Engineering secondary cell wall deposition in plants

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Summary
Lignocellulosic biomass was used for thousands of years as animal feed and is now considered a great sugar source for biofuels production. It is composed mostly of secondary cell walls built with polysaccharide polymers that are embedded in lignin to reinforce the cell wall structure and maintain its integrity. Lignin is the primary material responsible for biomass recalcitrance to enzymatic hydrolysis. During plant development, deep reductions of lignin cause growth defects and often correlate with the loss of vessel integrity that adversely affects water and nutrient transport in plants. The work presented here describes a new approach to decrease lignin content while preventing vessel collapse and introduces a new strategy to boost transcription factor expression in native tissues. We used synthetic biology tools in Arabidopsis to rewire the secondary cell network by changing promoter-coding sequence associations. The result was a reduction in lignin and an increase in polysaccharide depositions in fibre cells. The promoter of a key lignin gene, C4H, was replaced by the vessel-specific promoter of transcription factor VND6. This rewired lignin biosynthesis specifically for vessel formation while disconnecting C4H expression from the fibre regulatory network. Secondly, the promoter of the IRX8 gene, secondary cell wall glycosyltransferase, was used to express a new copy of the fibre transcription factor NST1, and as the IRX8 promoter is induced by NST1, this also created an artificial positive feedback loop (APFL). The combination of strategies—lignin rewiring with APFL insertion—enhances polysaccharide deposition in stems without over-lignifying them, resulting in higher sugar yields after enzymatic hydrolysis.

Introduction
Plant cell walls are virtually the only source of cellulose for the paper industry and will be a great source of sugars for the predicted lignocellulosic biofuels era (Carroll and Somerville, 2009; Simmons et al., 2008; Somerville et al., 2010). The utilization of plants to convert solar energy into transportable and storable energy will have positive impacts on the environment. It can help to reduce drastically the utilization of fossil-derived fuels, which will reduce carbon emissions into the atmosphere. Despite the environmental benefits of lignocellulosic biofuels, their production cost is unaffordably high. The raw sugar derived from plant cell walls is too expensive when compared to the price of crude oil. The main contributors to the high cost of cell wall–derived glucose are low sugar density of the biomass, cell wall recalcitrance to enzymatic hydrolysis and medium content in cellulose. Each factor either impacts transportation or requires intensive use of energy and chemicals for processing (Blanch et al., 2008; Klein-Marcuschamer et al., 2010; Searcy et al., 2007). Therefore, enhancement of polysaccharide accumulation in raw biomass and improvement of biomass digestibility will have important beneficial impacts on the cost of lignocellulosic biofuels production (Blanch et al., 2011; Klein-Marcuschamer et al., 2010).

By embedding the polysaccharide polymers and reducing their extractability and accessibility to hydrolytic enzymes, lignin is the major contributor to cell wall recalcitrance. There is usually a high negative correlation between lignin content and saccharification efficiency of plant cell walls (Chen and Dixon, 2007; Jørgensen et al., 2007; Vinzant et al., 1997). Unfortunately, most efforts to reduce lignin content during plant development resulted in severe biomass yield reduction particularly in dicotyledonous species (Franke et al., 2002; Shadle et al., 2007; Voelker et al., 2010), and therefore, there are very few crops exhibiting high lignin reduction. This relationship between secondary cell wall modification and plant growth is not unique to lignin modification, but is often correlated with loss of cell wall integrity causing vessel collapse as it is also observed when secondary cell wall genes involved in hemicellulose or cellulose biosynthesis are defective (Anterola and Lewis, 2002; Brown et al., 2005; Voelker et al., 2010). Vessels are essential for providing aboveground tissues with water and nutrients absorbed by the root system (Boyce et al., 2004; Déjardin et al., 2010; Gomez et al., 2008). However, lignin-related growth inhibition is not always related to vasculature collapse (Li and Chapple, 2010), it can be caused by constitutive induction of defence mechanism. Repression of the HCT enzyme (hydroxycinnamoyl CoA/shikimate hydroxycinnamoyl transferase) from the lignin biosynthesis pathway in Arabidopsis and alfalfa was showed to constitutively induce defence response and inhibit plant development, and both phenotypes were overcome by blocking the accumulation of the defence hormone salicylic acid (Gallego-Giraldo et al., 2011a,b). Hence, when silencing strategies are used to reduce lignin content in plants, the levels of gene repression to avoid biomass yield reduction are compromised.

