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Improved saccharification and ethanol yield from field-grown transgenic poplar deficient in cinnamoyl-CoA reductase

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dormancy, implying that they continuously develop a relatively large zone of lignifying—i.e., not fully lignified—xylem, whereas xylem of field-grown trees matures and becomes structurally different at the end of the season. Furthermore, field trials allow the trees to be grown as short-rotation coppice, a practice that is preferred when trees are cultivated for bioenergy purposes (19). Publicly accessible databases reveal that more than 700 field-trial applications with genetically modified (GM) trees (including forest trees, fruit trees, and wood perennials) have been made worldwide (20), but data on very few field trials have been published (21). Only one field trial, with transgenic 4-coumarate:CoA ligase (*4CL*)–down-regulated poplar, has been evaluated for biofuel applications, but no improvement in saccharification efficiency was observed (22). Improvements in ethanol yields have been demonstrated for *C3H*-deficient and *F5H*-overexpressing poplars although these were greenhouse-grown (23). Here, we show that reducing *CCR* expression in field-grown poplar in two locations in Europe improves biomass processing into glucose and ethanol, but the associated yield penalty has to be overcome to advance translation to industrial applications.

Results

Down-Regulation of *CCR* Improves Saccharification Yield. Down-regulation of *CCR* was obtained by transforming poplar with sense (lines FS3 and FS40) and antisense constructs (line FAS13) (13). These lines, along with the wild type (WT) controls, were micropropagated and grown in the greenhouse for 6 mo. Upon removing the bark, the typical red coloration of the xylem was visible and appeared in patches in some trees of line FAS13, whereas trunks of FS3 and FS40 were more uniform in color. Accordingly, preliminary saccharification experiments of debarked stems without and with an acid (HCl) pretreatment indicated that FS3 and FS40 had the highest saccharification yields (Fig. S1). To compare saccharification yields in red versus white areas of the same trunk, separate white- and red-colored xylem of FAS13 was scraped from the stems and compared with scraped xylem from the WT. Whether or not a pretreatment was performed, the saccharification yield of white xylem from the *CCR*-deficient trees did not differ substantially from that of WT (Fig. 1). In contrast, saccharification of red xylem yielded significantly more glucose compared with white xylem and xylem of WT, both with and without pretreatment. As expected, and in agreement with Leplé et al. (13), lignin content in the scraped red xylem ($10.9 \pm 1.1\%$) was 19% reduced compared with that of WT ($13.5 \pm 0.2\%$), whereas that of white xylem ($13.4 \pm 1.6\%$) was not reduced.

Field Trial in Belgium. Next, we requested regulatory permission to establish a field trial with lines FS3, FS40, and WT. The field was

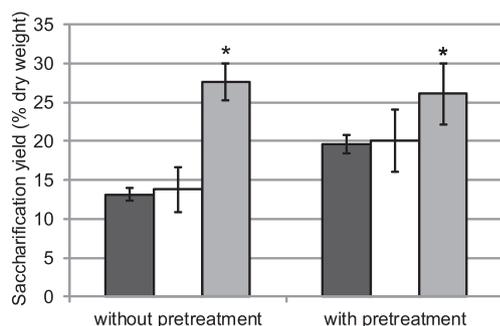


Fig. 1. Saccharification yields of xylem. Saccharification yields after 48 h, without and with acid (HCl) pretreatment, of scrapings of greenhouse-grown WT poplar ($n = 2$) and white and red xylem of greenhouse-grown FAS13 ($n = 7$). Error bars represent SDs. * $P < 0.01$. Dark gray, WT; white, white xylem of FAS13; light gray, red xylem of FAS13.

