Field evaluation of transgenic hybrid poplars with desirable wood properties and enhanced growth for biofuel production by bicistronic expression of \textit{PdGA20ox1} and \textit{PtrMYB3} in wood-forming tissue

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Abstract

Background: To create an ideotype woody bioenergy crop with desirable growth and biomass properties, we utilized the viral 2A-mediated bicistronic expression strategy to express both \textit{PtrMYB3} (\textit{MYB46} ortholog of \textit{Populus trichocarpa}, a master regulator of secondary wall biosynthesis) and \textit{PdGA20ox1} (a GA20-oxidase from \textit{Pinus densiflora} that produces gibberellins) in wood-forming tissue (i.e., developing xylem).

Results: Transgenic \textit{Arabidopsis} plants expressing the gene construct DX15::PdGA20ox1-2A-PtrMYB3 showed a significant increase in both stem fresh weight (threefold) and secondary wall thickening (1.27-fold) relative to wild-type (WT) plants. Transgenic poplars harboring the same gene construct grown in a greenhouse for 60 days had a stem fresh weight up to 2.6-fold greater than that of WT plants. In a living modified organism (LMO) field test conducted for 3 months of active growing season, the stem height and diameter growth of the transgenic poplars were 1.7- and 1.6-fold higher than those of WT plants, respectively, with minimal adverse growth defects. Although no significant changes in secondary wall thickening of the stem tissue of the transgenic poplars were observed, cellulose content was increased up to 14.4 wt% compared to WT, resulting in improved saccharification efficiency of the transgenic poplars. Moreover, enhanced woody biomass production by the transgenic poplars was further validated by re-planting in the same LMO field for additional two growing seasons.

Conclusions: Taken together, these results show considerably enhanced wood formation of our transgenic poplars, with improved wood quality for biofuel production.

Keywords: Bicistronic gene expression, Biofuel, Developing xylem promoter, Hybrid poplar, LMO field experiment, PdGA20ox1, PtrMYB3, Saccharification

Background

Interest in the development of sustainable energy using eco-friendly and renewable biomass is increasing \cite{1, 2}. Woody biomass offers economic and sustainable feedstock for bioenergy production \cite{3–5}. While both herbaceous (e.g., grass) and woody biomass are suitable...
forms of plant biomass for biofuel production [6, 7], grass biomass has the advantage of high saccharification efficiency as it is composed of polysaccharides that are easily converted to bioethanol [8]. However, the use of grass biomass for bioenergy must overcome certain logistics challenges stemming from low biomass density and limited period of harvest [9]. On the other hand, woody biomass (from perennial woody plants or trees) has environmental and economic advantages compared to herbaceous biomass because it can be produced in large quantities at high density even on marginal land and can be harvested at any time during the year [6, 10]. Furthermore, as trees grow, erosion is mitigated, carbon dioxide is captured, oxygen is produced, and biodiversity is supported. In addition, trees can provide food and raw materials for human [11].

Gibberellin 20-oxidase (GA20ox) is a key enzyme involved in the biosynthesis of bioactive gibberellic acids (GAs) that influences various aspects of plant growth and development, such as stem elongation, flowering, wood formation, and bud dormancy cycle [12–16]. Overexpression of GA20ox has been reported to increase plant height [17–20]. However, undesirable side effects, such as poor root/leaf development and slender stems, have been reported in many plants including transgenic poplar overexpressing GA20ox1 [21–23].

Perennial woody plants have evolved a circadian clock to synchronize their growth and development to the daily and seasonal cycles of the environment [24–27]. Bud dormancy onset relies on short-day (SD) length, low temperature, and metabolic cues in autumn [26, 28, 29]. In trees, accurate timing of growth arrest is critical for resistance to drought and/or freezing stresses in winter [30]. The onset of the growing season is marked by bud flushing in spring when days become warmer and longer. Timely completion of this important active growth-dormancy cycle is a prerequisite for survival of perennial woody plants, especially in temperate regions [13, 28]. Endogenous levels of GAs decrease during growth cessation and dormancy establishment. In poplar, GA pathways are downregulated early during growth cessation by SD photoperiod [30, 31]. Indeed, hybrid aspens overexpressing Arabidopsis GA20ox1 were unable to arrest growth for bud set and dormancy establishment, even during the SD photoperiod, due to high level of GA [31].

To address the issue of high GA content due to 35S promoter-driven constitutive overexpression of GA20ox1, we utilized a developing xylem (DX) tissue-specific promoter (i.e., DX15 promoter) to express PdGA20ox1, a GA20ox1 from Pinus densiflora [19], to avoid the undesirable phenotypes, and achieved up to threefold increased biomass production in hybrid poplars [22].

Woody biomass is primarily derived from secondary cell walls that comprise cellulose, hemicellulose, and lignin [32, 33]. Several MYB transcription factors (TF) have been identified as positive regulators of secondary wall formation, and MYB46 plays a pivotal role as a master switch for secondary wall biosynthesis in Arabidopsis [34, 35]. Overexpression of MYB46 induces ectopic secondary wall biosynthesis by activating cellulose, xylan, and lignin biosynthetic genes in Arabidopsis [36–38]. Consistent results were reported whenPtrMYB3, an ortholog of MYB46 (AT5G12870) in Populus trichocarpa, was overexpressed [39, 40].

In an effort to improve both quantity and quality of woody biomass without unwanted growth effects, we used a bicistronic gene expression system (e.g., 2A system) to express PdGA20ox1 and PrtMYB221 under the DX15 promoter [41]. The 2A system allows multiple genes to be encoded in a single open reading frame with a short intervening viral 2A sequence that has self-processing properties between the coding sequences [41–43]. Because PrtMYB221 is a negative regulator of lignin biosynthesis, the resulting transgenic poplars exhibited reduced lignin content but increased biomass production [41]. We then hypothesized that the use of PtrMYB3, a master regulator of secondary cell wall biosynthesis, in place of the PrtMYB221 in the previous construct design [41] might increase both overall tree growth and biomass density.