Because woody biomass is mostly composed of secondary cell walls, strategies that increase cell wall thickness will increase
biomass density. Such strategies would reduce transportation costs, which are significant contributors to the price of biomass delivered to the biorefinery (Aden et al., 2002; Kumar et al., 2005; Searcy et al., 2007). Furthermore, these improvements could also be used to reinforce stem strength to reduce lodging and increase wood quality for construction. However, to avoid undesired growth phenotypes, an increase in cell wall deposition needs to be developed cautiously and has to be designed to target specific cell types such as fibre and pith cells. It is well known, for example, that overexpression of secondary cell wall transcription factors is often associated with ectopic cell wall thickening and lignification. This has a negative effect on expanding cells and photosynthetic tissues, which is deleterious for plant growth (Goicoechea et al., 2005; Mitsuda et al., 2005; Zhong et al., 2008, 2011a,b). Recently, a transcription factor, belonging to the WRKY family, was isolated and shown to act as repressor of secondary cell wall deposition in the pith, and more interestingly, its repression induces thickening of the wall in the pith without impacting plant development (Wang and Dixon, 2011; Wang et al., 2011).

Secondary cell wall regulatory network is now partially understood and seems to be conserved across many species from dicot to monocot plants (Christiansen et al., 2011; Handakumbura and Hazen, 2012; Ruprecht et al., 2011). Interestingly, cell differentiation into vessel or fibre cells starts independently. Both are regulated by independent master transcription factors that rapidly share the same regulatory network to control the expression of many genes involved in the biosynthesis of the three major secondary cell wall components: cellulose, xylan and lignin (Cano-Delgado et al., 2010; Ohtani et al., 2011; Zhong et al., 2010, 2011b). This makes it challenging to manipulate cell wall composition or content of woody tissues without impacting cell wall integrity and plant development (Anterola and Lewis, 2002; Brown et al., 2005; Voelker et al., 2010). In this study, synthetic biology tools were used to rewire part of the secondary cell wall network and to develop two new and complementary strategies to manipulate cell wall biosynthesis in specific tissues. This strategy was designed to reduce lignin content in fibre cells (and thus cell wall recalcitrance) and to enhance polysaccharide deposition in fibres without impacting plant development. We first rewired the regulation of lignin biosynthesis by disconnecting it from many regulatory networks including that of the fibre and largely restricted its control to the one of vessels (Figure 1a). Next, to enhance secondary cell wall deposition in specific cell types, we created an artificial positive feedback loop (APFL) to boost the expression of the NST1 master transcription factor controlling secondary cell wall biosynthesis in fibres (Figure 1b). We applied this APFL to the low-lignin plants, engineered with a lignin biosynthesis that is disconnected from the fibre secondary cell wall regulatory network (Figure 1c). This engineering allowed us to generate healthy plants with reduced lignin and enhanced cell wall deposition, which—after various pretreatments—exhibit improved sugar releases from enzymatic hydrolysis as compared to wild type.

Results
Characterization of vessel-specific promoter pVND6
Because of the importance of vessels for transport of water and nutrients to photosynthetic organs, integrity of this tissue is required for good plant development. Both transcription factors, VND6 and VND7, have been characterized as master regulators for vessel formation, suggesting that they have a vessel-restricted expression pattern and would be independent of those that regulate fibre development (Kubo et al., 2005; Yamaguchi et al., 2008, 2010). To correlate the spatio-temporal activity of the promoter controlling the expression of these transcription factors with lignin biosynthesis, the promoter VND6 (pVND6) was used to express CADd to complement the cad-c cad-d mutant (Sibout et al., 2005, Figure S1a). The cad-c cad-d mutant was selected as a pre-screen tool because a homozygous mutant can be easily grown and displays an easily observable phenotype corresponding to partial vessel collapse as well as cell wall redness caused by the accumulation of a side-product derived from the accumulation of hydroxycinnamaldehydes. Stem cross-sections of two independent cad-c cad-d + pVND6::CADd lines were analyzed under bright light (Figure S1a). The reduction in redness seen

![Figure 1 Model of secondary cell wall engineering](image-url)
particularly in xylem and the restoration of the vessel integrity observed for both cad-c cad-d + pVND6::CADd lines met the acceptance criteria for use of this promoter in further investigations.

To compare the strength of VND6 and C4H promoters (pVND6 and pc4H, respectively), both were used to express the F5H1 gene in f5h1-1 null mutant background (Meyer et al., 1998). The use of this mutant as a tool to study the activity of these promoters was based on the absence of growth phenotype, vessel collapse, and sinapyl alcohol unit in the lignin that are easily detected by Mäule staining (Nakano et al., 1992). Two independent lines for both promoters were selected for analysis, and the activity of each promoter was compared by the amount of sinapyl alcohol units incorporated into the lignin using Mäule staining as the readout (Figure S1b). Cross-sections of stems from both lines expressing the F5H1 gene under the control of pVND6 show a much lower level of red coloration after the Mäule staining than that of the lines expressing F5H1 with pc4H. The lower coloration is more pronounced in interfascicular fibres of f5h1-1 + pVND6::F5H1 lines compared to those of f5h1-1 + pc4H::F5H1 lines and is caused by a lower accumulation of sinapyl alcohol. This observation supports that pVND6 activity is largely restricted to vessel cells in contrast to that of pc4H. Taken together with the cad complementation (Figure S1), these data demonstrate that pVND6 is a suitable promoter to manipulate lignin biosynthesis in vessels.

