DUF1399
1F47-4.2	2.4E−102	Potri.005G101400	AT1G20693	HIGH MOBILITY GROUP B2 (HMGB2)
2F1-1	0	Potri.014G045100	AT4G16780	HOMEOBOX LEUCINE ZIPPER PROTEIN 4 (HAT4)
2F6-5.1	1.3E−113	Potri.003G132000	AT1G63910	MYB DOMAIN PROTEIN 103 (MYB103)
2F6-5.2	2.4E−35	Potri.002G160200	AT2G45910	U-box domain-containing protein kinase family protein
2F58-1	5.4E−115	Potri.002G216000	AT1G54790	GDSL-motif esterase
3F10-6.1	0	Potri.002G051400	AT5G36740	Acyl-CoA N-acyltransferase with RING/FYVE/PHD-type zinc finger protein
3F10-6.2	2.4E−164	Potri.002G067600	AT1G21090	Cupredoxin superfamily protein
3F10-7.1	0	Potri.006G279100	AT4G31270	HARBINGER TRANSPOSON DERIVED PROTEIN 2 (HDP2)
3F10-8.2	4.5E−86	Potri.013G086700	AT5G36290	Uncharacterized protein family (UPF0016)
3F12-7	0	Potri.018G047700	AT2G26280	CTC-INTERACTING DOMAIN 7 (CID7)
3F16-5	2.1E−176	Potri.004G210800	AT2G28430	C3HC4 type family protein
3F16-6	3.5E−89	Potri.009G010800	AT2G28305	LONELY GUY 1 (LOG1)
3F17-7	8.9E−176	Potri.008G020900	AT3G54260	TRICHOME BIREFRINGENCE-LIKE 36 (TBL36)
3F90-4	0	Potri.008G012400	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*.

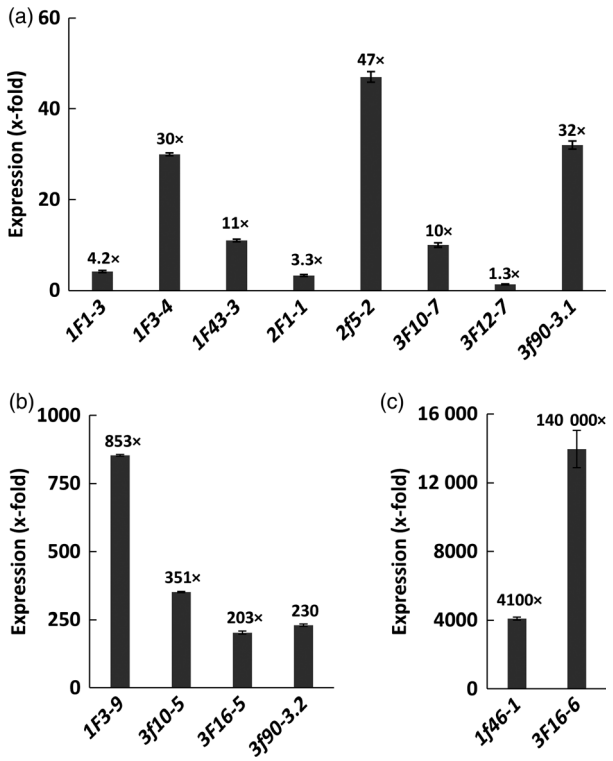

Figure 2 mRNA expression corresponding to inserted FLcDNAs show varying levels of gene up-regulation in FOX poplars. Overexpression tested in both mutant and transgenic lines (n = 3). FOX lines are organized based on levels of up-regulation: ≤100×-fold (a), ≤1000×-fold (b), ≥1000-fold (c).

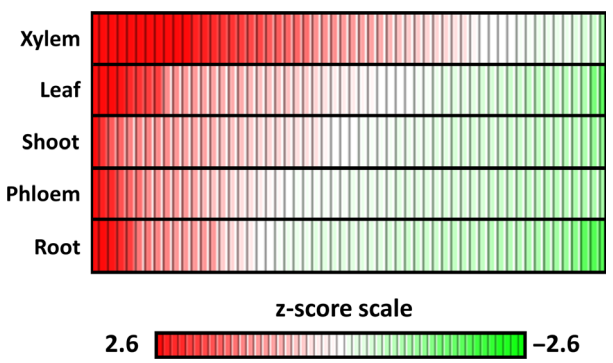

Figure 3 Native expression for poplar homologs for recovered FLcDNAs is often greatest in xylem tissue. Gene expression scaled per gene by z-scoring (n = 3) and arranged highest to lowest within respective tissue categories; red and green indicate z-scores that are higher and lower than average gene expression, respectively. Gene accession IDs found in Tables 2 and S2. Gene expression estimates based on published RNA-seq data by Shi et al. (2017). Heat map generated using Netwalker 1.0 software.