To test this hypothesis, we performed intensive analyses of the growth and biochemical characteristics of the resulting transgenic poplars grown under LMO field conditions for long periods of time to encompass different seasons. We report the findings and discuss their significance in this approach to improve woody biomass feedstock.

Results

Generation of transgenic Arabidopsis and hybrid poplars expressing both PdGA20ox1 and PrtMYB3

To express both PdGA20ox1 and PrtMYB3 in a developing xylem (DX) tissue-specific manner, we utilized the 2A peptide sequence and DX15 promoter as reported ([41]; see methods) and produced both transgenic Arabidopsis and hybrid poplars (i.e., DX15::PdGA20ox1-2A-PrtMYB3 plants). For detailed phenotypic analysis, we selected five T3 homozygous transgenic Arabidopsis plant lines (1–1, 3–2, 4–5, 5–2, and 6–2) and six transgenic hybrid poplar lines (3, 4, 5, 6, 7, and 9) (Figs. 1 and 2). Although the DX-specific expression capacity of the DX15 promoter has been confirmed [44], we verified stem tissue-preferential expression of PdGA20ox1 in transgenic poplar plants by both semi-quantitative RT-PCR and quantitative
real-time PCR (RT-qPCR). The results showed that expression of *PdGA20ox1* was detected in the stem tissues but not in leaves (i.e., major veins removed) of the DX15::PdGA20ox1-2A-PtrMYB3 transgenic hybrid poplars (Additional file 1: Fig. S1). However, in transgenic hybrid poplars constitutively overexpressing *PdGA20ox1* (i.e., 35S::PdGA20ox1 poplar), the *PdGA20ox1* gene was strongly expressed in both stems and leaves (Additional file 1: Fig. S1).

**Enhanced biomass formation by DX-specific bicistronic expression of *PdGA20ox1* and *PtrMYB3***

Consistent with our previous findings [41], transgenic plants harboring the DX15::PdGA20ox1-2A-PtrMYB3

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**Fig. 1** Enhanced biomass formation in transgenic *Arabidopsis* plants having DX15::PdGA20ox1-2A-PtrMYB3 construct. Growth and biomass formation were analyzed using 50-day soil-grown plants. **a** Overall growth phenotypes of transgenic *Arabidopsis* plants compared to wild-type (WT) plants. Representative photographs of five independent T3 homozygous transgenic lines (1–1, 3–2, 4–5, 5–2, and 6–2) are shown together with WT plants. **b** Expression of *PdGA20ox1-2A-PtrMYB3* transcripts in transgenic *Arabidopsis* plants. RT-qPCR was performed using cDNA templates generated from stem total RNAs. *AtActin8* was used as the internal quantitative control. **c** Increase in plant biomass in transgenic *Arabidopsis* plants compared to WT plants. Both stem length (upper) and stem fresh weight (lower) were measured. Error bars indicate S.D. (*n* = 3). **d** Quantification of cell wall thickness. Error bars indicate S.E. (*n* = 3, 150 cells per plant). **e** Histological analyses of stem tissue. Rosette level inflorescent stems from transgenic *Arabidopsis* plants (line 4–5) and WT plants were cross sectioned and stained with toluidine blue O. Interfascicular fiber cells were visualized. Scale bars indicate 25 µm. Unpaired Student’s t-test, *P*-value * (P < 0.05), ** (P < 0.01), *** (P < 0.001)
construct showed enhanced growth performance compared to WT plants (Figs. 1 and 2). We first confirmed an increase in transcript level of *PdGA20ox1-2A-PtrMYB3* in each T3 homozygous transgenic *Arabidopsis* line by RT-qPCR (Fig. 1b). Then we quantified biomass accumulation by measuring stem height and fresh weight of both transgenic and WT *Arabidopsis* plants (Fig. 1c). Stem height and fresh weight were dramatically increased in the transgenic *Arabidopsis* compared to the WT (up to 2- and threefold, respectively).

Consistent with the previous finding that overexpression of *Arabidopsis MYB46*, an ortholog of *PtrMYB3*, significantly increases in the thickness of the secondary walls of interfascicular fiber cells [35], our histological analysis of the stem cross sections revealed increased secondary wall thickening of interfascicular fiber cells (up to 1.27-fold) in all transgenic lines compared to WT control (Fig. 1d, e).

We measured the growth of transgenic hybrid poplars (i.e., DX15::PdGA20ox1-2A-PtrMYB3) and WT poplars
grown in a growth room for 60 days (Fig. 2). As expected, stem height and fresh weight of transgenic poplars were 2- and 2.5-fold higher, respectively, than those of WT poplars (Fig. 2a, b). The amount of \( PdGA20ox1-2A-PtrMYB3 \) transcript expressed in the stem tissue corresponded to the increase in the biomass of the transgenic hybrid poplars (Fig. 2c).

**LMO field-grown transgenic hybrid poplar trees showed increased biomass formation with minimal growth defects**

We evaluated the growth performance of DX15::PdGA20ox1-2A-PtrMYB3 transgenic poplars and WT poplars under LMO field conditions, along with 35S::PdGA20ox1 and DX15::PdGA20ox1 transgenic poplars, which are constitutive overexpression [19] or DX-specific expression of \( PdGA20ox1 \) [22], respectively (Fig. 3). After 3 months of active growth in the spring to summer seasons, we measured stem height and diameter growth of the transgenic poplars to quantify biomass accumulation (Fig. 3c). The height of 35S::PdGA20ox1 and DX15::PdGA20ox1 poplars was 1.65- and 1.55-fold greater than that of WT poplars, respectively, while there was no significant difference in diameter growth. However, height and diameter growth in DX15::PdGA20ox1-2A-PtrMYB3 transgenic poplars (line 3) were increased by 1.73- and 1.62-fold, respectively (Fig. 3c). The one-way ANOVA analysis showed that the diameter growth of the DX15::PdGA20ox1-2A-PtrMYB3 transgenic poplar (line 3) is significantly increased compared to WT as well as the 35S::PdGA20ox1 and the DX15::PdGA20ox1 poplars (Fig. 3c).