Restriction of lignin biosynthesis to vessels

Lignin biosynthesis pathway is well characterized and mutations in this pathway, particularly in the genes involved in the earlier enzymatic steps, affect drastically plant growth and fertility. Therefore, controlling the expression of one of these genes should be sufficient to control the entire production of monolignols. We selected the C4H gene, encoding for the second enzyme in the lignin biosynthesis pathway (Figure S2), as a target gene to control the flux through that pathway and consequently the production of monolignols. To control the expression of C4H, we used the c4h mutant (Ruegger and Chapple, 2001; Schlünder et al., 2009) and transformed the heterozygote line (due to the sterility of the homozygous line) with the pVND6::C4H gene construct. Several transformants harbouring the pVND6::C4H fragment were selected and genotyped for the presence of homozygous c4h allele and four independent lines were used for further analysis. In contrast to the non-transformed c4h homozygous plants, the c4h + pVND6::C4H plants did not show any obvious growth difference when compared to wild-type plants (WT). The c4h + pVND6::C4H plants were fertile and able to generate large rosettes and tall stems (Figure 2). However, old leaves from c4h + pVND6::C4H plants showed anthocyanin accumulation only in leaf vasculatures in contrast to those of wild-type plants that turned completely purple (data not shown), evidence that the activity of the pVND6 is more restricted than that of the native C4H promoter.

Using the acetyl bromide method, analysis of lignin content in senesced stems from several c4h + pVND6::C4H lines shows that it was approximately 2/3 that of the wild type (Figure 3a). To verify the lignin distribution in the stems, stem cross-sections were stained with phloroglucinol–HCl reagents. Cross-sections of both c4h + pVND6::C4H lines showed reduction of lignin in the interfascicular fibres compared to wild-type plants. In contrast to that of the homozygous c4h mutant, xylem tissues of c4h + pVND6::C4H lines show a strong purple coloration after phloroglucinol staining and no vessel collapse, which is similar to what is observed in wild-type plants (Figure 3b).

Design of an APFL to overexpress NST1

The transcriprtional network controlling secondary cell wall deposition in vessel and fibres has already been well investigated. Several transcription factors were identified as master switches for secondary cell wall deposition in vessel or fibre cells, but all regulate virtually the same downstream network by controlling the expression of the main secondary genes involved in the biosynthesis of cellulose, hemicelluloses, and lignin polymers. Thus, these master switches are potential targets for manipulation of cell wall thickness (Mitsuda and Ohme-Takagi, 2009). Unfortunately, without tight expression control they are more harmful to the plant. Several groups have shown that expressing them with a constitutive 35S promoter causes ectopic secondary cell wall, thus inhibiting plant growth (Goicoechea et al., 2005; Mitsuda et al., 2005; Yamaguchi et al., 2010; Zhong et al., 2008).

To develop a tighter over-expression system, we designed an APFL (Figure 1b) by expressing a new copy of a master

Figure 2 Pictures of lignin-engineered plant 6- (a) and 10-week-old (b) wild-type (WT), c4h mutant, and four c4h + pVND6::C4H complemented lines (135, 150, 324, 337). Plants were grown together on soil under short-day conditions for 5 weeks (10 h 14 h light/dark cycle) prior to be transferred under long-day growth conditions (14 h 10 h light/dark cycle) until maturity.
transcription factor under the control of one of its downstream induced promoters. The hypothesis was that this would enhance the overall expression of the master transcription factor because, when its native promoter turns on, it would also induce the expression of its downstream targets—including the APFL that will produce more of the master transcription factor. To verify this hypothesis, we tested for enhanced cell wall thickening. We selected the NST1 transcription factor that controls secondary cell wall deposition in fibres and used the promoter (pIRX8) of the secondary cell wall IRX8 glycosyltransferase gene that is known to be induced by NST1 (Mitsuda et al., 2005; Zhong et al., 2010). As it was also known that the NST1 transcription factor positively controls the lignin biosynthetic pathway but does not control the activity of pVND6 (used to control lignin biosynthesis in the c4h + pVND6::C4H plants (line 135) with the pIRX8::NST1 gene construct to generate new transgenic Arabidopsis lines c4h + pVND6::C4H-pIRX8::NST1. The lignin-engineered lines (c4h + pVND6::C4H) were selected as the genetic background to disconnect the overexpression of polysaccharide biosynthesis from lignin biosynthesis (Figure 1c).