established in Belgium and consisted of six randomized blocks for each line, each block consisting of 20 clonally propagated trees (Fig. 2A and Fig. S2A). Ten months after planting, stems were cut back at the end of the winter, and ~20 cm of the basal part of the stems was sampled, debarked, and scored for the red phenotype. As already observed in greenhouse-grown trees, the red xylem appeared often in patches, because of unequal levels of gene silencing in red and white areas (13). The data obtained from greenhouse-grown trees had indicated that saccharification yield was only increased in red xylem, but was comparable to that of WT in white xylem. Therefore, to investigate whether the red xylem that had developed in field-grown transgenic trees was also more easily converted into fermentable sugars, we first grouped the wood samples into six different redness classes, depending on the red surface area of the stem pieces (Fig. 2C). For FS40, every single debarked trunk piece had a red phenotype, either in patches or fully red, whereas FS3 appeared to be less down-regulated in *CCR* and had more trees that were completely white. Then, the saccharification yield was analyzed for debarked wood samples of WT and the three most red classes (class 3, 4, and 5) of both transgenic lines, thus bypassing the issue of unequal gene silencing. The highest saccharification yields, for both transgenic lines and independent of the applied treatment, were usually observed for debarked wood of fully red stems (i.e., class 5) (Fig. S3A). However, the harsher the NaOH pretreatment was, the lower was the increase in saccharification yield compared with the WT (Table 1 and Fig. S3B).

To test whether and to what extent (upper bound) the improved saccharification translated into higher ethanol yields, a selection was made of fully red tree samples, which were as much as possible derived from trees spread over the entire field (Table S1). These samples were analyzed by a simultaneous saccharification and fermentation (SSF) assay. The ethanol yields of FS3 and FS40 were 48% and 87% increased over that of the WT, respectively (Fig. 3).

Only trees that had fully red stems (i.e., class 5) were significantly reduced in height by 27% (FS3) and 17% (FS40), and the diameter was only decreased for stems of class 5 of line FS3, by 21% (Fig. S4A and B). The biomass with or without bark of the basal 20-cm stem segments was only decreased for FS3 class 5 by 48% and 51%, respectively (Fig. S4C and D). If the reduction in biomass of the 20-cm stem segments used for SSF was taken into account, FS3 and FS40 yielded the same amount of ethanol as WT.

Field Trial in France. *CCR*-deficient poplars were grown in another field trial in France (Fig. 2B). One transgenic line, FS3, was common to both field trials. The French field trial also included FAS13 and WT poplar. The field itself was divided into five blocks with 24 clonal replicates in each block for each line (Fig. S2B). Twenty months after planting, individual stems were harvested in a similar way as those from the Belgian field trial. Again, to circumvent the inequality of gene silencing and to determine the upper limit of improvement, for each of the five blocks in the field, the five most red trees for both transgenic lines and five WT trees were selected for saccharification and SSF analysis (Fig. 2D).

Similarly to in the Belgian field trial, both transgenic lines, FS3 and FAS13, had considerably increased saccharification yields per gram dry biomass compared with WT for all pretreatment conditions (Table 1). The increase in saccharification yield for FS3 was higher than that observed for FS3 trees from the Belgian field trial. However, FAS13 outperformed the other lines and even reached more than a doubling in saccharification yield without pretreatment. The increased saccharification yield translated into increased ethanol yields of 36% for FS3 up to 161% for FAS13, as demonstrated by SSF (Fig. 3). The weight of the main stems of all individual trees per line and per block was determined (Fig. S4E). The transgenic lines FS3 and FAS13 showed an average reduction in biomass yield of 16% and 24%. Considering the biomass penalty only for those trees that had been selected for the SSF experiments, i.e., 18% (FS3) and 40%