To assess any growth defects caused by transgenes, leaf growth was analyzed using the 10th-12th leaves from the apex of the main stem of the transgenic poplars (Fig. 4). Both 35S::PdGA20ox1 and DX15::PdGA20ox1 transgenic poplars exhibited a 50%-65% reduction in leaf area compared to WT poplars (Fig. 4), which is consistent with a previous report [22]. However, in the case of line 4 of the DX15::PdGA20ox1-2A-PtrMYB3 transgenic poplars, leaf size and chlorophyll content were similar to those of WT poplars (Fig. 4). Taken together, these findings indicate that the DX15::PdGA20ox1-2A-PtrMYB3 transgenic poplars exhibited better growth performance under LMO field conditions than did WT, 35S::PdGA20ox1, and DX15::PdGA20ox1 transgenic poplars.

**Measurement of bud dormancy phenology and survival rates of transgenic poplars in over-winter growth analysis**

GAs regulate the induction and release of bud dormancy in woody perennials [45–48]. Poplar species in our LMO field (see Methods) usually start winter bud set in October, and bud release occurs in April of the following year. The 35S::PdGA20ox1 and the DX15::PdGA20ox1-2A-PtrMYB3 transgenic poplars started bud set 19 and 3 days later, respectively, than did the WT poplars. However, the bud set timing of the DX15::PdGA20ox1-2A-PtrMYB3 poplars was similar to that of WT poplars (Fig. 5a). After winter, the 35S::PdGA20ox1 and the DX15::PdGA20ox1 poplars initiated the spring bud flush 6 and 2 days earlier and completed the bud flush 9 and 6 days earlier, respectively, than did the WT poplars (Fig. 5b). However, the DX15::PdGA20ox1-2A-PtrMYB3 poplars showed a similar bud set pattern to that of WT poplars (Fig. 5b). These results suggest that the increased level of GA in the 35S::PdGA20ox1 and the DX15::PdGA20ox1 poplars altered their bud dormancy phenology. Accordingly, we observed faster shoot development from the buds of the 35S::PdGA20ox1 and the DX15::PdGA20ox1 poplars, compared to WT and DX15::PdGA20ox1-2A-PtrMYB3 poplars in the spring (Additional file 1: Fig. S2).

In over-winter growth analysis of the trees in the LMO field experiment, all of the WT and the DX15::PdGA20ox1-2A-PtrMYB3 poplars survived the winter, and leaf production by the whole plant was observed (Fig. 5c, d). By contrast, the 35S::PdGA20ox1 and the DX15::PdGA20ox1 poplars showed reduced survival rates by 20–40% and 80–100%, respectively, with significantly delayed leaf growth (Fig. 5c, d).

**Transgenic poplars produce improved quality and quantity of woody biomass**

To validate enhanced woody biomass production in the DX15::PdGA20ox1-2A-PtrMYB3 transgenic poplars, we replanted the line 3 plants in the same LMO site in the spring and observed the plants for almost two years. As shown in the planting design (Additional file 1: Fig. S3), 26 WT and 30 DX15::PdGA20ox1-2A-PtrMYB3 (one-way ANOVA with Tukey’s test, \( P < 0.05 \))

(See figure on next page.)
Fig. 3 (See legend on previous page.)
Fig. 4 Leaf growth of transgenic poplar plants grown in an LMO field. Leaf growth and chlorophyll content were analyzed in transgenic poplar plants (35S::PdGA20ox1, DX15::PdGA20ox1 and DX15::PdGA20ox1-2A-PtrMYB3) and WT plants grown in an LMO field for 3 months (from spring to summer). a Representative photographs of the 10th–12th leaves from transgenic and WT poplars. b Measurement of leaf area (upper panel) and chlorophyll content (lower panel) in the transgenic and WT poplars shown in (a). Error bars indicate S.D. (n = 10). The same letters indicate non-significant differences among each line (one-way ANOVA with Tukey’s test, $P < 0.05$).
poplars were planted in a total of seven beds with different combinations (Additional file 1: Fig. S3b–d). After 23 months, the DX15::PdGA20ox1-2A-PtrMYB3 transgenic poplars (line 3) had outcompeted the WT poplars (Fig. 6a, b). The stem height and diameter were 1.46- and 1.20-fold greater than those of WT plants, respectively (Fig. 6a), and fresh weight of the main stem was 1.77-fold higher (Fig. 6b). The total biomass, including the weight of the branches (first and second branches), was still significantly higher (1.41-fold) in the DX15::PdGA20ox1-2A-PtrMYB3 transgenic poplar.
We examined stem tissues of the DX15::PdGA20ox-2A-PtrMYB3 poplars to examine any changes in wood formation. The stem cross section taken at the 20th internode from the 60-day-old poplars showed no significant differences, compared to that from a WT poplar, including xylem cell wall thickness (Additional file 1: Fig. S4). However, the cell wall composition analysis showed a significant increase in cellulose content in transgenic poplars relative to WT poplars (up to 14.4 wt%) (Fig. 7a). Similar results were obtained from both 3-month and 2-year-old poplars grown in LMO field (Fig. 7a).

Saccharification efficiency of the wood materials from the LMO field-grown DX15::PdGA20ox-2A-PtrMYB3 poplars was estimated by quantifying the amount of glucose released at different incubation times after hot water or alkali (NaOH) pretreatment (Fig. 7b). We observed a significant increase in saccharification efficiency in the NaOH-pretreated transgenic poplars compared to WT poplars (up to 9% at 24 h), but no significant change was found after hot water treatment (Fig. 7b). These results indicate that the transgenic poplars have enhanced wood formation and improved saccharification efficiency relative to WT poplars.