Several c4h + pVND6::C4H lines were generated and did not show any obvious growth difference when compared to wild type and c4h + pVND6::C4H plants (Figure 4). The c4h + pVND6::C4H-pIRX8::NST1 lines were fertile and able to generate large rosettes and tall stems. Like those of c4h + pVND6::C4H old leaves, leaf vasculatures from the c4h + pVND6::C4H-pIRX8::NST1 lines were purplish as a result of anthocyanin accumulation in contrast to wild-type old leaves that turned completely purple (data not shown). Expression analysis of both NST1 alleles (native and APFL allele) was verified by semi-quantitative RT-PCR and suggests that the native NST1 is expressed at the same level in wild-type plants and both c4h + pVND6::C4H and c4h + pVND6::C4H-pIRX8::NST1 lines (Figure S3). Expression of the new NST1 allele was only detected in the c4h + pVND6::C4H-pIRX8::NST1 lines, resulting in a higher general expression level of the NST1 gene (native and APFL allele) in c4h + pVND6::C4H-pIRX8::NST1 stems (Figure S3).

Artificial positive feedback loop increases cell wall deposition in fibre cells

To verify the impact of NST1 overexpression on lignin deposition, we first quantified lignin content in mature stems from the different engineered lines using the acetyl bromide method (Figure 3a). The c4h + pVND6::C4H-pIRX8::NST1 lines exhibited a slight increase in lignin content compared to the c4h + pVND6::C4H plants (parent line 135), but that level remained lower than

![Figure 3](image-url)
that of wild type. This analysis was extended with stem cross-sections that were stained with phloroglucinol–HCl. Cross-sections of the c4h + pvND6::C4H-pIRX8::NST1 lines (60 and 89) show no vessel collapse and less lignin in the interfascicular fibres compared to wild type (Figure 3b).

The next step was to analyze the impact of NST1 overexpression on cell wall deposition. Cell wall thickening was analyzed by transmission electron microscopy on stem cross-sections (Figure 5a). Compared to those of wild type, the c4h + pvND6::C4H line, fibre cells (xylary and interfascicular) from the c4h + pvND6::C4H-pIRX8::NST1 lines (line 60 and 89) showed an increase in cell wall deposition, although the cell wall thickening is more moderate and irregular for the c4h + pvND6::C4H-pIRX8::NST1 line 60 than for line 89 (Table S1). In contrast, no significant difference was observed for the vessel cells between the wild type and the different engineered lines. Furthermore, we measured stem diameters and weights of the engineered lines and found that both c4h + pvND6::C4H-pIRX8::NST1 lines exhibited higher biomass density (>10%) than the parental c4h + pvND6::C4H line (Table S2). To determine whether the increase was correlated to increased polysaccharide deposition, we further investigated c4h + pvND6::C4H-pIRX8::NST1 lines using confocal Raman microspectroscopy and immunofluorescence (Figures 5b,c). Cellulose distribution was analyzed by confocal Raman microspectroscopy by integrating the area between 1070 and 1140/cm of the collected Raman spectra at each position, allowing us to draw a cellulose map for each fibre cell (Figure 5b). As a result of NST1 overexpression, the c4h + pvND6::C4H-pIRX8::NST1 line showed thicker and denser cellulose distribution (represented by a higher intensity) compared to that of the wild type and c4h + pvND6::C4H lines in both xylary and interfascicular fibres. Surprisingly, the c4h + pvND6::C4H line displayed a lower cellulose density than wild type, particularly in its interfascicular fibres.

Immunofluorescence analysis was used as an alternative approach to analyze cellulose and xylan deposition in interfascicular fibre cells using CBM3a and mAb-LM10, respectively, on stem cross-sections of wild type, c4h + pvND6::C4H, and two lines (60 and 89) of the c4h + pvND6::C4H-pIRX8::NST1 lines (Figure 5c). CBM3a labelling analysis reveals that wall thickness was enhanced with crystalline cellulose for both c4h + pvND6::C4H-pIRX8::NST1 lines compared to that of the wild type and c4h + pvND6::C4H line. The increased cellulose deposition observed in c4h + pvND6::C4H-pIRX8::NST1 plants was more pronounced in line 89 than in line 60. The mAb-LM10 labelling analysis reveals that wall thickening is also correlated with an increase in xylan deposition, especially for the c4h + pvND6::C4H-pIRX8::NST1 line 89 when compared with that of the wild type and c4h + pvND6::C4H line. In contrast, the c4h + pvND6::C4H-pIRX8::NST1 line 60 shows a more moderate and irregular increase.