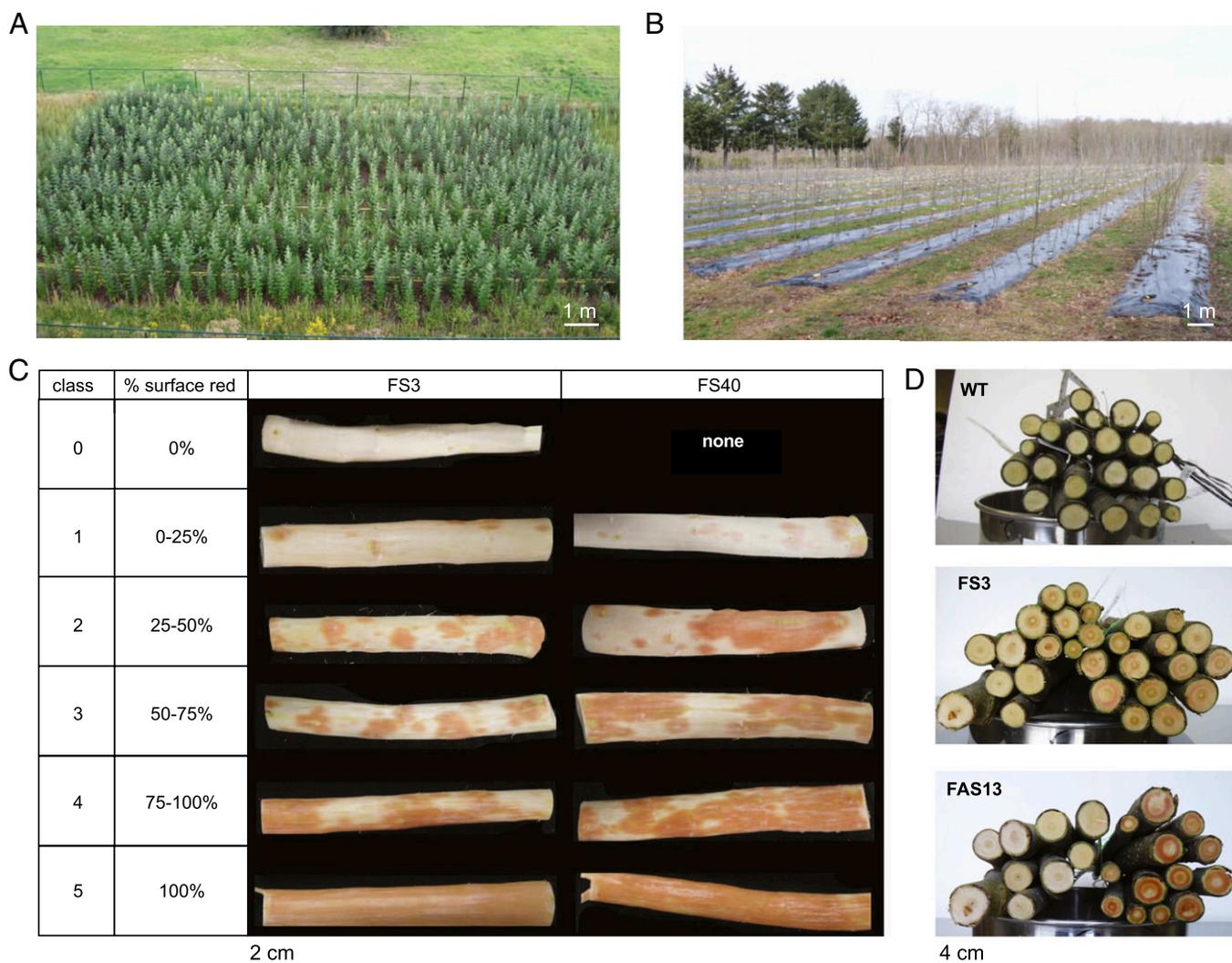


Fig. 2. Pictures of the Belgian and French field trials. (A) Field trial in Belgium (July 2009). (B) Field trial in France just before harvest (March 2010). (C) Classification and illustration of the variegated red phenotype observed for the transgenic trees in the Belgian field trial. (D) Illustration of the variegated red coloration in cross-sections for WT (Top), FS3 (Middle), and FAS13 (Bottom) from the French field trial.

(FAS13), and taking the SSF ethanol yield of the stem segments of these trees as a proxy for the SSF yield of the entire tree, the biomass yield penalty of FS3 outweighed the improvement in ethanol yield per gram biomass, similarly to FS3 grown in the Belgian field. However, FAS13 still had a 57% increase in ethanol yield compared with WT.

The Improved Saccharification Yield Is Due to a Higher Cellulose Conversion and Correlates with the Abundance of Ferulic Acid Markers. The improved saccharification yield observed could be due to higher cellulose levels in the transgenic lines or to a higher cellulose conversion. The cellulose content of the transgenic trees from the Belgian and French trials was similar to that of the WT (Table 2). Therefore, the cellulose fraction of all transgenic lines was more efficiently hydrolyzed than that of WT (Table 1). FAS13 had, under each pretreatment condition, the highest increase in cellulose conversion compared with WT and reached a doubled cellulose conversion when no pretreatment was included in the saccharification assays.