Discussion
To improve the wood and growth performance of poplars for biomass production, we expressed PtrMYB3 in a developing xylem (DX) tissue-specific manner together with PdGA20ox1, bicistronically. Overexpression of PtrMYB3 under the 35S promoter in both Arabidopsis and poplar results in ectopic secondary wall thickening through upregulation of the biosynthesis of cellulose, xylan, and lignin [39, 40]. As a proof-of-concept experiment, we created transgenic Arabidopsis plants expressing the DX15::PdGA20ox1-2A-PtrMYB3. The resulting transgenic Arabidopsis plants showed a significant increase in secondary wall thickening in the interfascicular fibers (up to 1.27-fold) compared to WT plants (Fig. 1d, e). In addition, expression of this construct increased the fresh weight of the stem by up to threefold (Fig. 1c). These results demonstrated the efficacy of our strategy of bicistronic gene expression of PtrMYB3 and
PdGA20ox1, further confirming our previous findings [41].

As shown in Fig. 2, the growth of the DX15::PdGA20ox1-2A-PtrMYB3 poplars exceeded that of WT considerably, with a 2.6-fold increase of stem fresh weight of the poplars grown in the growth room for 60 days. Next, we attempted to validate the growth room performance of the transgenic poplars under LMO field conditions after 3 months of active growth in spring and summer. The stem height and diameter of the DX15::PdGA20ox1-2A-PtrMYB3 poplars were 1.7- and 1.6-times greater, respectively, than those of WT poplars with minimal growth defects (Figs. 3 and 4). Finally, we confirmed enhanced woody biomass production by the DX15::PdGA20ox1-2A-PtrMYB3 poplars by re-planting them in the same LMO field for two years (from May 2019 to Mar. 2021) (Additional file 1: Fig. S3), resulting in 1.46- and 1.77-fold greater than those of WT plants in stem height and fresh weight, respectively (Fig. 6a, b). This is significant in that the growth room performance of the transgenic poplars was validated in actual field conditions.

It is notable that we could not find any significant histological changes in secondary wall formation in the DX15::PdGA20ox1-2A-PtrMYB3 poplars, including secondary wall thickening of stem tissue (Additional file 1: Fig. S4), in light of the finding that the transgenic Arabidopsis plants expressing the same construct had increased secondary wall thickening of their interfascicular fiber cells (up to 1.27-fold) (Fig. 1d, e). However, in cell wall composition analysis, the cellulose content of the DX15::PdGA20ox1-2A-PtrMYB3 poplars was 14.4 wt% greater than that of WT poplars (Fig. 7a). Thus, we hypothesized that the higher content of cellulose may contribute to the increase of saccharification efficiency. However, it is not known yet why only the cellulose content was increased. Additional studies are needed to address the possibility of post-transcriptional regulation of the biosynthesis of the other cell wall components. Nonetheless, it is noteworthy that transgenic rice plants with increased cellulose content had significant increase in saccharification efficiency regardless of the changes in the other cell wall components [49].
Survival and productivity of temperate perennial woody plants depend on proper timing of dormancy onset and release, which is largely regulated by a plant hormone, GA, in many woody plants [45–48]. GA20ox1 expression in *Populus* spp. is regulated by day length, and levels of bioactive GAs are downregulated by SD condition, by which mechanism ensures a rapid cessation of growth for bud set and dormancy establishment [11]. Indeed, hybrid aspens overexpressing *Arabidopsis GA20ox1* were unable to arrest growth even under SD conditions [31]. Previously, we reported transgenic poplars with enhanced wood formation due to constitutive or developing xylem-specific expression of the *PdGA20ox1* gene, which encodes a key enzyme involved in GA biosynthesis [19, 22, 41]. However, no intensive study has been conducted on the growth performance of them under the field condition for the entire period covering active growth-dormancy cycle.

In this study, we used three transgenic poplars, namely, 35S::PdGA20ox1, DX15::PdGA20ox1, and DX15::PdGA20ox1-2A-PtrMYB3 poplars, and their WT counterparts for evaluation of their growth performance in the LMO field condition that expands to two-growing seasons. As expected, the timing of bud dormancy onset and release (e.g., bud flush) was significantly different among these poplars (Fig. 5). The 35S::PdGA20ox1 and DX15::PdGA20ox1 poplars showed delayed bud set before winter but early bud flush the next spring compared to WT and DX15::PdGA20ox1-2A-PtrMYB3 poplars (Fig. 5). It is highly probable that the altered bud dormancy phenology was due to differences in the GA content among genotypes caused by the different *PdGA20ox1* expression. We speculate that the DX15::PdGA20ox1-2A-PtrMYB3 poplars may have adequately increased level of GA due to the significantly reduced *PdGA20ox1* transcripts in the stem tissues compared to the 35S::PdGA20ox1 poplars (Additional file 1: Fig. S1) and the DX15::PdGA20ox1 poplars. It should be noted that both the 35S::PdGA20ox1 and the DX15::PdGA20ox1 poplars were reported to have very high levels of *PdGA20ox1* expression in the stem tissues [22]. Interestingly, the significantly reduced *PdGA20ox1* expression was also found in our previous study with transgenic poplar lines expressing DX15::PdGA20ox1-2A-PtrMYB221, and the winter survival rate of this poplar was also similar to that of WT poplar [41].

For temperate perennial woody plants, timely bud set is an important protection mechanism to increase the probability of survival over winter, which is characterized by cold temperatures and abiotic stresses [13, 50]. In spring, the temperature difference between night and day is large, and the timing of bud flush has a strong influence on plant survival [51]. Our transgenic hybrid poplars, especially the 35S::PdGA20ox1 poplars, showed altered bud dormancy phenology, which might have contributed to their lower over-winter survival rate (Fig. 5d). However, the DX15::PdGA20ox1-2A-PtrMYB3 poplars showed a 100% over-winter survival rate with increased biomass up to 77% compared to WT poplars (Fig. 6), further validating our bicistronic expression strategy.