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/6/7a/8d 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 (TMO5; 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-Ito *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*, *TMO5*, *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 35S 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.

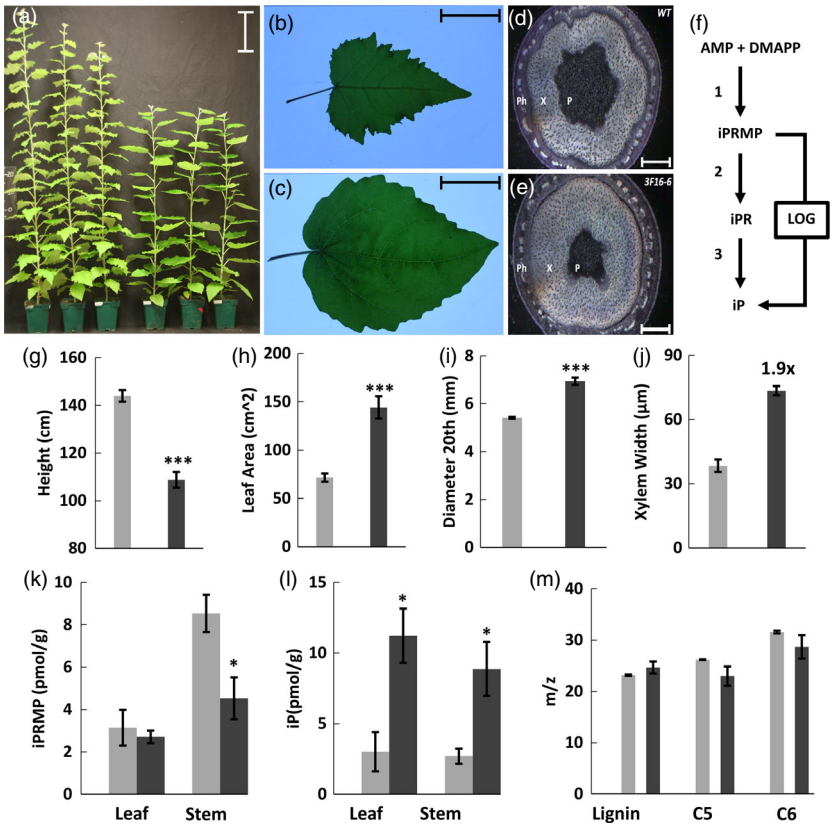

Figure 4 The *3F16-6* lines show dramatic phenotypic changes. Whole plant photograph with three ramets of WT (left) and *3F16-6* (right) (a). Fully developed leaves from 15th internode of WT (b) and *3F16-6* (c). Transverse stem sections taken from 15th internodes of WT (d) and *3F16-6* (e). Scale bars, 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: nucleotidase removal of inorganic phosphate from iPRMP generates iPR; (3) second step in the two-step pathway: nucleosidase removal of riboside 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—iP riboside, iPRMP—isopentenyl adenosine monophosphate, LOG—LONELY GUY, m/z—mass to charge ratio, P—Pith, Ph—phloem, X—xylem.

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). This is unsurprising since wood formation requires massive synthesis 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 dimeter 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 disproportionate and small 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 understandings 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 (Ioio *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 (TMO5; De Rybel *et al.*, 2013; Ohashi-Ito *et al.*, 2014). Both LHW and TMO5 encode bHLH proteins that heterodimerize and regulate vascular tissue organization (De Rybel *et al.*, 2013; Ohashi-Ito *et al.*, 2014). Moreover, the TMO5-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 tissue (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, TMO5 and respective 'LIKE' genes had similar cambial zone expression to multiple LOG genes including poplar *LOG1*. Finally, we observed multiple G/E-box 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 IFFL of *Arabidopsis* root to be also important for regulation of wood development (Fischer *et al.*, 2019; Figure 5c).

Woody biomass is a function of both stem girth and height and can be increased with biotechnological manipulations. In this experiment, ubiquitous overexpression of poplar *LOG1* simultaneously increased girth and decreased height growth. These negative effects to height might be overcome using a different promoter: an experiment overexpressing IPT (a CK two-step pathway gene) with a birch xylem-specific promoter in poplar was able to increase diameter without any pleiotropic effects (Immanen *et al.*, 2016).