Surprisingly, the acetyl bromide soluble lignin (ABSL) content was only lower for FAS13 (by 12%), but not for the other lines (Table 2). In contrast, for all transgenic lines in both field trials, Klason lignin content was significantly lower (by 5–24%) than that of WT (Table 2). All three transgenic lines incorporated

elevated amounts of ferulic acid into lignin (Table 2). The ferulic acid markers (14) are good indicators of the altered lignin structure because they have been fixed in the lignin polymer at the time of lignin deposition. In this sense, they even reflect the altered wood composition more accurately than the red coloration, which is only visible at the surface of the trunk. The abundance of the ferulic acid markers correlated with the saccharification yield for all pretreatment conditions, with Pearson correlation coefficients ranging from 0.53 to 0.92 (Fig. S5).

Saccharification and Fermentation of Samples with Bark. We next performed saccharifications of stems with bark. Saccharifications of wood–bark mixtures were performed with one alkaline pretreatment (6.25 mM NaOH) on the same samples as selected for SSF for both transgenic lines FS3 and FS40 of the Belgian field trial. The saccharification yield of the bark was roughly half of that of xylem tissue. Accordingly, when wood and bark material were combined, saccharification resulted in intermediate yields. However, the wood–bark mix still had a substantial increase in saccharification yield (+27% and +29% for FS3 and FS40) compared with that of WT (Fig. 4).

In addition, complete stems of the entire 20-mo-old French field trial, including bark, apical parts, and less red trees, were combined in three pools per line, saccharified, and fermented by

Table 1. Saccharification data of wood from both field trials

Line	Pretreatment	Saccharification yield, % dry weight	Increase in saccharification yield compared to WT, %	Cellulose conversion, % cellulose	Increase in cellulose conversion compared to WT, %
Belgian trial					
FS3	None	10.5 ± 2.5	14.1	23.3 ± 3.9	39.5
	0.4 M H ₂ SO ₄	26.5 ± 4.3	18.8	55.7 ± 5.2	28.3
	6.25 mM NaOH	32.0 ± 5.4	14.7	67.6 ± 7.0	31.8
	62.5 mM NaOH	39.3 ± 4.7	11.3	79.3 ± 6.8	15.1
FS40	None	12.8 ± 2.3	39.1	25.4 ± 4.6	52.1
	0.4 M H ₂ SO ₄	28.3 ± 3.2	26.9	56.7 ± 5.0	30.6
	6.25 mM NaOH	33.8 ± 4.9	21.1	68.7 ± 9.7	33.9
	62.5 mM NaOH	39.3 ± 4.8	11.3	79.7 ± 9.3	15.7
WT	None	9.2 ± 1.2		16.7 ± 1.7	
	0.4 M H ₂ SO ₄	22.3 ± 3.5		43.4 ± 4.5	
	6.25 mM NaOH	27.9 ± 3.3		51.3 ± 4.1	
	62.5 mM NaOH	35.3 ± 2.6		68.9 ± 4.0	
French trial					
FS3	None	9.9 ± 2.2	25.3	19.9 ± 7.2	17.1
	0.4 M H ₂ SO ₄	18.1 ± 3.5	33.1	36.2 ± 10.4	23.5
	6.25 mM NaOH	23.6 ± 3.1	24.9	46.4 ± 12.4	14.3
	62.5 mM NaOH	28.6 ± 3.3	14.9	56.4 ± 14.5	5.6
FAS13	None	18.9 ± 3.8	139.2	34.7 ± 8.2	104.1
	0.4 M H ₂ SO ₄	26.5 ± 2.9	94.9	48.3 ± 5.7	64.8
	6.25 mM NaOH	33.7 ± 2.6	78.3	60.6 ± 5.6	49.3
	62.5 mM NaOH	36.2 ± 2.1	45.4	65.2 ± 5.4	22.1
WT	None	7.9 ± 1.9		17.0 ± 5.2	
	0.4 M H ₂ SO ₄	13.6 ± 2.1		29.3 ± 6.8	
	6.25 mM NaOH	18.9 ± 2.6		40.6 ± 10.1	
	62.5 mM NaOH	24.9 ± 2.3		53.4 ± 12.0	

Saccharification yields are the mean (± SD) for all trees of redness class 5 for the Belgian field trial whereas cellulose measurements were performed on selected individual trees only (Table S1) (WT, $n = 6$; FS3, $n = 6$; FS40, $n = 7$). To calculate cellulose conversions, the specific saccharification yields of those individual trees for which cellulose was measured were used. For each of the five blocks in the French field trial, saccharification yield was determined for the five most red trees per transgenic line and five randomly chosen WT trees. For the French field trial, cellulose conversion was determined for pooled trees (Table S1) (WT, $n = 10$; FS3, $n = 10$; FAS13, $n = 10$). The cell-wall residue (expressed as % dry weight) was taken into account to calculate the cellulose conversions. Bold, significantly increased compared with WT.