**Conclusions**

The resulting DX15::PdGA20ox1-2A-PtrMYB3 poplars showed enhanced biomass production in both quantity and quality with sustained growth, which was evaluated in a field tests covering the entire active growth-dormancy cycle. Thus, our biotechnological tool can be expanded to various woody crops for production of desired multi-purpose biomass feedstock. Moreover, DX15::PdGA20ox1-2A-PtrMYB3 poplars represent a useful genetic background into which many useful traits may be stacked in order to produce a designer biomass feedstock.

**Methods**

**Plant materials and growth conditions**

*Arabidopsis thaliana*, ecotype Columbia (Col-0), was used in both wild-type and transgenic plant experiments. *Arabidopsis* was grown in soil in a growth room (14 h light; light intensity, 150 μmol m⁻² sec⁻¹) at 23 °C or on half-strength MS medium (Murashige and Skoog, Sigma-Aldrich) containing 2% sucrose with appropriate antibiotics for screening. Hybrid poplars (*Populus alba × P. glandulosa*, clone BH) were used as both WT controls and transgenic plants in this study. Plants were acclimated in soil and grown in a growth room (16 h light; light intensity, 150 μmol m⁻² s⁻¹; 24 °C) or in an LMO field at the Forest Bioresources Department of the National Institute of Forest Science, Republic of Korea (latitude 37.2 N, longitude 126.9E).

**Vector construction and plant transformation**

To construct a binary vector that can drive transgene expression in a developing xylem (DX)-specific manner, we modified the pMDC32 vector [52] as follows. The 2 x 35S promoter region of the pMDC32 vector was replaced with the DX15 promoter to create the DX15-pMDC32 vector [22, 44]. Full-length cDNAs encoding *PdGA20ox1* and *PtrMYB3* (Potri.001G267300.1) were amplified by polymerase chain reaction (PCR) from cDNA of *Pinus densiflora* and *Populus trichocarpa*, respectively. A virus-derived 2A peptide sequence was used to produce a fusion construct of *PdGA20ox1* (without the stop codon) and *PtrMYB3* and inserted downstream of the DX15 promoter in the DX15-pMDC32 vector using the Gateway cloning system as described
provided in Additional file 1: Table S1.

Histology and cell wall thickness measurements
Poplar main stems (20th internode) from 60-day-old soil-grown plants or rosette level stems of *Arabidopsis* plants were used to obtain hand-cut cross sections and stained with either 0.05% toluidine blue O or 2% phloroglucinol-HCl for 1 min as described in Ref. [22]. Images of stem sections were used to measure secondary cell wall thickness sintered glass crucible. After drying at 50 ºC, 30 mg extractive-free wood powder was recovered from a medium coarseness sintered glass crucible. After washing with 1% acetic acid and acetone, delignification was repeated for another 16 h using fresh reaction mixture. One microgram of total RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen) in 20 µl reaction volumes. Subsequent RT-PCR was performed with 1 µl of the reaction product as a template. Quantitative real-time PCR was performed using the CFX96TM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with iQTM SYBR Supermix (Bio-Rad, Hercules, CA, USA). Poplar Actin2 gene was used as the internal quantitative control, and relative expression was calculated by the 2−ΔΔCt method [57]. Sequences are provided in Additional file 1: Table S1.

RNA extraction and RT-PCR
Total RNAs of *Arabidopsis* were extracted using Trizol reagent (Life Technologies, Carlsbad, CA, USA) as described previously [22]. Total RNAs of poplar were extracted using the cetyltrimethylammonium bromide (CTAB) method with slight modification [56] as described [41]. In brief, fine powder from plant tissues was mixed with CTAB buffer followed by phenol:chloroform:miscellaneous alcohol (25:24:1) extraction. One microgram of total RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen) in 20 µl reaction volumes. Subsequent RT-PCR was performed with 1 µl of the reaction product as a template. Quantitative real-time PCR was performed using the CFX96TM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with iQTM SYBR Supermix (Bio-Rad, Hercules, CA, USA). Poplar Actin2 gene was used as the internal quantitative control, and relative expression was calculated by the 2−ΔΔCt method [57]. Sequences are provided in Additional file 1: Table S1.

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Growth measurements
Stem height was measured using a scale bar from the top of the plant to the soil level, and stem diameter was measured using digital calipers (Mitutoyo, Japan) at 3 cm above soil level. Both leaf area and chlorophyll content were measured in the 10–12th leaves from the top using an LI-3100 area meter (LI-COR Biosciences, Lincoln, NE, USA) and ethanol extraction method [58], respectively. Three biological replicates per line were analyzed.

Bud dormancy phenotypic measurements
Bud dormancy phenotype at the apex of the branch was scored every day to determine the dates of bud set and bud flush from September to October and April, respectively, in all poplar plants. Bud flush was recorded when the first unfolded leaf was observed at the apex of the branch. The bud set and flush dates of each line were counted and expressed as percentages relative to those of WT poplars.

Saccharification efficiency measurement
Saccharification efficiency of transgenic poplars grown for 3 months under LMO conditions was measured. Stem tissues were dried at 65 ºC for 3 days and ground to a fine powder. Reducing sugar content was determined following the procedure described by Ref. [59] with slight modifications. Briefly, for pretreatment, ground materials (~2 mg) were incubated in water or NaOH (1%, w/v) at 30 ºC for 30 min and then autoclaved at 120 ºC for 60 min. After neutralization, 300 µl of 0.1 M sodium acetate buffer (pH 5.0) containing 40 µg of tetracycline, 10 mg cellulase, and 1 mg β-glucosidase was added. After 24, 48, and 72 h of incubation at 37 ºC with shaking (180 rpm), samples were centrifuged (13,000 rpm, 3 min), and 5 µl of the supernatant was collected for reducing sugar measurement using the DNS (3,5-dinitrosalicylate) assay [60]. Reducing sugar content was quantified by measuring the absorbance at λ 550 with glucose solutions as standards.