Impact of secondary cell wall engineering on saccharification efficiency

Cell wall analysis of both engineered lines c4h + pvND6::C4H and c4h + pvND6::C4H-pIRX8::NST1 revealed that lignin content was reduced and the cell wall content in the c4h + pvND6::C4H-pIRX8::NST1 line was increased in comparison with wild type,

Figure 5 Impact of the cell wall engineering on polysaccharide deposition in stems. Stem cross-sections analysis from wild type (WT), c4h + pvND6::C4H line (135), and two c4h + pvND6::C4H lines harbouring the artificial positive feedback loop pIRX8::NST1 construct (60, 89). (a) TEM images, top panel represents xylary area with vessel and fibre cells (V and F, respectively), and bottom panel represents interfascicular area with fibre cells only. (b) Cellulose map defined by confocal Raman microspectroscopy of fibre cells from xylary area (first top panel: bright-field image, second top panel: cellulose map) and of interfascicular fibres (third top panel: bright-field image; fourth top panel: cellulose map). (c) Immunofluorescence micrographs of interfascicular fibre cells labelled with CBM3a for crystalline cellulose (top panel) and LM10 for xylan (bottom panel).
suggesting that biomass for the engineered lines should be less recalcitrant to enzymatic hydrolysis. Therefore, ball-milled stems from wild type, c4h, c4h + pVDN6::C4H and c4h + pVDN6::C4H-pIRX8::NST1 plants were subjected to saccharification after two different mild pretreatments (hot water and dilute alkali), and the amount of sugar liberated was measured after 24, 48 and 96 h (Figures 6 and S4). Results showed that in both pretreatments, sugar release was faster and much higher for the cell wall engineered plants than for wild type and was almost as good as a the c4h lignin mutant. For each time point, the sugar released from stems of c4h + pVDN6::C4H-pIRX8::NST1 lines was higher than that from the parental line (c4h + pVDN6::C4H line 135). It was more than 2.5 times higher than that from wild type (Figure S4) at 96 h after hot water pretreatment and two times higher than that from wild-type stems (Figure S4) at 96 h after dilute alkali pretreatment.

Discussion

Modifying the lignin content has always been a challenge in crops or trees, because the more severe the reduction, the more biomass yield is negatively affected. This reduction is also often associated with a loss of integrity in vessels, the tissues that are responsible for water and nutrient distribution from roots to the aboveground organs (Boyce et al., 2010; Gomez et al., 2008). Lignin is considered to be the main inhibitory factor for pulping, forage digestibility and efficient enzymatic hydrolysis of plant cell wall polysaccharides, but it cannot be easily removed (Chen and Dixon, 2007; Jorgensen et al., 2007; Vinzant et al., 1997). Therefore, the present strategy focused on reducing lignin in tissues other than vessels, so that vessel integrity is maintained. In addition, the strategy sought to disconnect lignin biosynthesis from NST1, one of the key transcription factor switches controlling secondary cell wall deposition in fibre cells, allows manipulation of its expression without affecting lignin biosynthesis. By re-engineering few control points of the secondary cell wall biosynthesis (Figure 1), we demonstrated that we are able to reduce the lignin content and to increase cell wall thickening in fibres without obvious alteration of plant development (Figures 2–5). We replaced the promoter driving the expression of the C4H gene, which controls a key step in lignin biosynthesis, by that of the VDN6 transcription factor (pVDN6). The activity of pVDN6 is largely restricted to vessel cells (Kubo et al., 2005; Zhong et al., 2008), allowing a preferable spatio-temporal control of lignin deposition (Figures 3 and S1). This suggests that the pVDN6 promoter could be replaced by several other vessel-specific promoters, such as pVDN7, pVNI, pMC9 and pACL5 (Bollhöner et al., 2012; Ko et al., 2012; Kubo et al., 2005; Vera-Sirera et al., 2010; Yamaguchi et al., 2010). In addition, the control of the lignin biosynthesis could also be performed by controlling the expression of PAL, 4CL1 or C3'H enzyme instead of C4H; as several lignin mutants from various plant species, affected for one these enzymes, exhibit lignin content reduction, vessel collapse and growth defect phenotypes (Anterola and Lewis, 2002; Brown et al., 2005; Voelker et al., 2010).

To transfer the lignin rewiring approach to crops, lignin mutants in a target crop need to be isolated or generated via a

Figure 6 Saccharification efficiency of biomass derived from engineered plants. Amount of sugars released from enzymatic digestion (after 24, 48 or 96 h) of mature stems derived wild-type (WT), c4h mutant, two c4h + pVDN6::C4H lines (135, 150), and four c4h + pVDN6::C4H lines harbouring the artificial positive feedback loop pIRX8::NST1 construct (50, 60, 89, 92) after hot water (a, b) or dilute alkali (c, d) pretreatments.
gene-silencing approach (Schwab et al., 2006; Voinnet et al., 1998). In these mutants, lignin biosynthesis will be restored in the vessels by using a vessel-specific promoter from the target crop or related species to express a new allele encoding a protein that exhibits the same function as the repressed protein. This new allele should have a different sequence than the silenced gene to be protected against silencing. This can be achieved by using an allele from a different plant species or via gene synthesis. For example, a new 4CL or C3'H encoding sequence could be expressed with a vessel-specific poplar promoter (Bollhöner et al., 2012; Ko et al., 2012) in 4CL antisense or C3'H RNAi poplar lines respectively to restore cell wall integrity and function of the vessels (Coleman et al., 2008a,b; Kitin et al., 2010; Voelker et al., 2010). Such engineering should restore growth and increase biomass yield of the silenced lines and retain great saccharification efficiency. Alternatively, the defective enzymatic step could be bypassed with alternative routes to synthesize the missing precursors. For example, the SmFSH gene from Selaginella could be expressed with a vessel-specific promoter to restore the integrity of vessel and plant growth of the C3'H RNAi poplar lines (Coleman et al., 2008a,b). This SmFSH gene was recently shown in Arabidopsis to be able to restore the growth of hct- and c3'h-deficient mutants lacking the ability to produce p-coumaroyl shikimate and to meta-hydroxylate p-coumaroyl shikimate, respectively (Figure S2; Weng et al., 2010).