SSF. After 36 h of SSF, WT, FS3, and FAS13 had ethanol yields of 3.8 (± 0.03), 4.7 (± 0.12), and 4.3 (± 0.08) g/L, respectively; i.e., the transgenic trees had a 14–26% higher ethanol yield than the WT. However, the biomass penalty (Fig S4E) outweighed the improved ethanol yields. For traits with a significant line effect, broad-sense heritabilities are shown in Table S2.

Discussion

Lignin is one of the most important factors limiting the processing of plant biomass into fermentable sugars (15–17). Here, we have evaluated *CCR*-down-regulated poplars, grown under greenhouse and field conditions, for their saccharification and ethanol yields. The fact that *CCR* down-regulation was associated with a visible phenotype allowed us to collect samples that were uniformly down-regulated in *CCR* from both the Belgian and the French field trials, thus bypassing the unequal gene silencing issue. This red phenotype-based sampling allowed us to answer our primary question on whether wood properties of dormant field-grown *CCR*-down-regulated trees were similarly improved as actively growing trees from the greenhouse.

CCR transgenic trees were evaluated at two field sites, which is necessary because of possible genotype × environment interactions (21). Although saccharification yields and cellulose conversions (Table 1) for the common FS3 line varied between the two field trials, a substantially increased saccharification and ethanol yield was obtained for red wood harvested from both field trials, indicating that the effects of *CCR* down-regulation on wood processing observed in the greenhouse were maintained in the two field environments. The one caveat is that the analyzed wood was

still juvenile (10-mo-old for the Belgian and 20-mo-old for the French field trial) and not derived from a full 3-y rotation.

Wood from the fully red field-grown trees from both field trials had higher saccharification yields than the WT for all transgenic lines under all tested saccharification pretreatment conditions, except for FS3 without pretreatment in the Belgian field trial (Table 1). Moreover, it is important to note that wood from *CCR* transgenic trees processed under a given pretreatment saccharified equally well as WT wood processed with a harsher treatment; for example, untreated FAS13 saccharified equally well compared with 6.25 mM NaOH-treated WT in the French field trial. Because pretreatment is one of the biggest costs in the production of biofuels (24), the processing of lignin-reduced or lignin-modified trees has the potential to decrease the amount of chemicals and water used and, thus, the overall production cost and environmental impact.

Previous publications and wet-chemistry assays on greenhouse-grown trees have shown that down-regulation of *CCR* resulted in a reduced lignin content, at least in the red-colored wood (13). Remarkably, ABSL content in field-grown *CCR*-down-regulated poplars was not (for FS3 and FS40) or only modestly (for FAS13) decreased (Table 2), even in samples that were red throughout the stem as seen in cross-sections. This result is in contrast with the Klason lignin content, which was significantly reduced in agreement with the improved saccharification and ethanol yield. Similarly, the abundance of oligolignols, also a proxy for lignin amount (25), was reduced in the field-grown samples (Fig S6). It is possible that the high ABSL levels were caused by increased amounts of UV-absorbing substances that are detected by the