Cell wall composition analysis
The main stems of LMO field-grown hybrid poplars (3 months and 2 years old) were used for cell wall composition analysis followed by the method described in Motiar et al. [61]. Stem tissues were dried (65 ºC/2 weeks) and ground to a fine powder. To determine extractives amounts, 400 mg of wood power was Soxhlet-extracted using acetone for 24 h and measured the weight of extractive-free wood powder. The lignin content was quantified using the acid hydrolysis procedure for Klasson lignin [62]. In brief, extractive-free wood powder (200 mg) was oven-dried at 105 ºC and weighed before treatment with 72% sulfuric acid at 20 ºC (stirring every 10 min for 2 h). Acid hydrolysis was followed in 4% sulfuric acid for 1 h in an autoclave (at 121 ºC) and the acid-insoluble lignin was determined gravimetrically of the residue remaining in medium coarseness sintered glass crucibles after filtration and oven-dried at 105ºC. For cellulose content, the standard method for alpha cellulose was used following delignification. Briefly, extractive-free wood powder (100 mg) was reacted with 20% sodium chlorite in sodium acetate buffer (60 mL/L glacial acetic acid and 1.3 g/L sodium hydroxide) at 50 ºC for 16 h and repeated for another 16 h using fresh reaction mixture. After washing with 1% acetic acid and acetone, delignified wood powder was recovered from a medium coarseness sintered glass crucible. After drying at 50 ºC, 30 mg...
of the recovered holocellulose was incubated with 17.5% sodium hydroxide for 30 min and another 30 min after dilution to 8.75% by adding distilled water at room temperature. After filtering and rinsing through a medium coarseness sintered glass crucible, the powder was soaked in 1 M acetic acid for 5 min and then rinsed again with distilled water. Then, the alpha cellulose content was measured gravimetrically after drying at 105 °C.

Statistical analysis
All experiments were performed in triplicate and repeated at least three times. The number of plants used in each experiment is indicated for each result presented. Statistical analysis was performed, and graphs were generated using SigmaPlot v12.0 (Systat Software, Inc., Chicago, IL, USA). The significance of differences between groups was calculated using Student’s t-test, and significance level is indicated by asterisks (*P < 0.05; **P < 0.01; and ***P < 0.001).

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s13068-021-02029-2.

Additional file 1: Table S1. Primers used in this study. Figure S1. Stem-specific expression of PdGA20ox1 transcripts in transgenic poplar plants. Gene expression in 60-day soil-grown poplar plants was analyzed. a Gene expression pattern of PdGA20ox1 by semi-quantitative RT-PCR using cDNA templates generated from either stem or leaf total RNA. b Quantification of PdGA20ox1 transcripts by qRT-PCR using cDNA templates generated from stem total RNA of the indicated hybrid poplars (i.e., WT, 35S::PdGA20ox1 #22, and DX15::PdGA20ox1-2A-PtrMYB003 #3). Error bars indicate S.E. (n = 3). Figure S2. Bud flushing status of transgenic hybrid poplars and WT poplars in spring. Shoot development from winter bud was faster in 35S::PdGA20ox1 and DX15::PdGA20ox1 transgenic poplars than in WT and DX15::PdGA20ox1-2A-PtrMYB3 poplars in spring. Figure S3. Planting design of WT and DX15::PdGA20ox1-2A-PtrMYB3 poplars in the LMO field. a Satellite photograph of the LMO field at the National Institute of Forest Science, Republic of Korea (latitude 37.2 N, longitude 126.9E). Hybrid poplars were planted in the yellow box. Bed numbers are indicated right. b Planting design of WT (green circles) and DX15::PdGA20ox1-2A-PtrMYB3 (red circles) poplars in each bed shown in (a). c Detailed planting map of each bed shown in (b). d Photograph taken two days after planting following the design. Figure S4. Secondary cell wall analysis of WT and DX15::PdGA20ox1-2A-PtrMYB3 transgenic poplar. a Histological analyses of secondary wall formation in DX15::PdGA20ox1-2A-PtrMYB3 and WT plants. The 20th internodes of stems from 60-day-old soil-grown poplar plants were used for cross-sectional analysis and stained with phloroglucinol-HCl. Scale bars indicate 25 mm. b Quantification of cell wall thickness. There was no difference in secondary cell wall thickness between transgenic poplar and WT plants. Error bars indicate S.D. of the mean of three biological replicates (30 cells were measured per plant).

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Authors' contributions
JSC, MHH, KHH, and JHK conceived the study. JSC, MHH, KHH, and JHK contributed to experimental design. JSC, MHH, ERB, YLC, and HWJ conducted all the experiments and coordinated the field test. ERB and YLC generated and processed the field test data. JSC, MHH, and HWJ performed the data analysis. KHH and JHK wrote the manuscript draft. All the authors read and approved the manuscript.

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The authors claim no conflicts of interest.

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References
1. Blunden J, Arndt DS. State of the climate in 2015. Bull Amer Meteor. 2016;97:1–275.
2. Fatma S, Hameed A, Noman M, Ahmed T, Shahid M, Tariq M, Sohail I, Tabassum R. Lignocellulosic biomass: sustainable bioenergy source for the future. Protein Pept Lett. 2018;25:148–63.
3. Castro E, Nieves IU, Mullinixin MT, Saques WJ, Hoffman RW, Fernández-Sandoval MT, Tian Z, Rockwood DL, Tamang B, Ingram LO. Optimization of dilute-phosphoric-acid steam pretreatment of Eucalyptus benthamii for biofuel production. Appl Energy. 2014;125:76–83.
4. Crawford JT, Shan CW, Budsberd E, Morgan H, Bura R, Gustafson R. Hydrocarbon bi-jet fuel from bioconversion of poplar biomass: technical-economic assessment. Biotechnol Biofuels. 2016;9:141.
5. Ko JH, Kim WC, Im JH, Kim JY, Patterson S, Han KH. Pathway-specific genetic pretreatment strategy to improve bioenergy feedstock. Bio-mass Bioenergy. 2018;115:253–9.
6. Somerville C, Youngs H, Taylor C, Davis SC, Long SP. Feedstocks for biofuel production. Appl Energy. 2014;125:76–83.
7. Wyman CE. Biomass ethanol: technical progress, opportunities, and commercial challenges. Annu Rev Energy Environ. 1999;24:189–226.
8. Rubin EM. Genomics of cellulosic biofuels. Nature. 2008;454:841–5.
9. Carroll A, Somerville C. Cellulosic biofuels. Annu Rev Plant Biol. 2009;60:165–82.
10. Kaylen M, Van Dyne DL, Choi YS, Blase M. Economic feasibility of producing ethanol from lignocellulosic feedstocks. Bioresour Technol. 2000;72:19–32.
11. Singh RK, Bhalerao RP, Eriksson ME. Growing in time: Exploring the molecular mechanisms of tree growth. Tree Physiol. 2021;41:657–78.