Another challenge was to manipulate general secondary cell wall biosynthesis using transcription factors to increase polysaccharide deposition without causing deleterious side-effects and over-lignification of the plant (Goicoechea et al., 2005; Mitsuda et al., 2005; Yamaguchi et al., 2010; Zhong et al., 2008, 2011a). The creation of an APFL using a secondary cell-wall-specific promoter (pirX8 in this study) and the NST1 transcription factor allowed us to increase secondary cell wall biosynthesis specifically in stems without altering plant development (Figures 1, 2 and 4). This APFL for the secondary cell wall is not restricted to the use of either pirX8 or NST1. Either could be replaced by different specific secondary cell wall promoters and transcription factors responsible for secondary cell wall deposition in fibre cells (Demura and Ye, 2010; Zhong et al., 2010). To our knowledge, this is the first APFL that has been developed in plants. There is only one example of an artificial negative feedback loop, which was developed in plants to delay senescence (Gan and Amasino, 1995). This negative feedback loop is based on the use of the early senescence-induced promoter (pSAG12) to express an IPT gene encoding for an isopentenyltransferase. It produces cytokinins at that specific developmental stage to delay senescence and keep the plant photosynthetically active much longer (Gan and Amasino, 1995). Furthermore, because of the conservation of senescence repression by cytokinins across plant species, this synthetic negative feedback loop was transferred into various crops (grasses and dicots) and was used to increase lifespan and improve plant biomass yield (Calderini et al., 2007; McCabe et al., 2001; Rolston et al., 2004). Interestingly, the secondary cell wall regulatory network falls into the same category of conserved mechanisms across plant species (Christiansen et al., 2011; Handakumbura and Hazen, 2012; Ruprecht et al., 2011) and was validated by the demonstration that NST1 like transcription factors from poplar and rice, were able to complement a double nst1/nst3 mutant from Arabidopsis using the NST1 promoter from Arabidopsis (Zhong and Ye, 2010; Zhong et al., 2011a). It demonstrates that poplar and rice NST proteins are able to regulate the same promoters as the Arabidopsis NST and control secondary cell wall network in Arabidopsis. Taken altogether, this strongly suggests that the APFL developed in Arabidopsis to overexpress NST1 transcription factor in fibres could be rapidly implemented into other vascular plant species to enhance secondary cell wall deposition. Therefore, this APFL technology could be used to increase cell wall content in plants designated not only for bioenergy, the pulping industry and forage production, but also to reinforce stem strength to reduce crop lodging and associated seed losses.

In summary, we presented two compatible approaches: (i) to narrow down lignin biosynthesis into vessels and (ii) to increase secondary cell wall thickening. Both were used to generate healthy plants with increased sugar yield upon saccharification. These approaches should open new ways for crop optimization and should benefit to lignocellulosic biofuels, paper and forage industries. Furthermore, we believe that the approach used to develop this APFL should be applicable to other metabolic pathways controlled by master transcription factors to boost their own expression in native tissues.

**Experimental procedures**

**Plant material and growth conditions**

Wild-type Arabidopsis thaliana plant (ecotype Columbia), c4h mutants (ecotype Columbia; ref3-2 mutant harbouring a poorly functional C4H allele; Ruegger and Chapple, 2001; Schmiller et al., 2009), c4h + pVND6::C4H lines and c4h + pVND6::C4H + pIRX8::NST1 lines were grown on soil from 8- to 10-day old seedling germinated either on soil or on sterile media. Because of its male sterility, the homozygote c4h mutants were identified by PCR-based genotyping from segregating populations derived from heterozygote c4h mutants. The c4h + pVND6::C4H lines were generated via floral dipping (Clough and Bent, 1998) of genotyped heterozygote c4h mutants with Agrobacterium tumefaciens GV3101 strain harbouring the pA6-pVND6::C4H binary vector. Selection of T1 and T2 c4h + pVND6::C4H lines was made on Murashige and Skoog medium supplemented with 1% sucrose, 1% agar and containing 30 μg/mL hygromycin followed by a c4h allele genotyping. Homozygote c4h mutants harbouring the pVND6::C4H DNA construct were named c4h + pVND6::C4H and used for downstream analysis or for agrobacterium-mediated transformation. The c4h + pVND6::C4H + pIRX8::NST1 lines were generated from the parent c4h + pVND6::C4H line (line 135) via floral dipping with Agrobacterium tumefaciens GV3101 strain harbouring the pKan-pIRX8::NST1 binary vector. Selection of T1 and T2 c4h + pVND6::C4H + pIRX8::NST1 transgenic plants was made on Murashige and Skoog medium supplemented with 1% sucrose, 1% agar and containing 30 μg/mL hygromycin and 50 μg/mL kanamycin. The homozygote c4h allele was verified for each generation.