Table 2. Wood composition of transgenics and WT from both field trials

Composition	Belgian trial			French trial		
	FS3	FS40	WT	FS3	FAS13	WT
ABSL (% CWR)	16.4 ± 1.2	16.6 ± 0.6	16.5 ± 0.9	18.2 ± 0.6	<u>16.3 ± 1.1</u>	18.6 ± 0.6
Klason (% CWR)	<u>19.6 ± 1.9</u>	<u>19.1 ± 1.8</u>	20.7 ± 1.9	<u>19.8 ± 1.9</u>	<u>16.6 ± 1.6</u>	21.7 ± 1.9
H + G + S (μmol/g Klason)	2096.4 ± 278.4	2078.0 ± 267.1	2305.8 ± 285.6	2254.0 ± 247.5	1588.0 ± 224.7	2430.4 ± 286.1
H/G/S (mol%)	1.3/33.6/65.1 ± 0.7/2.2/2.0	1.2/33.5/65.2 ± 0.5/2.4/2.1	1.7/33.7/64.6 ± 0.6/1.7/1.5	0.8/32.3/66.9 ± 0.2/1.1/1.2	0.8/ 34.4/64.8 ± 0.1/ <u>0.7/0.7</u>	0.8/32.8/66.4 ± 0.0/1.7/1.7
FA × 10 ⁻⁴ (area/(IS × mg CWR))	3.0 ± 2.1	6.2 ± 1.9	1.0 ± 0.3	4.3 ± 0.7	18.0 ± 4.1	1.2 ± 2.0
A _G × 10 ⁻⁴ (area/(IS × mg CWR))	9.4 ± 7.3	16.1 ± 6.6	3.7 ± 1.3	13.8 ± 4.6	25.6 ± 11.1	4.4 ± 1.8
Cellulose (μg/mg CWR)	538.0 ± 63.7	516.9 ± 60.3	509.0 ± 48.3	535.7 ± 55.8	560.5 ± 47.1	523.5 ± 49.3
Hemicelluloses (μg/mg CWR)	197.1 ± 20.3	208.8 ± 10.7	196.8 ± 16.0	197.2 ± 13.1	207.4 ± 18.1	192.4 ± 15.2

Wood composition (± SD) of transgenics and WT from both field trials. For the Belgian field trial, wood composition was determined on the individual trees that were selected for SSF (Table S1) (WT, *n* = 6; FS3, *n* = 6; FS40, *n* = 7). For the French field trial, wood composition was determined on pooled trees (Table S1) (WT, *n* = 10; FS3, *n* = 10; FAS13, *n* = 10). FA, ferulic acid; A_G, CCR marker; IS, internal standard; bold, significantly increased compared with WT; underlined, significantly decreased compared with WT.

acetyl bromide method used to quantify lignin (22), potentially by increased xylan degradation in tissues with lower lignin content (26). The increased saccharification yields correlated well with the abundance of the ferulic acid markers. At this point, we cannot conclude to what extent the incorporation of the ferulic acid contributes to the improved processing of the wood samples.

SSF of wood derived from FAS13 yielded 161% more ethanol than WT (Fig. 3). This experiment was performed on a selection of completely red, debarked basal trunks, thus avoiding stability issues of gene silencing. The value of 161% increase in ethanol yield can thus be considered as the maximum obtained in the ideal case of a uniformly and stably *CCR*-down-regulated tree and with focus on wood tissue only. Taking the yield penalty for these trees into account, FAS13 still yielded 57% more ethanol

than WT. On the other hand, the experiment in which all trees from the French field trial were pooled and analyzed by SSF, resulted in a 14–26% increase in ethanol yield compared with WT. This value can be considered as the lower bound because, in this experimental design, trees that were less efficiently down-regulated for *CCR*, were also included in the analyzed pool and not only wood, but complete trees with bark and apical parts. However, the 14–26% increase in ethanol yield per gram of dry wood was outweighed by the overall yield penalty. Obviously, one of the main future objectives will be to overcome the yield penalty that is often associated with modified lignin (27). Among the likely causes of the yield penalty are the structural abnormalities of the plant cell wall, such as collapsed xylem (Fig. S7), as was previously noticed in greenhouse-grown *CCR*-down-regulated poplars (13). In *4CL*-down-regulated poplar, the reduced growth has been attributed to collapsed xylem and the formation of tyloses in the vascular system, and it was shown that vascular tissue in the red areas is defective in its transport capacity (22, 28). One option to circumvent the negative consequences of lignin down-regulation on plant performance while still achieving strong down-regulation is to specifically target the down-regulation to fibers but leaving the vessels intact. Such strategies have already proven successful in *Arabidopsis thaliana* (29, 30).

In conclusion, although we have observed variability in the level of down-regulation in individual trunk parts, we were able to demonstrate a relationship between *CCR* down-regulation and saccharification yield because the visible phenotype allowed us to classify the material into highly and less down-regulated samples. We conclude that *CCR* down-regulation may become a successful strategy to improve wood processing (to fermentable sugars) if it can be stabilized and targeted to fibers only.