12. Biemelt S, Tschiersch H, Sonnewald U. Impact of altered gibberellin metabolism on biomass accumulation, lignin biosynthesis, and photosynthesis in transgenic tobacco plants. Plant Physiol. 2004;135:254–65.

13. Cooke J, Eriksson ME, Junttila O. The dynamic nature of bud dormancy in tree: environmental control and molecular mechanisms. Plant Cell Environ. 2012;35:1707–28.

14. Ridoutt BG, Pharis RP, Sands R. Fibre length and gibberellins A1 and A20 are decreased in Eucalyptus globulus by acyclocyclohexanedione injected into stem. Physiol Plant. 1996;96:559–66.

15. Rieu I, Ruiz-Rivero O, Fernandez-Garcia N, Griffoh C, Potvers SJ, Geng F, Linhartova T, Eriksson S, Nilsson O, Thomas SG, Phillips AL, Hedden P. The gibberellin biosynthetic genes AtGA20ox1 and AtGA20ox2 act, partially redundantly, to promote growth and development throughout the Arabidopsis life cycle. Plant J. 2006;53:488–504.

16. Zhuang W, Gao Z, Wang L, Zhong W, Ni Z, Zhang Z. Comparative proteomic and transcriptomic approaches to address the active role of G44 in Japanese apricot flower bud dormancy release. J Exp Bot. 2013;64:4953–66.

17. Carrera E, Bou J, Garcia-Martinez JL, Prat S. Changes in GA 20-oxidase gene expression strongly affect stem length, tuber induction and tuber yield of potato plants. Plant J. 2000;22:247–56.

18. Xu Y, Si, Raman AS, Ream, W, Fujiwara H. GRRR CE, Brown SM. Overexpression of oxidase confers a gibberellin-overproduction phenotype in Arabidopsis. Plant Physiol. 1998;118:773–81.

19. Park EJ, Choi HT, Choi YI, Lee C, Nguyen VP, Jeon HW, Cho JS, Funada R, Pharis RP, Kurepin LV, Ko JH. Overexpression of gibberellin 20-oxidase 1 from Pinus densiflora results in enhanced wood formation with gelatinous fiber development in a transgenic hybrid poplar. Tree Physiol. 2015;35:1264–77.

20. Xiao YH, Li DM, Yin MH, Li XB, Zhang M, Wang YJ, Dong J, Zhao J, Luo M, Luo XY, Hou L, Hu L, Pei Y. Gibberellin 20-oxidase promotes initiation and elongation of cotton fibers by regulating gibberellin synthesis. J Plant Physiol. 2010;167:829–37.

21. Eriksson ME, Israelsson M, Olsson O, Mortiz T. Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. Nat Biotechnol. 2000;18:784–8.

22. Jeon HW, Cho JS, Park EJ, Han KH, Choi YI, Ko JH. Developing xylem-preferential expression of PdGA20ox1 a gibberellin 20-oxidase 1 from Pinus densiflora improves woody biomass production in a hybrid poplar. Plant Biotechnol J. 2016;14:1161–70.

23. Mauriat M, Petterle A, Bellini C, Mortiz T. Gibberellins inhibit adventitious rooting in hybrid aspen and Arabidopsis by affecting auxin transport. Plant J. 2014;78:372–84.

24. Ibáñez C, Kozarewa I, Johansson M, Ögren E, Rohde A, Eriksson ME. Gibberellins inhibit adventitious rooting in hybrid aspen and Arabidopsis by affecting auxin transport. Plant J. 2014;78:372–84.

25. Maurya JP, Triozzi PM, Bhalerao RP, Perales M. Environmentally sensitive molecular switches drive poplar phenology. Front Plant Sci. 2018. https://doi.org/10.3389/fpls.2018.01873.

26. Lagercrantz U. At the end of the day: a common molecular mechanism for photoperiod responses in plants? J Exp Bot. 2009;60:2501–15.

27. Michelson JH, Ingvarsson PK, Robinson KM, Edlund E, Eriksson ME, Nilsson O, Janss O. Autumn senescence in aspen is not triggered by day length. Physiol Plant. 2018;162:123–34.

28. Zawacki C, Busov VB. Roles of gibberellin catabolism and signalling in growth and physiological response to drought and short-day photoperiods in Populus Trees. Plos ONE. 2014;9:e86217.

29. Eriksson ME, Hoffman D, Kuduk M, Maunat M, Mortiz T. Transgenic hybrid aspen trees with increased juvenile growth (GA) concentrations suggest that GA acts in parallel with FLOWERING LOCUS T2 control shoot elongation. New Phyto1. 2015;205:1288–95.

30. Chundawat SP, Beckham GT, Himmel ME, Dale BE. Deconstruction of lignocellulosic biomass to fuels and chemicals. Annu Rev Chem. 2011;1:121–45.

31. Zhong R, Cui D, Ye ZH. Secondary cell wall biosynthesis. New Phytol. 2019;221:1703–23.

32. Ko JH, Jeon HW, Kim WC, Kim JY, Han KH. The MYB46/MYB83-mediated transcriptional regulatory programme is a gatekeeper of secondary wall biosynthesis. Ann Bot. 2014;114:1099–107.