Plants designated for analysis were grown on soil under short-day condition for 5 weeks (10 h/14 h light/dark cycle) prior being transferred to long-day growth condition (14 h/10 h light/dark cycle) until maturity at 150 μmol/m2/s, 22 °C and 60% humidity. All the other plants were grown under long-day condition (14 h/10 h light/dark cycle) at 100 μmol/m2/s, 22 °C, and 55% humidity.

**Generation of pA6-pVND6::C4H and pTkan-pIRX8::NST1 binary vectors**

The C4H (REF3; At2g30490) and NST1 (At2g46770) encoding DNA sequences were amplified from Arabidopsis cDNA using gene-specific primers extended with gateway b1 and b2 sequences as described in the Gateway manual (Life
The presence of a genomic wild-type allele was recognized by the generation of a double 625/271 bp fragment followed by a HinfI restriction digest because the c4h allele has lost the restriction site (Ruegger and Chapelle, 2001; Schlimmiller et al., 2009, the presence of a genomic wild-type allele was recognized by the generation of a double 625/271 bp fragment after restriction). The presence of pVND6::C4H and pIRX8::NST1 transgenes was verified by PCR with the primer pA6-pVND6::GW and pTkan-pIRX8::GW (Appendix S1) by LR recombination to create pA6-pVND6::C4H and pTkan-pIRX8::NST1 binary vectors, respectively.

Genotyping by PCR

Genotyping was performed on purified genomic DNA (Appendix S1) extracted from wild type and plant harbouring the c4h allele and pVND6::C4H or pIRX8::NST1 genes. The genotyping of the genomic c4h allele was performed by PCR using the F-ref3-2/R-ref3-2 primer pair (Appendix S1) to amplify an 896 bp DNA fragment followed by a HinfI restriction digest because the c4h allele has lost the restriction site (Ruegger and Chapelle, 2001; Schlimmiller et al., 2009, the presence of a genomic wild-type allele was recognized by the generation of a double 625/271 bp fragment after restriction). The presence of pVND6::C4H and pIRX8::NST1 transgenes was verified by PCR with the primer pairs F1-pVND6/R1-C4H and F1-pIRX8/R1-NST1, respectively (Appendix S1).

Histochemical staining

For all analyzed lines, except the homozygote c4h mutant, base of equivalent primary stems (from approximately 20 cm tall plants and 8 cm for the homozygote c4h mutant) was embedded in 7% agarose before being transversally sectioned to a thickness of 100 μm using a vibratome (Leica VT1000S, Microsystems Inc., Buffalo Grove, IL). For bright-field and UV fluorescence analysis, sections were directly mounted in water. For Wiesner lignin staining (phloroglucinol–HCl staining), sections were incubated for 3 min in phloroglucinol–HCl 2% (w/v) solution composed of phloroglucinol (VWR International, Brisbane, CA) dissolved in LR white resin and transversely sectioned to a thickness of 100 nm with a Leica UC6 ultramicrotome as described in Yin et al. (2011). Sections were stained with the anti-xylan LM10 monoclonal antibody (McCarty et al., 2005) or with CBM3a (a probe to crystalline cellulose; Blake et al., 2006) and analyzed using a fluorescent microscope (Leica DM4000B; Microsystems Inc.). Images were captured with Microublisher Q-imaging camera coupled to Metamorph software (Molecular Devices, Sunnyvale, CA). With the LM10 antibody, the immunolabellings were carried out as described in Verhertbruggen et al. (2009). LM10 was provided as supernatant, used at a 10-fold dilution and the secondary antibody was an anti-rat coupled with FITC diluted 100-fold. The detection of crystalline cellulose with CBM3a was carried out as described in McCartney et al. (2004), and the primary and secondary antibodies were a mouse anti-HIS diluted 100-fold and an anti-mice coupled with FITC diluted 100-fold, respectively.