Our experimentation demonstrated the FOX method could be used with poplar to generate transgenic populations with high mutation rates with overrepresentation of mutants affected in a trait of interest. We conclude this method can be used for efficient gene discovery of woody perennial traits.

Methods

FOX binary vector construction

The CaMV 35S promoter and ocsCZ terminator cassette were cut from pART7 and inserted into pART27 binary vector using the NotI site. The multicloning site (MCS) between the promoter and terminator was further modified; to facilitate directional cloning of FLcDNAs, existing SfiI sites were replaced with SfiIAB sites (5′-GGC-CAT-TAC-GGC-CAA-CCT-TGA-TAT-CGG-CCG-CCT-CGG-CC-3′). These were inserted into the MCS between EcoRI and HindIII.

FOX library preparation

Xylem tissue was collected from an actively growing and approximately 15-year-old aspen (*Populus tremuloides*) tree near the campus of Michigan Technological University in June. Samples were frozen in liquid nitrogen and stored at −80 °C until further processed. Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). A FOX library was generated using the method described in Ichikawa *et al.* (2006).

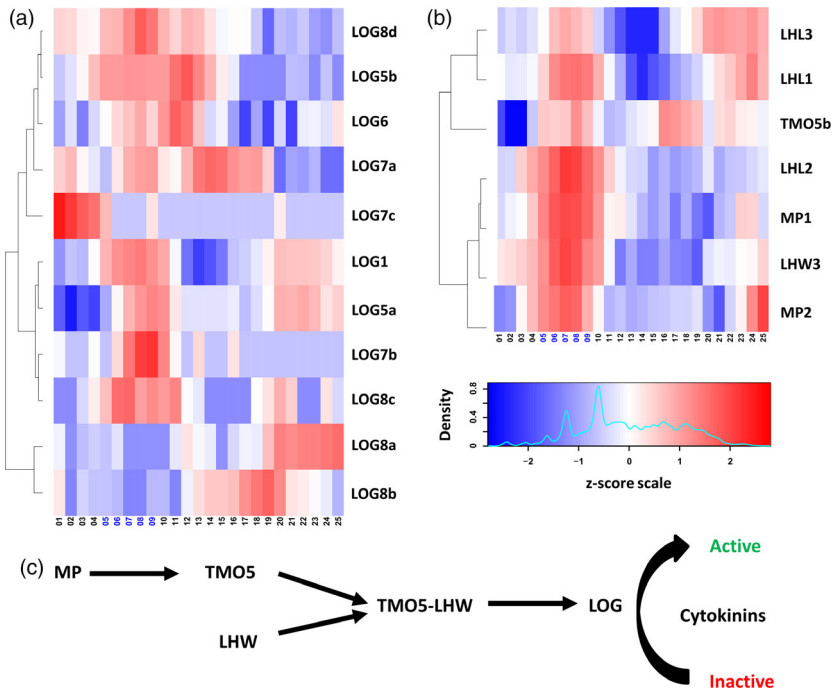

Figure 5 Poplar homologs of *Arabidopsis* genes involved in the LHW-TMO5 interconnected incoherent feed-forward loop (IFFL) show tissue-specific expression during wood formation. All poplar LOG genes with distinctive expression profiles in woody tissues (a). Various IFFL homologs with high gene expression 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 TMO5 transcription factor; TMO5 heterodimerizes with LHW; TMO5-LHW heterodimer induces LOG gene expression; LOG genes catalyse single step activation of CK precursors (De Rybel et al., 2014; Ohashi-Ito et al., 2014). LOG—LONELY GUY, LHW—LONESOME HIGHWAY, MP—MONOPTEROS, TMO5—TARGET OF MONOPTEROS 5. Gene accession IDs listed in Table S4.

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). ~1 μL of the extracted plasmid was transformed into Agrobacterium strain AGL1 via electroporation. Five individual transformations were performed and plated onto 150 mm × 15 mm Petri dishes. About 1.0×10^5 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 D1H10B 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.). 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/BSPI120I 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/cm^3), dry stem density (den-d) (g/cm^3), 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 for 24 h at 105 °C. Cell wall content was determined with 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, 2008; 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 Kamınek, 2002) followed by quantitation with LC-MS/MS (Dobrev and Vankova, 2012). Samples were extracted with extraction solvent (methanol/H_2O/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 supernatants 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 NH_4OH 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 phylogenetic 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 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.