33. Zhong R, Richardson EA, Ye ZH. The MYB46 transcription factor is a direct target of SND1 and regulates secondary wall biosynthesis in Arabidopsis. Plant Cell. 2007;19:2776–92.

34. Ko JH, Kim WC, Han KH. Ectopic expression of MYB46 identifies transcriptional regulatory genes involved in secondary wall biosynthesis in Arabidopsis. Plant J. 2009;60:649–65.

35. Kim WC, Ko JH, Kim JY, J, Bae HJ, Han KH. MYB46 directly regulates the gene expression of secondary wall-associated cellulose synthases in Arabidopsis. Plant J. 2013;73:26–36.

36. McCarthy RL, Zhong R, Ye ZH. MYB83 is a direct target of SND1 and acts redundantly with MYB46 in the regulation of secondary cell wall biosynthesis in Arabidopsis. Plant Cell Physiol. 2009;50:1950–64.

37. McCarthy RL, Zhong R, Fowler S, Lyskowsk P, Piysa‑haen S, Carleton K, Spencer C, Ye ZH. The poplar MYB transcription factors, PtrMYB83 and PtrMYB82, are involved in the regulation of secondary wall biosynthesis. Physiol Plant. 2013;147:253–69.

38. Zhong R, McCarthy RL, Haghjhit M, Ye ZH. The Poplar MYB Master Switches Bind to the SMRE Site and Activate the Secondary Wall Biosynthetic Program during Wood Formation. PLoS ONE. 2013;8:e69219.

39. Cho JS, Jeon HW, Kim MH, Vo TK, Kim J, Park EJ, Choi YI, Lee H, Han KH, Ko JH. Wood forming tissue-specific bicistronic expression of PdGA20ox1 and PdMYB221 improves both the quality and quantity of woody biomass production in a hybrid poplar. Plant Biotechnol J. 2019;17:1048–57.

40. Ryan MD, Drew J. Foot-and-mouth disease virus 2A oligopeptide mediated cleavage of an artificial protein. EMBO J. 1994;13:928–33.

41. Atkins JF, Willis NW, Loughran G, Wu CY, Parinaw K, Ryan MD, Wang CH, Nelson CC. A case for ‘StopGo’: reprogramming translation to August codon meaning of GGN by promoting unconventional termination (Stop) after addition of glycine and then allowing continued translation (Go). RNA. 2007;13:803–10.

42. Ko JH, Kim HT, Hwang ID, Han KH. Tissue-type-specific transcriptome analysis identifies developing xylem-specific promoters in poplar. Plant Biotech J. 2012;10:587–96.

43. Duan C, Lin X, Gao D, Liu H, Li M. Studies on regulations of endogenous ABA and GA3 in sweet cherry flower buds on dormancy. Acta Hortic Sinica. 2004;31:149–54.

44. Runne PLH, Welling A, Vahala J, Ripel L, Ruonala R, Kangasjärvi J, van der Schoot C. Chilling of dormant buds hyperinduces FLOWERING LOCUS T and recruits GA-inducible 1,3-b-glucanases to reopen signal conduits and release dormancy in Populus. Plant Cell. 2011;23:130–46.

45. Zheng C, A cheeks G, Shi Z, Halāy T, Kamiya Y, Ophir R, Galleirh DW, Or E. Distinct gibberellin functions during and after grapevine bud dormancy release. J Exp Bot. 2019;60:1635–48.

46. Zhuang W, Gao Z, Wen L, Hux O, Cai B, Zhang Z. Metabolic changes upon flower bud break in Japanese apricot are enhanced by exogenous GA4. Hortic Res. 2015;2:15046.

47. Sumiyoshi M, Nakamura A, Nakamura H, Hakata M, Ichikawa H, Hirochika H, Iishi T, Satoh S, Iwai H. Increase in cellulose accumulation and improvement of saccharification by overexpression of arabinoarabinofuranosidase in rice. PLoS ONE. 2013;8:e78269.

48. Weiser CJ. Cold resistance and injury in woody plants. Science. 1970;196:1269–78.

49. Inouye DW. The ecological and evolutionary significance of frost in the context of climate change. Ecol Lett. 2003;6:347–63.

50. Curtis MD, Grossniklaus U. A gateway cloning vector set for high-throughput functional analysis of genes in plants. Plant physiol. 2003;133:462–9.

51. Clough SJ, Bent AF. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. 1998;16:735–43.
55. Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT. A simple and general method for transferring genes into plants. Science. 1985;227:1229–31.
56. Logemann J, Schell J, Willmitzer L. Improved method for the isolation of RNA from plant tissues. Anal Biochem. 1987;163:16–20.
57. Pfaffl MW. Development and validation of an externally standardized quantitative insulin like growth factor-1 (IGF-1) RT-PCR using Light Cycler SYBR Green I technology. In: Meuer S, Wittwer C, Nakagawara K-I, editors. Rapid cycle real-time PCR. Springer: Heidelberg; 2001. p. 281–91.
58. Lichtenthaler HK. Chlorophylls and carotenoids—pigments of photosynthetic biomembranes. Methods Enzymol. 1987;148:350–82.
59. Yang F, Mitra P, Loqué D. Engineering secondary cell wall deposition in plants. Plant Biotechnol J. 2013;11:325–35.
60. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem. 1959;31:426–8.
61. Mottiar Y, Gierlinger N, Jeremic D, Master ER, Mansfield SD. Atypical lignification in eastern leatherwood (Dirca palustris). New Phytol. 2020;226:704–13.
62. Coleman HD, Park J-Y, Nair R, Chapple C, Mansfield SD. RNAi-mediated suppression of pcoumaroyl-CoA 3'-hydroxylase in hybrid poplar impacts lignin deposition and soluble secondary metabolism. Proc Natl Acad Sci USA. 2008;105:4501–6.

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