Lignin quantification

Senesced stems of wild-type, homozygote c4h mutant, c4h + pVND6::C4H and c4h + pVND6::C4H-pIRX8::NST1 plants were ball-milled using a Mixer Mill MM 400 (Retsch Inc., Newtown, PA) and stainless steel balls for 2 min at 30s. Extract-free cell wall residues (CWR) were obtained by sequentially washing 50 mg of ball-milled stems with 1 mL of 96% ethanol at 95 °C twice for 30 min and vortexing with 1 mL of 70% ethanol twice for 30 s. The resulting CWR were dried in vacuo overnight at 30 °C. Five milligrams of CWR was used to determine lignin content using acetyl bromide method. CWR were incubated in a shaking incubator for 2 h at 50 °C with 200 μL of 25% (V/V) of acetyl bromide glacial acetic acid (VWR International, Brisbane, CA) and then diluted to 1 mL with glacial acetic acid prior centrifugation. In a new 1.5-mL tube, 100 μL was mixed to 500 μL of glacial acetic acid and 300 μL of 0.3 M sodium hydroxide was added, followed by 100 μL 0.5 M hydroxylamine hydrochloride, and between each steps, samples were

Confocal raman microspectroscopy

Analysis of cellulose deposition in primary stems of wild type, c4h + pVND6::C4H and c4h + pVND6::C4H-pIRX8::NST1 plants was performed with a LabRam HR 800 confocal Raman system (Horiba Jobin Yvon, Edison, NJ) as described in Sun et al. (2011). Images were collected using a 785 nm diode laser and a high numerical aperture 100× (oil NA 1.40) objective to achieve a submicron spatial resolution. A 20 by 20 μm region was measured for each image with a mapping step of 0.5 μm, an integration time of 1 s and a spectral resolution of approximately 4/cm. A SWIFT mode was utilized for the raster mapping to significantly increase mapping speed. The LabSpec5 software (HORIBA Scientific, Edison, NJ) was used for setting up measurements and data processing. The spectra in the range of 1050–1150/cm were despaired and smoothed using the Savitsky–Golay algorithm. The spectra were then baseline corrected and further smoothed using Fourier smoothing coupled with cosine apodization function. The integrated intensity over the range of 1070–1140/cm of the processed spectra was used to generate the cellulose maps by OriginPro 8 (OriginLab, Northampton, MA).
mixed. An aliquot of the sample was withdrawn and transferred in a UV-star microplate (Greiner Bio-One North America, Inc; Monroe, NC) and mixed to 1 volume of glacial acetic acid prior measuring the absorbance at 280 nm. The absorption coefficient used was 15.69 L g/cm² (Foster et al., 2010) and adjusted to the pathlength based on the volume and the microplate wells (height of the liquid in the well).

**Cell wall pretreatments and saccharification**

Ball-milled senesced stems (5 mg) of wild type, homozygote c4h mutant, c4h + pVIN6::C4H and c4h + pVIN6::C4H+pRX8::NST1 plants were transferred into a 2-mL screw-cap tubes and mixed to 200 µL of water or 175 µL of NaOH (1%, w/v) for hot water or dilute alkaline pretreatments, respectively, incubated at 30 °C for 30 min and autoclaved at 120 °C for 1 h. After cooling down at room temperature, samples pretreated with dilute alkaline solutions were neutralized with 2.5 N HCl (25 µL). Saccharification was initiated by adding 300 µL of 83 mM sodium citrate buffer pH 6.2 containing 133 µg/mL tetracycline, 4.4% w/w cellulase complex NS50013 and 0.44% w/w glucosidase NS50010 (Novozymes, Bagsværd, Denmark). After 24, 48 or 96 h of incubation at 50 °C with shaking (800 r.p.m.), samples were centrifuged (20 000 g, 3 min) and 10 µL of the supernatant was collected for reducing sugar measurement using the DNS (3,5-dinitrosalicylate) assay (Miller, 1959). DNS reagent was prepared by dissolving 1 g of 3, 5-dinitrosalicylic acid in 50 mL of water at 40 °C, followed by the addition of 30 g of KNa tartrate and 1.6 g of NaOH subsequently, finally the volume was adjusted to 100 mL final and the buffer was kept in dark. The DNS reaction was performed by mixing 10 µL of sample to 90 µL of DNS reagent in a PCR tube followed by incubation at 95 °C for 6 min in a PCR machine to perform the colorimetric reaction. Reducing sugars were quantified by measuring the absorbance at λ540 and using glucose solutions as standards.

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**References**


**Supporting information**
Additional Supporting information may be found in the online version of this article:

**Figure S1** Correlation of VND6 promoter activity with the lignin biosynthesis pathway.
**Figure S2** Representation of the lignin biosynthesis pathway
**Figure S3** Expression analysis of C4H and NST1 genes in stems from the cell wall engineered lines.
**Figure S4** Saccharification efficiency of biomass derived from cell wall engineered plants.
**Table S1** Cell wall thickness of fibre cells.
**Table S2** Stem diameters and densities.
**Appendix S1** Supporting experimental procedures.