Materials and Methods

Belgian Field Trial. FS3, FS40, and WT trees were transferred to the field in May 2009, under a GM field trial authorization (B/BE/07/V2) provided by the Belgian competent authorities after a positive ruling from the Belgian Biosafety Advisory Council. Beginning of March 2010, the bottom ~20 cm of the stems was harvested, debarked, and immediately photographed.

French Field Trial. FS3, FAS13, and WT poplars were planted in the field in July 2008, after obtaining suitable authorization (Application B/FR/07/06/01, Authorization 07/015) from the "Direction Générale de l'Alimentation" from the French "Ministère de l'Agriculture et de la Pêche" (on September 21,

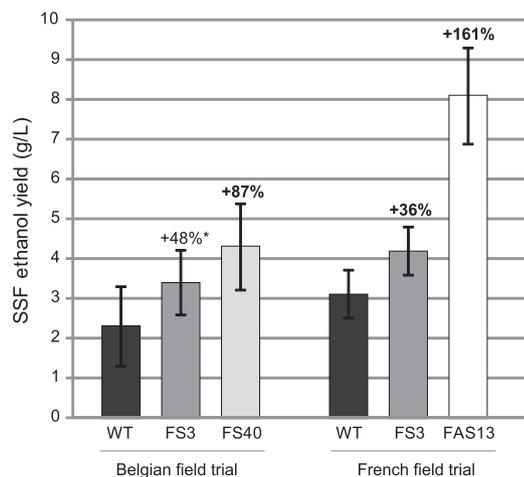


Fig. 3. Ethanol yield (g/L) for the Belgian and French field trials. For both locations, SSF was performed on pooled trees (Table S1) (Belgian trial: WT, *n* = 6; FS3, *n* = 5; FS40, *n* = 6; French trial: WT, *n* = 10; FS3, *n* = 10; FAS13, *n* = 10). Ethanol yields were measured after 31 h and 28 h of SSF for the Belgian and French field trials, respectively. Error bars represent SDs. Bold, significantly increased compared to WT. **P* = 0.08.

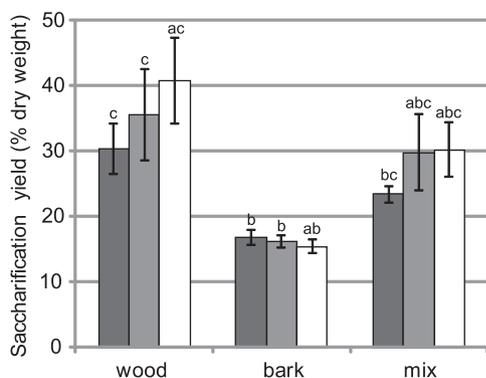


Fig. 4. Saccharification yields of wood and bark analyzed separately and of wood–bark mixtures of transgenic trees and WT of the Belgian field trial. The same trees that were selected for SSF and wood compositional analysis were used (WT, $n = 6$; F53, $n = 6$; F540, $n = 7$). A pretreatment with 6.25 mM NaOH was applied. Error bars represent SDs. Dark gray, WT; light gray, F53; white, F540. ^aSignificantly different compared with WT within the same tissue; ^bsignificantly different compared with wood within the same line; ^csignificantly different compared with bark within the same line.

2007 for a 5-y period) after a positive ruling from the French “Commission du Génie Biomoléculaire”. In March 2010, an ~20-cm segment at the base of each stem was harvested, debarked, and photographed.

Saccharification Assays. For a detailed description of the saccharification assay for greenhouse-grown samples, see *SI Materials and Methods*. For

field-grown poplar samples, the basal part of the harvested stems were handled with the iWALL custom-designed robot (Labman Automation Ltd.) (31).

Simultaneous Saccharification and Fermentation. Poplar biomass was pretreated with lime, saccharified, and fermented as described (32, 33).

Wood Compositional Analysis. Lignin analyses included the acetyl bromide (34) and Klason method (35) and thioacidolysis (14, 36); polysaccharides were analyzed by the Updegraff method and the alditol-acetate assay (37).

Metabolomics. Gradient elution and MS analysis were performed as described (38).

Statistical Analyses. Associated P values were adjusted with the Dunnett test (two-sided). Differences with a Dunnett adjusted P value <0.05 were considered significant.

Details on all methods are described in *SI Materials and Methods*.

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